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Gaetano Santulli *Editor*

Mitochondrial Dynamics in Cardiovascular Medicine

Foreword by Dr. Wilson S. Colucci

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Mitochondrial Dynamics in Cardiovascular Medicine

Foreword by Dr. Wilson S. Colucci

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Foreword

The field of mitochondrial biology is rapidly changing. Mitochondria certainly play a fundamental role in providing energy for all cell types, and are of particular importance in meeting the finely tuned energetic demands of the cardiovascular system. In addition, it is increasingly recognized that mitochondria are also key participants in a large number of nonenergetic biological pathways that, on the one hand, are essential to normal cellular function, and on the other hand, are central to a plethora of pathological processes that lead to cardiovascular disease.

The goal of this compendium is to provide a single place where the reader can obtain timely and authoritative information in this evolving field. Thanks to the informed guidance of the editor, Dr. Gaetano Santulli, *Mitochondrial Dynamics in Cardiovascular Medicine* brings together articles from prominent experts in the areas of mitochondrial biology, physiology, and disease – all with a focus on the cardiovascular system.

Basic areas of physiology are summarized and provide the background for detailed, cutting-edge reviews of the role of mitochondria in a wide array of cardiovascular processes from cardiac hypertrophy and heart failure to arrhythmias and ischemia. This book concludes with chapters on potential new therapeutic approaches – mitochondrial transplantation and mitochondria-targeted antioxidants. The liberal use of color figures, diagrams, and tables add to the value of this resource.

This is a book that will appeal to readers of all backgrounds, from student to scholar. Individually, the chapters in this book are a valuable resource. Together, they provide a comprehensive state-of-the-art treatment of the role of this dynamic and exciting organelle in the cardiovascular system.

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Chapter 1
Functional Implications of Cardiac Mitochondria Clustering

Felix T. Kurz, Miguel A. Aon, Brian O'Rourke, and Antonis A. Armoundas

Mitochondria in cardiac myocytes are densely packed to form a cell-wide network of communicating organelles that accounts for about 35% of the myocyte's volume [1]. The lattice-like arrangement of mitochondria, mostly in long and dense rows parallel to the cardiac myocyte myofilaments, has the structural features of a highly ordered network [2]. This specific mitochondrial network architecture ensures that the large demand of adenosine triphosphate (ATP) of cardiac myocytes is met and appropriately distributed. Mitochondria synthesize approximately 30 kg of ATP each day to both provide energy for the basic cellular metabolism and to secure basic physiological functions of the cardiovascular system such as the maintenance of pulmonary and systemic blood pressure during heart contractions [3]. Their intracellular position is closely associated with the sarcoplasmic reticulum and the myofilaments to facilitate cellular distribution of ATP [4]. However, the role of mitochondrial structure and function is not only limited to ATP generation;

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mitochondria participate in and control numerous metabolic pathways and signaling cascades such as the calcium signaling, redox oxidation, β-oxidation of fatty acids, oxidative phosphorylation, the synthesis of aminoacids, heme and steroids, and cellular apoptosis [5]. Their structural and functional diversity thus surpasses any other cellular organelle.

Specifically, the control of mitochondrial oxidative phosphorylation is dependent on the arrangement, interplay and interdependence of mitochondrial ion channels, transporters, and pumps that are located in an inner membrane that is selectively permeable to enable the adjustment of mitochondrial volume, inner membrane potential $\Delta\Psi_m$ and mitochondrial redox potential [6]. Thus, the mitochondrial membranes facilitate and balance cellular energy supply and demand.

The Mitochondrial Network in Cardiac Myocytes

Mitochondrial Inner Membrane Oscillations

When subjected to critical amounts of oxidative stress or substrate deprivation, the mitochondrial network in cardiac myocytes may pathophysiologically transition into a state where the inner membrane potential $\Delta\Psi_m$ of a large amount of mitochondria depolarizes and oscillates [6–9]. Oscillations of individual mitochondria in intact myocytes were first documented in the early 1980s when Berns et al. excited quiescent cardiac myocytes with a focal laser beam to induce transient cycles of alternating $\Delta\Psi_m$ depolarization and repolarization [10]. Mitochondrial inner membrane depolarizations were also detected in smooth muscle cells [11], cultured neurons [12], and in isolated mitochondria [13]. A functional link between metabolic oscillations and cardiac function was demonstrated when O'Rourke et al. [14] showed that cyclical activation of ATP-sensitive potassium currents in guinea pig cardiac myocytes under substrate deprivation was associated with low frequency oscillations in the myocyte's action potential duration and excitation-contraction coupling. These oscillations were accompanied by a synchronous oxidation and reduction of the intra-cellular nicotinamide adenine dinucleotide (NADH) concentration.

While it was initially believed that this mechanism was due to changes in glucose metabolism, subsequent studies detected an association of the oscillations with redox transients of mitochondrial flavoprotein and waves of mitochondrial $\Delta\Psi_m$ depolarization [15], therefore implying a mitochondrial origin of the phenomenon. Network-wide mitochondrial membrane potential oscillations could be initiated reliably by the application of a localized laser-flash that induced a depolarization of $\Delta\Psi_m$ in just a few mitochondria [16]. The association of $\Delta\Psi_m$ oscillations with reactive oxygen species (ROS) was discovered by Zorov and Sollot who showed that a localized laser-flash produced a high number of free radicals in cardiac mitochondria in an autocatalytic manner, concomitant with depolarization, referred to as ROS-induced ROS release [17, 18]. Experimentally, synchronous $\Delta\Psi_m$ oscillations

were observed in small contiguous mitochondrial clusters as well as in large clusters that span the whole myocyte [16], or as mitochondrial $\Delta\Psi_m$ depolarization waves that could pass between neighboring cardiac myocytes through intercalated discs [15]. Mitochondrial communication may also be facilitated through the formation of intermitochondrial junctions [19], which could facilitate wave propagation.

Generally, mitochondrial network coordination is heavily influenced by the intricate balance of ROS generation and ROS scavenging within the myocyte: when a critical amount of network mitochondria exhibits a destabilized inner membrane potential, the increased presence of ROS can lead the network into a state of ROS-induced ROS release [16, 20]. It is likely that the antioxidant flux is lowered to a point at which ROS production cannot be scavenged fast enough – this causes a flux mismatch that rapidly goes out of control such that multiple mitochondria form a myocyte-wide cluster of synchronously oscillating mitochondria, also called spanning cluster; these mitochondria lock into an oscillatory mode with low frequencies and high amplitudes [6, 8, 20].

The cardiac mitochondrial network can also behave in an oscillatory mode in the absence of metabolic or oxidative stress. A computational model of the mitochondrial oscillator has predicted mitochondrial behavior that was characterized by low-amplitude, high-frequency oscillations [21], which was later demonstrated experimentally [8]. Since, the collective mitochondrial behavior happened (i) in the absence of metabolic/oxidative stress, (ii) did not involve collapse or large excursions of the membrane potential (i.e., microvolts to a few millivolts), and (iii) could be clearly distinguished from the high-amplitude, low-frequency oscillations involving strongly coupled mitochondria through elevated ROS levels, this regimen was dubbed "physiological" [6, 8]. The transition between normal and pathological behavior in mitochondrial function can be determined by the abundance and interplay between cytoplasmic (Cu, Zn SOD) and matrix (Mn SOD) superoxide dismutases with ROS generation in the respiratory chain, as recently demonstrated [22].

In intact cardiac myocytes, ROS generation from mitochondrial respiration mainly originates in complex III and complex I, and is favored by highly reduced redox potentials and high $\Delta\Psi_m$, typically corresponding to either inhibition or low respiratory flux [6, 16]. The occurrence of $\Delta\Psi_m$ limit-cycle oscillations could be suppressed by adding inhibitors of mitochondrial respiration or ligands of the mitochondrial benzodiazepine receptor, but not adding inhibitors of the mitochondrial permeability transition pore (mPTP) [23]. This class of $\Delta\Psi_m$ oscillations was consistent with a ROS-induced ROS release mechanism involving the activation of mitochondrial inner membrane anion channels (IMAC), see also below and [6, 16, 24].

Mitochondrial Morphology, Dynamics and Morphodynamics

Changes in the morphology of mitochondria or their dynamic characteristics can influence their collective behavior. In cardiac myocytes, mitochondria can be perinuclear, intermyofibrilar and subsarcolemmal, and possess different morphologies

as well as proteomic and physiological status [25–29]. This (static) morphological heterogeneity is associated with different mitochondrial functions [30, 31]; for instance, mitochondria in intermyofibrillar locations show a reduced content of flavoproteins compared to those beneath the sarcolemma, thus resulting in different oxidative capacities for NADH- and FADH-generating substrates [30, 32].

The role of dynamic heterogeneity becomes important for pertubations of the mitochondrial network during oxidative or metabolic stress: the mitochondrial network's response to stress can affect not only intracellularly clustered mitochondria, but scale to the majority of cellular mitochondria and even to the whole-organ level, thus leading to emergent phenomena, such as ventricular arrhythmias [20, 33–35]. In many non-cardiac myocytes, mitochondrial mobility and morphological plasticity or morphodynamics is affected by proteins that facilitate fusion and fission processes, by cytoskeletal proteins (through disruptive structural changes of the cytoskeleton) [36], and changes in myocyte energy metabolism or calcium homeostasis [37, 38]. Recently, it was shown that redox signaling also occurs in clusters of morphologically changing axonal mitochondria, suggesting a morphological and functional inter-mitochondrial coupling in neurons [39, 40]. However, apart from "pre-fusion" events at inter-mitochondrial junctions [19], fusion and fission events as well as mitochondrial mobility have not been observed in adult cardiac myocytes so far, although mitochondrial fusion has been observed in skeletal muscle [41]. However, there is growing evidence from mitochondrial protein gene studies that mitochondrial morphodynamics may play a role in the maintenance of mitochondrial fitness [42].

Spatio-Temporal Behavior of Individual Mitochondrial Oscillators

While many studies have investigated the response of the whole mitochondrial network in external stress stimuli (i.e. ischemia-reperfusion injury or oxidative stress) at the inner mitochondrial membrane [8, 16], more recent ones have focused on the role of individual mitochondria and their spatio-temporal behavior [9, 35, 40, 43, 44].

Alterations of the mitochondrial membrane potential $\Delta\Psi_m$ can be monitored with the positively charged lipophilic fluorescent dyes tetramethylrhodamine methyl or ethyl ester (TMRM or TMRE), that redistribute from the matrix to the cytoplasm with a depolarization of $\Delta\Psi_m$ [45] (see Fig. 1.1). Myocyte-wide $\Delta\Psi_m$ oscillations can then be triggered by a perturbation of the mitochondrial network through thiol-oxidizing substances such as diamide, or by a highly localized laser flash [16, 46]: the ensuing sequence of locally confined ROS generation and induced autocatalytic ROS release [16, 17, 47] can lead to a critical accumulation of ROS that initiates a propagating wave of $\Delta\Psi_m$ depolarization [7]. However, the reaction of each individual mitochondrion within the myocyte is highly variable: while a major-

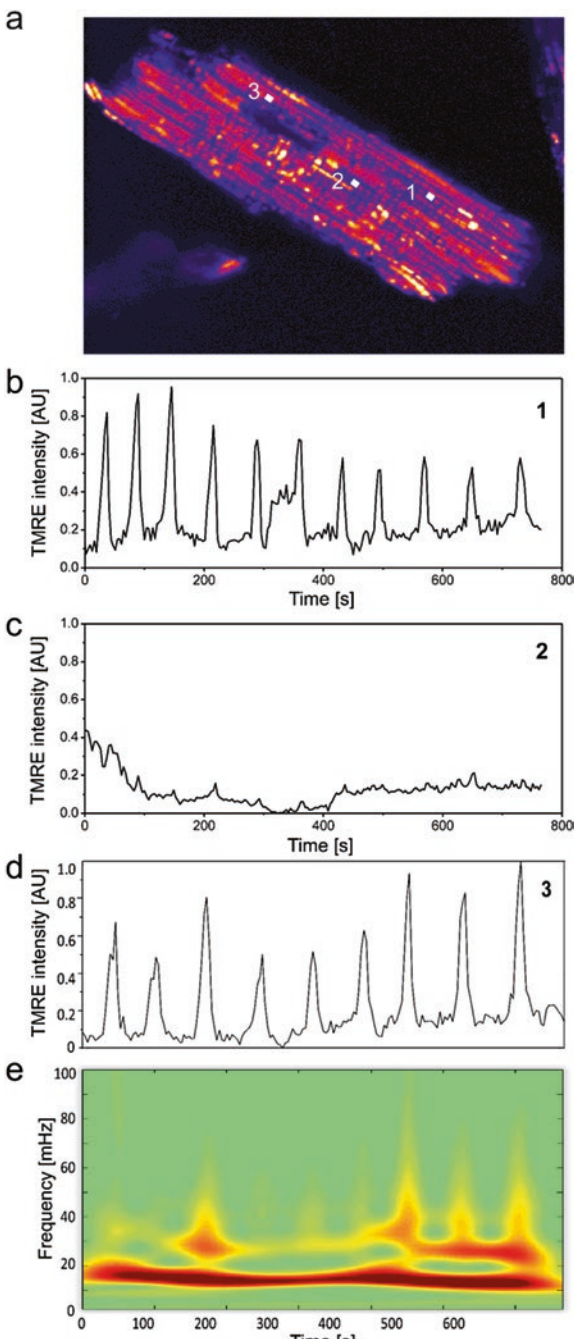

Fig. 1.1 Dynamic mitochondrial oscillations and wavelet transform analysis. (**a**) Densely packed mitochondria in an adult cardiac myocyte, monitored with the fluorescence dye TMRE. (**b**–**d**) Perturbations of the mitochondrial network can result in oscillatory patterns of individual mitochondria that differ in occurrence and frequency content. (**e**) Dynamic frequency content of oscillating mitochondria can be analyzed with the absolute wavelet transform: the major frequency component of mitochondrion 3 (**d**) varies between 15 and 20 mHz (Reprinted with permission from Ref. [81]. Copyright 2014)

ity of mitochondria synchronize to cell-wide $\Delta\Psi_m$ oscillations, some mitochondria do not participate whereas others participate only temporarily (see Fig. 1.1b–d) [9, 43]. This diversified mitochondrial behavior (in frequency and amplitude) is directly associated with the scavenging capacity of intracellular ROS on a local level within the myocyte, as well as the inner membrane efflux of superoxide ions.

A large cluster of mitochondria with synchronized $\Delta\Psi_m$ oscillations can recruit additional mitochondria in analogy to a network of weakly coupled oscillators (see below), whereas some mitochondria may exit the cluster, i.e. show $\Delta\Psi_m$ oscillations that are not synchronized with the cluster, because their local ROS defense mechanisms are exhausted [9, 43]. Thus, this complex interplay of ROS generation and scavenging determines the individual mitochondrial frequency and amplitude.

Since the frequencies of these oscillations change in time, they are best described by methodologies that do not assume an a priori stationarity of $\Delta\Psi_m$ signals, such as wavelet transform analysis. Unlike standard methods of statistical analysis, that can only be applied if the nonstationarity of the signals is associated with the low-frequency part of the observed power spectrum, wavelet analysis allows analyzing the full range of frequencies of the nonstationary mitochondrial network. The wavelet analysis provides a time-resolved picture of the frequency content of each mitochondrial signal, see Fig. 1.1e. Earlier methodologies, e.g. relative dispersion analysis or power spectral analysis, although they provided a glimpse of the functional mitochondrial network properties such as the long-term memory of mitochondrial oscillatory dynamics [7], probed the average behavior of the whole myocyte (single signal trace) [8], without discriminating individual mitochondria. Therefore, application of wavelet analysis on individual mitochondria to characterize the dynamic behavior of the mitochondrial network across the myocyte, was a significant step forward in the systematic analysis of the whole population of mitochondrial oscillators, accounting for both their frequency dynamics and spatio-temporal correlations [9, 43].

Mitochondrial Clusters with Similar Oscillatory Dynamics

It has been shown that mitochondria whose signals are highly correlated during a specific time range, i.e. mitochondria with a similar frequency content, could be grouped into different clusters [9]. Then, the largest cluster of similarly oscillating mitochondria forms a highly correlated and coherent $\Delta\Psi_m$ signal that drives the cell-wide oscillations which are observed during the recording. These major clusters do not necessarily contain mitochondria that demonstrate strong spatial contiguity (albeit the data is showing only a cross section of the network, therefore synchronized clusters that are "non-contiguous" could be connected as a 3D structure through other planes), nor do they have to coincide with local ROS levels as in the case of a "spanning cluster" as proposed in [8]. In fact, the spatial contiguity of major clusters is substrate dependent (such as glucose, pyruvate, lactate and β-hydroxybutyrate) through their influence on the cellular redox potential [35].

The size of mitochondrial clusters is related to the cluster frequency and phase: mitochondria within the cluster adjust their frequencies and phases to a common one, while large clusters comprising many (coupled) mitochondria need a longer time to synchronize [48, 49]. Similarly, clusters with large separation distances among their components or a low contiguity, take longer to synchronize compared to clusters with smaller separation distances among their components. In both cases, the common cluster frequency remains low and is characterized by an inverse relation between common cluster frequency and cluster size [9, 35].

For cardiac myocytes superfused with glucose, pyruvate, lactate or β-hydroxybutyrate, local laser-flashing still leads to synchronized cell-wide oscillations of mitochondrial clusters. However, the network's adaptation to the specific metabolic conditions leads to differences in mitochondrial cluster synchronization and dynamics. These differences can be attributed to the altered cardiac myocyte catabolism and ROS/redox balance [35]. For substrates such as pyruvate and β-hydroxybutyrate that generally lead to strongly reduced redox potential (in comparison with glucose or lactate), the rate of change of cluster size versus averaged cluster frequency is higher than for perfusion with glucose or lactate, see Fig. 4 in [35]; yet, typical cluster frequencies for β -hydroxybutyrate were found in a much smaller range (5–20 mHz) than those for pyruvate (10–70 mHz) [35]. A large range of mitochondrial frequencies is a strong indicator of desynchronization processes that are based on dynamic heterogeneity. Likewise, an increased dispersion in cluster size, as observed for perfusion with pyruvate and β-hydroxybutyrate, is a signature of an increasingly fragmented mitochondrial network with several smaller clusters of similar size that have different frequencies. In contrast, a low mitochondrial frequency dispersion is an indicator of a rapid increase in the formation of mitochondrial network clusters during ROS-induced ROS release, such that an increasingly rapid recruitment of mitochondrial oscillators would result in a decrease of clusters with different frequencies. Since in a pathophysiological state, a slow growth in cluster size after the rapid onset of a synchronized cell-wide cluster oscillation is a sign of exhausted ROS scavenging capacity (reflected by a decrease of $\Delta\Psi_m$ amplitude), quantification of cluster synchronization and contiguity properties provide important information about the myocyte's propensity to death [9]. These findings help provide a better understanding of the altered cellular metabolism observed in diabetic cardiomyopathy or heart failure, where the myocyte's response to the switch in substrate utilization, e.g. through an increased utilization of glucose, can be protective or maladaptive [49–51].

Apart from isolated cardiac myocytes, such a relation could also be observed in mitochondria in intact hearts preparations, where neighboring cardiac myocytes are linked electrically and chemically via gap junctions [35, 52]. In those experiments, mitochondrial populations of several myocytes show cell-wide $\Delta\Psi_m$ signal oscillations following a laser flash, similar to those observed in isolated or pairs of cardiac myocytes [15, 52]. Such behavior could also be observed in rat salivary glands in vivo [53]. Generally, mitochondrial oscillations in intact tissue are faster than in isolated myocytes with an approximate frequency range of 10–80 mHz in cardiac

tissue and 50–200 mHz in salivary glands [35, 53], and their frequency is not as strongly influenced by the size of the cluster as in isolated myocytes [35].

The Mitochondrial Network as a Network of Coupled Oscillators

The intricate spatio-temporal dynamics of mitochondrial networks results from many forms of inter-mitochondrial interactions that involve several metabolic pathways. Such pathways include the modification of gene expression, electrochemical interactions that are mediated by ROS through ROS-induced ROS release, other (e.g. exogenous) forms of control exerted on the local redox environment [54], and morphological changes in neighboring mitochondria [19]. In general terms, signaling or communication between (intra-cellular) organelles modulates the relation between cellular networks that involve the transformation (generation) of mass (energy), as represented by the metabolome, and those networks that carry information as represented by the proteome, genome, and transcriptome. A unique phenotypical signature of a myocyte is then given by the fluxome which represents the network of all cellular reaction-fluxes: it interacts with all other networks and provides a description of the dynamic content of all cellular processes [55]. Emergent properties of the cardiac mitochondrial network, i.e. properties that cannot be derived from the simple sum of behavior of the network's individual components represent a distinguishing trait of biological complexity and provide the opportunity to study the dynamical coordination of functional intra-cellular relations.

Emergent Properties of the Mitochondrial Network: Mitochondrial Criticality

Biological networks exhibit mostly scale-free dynamics and topologies: the structure of the network is represented by several network hubs, i.e. nodes with a large number of links to other nodes, and a large number of nodes with low connectivity [56]. Analysis of emergent properties of these networks, e.g. large-scale responses to external or internal stimuli (perturbations), synchronization, general collective behavior, vulnerability to damage and ability to self-repair, has been the subject of intensive research endeavors [57–59]. In those studies, the scale-free character of the networks is revealed by the presence of network constituents with different frequencies on highly dependent temporal scales [60]. However, as pointed out by Rosenfeld et al. [61], the network's robustness to perturbations and modularity cannot be determined using a reductionist approach from knowledge of all elementary biochemical reactions at play, but instead requires network conceptualizations known from systems biology.

In the cardiac network of mitochondrial oscillators, the synchronized, myocyte-wide, low-frequency, high-amplitude depolarizations of $\Delta\Psi_m$ represent an emerging self-organized spatio-temporal process in response to a perturbation of individual mitochondrial oscillators that are weakly or strongly coupled in the physiological or pathophysiological state, respectively [8, 62–64]. The oscillation period of an individual mitochondrial oscillator is therefore not static but can be modulated by long-term temporal correlations within the mitochondrial network [8, 21]. Such synchronization processes in the form of biochemical rhythms, as observed in many systems of chemically, physically or chemico-physically coupled oscillators, serve the purpose of generating and sustaining spatio-temporal organization through numerous regulatory mechanisms: the resulting dynamically changing oscillations are used by cardiac myocytes to achieve and control internal organization [62–65]. During oxidative or metabolic stress, the mitochondrial network's response can affect not only intracellularly clustered mitochondria, but scale-up to affect the majority of cellular mitochondria and even the whole-organ, thus leading to myocardial malfunction, and ultimately to ventricular arrhythmias [24, 33, 34]. For example, during reperfusion following ischemic injury, cardiac myocytes become oxidatively stressed due to substrate deprivation (e.g., oxygen, glucose), leading to mitochondrial membrane potential collapse at the onset of oscillations [16, 46], transient inexcitability [16] and, eventually, ventricular fibrillation [24, 34, 66]. Experimental measures to prevent these effects include the application of the mBzR antagonist 4′-chlorodiazepam that modulates IMAC activity [34]. The prominent role of IMAC opening in ROS-induced ROS release and $\Delta\Psi_m$ depolarization was further supported by blocking mPTP via cyclosporin A, which had no effect on ensuing post-ischemic arrhythmias [24, 34] (see also below and Fig. 1.2).

As was previously demonstrated [9, 20], synchronization of mitochondrial oscillations can be achieved by a critical amount of spontaneously synchronized oscillators, a concept termed 'mitochondrial criticality' [7, 20]: a number of synchronously oscillating mitochondria recruits more mitochondria to eventually lead the majority of network mitochondria into myocyte-wide synchronized $\Delta\Psi_m$ oscillations. A transition of this critical threshold into a state of collective, synchronized mitochondrial behavior is equivalent to that of a phase transition between states of matter in thermodynamics. However, in the mitochondrial network it describes a transition from a physiological state into a pathophysiological state. Such transitions among large populations of locally connected mitochondria can be described with the percolation theory which takes into consideration the long-range connectivity of (random) systems and employs a probabilistic model to assess the collective dynamics of the inter-connected network of mitochondria [67]. For ideal lattice-like networks, which is a convenient description of the dense, quasi-uniform and spatially arranged cardiac mitochondria, the theoretical percolation threshold can be identified as pc = 0.59; this threshold is close to experimentally obtained values of mitochondrial criticality where a transition into myocyte-wide $\Delta\Psi_m$ oscillations was observed in about 60% of all mitochondria within the mitochondrial network with significantly

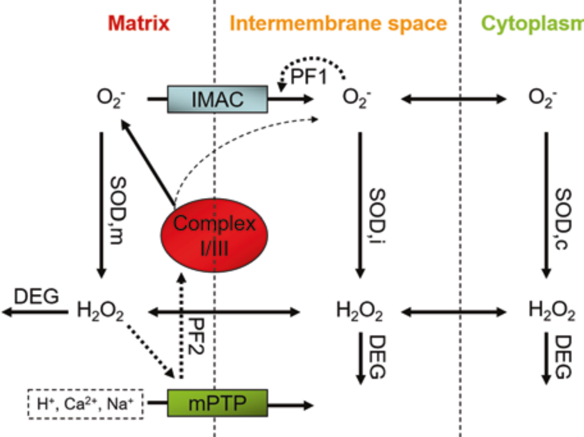

Fig. 1.2 Schematic description of mitochondrial ROS-induced ROS release. The mitochondrial respiration complexes I and III produce superoxide anions (O_2^-) and release them in the mitochondrial matrix. The anions can then reach the intermembrane space (IS) through IMAC, although small quantities directly exit complex III (dashed arrow that originates from complex III). Superoxide dismutases (SOD) in matrix, IS and cytoplasm convert superoxide anions into hydrogen peroxide that can diffuse freely between all compartments. Its degradation is effected by peroxidases (DEG). One positive feedback loop (PF1) is initiated by the activation of IMAC through superoxide anions in the IS to eventually depolarize the mitochondrial inner membrane potential [16, 21]. The resulting stimulation of SODs can, if passing a critical threshold, deactivate IMAC to allow electron transport and repolarization of the membrane potential. Byproducts of hydrogen peroxide-initiated redox processes can trigger mPTP opening (e.g. oxidized lipids or hydroxyl radicals), thus leading to (temporarily) increased production of superoxide anions in the respiratory chain to further open mPTPs in a positive feedback loop (PF2). (This diagram has been adapted from its original version in Yang et al. [71]).

increased ROS levels [20]. For mitochondria in close vicinity to the percolation threshold, small perturbations can thus provoke a transition into global limit cycle oscillations.

Stochasticity and Mitochondrial Network Modeling

Computational models may help obtain a deeper understanding of the intricate interdependencies of mitochondrial networks that lead to emergent behavior. Today, there are several computational models that describe mitochondrial network dynamics, see e.g. [68] for a detailed review.

A mathematical model that has employed ordinary differential equations to represent matrix concentration time rates of ADP, NADH, calcium, Krebs cycle intermediates and $\Delta\Psi_m$ has been recently reported by Cortassa et al. [69]. In this model, the role of IMAC opening in ROS-induced ROS release and ROS diffusion dynamics were incorporated to correctly reproduce mitochondrial respiration, calcium

dynamics, bioenergetics, and the generation of $\Delta\Psi_m$ limit cycle oscillations [21]. The model provided evidence for the existence of synchronized clusters of mitochondria that entrain more mitochondria to participate in synchronous $\Delta\Psi_m$ oscillations [47]. Other models have been developed based on the hypothesis of mPTP-mediated ROS-induced ROS release [18, 70] or a combination of mPTP- and IMAC-mediated ROS-induced ROS release [71] (see also Fig. 1.2 for physiological details). Another probabilistic, agent-based model has considered the switch of superoxide anions to hydrogen peroxide, to model ROS signaling for increasingly distant neighboring mitochondria, thus facilitating the inhibition of ROS-induced ROS release through gluthathione peroxidase 1 in the cytosol [72].

Dynamic tubular reticulum models of mitochondrial networks that involve the modeling of molecular fission and fusion events have been recently proposed [73, 74]. Some use graph-based models of the mitochondrial network with a set of simple rules of network connectivity, see e.g. [75], which predict topological properties of the mitochondrial network. However, this approach does not take into account spatial information about the proximity and positioning of neighboring mitochondria.

Overall, the dynamic response of mitochondrial networks to stimuli is due to the stochastic nature of the underlying processes. These processes can be modeled by the addition of intrinsic noise (that emerges from the stochastic processes at small timescales), into system-defining differential equations [61, 76, 77]. However, although in low-dimensional non-linear systems the introduction of random noise may lead to deterministic chaos [78], in high-dimensional non-linear systems with strong links among their components stochasticity may exhibit synchronized behavior [79], as is the case for cardiac mitochondria.

In this case, criteria for stochasticity of high-dimensional non-linear systems are easily fulfilled for strong links between components [79]. This is especially the case for mitochondria in cardiac myocytes that are morphologically and functionally coupled to reveal complex spatio-temporal dynamics [19, 80, 81], see also below.

Mitochondrial Coupling in a Stochastic Phase Model

Synchronization phenomena in networks of coupled nonlinear oscillators were first studied mathematically a few decades ago by Winfree and Kuramoto [82, 83]. The Kuramoto model assumes a (large) group of identical or nearly identical limit-cycle oscillators that are weakly coupled and whose oscillations are driven by intrinsic natural frequencies which are normally distributed around a mean frequency [84]. It provides an exact solution for the critical strength of coupling that allows a transition into a fully synchronized state [85]. The simplicity of the model and its analytical power have made it a frequently applied model in the context of circadian biology, but also in many other systems that contain biologically, physically or chemically coupled oscillators (reviewed in detail in [84, 85]).

The coupling constant in Kuramoto models is time-independent and identical for every pair of coupled oscillators [84]. In cardiac myocytes, however, the dynamic behavior of mitochondrial oscillating frequencies, as well as the presence of structurally varying areas of mitochondrial density or arrangement within the myocyte suggest a position-dependent and time-varying mitochondrial coupling.

We have recently studied the cardiac inter-mitochondrial coupling by adopting an extended Kuramoto-type model, to determine the time-dependent coupling constants of individual mitochondrial oscillators [35]. In this model, we combined wavelet analysis results with a set of stochastic differential equations that describe drifting frequencies with the intention to develop a minimum-order model that uses only two parameters to characterize inter-mitochondrial coupling: the intrinsic oscillatory frequency and the mitochondrial coupling constant [35]. By mapping and identifying each mitochondrion within the mitochondrial network, the exact nearest neighbors of each mitochondrion were used to describe the spatial variance of mitochondrial coupling, i.e. inter-mitochondrial coupling constants were considered for the local nearest-neighbor environment. The concept of local coupling acknowledges inter-mitochondrial coupling through physical connections, i.e. coupling through diffusion of chemical coupling agents such as ROS and/or forms of inter-mitochondrial communication at inter-mitochondrial junctions [19]. It also emphasizes the role of local ROS scavenging mechanisms that prevent long-range diffusion. Using this approach, we were able to determine the averaged dynamic coupling constant (of local mean field type) of each mitochondrion to all of its nearest neighbors, see Fig. 1.3. Quantitative understanding of local myocardial coupling has important implications in the study of the myocyte's response to metabolic and oxidative challenges during its transition to a pathophysiological state.

Coupling Dynamics in Mitochondrial Frequency Clusters

Inter-mitochondrial coupling within the cardiac myocyte can be studied by comparing the spatio-temporal distribution of inter-mitochondrial coupling constants of mitochondria that belong to the (largest) mitochondrial cluster with similar frequencies, against those of the remaining ("non-cluster") mitochondria. Cellular conditions that promote low coupling constant values for the cluster mitochondria versus the remaining oscillating mitochondria (that may also be organized in clusters), indicate similar structural organization principles [35].

By relating the inter-mitochondrial coupling within a cluster with the coherence (a measure of the similarity of the mitochondrial $\Delta\Psi$m oscillations) of the cluster (Fig. 1.3d), we have shown that larger clusters show stronger cluster coupling constants rendering more coherent cluster oscillations. However, for small cluster areas that occur when a myocyte is superfused with lactate (see also Fig. 4 in [35]), an increase in cluster coherence is not accompanied by an increase in cluster coupling. Such behavior is indicative of a greater influence of spatial rather than temporal coupling on the network of mitochondrial oscillators. During superfusion with

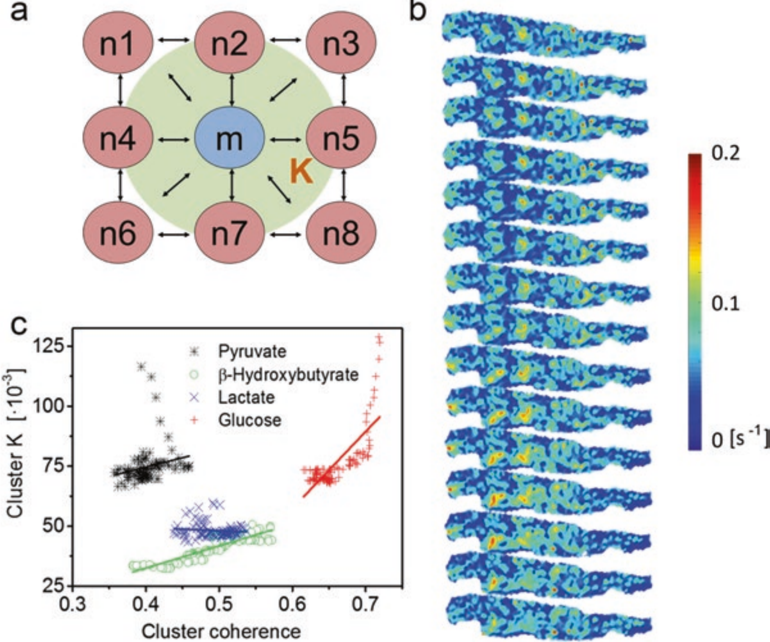

Fig. 1.3 Dynamic inter-mitochondrial coupling and relation to frequency cluster coherence. (**a**) Dynamically changing inter-mitochondrial coupling can be described with a stochastic phase model that provides local coupling constants K of mean-field form (*green cloud*) for each mitochondrion *m* to its nearest neighbors *n*. Inter-mitochondrial coupling is mediated by diffusive coupling agents that interact with the inner mitochondrial membrane, see also Fig. 1.2. (**b**) Maps of coupling constants at different time-points (from *top* to *bottom* with time intervals of 17.5 s): strong coupling is indicated in yellow and red colors, weak coupling in green and blue colors. Clustered nests of highly coupled mitochondria indicate synchronously oscillating mitochondria, whereas regions with low coupling may be associated with a local exhaustion of antioxidant defense systems. (**c**) Coupling constant K versus coherence for mitochondria in a large frequency cluster (details see main text) for cell-superperfusion with different substrates. Generally, cluster coupling increases with coherence, whereby the rate of increase is associated with the size of the cluster (see also Fig. 4 in [35]). Lactate-perfusion is associated with small cluster areas such that cluster coupling is not increasing with cluster coherence. Linear fit curves are shown in their respective substrate color (Reprinted with permission from Ref. [35]. Copyright 2015)

pyruvate an early decrease of coupling strength (Fig. 1.3d) can be attributed to a strong drift towards the common cluster frequency of those mitochondria with the initially largest difference from the common cluster frequency. When cluster coherence levels at this point are low, which is likely is for small clusters (as experimentally verified for pyruvate perfusion (Fig. S5 in [35]), mitochondria might rapidly drop in and out of the cluster. In addition, the distribution of mitochondrial oscillator frequencies is narrowed during their rapid recruitment into the major frequency cluster, leaving fewer clusters with different frequencies. In contrast, high frequency distributions and high rates of change of cluster size compared to the common cluster frequency are a sign of desynchronization through dynamic heterogeneity.

In this system, the observed strong drift of oscillatory frequencies relates to the fundamental concept of synchronization within the mitochondrial oscillator network: its mechanism can be explained by the dynamics of local ROS generation and scavenging mechanisms that involve the mitochondrial matrix as well as extramitochondrial compartments [86]. Then, the slow drift of each mitochondrial oscillator towards the actual mitochondrial frequency is in agreement with the notion of diffusion-mediated signaling, with ROS as the primary coupling agent that is locally restricted and mainly dependent on local ROS density changes.

For small areas of structurally dense, phase-coupled mitochondrial oscillators, local ROS concentration is strongly increased through ROS-induced ROS release, thus leading to the recruitment of neighboring mitochondria. Consequently, these growing clusters lose spatial contiguity and, therefore, show a decrease in their common frequency which, in turn, leads to a decrease of the frequency of local ROS release and to declining local ROS levels, especially along the cluster perimeter. This effect naturally decreases inter-mitochondrial coupling. The formation of spanning clusters, however, provides for a slight increase in the overall basal ROS level to coincidentally increase local inter-mitochondrial coupling. Generally, these observations indicate a preference of spatial coupling over temporal coupling that affect synchronized mitochondrial oscillator networks: early cluster formation mainly drives the correlation between common cluster frequency and local mitochondrial coupling. During this time, the mean cluster coupling constant is large (see Fig. 5 in [15]). Cluster size and local coupling, however, mainly show correlative effects when the cluster of synchronized mitochondrial oscillators involves a majority of the mitochondrial network. At this point, a spanning cluster has already been formed and most mitochondria participate in cell-wide $\Delta\Psi_m$ oscillations.

ROS as primary inter-mitochondrial coupling agents and products of mitochondrial respiration take a central role in cell homeostasis and its response to oxidative or metabolic stress. Under physiological conditions, mitochondria minimize their ROS emission as they maximize their respiratory rate and ATP synthesis according to the redox-optimized ROS balance hypothesis [87]. Stress alters this relationship through increased ROS release and a diminished energetic performance [88]. Thereby, the compartmentation of the redox environment within the cellular compartments such as the cytoplasm and mitochondria, is important in the control of ROS dynamics and their target processes [86]. Within physiological limits, increased mitochondrial metabolism resulting in enhanced ROS generation can function as an adaptive mechanism leading to increased antioxidant capacity and stress resistance, according to the concept of (mito)hormesis [89, 90]. Generally speaking, controlled ROS emission may serve as a robust feedback regulatory mechanism of both ROS generation and scavenging [88]. Dysfunctional mitochondria alter their redox state to elicit pathological oxidative stress, which has been implicated in ageing [91, 92], metabolic disorders like diabetes [93], as well as in cardiovascular [34, 94], neoplastic [95], and neurological diseases [40, 96]. Chronic exposure to increased ROS levels can affect excitation-contraction coupling and maladaptive cardiac remodeling that may lead or increase the propensity to cardiac arrhythmias [34, 97, 98].

Mitochondrial Scale-Free Network Topology and Functional Clustering

Self-Similar Dynamics and Fractal Behavior in Mitochondrial Networks

The defining feature of time series signals with scale-free dynamics is the similarity of frequency-amplitude relations on small, intermediate, and large time scales. In a network of coupled oscillators this translates into new links between oscillators that form in higher numbers depending upon the number of already existing links. In scale-free networks, the occurrence of hubs (i.e., nodes with multiple input and output links) increases the probability of new links being formed [99], an effect known as *preferential attachment* [58] or the "Matthew effect" in the sociological domain [100].

Such fractal or self-similar dynamics develop because of a multiplicative interdependence among these processes and has been observed in cardiac myocytes subjected to oxidative stress [20, 34, 101], in self-organized chemostat yeast culture [62, 102, 103] and in continuous single-layered films of yeast cells exhibiting large-scale synchronized oscillations of NAD(P)H and $\Delta\Psi_m$ [62, 104]. Using wavelet analysis, and as predicted by fractal dynamics, one observes an intricate scale-free frequency content with similar features on different time scales (Fig. 1.4) [60].

For cardiac mitochondrial networks in the physiological regime, it could be demonstrated that the failure or disruption of a single element beyond a critical threshold can result in a failure of the entire network; such scaling towards higher levels of organization in the mitochondrial network obeys an inverse power law behavior that implies an inherent correlation of cellular processes operating at different timescales [8, 58]. The observation of scale-free dynamics in mitochondrial oscillator networks is in agreement with their representation as weakly coupled oscillators in the physiological domain (in the presence of low ROS levels), and their role as energetic, redox and signaling hubs [105].

Functional Connectivity and Mitochondrial Topological Clustering

Dynamic changes in the network's topology during synchronized myocyte-wide oscillations in the pathophysiological state reveal important information about functional relations between distant mitochondria displaying similar signal dynamics. These relations cannot be explained alone by local coupling through diffusion or structural connections. As in networks of communicating neurons, the spontaneous occurrence of changes in the clusters' oscillatory patterns observed at markedly different locations, indicates strong and robust functional correlations; interestingly,

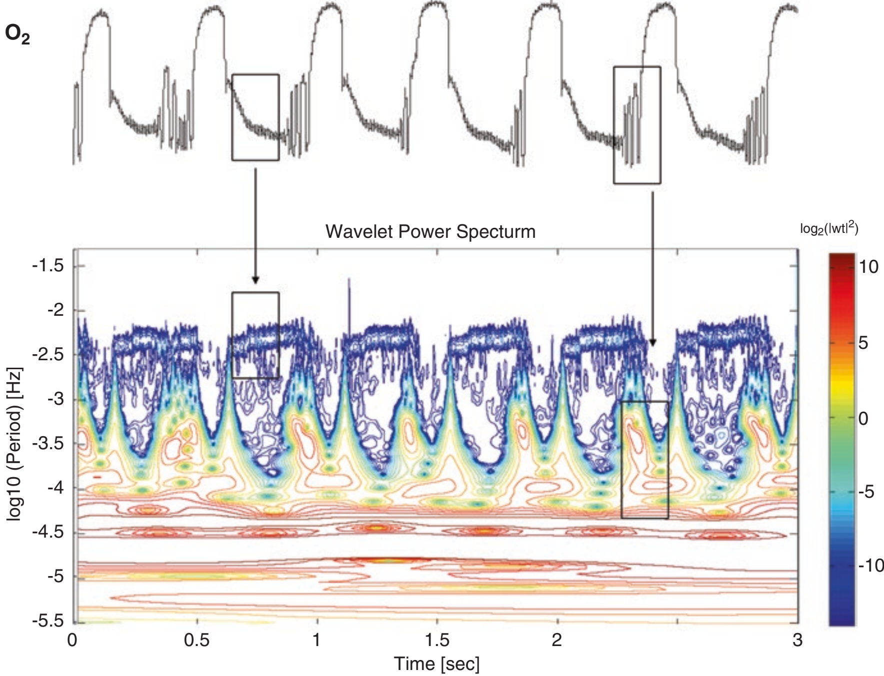

Fig. 1.4 Wavelet analysis of the O_2 signal time series obtained from the self-organized multioscillatory continuous culture of S. cerevisiae. (**a**) Relative membrane inlet mass spectrometric signal of O_2 versus time for S. cerevisiae. The time scale is not shown, but corresponds to hours after the start of continuous fermentor operation. Large-amplitude oscillations show substantial cycle-to-cycle variability with 13.6 ± 1.3 h (N = 8). Other evident oscillation periods are ~40 min and ~4 min. This figure was provided courtesy of Prof. David Lloyd, affiliated with Cardiff University, United Kingdom. (**b**) Logarithmic absolute squared wavelet transform over logarithmic frequency and time. At any time, the wavelet transform uncovers the predominant frequencies and reveals a complex and fine dynamic structure that is associated with mitochondrial fractal dynamics. *Boxes* and *arrows* show the correspondence between the time series and the wavelet plot

the time-scales of these changes are similar in mitochondrial and neuronal networks [106, 107].

Mitochondrial network functionality can be assessed by measuring the *functional connectedness* between mitochondrial network nodes, i.e. by obtaining a quantitative measure of the functional relations that span the dynamically changing mitochondrial network topology [108].

Such a measure of mitochondrial functional connectedness can be the clustering coefficient, similar to that employed by Eguiluz et al. for scale-free functional brain networks [109]. In that case, for a specific network constituent, the clustering coefficient provides a measure of connectedness between neighbors [110]. For instance, clustering plays an important role in neuronal phase synchronization [111]. In the case of mitochondrial networks, dynamic functional clustering significantly differs from random Erdös-Rényi networks [112], even in the presence of similar number

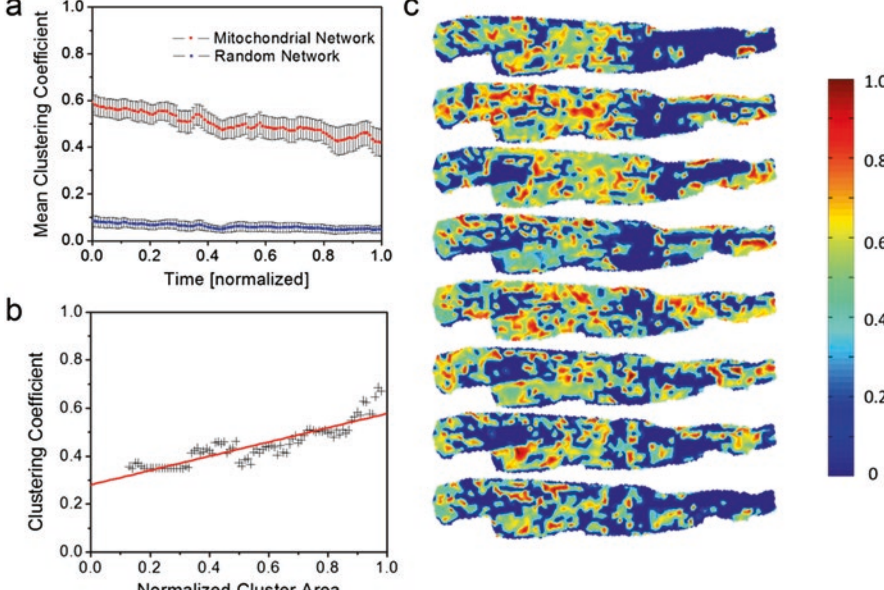

Fig. 1.5 Dynamic functional clustering in mitochondrial networks. (**a**) Comparison of dynamic functional clustering for mitochondrial networks (*red line*) and random networks (*blue line*) with the same number of links on the same set of vertices. Mitochondrial networks are scale-free networks whose functional organization is significantly different from random networks. (**b**) Functional clustering for a large cluster of similarly oscillating mitochondria versus the cluster's area. Functional clustering is increasing with the number of synchronously oscillating mitochondria. (**c**) Consecutive maps of distributions of functional clustering coefficients in a cardiac myocyte. Some regions of the cell maintain a high functional clustering during the recording (*middle left* area of the cell) whereas other regions only temporarily display functional clustering (*far right area* of the cell) (**a** and **b** reprinted with permission from Ref. [81] . Copyright 2014)

of links [81] (Fig. 1.5). This result supports the concept that mitochondrial networks exhibit structural-functional unity [113]. Therefore, dynamic mitochondrial clustering is consistent with its functional properties that are associated with mitochondrial positioning within the network, as previously described [30, 31]. In Fig. 1.5c, the dynamic nature of topological clustering is shown for a glucose-perfused cardiac myocyte during synchronized membrane potential oscillations. While some regions on the left part of the myocyte maintain high clustering, mitochondria on the right side show higher clustering only during the middle portion of the recording, as the spatio-temporal topology evolves.

To further investigate the mitochondrial network dynamics, future studies will need to identify and quantify functional ensembles of strongly interconnected mitochondria [80, 114]. These studies may shed new light into populations of coexistent subsarcolemmal and intermyofibrillar mitochondria that exhibit different roles in the myocyte (patho)physiology [28, 29, 115].

Concluding Remarks

Biological networks within one cell, organ or organism are not isolated entities but exhibit common nodes. Introducing higher-dimensional node degrees generates multi-layer, interdependent, networks. So far, our understanding of such networks is still in its infancy (see [116, 117] for detailed reviews). For example, within the cardiac mitochondrial network, at least two layers can be distinguished, corresponding to structural connections via inter-mitochondrial junctions, and communication through mechanisms that control local ROS balance [19, 35, 80, 118].

Stressful events can trigger synchronized mitochondrial oscillations in which mitochondria functionally self-recruit in frequency clusters [9, 16, 35, 81]. In functional terms, mitochondria can be seen as alternating between states of excitability and quiescence modulated by ROS signaling, thus modulating myocyte's function and energy metabolism [119]. In cardiac myocytes under critical situations, e.g. at myocyte-wide ROS accumulation close to the percolation threshold, inter-mitochondrial functional, structural and dynamic coupling can lead to electrical and contractile dysfunction, and ultimately to myocyte death and whole heart dysfunction. Eventually, under criticality conditions, perturbation of a single mitochondrion could propagate to the remaining mitochondria, leading to myocyte death.

A mitochondrion's gatekeeper role, at the brink of cardiac myocyte death, can only be understood when its function in the mitochondrial network can be predicted either experimentally or through quantitative modeling in which case the mitochondrial parameters are inferred from the available experimental data. Although an increase in computational efficiency has helped modeling make impressive progress in recent years [68], a complete description of the mitochondrial network is still evolving. Stochastic models have been well suited to provide system-wide mitochondrial parameters, such as dynamic coupling constants and functional clustering coefficients in large networks of coupled mitochondrial oscillators.

Synergistic, experimental and computational investigations have unveiled emergent properties of the complex mitochondrial oscillatory networks that describe cluster partitioning of synchronized oscillators with different oscillatory frequencies [120, 121], as well as the chimeric co-existence of synchronized and non-synchronized oscillators [122, 123]. These results coincide with our observations of mitochondrial oscillations in cardiac myocytes, thus supporting the view of network-coupled mitochondrial oscillators. The computational approach promises further discernment of the dynamic spatio-temporal relations within biological networks, while aiding our understanding of its functional implications for cell survival. Therefore, the quantitative description of inter-mitochondrial coupling and functional connectedness provides parameters whose dynamic behavior under pathological conditions may help evaluate the impact of, e.g., metabolic disorders, ischemia-reperfusion injury, heart failure, on mitochondrial networks in cardiac, cancer and neurodegenerative diseases.

To further investigate the mitochondrial network dynamics, future studies will need to identify and quantitate functional ensembles of strongly interconnected

mitochondria [80, 114]. These studies may shed new light into populations of coexistent subsarcolemmal and intermyofibrillar mitochondria that exhibit different functional outputs [28, 29, 115].

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Chapter 2
Mitochondrial Calcium Handling in Physiology and Disease

Veronica Granatiero, Diego De Stefani, and Rosario Rizzuto

Calcium as Important Second Messenger

In all multicellular organisms, a precise and efficient cell-to-cell communication is required for coordinating the vast majority of physiological processes. Hundreds of diverse molecules (e.g. ions, metabolites, peptides and proteins) can carry information from one cell to another, thus acting as signaling entities that interact with specific receptors generally located on the plasma membrane. Despite this huge complexity, only a small number of intracellular transduction pathways (e.g. Ca^{2+}, cAMP or phosphorylation cascades) can ensure the proper decoding of all these diverse signals. One of the most important intracellular messengers is Ca^{2+}. Indeed, between cytosol and the extracellular environment there are both chemical and electrical gradients that represent the main driving force for changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]$). Cells invest a substantial quota of their energy to guarantee this electrochemical gradient to allow rapid and transient changes in $[Ca^{2+}]$ that can largely differ through space and time [1], thus generating a plethora of signaling patterns that can be differentially decoded. Ca^{2+}-dependent signal transduction requires the binding to buffering proteins, the compartmentalization into intracellular compartments and the extrusion outside the cells [2]. Ca^{2+} binding triggers changes in protein shape and charge and consequently activates or inhibits protein functions. The best known Ca^{2+}-binding protein is calmodulin that, in concert with other Ca^{2+} buffers and intracellular compartments, controls the spatiotemporal patterning of Ca^{2+} signals [3]. These dynamic changes of $[Ca^{2+}]$ thus trigger a number of cellular events, including muscle contraction, hormone secretion, synaptic

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transmission, cellular proliferation, apoptosis and others [4–7]. The universality and versatility of Ca^{2+} signaling is thus guaranteed by a sophisticated machinery of ion channels, pumps, and exchangers that drive the flux of Ca^{2+} ions across the plasma membrane and across the membrane of intracellular organelles, such as the Endoplasmic Reticulum (ER) and mitochondria (see Chap. 10). During last decades, an increasing number of researchers focused their efforts in the identification and characterization of all the import/export mechanisms of Ca^{2+} homeostasis, but the whole scenario is still not complete. Ca^{2+} signaling can be activated through two main mechanisms. The first is the Ca^{2+} entry from the plasma membrane channels, which can be classified into four groups, Store Operated Ca^{2+} Channels (SOCs), which open following intracellular store depletion [8], Receptor Operated Ca^{2+} Channels (ROCs), which open after an external ligand binds the receptor [9], Voltage Operated Ca^{2+} Channel (VOCs), which are sensitive to decreases of membrane potential and Second Messenger Operated Ca^{2+} Channels (SMOCs) which open after binding of second messenger on the inner side of the membrane [10, 11]. The second mechanism implies Ca^{2+} release from intracellular depots. The most important store is the ER, but recent works demonstrated that also other organelles, such as Golgi apparatus, endosome and lysosome are able to participate in Ca^{2+} signaling [12, 13]. In this scenario, the best described pathway involves the release of IP_3 after activation of a G-protein coupled receptor, and the consequent Ca^{2+} release from the ER through the binding to the IP_3R. Other intracellular Ca^{2+}-releasing channels are the Ryanodine Receptor (RyR) [14] or the Two-pore channels (TPCs) activated by NAADP [15]. Termination of Ca^{2+} signals is finally completed through Ca^{2+} extrusion from the cytoplasm by various pumps and exchangers, e.g. SERCA (sarco-endoplasmic reticulum ATPase) or PMCA [16].

This chapter is primarily focused on the role of mitochondria in cellular Ca^{2+} handling. Indeed, many subcellular compartments, including mitochondria, are key regulators of Ca^{2+} signaling, by actively participating to shape global Ca^{2+} waves and determining specific cellular functions.

Biophysical and Structural Basis of Mitochondrial Ca^{2+} Handling

The relationship between mitochondria and Ca^{2+} dates back to more than 50 years ago, when Engstrom's group, in the 1961, demonstrated for the first time that energized mitochondria, when exposed to Ca^{2+} pulses, were able to rapidly and efficiently take up large quantity of this cation [17]. These organelles are characterized by a distinctive structure. They are double membrane-bounded organelles thought to be derived from an proteobacterium-like ancestor, presumably due to a single ancient invasion occurred more than 1.5 billion years ago [18, 19]. The basic evidence of this endosymbiont theory is the existence of the mitochondrial DNA (mtDNA), with structural and functional analogies to bacterial genomes. Mitochondria are defined by two structurally and functionally different membranes,

the outer membrane (OMM) and the inner membrane (IMM), characterized by invaginations called "cristae", which enclose the mitochondrial matrix. The space between these two structures is traditionally called intermembrane space (IMS). Cristae are not random folds, but rather internal compartments formed by profound invaginations, originating from very tiny "point-like structures" in the inner membrane [20]. These narrow tubular structures, called cristae junctions, can limit the diffusion of molecules from the intra-cristae space towards the IMS, thus creating a micro-environment where respiratory chain complexes, and also other proteins, are hosted and protected from random diffusion. The OMM contains high amount of a specific transport protein, VDAC (Voltage-Dependent Anion Channel), which is able to form pores on the membrane, becoming permeable to ions and metabolites up to 5 kDa [21]. Conversely, the IMM is a highly selective membrane that tightly controls the exchange of ions and metabolites through specialized molecular machineries. The chemiosmotic theory of energy transfer was first proposed by Peter Mitchell [22], who suggested that the electrochemical gradient across the IMM is utilized by the F_1/F_0 ATPase to convert the energy of NADH and $FADH_2$, generated by the breaking down of energy rich molecules, such as glucose or lipids, into ATP. This gradient ($\Delta\mu$) is composed by both electrical ($\Delta\Psi$) and chemical components (ΔpH). This potential represents a huge driving force that allows the accumulation of cations into the matrix, and is normally estimated to be approximately −180 mV [23]. Another key feature of mitochondria is their spatial organization in the cells. They are not solitary organelles, but they make contacts with several other structures, especially with the ER. Indeed, the physical and functional coupling of these two organelles in living cells was originally found to determine the Ca^{2+} and lipid transfer between the two organelles. Several works indeed underlined the presence of overlapping regions of two organelles and allowed to estimate the area of the contact sites as 5–20% of total mitochondrial surface [24, 25]. More recently, electron tomography techniques allowed to estimate an even smaller distance (10–25 nm), as well as the presence of trypsin-sensitive tethers between the two membranes [26].

In mammals, many proteins have been identified to be indirectly involved in the regulation of ER–mitochondria functional interaction, such as some chaperones, PACS-2, BAP31 and NOGO-A. Searching for the long-sought direct tether, de Brito and Scorrano and co-workers have recently identified MFN2 as important mammalian protein directly bridging the two organelles. MFN2 is enriched in the ER–mitochondria interface and connects ER with mitochondria via direct interactions between the protein localized in the ER and MFN1 or MFN2 present in the OMM. They also showed that genetic ablation of MFN2 causes an increase in the distance between the two organelles with a consequent impairment of mitochondrial Ca^{2+} uptake [27]. The mechanistic role of MFN2 in tethering the two organelles was confirmed in different systems [28–30], but this notion was also recently challenged [31, 32]. Nevertheless, there is a broad agreement in considering ER/mitochondria contact sites as critical determinant of the rapid transport of Ca^{2+} into the matrix. Indeed, some mitochondria are strategically positioned near the ER Ca^{2+} releasing sites, and are thus exposed to a microdomain where the local [Ca^{2+}] is

significantly higher compared to the bulk cytosol [24, 25, 33–37]. In addition, a specific direct coupling between the IP_3R and Ca^{2+} channels of the OMM has been described [38], thus further underlining the pivotal role that organelle tethering play in cellular Ca^{2+} homeostasis.

Mitochondrial Ca^{2+} Homeostasis

Mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_{mt}$) is the result of the dynamic equilibrium between two continuous and opposite processes, i.e. mitochondrial Ca^{2+} influx vs efflux. The basic functional characteristics of mitochondrial Ca^{2+} uptake and release have been firmly established in the 1960s and 1970s. Ca^{2+} influx requires an intact mitochondrial membrane potential (negative inside) and a so called electrogenic Ca^{2+} uniporter (i.e. resulting in the net transport of two positive charges the matrix) [17, 39, 40]. Conversely, the efflux from the matrix depends on two distinct mechanisms, (i) an ubiquitous H^+/Ca^{2+} exchange (likely to be electroneutral) [41] and (ii) a Na^+/Ca^{2+} exchange (likely to be electrogenic, with 3 or 4 Na^+ ions per Ca^{2+}) [42, 43].

Mitochondrial Ca^{2+} Uptake

3.1.1.The Mitochondrial Ca^{2+} Uniporter (MCU)

In the last 50 years, several efforts have been spent to identify the channel responsible for ruthenium red (RuR)-sensitive mitochondrial Ca^{2+} entry. However, only in the last 10 years significant successes have been accomplished. The first fundamental step was indeed obtained in Clapham's lab in the 2004, when the Ca^{2+} channel of the inner mitochondrial membrane was finally directly measured. In this work, single mitoplasts (i.e. mitochondria lacking the outer mitochondrial membrane) were isolated from COS7 cells and patch clamp was performed. Accordingly, it was unambiguously shown that organelle Ca^{2+} entry was mediated by a Mitochondrial Ca^{2+} Uniporter (MCU), here also named MiCa. This channel is inward rectifying, highly Ca^{2+}-selective, sensitive to RuR and characterized by an enormous Ca^{2+} carrying capacity (half-saturation at 19 mM) with no Ca^{2+}-dependent inactivation [44]. Nonetheless, despite the detailed electrophysiological characterization of MCU, its molecular identity remained a mystery. The first step in the right direction came in 2010, when the group of Vamsi Mootha identified a mitochondrial protein, named MICU1, specifically affecting mitochondrial Ca^{2+} uptake [45]. However, it was immediately evident that MICU1 was a regulator rather than the channel per se. Only 1 year later, our group and Mootha's lab independently identified another previously uncharacterized mitochondrial protein acting as the bona fide mitochondrial Ca^{2+} uniporter [46, 47]. The MCU gene (previously known as CCDC109A) is

broadly conserved among metazoans and plants, but absent in yeasts [48, 49], and encodes for a 40 kDa protein (running at 35 kDa due to the cleavage of the organelle localization peptide) with two coiled-coil domains and two transmembrane domains separated by a short loop (EYSWDIMEP). The loop is enriched in acidic residues and exposed to the intermembrane space, while the vast majority of the protein is located inside the matrix [50]. Residues E256, D260 and E263 are critical for Ca^{2+} channeling function, since their replacement with both uncharged and positively charged amino acids result in the loss of Ca^{2+} permeation [47, 51]. Moreover, substitution of Ser^{258} leads to decreased ruthenium red sensitivity [46, 52]. The recent elucidation of the 3D structure revealed that the functional channel is a homopentamer stabilized by the coiled-coil motif protruding into the mitochondrial matrix [53]. However, it is now clear that MCU is the key component of a higher order macromolecular complex, named the MCU complex, that represents the whole molecular machinery mediating the electrophoretic transport of Ca^{2+} cations inside the organelle matrix [54, 55]. MCU alone is necessary and sufficient (at least *in vitro*) for mitochondrial Ca^{2+} uptake, thus indicating that it represents the pore forming subunit [47], but what happens in vivo is still under intense investigation.

The MCU Complex

The first indication that MCU is part of a bigger complex was revealed even before the discovery of the channel itself, with the identification of MICU1 [45]. In the last 5 years, a growing amount of proteins have been shown to interact and modify MCU activity. At the moment, the general consensus is that the MCU complex can be composed by three different membrane components, MCU [46, 47], its isoform MCUb [51] and the recently identified EMRE (essential MCU regulator, previously known as C22orf32) [56], and some associated regulators (see later). The MCUb protein has a high similarity in sequence with MCU, but at least a couple of amino acid substitutions in the loop domain are predicted to be critical for ion permeation and suggest that this protein has a lower efficiency in Ca^{2+} transport. Accordingly, MCUb overexpression drastically reduces the mitochondrial Ca^{2+} uptake, while its silencing exerts the opposite effect, thus indicating that MCUb is the endogenous dominant-negative isoform of MCU [51]. Intriguingly, the MCU/MCUb expression ratio widely varies among tissues, suggesting that this mechanism could account at least in part for the differences in MCU activity displayed by different tissues [57]. On the other hand, EMRE is a small protein of about 10 kDa, with a predicted mitochondrial targeted sequence containing a single transmembrane domain and a highly conserved C-terminus rich in aspartate residues. This protein was identified in the 2013 using a quantitative mass spectrometry approach in cell culture (SILAC) [56]. As to its function, EMRE appears to be required for Ca^{2+} channeling activity in vivo and to mediate the binding of MICU1 to the channel. In the contrast to its essential role, EMRE homologs are not present in plants, fungi or protozoa in with MCU and MICU1 are highly conserved. However, knockout of EMRE is able to abrogate the mitochondrial Ca^{2+} uptake and that the overexpression of MCU in

these conditions was not able per se to restore the normal mitochondrial Ca^{2+} uptake. More recently, Foskett and colleagues proposed that the C-terminal region of EMRE acts as Ca^{2+} sensor and ensures the inhibition of uniporter activity under high matrix [Ca^{2+}] [58]. However, the membrane topology of EMRE is still debated and additional studies will be necessary to carefully dissect its contribution to the whole MCU complex. In addition to these core membrane components, the MCU complex also includes soluble components localized in the intermembrane space that accounts for the so-called sigmoidicity of the mitochondrial Ca^{2+} uptake system.

The Sigmoidicity of Mitochondrial Ca^{2+} Uptake

One well established feature of organelle Ca^{2+} handling is the sigmoidal relationship between extramitochondrial [Ca^{2+}] and Ca^{2+} uptake into the matrix. In resting cells, [Ca^{2+}] in the cytosol ([Ca^{2+}]$_{cyt}$) is approximately 100 nM, thanks to the activity of Ca^{2+}-ATPases (e.g. PMCA, SERCA, SPCA). In this condition, MCU activity is very low even in front of the huge driving force represented by the mitochondrial membrane potential, in order to prevent vicious cycling of the cation and consequent energy drain. As soon as Ca^{2+}-signaling is activated, [Ca^{2+}]$_{cyt}$ rises and MCU gets activated and leads to a very large cation accumulation inside the matrix, thus ensuring rapid mitochondrial Ca^{2+} uptake. This behavior requires both a gatekeeper (i.e. a MCU inhibitor working at resting [Ca^{2+}]$_{cyt}$) as well as cooperative activator (i.e. a MCU enhancer that becomes active after Ca^{2+}-signaling activation). Importantly, these features are not part of the MCU channel per se but they are rather provided by the MICU protein family, that includes three distinct members (MICU1, MICU2 and MICU3). Originally, MICU1 was shown to possess EF-hand domains and be associated with the inner mitochondrial membrane. Its silencing compromises mitochondrial Ca^{2+} uptake in intact cells and isolated organelle, without affecting mitochondrial membrane potential or respiration [45]. Few years later, Madesh and coworkers demonstrated that MICU1 inhibits channel function in unstimulated cells, thus proposing MICU1 as the essential gatekeeper of MCU-mediated mitochondrial Ca^{2+} uptake. Indeed, MICU1 silencing causes an increase of mitochondrial Ca^{2+} levels in basal conditions, sensitizing the cells to excessive ROS production and cell death [59]. In parallel, Hajnoczky's group confirmed the role of MICU1 as MCU gatekeeper, and further demonstrated that MICU1 also act as a cooperative activator of MCU activity at high cytosolic [Ca^{2+}] [60]. Finally, we recently added another level of complexity to this picture. Indeed, we found that MICU1 forms dimers with its isoform MICU2 through a disulfide bridge. Thus, according to our hypothesis, MICU1 and MICU2 represents a unique entity, widely expressed in all tissues, and represent the molecular machinery underlying the sigmoidicity of mitochondrial Ca^{2+} uptake. In this model, MICU1 acts predominantly as an MCU activator, although it can still retain some residual gatekeeping function in some situation. Conversely, MICU2 act as a genuine MCU inhibitor, thus guaranteeing the normal physiological activation threshold. Accordingly, at resting [Ca^{2+}], the prevailing

inhibitory effect of MICU2 ensures minimal Ca^{2+} accumulation in the presence of a very large driving force for cation accumulation, thus preventing the deleterious effects of Ca^{2+} cycling and matrix overload. As soon as extramitochondrial [Ca^{2+}] increases, Ca^{2+}-dependent inactivation of MICU2 and the concomitant activation of MICU1 guarantee the prompt initiation of rapid mitochondrial Ca^{2+} accumulation, thus stimulating aerobic metabolism and increasing ATP production [61].

Finally, other MCU regulators have been proposed, including MCUR1 (also known as CCDC90A) and SLC25A23 (also known as SCaMC-3). They are both are membrane proteins shown to interact with MCU and modulate its activity [62, 63]. However, these two protein are also involved in other processes, such as the assembly of complex IV [64] or ATP/Pi exchange [65–67] respectively. This opens the possibility that the functional effect of these proteins on mitochondrial Ca^{2+} homeostasis could be an indirect secondary effect, as better discussed elsewhere [68].

Mitochondrial Ca^{2+} Efflux

As to the Ca^{2+}-release pathways from mitochondria, their functional existence has been characterized already in the 1970s [41, 43]. Ca^{2+} efflux from mitochondria depends on two different mechanisms, one mediated by a $2H^+/Ca^{2+}$ antiporter (mHCX) and expressed in most of the cells and the other by a 3 (or 4) Na^+/Ca^{2+} exchanger (mNCX), predominant in excitable tissues. The molecular identity of the $2H^+/Ca^{2+}$ exchanger is still a matter of debate, but the recent works by Clapham and coworkers suggests that Letm1 is a strong candidate [69, 70], although this notion is not yet widely accepted [71, 72]. Conversely, a universal consensus exists on the identity of the mitochondrial Na^+/Ca^{2+} exchanger. Indeed, in 2010 Sekler and coworkers demonstrated that the gene NCLX (also known as NCKX6, SLC24A6 or SLC8B1) encodes for a mitochondrial protein necessary for Na^+-dependent Ca^{2+} clearance from the matrix [73]. Interestingly, many years before, an unidentified mitochondrial protein, but similar in molecular weight to NCLX, was reported to exhibit Na^+/Ca^{2+} exchange activity when purified and reconstituted in vitro [74, 75]. The NCLX protein shares only low sequence homology (approximately 20%) to Na^+/Ca^{2+} exchangers of the plasma membrane and the exact stoichiometry of Na^+/Ca^{2+} exchange is still unknown. Based on thermodynamic considerations, it should catalyze the exchange of 3–4 Na^+ every Ca^{2+}, thus causing the net import of one or two positive charges into the matrix. In line with this, molecular dynamics simulation based on the crystal structure of a bacterial Na^+/Ca^{2+} exchanger [76] suggests a 3:1 stoichiometry [77], that should apply also to mNCX. Up to now, the contribution of NCLX to mitochondrial Ca^{2+} extrusion has been confirmed in a variety of cellular models, including B lymphocytes, pancreatic beta cells, cardiomyocyte-like cell lines and astrocytes. Unfortunately, animal models lacking the NCLX gene are still missing at this moment, but in the future they will provide essential information on the physiopathological role of mitochondrial Ca^{2+} homeostasis.

The Pathophysiological Role of Mitochondrial Ca^{2+} Signaling

The molecular identification of the components of the MCU complex finally opened the possibility to address the physiological role of mitochondrial Ca^{2+} uptake by genetic approaches, thus overcoming the limits of classical pharmacological approaches, in particular considering the lack (or, at least, the poor performance) of MCU-targeting drugs suitable for in vivo applications. However, in the last three decades a huge amount of experimental work has been carried out using either pharmacological or indirect genetic tools to target mitochondrial Ca^{2+} homeostasis and dissect its patho-physiological role. According to these studies, the general consensus is that Ca^{2+} inside mitochondria acts a pleiotropic signal, with different cellular outcomes that depend on the investigated cell type, the metabolic state and the concomitant presence of other stress signals. On one hand, Ca^{2+} plays a regulatory role within the organelle itself ranging from the regulation of ATP production to the release of caspases cofactor with consequent cell death. On the other hand, mitochondrial Ca^{2+} can exert its function at the whole cell level, e.g. by regulating cation homeostasis both locally and globally [78]. However, in all the studies that preceded the molecular identification of MCU, the experimental approaches used to address the physiological relevance of mitochondrial Ca^{2+} homeostasis was (i) the use of drugs interfering, more or less specifically, with the Ca^{2+} uptake (e.g. uncouplers, ruthenium red, Ru360) or release (e.g. CGP37157) processes and (ii) genetic manipulations indirectly modifying the amount of Ca^{2+} that mitochondria can accumulate (e.g. regulation of Bcl2 family proteins or MAM-localized proteins). However, none of these approaches is specific, since they either modify other organelle functions (e.g. ATP synthesis), or affect extramitochondrial processes (e.g. Ca^{2+}-depletion of intracellular stores, IP_3R activity).

Even the data obtained with the most specific MCU inhibitors, i.e. ruthenium red and Ru360, should be interpreted with caution. Indeed, it has been shown that in several cellular models, the functional effects of these two compounds are not dependent on their inhibition of MCU [79], in line with the predicted poor membrane solubility of these drugs. Fortunately, the recent identification and elucidation of the MCU complex, although not yet definitive, has opened the field to genetic loss- or gain-of function approaches to unambiguously determine the role of mitochondrial Ca^{2+} uptake in organism pathophysiology. Due to the intrinsic novelty of these discoveries, the picture is still largely incomplete, and apparently contrasting data are being published. While the studies carried out in cultured cells concern both the overexpression and downregulation of all the proteins in the complex, in vivo models (at the moment this chapter was written) are available only for MCU KO and overexpression. Here, we will critically synthetize the main findings on the role of mitochondrial Ca^{2+} transport in both physiological and pathological conditions.

Regulation of Cellular Bioenergetics and Autophagy

In the last 5 years, the genetic manipulation of MCU complex components has been carried out in several cultured cells. Overall, the relationship between mitochondrial Ca^{2+} uptake and the regulation of energy production has been confirmed in most of them. For example, in pancreatic β-cells, glucose-induced insulin release is dependent on the stimulation of mitochondrial oxidative metabolism, and it involves both K_{ATP}-dependent [80] and K_{ATP}-independent [81] regulation of exocytosis. Stimulation of mitochondrial dehydrogenases by organelle matrix [Ca^{2+}] is a critical step critical in this process [82]. Indeed, MCU silencing impairs the Ca^{2+}-dependent phase of glucose-induced ATP increase [83, 84], and hence insulin secretion [85, 86]. In line with this, NCLX silencing temporally enhances the glucose-induced increase in oxidative phosphorylation [87, 88] and cellular ATP levels [84]. Interestingly, also silencing of MICU1 (that increases basal $[Ca^{2+}]_{mt}$ but reduces agonists-induced transients) has a similar effect in this cellular model [85], thus suggesting that tonic rises in $[Ca^{2+}]_{mt}$, and not constitutively high resting $[Ca^{2+}]_{mt}$, are decoded and translated into a boost of ATP production. This is further supported by the observation that, at least in HeLa cells, silencing of MICU1 do not modify basal respiration levels, but it rather dampens agonists-induced increase in oxygen consumption [59]. Overall, these data demonstrated that mitochondrial Ca^{2+} accumulation, mediated by MCU and modulated by NCLX, is thus required for normal glucose sensing by pancreatic β-cells. Moreover, direct coupling of mitochondrial Ca^{2+} transients and oxidative metabolism has also been confirmed in other cellular model (as well as in vivo, see below). Overexpression of MCU in human fibroblasts derived from both healthy subjects and patients affected by mitochondrial disorders can efficiently decrease AMPK phosphorylation levels [89], i.e. the major cellular metabolic sensor of AMP/ATP ratio. Similarly, MCU silencing enhances starvation-induced increase of phospho-AMPK levels in MDA-MB231, a breast cancer derived human cell line [90].

Autophagy is a broadly conserved adaptive response to energetic defects [91], as well as the leading mechanism for eliminating damaged organelles [92, 93]. Recently, a seminal work by Foskett and colleagues clearly demonstrated that mitochondrial Ca^{2+} signaling has a fundamental role in the regulation of autophagy [94]. In addition, the removal of damaged or non-functional mitochondria is regulated by a specific autophagic process named "mitophagy", that acts as a critical organelle quality control pathway [95]. In this context, mitochondrial Ca^{2+} has been recently shown to be a potential specific signal in the control of mitophagy. Indeed, downregulation of ER to mitochondrial Ca^{2+} transfer can effectively decrease parkin-mediated organelle removal [96]. In addition, we also demonstrated a causal link between mitochondrial Ca^{2+} signaling and autophagy/mitophagy in a cellular model of mitochondrial disease [89]. Mitochondrial disorders are indeed a wide range of clinical syndromes caused by inherited mutations in the mitochondrial DNA (mtDNA). Given the complexity of mitochondrial genetics and biochemistry, the

clinical manifestations of mitochondrial disorders are extremely heterogeneous. They range from lesions of single tissues or structures, such as the optic nerve in Leber's hereditary optic neuropathy (LHON), to more widespread lesions including myopathies, encephalomyopathies, cardiomyopathies, or complex multisystem syndromes with onset ranging from neonatal to adult life, such as MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes [97, 98]. In this pathological context, the direct correlation between mitochondrial Ca^{2+} and autophagy is supported by the demonstration that genetically or pharmacological approaches that enhance mitochondrial Ca^{2+} uptake can efficiently slow down the hyperactivated autophagic flux, through an AMPK-dependent mechanism [89]. Overall, the emerging picture consistently shows that the regulation of the autophagy is dependent on the modulation of oxidative metabolism by $[Ca^{2+}]_{mt}$. Accordingly, the silencing of MCU, MICU1 and MCUR1, all conditions that decrease organelle Ca^{2+} accumulation after IP_3-coupled stimuli, increase the formation of autophagosomes and are accompanied by higher AMPK phosphorylation in both HeLa and HEK293 cells [59, 63]. In conclusion, the positive role that $[Ca^{2+}]_{mt}$ exerts on energy production has been well confirmed by genetic approaches targeting the MCU complex in cultured cells.

Regulation of Cell Death

As to the Ca^{2+}-dependent regulation of cell death, the situation is more complex, with apparently slightly contrasting results. As reported above, all conditions that decreases $[Ca^{2+}]$ should in principle protect cells from toxic stimuli. In line with this view, MCU overexpression enhances cell death triggered by a variety of stimuli (including H_2O_2, C2-ceramide and NMDA) in HeLa cells [47, 99], cerebellar granule neurons (CGNs) [99] and primary cortical neurons [100]. Notably, in cortical neurons overexpression of MCU enhances cell death per se, even in the absence of excitotoxic agents [100], thus confirming the idea that different cell types have different susceptibility to mitochondrial Ca^{2+} overload. Accordingly, in the same experimental settings, MCU silencing exerted the opposite effect, i.e. protected cells from apoptosis [99, 100]. However, in one report, primary human epithelial cells (HMEC), but not HeLa or MDA-MB-231 (a breast cancer-derived line), displayed reduced survival after C2-ceramide treatment when MCU was downregulated. MCU was also dispensable for apoptosis triggered by paclitaxel (a cytoskeletal drug) and ionizing radiations in MDA-MB-231 [90]. In the same cell line, MCU silencing was even shown to enhance caspase-independent cell death when moderate dose of ionomycin (3 μM) was used as stimulus, and ineffective at higher doses (10 μM) or after Bcl-2 inhibition with ABT-263 [101]. Conversely, in a similar cell line, the MDA-MB-435S, silencing of MCU slightly protected from a different form of cell death, named paraptosis, induced by celastrol [102]. However, it must be stressed that (i) not all death stimuli depend on organelle Ca^{2+} overload and (ii) mitochondrial Ca^{2+} uptake is not an proapoptotic signal per se, but it rather requires the concomitant activation of other signaling pathways that can be cell-type

specific. It was recently shown that MCU mRNA is one of the targets of miR-25 [103]. Therefore, overexpression of miR-25 leads to a decrease of MCU at protein level and of mitochondrial Ca^{2+} transients. Accordingly, upregulation of miR-25 protects both HeLa [103] and H9c2 (derived from rat myocardium) [104] cells from death induced by H_2O_2 and C2-ceramide, but not by staurosporine (a stimulus that does not strictly rely on mitochondrial Ca^{2+} overload). In addition, treatment of PC3 (derived from prostate cancer) cells with a specific miR-25 antagonist, efficiently enhances cell death [103], thus further reinforcing the notion that mitochondrial Ca^{2+} is a sensitizing element for apoptosis.

Some additional insights into the relationship between mitochondrial Ca^{2+} homeostasis and apoptosis can be obtained by looking at the other components of the MCU complex. Indeed, it has been demonstrated that in HeLa [59, 60, 90], HMEC and primary hepatocytes [60], but not in MDA-MB-231 [90], silencing of MICU1 enhances cell death triggered by C2-ceramide or H_2O_2. Comparable results have been obtained also in primary endothelial cells exposed to lipopolysaccharide and cycloheximide [59]. It is here worth noting that loss of MICU1 leads on one hand to higher mitochondrial Ca^{2+} uptake at low $[Ca^{2+}]_{cyt}$, but on the other it also lowers organelle cation accumulation when $[Ca^{2+}]_{cyt}$ rises [60, 61]. As a consequence, in MICU1 silenced cells, the transfer of low amplitude Ca^{2+} signals should be favored, while large and rapid increases of $[Ca^{2+}]_{mt}$ triggered by temporally restricted Ca^{2+} signals should be impaired. In line with this, Ca^{2+}-dependent apoptotic stimuli generates increases in $[Ca^{2+}]_{cyt}$ that are small in amplitude but prolonged in time [105–107], as opposed to normal physiological agonists (e.g. IP_3-mobilizing agents), which triggers large but transient rises in $[Ca^{2+}]_{cyt}$. Indeed, silencing of MICU1 potentiates the transfer of apoptotic stimuli (i.e. small and prolonged signals) and dampens the burst on oxidative metabolism triggered by IP3-coupled stimuli (i.e. large and fast signals) [59]. Finally, also the knockdown of another putative MCU modulator, SLC25A23, a condition that inhibit mitochondrial Ca^{2+} accumulation, protect from cell death induced by oxidative stress [62]. Regarding the exact cell death mechanism downstream to $[Ca^{2+}]_{mt}$ rises, generation of reactive oxygen species (ROS) is one of obvious candidate, although different results have been obtained. According to Madesh and coworkers, silencing of MICU1 leads to an increase of basal mitochondrial ROS levels that in turn enhances the susceptibility to apoptotic stimuli [59]. Conversely, Hajnoczky and coworkers showed that the basal ROS levels are not affected by MICU1 deficiency. However, during prolonged store operated Ca^{2+} entry, a gradual increase in mitochondrial H_2O_2 production was detected when MICU1 was downregulated, maybe contributing to enhanced cell death [60]. Finally, no differences in ROS levels were detected after regulation of either MICU1 or MCU in MDA-MB-231 cells [90], a model were MCU appears to be dispensable for cell death. However, we also recently reported that the genetic inhibition of MCU in this cell line limits tumor progression both in vitro an in vivo through a ROS/HIF1α-dependent mechanism [108]. Overall, the available data obtained in cultured cells support the idea that mitochondrial Ca^{2+} overload can be a trigger of cell death, although its contribution can largely vary depending on the cell type.

Targeting Mitochondrial Ca^{2+} Uptake In Vivo

The vast majority of studies cited so far agrees on the fact that mitochondrial Ca^{2+} is a pleiotropic signal that regulates many essential aspects of cellular physiology. Starting from these observation, one could easily expect that the genetic ablation of the MCU gene would be detrimental for so many functions at whole organism level to be incompatible with life (i.e. leading to embryonic lethality). However, the first attempt to target MCU in *Mus musculus* showed a very surprising result that puzzled most of the scientists in the field. Indeed, in 2013 Finkel and coworkers published a paper showing a viable MCU knockout (KO) mouse, characterized by the lack of any significant phenotype except for a slight decrease of skeletal muscle peak performance [109]. MCU-KO mice showed no histological aberrations or evident dysfunctions in any organ, neither in high energy demanding tissues such as heart, skeletal muscle and brain. In this model, differences are lacking not only in resting conditions, but also under both physiological (e.g. isoprenaline) or pathological (e.g. ischemia/reperfusion injury in the heart) stimuli [109, 110]. However, it became evident that ablation of MCU in the standard C57BL/6 strain actually leads to embryonic lethality, and this notion has been confirmed in different and independent mouse models (see below). Viable mice could indeed be obtained only in a mixed C57BL/6×CD1 background, and even in this strain the birth ratio of the homozygous MCU null mice is half of the expected [111], thus indicating the requirement for mitochondrial Ca^{2+} uptake during embryo development. How can the genetic background have such a profound impact remains an enigma, although similar results are not new [112]. For example, knockout of the transforming growth factor-β1 (TGF-β1) leads to different outcomes that largely depend on the mouse strain. Embryonic lethality is complete when backcrossed in C57/BL6, 50% in a mixed 129×CF1 and just 20% in BALB/c [113]. Similarly, endothelial growth factor (EGF)-receptor-deficient mice show large variation in survival, ranging from peri-implantation to postnatal lethality, according to the strain of the developing embryos [114, 115]. In any case, the lack of any phenotype of the viable MCU-KO mice is still puzzling. The obvious explanation is that some kind of compensation could take place, for example due to the activation of alternative routes for mitochondrial Ca^{2+} uptake. However, the evidences provided by Finkel and coworker are compelling and demonstrate that MCU is the only mechanism leading to the fast, energy-driven organelle Ca^{2+} uptake in a variety of experimental setups (see Chap. 3). The only concern lies in the observation that in MCU-KO mitochondria, in steady state, matrix Ca^{2+} content, although greatly reduced, is still measurable, maybe suggesting that alternative pathways for slow Ca^{2+} uptake could exist. Again, also the absence of the "expected" phenotype is not new when dealing with loss-of-function models [116]. For example, knockout of creatine kinase, an enzyme that control a critical parameter such as the intracellular ATP levels, leads to no evident phenotype [117]. Even more strikingly, genetic ablation of myoglobin, the oxygen-binding protein of striated skeletal muscle, resulted in no consequences [118].

Interestingly, in myoglobin-KO mice non-obvious multiple compensatory mechanisms take place, all converging to steepen the oxygen pressure gradient to the mitochondria, including higher capillary density, smaller cell width, elevated hematocrit and increased coronary flow and coronary flow reserve [119–121]. Other examples of unpredictable compensations include cGKII- [122] and GDNF-KO [123] mice. More generally, biological robustness should be taken into account when evaluating these models. For example, in yeasts only 13% of genes are essential when mutations in single genes are analyzed [124, 125], but most of the genes are only apparently dispensable, as demonstrated when the functional buffering of biochemical pathway is taken into account [126]. In our biased opinion, a compensation in the downstream signals activated by mitochondrial Ca^{2+} transient (that are still largely unknown) must be present in these mice, a concept that is supported also by results obtained in other related genetic models. Indeed, Anderson and coworkers generated a heart-specific transgenic mouse model expressing a dominant negative MCU isoform, $MCU^{D260Q,E263Q}$ (DN-MCU), in the same mixed C57BL/6×CD1 background of the constitutive MCU-KO model [127]. In cultures cells this mutant is not able to completely abolish organelle Ca^{2+} accumulation [47], but mitochondria from DN-MCU expressing hearts show no measurable Ca^{2+} uptake. In any case, this genetic model shows a clear phenotype, i.e. the lack of isoprenaline-induced fight or flight response. In particular, these mice are incapable of heart rate acceleration induced by β-adrenergic stimulation, an effect completely absent in the constitutive MCU-KO model [110]. The mechanism behind this effect depends on the enhancement of oxidative phosphorylation triggered by $[Ca^{2+}]_{mt}$ increase. Indeed, while MCU appears to be dispensable for normal heart function, under physiological stress, the MCU-dependent boost of ATP production is necessary to sustain the increased demand of SERCA pumps and the proper Ca^{2+} load of the sarcoplasmic reticulum in pacemaker (SAN) cells. In line with this, DN-MCU expressing hearts exhibit impaired performance at increasing workloads (lower ventricular developed pressure at >600 bpm) and a higher oxygen consumption rate (OCR) at any heart rate [127]. Moreover, DN-MCU expressing cardiomyocytes display a clear extramitochondrial adaptation (i.e. higher diastolic $[Ca^{2+}]_{cyt}$) that depends on the reduced ATP availability. However, despite this phenotype, also in this model the susceptibility to ischemia reperfusion injury is comparable to control animals [128]. Conversely, this notion has been recently challenged by a new mouse model developed by Molkentin and coworkers. They indeed generated a conditional MCU knockout model with two LoxP sites between exons 5 and 6 ($MCU^{fl/fl}$) that were crossed with mice expressing a tamoxifen-inducible Cre recombinase (MerCreMer [MCM]) driven by the cardiomyocyte-specific α-myosin heavy chain promoter. MCU gene deletion was induced in adult (i.e. 8 weeks) mice and the cardiac function was evaluated [129]. Interestingly, in a parallel study, Elrod and colleagues crossed the $MCU^{fl/fl}$ animals with mice constitutively expressing Cre recombinase (B6.CMV-Cre). No homozygous $MCU^{-/-}$ mice could be obtained due to complete embryo lethality, thus confirming the requirement for MCU during development [130]. Ablation of MCU in adult heart leads to an 80% decrease of MCU at the protein level, most likely due to the mosaicism of the MCM strain [131,

132]. Therefore, this lead to a drastic, but not complete, reduction of mitochondrial Ca^{2+} uptake has been observed, as can be easily appreciated when looking to intra-mitochondrial Ca^{2+} dynamics (as opposed to indirect extramitochondrial [Ca^{2+}] measurement in isolated organelle, which is a less sensitive method especially when dealing low mitochondrial Ca^{2+} uptake rates). Cardiac-specific MCU-KO mice show no major defects, even 40 weeks after gene ablation, in both normal conditions and after cardiac pressure overload induced by transverse aortic constriction (TAC) [129]. However, this genetic model not only show the lack of any fight or flight response, as previously reported [127], but it also display a clear additional phenotype. Indeed, MCM-MCU-KO mice are strongly protected from I/R injury [129, 130], in line with the predicted role that mitochondrial Ca^{2+} plays in cell death, but in sharp contrast to other MCU-KO models [109, 128]. In support of this notion, mitochondrial Ca^{2+} overload has been shown to be a key determinant of cardiac cell death also in different models of heart failure [133]. However, these discrepancies are difficult to solve, in particular considering the lack of standardization among these models. On one hand, the mice were generated in two different strains (pure C57BL/6 and mixed C57BL/6×CD1), and on the other inhibition of MCU during development is likely to activate compensatory networks. In order to solve some of these issue, it is worth looking to data produced by the International Mouse Phenotype Consortium (IMPC) with the aim to generate standardized procedures for phenotyping and provide reliable insights into the function of the whole mammalian genome [134–137]. The consortium already generated KO models for MCU (http://www.mousephenotype.org/phenotype-archive/genes/MGI%3A3026965), MCUb (http://www.mousephenotype.org/phenotype-archive/genes/MGI%3A1914065) and EMRE (http://www.mousephenotype.org/phenotype-archive/genes/MGI%3A1916279). Some phenotypic data are already available and will be discussed here, although it must be stressed that overall the picture is not yet complete and some of the data must still undergo to quality control processes, and should be thus taken with caution. According to these data, genetic ablation of MCU or EMRE (both conditions that completely block mitochondrial Ca^{2+} uptake) leads to embryonic lethality, thus further reinforcing the requirement of mitochondrial Ca^{2+} signaling during embryogenesis. Unexpectedly, $MCU^{+/-}$ heterozygous mice show several impaired physiological parameters, including a decreased cardiac stroke volume, abnormal fasting glucose level and fat amount (although for these last two parameters, males and females behave differently, thus indicating sexual dimorphism). $EMRE^{+/-}$ heterozygous mice display instead neurological defects (e.g. lower center distance travelled) and impairment of blood glucose homeostasis near statistical significance. Finally, $MCUb^{-/-}$ homozygous mice are viable (as one would expect) but show vestibular impairment (low contact righting score) and electrocardiogram defects. Interestingly, considering the high expression of MCUb in the immune system, a tendency (although not significant) to higher lymphocytic count is also present. Overall, these data suggest that the modulation of MCU complex components has a broad potential impact on physiology at whole organism level.

In line with this view, mitochondrial Ca^{2+} uptake has been shown to play a central role in skeletal muscle pathophysiology. In this case, in order to avoid potential compensatory effects activated during embryonic development, adeno-associated viral (AAV) particles for both the overexpression and silencing of MCU were used in either newborn or adult mice. MCU overexpression and downregulation causes muscular hypertrophy and atrophy, respectively. Moreover, MCU overexpression also protects from denervation-induced muscle atrophy triggered by sciatic nerve resection. Regarding the mechanisms behind this phenotype, they appear to be independent of the control of aerobic metabolism, since (i) PDH activity, although defective in MCU-silenced muscles was unaffected in MCU-overexpressing muscles, (ii) the hypertrophy was comparable in both oxidative and glycolytic muscles (in the latter the contribution of mitochondrial metabolism should play a relatively marginal role) and (iii) analysis of aerobic metabolism revealed no major alterations. Conversely, this effect rather relies on two major hypertrophic pathways of skeletal muscle, PGC-1α4 and IGF1-Akt/PKB, leading to marked increase in protein synthesis. These results indicate the existence of a Ca^{2+}-dependent mitochondria-to-nucleus signaling route that links organelle physiology to the control of muscle mass. It also should be underlined here that the apparent differences in phenotype between gene knockout and gene silencing approaches are not new [138–140] and are thought to be due to the activation of a compensatory network to buffer against deleterious mutations (i.e. gene knockout), which are not observed after gene knockdown [141].

Genetic manipulation of MCU has also been carried out in other organisms. Indeed, both silencing and conditional knockout of MCU in *Trypanosome brucei* impairs energy production, enhances cellular recycling through autophagy and produces marked defects of growth *in vitro* and infectivity in mice [142]. Similarly, developmental defects are also evident in *Danio rerio* injected with anti-MCU morpholinos [143], underlining the physiological requirement of an efficient organelle Ca^{2+} uptake machinery in simple vertebrates. Conversely, knockout of MCU in *Caenorhabditis elegans* leads to apparently viable and fertile worms, although the productions of ROS and wound healing are impaired [144].

Also in the case of the MCU regulators, the situation is still controversial. Very recently, Hajnoczky and coworkers described the first attempt to target MICU1 in vivo [145]. Embryonic ablation of MICU1 causes perinatal lethality in mice without causing gross anatomical defects. Most importantly, liver-specific MCU deletion determines the striking failure in organ regeneration after partial hepatectomy. MICU1-deficient hepatocytes show exaggerated Ca^{2+} overload-induced mitochondrial permeability transition pore (PTP) opening, and indeed the phenotype is completely reversed in vivo by using the PTP inhibitor NIM811 [145]. Conceptually similar results have been obtained in a different model of MICU1-KO mice generated by Finkel and coworkers (see Chap. 3). However, in this model the perinatal mortality is only partial, and the phenotype of surviving MICU1-KO animals spontaneous improves during aging, most likely due to a compensation through a decreased expression level of EMRE [146].

Finally, the pleiotropic role of mitochondrial Ca^{2+} uptake has also been confirmed in human pathophysiology. Few families carrying a loss-of-function mutation of MICU1 have been identified. Homozygous individuals for this mutation are characterized by early-onset proximal muscle weakness with a static course, moderately or grossly elevated serum creatine kinase levels accompanied by learning difficulties and a progressive extrapyramidal movement disorder. Fibroblasts derived from these patients show the expected impairment of MCU gating accompanied by mitochondrial fragmentation and decreased oxidative metabolism [147, 148]. Although the pathogenesis lying behind this disease is impossible to dissect at the moment, it needs stressing that this finding underlines the importance of mitochondrial Ca^{2+} transport in humans, as a change in one of the regulatory proteins in the complex results in a genetic disease with devastating symptoms.

Overall, it is still difficult at the moment drawing any definitive conclusion on the role of mitochondrial Ca^{2+} handling in pathophysiology. Still, despite the initial setback caused by the lack of significant phenotype of the MCU-KO, the new available models are starting to uncover the real contribution of mitochondrial Ca^{2+} signals to physiological processes. New questions need to be addressed and new cellular pathways are waiting to be discovered, in an unexpectedly exciting exploding field.

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Chapter 3
The In Vivo Biology of the Mitochondrial Calcium Uniporter

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Decades of research have established the importance of regulating mitochondrial calcium both for stimulating ATP production [1], as well as modulating cell death through the calcium-dependent opening of the mitochondrial permeability transition pore (mPTP) [2]. How calcium passes through the inner mitochondrial membrane remained elusive until 2011, when two groups simultaneously identified CCDC109A as the gene encoding the bona fide mitochondrial calcium uniporter, renaming this previously uncharacterized gene product MCU [3, 4]. In initial cell culture experiments, MCU silencing severely attenuated rapid mitochondrial calcium uptake [3, 4]. Additional analysis revealed that in mammalian cells the calcium uniporter complex comprises not only MCU but several additional proteins that are thought to regulate MCU-mediated calcium entry into the mitochondria [5]. The first member of the uniporter complex to be described was MICU1 [6]. MICU1 and its paralogues MICU2 and MICU3 are thought to be localized in the intermembrane space and involved in the gatekeeping of the uniporter [7, 8]. Based on work done in cell culture models, MICU1 appears to inhibit MCU opening at low cytoplasmic calcium levels [7–10] and may also contribute to channel activation at high

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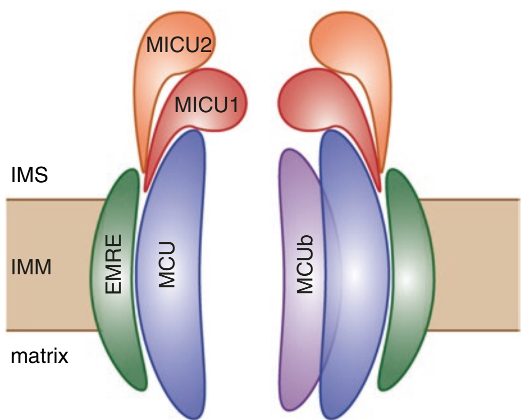

Fig. 3.1 The uniporter is a multiprotein complex. Representative diagram of some of the known components of the uniporter. The predominance of evidence suggests that MICU1 and MICU2 are located in the inner mitochondrial space (*IMS*), while MCU, MCUb, and EMRE span the inner mitochondrial membrane (*IMM*). See text for details.

calcium levels [8]. Recently, a single pass membrane protein named Essential MCU Regulator (EMRE) was described to be necessary for mitochondrial calcium uptake [11]. EMRE is proposed to act as a scaffold, mediating the interaction between MCU and other proteins belonging to the complex, including MICU1 and MICU2. Furthermore, the pore is formed by multimers of MCU as well as MCUb, a protein with high sequence homology with MCU that appears to negatively regulate calcium uptake. The relative stoichiometry of MCU and MCUb potentially contributes to variable uniporter activity among tissues [12]. Together, these genetic, biochemical, and structural studies have provided a relatively complete picture of the organizational structure of the uniporter complex (Fig. 3.1). However, the limited energetic demand of cells in culture suggested that to better understand the physiological role of mitochondrial calcium regulation, in vivo models would be needed. Here, we review the initial in vivo observations characterizing the effects of disrupting uniporter components in non-mammalian and mammalian model organisms.

Analysis of the Uniporter in Model Organisms

Homologues of MCU are present in a wide array of organisms including most plants and metazoans (Fig. 3.2). In contrast, many fungi and protozoans lack evidence for either MCU-like or MICU1-like proteins [13]. Similarly, there is no apparent uniporter activity evident in yeast [14]. Interestingly, the filamentous fungus *Neurospora crassa* has an MCU homologue [13], yet does not appear to have canonical mitochondrial calcium uptake activity [14], raising questions regarding what function this MCU homologue actually performs. Most organisms that have MCU-like proteins also have MICU1-like proteins, with the exception of the fungi possessing MCU homologues, including *N. crassa*, which typically appear to lack MICU1 homologues [13]. The wide diversity in domain structure of the MCU homologues in these fungi [13], coincident with the absence of MICU1, suggests that there are

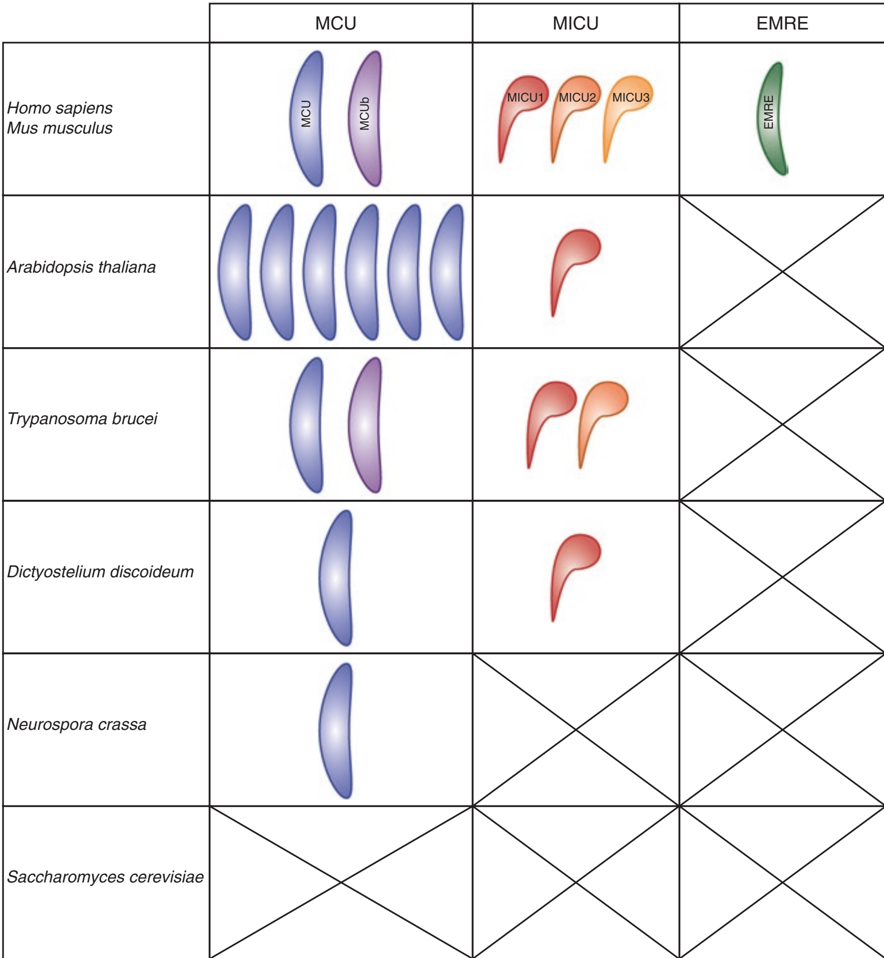

Fig. 3.2 Evolutionary diversity of the uniporter components. In both humans and mice there are two forms of MCU (MCU and MCUb), three MICU family members, and one EMRE. In other species, the quantity of these elements can vary considerably, including the occurrence of EMRE only in metazoans and the complete absence of a uniporter in yeast.

multiple evolutionary approaches to regulate mitochondrial calcium entry. In contrast to the broad evolutionary range of MCU and MICU1, EMRE is specific for metazoans [11]. These evolutionary differences have allowed for heterologous systems to explore functional requirements of the uniporter. For instance, the yeast *S. cerevisiae,* which lacks any uniporter, has been used as a platform for in vivo reconstitution [15]. Interestingly, this analysis has demonstrated that human MCU requires EMRE for calcium uptake, while the structurally distinct MCU found in *Dictyostelium discoideum* (DdMCU) does not [15]. Indeed, DdMCU restores calcium uptake in human cells lacking either MCU or EMRE. As such, these results

establish two important principles. First, lessons gleaned from organisms such as yeast that lack any uniporter tell us that there is no absolute requirement for uniporter activity to maintain mitochondrial integrity or function. Second, in organisms that contain uniporter activity, there is a strong predilection for the occurrence of both MCU and MICU1 homologues to be present although evolutionary-derived variations in the structure of MCU can obviate the requirement for either MICU1-like or EMRE-like proteins.

Analysis of model systems has revealed significant information regarding the physiological function of the uniporter. One early example involved the role of the uniporter in the biology of *Trypanosoma brucei*, the parasite responsible for African sleeping sickness [16]. This parasite can exist in several distinct life cycle stages, including the procyclic form (PCF), in which the parasite resides within its normal vector the tse tse fly, and the bloodstream form (BSF), in which it circulates in the blood of its mammalian host. Remarkably, both the PCF and BSF forms contain a single mitochondrion. Moreover, the PCF mitochondrion has intact and fully functional oxidative phosphorylation capacity, while the BSF form does not. This system therefore is unique in that the two different forms of the parasite each have a single mitochondrion, yet the activity of that single mitochondrion is completely different depending on the lifecycle of the organism. When these authors ablated the *Trypanosoma brucei* uniporter (TbMCU) they were able, as expected, to reduce calcium uptake into the mitochondria. In addition, growth of both the PCF and BSF forms was inhibited in the absence of TbMCU. Similarly, mice injected with *Trypanosoma* containing a conditional knockout of TbMCU had decreased parasitemia and increased survival [16]. Given that the BSF form of *Trypanosoma brucei* does not rely on its mitochondrion for ATP (this form is entirely dependent on glycolysis) [17], this would suggest that mitochondrial calcium is required for some other function than simple bioenergetics in order to maintain viability of the parasite. The authors provide at least supportive evidence that in the absence of the uniporter there is an inability to generate acetyl-CoA due to a calcium-mediated reduction in pyruvate dehydrogenase (PDH) activity [16]. Interestingly, this impairment in PDH is also seen in mammalian models where MCU is deleted [18].

There has been some limited analysis of the requirement of the uniporter and its components in plants. In *Arabidopsis*, neither EMRE or MCUb exist, while there appear to be at least six MCU homologues present [19]. One of these homologues (At5g66650) appears to be targeted to the chloroplast, consistent with past observations that light-dependent calcium influx occurs in chloroplasts and this influx can be blocked by ruthenium red, a pharmacological inhibitor of the uniporter [20].There also appears to be differential tissue expression of these plant MCU homologues with some expressed highly in the root, while others are more abundant in the flower or guard cells [19]. This would imply that the diversification of MCU homologues might provide specialized calcium-dependent roles for individual tissues within the plant. Interestingly, while most plant species contain two MICU1 homologues (similar to MICU1 and MICU2), *Arabidopsis* contains only one such

family member [21]. This single *Arabidopsis* MICU1 family member (At-MICU) appears to contain three EF hands, rather than the two EF hands found in both MICU1 and MICU2. As such, it would appear that with At-MICU, *Arabidopsis* has incorporated MICU1 and MICU2 function within a single molecule. Plants lacking At-MICU appear to develop normally but have an increase in steady state mitochondrial calcium levels [21]. This same calcium-overloaded phenotype is seen in tissues or mitochondria derived from mice lacking MICU1 [22, 23].

Analysis of model organisms has also revealed an interesting relationship between the uniporter and mitochondrial reactive oxygen species (mtROS) production. This was evident in the previously discussed *Trypanosoma* model where overexpression of TbMCU was demonstrated to increase mtROS production [16]. In *C. elegans*, skin wounding causes local superoxide production that appears to be required for actin-based wound closure [24]. Previous work had shown that a sustained rise in cytosolic calcium was also required for wound repair in this model [25]. The mechanistic link between these two phenomena came from the observation that MCU-null worms show reduced mitochondrial calcium uptake after wounding, as well as reduced mtROS production. Thus, a pathway induced by skin wounding leads to increased mitochondrial calcium uptake, increased mtROS production and the subsequent redox-dependent activation of the actin cytoskeleton [24]. While in this case the uniporter-dependent production of mtROS was beneficial, in other cases it appears to confer toxicity. For instance, a recent study sought to examine the toxicity of aminoglycoside antibiotics using a zebrafish model. Just as in human patients, in zebrafish, aminoglycosides can induce inner ear damage by permanently damaging the mechanosensory hair cells within the ear. Again, previous evidence suggested that this damage was mediated, at least in part, by ROS [26, 27]. However, in this case, it appears that in the dying hair cell, ROS production is dependent on mitochondrial calcium uptake through the uniporter [28]. As such, while calcium uptake through the uniporter appears to generate increased mtROS production in both worms and zebrafish, these two examples suggest that this can either be beneficial or harmful depending on the context.

Finally, one recent, fascinating report analyzed the role of the uniporter in *Drosophila*. This work was stimulated by a previous screen looking for genes that affected olfactory memory in the fly. Interestingly, this analysis suggested that silencing the *Drosophila* form of MCU or MICU1 impaired memory formation [29]. Following up on this observation, a recent study demonstrated that knockdown of *Drosophila* MCU (dMCU) within mushroom body neurons, a region essential for olfactory memory, resulted in impaired memory formation [30]. Interestingly, this defect was selective, as it did not affect the capacity to learn; instead, it only altered the capacity to remember at one or three hours after the conditioning stimulus [30]. Moreover, knockdown of dMCU only altered adult memory if performed during pupation, as a similar knockdown in adult flies had no effect [30].

Mouse Models

The generation of the first mouse model of MCU deletion was described in 2013 and represented an important step toward better defining the physiological impact of mitochondrial calcium [18]. While embryonically lethal in a pure genetic background such as C57BL/6, MCU-deficient mice could be generated in a mixed genetic background (e.g. CD1). Mitochondria derived from these $MCU^{-/-}$ mice were unable to rapidly uptake calcium, confirming results from cells lacking MCU. Unexpectedly, these mice displayed a seemingly modest phenotype and were only slightly smaller than wild-type littermates [18]. Despite reduced calcium in the mitochondrial matrix of $MCU^{-/-}$ skeletal muscle, there was no significant change in basal metabolism measured in either mouse embryonic fibroblasts (MEFs) or isolated mitochondria. When challenged with strenuous exercise requiring a rapid increase in skeletal muscle work load, however, $MCU^{-/-}$ mice were measurably impaired in exercise capacity (Fig. 3.3). Furthermore, addition of extra-mitochondrial calcium to wild-type cardiac mitochondria induced mPTP opening as evidenced by mitochondrial swelling, and this response was, as expected, inhibited by cyclosporine A (CsA), an mPTP inhibitor, or by Ru360, a ruthenium red analog and uniporter inhibitor. In contrast, $MCU^{-/-}$ mitochondria were completely resistant to calcium-induced mPTP opening, even at high extra-mitochondrial calcium concentrations (e.g. 0.5 mM). On the other hand, $MCU^{-/-}$ MEFs were not more sensitive than wild-type cells to death-inducing agents including hydrogen peroxide, tunicamycin, doxorubicin, and thapsigargin [18]. Since calcium overload is reported to play a role in injury and necrosis in the setting of ischemia-reperfusion (I/R), hearts from wild-type and $MCU^{-/-}$ mice were subjected to an ex vivo Langendorff model of I/R injury. Surprisingly, overall $MCU^{-/-}$ hearts were not protected. However, while CsA conferred significant protection to wild-type hearts, it did not protect $MCU^{-/-}$ hearts. These results suggest that cell death in the absence of MCU may be mediated through a mPTP-independent pathway, begging the questions of whether and how cell death signaling may be rewired in the setting of chronic MCU inhibition.

A subsequent study analyzed cardiac function in the same strain of $MCU^{-/-}$ mice [31]. This analysis revealed that despite the fact that $MCU^{-/-}$ heart mitochondria had attenuated calcium uptake and reduced matrix calcium levels, basal cardiac function was normal at both 12 and 20 months of age. Moreover, the ability of $MCU^{-/-}$ hearts to respond to certain stresses – either pharmacological (isoproterenol stimulation) or physiological (surgical transverse aortic constriction) – appeared indistinguishable from wild-type littermates. This lack of a basal or stress-induced phenotype was quite unexpected given the numerous important roles mitochondrial calcium was believed to play in cardiac physiology [32].

To further investigate the role of MCU in the heart, other groups have generated mice with transgenic expression of a dominant-negative (DN) MCU [33]. These mice were used to focus on the involvement of MCU in increasing ATP production in order to fuel demand-dependent heart rate increases. To inhibit MCU-mediated

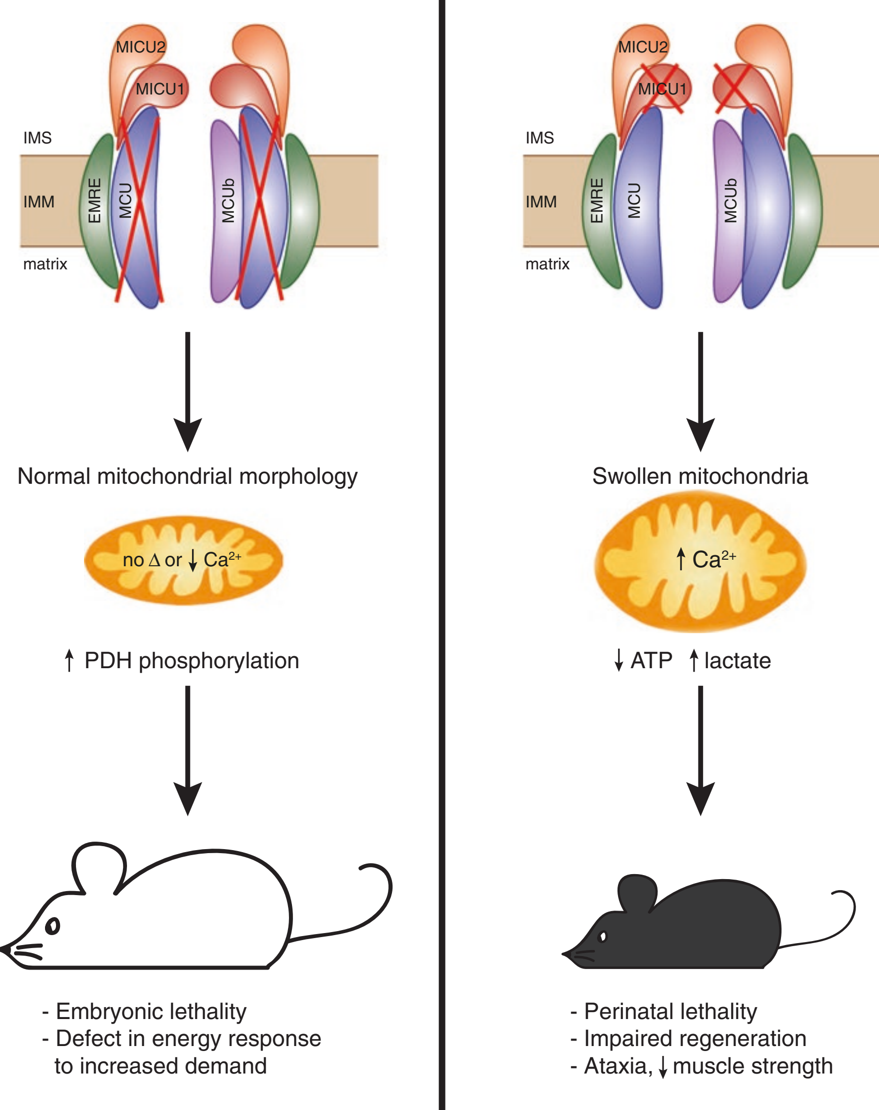

Fig. 3.3 Insights from genetic manipulations of MCU and MICU1 in mouse models. Summary of some of the findings from reports in which mice lacking either MCU (*left panel*) or MICU1 (*right panel*) have been characterized. These include effects on resting mitochondrial calcium levels and mitochondrial morphology, as well as alterations at the tissue and organ level. See text for additional details.

mitochondrial calcium uptake in the myocardium, a mouse containing a DN-MCU construct driven by the α-myosin heavy chain promoter was generated. This dominant negative mutation is in the pore-forming region and takes advantage of the observation that within the inner mitochondrial membrane, MCU forms oligomers [34]. In vivo expression of DN-MCU prevented rapid, MCU-mediated, mitochon-

drial calcium uptake. Nonetheless, under basal conditions, DN-MCU mice had hearts with normal chamber size and function. These animals did exhibit a reduction in atrial ATP levels when compared with littermate controls. While basal heart rates were only mildly slower in DN-MCU hearts, in unrestrained and un-sedated mice, the heart rate increase following isoproterenol stimulation was less pronounced in the DN-MCU mice. Cardiac sinoatrial nodal (SAN) pacemaker cells isolated from DN-MCU mice also showed reduced sarcoplasmic reticulum (SR) calcium content after physiological stress (isoproterenol stimulation) compared with wild-type SAN cells. This defect could be rescued by exogenous ATP. These data suggested that in this model, mitochondrial calcium uptake through MCU plays a role in ATP synthesis needed for maintaining overall calcium homeostasis. As such, the study concluded that MCU is required for the physiologically important 'fight or flight' stress response.

In a second study, the same mouse model of dominant-negative MCU was used to examine certain stress responses in the absence of mitochondrial calcium uptake [35]. Unexpectedly, isolated Langendorff-perfused DN-MCU hearts exhibited higher oxygen consumption rates (OCR) than WT. As mitochondrial morphology, protein and DNA content seemed unaltered; a potential explanation would be that DN-MCU hearts were energetically less efficient than WT. In contrast, permeabilized myocardial fibers or isolated mitochondria from DN-MCU mice exhibited only a modest reduction, if any, in oxygen consumption. Similar to what was reported in the whole-body MCU knockout [18], phosphorylation of PDH was significantly increased, and hence PDH was less active in DN-MCU hearts compared with WT. Monitoring membrane potential and changes in reactive oxygen species (ROS) during I/R revealed significantly reduced ROS in DN-MCU hearts, but no difference in infarct area following I/R was found [35]. As such, this lack of protection was consistent with that seen in total body MCU knockout mice [18].

In contrast to these models of constitutive MCU deletion or inhibition, two collaborative groups recently developed a conditional knockout mouse model in which MCU can be deleted in specific tissues by Cre-mediated methods [36, 37]. When $MCU^{fl/fl}$ mice were crossed to a transgenic germline-Cre model, no $MCU^{-/-}$ pups were obtained, implying that as previously discussed homozygous MCU deletion results in embryonic lethality in inbred mice. Crossing $MCU^{fl/fl}$ to αMHC-Cre to specifically delete MCU in cardiomyocytes resulted in about 80 % reduction of MCU protein in adult cardiomyocytes. Unlike in the global MCU knockout [31], matrix calcium in adult cardiomyocytes did not decrease with MCU deletion, although mitochondrial calcium uptake was eliminated [36, 37]. Using a tamoxifen-inducible heart-specific Cre, the authors next assessed I/R injury using a left coronary artery (LCA) ligation model. As would be expected if calcium uptake caused mPTP opening, MCU conditional knockout (cKO) mice were protected from injury, exhibiting reduced TUNEL staining and infarct sizes that measured roughly half of WT littermates. The discrepancy between these positive results and the lack of protection seen in MCU knockout or DN-MCU mice is currently unexplained. Possibilities include technical differences such as differences in the model of I/R injury (e.g. in vivo LCA ligation as opposed to an *ex vivo* Langendorff model).

More likely, these disparities reflect differences in the timing and duration of MCU deletion. In the MCU cKO, tamoxifen was administered to adult mice, several weeks before the response to I/R was assayed. In contrast, in the $MCU^{-/-}$ and DN-MCU mice, MCU activity was absent throughout development. This suggests that there might be important physiological differences between acute and chronic deletion of MCU.

Further analysis of the MCU cKO mice revealed a number of interesting phenotypes. Similar to what has been observed in other models, even 1 year after MCU deletion, no pathological effects on mitochondrial, cellular, or cardiac structure were observed [36]. When acute cardiac stress was induced by intravenous infusion of isoproterenol, PDH phosphorylation was higher in the MCU cKO versus WT, and correspondingly isoproterenol-stimulated PDH enzymatic activity in MCU cKO was decreased [37]. This finding was consistent with observations in both non-mammalian model organisms [16] and other MCU deletion models [18]. Regarding metabolism, no differences in basal respiration were found in adult cardiomyocytes, although the MCU cKO cells were refractory to isoproterenol-induced maximal oxygen consumption rates [37]. In another cardiac stress test, transverse aortic constriction was performed on MCU cKO and control mice, but led to no differences in cardiac pathology or function [36], consistent with the data from the $MCU^{-/-}$ mice [31]. When mice were challenged to sprint on a treadmill, when only two minutes of warm-up time was allowed, MCU cKO mice displayed significantly reduced running capacity compared with controls. This difference disappeared when 30 min of warm-up was allowed. Hence, while subtle, it would appear that adult cardiac-specific deletion of MCU impairs the ability of the heart to adapt to certain acute stresses that require acute increases in mitochondrial metabolism.

Studies of the mitochondrial calcium uniporter in vivo have not been limited to MCU, the channel-forming unit, as the multi-protein complex includes other components critical for its regulation. In particular, the impact of MICU's gatekeeping function on the uniporter in vivo is of great interest. The first two studies investigating MICU1 deletion in mouse models were recently published (Fig. 3.3) [22, 23]. In the first study, MICU1 whole body deletion resulted in complete perinatal lethality shortly after birth [22]. Permeabilized MEFs derived from the animals showed increased calcium uptake at low concentrations of cytoplasmic calcium. No gross anatomical or morphological defects were observed in the knockout animals, despite a trend toward fewer neurons in the nucleus ambigous and nucleus facialis which regulates breathing. The authors suspected this defect might have contributed to the observed perinatal mortality. The authors then deleted MICU1 in the liver through tail-vein injection of hepatocyte-specific Cre. Following a 70% partial hepatectomy, they observed that liver damage was more extensive with MICU1 deletion and regeneration was abrogated. This was hypothesized to occur from mitochondrial calcium overload, with subsequent increased mPTP opening. Thus, in this model, mitochondrial calcium overload resulting from MICU1 deletion was linked to sustained inflammation and injury.

Shortly thereafter, another report described an independently generated mouse model of MICU1 deletion [23]. In this strain, which was a slightly different back-

ground than the other MICU1 mouse model, homozygous deletion of MICU1 resulted in high but not total postnatal lethality. Analysis of over 1000 live births suggested that roughly one in seven MICU1$^{-/-}$ mice survived beyond the first postnatal week [23]. These rare surviving MICU1$^{-/-}$ mice initially exhibited a severe phenotype reminiscent of human patients with loss-of-function MICU1 mutations that included abnormal mitochondrial morphology, decreased muscle ATP levels, elevated serum lactate, and neurological features such as ataxia [38]. Confirming the results of the previous study, mitochondria isolated from these MICU1$^{-/-}$ mice exhibited increased calcium uptake at low extramitochondrial calcium concentrations, and in addition had elevated resting mitochondrial matrix calcium levels. Hence, the predominant in vivo function of MICU1 appears to be gatekeeping of the uniporter to prevent mitochondrial calcium overload. Notably, as these MICU1$^{-/-}$ mice aged, their symptoms improved, coincident with reduced EMRE gene expression. As EMRE is reported to be essential for mitochondrial calcium uptake, a reduction in EMRE would potentially limit entry of calcium into mitochondria, helping to relieve calcium overload. Remarkably, deleting one allele of EMRE in MICU1$^{-/-}$ mice rescued mitochondrial calcium handling and perinatal mortality [23]. These results suggest that the relative expression of uniporter components can modulate mitochondrial calcium uptake with profound physiological consequences. Interestingly, it would appear that MICU1 deletion, characterized by calcium overload, is perfectly compatible with embryogenesis but rapidly fatal immediately after birth. In contrast, MCU deletion, which presumably represents a condition of insufficient calcium levels, is embryonically lethal in a pure background, while mice born in a genetically mixed background have no difficulties after birth, no significant basal phenotype and only a modest defect following various stress conditions (Fig. 3.3).

The Role of the Uniporter in Human Disease

There is relatively little direct information regarding the role of the uniporter in human disease. This most likely reflects the relative recent discovery of the molecular basis for mitochondrial calcium entry. One area in which there is already some, albeit conflicting, data is the role of MCU in cancer. Given the potential role of mitochondrial calcium in mediating cell death, it would appear likely that alterations in uniporter activity could alter tumor cell resistance to apoptotic or necrotic cell death. One early report demonstrated that expression of MCU was negatively regulated by miR-25, a microRNA that is often upregulated in human tumors (see Chap. 23) [39]. Indeed, analyzing human colon samples, and the corresponding surrounding normal mucosa, these authors were able to identify that tumors had high levels of miR-25, and corresponding low levels of MCU protein expression [39]. In heterologous tumor cell lines, expression of miR-25 resulted in decreased MCU expression and reduced sensitivity to apoptotic stimuli. This pathway may also be operative in non-malignant diseases. A recent report has also demonstrated

reduction of MCU in pulmonary arterial hypertension (PAH), a devastating and ultimately fatal disease characterized by excessive smooth muscle proliferation in the pulmonary vasculature. This report described a reduction in MCU expression in smooth muscle cells (SMCs) derived from patients with PAH when these cells were compared to SMCs from healthy controls [40]. Again, this reduction appeared to be mediated by increased expression of miR-25, as well as another microRNA, miR-138. Remarkably, administering nebulized anti-miRs directed against miR-25 and miR-138 restored MCU expression and reversed PAH in an animal model of this condition [40]. While the previous reports would indicate that reduced MCU expression appears to contribute to cancer cell growth, or the abnormal proliferation of SMCs, there is also evidence for the opposite phenotype. For instance, analysis of human breast cancer samples revealed that there was a positive correlation between MCU expression and breast cancer tumor size and lymph node infiltration [41]. Concurrently, this same clinical data set revealed a negative correlation between expression of the dominant negative MCUb and tumor size. Thus, in breast cancer, it would appear that MCU expression might fuel disease progression. The authors propose that this may result from increased MCU expression leading to augmented mtROS production and the subsequent redox-dependent stabilization of HIF-1α [41]. Again, in a clinical data of some 500 human breast cancer patients, a significant correlation was found between MCU expression and the expression of both HIF-1α and multiple, potentially oncogenic, HIF targets.

The strongest connection between the uniporter and human disease comes not from alterations in MCU expression, but rather from analysis of inherited mutations in MICU1. A cohort of pediatric patients was shown to have loss-of-function mutations in MICU1 leading to the absence of detectable MICU1 protein expression in these individuals [38]. The manifestation of MICU1 loss was characterized by proximal muscle weakness, with evidence of ongoing muscle damage (e.g. elevated serum creatine kinase levels). This was confirmed by muscle biopsies which demonstrated increased central nuclei with clusters of regenerating fibers [38]. Over half of the subjects exhibited extrapyramidal signs that included tremors, chorea, and dystonic posturing. In some patients, there was evidence of ataxia, microcephaly, and ocular dysfunction (i.e. ptosis, opthalmaplegia, and optic atrophy). While the constellation of myopathic and neurological symptoms suggests a potential mitochondrial basis, it is of interest to note that none of the patients appeared to have other common abnormalities such as deafness, heart failure, or diabetes, often associated with primary mitochondrial diseases. Sequencing of affected individuals revealed various MICU1 mutations, often affecting splice donor sites and leading to nonsense decay of the mutant mRNA. Fibroblasts derived from these individuals revealed evidence for increased mitochondrial calcium levels, with evidence of mitochondrial fragmentation [38]. A second, more recent report described two cousins, each of whom contained a large deletion in exon 1 of MICU1. Their clinical course was slightly different, although it did include muscle weakness with high serum creatine kinase levels [42]. For these two cousins, however, most of their symptomatology was episodic. In one case, minimal exercise would precipitate becoming pale and sweaty, followed by lethargy to the point of being noncommuni-

cative and unarousable. The other cousin had similar exercise-induced lethargy and confusion that would last for hours, if not days. Again, fibroblasts from these affected patients showed no MICU1 expression [42]. These cells exhibited normal mitochondrial function but altered mitochondrial calcium uptake. Interestingly, the episodic nature of these symptoms is reminiscent of other channelopathies that often underlie other paroxysmal conditions such as migraines, epilepsy and sudden cardiac death [43].

Conclusion

Since its molecular discovery a mere 5 years ago, remarkable progress has been made in discerning the in vivo role of the mitochondrial calcium uniporter. This analysis has ranged from analyzing uniporter function in a variety of model organisms, to more standard mouse model systems. While important phenotypes have been discerned, perhaps the most surprising result is that viable mice can be produced that lack any functional uniporter [44, 45]. Perhaps, in retrospect, the absence of a uniporter in yeast might have provided some guidance that mitochondria can function without this element of acute calcium regulation. Additional models and analysis are clearly required to understand the other components (e.g. EMRE, MICU2, etc.), as well as how all these components interact. The observation that the perinatal lethality of MICU1 can be rescued by deleting one allele of EMRE [23] suggests that the creation of additional mouse models will allow for important and potentially insightful combinatorial models. Additional information is also needed to explain what appears to be a clear distinction between acute and chronic deletion of uniporter components. There seems to be some functional remodeling and rewiring that occurs following chronic MCU deletion, for example, that allows cells to physiologically adapt. In some ways, this may represent a form of retrograde signaling, namely a pathway by which a mitochondrial perturbation can ultimately result in nuclear transcriptional changes [46]. All these and other related questions suggest that the next 5 years may provide further insight into the in vivo role of the uniporter in addition to the exciting progress already made.

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Chapter 4
Mitochondrial Bioenergetics and Dysfunction in Failing Heart

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In order to perform work, the myocardium must obtain a constant supply of cellular energy in the form of ATP. The heart is one of the highest ATP-consuming organs in the body, turning over more than its weight in ATP per day. Approximately 95% of ATP is produced by the mitochondrion, primarily through oxidative phosphorylation and to a lesser extent, oxidation of glucose and amino acids [1, 2]. Thus mitochondria play a crucial bioenergetic role in healthy heart function. Intertwined with this, mitochondria are dynamic regulators of cellular adaptation and homeostasis, cell survival and cell death [3].

Heart failure due to systolic dysfunction of varying etiology has been generally defined as a syndrome featuring progressive inability of the heart to supply sufficient blood flow to the body, resulting in symptoms such as shortness of breath and exercise intolerance as the body becomes starved of oxygen and essential nutrients. The idea that a shortage of energy supply was at the centre of cardiac dysfunction in heart failure has been proposed for decades, but was brought to the forefront by a series of papers published by Ingwall [4–7] and others [8, 9] in the 1990s, enabling real-time patient measures through the use of nuclear magnetic resonance (NMR) techniques. Through these studies it was demonstrated that levels of ATP and the energy storage molecule creatine phosphate (PCr) were around 30% lower in the tissues of heart failure patients [9, 10], correlating with a decline in cardiac function [9, 10], with PCr:ATP ratios shown to be an strong predictor of patient mortality, similar to NYHA class alone [5].

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Although mitochondrial dysfunction, as a key contributor to the energy deficit underlying diminishing cardiac output, was identified early from animal studies, direct evidence from humans studies has been more limited and hampered by sampling access, methodological approach and biopsy quality [11]. Indeed studies of isolated mitochondrial preparations from human failing myocardium have reported mixed findings in that high quality, freshly isolated mitochondrial preparations can exhibit tightly coupled respiration under strictly regulated in vitro conditions [11]. Such findings suggest that meticulous isolation methods may select for high quality mitochondria with relatively intact structural and functional respiratory chain

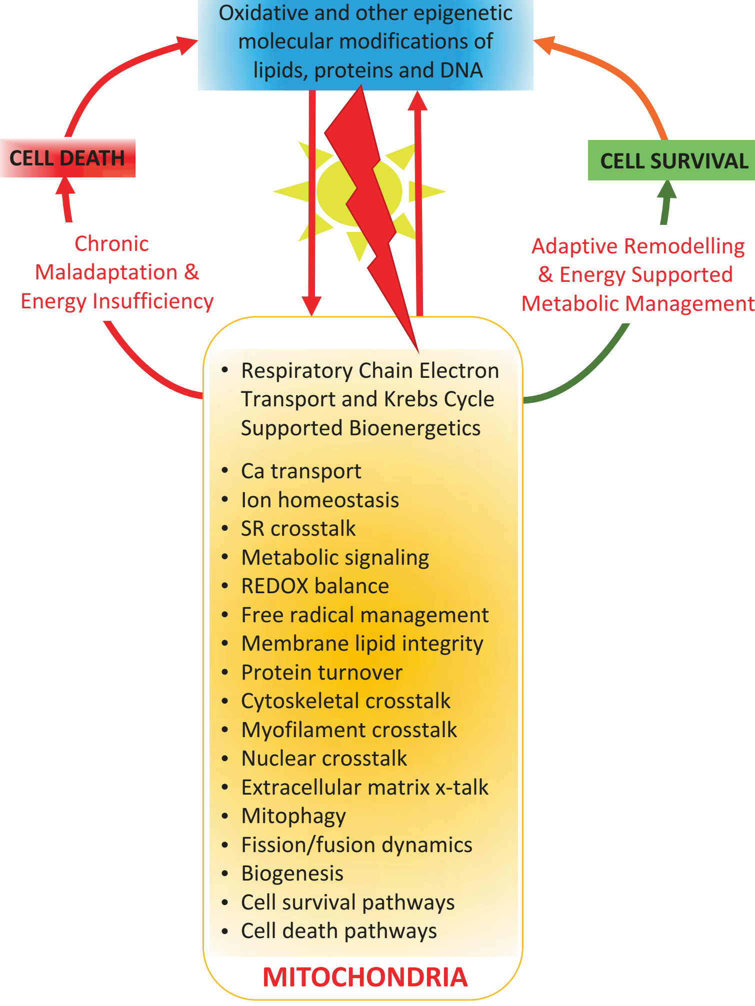

Fig. 4.1 The general scheme highlighting post-translational modifications contributing to mitochondrial and myocardial contractile dysfunction in the progression of systolic heart failure

integrity in vitro. However, this also indicates that in situ study of mitochondria may be more revealing in heart failure because there are numerous other mitochondrial and extra-mitochondrial components beyond respiratory protein complex electron transport regulating bioenergetic function that may be dysfunctional in systolic heart failure.

Progressively worsening energy deficit has profound effects on cellular homeostasis which compounds myocardial dysfunction, further contributing to loss of cardiac function. More than two thirds of energy produced by the cell is utilized for contractile function, with the remainder required for regulation of Ca^{2+} homeostasis, membrane potential, sarcoplasmic reticulum Ca^{2+} handling, as well as other ion channel function, cell maintenance and repair [2]. Restricted energy for contractile function potentially limits capacity for calcium handling, resulting in accumulation of calcium in the sarcoplasmic reticulum, decreased activation of Ca^{2+} -dependent mitochondrial Krebs enzymes as well as altered cell signalling pathways such as SIRT1 which are involved in mitochondrial turnover and biogenesis [12]. Furthermore, increased Ca^{2+} also predisposes the mitochondrion to activation of the permeability transition pore, leading to cell death. All of these above indicators have been reported in heart failure [13, 14]. As seen, disruption of one pathway potentially has a multitude of effects on other signalling, altering the normal regulatory homeostasis of cell function.

In this chapter, we briefly discuss the various aspects contributing to a decline in energy production in the failing heart with particular emphasis on the role of mitochondria. As generally summarized by the scheme in Fig. 4.1, specific focus will include post-translational modification and dysfunction of proteins of the oxidative phosphorylation and Krebs pathways, the role of reactive oxygen species in targeting membrane proteins and lipids, metabolic substrate 'switch', mitochondrial membrane lipid composition as well as the contribution of these factors to cell signalling involving mitochondrial turnover and cell death. While the chapter provides a brief introductory overview, it also highlights that considerable more work is required to augment our understanding of the complex, multiple functional roles of mitochondria, and to target their specific components in the translation of therapeutics to counter heart failure.

Post-Translational Modification of Mitochondrial Proteins Leading to Dysfunction

Mitochondrial ATP synthesis involves a number of distinct pathways within various cellular compartments and is regulated at numerous hierarchical levels [1–3]. Glucose is initially oxidized to form pyruvate by glycolysis within the cytoplasm, resulting in the formation of two ATP molecules. Pyruvate then enters the mitochondrion through the inner membrane mitochondrial pyruvate carrier (MPC), which forms a major regulatory checkpoint for choice of substrate metabolism. Pyruvate is then further broken down through eight enzymatic steps of the Krebs cycle within the mitochondrial matrix, resulting in the reduction of metabolic

intermediates NADH and $FADH_2$. Oxidative phosphorylation completes the production of 38 molecules of ATP per mole of glucose through a series of four electron transporting complexes (CI, CII, CIII, CIV), whereby NADH or $FADH_2$ is oxidized by complexes I or II respectively, resulting in the flow of electrons through the chain to convert O_2 to water (complex IV). Protons are extruded at complexes I, III and IV into the intermembrane space, which are subsequently channelled back into the matrix via ATP synthase, driving the synthesis of ATP. This process is tightly regulated, with close coupling of electron transport, membrane potential and ATP synthesis.

Lowered rates of coupled ATP synthesis have been reported in both animal models [15] and human heart failure tissues [16]. As mitochondrial metabolism contributes to around 95% of cardiac ATP supply, predominantly through oxidative phosphorylation, it could reasonably be surmised that disruption of mitochondrial energetics and metabolism lies at the heart of ATP deficit. Decreased activities of individual complexes, namely I, III and IV have been reported in human heart failure tissues [17–21]. Although altered gene expression of electron transport chain protein subunits has not been uniformly reported [18], whether lowered activity was a result of reduced mitochondrial content overall or due to specific protein modification causing dysfunction was unclear. However, we recently reported that the diminished activities of mitochondrial complexes I and IV in end-stage heart failure were due to specific post-translational modifications of key protein subunits, rather than a loss of mitochondrial content per se [17].

Raised Oxidative Stress in Heart Failure Contributing to a Decline in Mitochondrial Function

Under normal cellular conditions, around 2–4% of oxygen is incompletely converted to water, resulting in the formation of superoxide, predominantly at complexes I and III [22–24]. In the healthy cell, endogenous antioxidant enzyme systems match the rate of reactive oxygen species (ROS) production by converting superoxide to hydrogen peroxide and then further processed to form water and oxygen. However, in the scenario of excessive ROS production or a deficit of antioxidant systems, as occurs in advanced age, oxidative damage to cellular proteins, lipids and DNA can accumulate. Under certain conditions, superoxide is able to react with nearby membrane phospholipids, including mitochondrial membranes, resulting in the formation of lipid-soluble, highly reactive aldehyde products such as malondialdehyde (MDA) or 4-hydroxy-2-nonenal (HNE) inducing further damage to lipids, proteins and even mitochondrial DNA. Raised levels of ROS and lipid peroxidation products have been measured in the tissues [17, 25, 26] and pericardial fluid [27] of heart failure patients, and have shown to correlate with contractile dysfunction [27]. In addition, protein carbonyls, formed by the oxidation of sensitive amino acid groups such as the cysteine and lysine residues, have been reported to be elevated in a range of human diseases, but only more recently has a strong correlation between

myofilament oxidation and systolic failure been reported in human end-stage heart failure [28]. The most abundant myofilament modifications augmented in end-stage systolic failure patients have been identified to involve carbonylation, disulphide cross-bridge formation and S-nitrosylation of actin and tropomyosin. These structural modifications of the contractile apparatus impede contractile function which is progressively exacerbated as more mitochondria are themselves impacted by oxidative modifications and reactive intermediates that can no longer be adequately cleared in the face of elevated oxidative work stress.

As HNE is formed by superoxide-induced oxidation of membrane polyunsaturated fatty acids (PUFA) such as arachidonic acid, not surprisingly, raised HNE levels have been measured after ischemia-reperfusion [29–31] and in LV tissues and plasma of heart failure patients [32–34]. HNE attacks proteins at histidine, cysteine and lysine residues, resulting in disruption of three-dimensional folding and interfering with enzymatic function [29]. When applied directly to mitochondrial preparations, HNE inhibits state III coupled respiration, resulting in a loss of ATP production [33]. In isolated rat cardiomyocytes, HNE treatment increased ROS levels, resulting in calcium overload leading to hypercontracture [34]. In our study, we demonstrated that specific protein subunits within complexes I, III and IV were targets of post-translational oxidative modification by HNE, carbonylation and protein nitration [17], particularly those associated with redox centres. Selective targeting of specific oxidative phosphorylation protein subunit sites has also been demonstrated in other models of cardiomyopathy, while increased binding by HNE has been measured in several Krebs cycle enzymes, particularly those associated with complex I, following ischemia-reperfusion injury [35–37]. Notably, in fibroblasts isolated from patients with complex I deficiency, increased superoxide levels inversely correlated with CI activity and were also linked to loss of mitochondrial membrane potential [38].

Influence of Krebs Cycle Enzymes on Metabolic Flux

As remarked by Sadek et al. [37], loss of oxidative phosphorylation activity alone is not sufficient to fully account for the deficit in respiratory rates, rather previous studies indicate that NADH levels have a more direct effect on respiration as NADH ‘feeds’ the respiratory chain [39]. Three of the eight Krebs cycle enzymes; α-ketoglutarate dehydrogenase (KGDH), isocitrate dehydrogenase (ICDH) and malate dehydrogenase (MDH) directly supply NADH to complex I, of which the first two are also regulators of Krebs cycle flux. Due to the close spatial arrangement between complex I and these particular Krebs enzymes, we postulated that in the failing heart, ROS produced from complex I may also affect closely-associated Krebs cycle enzymes. Indeed, as predicted, activities of ICDH and MDH were lower in human failing tissues [17], consistent with impairment seen following ischemia-reperfusion injury [37]. Studies in spontaneously-hypertensive rat strains which progress to failure have demonstrated a loss of ICDH activity in the early

hypertrophic phase, which was associated with increased HNE modification. Furthermore, dose-response studies in isolated mitochondria show a direct correlation between HNE binding and loss of ICDH activity, highlighting that ICDH is a direct target and is inactivated by HNE in hypertrophy [40]. Myocardial adaptation following myocardial infarction in rats involves early perturbations of Krebs cycle flux and concomitantly correlates with diminished contractile function, which subsequently progresses to also involve a loss of pyruvate dehydrogenase flux and worsening systolic ejection fractions [41]. Activity of aconitase, a Krebs cycle enzyme that is highly vulnerable to oxidative stress, is also significantly lower in the failing heart [17], highlighting the role of oxidative stress in the pathology of heart failure. Notably, these studies highlight specific loss of activity in both oxidative phosphorylation and Krebs cycle enzymes that is selective and not due to changes in protein abundance [17], but rather post-translational modifications such as those induced by oxidative injury.

In addition to the overall energetic flux, alterations to the NADH/NAD ratio has a profound influence on REDOX status and other downstream pathways, in particular that of the sirtuin pathways. NADH also serves to replenish NADPH stores, which are essential for the reduction of glutathione, one of the most important cellular antioxidant systems. Also crucial to REDOX and mitochondrial NADPH pools is a mitochondrial protein complex that has had limited study in the human heart failure setting, nicotinamide nucleotide transhydrogenase (NADPH-transhydrogenase, Nnt) [42]. One role of Nnt is the maintenance of NADPH-dependent ratio of reduced to oxidised glutathione, and other NADPH-dependent thiols such as thioredoxin, peroxiredoxins and glutaredoxin, in the regulation of cellular oxidative stress. Nnt activity is coupled to the mitochondrial proton motive force during ATP synthesis and is a major source of NADPH. We have previously reported that relative to non-failing myocardium, Nnt activity is markedly diminished due to protein carbonylation, concomitant with diminished glutathione reductase activity, lower NADPH and GSH/GSSG and augmented oxidized glutathione in chronic end-stage heart failure patients [42].

Sirtuin 1 (SIRT1) also has an important role in regulating energy metabolism, as a nuclear metabolic sensor, SIRT1 directly couples the cellular metabolic status (via NAD^+) to the regulation of gene expression via deacetylation of histones, transcription factors and co-factors [12]. SIRT1 can also shuttle between the nucleus and cytosol in response to various environmental stimuli, thus acting as a sensor for the overall energetic status of the cell, primarily through the availability of NAD^+, which is involved in both redox and metabolic reactions. SIRT1 also plays an important role in activating cell survival machinery. SIRT1 expression has shown to be reduced in mouse hearts subjected to ischemia-reperfusion (IR), while preconditioning increased SIRT1 and antioxidant levels and lowered expression of pro-apoptotic factors. In the same study, mice overexpressing SIRT1 showed improved post IR recovery, while SIRT1 induced expression of protective factors and antioxidants such as Mn-SOD, thioredoxin 1, and Bcl-xL, while negatively regulating pro-apoptotic factors such as Bax and cleaved caspase-3 [43]. Intravenous infusion of 10–20 mg/kg NAD^+ into rats has furthermore shown to reduce post IR infarct size

by up to 85%, and significantly reduce apoptosis following ischemia-reperfusion by decreasing levels of Bax, cleaved caspase 3 and TUNEL staining, while increasing levels of pro-survival Bcl [44].

Inhibition of oxidative phosphorylation and Krebs cycle enzymes, as may occur following IR injury or heart failure, may prevent the downstream oxidation of NADH by the respiratory chain, resulting in a chemically reduced system and a build-up of NADH. This could be predicted to result in a lowering of the NAD^+/NADH ratio, which in turn would lower expression of SIRT1. Indeed, measures of SIRT1 expression in cardiomyocytes isolated from human failing hearts have shown SIRT1 levels to be reduced to around 55% of that in healthy controls. This was accompanied by increases in pro-apoptotic markers such as Bax, increased numbers of TUNEL-positive cardiomyocytes, oxidative stress (8-OH-dG) and lowered levels of antioxidants such as Mn-SOD, thioredoxin 1 and anti-apoptotic Bcl-xL in advanced failure [14]. Furthermore, extensive studies in complex I-deficient (NDUFS4$^{-/-}$ mice showed a significant decrease in the NAD^+/NADH ratio, despite similar levels of NAD^+, which was accompanied by increased sensitivity to calcium-induced mitochondrial permeability transition and pore opening. Mitochondrial permeability transition sensitivity could be alleviated by treatment with nicotinamide mononucleotide (NMN), an NAD^+ precursor which has been shown to raise NAD^+ levels, thus restoring the NAD^+/NADH ratio [45]. Hence, NAD^+ and energetic status may have a profound effect on cell survival and contribute to the pathogenesis of heart failure. An important implication in the underlying dysfunction that contributes to the augmented NADH and decreased NAD^+/NADH ratio in advanced left ventricular failure relates to the sirtuin deacetylases which require NAD+ for their activity. Subsequent decline in sirtuin deacetylase function may limit the regulation of acetylation such that a multitude of lysine-rich proteins eventually become hyperacetylated in human heart failure, in particular these include regulators of the mitochondrial permeability transition pore, (cyclophilin D, oligomycin sensitive conferral protein site of the adenosine triphosphate synthase) and components of the malate-aspartate shuttle [45, 46]. Thus hyperacetylation of critical mitochondrial proteins further exacerbates loss of REDOX regulation in heart failure.

PDH and Substrate Availability

One of the characteristic effects of cardiac hypertrophy, and to a greater extent, heart failure is the shift towards glycolysis, and away from fatty acid oxidation. The effect of this change is to create a more efficient means of energy supply. However, this comes at the expense of energy output, with glycolysis producing two molecules of ATP per molecule of glucose as opposed to 38 ATPs per molecule during oxidative phosphorylation. Substrate selection within the cell is governed at multiple levels; at the genetic level through expression of metabolic enzymes and receptors, as well as allosteric and substrate feedback regulation. One of the major regulators of this process is the peroxisome proliferator-activated receptors (PPAR),

which govern expression of key enzymes and receptors involved in energy metabolism such as glucose receptors GLUT1 and GLUT4, fatty acid oxidation enzymes and pyruvate dehydrogenase kinases (PDKs) [47, 48]. Choice of substrate selection also occurs at ‘gateways’ to major metabolic pathways, such as pyruvate entry into the mitochondrion and at key crossroads between two pathways.

Pyruvate dehydrogenase (PDH) sits at the crossroad between the glycolytic and fatty acid oxidation pathways, catalysing the conversion of pyruvate from glycolysis to Acetyl CoA, and is therefore a major regulator of substrate selection for ATP synthesis. Pyruvate entry into the mitochondrion is regulated via the inner membrane MPC, whose molecular identity has only recently been discovered [49–51]. The PDH enzyme complex within the mitochondrial matrix is itself regulated by a group of four inhibitory kinases, each of which has varying affinities for various metabolic intermediates such as NADH, ATP and Acetyl CoA. When the intermediates are in a highly reduced state (e.g. high [NADH]/[NAD] or [Acetyl CoA]/CoA], PDKs are activated, inhibiting the PDH complex and thus limiting downstream metabolism through the Krebs cycle [51]. In turn, PDH phosphatases (PDPs) phosphorylate and hence activate PDH. PDP1 is activated in response to Ca^{2+}, producing an increase in PDH activity and therefore energy availability in times of high energy demand such as muscle contraction. PDH can therefore provide rapid metabolic regulation in response to altered energy demands.

In addition to regulatory and allosteric feedback mechanisms, post-translational regulation of proteins through acetylation and other mechanisms has only been recognized more recently and plays a pivotal role in the regulation of mitochondrial energetics. One such protein under this form of regulation is the MPC protein. MPC regulates the entry of pyruvate into the mitochondrion as an inner membrane channel and thus serves as a checkpoint to regulate metabolic flux. Protein binding and deacetylation of MPC1 by sirtuin 3 (SIRT3), which is involved in mitochondrial homeostasis, enhances its activity [52]. MPC1 protein expression is deleted or underexpressed by up to 85% in a number of cancer cell lines, showing a strong correlation with poor prognosis, which is consistent with an increased reliance on glycolytic metabolism in these cells [53]. Recent results from our own lab indicate a decrease in MPC1 and MPC2 expression in the failing heart (unpublished- in preparation). Notably, MPC protein expression is crucial to conferring a survival advantage in post-ischemic infarcted tissues [54]. In a separate study, suppression of MPC1 produced cells which were able to maintain Krebs cycle activity and cell growth via alternative pathways to Acetyl CoA, demonstrating that inhibition of the MPC pathway produced greater metabolic flexibility compared to other downstream pathways [55]. Loss of the MPC1 protein in cardiac-restricted knockout mice has reportedly resulted in mice with normal cardiac function at 8 weeks [56], despite several markers of hypertrophy, but which only develop overt systolic dysfunction by the age of 18 weeks. Thus this study demonstrates that pyruvate uptake is essential for normal cardiac function [56].

Increased expression of PDK2 and PDK4 has been measured in the tissues of heart failure patients, reflecting an increased reliance on glycolytic metabolism,

similar to that of fetal phenotype [57]. However, gene expression profiling reveals that it is not a reversion to a fetal profile per se which occurs in heart failure, but rather a downregulation of adult heart isoforms which occurs, allowing the fetal markers to dominate [57]. Conversely, overexpression of PDK4 in a mouse model in itself showed no noticeable effect on cardiac function. However, when introduced in a mouse strain which constitutively expressed calcineurin, which causes hypertrophy, this resulted in increased fatty acid oxidation, cardiac fibrosis and a dramatic rise in mortality [58]. Doxorubicin, a cancer drug known to cause cardiomyopathy, inhibits pyruvate transport, possibly by interacting with cardiolipin molecules surrounding the pyruvate carrier [59], while a decline in pyruvate transport concomitant with lowered cardiolipin levels reportedly occurs in the hypothyroid rat [60]. Thus loss of metabolic flexibility can have profound effects on cardiac function and survival.

Metabolic remodelling involves a progressive alteration in substrate supply, initially by activation and inactivation of pathways involved in fuel selection to provide the most efficient source for that particular environment. At the second level, genetic alterations lead to adaptation and eventually maladaptation of the heart involving metabolic inflexibility, such that that heart is no longer able to modify its metabolism based on it metabolic state and condition [61]. The shift in gene expression to a fetal-like pattern is associated with concomitant limited energy availability due to increased reliance on glucose-based metabolism that is unable to supply sufficient ATP to meet total energy needs.

Cardiolipin and Oxidative Phosphorylation Supercomplex Proteins

Cardiolipin is synthesized de novo within the mitochondrion and is resides almost exclusively within the mitochondrial inner membrane [62, 63]. Cardiolipin (CL) contains four acyl chains attached to a glycerophosphate backbone. Newly synthesized CL contains a mixture of acyl chains, but is enriched post-synthesis to contain four linoleic acid (18:2) side chains by two separate pathways, one of which is the acetyltransferase Tafazzin (TAZ), to form the predominant species tetralinoleoyl-cardiolipin (L_4CL). As they are embedded within the mitochondrial inner membrane, many membrane proteins including complexes I, III, IV, the adenine nucleotide translocase (ANT) and ATP synthesis require a close molecular association with CL for optimal function, in fact, detergent solubilized protein/CL complexes stripped of CL result in loss of enzymatic function [62, 63]. Furthermore, ischemia-reperfusion in isolated rat hearts results in losses of CI, CIII or CIV activity concurrent with decreased CL levels [64–66], with raised mitochondrial ROS following reperfusion contributing to both protein and lipid peroxidation. Notably, in these same studies, enzyme activity could be restored following enrichment of mitochondrial preparations with CL liposomes, indicating CL played a critical role

in respiration. Not only is CL required for enzymatic activity, but CL must be in the L_4CL configuration for optimal activity to occur. It is precisely for this reason that hearts from Barth syndrome patients, in which there is a mutation in the TAZ gene, often suffer from severe dysfunctional oxidative phosphorylation leading to cardiomyopathy [67, 68]. Lowered total cardiolipin, in particular the L_4CL species, have been reported in the tissues of heart failure patients [69].

Cardiolipin is not only required for optimal activity of electron transport chain protein complexes, but has more recently been attributed as the structural 'glue' which holds the mitochondrial respiratory chain together. As opposed to earlier stepwise 'linear' arrangement models of electron transfer, these mitochondrial protein complexes appear to be arranged as 'supercomplexes', in particular those of complexes I/III/IV and the ANT/ATP synthase [70, 71]. These supercomplexes provide stability and maximize efficiency of electron transfer, providing optimal spatial orientation between various enzyme components of the respiratory chain [70]. Studies in a canine model of heart failure have demonstrated an increased dissociation of supercomplex formation and a greater proportion of ETC complex enzymes in loose formation, resulting in decreased capacity for electron transfer and ATP synthesis [72]. Together with a decreased enzyme activity by virtue of lowered CL availability, CL loss in heart failure further exacerbates the problem by decreasing supercomplex formation.

Cardiolipin and 'the Pore'

Mitochondria possess an intrinsic mechanism which initiates cell death following lethal cell damage. Cell death may occur by necrotic or apoptotic cellular pathways, of which the latter occurs by a programmed pathway involving numerous cellular messengers and activators. One mitochondrial pathway involves the opening of a large pore spanning the outer and inner membranes, resulting in the release of cytochrome *c* from the intermembrane space and the initiation of cell death by apoptosis. The mitochondrial permeability transition pore is understood to involve the adenine nucleotide translocase (ANT) and voltage-dependent anion channel (VDAC) as well as attached cyclophilin D [73]. Trigger factors that augment sensitivity for permeability transition pore opening include high [Ca^{2+}], ROS, hyperacetylation or ATP depletion, all of which may be present under conditions of heart failure [13, 46, 73]. Recent studies have also attributed a role for cardiolipin in the regulation of mitophagy, the controlled process of removal of defective mitochondria. Studies in neuronal cells have demonstrated cardiolipin externalization to the outer membrane acts as a trigger for mitophagy-induced cell death, with knockout of CL synthesis decreasing the delivery of mitochondria to autophagosomes [74]. Furthermore, knockout of the TAZ gene in mouse embryonic fibroblasts (MEFs), results in defective mitophagy and reduced mitochondrial degradation in these cells, accompanied by impaired oxidative phosphorylation and increased oxidative stress,

which may partially explain the raised numbers of dysfunctional mitochondria present in tissues of Barth Syndrome patients [75].

Biogenesis Versus Mitophagy

Whether mitochondrial biogenesis and their breakdown (mitophagy) play a significant role in heart failure has only recently been examined [3]. Gross morphological changes such as tissue fibrosis and hypertrophy have been well described, however whether the surviving tissue contains a reduced proportion of mitochondria has only recently been determined. Karamanlidis [76] and associates have described a loss of mitochondrial DNA in human heart failure, attributed not to an alteration in genetic control (expression of PGC-1α), but rather due to increased oxidative damage to DNA, as measured by a 50% increase in 8-OH-dG. Furthermore, mitochondrial content depends on the etiology of heart failure, with mitochondrial DNA content being increased in patients with dilated cardiomyopathy, yet unchanged in those from ischemic heart disease [77]. Mitochondria also had a greater percentage of mutations and deletions caused by oxidative damage in the dilated cardiomyopathic hearts compared to ischemic heart disease. Despite this, both heart failure subgroups showed lower oxidative phosphorylation capacity. It could be surmised that increasing mitochondrial content serves to increase oxidative phosphorylation capacity, however, in the case of the abovementioned study [77], increasing mitochondrial content actually served to raise ROS levels, further exacerbating the damage. Patients with defective oxidative phosphorylation capacity due to a genetic mutation in one of the electron transport chain proteins frequently display increased numbers of morphologically irregular mitochondria as a compensatory mechanism [77, 78]. However, due to the tight spatial arrangement of the myofibrils, the increased number of mitochondria may push myofibrillar proteins out of alignment, thus providing additional interference with contractile function [78]. Thus heart failure progression can feature a heterogeneously localized increase in the total numbers of defective mitochondria, interspersed with a gradually diminishing pool of normally functioning mitochondria, struggling to sustain cardiac output.

Conclusion

The failing heart is characterized by a complex interplay between defective mitochondrial proteins, oxidative damage and other molecular modifications to cellular proteins, lipids and DNA and altered signalling pathways, resulting in insufficient energy production. This state progressively diminishes the capacity of the cell to carry out normal energy turnover, cellular membrane and extracellular matrix turnover, ion homeostasis, signalling and contractile function. Restoring the energetic status, reducing cellular damage and augmenting lipid and protein turnover are key

to targeting treatments for systolic heart failure. Our current understanding of mitochondrial dysfunction in heart failure identifies a plethora of targets that must be tackled concomitantly, many specifically mitochondrial, only some of which have been discussed in this brief review.

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Chapter 5
Mitochondrial Mutations in Cardiac Disorders

Sung Ryul Lee and Jin Han

Mitochondria are indispensable organelles for generating adenosine triphosphate (ATP) in eukaryotic cells via the electron transport chain (ETC) and oxidative phosphorylation system (OXPHOS). Additionally, numerous biological functions, including ATP transport, production of heat, metal homeostasis, and stress signaling and defense responses, involve mitochondria [1–4]. The rhythmic contractions of the heart ensure oxygenation of the organs in the body and their functions depend on mitochondrial aerobic energy production by ETC and OXPHOS [5]. Thus, mitochondrial diseases preferentially affect the heart, as impaired cardiac conditions are associated with mitochondrial dysfunction due to defects in either OXPHOS or ETC [6–8]. Numerous efforts to identify the genetic determinants of cardiovascular disease (CVD), including high blood pressure, coronary heart disease, cardiomyopathy, heart failure, and stroke, have been directed primarily to nuclear genomes [9]. For example, nuclear gene mutations that regulate mtDNA maintenance and replication, such as mitochondrial transcription factor A (TFAM), mtDNA polymerase γ (POLG) as a Mutator model, and PEO1 (Twinkle) as a Deletor model [10], have been increasingly recognized for their cardiac involvement. The occurrence and progress of CVD is affected by a combination of genetic, environmental, and behavioral factors that influence an individual's susceptibility to known disease risk factors [11]. In addition, numerous forms of CVD are not completely understood by the anatomic paradigm of medicine and Mendelian genetics [11–13]. Therefore, the

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common disease-common variant hypothesis is difficult to apply to most common human age-related diseases such as diabetes, metabolic syndrome, and neuronal disorders [11, 12, 14]. Mitochondrial dysfunction due to mtDNA mutations appears to play a role in the development of cardiac disorders and severe exercise limitation [6, 8, 15–18]. Remarkable advances have been made in understanding the basic mitochondrial genetics and the relationship between inherited mutations and/or sporadic mutations and disease manifestation by mitochondrial diseases, as well as in identifying acquired mtDNA mutations in CVD, cancer, and aging [19]. However, the phenotypic display and genetic background of mitochondrial disorders are extraordinarily diverse [13].

Mitochondrial cardiomyopathy (MCM) is defined as an OXPHOS disease characterized by abnormal number, structure, or functions of cardiac mitochondria [7, 20–22] and may be resulted in cardiac manifestations, such as: (1) structural alterations (hypertrophic or dilated cardiomyopathy, left ventricular myocardial noncompaction), (2) disturbance in heart rate and/or rhythm (ventricular tachycardia, Wolff-Parkinson-White syndrome, heart block, or arrhythmias), and (3) final functional impairment as heart failure [23–27]. Mitochondrial diseases and mtDNA mutations are not restricted to mitochondria. Two pathways have been suggested to explain the etiology of MCM. Primary MCMs are caused by mutations in mtDNA or nuclear DNA [28]. Secondary MCMs are induced by a primary insult affecting mitochondrial function either indirectly or by damaging the mitochondrial genome. Inadequate levels of mtDNA and its mutations can cause defects in the synthesis of key subunits of ETC complexes. In this chapter, we summarize the current understanding of mtDNA mutations, focusing on cardiac disorders, and briefly address the epigenetic modifications of mtDNA for future studies on cardiac disorders. Finally, current strategies for overcoming the effects of mitochondrial mutations and/or dysfunction on cardiac disorders are addressed.

Mitochondrial DNA and Its Genetics

General Aspects of Mitochondria

Mitochondria are spherical, double membrane-bound semiautonomous organelles. Unlike developing non-cardiac cells or cardiomyocytes, mitochondria in adult cardiac myocytes show distinct distributions: (1) interfibrillar, (2) subsarcolemmal, and (3) perinuclear populations [29]. Thus, adult cardiac mitochondria may be relatively firmly fixed and are subjected to very low-amplitude fluctuations or vibrations [30, 31]. In the adult heart, the machinery required to alter mitochondrial shape in terms of mitochondrial fission involves fission proteins, but their physiological functions are extensively under investigations [29]. Mitochondria have their own DNA located in its matrix within the inner membrane (Figs. 5.1 and 5.2 and Table 5.1).

Since most mtDNA-encoded proteins are extremely hydrophobic peptides, they cannot easily enter the mitochondria without the help of mitochondrial translocation

machinery [32]. Molecules smaller than 5 kD can pass through the mitochondrial outer membrane through porins. However, larger proteins containing mitochondria signal sequence can only pass through multiprotein translocase complexes such as the transporter inner membrane, transporter outer membrane, and other transporter proteins [33–35]. The TCA cycle, which produces NADH and $FADH_2$, moves electrons into the ETC. All the components of the ETC and ATP synthase complex are located in the inner mitochondrial membrane [36]. Energy production in the mitochondria is coordinated by these steps and thus any defects in this complex process, in terms of quantity and/or functionality, can cause mitochondrial dysfunction [36].

Mitochondria are composed of over 1,000 proteins [37–39], but mtDNA encodes only 13 proteins (Fig. 5.1 and Table 5.1). Nearly all protein components involved in mitochondrial biogenesis, including transcription, replication, and protein synthesis, are encoded by nuclear DNA (Fig. 5.3). Mitochondria also contain several proteases that control protein turnover to match the need for removal of defective peptides such as misfolded proteins, and regulate gene expression in response to various cellular signals [34, 40, 41]. Mitochondrial dysfunction can be caused by the mitochondria, nucleus, or both (Tables 5.1 and 5.2), as the maintenance of mitochondrial function is finely controlled by protein synthesis and degradation. Therefore, mitochondrial dysfunction can result from; (1) single OXPHOS complex deficiencies due to mtDNA or nuclear mutations, (2) multiple OXPHOS deficiencies, (3) coenzyme Q10 deficiency, (4) mitochondrial depletion syndrome, (5) disorders of 3-methylglutaconic aciduria, (6) disorders of mitochondrial fatty acid β-oxidation, and (7) disorders of intramitochondrial organic acid metabolism (due to deficiency of propionyl-CoA carboxylase or methylmalonyl-CoA mutase or its cofactor adenosylcobalamin) with secondary mitochondrial dysfunction [1, 45, 46]. These mitochondrial dysfunctions inhibit mitochondrial O_2 consumption, collapse the membrane potential, and reduce ATP levels by causing an imbalance between energy intake and expenditure [47]. Additionally, mitochondrial dysfunction can impair other numerous aspects of cell metabolism and homeostasis, such as cellular calcium regulation and apoptosis [48]. Dysfunctional mitochondria can be removed from the cell by mitophagic processes, which are controlled by nuclear-encoded proteins [13, 49, 50].

mtDNA

Many aspects of mtDNA differ from those of nuclear DNA [19, 51]. As shown in Fig. 5.1, the human mtDNA is 16,569 base pair (bp) in length and is characterized by high gene density (nuclear DNA and mtDNA; ~1 per 40,000 bp and ~1 per 450 bp, respectively) and differences in codon use [19, 40, 44, 52]. Except for the D-loop containing the control elements for the transcription and replication of mtDNA, mammalian mtDNA is highly compact without introns. mtDNA is packaged into nucleoid structures (Fig. 5.2) composed of TFAM, single-stranded DNA-binding protein (mtSSB), and twinkle, which is a mitochondrial DNA helicase [1, 40, 53, 54]. Typically, one or two copies of mtDNA are packaged with the help of

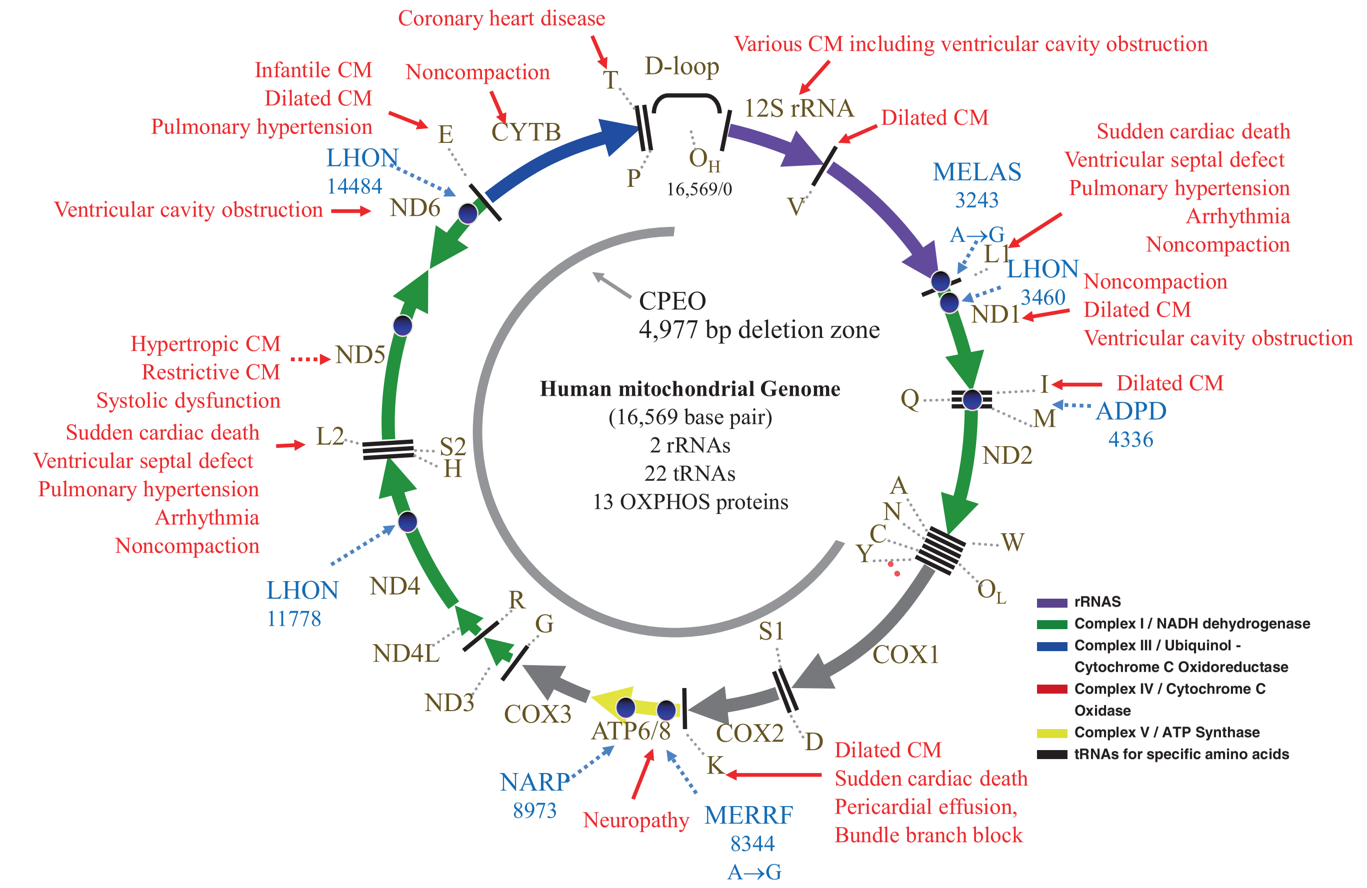
Coronary heart disease
Various CM including ventricular cavity obstruction
Infantile CM
Dilated CM
Pulmonary hypertension
Noncompaction
T
D-loop
12S rRNA
Dilated CM
E
CYTB
LHON
14484
P
O_H
16,569/0
V
MELAS
3243
Sudden cardiac death
Ventricular septal defect
Pulmonary hypertension
Arrhythmia
Noncompaction
Ventricular cavity obstruction
ND6
A→G
L1
LHON
3460
ND1
Noncompaction
Dilated CM
Ventricular cavity obstruction
CPEO
4,977 bp deletion zone
Hypertropic CM
Restrictive CM
Systolic dysfunction
ND5
Human mitochondrial Genome
(16,569 base pair)
2 rRNAs
22 tRNAs
13 OXPHOS proteins
Q
I
Dilated CM
M
ADPD
4336
ND2
Sudden cardiac death
Ventricular septal defect
Pulmonary hypertension
Arrhythmia
Noncompaction
L2
S2
H
A
N
C
Y
W
O_L
LHON
11778
ND4
R
G
ND4L
ND3
COX3
S1
COX1
ATP6/8
COX2
D
K
Dilated CM
Sudden cardiac death
Pericardial effusion,
Bundle branch block
NARP
8973
Neuropathy
MERRF
8344
A→G
rRNAS
Complex I / NADH dehydrogenase
Complex III / Ubiquinol - Cytochrome C Oxidoreductase
Complex IV / Cytochrome C Oxidase
Complex V / ATP Synthase
tRNAs for specific amino acids

Fig. 5.1 Mitochondrial DNA (*mtDNA*) and pathogenic mtDNA mutations associated with cardiac manifestations. Mitochondrial respiratory chain complexes are composed of approximately over 80 polypeptides. Human mtDNA codes for 7 of the 43 subunits of complex I, one of the 11 subunits of complex III (cytochrome b, CYTB), 3 of the 13 subunits of complex IV (COX I, II, III), and 2 of 16 subunits of complex V (ATPases 6 and 8). It also codes for 2 rRNAs and 22 tRNAs for mitochondrial protein synthesis. Mutations in mtDNA can occur in any regions, while mutations in the D-loop region are typically not retained because of its important regulatory roles in transcription, translation, and replication. Some examples of mtDNA mutations associated with known human genetic diseases and cardiac manifestations are marked as *blue* (*broken arrow*) and *red* (*solid arrow*), respectively. *Abbreviations: ADPD* late-onset Alzheimer's disease, *COX* cytochrome C oxidoreductase, *CPEO* chronic progressive external ophthalmoplegia, *LHON* Leber's hereditary optic neuropathy, *MELAS* mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, *MERRF* myoclonic epilepsy with ragged red fibers, *NARP* neuropathy, ataxia, and retinitis pigmentosa, *ND1-6* component of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I), O_H heavy strand origin of replication, OXPHOS mitochondrial oxidative phosphorylation, *P* promoter of heavy and light strands

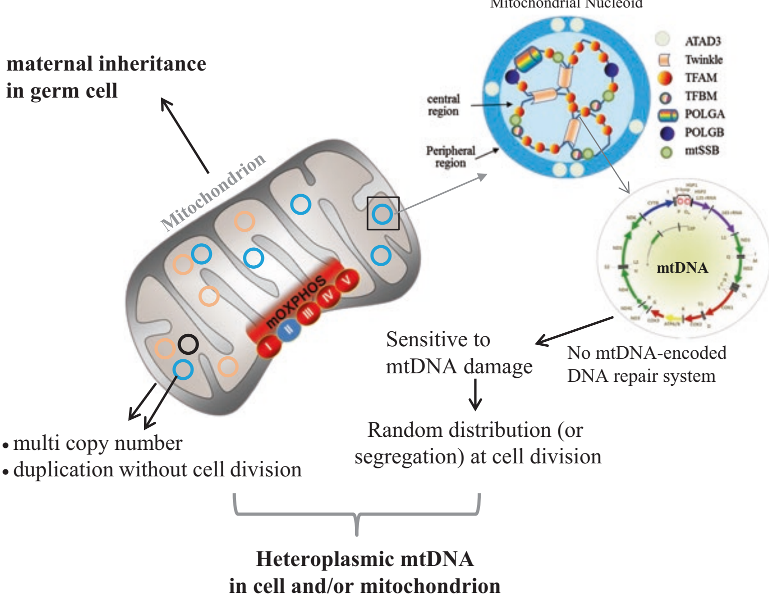

Fig. 5.2 Characteristics of mitochondrial genetics. One cell may contain several thousand mitochondria, and each mitochondrion contains one to ten copies of mtDNA. The replication of mtDNA is less dependent on nuclear DNA division and thus shows continuous replicative behavior. The mtDNA is encapsulated by the mitochondrial nucleoid. In the nucleoid structure, mitochondrial transcription factors, such as TFAM, TFBM, POLGA, POLGB, MTSSB, and Twinkle, are located in the central region along with the mtDNA, whereas proteins have an indirect relationship with mtDNA (e.g. ATAD3) and are arranged peripherally. This nucleoid structure results in a random distribution of mtDNA during cell division. mtDNA encounters more mutation-prone conditions because of its proximity to ROS-producing sites, limited presence of mtDNA repair proteins encoded by nuclear DNA, continuous replication state, and morphological changes during fission and fusion processes. In the mitochondria, there is less selective pressure to propagate wild-type mtDNA, and thus mtDNA mutations easily survive and are randomly distributed. Additionally, over 1,000 mitochondrial proteins and substrates are supplied from the cytosol, and the nucleus can modulate mitochondrial homeostasis, and therefore mitochondrial dysfunctions are not solely affected by mtDNA mutations. These factors result in more complexity in mitochondrial genetics, unlike Mendelian genetics of the nucleus. This characteristic of mitochondrial genetics results in complex phenomena as well as maternal inheritance in germline cells as a heteroplasmy and threshold effect in phenotypic presentations. *Abbreviations: ATAD3* ATPase family AAA Domain-containing protein 3, *mtDNA* mitochondrial DNA, *mtSSB* mitochondrial single-stranded DNA-binding protein, *POLGA* mtDNA-specific DNA polymerase gamma A (catalytic subunit), *POLGB* mtDNA polymerase γ accessory subunit, *TFBM* mitochondrial transcription factor B

interacting factors into a mean size of ~100 nm nucleoid [55, 56]. Within mitochondria, mtDNA is associated with a variety of interacting proteins involved in its packaging, replication, and transcription. Through the action of these proteins, mtDNA is integrated into mitochondrial bioenergetics and exhibit signaling capabilities [57, 58]. If these mtDNA-interacting proteins are affected by insult or stress, mtDNA can be lost.

Table 5.1 Genes in the mitochondrial respiratory chain. Mitochondrial respiratory chain complexes are composed of approximately 80 polypeptides constituting the electron transport chain: 13 are encoded by mtDNA and the remaining are synthesized in the cytosol and translocated into the mitochondria. Mutations in respiratory chain complexes result in single or multiple complex deficiencies originating from either sporadic mutations or inheritance. Italic characters indicate disease-causing mitochondrial mutations

	Mitochondrial respiratory chain				
	Complex I	Complex II	Complex III	Complex IV	Complex V
	NADH ubiquinone oxidoreductase	Succinate ubiquinone oxidoreductase	Ubiquionol-cytochrome c oxidoreductase	Cytochrome c oxidase	F_0F_1 ATPase
mtDNA-encoded	7	0	1	3	2
Nuclear DNA-encoded subunit	~40	4	10	10	~15
Diseases or phenotypes related to defects	DCM HCM Leigh's Syndrome Leukodystrophy *LHON* LHON and dystonia LVNC *MELAS* WPW	DCM HCM Leigh's Syndrome LVNC Paraganglioma Pheochromocytoma	*DCM* Encephalomyopathy GRACILE *HCM* *HiCM* Leigh's Syndrome Septo-optic dysplasia Sporadic myopathy	*ALS-likesyndrome* Cardioencephalomyopathy DCM Encephalomyopathy HCM Hepatopathy HiCM Leigh's Syndrome Leukodystrophy and tubulopathy *Sporadic anemia* *Sporadic Myopathy*	*FBSN* *HCM* *LVNC* *MILS* *NARP*

ALS amyotrophic lateral sclerosis, *FBSN* familial bilateral striatal necrosis, *DCM* dilated cardiomyopathy, *GRACILE* growth retardation, aminoaciduria, lactic acidosis, and early death, *HCM* hypertrophic cardiomyopathy, *HiCM* histiocytoid cardiomyopathy, *LHON* Leber's hereditary optic neuropathy, *LVNC* left ventricular noncompaction, *MELAS* Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome, *MILS* maternally inherited Leigh's syndrome, *NARP* neurogenic weakness with ataxia and retinitis pigmentosa, *WPW* Wolff-Parkinson-White syndrome

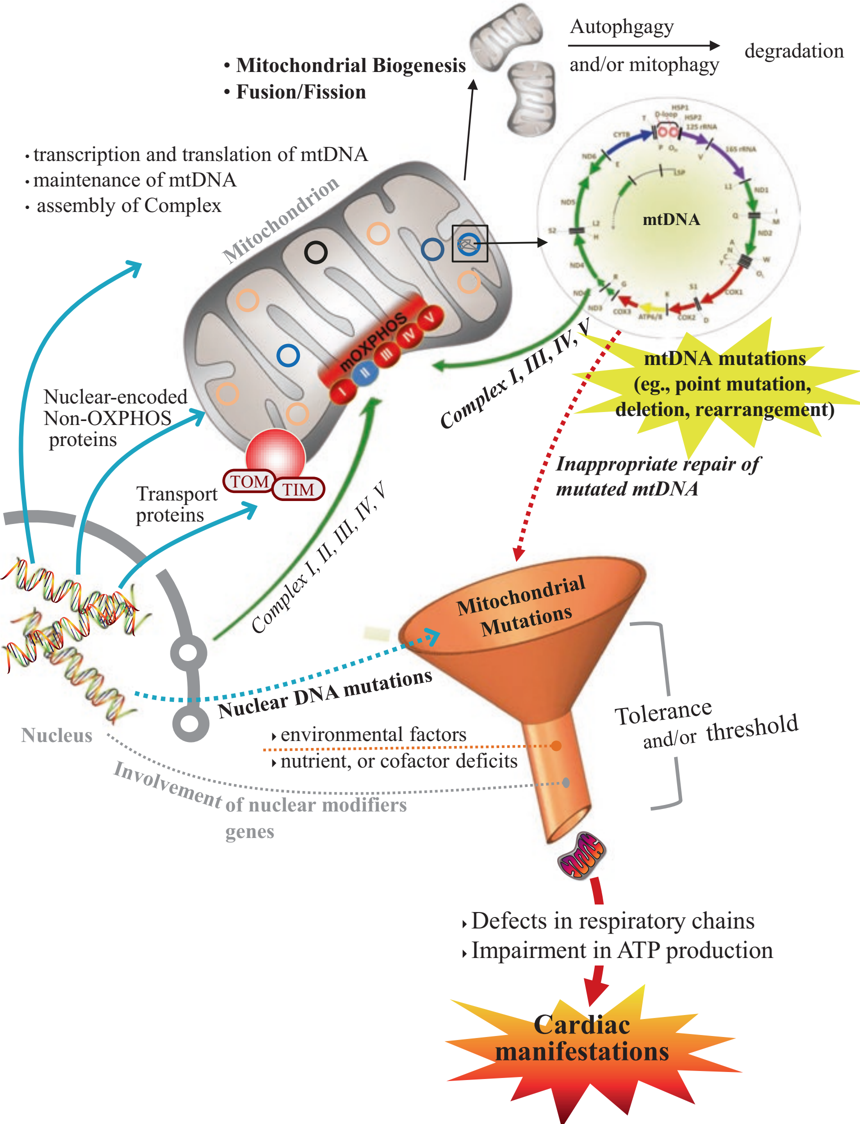

Fig. 5.3 Mitochondrial biogenesis and threshold effects of mitochondrial mutations. Mitochondrial mutations originate from nuclear DNA, mtDNA, or both. Most mitochondrial proteins are encoded by nuclear DNA and imported into mitochondria. In addition to the mitochondrial origin of mtDNA mutations, the occurrence of mtDNA mutations is highly affected by the status of nuclear DNA. Mitophagy can remove damaged mitochondria, but currently the specific removal of mitochondria containing mtDNA mutations is not well-understood. mtDNA mutations can be easily produced in a sporadic manner and these mutations do not always cause mitochondrial dysfunction because of threshold effects, indicating that phenotypic expression of mtDNA mutations must accumulate in mtDNA before presenting as phenotypic or functional anomalies. This threshold level varies among different tissues. Perturbations in mitochondrial metabolism also causes dysfunction in the OXPHOS system, or mtDNA mutations may stimulate increased mitochondrial biogenesis to compensate for mitochondrial dysfunction. The incomplete or indirect exhibition of mtDNA mutations can be influenced by pharmaceutical or environmental exposures and nutrient or cofactor deficits. In addition, nuclear modifier genes, which often have at least two alleles, one that exacerbates disease and another that suppresses the disease, have been investigated to better understand the incomplete penetrance of mitochondrial mutations

Table 5.2 Diagnosis of suspected mitochondrial diseases

Mutation or deficiency	Method	Sample
Mitochondrial DNA mutations	Affymetrix MitoChip 2.0 DGGE dHPLC NGS PCR/RFLP analysis PCR/ASO dot blot analysis Southern blot hybridization analysis	Tissue biopsy
Nuclear DNA mutations	Exome sequencing PCR/RFLP analysis [42–44] Southern blot hybridization analysis	Tissue biopsy
NADH-coenzyme Q reductase (complex I) deficiency	Oximetry (polarographic measurement)	Tissue biopsy, permeabilized fiber, mitochondria
NADH ferricyanide reductase (complex I)	Spectrophotometry	
Succinate cytochrome-c reductase (complex II and/or III) deficiency	Spectrophotometry Oximetry (polarographic)	
Co Q-cytochrome-c reductase (complex III) deficiency	Spectrophotometry Oximetry (polarographic)	
Cytochrome-c oxidase (complex IV)	Spectrophotometry Oximetry (polarographic) Enzyme histochemical assay	Tissue biopsy
>2% subsarcolemmal collection of mitochondria (ragged red fiber) in skeletal muscle	Gomori trichrome staining	Tissue biopsy (frozen section)
Structural abnormalities in mitochondria (number, shape, or size)	Electron microscopy	Tissue biopsy
Other biochemical tests		
Lactate, pyruvate, phosphocreatine measurement	Enzymatic method	Blood or CSF
Amino acid analysis	Tandem mass spectrometry (MS/MS)	Blood or CSF
Organic acid analysis	Column gas chromatography with mass spectrometry	Blood or CSF

ASO allele-specific oligonucleotide, *CSF* cerebrospinal fluid, *DGGE* denaturing gradient gel electrophoresis, *dHPLC* denaturing high-performance liquid chromatography, *NGS* next-generation sequencing, *PCR* polymerase chain reaction, *RFLP* restriction fragment length polymorphism

Biogenesis of Mitochondria: Replication, Translation, and Transcription of mtDNA

Mitochondrial biogenesis can be defined as an increase of mitochondrial mass and number of pre-existing mitochondria [59]. Mitochondrial biogenesis (Fig. 5.3) is coupled with several quality control pathways to maintain mitochondrial integrity via antioxidants, mtDNA repair systems [60], and mitochondrial unfolded protein

response systems [49, 61–64]. Albeit nuclear DNA synthesis is highly dependent on the cell cycle, replication of mtDNA is not dependent on cell cycle [40, 65]. The entire mtDNA molecule can be replicated within 90 min (DNA synthesis rate; 180–270 bp/min), based on in vitro minimal mtDNA replisomes consisting of nuclear-encoded POLG consisting of a catalytic subunit with 5→3′ exonuclease activity (PolgA) and processivity subunit (PolgB), mtSSB, and twinkle [40, 44, 66, 67]. Two possible models of mtDNA replication have been suggested: one is a strand-asynchronous mode initiating at the heavy strand origin (O_H) [66], while the other is a leading-lagging strand initiating at the same location [68].

However, the exact mechanism of human mtDNA replication should be further elucidated. Because numerous proteins are involved in mtDNA replication, a loss in mtDNA number may reflect defects in mtDNA replication factor activity [69]. The transcription of mtDNA is initiated from one of the two promoters on the heavy strand (HSP_1 and HSP_2) and from a single promoter on the light strand through dedicated mitochondrial RNA polymerase, TFAM, and 2B [40, 70]. Except for the D-loop region, approximately 93% of mtDNA must be entirely transcribed to produce properly functioning OXPHOS in cells [52]. Mature mRNA is produced by subsequent processing and modifications in the polycistronic transcript [40, 71]. The quantity of each mRNA, rRNA, and tRNA is not equal, possibly because of post-transcriptional modifications affecting transcript processing, maturation, and stability [72, 73]. Conceivably, coincidence of replication and transcription of mtDNA, which may involve head-on collisions between transcription and replication machineries, can be coupled via the action of a mitochondrial transcription elongation factor (TEFM) [40, 72, 74–76]. This factor may serve as a molecular switch that controls the replication of mtDNA or regulates the number or increases the transcription rate [76]. Translation of bicistronic transcripts [42] from mtDNA are produced by RNA-poor mitoribosomes [50]. In addition, partial stop codons in some transcripts that are polyadenylated [73] and deviate from the standard genetic code [19] are present in mitochondria.

Mitochondrial Genetics

Typically, cell contains a single nuclear genome; in contrast, the mtDNA population in cell or mitochondrion is not homogeneous. Unlike Mendelian genetics governing the nuclear genome, the characteristics of mitochondrial genetics are as follows: (1) nearly all maternal inheritance of disease mutation, (2) polyploidy nature of mitochondrial genome, (3) structure of mitochondrial nucleoid, (4) clonal expansion of mtDNA even in the same mitochondrion, and (5) random mitochondrial segregation [77] occur even in the same cell and thus the ratio of mutant to normal mtDNA can be shifted during the segregation (Fig. 5.2). Clonal expansion of mtDNA and random segregation of mitochondria lead to heteroplasmy, which is defined as the presence of at least two populations of mtDNA molecules such as normal (wild-type)

and mutated DNA [34]. These characteristics of mitochondrial genetics may explain the age-related and even tissue-related variability in mtDNA-related disorders [78].

Mitochondrial DNA Mutations

The heart must effectively respond to variations in the physiological demand by balancing ATP production and metabolic responsiveness [79]. The first mtDNA mutations were identified several decades ago [80, 81] and significant advances have been made in understanding mtDNA defects involved in the progress of mitochondrial disease [27, 44]. Examples are depicted in Table 5.3. mtDNA variants can be classified according to clinical relevance as recent deleterious mutations, ancient adaptive mtDNA mutations, or somatic mtDNA mutations accumulated over time in tissues [82].

In population genetics, the variability introduced into mtDNA sequences by mutations has been used to identify family members from various points in human history. In disease-based epidemiological studies, the prevalence of mtDNA disease was found to be ~1:5,000 and heteroplasmic mtDNA mutations were found in 1:200 newborns [84, 85]. Compared to nuclear DNA genes, mtDNA genes have a very high sequence evolution rate and mutational load (e.g., nuclear DNA vs. mtDNA; 2.5×10^{-8} vs. 3×10^{-6}, $\sim 2.7 \times 10^{-5}$ per base per generation) because of the continuous replication state and high number of mtDNA [82, 86–88]. Both nuclear DNA- and mtDNA-encoded factors co-evolve in response to mutation rate differences through protein–protein interactions in the OXPHOS system, protein-RNA within the mitochondrial ribosome, and protein-DNA involved in mitochondrial replication and transcription [89].

Possible Causes of mtDNA Mutations

Although DNA is a relatively stable molecule, spontaneous decomposition of the nucleic acid structure of mtDNA and numerous reactive oxygen species (ROS) and resulting products may be involved in DNA modifications and mutagenesis [90–92]. Oxidative stress, which is defined as increased production of ROS and accumulation of their chemically active metabolites over the threshold level, leads to spontaneous deamination of mtDNA, base loss, and other modifications [93–96]. Among these, oxidative modification of mtDNA in either sugar (e.g., 8-oxoguanine) or DNA bases is the most common and prevalent type [92]. Oxidative mtDNA damage has been suggested as a causal factor in mtDNA mutation, but this hypothesis is not consistent with the results of recent studies [97, 98]. For example, in oxoguanine DNA glycosylase (OGG1) null mice, OGG1 deficiency did not cause mitochondrial respiratory dysfunction in the heart and liver, even when 8-oxo-2′-deoxyguanosine was produced in excess [99]. Moreover, mice expressing POLG with low fidelity

Table 5.3 Syndromic mitochondrial mutations and onset of cardiovascular manifestations. Genetic defects in nuclear DNA and mtDNA lead to mitochondrial dysfunction. The clinical spectrum in mtDNA disease is wide within both individuals and families. The clinical phenotypes of mitochondrial disorders are extremely heterogeneous and the onset of clinical manifestations is largely depended on the context of mutations in mtDNA and/or nuclear DNA

Mitochondrial diseases	mtDNA mutations (status)	Phenotype or characteristic	Onest
mtDNA depletion syndromes	Nuclear-encoded DNA mutation	Severe muscle weakness, progressive encephalopathy, or liver failure	Early infancy/childhood
Chronic progressive external ophthalmoplegia (CPEO)	Single or multiple mtDNA deletions (heteroplasmic)	Arrhythmia myopathy and fatigue	Late childhood or adult life
Kearns-Sayre syndrome (KSS)	Single, large-scale mtDNA deletion (heteroplasmic)	Arrhythmia, Third-degree heart block	Early infancy/childhood
Leber's Hereditary optic neuropathy (LHON)	Complex I gene mutations (hetero- or homoplasmic)	Cardiomyopathy accompanied by Wolff-Parkinson-White (WPW) syndrome (an aberrant conduction defect)	Late childhood or adult life
Leigh Syndrome (subacute necrotizing encephalomyelopathy)	Complex I, IV, and V gene mutations (heteroplasmic)	Cardiomyopathy and arrhythmia	Early infancy/childhood
Mitochondrial encephalomyopathy with lactic acidosis and stroke like episodes (MELAS)	mt-tRNA gene mutations (heteroplasmic)	Dilated cardiomyopathy and left ventricular hypertrabeculation, Hypertrophic cardiomyopathy	Late childhood or adult life
Myoclonic epilepsy with ragged red fibers (MERRF)	mt-tRNA gene mutations (heteroplasmic)	Arrhythmia, Dilated cardiomyopathy	Late childhood or adult life
Maternally inherited diabetes and deafness (MIDD)	mt-tRNA gene mutations (heteroplasmic)	Left ventricular hypertrabeculation and arrhythmia	From childhood to late adulthood
Neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP)	Complex V mutations (heteroplasmic)	Cardiomyopathy	Late childhood and adult life
Barth Syndrome (lethal infantile cardiomyopathy)	Nuclear-encoded tafazzin gene mutation	Arrhythmia, Left ventricular non-compaction cardiomyopathy	Early infancy/childhood
Exercise intolerance [83]	mt-tRNA gene mutations and/or Complex III defects (heteroplasmic)	Fatigue, muscle weakness	Late childhood and adult life

showed significant accumulation of mtDNA mutations, which shortened the lifespan of mice without remarkable levels of oxidative stress [6, 100, 101].

Several mitochondrion-specific conditions may cause mtDNA to be particularly vulnerable to mutations [92, 102]: (1) the mtDNA is localized close to the inner membrane, in which ROS are produced. During mtDNA replication, the lagging strand of mtDNA is single-stranded for a long time, and thus spontaneous mutations can be easily introduced compared to that in double-stranded DNA. (2) mtDNA in the nucleoid is not extensively condensed and cannot be efficiently protected from deleterious insults. Whether the mitochondrial nucleoid is in proximity to the site of ROS production remains controversial [55]. (3) Covalent modifications of mtDNA in mitochondria cannot be readily detected and repaired by the mtDNA repair system because mtDNA does not encode for proteins involved in mtDNA repair, and mitochondrial POLG has low fidelity [13, 103]. As described above, the continuous replicative nature of mtDNA makes it susceptible to mutations, which could be passed on by clonal expansion and mitochondrial segregation during cell division or renewal [65]. Thus, it has been speculated that the continuous replication of mtDNA is of pathogenic importance not only in patients with mtDNA depletion, but also in patients with different pathogenic mtDNA mutations [104]. Independently, metabolic impairments lead to alterations in mitochondrial bioenergetics, strengthening mtDNA damage, increasing the mutation rate, perturbing mtDNA replication, and mitophagy, and eventually leading to the accumulation of somatic mtDNA mutations [82]. In diabetes, metabolic stress mediated by cytokines, inflammation, and oxidative insults cause a loss in mtDNA number rather than rearrangement and/or point mutation of mtDNA [57]. In addition, an imbalance of nucleotide pools can induce mtDNA depletion, as large portions of nucleotides are imported into the mitochondria and the mitochondrial inner membrane is less permeable to charged molecules [105]. Therefore, an insufficient supply of nucleotides in the mitochondria may lead to misincorporation of bases in replicating mtDNA.

mtDNA Repair System in Mitochondria

Clearly, a reduced repair capacity will result in an increased number of mtDNA mutations and risk of disease progression [106]. Several DNA repair systems function in the nucleus: direct reversal of mutated bases [107], base excision repair [92, 103], mismatch repair, nucleotide excision repair, and DNA double-stranded break repair [103]. However, mitochondria do not contain all of these repair systems. The major repair pathway in mitochondria is the base excision repair system which involves OGG1, POLG, and DNA ligase for recognition and incision, DNA synthesis, and final ligation of the single-stranded nick, respectively [92, 103, 108]. Considering the high rate of mtDNA mutations, oxidative stress may directly or indirectly deteriorate the mtDNA repair system. For example, when cells are exposed to H_2O_2, or possibly other oxidants, the potential of the repair system is suppressed, indirectly increasing the mutation rate [109]. In addition, several polymorphisms in antioxidant enzyme-encoded genes have been identified, which result

in a range of effects such as no effect on enzyme activity to complete absence of the gene product. This indicates that polymorphisms in antioxidant defense genes modify the risk of disease development due to mtDNA mutations [110].

mtDNA Rearrangement

The occurrence of mtDNA rearrangement events may be a semi-random process that is relatively unique to each cell. Possible mtDNA rearrangements can be categorized into three types: (1) deletion mainly occurring between the origins of replication, O_H and O_L, which are typically flanked by two 13-bp direct repeats [111], (2) F-type rearrangement (e.g., tandem duplication), and (3) R-type rearrangement that joins two differently oriented mtDNA strands [112–116]. A large-scale rearrangements of mtDNA can result in partial deletions (ΔmtDNA), which vary in size from 1.3 to 8 kb and encompass several genes [117], partial duplications, or rarely triplication; these are typically heteroplasmic [118]. As shown in Fig. 5.2 and Table 5.4, deletions in mtDNA without duplication are more frequently detected in nondividing tissue, such as cardiac muscle cells rather than blood cells, and frequently results in a mosaic pattern [34, 115, 119–121]. mtDNA can be deleted during replication by slipped-strand homology in repetitive sequences or the failure to repair double-stranded breaks [92]. Irradiation [122, 123], chemical agents such as *t*-butyl hydroperoxide [124], and ultraviolet light can also generate double-strand breaks and thus mtDNA rearrangements [113–116]. Loss of *cis*-acting elements required for mtDNA replication, such as the D-loop, may be related to the frequency of mtDNA deletion [34]. Although numerous mtDNA deletions have been detected, the molecular mechanism leading to deletion of mtDNA, particularly large-scale deletions, is poorly understood. Partial mtDNA duplication, which is produced from coexisting ΔmtDNA, is not deleterious. However, at least one family with mitochondrial disease showed duplicated mtDNA with no detectable ΔmtDNA [118].

mtDNA Point Mutations

Point mutations can result in deletion, insertion, or substitution to affect single sites. DNA substitution mutations include transitions and transversions. Transitions are interchanges of bases of similar shape, either between purines or between pyrimidines. Transversions are interchanges of purine for pyrimidine bases. In previous studies, the most frequently detected mutations are transition mutations (http://www.mitomap.org), which is consistent with misincorporation by POLG [125] or deamination of cytidine and adenosine [126] as primary mutagenic events in mtDNA abasic sites [116]. Single- and double-strand breaks as well as 8-oxoguanine DNA adducts are considered to be major mutagenic lesions generated by ROS [116]. The most rapid point mutation observed is a G→A transition at bp 10,068 of the mitochondrial genome. This mutation occurs at a rate of 10^{-6} mutations per

genome doubling [127]. For point mutations related to cardiac disorders, mutations in tRNA genes of mtDNA are often detected (Fig. 5.1 and Table 5.4), but are much better tolerated than those that directly affect the functioning of complex I, whose defects are the most frequently observed [128]. In the steady state, all new mutations will be copied, destroyed, and therefore stochastically redistributed as in post-mitotic cells. In addition to the occurrence of functional changes, primary mutations occurring in mtDNA can affect nearly any step of mt-mRNA maturation, including mt-tRNA, mt-rRNA, and their modification processing, which are governed by nuclear-encoded proteins [73].

Table 5.4 Cardiac manifestations with primary mtDNA mutations. Cardiac manifestation are structural, functional, or both. The most frequent cardiac manifestation mediated by mtDNA mutations is cardiomyopathy [7] such as hypertrophic-, dilated-, or restricted cardiomyopathy. The list below does not show the full spectrum of mtDNA mutations, but rather some examples

Cardiac manifestation	mtDNA mutation
Hypertrophic cardiomyopathy	mt-tRNA$^{\text{Leu, Glu, Lys, Val, or Glu}}$, m.4797 C→M, m.8728 T→Y, mt-12s rRNA, NADH:Ubiquinone Oxidoreductase 1(ND1), ND5, ATP Synthase 8, other mutations in nuclear genes
Dilated cardiomyopathy	mt-tRNA$^{\text{Glu, Ile, or Val}}$, mtDNA deletion and/or depletion, ND1
Restrictive cardiomyopathy	mtDNA deletion, ND5
Noncompaction (left ventricular hypertrabeculation)	mt-tRNA$^{\text{Leu}}$, m.8381 A→G, ND1, cytochrome B
Histiocytoid cardiomyopathy	m.8344A→G, cytochrome B
Myocardial fibrosis	mtDNA deletion
Arrhythmia	
Sinus arrhythmias	mt-tRNA$^{\text{Leu}}$
Atrial fibrillation/atrial flutter (AFIB/AFLU)	Mt-tRNA$^{\text{Leu}}$, mtDNA depletion
Wolff–Parkinson–White (WPW)-syndrome	Mt-tRNA$^{\text{Leu or Lys}}$, ND5
Atrio-ventricular-Block	Single mtDNA deletion, Mt-tRNA$^{\text{Leu}}$
Bundle branch block	mt-tRNA$^{\text{Leu or Lys}}$, single mtDNA deletion
QT-prolongation	Single mtDNA deletion
Ventricular tachycardia	Single mtDNA deletion
Heart failure/systolic dysfunction	mt-tRNA$^{\text{Leu}}$, ND5
Pulmonary hypertension	mt-tRNA$^{\text{Leu, or Glu}}$, mtDNA deletion
Dilation of aortic root	mtDNA deletion and/or mtDNA depletion
Pericardial effusion	mt-tRNA$^{\text{Lys}}$
Coronary heart disease	mt-tRNA$^{\text{Thr}}$
Autonomic nerve fibers	mt-tRNA$^{\text{Leu}}$
Patent foramen ovale	mt-tRNA$^{\text{Leu}}$
Ventricular septal defect	mt-tRNA$^{\text{Leu}}$, mtDNA depletion
Hypoplastic left heart syndrome	mtDNA depletion
Ventricular cavity obstruction	mt-12sRNA, ND1, ND4, ND6
Sudden cardiac death	mt-tRNA$^{\text{Leu or Lys}}$, mtDNA depletion

Depletion of mtDNA or Reduction in mtDNA Number

Controlling the amount of mtDNA per cell may be the first line regulation for OXPHOS activity during development [129]. Mutation of nuclear genes involved both in mtDNA replication and maintenance can increase quantitative (e.g., mtDNA depletion) or qualitative (e.g., multiple mtDNA deletions) mtDNA mutations [130]. Interestingly, human diseases caused by excess mtDNA content compared to nuclear DNA are rare [131]. A reduction or loss in mtDNA number (or content) plays a role in a range of cardiac manifestations (Tables 5.3 and 5.4) and aging [132, 133]. Different deletions may accumulate in different tissues of the same individual with a variable range [134], and thus phenotypic presentation varies between individuals (Table 5.4). Although mtDNA deletion is common, it is unclear how mtDNA deletions are tolerated and propagated in somatic cells (Fig. 5.3). One proposed mechanism involves the mitochondrial unfolded protein response, which is regulated by the transcription factor ATFS-1. The activation of ATFS-1 caused by OXPHOS defects transmits or maintains the deleterious mtDNA in an attempt to recover OXPHOS activity by promoting mitochondrial biogenesis and dynamics [135].

Epigenetic Modification in mtDNA

Epigenetics is defined as a stably heritable phenotype resulting from changes in a chromosome through posttranslational modifications, histone variants, and DNA methylation without direct alterations in the DNA sequence [136–138]. For example, disruption of nuclear DNA methylation are widely detected in human diseases [139]. Similarly to in the nucleus, the genetic expression of mtDNA can be altered by epigenetic modification of mtDNA or nucleoid structure-forming components, as mtDNA molecules are organized into nucleoid structures and opening of the closed structure may be involved in regulating mtDNA transcription and replication [40]. In addition, an increasing number of studies has examined mtDNA methylation produced in response to oxidative stress, while other mitochondria epigenetic fields, such as the study of mitochondrial non-coding RNAs, are predominantly limited to basic science studies [136, 137]. However, it is necessary to understand these epigenetic modifications in mtDNA to fine-tune mitochondrial genetics and its disease involvement.

Possible Outcome of mtDNA Mutations and Its Considerations

The distinction between variants with point mutations and variants with large deletions is important for interpreting mitochondrial mutations (Fig. 5.1 and Table 5.2). Inheritance of mitochondrial DNA mutations depends on the mutation

type, such as only blockage of replication or possible propagation of a novel mutation [92, 106, 140], and requires months or years for manifestation [34]. In the absence of amino acid substitution, conservative amino acid changes, or mutation in noncoding regions of the mitochondrial genome, with the exception of specific promoters or replication origin region [34, 141], a large number of base changes in mtDNA will not be manifestoed. In addition, tolerable mutations in mtDNA do not impair mitochondrial function (Fig. 5.3) because normal mitochondria remain present, and a tissue-specific threshold effect occurs until the transition to a pathological condition [142, 143]. Therefore, discriminating whether an identified mtDNA variant is a deleterious mutation or polymorphism is complex. For mtDNA mutations involved in cardiac disorders, point mutations in mitochondrial protein-coding genes (Fig. 5.1 and Table 5.4) preferentially affect OXPHOS function, particularly in complexes I and IV [144]. A large number of mutations in non-protein coding genes of mtDNA have been mapped to mt-tRNA loci possessing a high frequency of point mutations (Fig. 5.1), despite accounting for just 5–10% of the mtDNA (Fig. 5.1). Mutations in mt-tRNA may impair overall mitochondrial translation by reducing the availability of functional mt-tRNAs [73, 145] and are functionally recessive. Furthermore, mischarging of mt-tRNAs occurs more frequently as a consequence of certain mutations and leads to a gain or loss of function of the protein involved [73, 146]. Notably, a dominant form of mt-tRNA mutation exists [147] and certain nucleotide changes that are not pathogenic may modulate the effects of other deleterious mtDNA mutations [148]. Interestingly, point mutations in mtDNA-encoded OXPHOS often escape the rules of mitochondrial genetics in that they affect single individuals without maternal inheritance (Fig. 5.3) and single tissues such as the skeletal muscle [149]. Several human disease-causing mutations both in the mtDNA and nuclear DNA were identified as common polymorphisms, suggesting that the pre-occurrence of compensatory mutations either within the same gene or in epistatically interacting genes exist [89]. When mitochondrial metabolism is perturbed because of a dysfunctional OXPHOS system or mtDNA mutations, mitochondrial biogenesis coupled with a peroxisome proliferator-activated receptor γ coactivator 1α may be increased to compensate for mitochondrial dysfunction [150]. In addition, nuclear modifier genes, which often have at least two alleles, one of which exacerbates disease and the other which suppresses the disease, have been investigated to better understand the incomplete penetrance of mitochondrial mutations [151].

Mitochondrial DNA Mutations and Cardiac Disorders

The heart is primarily composed of cardiomyocytes, which contain high numbers of mitochondria (thousands per cell), accounting for approximately 20–40% of the cell volume [36]. When the energy supply becomes limiting due to mitochondrial dysfunction, the infrastructure slowly deteriorates, and more severe consequences may

be triggered by catastrophic failures at localized sites [134]. Accordingly, an impaired energy balance from mitochondria can be broadly conceived as both a cause and effect of heart dysfunction [152, 153]. Using advanced technologies, the number of detected mitochondrial diseases has increased (Table 5.2). Recently, it has been increasingly recognized that primary and secondary mitochondrial disorders are closely associated with cardiac defects [7, 8, 21, 141, 154–157]. mtDNA mutations are clearly an important cause of genetic diseases [7, 147]. Various mtDNA mutations have been detected in heart tissue (Tables 5.3 and 5.4); the frequent symptoms found in cardiac manifestations are muscle fatigue and weakness, which do not always receive attention in busy clinics [26]. Patients with specific mtDNA mutations may present with different cardiac phenotypes and similar cardiac involvement can occur in patients with different mtDNA mutations [154]. In addition, the frequency, sex distribution, age at onset, other affected organs, or affected cardiac tissue of mtDNA mutations are diverse, even for similar mtDNA mutations [6, 7, 84, 141]. A more detailed description of all known pathogenic mtDNA mutations is beyond the scope of this chapter and a current compendium can be found on the MitoMAP database [158] or other resources including Support and Advocacy groups, databases and analysis pipelines, research and patient networks. For mtDNA mutations associated with cardiac involvement, there are three main clinical groups: asymptomatic, arrhythmias, and cardiomyopathies. Generally, cardiac disorders involving mtDNA mutations including mtDNA depletion may be in the form of structural defects, functional lesions, or both [6, 7, 129]. The frequency of cardiomyopathy in children with mitochondrial disease is greater than 20–40% [45, 159]. Among cardiac manifestations associated with mtDNA mutations (Table 5.4), hypertrophic cardiomyopathy is a common mitochondrial mutation, occurring in 40% of patients, which is higher than in dilated cardiomyopathy [45, 159, 160]. Mitochondrial cardiomyopathies may also present as left ventricular noncompaction or as dilated, histiocytoid, or restrictive cardiomyopathies (Tables 5.3 and 5.4), or may be associated with endocardial fibroelastosis [45]. Histiocytoid cardiomyopathy, previously regarded to result from abnormal Purkinje fibers within the cardiac conduction system, is now considered to be a primary genetic cardiomyopathy [161] because of its cardiac manifestation of a mitochondrial disorder caused by mtDNA mutation.

Mitochondrial disorder-associated cardiac manifestations can be categorized as hereditary or sporadic disorders [162]. Unlike mitochondrial disorders associated with neurodegeneration or neurological disorders that are largely noticeable [107, 143, 163], the onset the mitochondrial disorders due to mutations in nuclear genes occurs in early childhood, whereas disorders caused by mtDNA mutations are observed in late childhood or adulthood [4, 5]. Mitochondrial diseases occurring in children support the hypothesis that mitochondrial disease can be manifested any tissue and show various signs at different ages [118]. Childhood mitochondrial disease is typically more severe than adult-onset disease and includes progressive neurological, cardiac, and liver dysfunction. The onset of clinical symptoms, phenotypic variability, and variable penetrance of mitochondrial diseases are governed by a number of factors [44]. First, because of the presence of heteroplasmy, a minimum critical proportion of mutated mtDNA (60–90% mutant to wild-type DNA) is necessary before biochemical defects and tissue dysfunction become apparent (Fig. 5.2).

This difference in the threshold level of individuals is related to the low correlation between clinical severity and proportion of mtDNA mutations [151]. Second, eukaryotic cells contain genetically different mitochondria and thus randomly segregate during mitosis to generate some cells containing only mutant or nonmutant organelles. Considering the continuous replicative state of mitochondria and their DNAs, the proportion of mtDNA mutations in cells differs. Third, during aging, mtDNA mutations can accumulate to very high level in nondividing cells such as muscle and neuron cells. This can be achieved by the lower regulation of mtDNA replication through random intracellular drift, resulting in the clonal expansion of single mutant events during human life [164]. Finally, the phenomenon of mtDNA bottleneck occurs in germline cells. In addition, mtDNA aggregates or nucleoids may be involved in rapid changes in the mitochondrial DNA allele frequency between generations [145]. Diseases caused by nuclear genes that affect mtDNA stability are an interesting field of mitochondrial disorders, involving both cellular genomes [1, 85, 165, 166]. Therefore, a primary nuclear gene defect can cause secondary mtDNA loss or deletion formation. The mitochondrial manifestations originated from nuclear gene defect may resemble those caused by mtDNA mutations, but follow a Mendelian inheritance style, such as autosomal dominant progressive external ophthalmoplegia and paralysis of the motor nerves of the eye [166]. Overall, precise prediction or interpretation from mtDNA mutation to malfunction is not an easy task because of mtDNA heteroplasmy, poor genotype-phenotype correlation in many patients, and complex interactions between the nucleus and mtDNA [19, 167–170].

Diagnostic Approaches and Their Considerations

The human heart contains a greater number of mitochondria and exhibits higher ETC activity than the skeletal muscle, but the production of ATP by OXPHOS activity is lower than in muscle (heart; 40% vs. muscle; 80%) [36]. This is because of the much higher each-step activation (ESA) of OXPHOS in the skeletal muscle compared to in the heart [171]. In rodents, the heart shows higher OXPHOS activity than the muscle. In addition, human mitochondria show lower calcium accumulation than that in rodents [36]. These differences between human and rodent hearts must be taken into account. Compensatory mechanisms can maintain cardiac output, even in cases of heart failure. Thus, the time of diagnosis or identification of mtDNA is a clinically critical factor because it is not clear whether alterations in mitochondrial function are a primary or a secondary effect [36].

Based on previously described causes of mitochondrial dysfunction, several diagnostic approaches have been suggested (Table 5.2). The best approach for assessing dysfunction depends on the sample source (e.g., mitochondria, cell, or in vivo), qualitative or quantitative assessment, and routine accessibility [36, 43, 44, 119, 172]. Biochemical analysis for suspected mitochondrial mutations includes: (1) lactate, pyruvate, and phosphocreatine analysis. Clinical testing can be performed to identify a mitochondrial disease by serial measurement of lactate, pyruvate, and alanine after carbohydrate loading with glucose or fructose [43]. Fasting studies can

also provide clues to reveal a tendency towards hypoglycemia or secondary fatty acid oxidation defects; (2) amino acid analysis; (3) organic acid analysis; and (4) quantification of coenzyme Q10 and carnitine [43]. More directed biochemical assays can find deficiencies in NADH-coenzyme Q reductase (complex I) and cytochrome-c oxidase (complex IV), whereas the activity of complex II is less affected because it is entirely encoded by nuclear DNA [173, 174]. However, the activities of complexes I and III can be easily assessed using histochemical methods [119]. The presence of cytochrome C oxidase-negative myocytes and increased staining for succinate dehydrogenase may reveal respiratory chain defects and mitochondrial proliferation. Based on the morphological changes induced by possible mitochondrial dysfunction or compensation responses, myofibrillar myopathies are characterized by myofibrillar disorganization beginning at the Z-discs and are genetically caused by mutations in nuclear genes [160, 175, 176]. Cardiomyocytes in affected myocardial tissue show paracrystalline inclusions, enlarged mitochondria, or concentric onion-like cristae [23]. Using transmission electron microscopy and serial block face scanning electron microscopy with 3D reconstruction, more detailed ultrastructural abnormalities of the mitochondria including matrix compartmentalization, nanotunelling, or donut-shaped mitochondria were identified [22]. As depicted in Table 5.2, the molecular diagnosis of suspected mitochondrial disease using cutting-edge sequencing techniques has evolved rapidly over the past two decades [34, 120, 177]. However, these techniques cannot resolve some diagnostic dilemmas found in the genetics of mitochondria because of difficulties in determining the potential pathogenicity of mtDNA variants [82, 178]. Restricting the loss of mtDNA to specific tissues remains challenging, as variable tissue involvement has been reported in a single family [166]. Apart from mutations in the mtDNA sequence, metabolomics, which is based on nuclear magnetic resonance or mass spectrometry, has been applied to understand the comprehensive profiling of metabolites and other small molecules in cells and tissues. This method identifies clinical biomarkers of mtDNA mutation related to established cardiometabolic risk factors [179, 180]. As a less specific test for determining mitochondrial capacity, exercise testing, including bicycle and treadmill ergometry with a combination of biochemical and oxygen consumption tests, can be helpful for identifying possible mitochondrial defects [181]. Experimentally, a transmitochondrial hybrid, which can be generated by fusing a cytoplast containing mutated mtDNA with a cell line lacking mtDNA, can be used to identify defects in OXPHOS and the threshold effect of newly identified mtDNA mutations [142, 182].

Therapeutic Approach

When mitochondrial disease is known or suspected, cardiac examination should be directed towards eliciting signs of cardiac dysfunctions including heart failure (Table 5.4) and signs of hypertension [23]. The susceptibility to cardiac disorders, rate of disease progression, or response to pharmacological therapy depends on the

mutations in nuclear DNA, mtDNA, or both [183]. For the management of mtDNA mutation-associated cardiac disorders, treatment strategies are currently based on supportive or imaginary treatment of the symptoms without genetic corrections of the affected mitochondria or cells [6, 26, 46, 64, 183–186]. First, based on the mitochondrial heteroplasmy, mitochondrial biogenesis is considered to be a set of molecular events by which cells replace or increase their mitochondria through enhanced mitochondrial proliferation. At this point, the modulation of peroxisome proliferator-activated receptor γ-coactivator 1α and AMP-dependent kinase are thought to increase mitochondrial biogenesis [6, 60, 187, 188]. Another method may exist for regulating mitochondrial biogenesis through putative mitochondrial-localizing hormone receptors [64, 188–190]. Second, interventional efforts targeting the direct modulation of OXPHOS activity have been extensively investigated in an experimental setting and studies are being conducted to translate these results to human applications [46, 188]. To relieve biochemical defects in the mitochondria, studies have attempted to bypass the pathogenic condition by removing noxious metabolites or suppressing their production [191]. Third, many mitochondrial disorders exhibit mutations in mt-tRNA genes and thus screening of regulatory molecules that can stabilize mt-tRNAs should be considered [25, 187, 192]. Finally, various genetic tools for gene therapy aimed to correct, bypass, switch heteroplasmy, and avoid mtDNA transmission have gained attention in recent years and have shown promising results [18, 25, 188, 192–195]. However, the clinical applications of these methods are limited because safe delivery of the therapeutic agent into mitochondria in an organ-specific manner is required [195, 196]. As a non-pharmacological intervention, exercise can be used as another interventional regime for treating mitochondrial mutations-related cardiac disorders because exercise training improves enzyme activity, mitochondrial biogenesis, and mitochondria quality [19, 197]. Recent findings support that exercise can induce translocation of tumor suppressor protein p53 to the mitochondria and stimulate mtDNA mutation repair independent of POLG and mitochondrial biogenesis [198]. From the perspective of genetic counseling, improving genetic advice through prenatal analysis using amniocentesis and chorionic villus biopsy and the development of new strategies for preventing the transmission of mtDNA into next generation [199, 200], such as oocyte manipulation including spindle transfer, pronuclear transfer, or polar body transfer, is a very important aspect of disease management [201]. Overall, current therapeutic regimes have not been successful. Thus, therapeutic regimens for mitochondrial disorders manifesting in cardiac disorders require more extensive development based on mitochondrial genetics, nuclear modifier genes, and quality control systems [202].

Conclusion

Error-prone conditions in the mitochondria can result in a continuously increasing mutational load of mtDNA. Despite the high variability of phenotypic presentations, studies focused on cardiac manifestation caused by mtDNA mutation are

clinically important [7]. At least three factors can perturb mitochondrial bioenergetics and result in cardiac disorders: (1) variation in the mtDNA sequence (e.g., inherited or spontaneous mutations), (2) variation in sequences of over 1,000 nuclear DNA-encoded mitochondrial proteins or in the expression of these genes (e.g., deleterious mutations and epigenetic changes) [203], or (3) susceptibility to varied environmental conditions (e.g., energy resource and demand, mitochondrial toxins). The critical role of mutations in mtDNA should be stressed with respect to human genetic diseases and cardiac disorders. Further studies are required to investigate the genetics of mtDNA, and continued study of this fascinating genome will expand our understanding of cardiac disorders and other mtDNA mutation-associated complications.

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Chapter 6
Mitochondrial Function in Non-ischemic Heart Failure

Anisha A. Gupte and Dale J. Hamilton

Non-ischemic heart failure (HF) is a complex disorder arising from cardiac stressors such as pressure overload that are unrelated to coronary artery disease. The syndrome comprises of several features including metabolic abnormalities, energy deficit and concomitant myocardial contractile dysfunction. HF affects about 6–10% of the population worldwide, and the 5-year prognosis is >50% [1]. Non-ischemic HF is a broad syndrome comprising of various etiologies, differences in chronicity of the condition and many idiopathic etiologies that are never understood. However, the end-stage manifestation of impaired cardiac function is common and similar to ischemic HF. Current treatments of neurohormonal blockage or end-stage unloading have significantly improved quality of life but prognosis still remains poor, worse than that of some cancers. However, some of these current treatments have indicated to us that the failing myocardium although metabolically impaired, is capable of reinvigorating once provided with the appropriate metabolic conditions. It is therefore important to understand the metabolic alterations in the failing myocardium and the function of mitochondria that determine the major aspects of cardiac metabolism.

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Mitochondrial Function in Different Types of Non-ischemic Cardiomyopathy

Non-ischemic heart failure is categorized into four major types, all of which are unrelated to coronary artery disease: (1) Dilated cardiomyopathy (DCM), (2) Hypertrophic Cardiomyopathy, (3) Restrictive cardiomyopathy, (4) Arrhythmogenic Right Ventricular Dysplasia.

1. **Dilated cardiomyopathy (DCM)** DCM, the most common type of non-ischemic CM, is characterized by dilation of the left ventricle (LV) and eventual weakening of the LV wall. It may also be accompanied by fluid retention or congestive HF. DCM can result from heart valve diseases, chronic hypertension, viral infections, diabetes, and peripartum cardiomyopathy but most cases are diagnosed as idiopathic.

Mitochondrial dysfunction has been described in human DCM, stemming from multiple foci, including reduced specific activities of mitochondrial enzymes [2], mtDNA damage [2], mitochondria copy number [3] etc. It has been suggested that suppression of mitochondrial complex activities in DCM may be secondary to metabolic insults associated with DCM [4]. Diabetic cardiomyopathy is classified in this category of non-ischemic HF and has been shown to have mitochondrial dysfunction in both human and animal studies. In diabetic and healthy men, Diamant and colleagues [5] demonstrated diastolic dysfunction which was associated with a reduction in cardiac phosphocreatine/ATP (PCr/ATP) ratios. A similar reduction in cardiac PCr/ATP ratio has also been shown in diabetic patients with normal cardiac function [6], indicating that changes in mitochondrial function may precede impairment of cardiac function. Altered lipid metabolism, increased oxidative stress and altered mitochondrial structure has been reported the diabetic human myocardium, much of which is caused by the diabetic metabolic environment. Using permeabilized atrial cardiac myofibers, Anderson et al. demonstrated impaired mitochondrial respiratory capacity in patients with type 2 diabetes [7]. Even those with HbA1c as low as 6% showed mitochondrial dysfunction in cardiac myofibers, and there was an inverse relationship between HbA1C and mitochondrial respiratory capacity. Mitochondrial dysfunction was associated with greater H2O2 emission, depletion of glutathione, and increased levels of hydroxynonenal-modified proteins and 3-nitrotyrosine-modified proteins [8]. Further, a metabolic shift from fatty acid + glucose to high dependence on fatty acids as the primary fuel has been shown in obese and diabetic hearts [9]. Increased dependence on fatty acids has been postulated to be an underlying cause of increased oxidative stress, incomplete fatty acid oxidation, and thereby inefficient mitochondrial energy production [10].

2. **Hypertrophic cardiomyopathy (HCM)** is characterized by hypertrophy or thickening of the ventricular walls primarily at the septum, left ventricular stiffness, mitral valve insufficiency and disarrayed myocyte structural organization. HCM can be caused by gene mutations, chronic high blood pressure, aging or

unknown causes. Although most mutations associated with HCM are in genes coding for sarcomeric proteins, the PCr/ATP ratios are reduced in HCM both with pre-hypertrophic stage and established LV hypertrophy. This suggests bioenergetics deficiency as a cause of myocardial remodeling [11]. Patients with mutations in mtDNA can have similar cardiac phenotypes as HCM patients with sarcomeric protein mutations [11], further supportive of a mitochondrial role in HCM pathogenesis.

3. **Restrictive cardiomyopathy** and
4. **Arrhythmogenic Right Ventricular Dysplasia** are rare forms of cardiomyopathy characterized by extensive fibrosis. Very little is known about mitochondrial function of these classes of cardiomyopathy, and will not be discussed in this chapter.

Mitochondrial Function in Diastolic Heart Failure

Diastolic heart failure or heart failure with preserved ejection fraction (HFpEF) is characterized by extensive fibrosis, myocardial and myocyte stiffening, abnormal diastolic relaxation and left ventricular (LV) filling [12]. It is commonly seen in post-menopausal women and the elderly. Although it is as prevalent as HF with reduced EF (HFrEF), in contrast to HFrEF, there are no effective treatments for HFpEF. Mitochondrial function is undoubtedly important for myocardial contraction, but diastolic relaxation is also an active, ATP-dependent process that requires efficiently functioning mitochondria. Multiple studies have reiterated the fact that the creatine phosphate/ATP ratio, reflecting the balance of energy consumption and supply in the heart, is reduced in patients with HFpEF [13], hypertensive DD [14], diabetic patients with DD [6] and normotensive patients with DD but well-controlled diabetes [5]. In support, mitochondrial dysfunction has been implicated in DD in animal models [15]. Availability of ATP can influence the rate and extent of myocardial relaxation in the following ways: (1) ATP is required for function of the ion pumps (sarcoplasmic reticular Ca2+ ATPase) that transfer Ca2+ out of the cytosol during diastole; (2) ATP provides energy for the sarcolemmal Na-K-ATP pump required for achieving CM relaxation; (3) ATP interacts with actomyosin to cause its dissociation to actin and myosin, a step critical to relaxation [16]. ATP is indeed required for both contraction and relaxation, but when there is a small depletion in amounts of ATP, the actin-myosin complexes can still form due to the high affinity of the myosin head for ATP, but the dissociation of actin and myosin is reduced since it requires higher concentrations of ATP. Thus, smaller depletions in ATP have a greater impact on diastolic than systolic function, whereas larger depletions in ATP affect contraction and relaxation. Further, increases in cytosolic ADP levels in absence of any other myofilament function regulators are sufficient to slow cross-bridge cycling and cause impaired diastolic relaxation [17].

Differentiating Mitochondrial Function in Ischemic and Non-ischemic Heart Failure

While it is well known that the failing is energy deprived in both ischemic and non-ischemic etiologies of HF, it has been difficult to differentiate whether the mitochondrial bioenergetics in the two etiologies are affected similarly. Given the differences in the underlying pathologies- ischemic oxygen deprivation versus non-ischemic pressure overload, acute ischemic insult versus chronic non-ischemic stress, and more, it would be expected that the mitochondrial function in two etiologies would be widely different. However, distinguishing it has not been easy, primarily due to availability of animal models that mimic the co-morbidities and chronicity of metabolic stress that is seen in human non-ischemic HF, the difficulty in timely and sufficient procurement of human heart tissue for detailed mitochondrial analyses, the complicated data interpretation due to various co-morbidities associated with non-ischemic HF, and finally the end-stage view of human heart tissue samples that may be ultimately similar in mitochondrial function despite following different pathways.

However, some recent studies have shed light on the details of mitochondrial functional aberrations in non-ischemic and ischemic HFs. Park et al. [18] were the first to make a direct comparison of mitochondrial function between ischemic and non-ischemic HF in humans. Using non-failing hearts from rejected donors as controls, Park and colleagues found decreasing mitochondrial OXPHOS from control to non-ischemic to ischemic HF mitochondria when function was expressed per milligram of heart tissue [18]. However, when normalized to the citrate synthase activity, a measure of mitochondrial numbers, the difference between non-ischemic HF and ischemic HF mitochondria was negated. Therefore, more than differences in OXPHOS, the difference in mitochondrial content is more significant between the etiologies. The mitochondria from ischemic HF also had greater proton leak, attenuated OXPHOS efficiency, and greater levels of free radicals. However, the OXPHOS normalized to citrate synthase activity was less than control mitochondria for both non-ischemic HF and ischemic HF mitochondria. While comparing mitochondrial function from well-perfused and chronically ischemic regions of the same heart, Stride et al. [19] found that maximal oxidative phosphorylation capacity was diminished, levels of reactive oxygen species (ROS) were increased and expression of antioxidants were decreased in ischemic compared with non-ischemic myocardial regions, although the degree of mitochondrial coupling was the same. Lai et al. [20] performed myocardial transcriptomic and metabolomic profiling of compensated hypertrophy achieved by transverse aortic constriction (TAC) versus decompensated hypertrophy and heart failure achieved by TAC plus apical myocardial infarction. They found that expression of most mitochondrial genes did not differ between the two conditions, with the exception of decreasing fatty acid transport and oxidation genes in ischemic HF. Greater differences were seen in the metabolomics profiles than transcriptomic profiles of the two conditions, suggesting more post-translational differences that result in different metabolic phenotypes. Similar to our studies in human myocardial mitochondria [21],

Lai et al. [20] also suggest bottlenecks of carbon substrate flux into the Krebs cycle rather than overt mitochondrial failure in HF.

Changes in Myocardial Substrate Utilization in Non-ischemic HF

Most forms of non-ischemic HF are associated with substrate switching to greater glucose utilization [22–24]. These changes are driven by maladaptive changes of the myocardium such as altered enzymes and substrate flux, and not due to limited availability of fatty acid substrates, which may actually be in excess [25]. On the other hand, the diabetic heart may show greater dependence on fatty acids due to cardiac insulin resistance (Fig. 6.1). The shift towards glucose metabolism improves myocardial contractile efficiency by increasing the stoichiometric ratio of ATP production to oxygen consumption in addition to minimizing oxidative losses through mitochondrial respiratory chain uncoupling associated with free fatty acid (FFA) metabolism [26]. This adaptation may have greater relevance in ischemic HF where oxygen supply is limited. Impaired ability of glucose uptake or utilization is

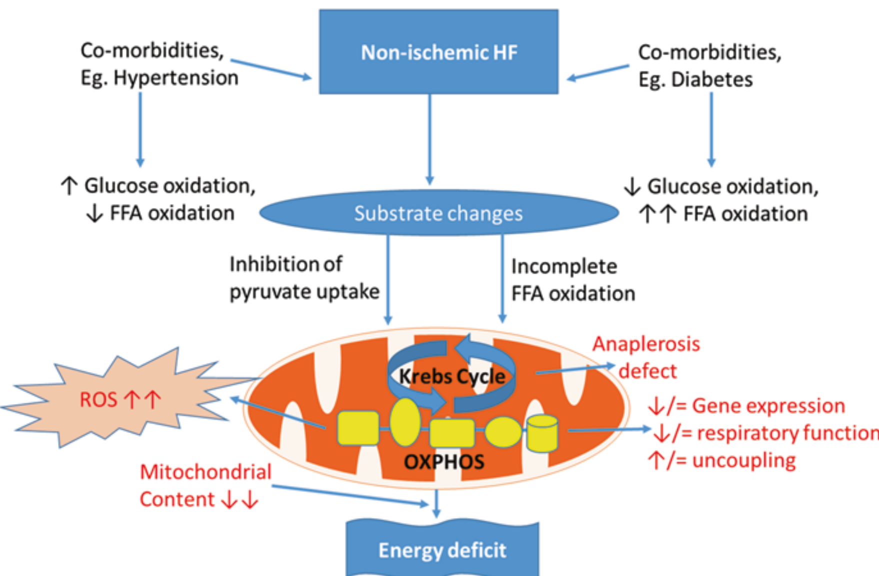

Fig. 6.1 Nodes at which mitochondrial function is impacted in non-ischemic heart failure. Non-ischemic HF and the associated co-morbidities have varied impacts on the substrate preference at the level of cellular uptake and mitochondrial oxidation. While there may or may not be gross mitochondrial OXPHOS limitations in non-ischemic HF, the combination of altered substrates, reduced mitochondrial content and altered mitochondrial flux together result in significant cardiac energy depletion

detrimental to myocardial function as shown in animal studies of GLUT4 deletion [27]. With progression of HF, a compensatory hyperadrenergic state leads to elevation of blood free fatty acid levels and subsequent onset of insulin resistance (see Chap. 25). Increased dependence on fatty acid metabolism increases oxygen consumption, decreased cardiac efficiency and feeds into a vicious cycle of metabolic impairment. Despite increased fatty acid oxidation, the myocardium remains energy depleted and excessive ROS production associated with FFA oxidation may partly underlie mitochondrial dysfunction in HF. Also, FFA oxidation is not complete in the failing myocardium, leading to incomplete FFA oxidation and changes in levels of intermediates of FFA oxidation such as acylcarnitines that further impair mitochondrial function [10, 21]. Finally a decline in utilization of both fatty acids and glucose in invariably observed with further progression of HF, with increased myocardial oxygen consumption but enhanced energy depletion in the myocardium.

Mitochondrial Function Determination in Animal Models of Non-ischemic Heart Failure

Transverse Aortic Constriction (TAC) is the most common animal model of heart failure, in which a fixed aortic constriction causes an abrupt increase in LV afterload, acute hemodynamic instability with reduced EF and early post-operative mortality [28]. This technique allows the study of effects of pressure overload to isolated to the heart, independent of systemic changes. However, the lack of slow progressive pressure overload and absence of systemic metabolic stressors commonly seen in non-ischemic HF, reduces its relevance to humans.

The myocardium consists of two populations of mitochondria, classified based on location: sub-sarcolemmal mitochondria (SSM) and intra-myofibrillar mitochondria (IFM). Previous studies [29, 30] have indicated a role for SSMs in ATP production for non-contractile, supportive cellular functions such as protein synthesis etc., whereas IFMs are the subpopulation of mitochondria that provides ATP for contraction of the myofibrils. To get a complete understanding of the bioenergetics of the failing myocardium, it is therefore important to determine the function of both SSM and IFM. Pressure overload differentially affects respiratory capacity of IFM and SSM in a rat model of TAC [30]. At the basal level, mitochondrial state 3 levels were found to be 50% higher in the IFM than the SSM, whereas ADP:O ratio and ADP-limited state respiration were comparable in both populations. However, TAC significantly impaired respiratory rates in both IFM and SSM, but only to a small extent in the SSM. Thus, after 20 weeks of pressure overload, no differences were seen in the respiratory levels of IFM and SSM. Pressure overload also decreased mitochondrial content as demonstrated by reduced total citrate synthase activity. These studies confirm the effects of pressure overload on cardiac mitochondria and also indicate that the effects are differential among the two subpopulations. Interestingly, electron microscopy showed that the cardiac IFM were smaller in size than SSMs in rats, and FACS analyses showed that IFM had less internal structural complexity than SSM [30].

The role and differential effects of HF on the mitochondrial subpopulations has been controversial, largely due to the isolation techniques and methods of respiration assessment. It is also evident that different co-morbidities associated with the non-ischemic HF may have different effects on the function of the subpopulations of cardiac mitochondria. For instance, type 2 diabetes, calcium stress, volume overload in the heart primarily affect the respiratory capacity of the SSM and not that of the IFM [31–33], whereas type 1 diabetes and cardiomyopathy affect only IFM [34, 35], and ischemia and aging affect both populations with a greater influence of ischemia on SSM [36], and of aging on IFM [37]. One disadvantage of studying isolated IFM or SSM has been due to the differential centrifugation and enzyme treatment process used in the isolation. It is likely that these processes selectively isolate structurally intact mitochondria that are not damaged in HF [38]. To circumvent this problem, many studies use permeabilized fibers or skinned fibers assuming assessment of function of both populations [39, 40]. However, this technique may preferentially assess SSM due to their peripheral location right below the cell membrane whereas the IFM may be readily accessible for substrates due to the greater diffusion distance [41]. There is also a significant increase in production of ROS by mitochondria in non-ischemic HF [42]. Consequently, scavenging mitochondrial ROS by mitochondrial targeted catalase in mice with TAC-induced heart failure, showed reversal of most of the mitochondrial proteome changes in heart failure. The mtCatalase also induced a subset of unique changes that represent processes adaptive to increased workload and metabolic demands of pressure overload which included preservation of fatty acid metabolism, attenuation of apoptosis etc. [43]. However, while implementing pressure overload can induce compensated hypertrophy, it is thought that it may not necessarily result in HF. Thus, Faerber et al. tested a minimally invasive TAC through a ministernotomy without intubation to show induction of hypertrophy, HF symptoms in 57% of the mice undergoing the surgery, accompanied by reduced oxidative capacity and suppression of PGC1a expression [44].

Non-surgical Mouse Model of Non-ischemic Heart Failure to Study Mitochondrial Function

Most of our understanding of the mitochondrial mechanisms underlying HF comes primarily from animal models of acute, ischemic HF induced by coronary artery ligation, intermittent ischemia-reperfusion, pressure overload hypertrophy induced by TAC and genetically engineered models of cardiomyopathy. Recently, our group characterized the transcriptomic and mitochondrial functional profile of mice treated with a combination of the hypertensive agent Angiotensin II and a nitric oxide inhibitor L-N^G-nitroarginine methyl ester (L-NAME) [42]. This model is independent of any surgical trauma and develops heart failure (Ejection Fraction, EF <40%) in 4 weeks with the combination treatment. Either treatment alone did not result in the same severity of heart failure. The mice with L-NAME_+ AngII treatment served as a good mouse model of non-ischemic HF induced by pressure overload to study detailed changes in cardiac mitochondrial function.

The SSM from the myocardium of the non-ischemic HF mice with AngII + L-NAME treatment showed markedly reduced state 3 rates in response to pyruvate-malate (PM) and palmitoyl-carnitine-malate (PCM) substrates [42]. There was an overall reduction in Respiratory Control Ratios (a measurement of mitochondrial efficiency) in response to PM, PCM and glutamate-malate (GM). However, SSM function was unchanged between control and HF mice for the complex II substrate succinate for both SSM and IFM. Although the IFM from HF mice showed reduced state 3 respiratory rates compared with control mice for PM, PCM and GM, the overall RCR was not significantly different, likely to due to compensatory changes in the basal state 2 respiratory rates. Therefore, although the heart is energy-deficient in these HF mice, the greatest suppression of mitochondrial activity is seen in SSM mitochondria which are believed to have a more supportive role rather than the IFM which are thought to have a direct impact on contraction. However, permeabilized cardiac fibers which consist of a combination of IFMs and SSMs showed decreased mitochondrial function in HF mice than control mice [42], confirming an overall compromise of mitochondrial function that contributes to energy depletion in these mice. However, these data also showed that there wasn't a dramatic inhibition of all mitochondrial function, since once the isolated mitochondria were provided with the appropriate buffers and abundant substrates ex vivo, their function was moderately reduced compared with control mice, but not completely blocked.

Thus, the dramatic reduction in contractility of the heart in non-ischemic HF cannot be explained by small changes in mitochondrial function measured in isolated mitochondria or fibers. It is therefore likely that the energy deficit stems from a combination of direct mitochondrial deficiencies and impairment of upstream processes that lead to formation of intermediates in the Krebs cycle and NADH or FADH2 into the electron transport chain. The decrease in overall cardiac bioenergetics in the failing non-ischemic myocardium is also due to reduced mitochondrial content, which has been reported by several groups [18, 19, 45]. This is further accompanied by greater ROS production and overall increase in oxidative stress in the myocardium, which in most part is attributed to the inefficient mitochondrial function [43]. This is supported by the fact that mice with mitochondrial targeted catalase but not peroxisomal catalase are protected from AngII-induced oxidative stress and cardiomyopathy [46]. But oxidative stress is also contributed by impaired anti-oxidant mechanisms, however it is thought that dysfunctional mitochondria are the initial stimulus to initiate the vicious cycle of excessive ROS [47]. It is also likely that in comparison with ischemic HF, the chronicity of non-ischemic HF allows adaptation of the mitochondria to the changing metabolic milieu. In support, many pathways leading to substrates for mitochondria have been shown to be down-regulated in non-ischemic HF. For instance, fatty acid metabolism, including fatty acid oxidation has been shown to reduce in non-ischemic HF independent of changes in OXPHOS genes or function of the mitochondria.

Human Studies in Non-ischemic HF

The understanding of the mechanisms and consequences mitochondrial functional changes in HF would form the basis for innovative personalized metabolic recovery therapies for human HF. Stride and colleagues reported markedly diminished OXPHOS capacity in human HF [45]. Other investigators reported similar findings [7, 48]. However these studies included respiratory assessment of mitochondrial function on atrial or atrial appendage samples rather than ventricular wall specimens. In the study by Sharov et al. [49], investigators procured sub-endocardial and sub-epicardial samples from explanted hearts and examined skinned fibers which were saponinized and studied with a Clark electrode in an oxygraph cell. They demonstrated no difference between control and HF samples in basal respiration rates but showed depressed state 3 ADP stimulated respiration in HF samples. Coupled respiration however was present [49]. Our studies of mitochondrial isolates from left ventricular wall tissue demonstrated persistence of a population of mitochondria that were capable of highly coupled oxidative phosphorylation (OXPHOS) [50, 51]. No major differences were noted in mitochondrial function for end-stage non-ischemic and ischemic HF. However, metabolomics and gene expression studies showed an overall marked down-regulation of the myocardial bioenergetics in human HF [21]. In support, some investigators have documented functional mitochondria in failing myocardial tissue but with reduced OXPHOS capacity due to lower mitochondrial content [3, 18]. Although reduced in number and capable of function these mitochondrial are not meeting energy requirements for the failing heart. The fact remains that the failing myocardium is energy deprived [52] and PCr/ATP ratios progressively decreased as HF severity increases. However, as noted by a letter commentary to a review of the topic, it is not clear as to whether there is a fuel supply or energy transfer problem [53, 54]. What is becoming clear is that there remains a population of mitochondria capable of coupled respiration and therefore there is potential for recovery. Using pre- and post- paired samples, it has been shown that mechanical unloading with left ventricular assist devices results in metabolic recovery [21]. It is thus likely, that the energy deficit is initiated in mechanisms upstream of the mitochondria, perhaps at the levels of substrate oxidation/uptake and the overall number of mitochondria, rather than the biochemical properties of the mitochondria themselves.

Mechanistic understandings of the source of energy depletion could lead to therapeutic metabolic interventions. As example metabolomic studies from our laboratory revealed impaired pyruvate handling and resulting in an anaplerosis problem [21]. In ventricular wall tissue from failing human hearts, pyruvate concentrations were elevated while downstream Krebs cycle intermediates citrate, succinate, fumarate and malate were depleted. This in turn results in reduced delivery of reduced compounds NADH and FADH to complex I and succinate to complex II of the mitochondrial electron transport chain. The mitochondrial isolates from the same tissue were coupled and intact [50]. Mechanical unloading with a left ventricular device reversed the problem [21]. The tissue pyruvate concentration on pre- and

post-LVAD paired samples declined and the Krebs intermediate increased. Perhaps then with mechanistic understandings, we can hope to improve substrate processing and energy transfer to fuel the failing heart.

Therapeutic Implications of Understanding Mitochondrial Function in Non-ischemic HF

The rapidly increasing prevalence and limited success of conventional treatments urgently calls for novel treatment strategies. Given the large metabolic changes in non-ischemic HF, metabolism-targeted therapeutic strategies are very attractive. We and other have shown that the failing myocardium although metabolically impacted, remains viable with the possibility of reversal of metabolic function [21, 55]. Contractile failure is largely a manifestation of adaptive responses of viable myocytes to various forms of non-lethal stressors which is reversible either upon the removal of stressors or reversing the mal-adaptive changes [55]. Treatments such as left ventricular assist devices have shown improvement in mitochondrial and metabolic function in the failing myocardium [21]. However, it might be important to apply these metabolism-targeted therapies before attaining end-stage HF which may be more difficult to reverse completely at the level of contractile function. The following important areas of potential metabolism-targeted therapies have the greatest likelihood of success: enhancing mitochondrial biogenesis, decreasing mitochondrial oxidative stress, rescuing iron and calcium homeostasis, reversing changes in substrate preferences [55, 56]. Strategies to enhance mitochondrial function may include activation of the master regulator of mitochondrial biogenesis Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) [57], activation of adenosine monophosphate-activated kinase (AMPK) which drives mitochondrial biogenesis in exercise-induced non-pathological hypertrophy [58], activation of the nitric oxide pathway [59], increasing estrogen receptor signaling [60]. To improve mitochondrial oxidative stress status, direct targeting of ROS inside the mitochondria rather than systemically would have greater benefits, since results from large scale clinical trials of systemic antioxidants such as Vitamin E were disappointing [61, 62]. Mitochondrial ROS scavengers or inhibitors of ROS production such as MitoQ [63], Szeto-Schiller peptides [64] and manganese superoxide dismutase [65] or mitochondrial catalase mimetics [43] have shown potential of cardiac protection in animal models of non-ischemic HF. Selective chelation of mitochondrial free iron can be achieved with compounds such as deferiprone, with some benefits reported in improvement of mitochondrial function in non-cardiac conditions of mitochondrial dysfunction [66]. Another metabolic approach to improve cardiac function in non-ischemic HF will be to target the substrate changes. Enhancing glucose utilization and lowering fatty acid utilization in HF has been shown to be beneficial in HF progression. Inhibitors of fatty acid metabolism such as CPT1 inhibitors Etomoxir and Perhexiline have shown limited but promising effects in humans [67, 68]. Other inhibitors of fatty acid oxidation such as Dichloroacetate, Ranolazine, trimetazidine, and activators of the metabolism regulator AMPK such as AICAR may be

considered as potential therapies. Taken together, detailed studies of mitochondrial function in non-ischemic HF myocardium present a strong potential in designing personalized medicine based on the degree of mitochondrial function, mitochondrial content and substrate changes.

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Chapter 7
Mitochondria in Ischemic Heart Disease

L. Maximilian Buja

Ischemic heart disease (IHD) is a leading cause of morbidity and mortality worldwide. IHD generally results from pathological interaction between diseased coronary arteries and the myocardium that requires abundant blood flow. Imbalance in coronary blood flow and myocardial demand leads clinically to the development of an acute coronary syndrome (ACS) [1]. At a fundamental level, IHD involves altered biology of the cardiomyocyte (CMC). Increasing evidence has focused on the mitochondrion as a key organelle in the response of the CMC to ischemic injury [2]. This evidence is reviewed in this chapter in the context of the overall pathobiology of IHD.

Basic Concepts of Ischemic Heart Disease

Thrombotic occlusion of an atherosclerotic coronary artery leads to ischemia to the subtended segment of myocardium due to marked reduction in oxygen and metabolic substrate delivery [1]. The ischemic myocardium is subject to complex metabolic and functional changes that lead to progressive injury to CMC and the microvasculature. Contractile function is lost within seconds. Ischemic CMC can become electrically unstable and become the source of an ectopic focus which can lead to ventricular fibrillation and sudden death. With profound ischemia, CMC become progressively impaired and transition from reversible to irreversible injury by 15–20 min. Irreversible injury then progresses in a wavefront pattern extending out from the ischemic subendocardium to the subepicardium leading to a complete myocardial infarct after about 3 h [3–5].

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Some processes can profoundly alter the response of the myocardium during evolving myocardial infarction [6–11]. If instituted in a timely manner, reperfusion significantly limits the magnitude of irreversible myocardial damage. However, there may be some reperfusion injury in the form of impaired contractile function of spared myocardium (stunning) and lethal reperfusion injury to a population of severely impaired CMC at the time of reperfusion. Another process with a major impact on myocardial ischemia is myocardial conditioning. Myocardial preconditioning refers to the retardation of the development of irreversible myocardial injury when brief periods of coronary occlusion and reperfusion precede a prolonged coronary occlusion. After a latent period, the phenomenon returns 24 h later as a second window of protection (SWOP). Postconditioning also achieves some degree of protection when gradual or intermittent reperfusion rather than immediate full reperfusion is provided after a bout of coronary occlusion. Preconditioning and postconditioning at a distance refer to the salutary effects on the evolution of myocardial ischemic injury produced by episodes of transient ischemia in skeletal muscle.

Myocardium and Its Cardiomyocytes

The myocardium is organized around CMCs and supporting structures [9]. CMCs are large cells (average diameter of 14 μm and length three to four times diameter) which constitute 80% of the volume of the myocardium but 20% of the total number of cells, most of which are non-CMC [9]. The myocardium contains a microvasculature including arterioles, capillaries and venules, all lined by endothelium. The capillaries are tightly aligned with the CMC in a one-to-one ratio. The CMC have one or two (25%) central nuclei containing chromosomes with deoxyribonucleic acid (DNA), abundant myofibrils arranged in sarcomeric contractile units and abundant mitochondria for energy production (Fig. 7.1). The plasma membrane (sarcolemma) has invaginations, the T tubules, at the level of each sarcomere, the T tubules with adjacent elements of smooth endoplasmic reticulum arranged in diads and triads to facilitate calcium flux and excitation-contraction coupling. Adjacent CMC are connected end-to-end by specialized plasma membrane, the intercalated discs, and side-to-side by desmosomes.

Because the CMC are specialized for continuous contraction and relaxation, the CMC have a very high energy requirement. This energy requirement is met by the large number of mitochondria which are organelles specialized to conduct oxidative phosphorylation. The mitochondria of CMC have large numbers of tightly paced cristae formed from invaginations of the inner mitochondrial membrane reflecting their high energy output. Maintenance of the electrical potential difference ($\Delta_{\Psi m}$) across the inner mitochondrial membrane is essential for oxidative phosphorylation and ATP generation [9].

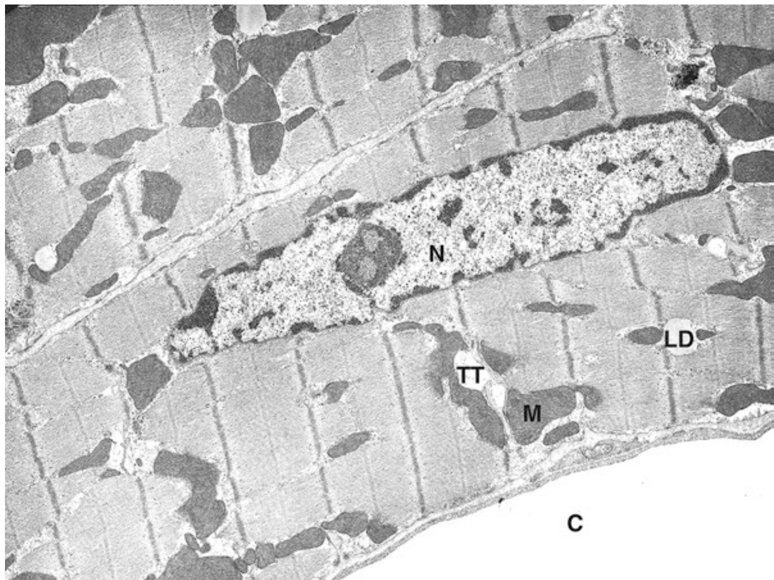

Fig. 7.1 Ultrastructure of the normal mammalian myocardium. The typical cardiomyocyte has a central nucleus (*N*), compact cytoplasm, a sarcolemma (plasma membrane) with invaginations called transverse tubules (*TT*), myofibrils arranged into sarcomeres, a few lipid droplets (*LD*), glycogen granules and numerous mitochondria (*M*) containing numerous densely packed invaginations of the inner membranes, the cristae. In close proximity to the cardiomyocytes are capillaries (*C*). Electron micrograph, ×11,000 (This figure is reproduced from reference [2] with permission)

Modes of CMC Injury and Death

In the last several years, research has shown that CMC have the molecular mechanisms to undergo all of the major modes of cell injury and death. These include oncosis, apoptosis and autophagy [9, 10, 12, 13]. The two of major importance in myocardial ischemic injury and infarction are oncosis and apoptosis [9, 10, 12–15]. Oncosis is manifest by cellular and organellar swelling due to progressive membrane dysfunction and damage due to ischemic or toxic injury coupled with rapid energy depletion reflected by rapid loss of adenosine triphosphate (ATP). Apoptosis is triggered by various physiologic and pathologic stimuli and is characterized by cellular and organellar shrinkage with subsequent fragmentation. Apoptosis is mediated by activation of a cascade of caspase enzymes (cysteine-aspartate proteases) via extrinsic or intrinsic pathways; at least partial preservation of ATP is required. Cell death due to apoptosis is characterized morphologically by cell shrinkage with intact plasma membrane and biochemically by caspase activation and discrete double-stranded DNA breaks. Although oncosis and apoptosis have been termed accidental and programmed cell death, respectively, oncosis can be a relatively uncontrolled process (oncotic necrosis) but often involves

signaling pathways that mediate a more regulated process of necroptosis. Many forms of oncosis as well as apoptosis follow programmed patterns mediated by the activation of cell surface receptors, and both involve activation of distinctive gene profiles [14, 15].

Autophagy involves segregation of cellular components, including proteins and mitochondria, in autophagic vacuoles, merger of the autophagic vacuoles with lysosomes to form autophagolysosomes, and subsequent degradation of the constituents in the vacuoles [9]. Depending on whether the autophagic process is regulated or not, autophagy can serve as a mechanism of cell survival following stress or another distinctive form of programmed cell death [9].

Basic Pathobiology of Myocardial Ischemic Injury

Basic information about modes of cell injury and death have been integrated with our knowledge of CMC injury occurring in myocardial infarction. Ischemic cardiomyocyte injury is characterized by progressive membrane damage with a component of cell swelling [3–5]. Ischemic membrane damage has been shown to progress from discrete alterations in specific membrane pumps and ion channels to an intermediate stage of less selective and increasing membrane permeability with more severe ionic disturbances including increased influx of calcium ions to a final stage of membrane rupture [12]. The membrane damage is mediated by activation of phospholipases and proteases and accumulation of toxic metabolites. Mitochondrial swelling and calcium accumulation are prominent components of evolving CMC injury (Figs. 7.2 and 7.3). This pattern of cell injury is typical of the oncotic pattern of cell injury and death [12–15]. With the recognition of apoptosis as another important mode of cell injury and death, the role of apoptosis in ischemic CMC injury also must be considered [12–15].

CMC have been demonstrated to contain the molecular mechanisms to activate apoptotic pathways as well as pathways leading to progressive membrane damage even though CMC undergoing irreversible ischemic injury do not show the classic morphological features of apoptosis [14–21]. This has led to the conclusion that cardiomyocyte ischemic injury and death is a hybrid form of cell injury in which the terminal events are dominated by oncotic ultrastructure [10, 14, 15]. Perturbation of apoptosis with caspase inhibitors and genetic manipulation produces partial but not complete reduction in infarct size [16]. Thus in evolving myocardial infarction, multiple modes of cell death participate including oncotic necrosis, necroptosis, and apoptosis.

Autophagy has been shown to be capable of modulating cardiomyocyte cell injury and acute myocardial ischemic injury. Autophagy has also been found to have a role in cardiomyocyte survival in the setting of hibernating myocardium, a state of chronic ischemic myocardium associated with decreased myocardial function [22–24]. In heart failure, increased loss of CMC occurs by multiple modes of injury, namely, oncotic necrosis, necroptosis, apoptosis and autophagy, and this CMC loss

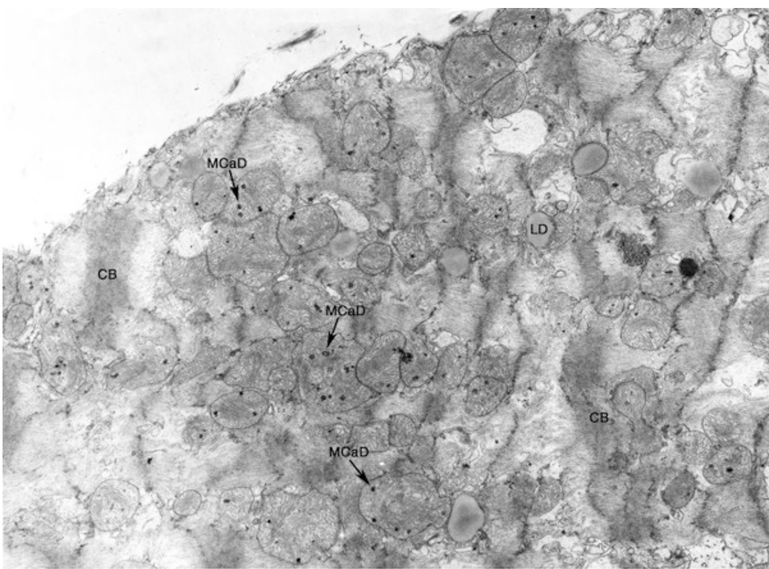

Fig. 7.2 Ultrastructure of ischemic cardiomyocyte with features of contraction band formation and calcium overload as seen in the peripheral zone of an evolving canine myocardial infarct. Note the foci of myofibrils condensed into bands (*CB*), the lipid droplets (*LD*), and granular mitochondrial calcium phosphate deposits (*MCaD*). Electron micrograph, ×6,500 (This figure is reproduced from reference [8] *Dialogues in Cardiovascular Medicine* with the permission of the publisher Les Laboratories Servier, Suresnes, France)

contributes importantly to the progression of heart failure [25–28]. Both in ischemic injury and heart failure, the rate and magnitude of ATP depletion is a key determinant of the pathway of CMC injury and death [14, 15, 28].

Key Role of Mitochondria in CMC Injury

A number of distinct and overlapping subcellular pathways involving cell membrane death receptors, endoplasmic reticulum and mitochondria can be involved in the development of oncotic and apoptotic cell death [14–21]. Nevertheless, recent studies have implicated the mitochondria have a critical role in the pathogenesis of cell injury (Figs. 7.4 and 7.5) [21–28]. The biochemical and ultrastructural changes occurring in cardiac mitochondria during the evolution of myocardial ischemic injury are well documented [3–5]. Mitochondria are dynamic organelles that constantly undergo regulated processes of fusion, fission and substrate trafficking [29, 30]. In stressed cells, mitochondria can be subject to deleterious and beneficial effects due to activation of death channels and salvage pathways, respectively [2, 16–21, 28, 31–33]. The mitochondrial death channels include the mitochondrial permeability transition pore (mPTP) and a putative mitochondrial apoptosis channel

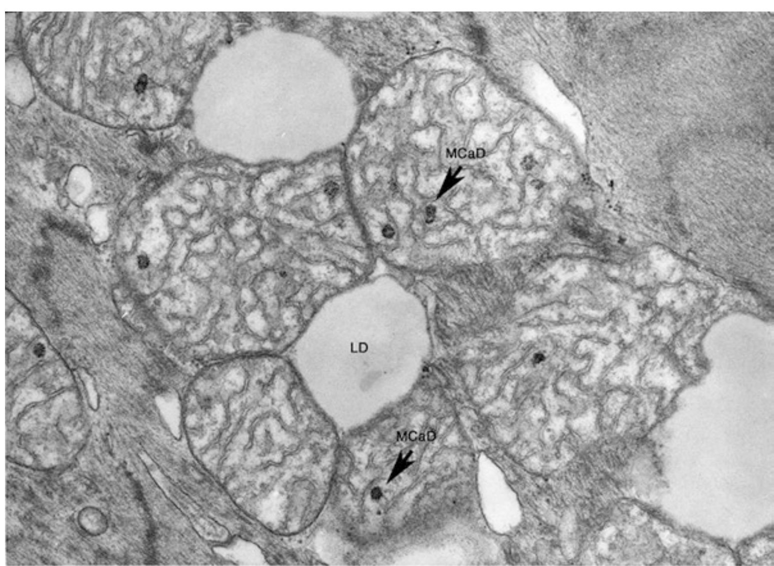

Fig. 7.3 Ultrastructural detail of ischemic cardiomyocyte with lipid deposits (*LD*) and swollen mitochondria containing very electron-dense, annular-granular calcium phosphate deposits (*MCaD*) Electron micrograph, ×26,000. As ATP is reduced, mitochondrial oxidative capacity is decreased, leading to an accumulation of reesterified fatty acids as lipid droplets. As the sarcolemmal function becomes impaired, an increase in calcium influx occurs. With reperfusion, further calcium influx is coupled with an oxidative burst generating toxic oxygen-based radicals. The excess calcium triggers hypercontraction of the myofibrils manifest as contraction bands. The overload of calcium ions and toxic oxygen-based radicals leads to opening of the mitochondrial permeability transition pore (*mPTP*) , loss of membrane potential, swelling and collapse of ATP generation (This figure is reproduced from reference [8] *Dialogues in Cardiovascular Medicine* with the permission of the publisher Les Laboratories Servier, Suresnes, France)

(mAC). The mPTP is a voltage-dependent channel that is regulated by calcium and oxidative stress. The function of the mPTP is influenced by several proteins, especially the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT) and cyclophilin D (CypD). The VDAC is located in the outer membrane, the ANT in the inner membrane, and CypD on the matrix side of the inner membrane. These proteins span the inner and outer mitochondrial membranes and provide a path for transport between the mitochondrial matrix to the cytoplasm. The opening of the mPTP results in the immediate loss of the electrical potential difference across the inner membrane with resultant cessation of ATP synthesis, influx of solute, and mitochondrial swelling [2, 28, 31–33]. Recent studies have determined a key role for another molecule, the F_0F_1ATPase, in interacting with the other component molecules and leading to the mPTP formation [28]. Modulation of mitochondrial proteins, including a modest increase in expression of uncoupling protein 2 (UCP-2), can cause a rapid decline in mitochondrial membrane potential and ATP level resulting in oncosis [34].

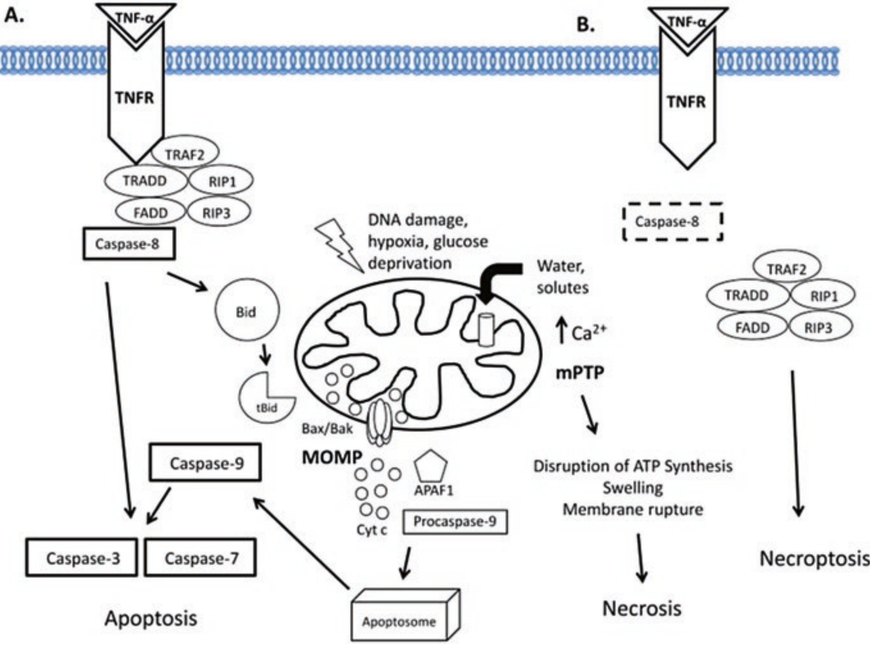

Fig. 7.4 Cell death pathways. (**a**) Apoptosis. In the extrinsic death receptor pathway, binding of an extracellular ligand such as TNF-α to its death receptor TNFR recruits adaptor molecules to form the death-inducing signaling complex (*DISC*). Caspase-8 cleaves and activates effector caspase-3 and -7. In the intrinsic mitochondrial pathway, intracellular stress signals such as DNA damage, hypoxia, or glucose deprivation activate the pro-apoptotic Bax and Bak. During mitochondrial outer membrane permeabilization, Bax and Bak form pores that allow the release of apoptogens such as cytochrome *c* into the cytosol. Cytochrome *c* and APAF1 form the apoptosome which recruits and activates caspase-9 and leads to caspase-3 and -7 activation. Death-receptor mediated caspase-8 activation can also cleave Bid to form truncated Bid (tBid) and activate the intrinsic pathway. (**b**) Necrosis/Necroptosis. Excessive influx of Ca^{2+} into the mitochondria leads to opening of the mitochondrial permeability transition pore (mPTP) and disruption of the proton gradient and ATP synthesis. The influx of water and solutes into mitochondria leads to swelling and membrane rupture. Regulated necrosis, or necroptosis, occurs in the absence of caspase-3 where the RIP1-RIP3 complex facilitates necroptosis (This figure is reproduced from reference [21] with permission)

Apoptosis can be initiated by an extrinsic pathway involving activation of certain membrane receptors with death domains and intrinsic pathways involving the endoplasmic reticulum and the mitochondria. There are levels of interaction between these pathways. A key event in many forms of apoptosis is mitochondrial outer membrane permeabilization (MOMP) produced by activation of the mAC [2, 16–21, 28, 31–33]. Outer mitochondrial membrane integrity, including the putative mAC, is regulated by multiple interactions between proteins of the Bcl-2 faimly, including Bax, Bid, Bcl-2 and Bcl-X_L. Opening of the MOMP leads to release of cytochrome c and other molecules which join with cytoplasmic components including procaspase 9, apoptotic protease activating factor-1 (Apaf-1) and dATP to form

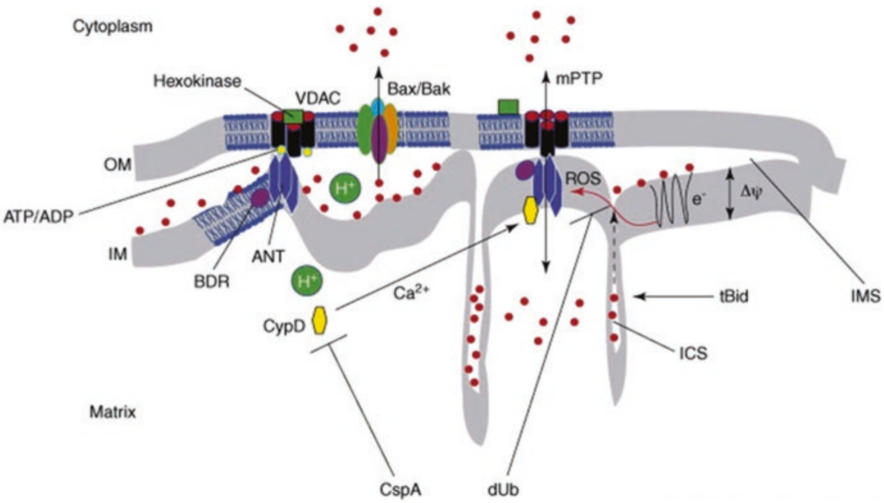

Fig. 7.5 The mitochondrial death channels. The mPTP involved in oncotic necrosis and necroptosis has several component or associated proteins including the voltage-dependent anion channel (*VDAC*), adenine nucleotide translocase (*ANT*), cyclophilin-D (*CypD*), the benzodiazepine receptor (*BDR*) and hexokinase. The specific composition of a mitochondrial apoptosis channel (*mAC*) has not been determined but it is clear that the interactions of the Bcl-2 family of proteins, including Bax, Bak, and tBid with the outer mitochondrial membrane control the mitochondrial outer membrane permeabilization (*MOMP*). The mPTP and mAC (or Bax channel) are both regulated by Bcl-2 proteins and are opened by calcium ionophores and oxidative stress. When the mAC is opened and cytochrome c is released, this triggers apoptosis. When ischemia-reperfusion leads to an increase in matrix calcium levels above a certain threshold, the mPTP opening occurs. CypD and reactive oxygen species (*ROS*) can lower this threshold. Once the mPTP is opened, the mitochondrial membrane potential is rapidly lost ($\Delta_{\Psi m}$) (This figure is reproduced from reference [31] with permission)

the apoptosome. The apoptosome then triggers the activation of caspase 3 and other effector caspases in the cytoplasm leading to downstream effects on the nucleus [2, 21, 28].

The mitochondrial pathway rather than the extrinsic pathway appears to be dominant in the apoptosis component of myocardial ischemic injury. Confirmatory evidence has been provided to show that the extent of myocardial ischemia and reperfusion damage was reduced in transgenic mice overexpressing the anti-apoptotic Bcl-2 but not in transgenic mice overexpressing a truncated form of the surface death receptor [35]. The interrelationships between apoptosis and oncosis has been confirmed by models involving initial activation of apoptosis followed by oncosis and, conversely, oncosis followed by apoptosis [16–19]. At the level of the mitochondria, the oncotic trigger of loss of the mPTP can occur in close temporal relationship to cytochrome c release via the mAC.

In ischemic CMC, when the electron transport system in mitochondria stops functioning due to lack of oxygen, this state leaves various components of the electron transport chain primed to generate oxygen free radicals when oxygen returns.

Indeed with reperfusion a massive diversion of electrons from the electron transport system leads to the generation of oxygen radicals, the oxidative burst. Simultaneously, a large influx of calcium occurs. When the mitochondria are exposed to excess oxygen radicals and calcium, the result is opening of the mPTP and collapse of mitochondrial function [11]. This is a key event in ischemic reperfusion injury. A dual role for autophagy also has been identified. Activation of autophagy during the early phase of ischemia is cardioprotective, whereas delated or late activation of autophagy during reperfusion is detrimental and promotes cell death [28].

Conditioning is a complex phenomenon initiated by activation of various G-protein coupled receptors by various autocoids, including adenosine, bradykinin, and opioids, that are released during the brief periods of ischemia and reperfusion [36–38]. Activation of these receptors is followed by activation of a complex signaling cascade, including multiple kinases, that leads to opening of potassium channels in the mitochondrial membrane and maintenance of the mPTP and the electrical potential of the inner mitochondrial membrane. Preservation of mitochondrial function and ATP production is an absolute requirement for the protective effect of conditioning.

A component of the protective effect of conditioning involves the activation of a reperfusion injury salvage kinase (RISK) pathway in the mitochondria [10, 11, 39, 40]. One component of the pathway involves phosphatidylinositol-3 kinase (PI-3 K) acting on Akt (protein kinase B) and the mammalian target of rapamycin (mTOR). The other component involves mitogen-associated protein kinase (MAPK) and p42/p44 extracellular signal-related kinase (ERK). The two arms of the pathway converge on p70s6 kinase to activate glycogen synthase kinase β which acts to prevent opening of the mPTP. An isoform of the ATP-sensitive potassium channel (K^+_{ATP}) also regulates mPTP opening. A component of autophagy, mitophagy, contributes to the protective effect of ischemic preconditioning [28–30].

Therapeutic Considerations

Because of the clinical importance of preserving myocardium, extensive effort has been aimed at developing pharmacological approaches to limiting myocardial ischemic damage. This area of research has been frustrated by initially promising experimental work being followed by negative or equivocal clinical trials in patients. Although myocardial ischemia – reperfusion injury is clearly mediated by calcium overload, toxic oxygen-based molecules, inflammatory mediators and neutrophils, clinical trials of agents aimed against these components of ischemic injury have not been found to be convincingly effective [10, 11]. Deficiencies in the design of the experimental work and complexities of confirming efficacy in clinical trials are important factors for these results [10, 11, 41]. The major advances in salvage of ischemic myocardium have come from the introduction of thrombolytic therapy and percutaneous coronary interventions (PCI) rather than from pharmacological treatments. Nevertheless, increased understanding of the pathobiology of

Table 7.1. Targeting mitochondria in cardiomyocytes and the heart

Target	Inhibitors	Mechanism
Direct action via mPTP	CsA an its analogues	Prevents opening of mPTP pores via mPTP via cyclophilin D (CypD) interaction; side toxicity due to interaction with other cyclophilins
	N-Me-4-Ile-CsA (NIM811)	Prevents mPTP opening via CypD interaction; more specific than CsA
	Sangliferhrin A	Prevents opening of mPTP pores via CypD interaction at different sites from CsA
	BKA and ADP	Induces *m* conformation of ANT
	H^+ and divalent cations (Mg^{2+}·Mn^{2+}·Sr^{2+}·Ba^{2+})	Antagonize Ca^{2+} binding to ANT
Indirect action – mPTP opening	Reactive oxygen species (ROS) scavengers (e.g., propofol, pyruvate, MCI-186)	Prevent accumulation of ROS and oxidation of critical thiol groups on ANT
Pharmacological preconditioning		Prevent oxidative stress, normalize mitochondrial metabolism
	Adenosine	Induces desensitization of mPTP by causing ischemic preconditioning (IPC)
	Nicorandil	Mimics IPC, induces desesitization by opening $mito_K$ATP
	Diazoxide	Similar mechanism
	Bepridil	Similar mechanism
	Isoflurane	Induces IPC-like condition, inhibits complex I and glycogen synthase kinase 3p
Mitochondrial dysfunction	Coenzyme Q	Cofactor in electron transfer; reduces mitochondrial ROS
	L-carnitine	Involved in fatty acid transport into mitochondrial inner membrane
	MITO Q (mitoquinone)	Antioxidant
Other targets	SS-31 (i.e.,MTP-131 and Bendavia)	Binding to inner membrane cardiolipin, preserving oxidativePhosphorylation reducing ROS
	TMD#7538	Inhibits both MOMP and mPTP

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myocardial ischemic injury and the key role of the mitochondria has led to renewed interest and promise in novel therapeutic approaches, used alone or in combination with PCI, for the treatment of patients with acute ischemic heart disease [9–11, 28, 31–33, 42–58].

The prototype for a pharmacological approach targeting the mitochondria is cyclosporine A (CsA) (Table 7.1) [28]. CsA is known to inhibit the formation and opening of the mPTP by binding to CypD. Positive results were obtained in a proof-of-concept small clinical trial of patients with evolving myocardial infarction

[48, 49]. Other pharmacological agents are being designed and tested for their ability to activate the RISK pathway in the mitochondria and to enhance mitochondrial energy metabolism [11, 40, 54, 55]. Pharmacological agents under recent or active investigation include adenosine, atrial naturetic peptide, beta adrenergic blocking drugs, nitrates, phosphodiesterase-5 inhibitors, and supersaturated oxygen therapy, and antioxidant peptides targeted at mitochondria [54–58].

In experimental models, caspase inhibitors and other supressors of apoptosis have been shown to reduce myocardial infarct size [50]. However, approaches to retard apoptosis during evolving myocardial ischemic injury in patients have not yet been demonstrated. Other agents, designated as necrostatins, are being developed for activity against receptors involved in the hybrid necroptosis form of injury [50, 51]. Other approaches directed to the non-myocytic components of the myocardium, including endothelial cells, fibroblasts, and extracellular matrix are being explored [52].

The resurgence of interest in reducing ischemic and reperfusion injury has led to the formation of an NIH-sponsored consortium for preclinical assessment of cardioprotective therapies (CAESAR) [59–61]. Recently, however, several larger multicenter trials have reported lack of protective effect with CsA and remote ischemic pre-conditioning [62–66]. It has been pointed out that the effect of CsA is indirect and mediated by binding of CsA to the matrix protein CyP-D with the net effect being desensitization rather than true block of the pore. This has given rise to strong interest in developing potentially more effective inhibitors of the mPTP (Table 7.1) [28].

Conclusions

The cumulative effects of episodes of ACS and subsequent extent of myocardial infarction have major significance for the prognosis of patients with ischemic heart disease. Acutely, extensive myocardial infarction is associated with acute heart failure, cardiogenic shock and malignant arrhythmias [1]. Chronically, the cumulative effect of ischemic damage leads to chronic heart failure [9]. Chronic heart failure is mediated not only by the loss of myocardium but by progressive pathological remodeling in the viable myocardium [6–10]. Coronary artery stenting and coronary artery bypass grafting, unless introduced early, may not be effective in stabilizing or reversing the process which leads to fixed structural dilatation of the heart and is manifest clinically as ischemic cardiomyopathy [9]. Contradictory or negative results of interventional studies highlight the difficulties of translating basic knowledge of pathobiology into effective clinical therapy and the need for continuing effort in that regard [67, 68]. Nevertheless, continued effort at developing pharmacological and pathophysiological approaches to reducing ischemic damage is worthy of pursuit. Further development of more effective approaches to preserving mitochondrial integrity should continue to be pursued.

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Chapter 8
Mitochondrial Bioenergetics During Ischemia and Reperfusion

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During recent years many works have described the morphological interaction between mitochondria and sarcoplasmic reticulum (SR) with different methodologies, such as co-localization with Ca^{2+} fluorofores and confocal microscopy in cardiomyocytes or detecting electron-dense bridges between the terminal cisternae of the SR and mitochondria by transmission electron microscopy which remain still during the isolation [1–4]. Nevertheless, these models are far from the pathophysiological situation of ischemia/reperfusion (I/R) and they do not provide a good estimation of the functional interaction between mitochondria and SR. Alternatively, the mechano-calorimetrical approach provides a tool to study this interaction in whole hearts exposed to no-flow ischemia and reperfusion, by means of selective pharmacological tools.

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Calorimetry was first used to estimate cardiac energetic of beating and resting state in papillary muscles from different species [5, 6] and then in entire perfused hearts [7]. The heat fractions associated to the Ca^{2+} activation (binding to troponin-C and ATP hydrolysis for cytosolic removal), to the actomyosin ATP-cycling and to the metabolic recovery of ATP levels were recognized by the different methodologies [6, 8–11]. The flow calorimeter provided a tool to understand Ca^{2+} movements [7, 12–14]. The thermopiles were used with papillary muscles under hyperthyroid state to study cardiac heat fractions under this pathological influence [15] and in senility [16]. More recently, a new work-loop microcalorimeter is being used with trabeculae from diabetic rabbits to study the influence of this pathology on the efficiency-afterload relationship [17, 18]. Nevertheless, only the flow calorimeter for entire perfused hearts is able to measure heat rate under no-flow or low-flow ischemia, as models to study the consequences of ischemia/reperfusion in entire hearts [19–26].

Mitochondria release an important fraction of the total heat flow, both in resting and during steady-state beating, because they provide metabolism and resynthesis of the ATP continuously consumed by ionic pumps and myofilaments. But part of the mitochondrial heat output is related to the maintaining of their own electrochemical gradient (ΔΨm) and Ca^{2+} transport. The control of mitochondrial calcium concentration ($[Ca^{2+}]$m) is a key to regulate the activity of some enzymes of the tricarboxylic acid cycle (TCA) and adjust metabolism and ATP synthesis to the cardiac cytosolic demand [16, 27]. The $[Ca^{2+}]$m mainly depends on the uptake by the uniporter (UCam) driven by the ΔΨm and the extrusion by the mitochondrial Na^+/Ca^{2+} exchanger (mNCX), in a balance which finally determines that $[Ca^{2+}]$m oscillates in beat-to-beat transients [2, 28, 29]. There are species-dependent differences in the level of free mitochondrial Ca^{2+}, which have been attributed to the cytosolic $[Na^+]$, since this ion is the main driver of mitochondrial Ca^{2+} extrusion via the mNCX [30, 31]. Maack and colleagues demonstrated that inhibition of UCam with Ru360 in guinea-pig myocytes reduced the mitochondrial Ca^{2+}-transients but symmetrically increased the cytosolic Ca^{2+}-transients measured respectively by confocal microscopy with the fluorofores Rhod-2 and Indo-1 in a patch-pipette. In addition, the inhibition of mNCX with CGP-37157 produced the opposite changes [30]. Considering the interaction between mitochondria and SR, it was shown that Ca^{2+} released by the RyR2 at about 100 μM is able to induce the UCam uptake with the consequent increase in $[Ca^{2+}]$m [32]. Also, physiologically induced mitochondrial reactive oxygen species (ROS) can regulate the SR release by RyR2 [33]. Thus, Ca^{2+} and ROS participate in the functional interaction as parts of a fine and adjusted control of energetic balance between metabolism and ATP consumption.

During ischemia, the total heat rate (Ht) is reduced at the time in which cellular metabolism and oxidative phosphorylation are inhibited. During reperfusion, the reintroduction of oxygen allows the ATP synthesis, so that the ionic pumps can be reactivated. But cytosolic Ca^{2+} is quickly increased, mostly by activation of the Na^+/H^+-exchanger (NHE) and the consequent rise in cytosolic $[Na^+]$ and Ca^{2+} influx by the reverse mode of sarcolemmal NCX (SL-NCX) [34, 35]. These events become in diastolic contracture during the first minutes of reperfusion, and very low contractility (P) with a great increase in total heat release (Ht), thus resulting in a heart with very low

total muscle economy (P/Ht). The rise in diastolic cytosolic Ca^{2+} during the ischemia and reperfusion period leads to mitochondrial Ca^{2+} accumulation, particularly when the aerobic metabolism is recovered. The consequent dysfunction depends mostly on the period of ischemia, and goes from the myocardial stunning, when post-ischemic contractility is partially reduced, to the myocardial infarction, when contractility is strongly reduced and cellular necrosis and apoptosis are developed. Also, the ischemic insult on the electron transport chain results in an increased mitochondrial ROS generation. The Ca^{2+} overload and ROS could open the mitochondrial permeability transition pore (mPTP), which further compromises the cellular energetic [36]. In long periods of ischemia mitochondria have a central role in cell death, since they are accumulating Ca^{2+} and they are responsible for the synthesis of ROS, events that could trigger the activation of mPTP [37]. Nevertheless, certain role of the mitochondrial Ca^{2+} transporters to regulate cellular Ca^{2+} homeostasis was proposed to induce cardioprotection, such as the inhibition of the mNCX [38], the UCam blockade or the activation of channels mK_{Ca} [39] and mK_{ATP} [40]. The common mechanism is the prevention of the mitochondrial depolarization and the matrix Ca^{2+} overload, which in turn reduce mPTP activation [39]. Recently, the interaction between SR and mitochondria was characterized by reducing the functional contact points at the complex between IP3R1 receptors and the mitochondrial VDAC1/Grp75 Ca^{2+} channels, which protected the cardiomyocytes by preventing the mitocondrial Ca^{2+} overload and apoptosis [41]. On the other hand, in HL-1 type cardiomyocytes it was demonstrated that the inhibition of the mNCX reduced the SR Ca^{2+} content and slowed the spontaneous SR Ca^{2+} leak, which is a trigger of automaticity and arrhythmogenesis [42]. In conclusion, different evidences suggest two directions for Ca^{2+} regulation between SR and mitochondria, which must be dependent on the degree of dysfunction: that from the SR to mitochondria generally triggers Ca^{2+} overload, while that from mitochondria to SR seems towards regulate Ca^{2+} release and leak.

Energetical Approach to the Functional Interaction Between Mitochondria and Sarcoplasmic Reticulum in Post-ischemic Stunning

In previous works we studied the cardiac stunning induced by a relatively short period of ischemia and reperfusion, and the mechanisms of cardioprotective strategies. Here, a new analysis was done to revise the several evidences about the functional interaction between mitochondria and SR. For this purpose, perfused entire hearts inside a flow-calorimeter were treated with selective mitochondrial inhibitors before ischemia and then reperfused with Krebs containing 10 mM caffeine-36 mM Na^+ (Krebs-caff-low Na) without changing [Ca^{2+}] of initial perfusion (2 mM). The high concentraction of caffeine was frequently used in cardiomyocytes to estimate the content of Ca^{2+} in SR, because it induces a permanent open state in the ryanodine receptors (RyR2) [43]. In rat and guinea-pig perfused hearts, 10 mM caffeine

induces a contracture which is potentiated by reducing [Na^+] to 36 mM, the minimal needed to maintain membrane functionality in whole hearts [20]. The condition of low Na^+ minimizes Ca^{2+} removal by the sarcolemmal sodium/calcium exchanger (SL-NCX). Thus, the slow relaxation of the contracture mainly depends on mitochondrial Ca^{2+} uptake and less on the sarcolemmal Ca^{2+}-ATPase, since neither SL-NCX nor the SR are functionally efficient to remove cytosolic Ca^{2+} under 10 mM caffeine-36 mM Na^+. Nevertheless, SERCA is continuously stimulated to uptake the Ca^{2+} lost by the leaky state of RyR2, producing a Ca^{2+} cycling with ATP consumption and heat release. Figure 8.1 shows the ventricular of contracture (ΔLVP) and heat rate (ΔHt) produced over the ischemic levels by reperfusing hearts with Krebs-caff-low Na. In this protocol, the caffeine-induced contracture (CIC) during the reperfusion followed a period of 45 min ischemia at 30 °C. This period was enough to induce stunning in reperfused hearts, since contractility was recovered to 64% ± 10% of initial at the end of R, without infarct [20]. The ratio of area-under-curves during the CIC (AUC-ΔHt/AUC-ΔLVP) allow to estimate the energetical consumption (EC) necessary to maintain the CIC (Table 8.1). When hearts were treated before ischemia with 10 μM clonazepam (Clzp), a selective inhibitor of the mNCX [38], the stunning was slightly worsen because the post-ischemic contractile recovery (PICR) reached 46% ± 8% of initial P. Also, the CIC of these hearts treated with Clzp was reduced and the relaxation accelerated, while Ht was simultaneously increased (Fig. 8.1a, b). In consequence Clzp reduced the AUC- ΔLVP and increased both the AUC-ΔHt and the EC (Table 8.1). These results suggest that in the post-ischemia mitochondria must be regulating the Ca^{2+} content of SR through the extrusion by the mNCX. A Ca^{2+} cycling from the mNCX to SERCA and from RyR2 to the UCam may be happening in the myocytes, and it will be exacerbated under 10 mM caffeine-36 mM Na^+.

The partial blockade of mNCX seems to unbalance this cycling, with the consequent predominance of the mitochondrial Ca^{2+} uptake which drives to reduction of cytosolic Ca^{2+} and SR uptake (Fig. 8.2). This mechanism explains the quicker relaxation of CIC and the increase in the heat rate associated to a higher mitochondrial metabolism necessary to maintain the ΔΨm dissipated by the higher [Ca^{2+}]m. This direction of Ca^{2+} regulation in stunning is opposite to that described in more severe situations of ischemia/reperfusion, explained as a SR Ca^{2+} overload that triggers mitochondrial dysfunction with opening of the mPTP [44]. Nevertheless, we have found several evidences of the regulation from mitochondria to the SR content that will be described here. The experimental differences are mainly due to the duration of ischemia, and express different degrees of mitochondrial response to hypoxia or ischemia, which go from prevention of dysfunction in short periods of oxygen-deficiency to the triggering of mPTP opening and cellular apoptosis in situations of Ca^{2+} overload or long periods of oxygen deprivation. In our results of hearts treated before ischemia with Clzp in control Krebs (C + Clzp), the fall in the SR Ca^{2+} content (suggested by the fall in CIC) explains a tendency to reduce the PICR in beating hearts [20].

The same direction of functional interaction from mitochondria to SR was better characterized in the cardioprotective effects of a model of cold cardioplegic solution, which is used in surgery to stop heart in diastole, consisting in perfusing

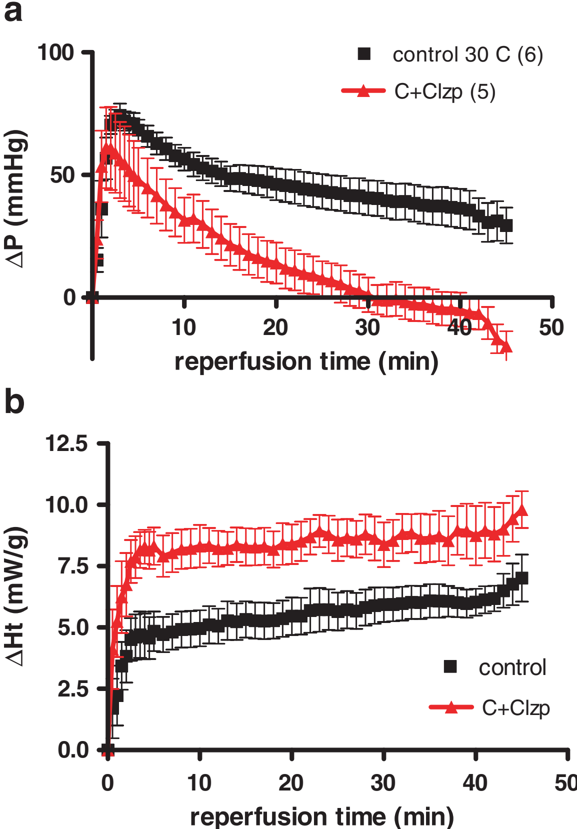


Fig. 8.1 Effects of reperfusing ischemic rat hearts at 30 °C with Krebs-10 mM caffeine-36 mM Na^+, after treatment with 10 μM clonazepam (*Clzp*) or without treatment (*control*). In (**a**) changes in left ventricular pressure (ΔLVP, in mm Hg), in (**b**) changes in the heat rate production (Δ*Ht*, in mW/g), both over the ischemic value

Krebs-25 mM K-0.5 mM Ca^{2+} (CPG) before ischemia, in rat and guinea-pig isolated hearts [20, 24]. In rats, CPG improved the PICR up to 90 % ± 10% of initial pressure development (P) in the model of moderate stunning (non-treated C-hearts recovered up to 64% ± 10% of initial P) during the steady beating in reperfusion; CPG also increased total muscle economy (P/Ht) and prevented the diastolic contracture [20]. Moreover, when ischemic hearts previously treated with CPG were reperfused with Krebs-caff-low Na, the peak of the CIC and AUC-ΔLVP were similar to those of non-treated C-hearts (Fig. 8.3a, b), while the simultaneous AUC-ΔHt and the EC were slightly reduced in comparison to the C group (Table 8.1). Comparison of Figs. 8.1a and 8.3a suggests that the peak of SR Ca^{2+} release was slightly increased by CPG, which can explain the increase in PICR. But Ca^{2+} cycling between cytosol and SR (AUC-ΔLVP) and the energetic consumption (EC) for maintaining the CIC during R remain similar in C and CPG hearts. Nevertheless, a Ca^{2+}-cycling dependent of $[Ca^{2+}]$o has been suggested from the increased resting heat rate in cardioplegic non-ischemic hearts, which was sensitive to verapamil and hypoxia. It was interpreted as a cytosolic Ca^{2+} removal in response to Ca^{2+} entered through L-channels upon depolarization [22, 45].

Table 8.1 Area under the curves of intraventricular pressure (ΔLVP) and heat rate (ΔHt) increments over the value of the end of ischemia, during the reperfusion with Krebs-10 mM caffeine- 36 mM Na^+ on rat hearts with the different treatments described in the text

Treatment	AUC-ΔLVP (mmHg. min)	AUC-Δ*Ht* (mW. min/g)	EC: AUC-Δ*Ht*/AUC-ΔLVP
Moderate stunning at 30 °C			
C (6)	2076.1 ± 263.0	243.9 ± 30.0	0.13 ± 0.03
C + Clzp (5)	831.9 ± 298.2	376.2 ± 33.7	0.80 ± 0.29
CPG non-ischemic (4)	2835.6 ± 624.8	357.9 ± 215.4	0.13 ± 0.07
CPG (13)	2389.1 ± 191.1	206.5 ± 27.8	0.10 ± 0.02
CPG + Clzp (5)	1675.0 ± 393.4	203.2 ± 64.7	0.10 ± 0.02
CPG + KBR (10)	3082.9 ± 427.0	260.1 ± 23.6	0.096 ± 0.01
CPG + KBR + Pyr (6)	2791.9 ± 371.3	265.3 ± 31.5	0.10 ± 0.01
C + Gst females (6)	2419.6 ± 544.0	237.7 ± 36.8	0.21 ± 0.11
C + Gst males (10)	2336.1 ± 255.9	169.6 ± 21.5	0.08 ± 0.01
C + OV + Gst males (9)	2580.0 ± 376.5	479.1 ± 89.1	0.21 ± 0.05
C + Gst + Pyr females (6)	2553.0 ± 481.6	392.5 ± 46.9	0.18 ± 0.03
Moderate stunning at 37 °C			
C (12)	1705.9 ± 236.5	455.9 ± 95.8	0.26 ± 0.02
C + Clzp (5)	568.3 ± 32.7	427.0 ± 7.2	0.76 ± 0.05
C HpT (6)	918.3 ± 17.3	455.2 ± 16.6	0.49 ± 0.01
C+ Clzp HpT (5)	569.9 ± 28.75	353.0 ± 34.7	0.64 ± 0.09
C HypoT	1129.6 ± 108.7	413.1 ± 25.2	0.38 ± 0.04
Severe stunning at 37 °C			
C (11)	2428.9 ± 193.7	296.8 ± 49.0	0.13 ± 0.02
C+ GST 5 mg/kg (ip) males (5)	1865.6 ± 184.4	349.5 ± 30.9	0.19 ± 0.02

Control (C)

How does ischemia modify the SR Ca^{2+} content? Evidences were obtained by comparing the effects of perfusing Krebs-caff-low Na in hearts treated with CPG, with and without an ischemic period [20]. The CIC in non-ischemic CPG hearts had a profile different to that of ischemic hearts, with a low initial peak growing afterwards (Fig. 8.3a, b) in a pattern with higher AUC-ΔLVP, AUC-ΔHt and EC than those from the ischemic CPG hearts (Table 8.1). This pattern suggests that ischemia reduced the SR Ca^{2+} content for reperfusion, which may be caused by a leak of Ca^{2+}. This mechanism explains the diastolic contracture seen during the end of I and still more during the first minutes of R. This interpretation agrees with that of Valverde et al. [46] who demonstrated that in the mice hearts ischemia induced the leak of Ca^{2+} from SR during the first minutes of reperfusion, which becomes in diastolic contracture and reduced contractility (PICR). The fact that in non-ischemic rat hearts the relaxation of the CIC was slower than in the reperfused ischemic hearts indicates that restitution of mitochondrial metabolism after a brief ischemia would stimulate Ca^{2+} uptake, thus reducing cytosolic Ca^{2+}. The increase in mitochondrial

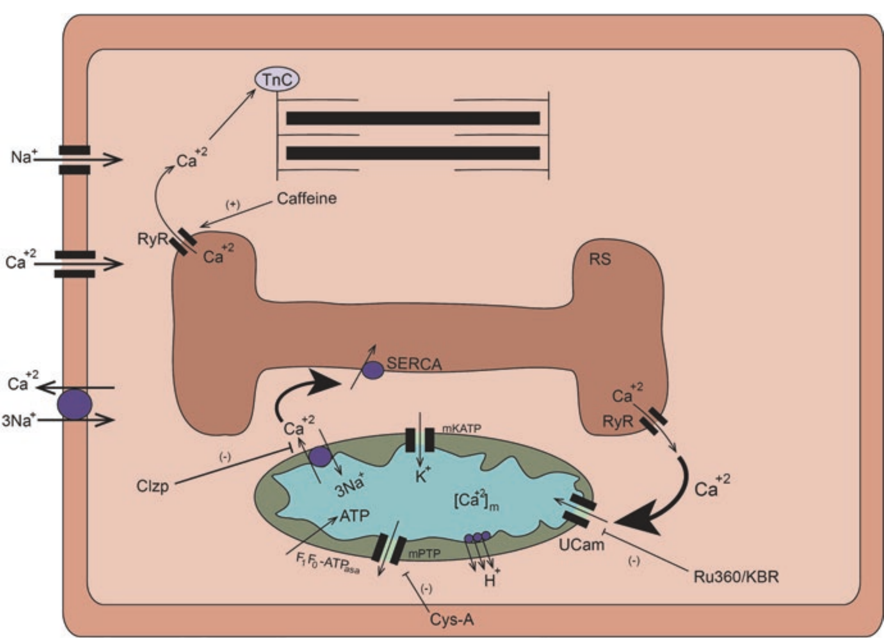


Fig. 8.2 Schematic representation of a cardiac cell with the cellular transporters which could influence in the functional interaction between mitochondria and sarcoplasmic reticulum (SR) and the specific drugs described in this work

Ca^{2+} uptake induces the activity of the metabolic enzymes with the consequent rise in the heat release. This was evidenced from the higher AUC-ΔHt in ischemic than in non-ischemic CPG hearts (Table 8.1).

The inhibition of mNCX with Clzp also reduced the cardioprotection of CPG, since PICR reached up to 50 % ± 6.4% [20]. This effect can be attributed to a reduction in the SR Ca^{2+} content, since Clzp reduced the CIC (Fig. 8.3a) and the AUC-LVP of hearts with CPG, without changing the EC (Table 8.1). Again, results show that PICR depends on the SR Ca^{2+} content regulated by the mitochondrial Ca^{2+} extrusion through the mNCX. Moreover, blockade of UCam with 1 μM Ru-360 during the CPG perfusion also reduced PICR of rat hearts (to 39.9% ± 11.1%) and increased Ht, with reduction of muscle economy (P/Ht) to about 24% of initial economy during R (CPG hearts recovered up to 59% of initial muscle economy) but without an important diastolic contracture [23]. The effects suggest that uptake of mitochondrial Ca^{2+} by UCam (triggered by the cytosolic Ca^{2+} transient) is essential to determine $[Ca^{2+}]$m, metabolism and ATP resynthesis, which in turns maintains contractility. But simultaneously, the UCam blockade may be increasing cytosolic Ca^{2+} whose removal seems to be energetically more expensive. In another work [22] we found that in cardioplegic hearts whose UCam was blocked with 5 μM KB-R7943 (CPG + KBR) the CIC was higher than that obtained in CPG hearts, with similar Ht (Fig. 8.3c, d) and AUC-ΔHt, by which the EC was reduced from 0.12 to 0.05

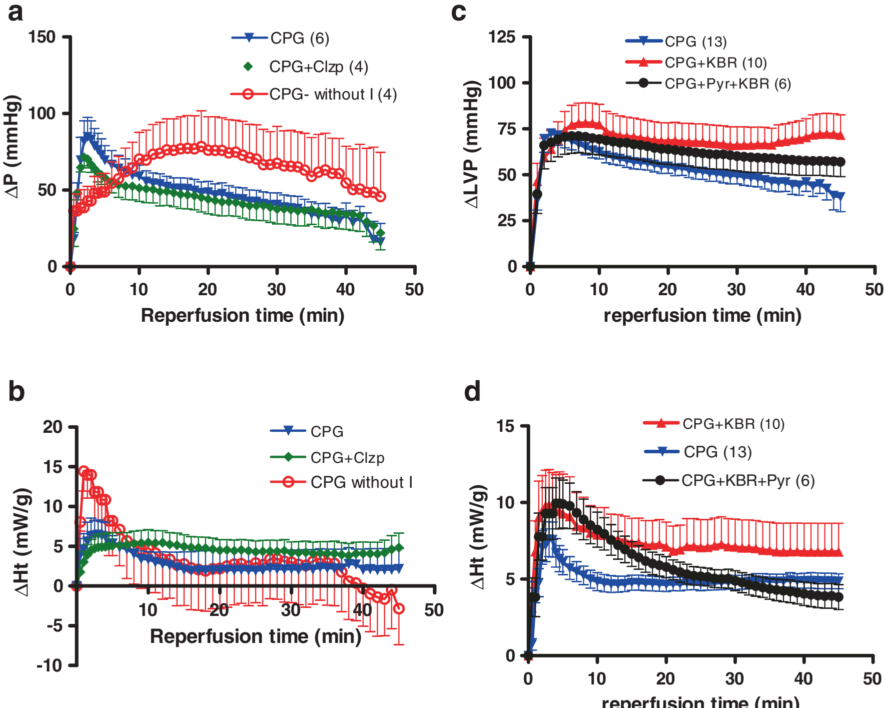


Fig. 8.3 Effects of cardioplegia (*CPG*, Krebs-25 mM K-0.5 mM Ca) and of mNCX blockade with 10 μM clonazepam (Clzp), non-ischemia, UCam blockade with KBR or UCam stimulation with pyruvate (*Pyr*) on the contracture (ΔLVP, in **a** and **c**) and the heat rate (Δ*Ht*, in **b** and **d**) produced during reperfusion with Krebs-10 mM caffeine-36 mM Na^+

(Table 8.1). The increase of CIC can be explained by the fact that UCam inhibition leaves more Ca^{2+} available in cytosol for myofilaments during CIC [22]. Although KBR was first known as a blocker of the sarcolemmal NCX, then it was described as inhibitor of the UCam [47] and blocker of L-type Ca channels [48] all of them at the same IC50 of 5 μM, but only the inhibition of UCam explains this effect. Then, both types of results obtained when UCam is blocked under CPG with Ru360 or KBR suggested that the UCam is necessary to determine ATP resynthesis and contractility and contributes to reduce diastolic cytosolic Ca^{2+}. The blockade of UCam with KBR reduced the EC in agreement with the lower metabolic rate and Ca^{2+} removal both of which become in a higher contracture.

When adding the aerobic substrate **pyruvate** (Pyr, at 10 mM) to the perfusion with CPG + KBR, the effect due to UCam blockade was partially reverted, since the CIC and the AUC-ΔLVP were reduced from the value of CPG + KBR towards the value of CPG (Fig. 8.3c). The AUC-ΔHt was not different to that of CPG + KBR but it was higher than that of CPG, while the EC was similar for the three conditions

(Fig. 8.3d and Table 8.1). Results suggest that Pyr reduced the CIC due to a lower $[Ca^{2+}]i$ consequent to the stimulation of mitochondrial Ca^{2+} uptake. Pyr did not induce changes in the energetic consumption of CPG + KBR because the extra $[Ca^{2+}]m$ also increases the mitochondrial metabolism. The effects of pyruvate agree with those attributed to the increase in the cytosolic phosphorylation potential [49, 50]. Thus, a better cardiac energetic state improves the SR calcium handling and release, with the consequent positive inotropic effect [51, 52]. Also, Pyr increased myofilament calcium sensitivity [53]. Nevertheless, in rat hearts perfused at 30 °C, Pyr had induced negative inotropism with prolongation of the relaxation, both of which were reversed by previous treatment with KBR. The negative inotropic effect of Pyr was interpreted by a combination of acidosis and stimulation of UCam [54]. At the metabolic level, Pyr is an aerobic substrate that induces acidosis caused by a symporter for H^+ and Pyr uptake, a process similar to the mitochondrial pyruvate carrier (MPC) [55]. Intracellular acidification produces direct inhibition of the RyR2 channel [52, 56], prolonged relaxation [57] and lenghtening of the decay of Ca^{2+} signals in the transient and in the caffeine-induced rapid cooling contractures in guinea-pig cardiomyocytes, associated to a reduced Ca^{2+} efflux through the SL-NCX [58]. Interestingly, the reversion of the negative inotropism of Pyr by KBR suggested that Pyr must be decreasing the Ca^{2+} available for contraction when it stimulates the mitochondrial Ca^{2+} uptake through the UCam [54]. On mithocondria Pyr acts as a substrate to the electron transport chain and contributes to generate of the proton-motive gradient, which is the driving force for the Ca^{2+} uptake by the UCam. The rise in $[Ca^{2+}]m$ stimulates the mitochondrial metabolism, which is evident from the increase in Ht [54]. The mitochondrial mechanism of Pyr was also evidenced in isolated cardiomyocytes loaded with Rhod-2 (in conditions of 4 °C to favour mitochondrial accumulation). The increase of $[Ca^{2+}]m$ after perfusion Pyr 10 mM was interpreted from the increase of the relative fluorescence signal (F/Fo). When myocytes were perfused with CPG, both signals, Rhod-2 (mitochondrial Ca^{2+}) and Fluo-4 (cytosolic Ca^{2+}), were also increased in agreement to the activation of L-type Ca^{2+} channels during the cardioplegic depolarization and consequent Ca^{2+} influx to cytosol and mitochondria [22, 24, 54]. But the addition of Pyr 10 mM to CPG induced reduction of Fluo-4 signal and further increase of Rhod-2 signal [54], suggesting that Pyr favoured the UCam uptake. In conclusion, in the effects of Pyr, mitochondria influences on Ca^{2+} movements and contractility, with energetic consequences. The cardioprotective effects were strongly improved when 10 mM Pyr was present before, during, and after ischemia in hearts at 37 °C [54], in accordance with the temperature dependences of metabolic reactions, phosphorylation potential, and Ca^{2+} uptake of SR.

In another work we studied the effects of the **phytoestrogen genistein** (Gst) in the models of stunning consequent to I/R. This phytoesterol is one of the soy isoflavones, recommended for states of estrogenic deficiency. It had been described that Gst reduced myocardial infarct in ovariectomized rats [59], and in rabbit hearts with coronary occlusion when it was used as post-conditioner [60]. In ischemic rabbit hearts Gst had also reduced the infarct size but without improving contractility [61].

There were not previous studies of Gst on the stunning models, while in oxygenated papillary muscles or cardiomyocytes there were described negative or positive inotropism depending on the animal species and attributed to blockade of Ca^{2+} and K^+ channels [62] or increase in sarcorreticular Ca^{2+} uptake [63, 64]. First we evaluated the effects on female and male rat hearts with the model of stunning at 30 °C. Unexpectedly, Gst reduced PICR in males and recovered the pre-ischemic P in females [26]. Looking forward the mechanism, the PICR of males was increased by previous blockade of phosphatases with sodium ortho-vanadate (OV), suggesting that the negative inotropism was due to the typical inhibition of tyrosine kinases by Gst, which affects some mechanisms especially Ca^{2+}-channels. Nevertheless, this effect was not seen in females, because it was counteracted by Gst stimulation of the SR Ca^{2+} content. When it was evaluated by reperfusing hearts with Krebs-10 mM caffeine-36 mM Na^+, we found that AUC-ΔLVP of the CIC was similar for females and males, in the abscence and the presence of Gst (Table 8.1). But the heat release was lightly higher with Gst, as well as the AUC-ΔHt and the EC (Table 8.1). Still more they increased in hearts treated with OV and with 10 mM pyruvate (Pyr), the last one due to the stimulation of Ca^{2+} uptake and metabolism [26]. Gst reduced the relaxation rate of CIC in both female and male hearts, which indicates that Gst inhibits the UCam, since it was partially reduced by Pyr. Results suggest that inhibition of UCam by Gst contributes to the diastolic contracture during I/R, among the other mechanisms of Gst [26]. More recently, results still not published showed that Gst was really cardioprotective, more in males than in females rats, in a model of severe stunning at 37 °C when it was administered by injection at 5 mg/Kg the day before (Fig. 8.4a). Nevertheless, when evaluated whether the effect was due to an increased SR Ca^{2+} content, the hearts from rats treated with 5 mg/Kg Gst and reperfused with Krebs-caff-low Na slightly reduced the CIC (Fig. 8.4b), and increased EC (Table 8.1) suggesting that Gst could contribute to a lower diastolic Ca^{2+} leak from SR during I/R, thus reducing the diastolic contracture and dysfunction. But this mechanism seems to be less important by comparison with the improvement of contractility observed during reperfusion. So, other mechanisms in vivo have to be explored to understand Gst cardioprotection.

Energetical Comparison Between Ischemia/Reperfusion and Hypoxia/Reoxygenation

Certainly, there are differences between I/R and hypoxia-reoxygenation (H/Rox). Although both pathological situations share the decrease in oxygen supply as a common event, there are some others such as accumulation of metabolites, profound acidosis and strong calcium overload which are the heritage of the I/R injury [65, 66].

A reduction in oxygen supply to the myocardium (hypoxia) drives to an energetic dysfunction at mitochondrial level so that ATP production becomes insufficient

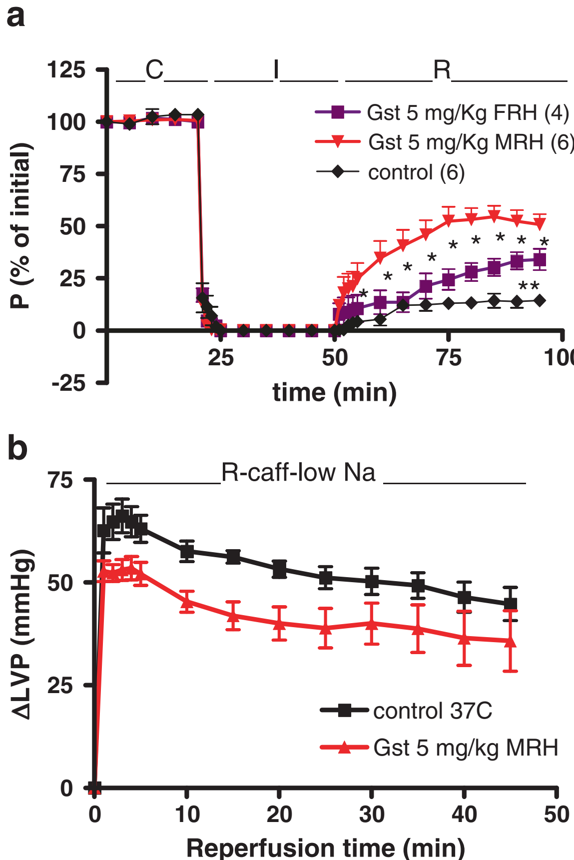


Fig. 8.4 Effects of 5 mg/Kg of the phytoestrogen genistein on the maximal pressure development (P, as % of initial) in female (FRH) and male (MRH) rat hearts exposed to severe stunning (30 min ischemia at 37 °C) (**a**), and on the contracture (ΔLVP) produced during reperfusion with Krebs-10 mM caffeine-36 mM Na^+ of the ischemic rat hearts (**b**)

for the imposed demand. The energetic dysfunction affects not only post-ischemic or post-hypoxic myocardial contractile function but also ionic homeostasis, being that of calcium the most relevant. The heart is able to change from aerobic to anaerobic metabolism for obtain energy from the ATP, producing lactic acid and hence cytosolic acidosis. While perfusion is maintained, metabolites and protons produced are continuously eliminated from the cells and consequently the possibility for a strong cytosolic and mitochondrial calcium overload during reoxygenation is decreased respect to post-ischemic reperfusion. Nevertheless, the generation of reactive oxygen species (ROS) during hypoxia and reoxygenation or I/R [67] would contribute to modify the activity of some calcium transporters and proteins that are involved in contractile recovery after a hypoxic or ischemic episode. On this connection, it was described that the activity of the ryanodine receptor (RyR) release channel in the SR is increased by oxidizing conditions [68, 69]. In addition it was reported that although low levels of ROS increases SERCA2a activity, in the model of myocardial severe I/R ROS contributes to

inactivate it [70, 71]. Furthermore, the reported direct oxidizing effect of ROS on the protein titin increases the passive stiffness of heart myofibrils [72]. Of course, the irreversible modification of these activities depends on the extension and time of the injury.

The model of H/Rox would allow to study only the oxygen-dependent processes that are included within the I/R event. Since I/R and H/Rox differ from one another, the role of mitochondria and its relationship with the sarcoplasmic reticulum in Ca^{2+} handling is expected to be different accordingly.

At the first sight, the model of H/Rox appears to be less dangerous than that of I/R. In fact, unpublished experiments carried out in our laboratory showed that to get similar post hypoxic contractile recovery than that obtained after 45 min ischemia at 30 °C, it was necessary to spin out the time of exposure to hypoxic perfusion to 60 min. On the other hand, at the end of the hypoxic period contractile activity was still remaining (about 25%, see Fig. 8.5a) while contractility decreased to zero after a few minutes of ischemia. In both situations the total muscle economy at the end of reoxygenation/reperfusion was similarly decreased (see Figs. 8.4 and 8.5b and reference [54]) although in the H/Rox model it seems to be faster. The mechanical and energetic responses to reoxygenation in the presence of Krebs caff-low Na^{+} also differ from that of I/R responses. Thus, the CIC on reoxygenation increased during the first minutes and then decreased toward the last hypoxic value (Fig. 8.5c). The relaxation rate of the CIC was higher than that observed in post-ischemic similar perfusion in identical period (compare Figs. 8.1a and 8.5c). Unlike that observed in post-ischemic CIC, after 10 min of post-hypoxic CIC spontaneous contractile activity appeared and stayed during the rest of reoxygenation period. This mechanical performance was accompanied by a transient increase in total heat production which reached a peak, then decreased (Fig. 8.5d) and later increased again at the time in which spontaneous contractile activity was developed. Once again, Ht behavior was different from that seen in post-ischemic perfusion (compare Figs. 8.1b and 8.5d).

These results suggest the following scenario that is compatible with a lesser mitochondrial calcium overload under H/Rox as compared with I/R [73, 74], yielding more feasible conditions for faster recovery. On reoxygenation with caffeine and low sodium, SR calcium content is massively released activating both, contracture and metabolism at mitochondrial level, which increase heat production. Afterwards, contracture relax quickly accompanied by a partial decrease of Ht suggesting that cytosolic Ca^{2+} is removed not only by mitochondria (without calcium accumulation above a deleterious value for uncoupling) but also partially by sarcolemmal Ca^{2+} -ATPase, according that mitochondrial ATP production is reestablished. Although SERCA may be also removing Ca^{2+} and releasing heat by ATP consumption, the effect of caffeine on RyR must be reducing the net uptake of Ca^{2+} by the SR. The relaxation of CIC suggest that H/Rox affect less the Ca^{2+} homeostasis than I/R, thus permitting the development of spontaneous contractile activity later on reoxygenation even in the presence of caffeine, which was absent in the I/R model.

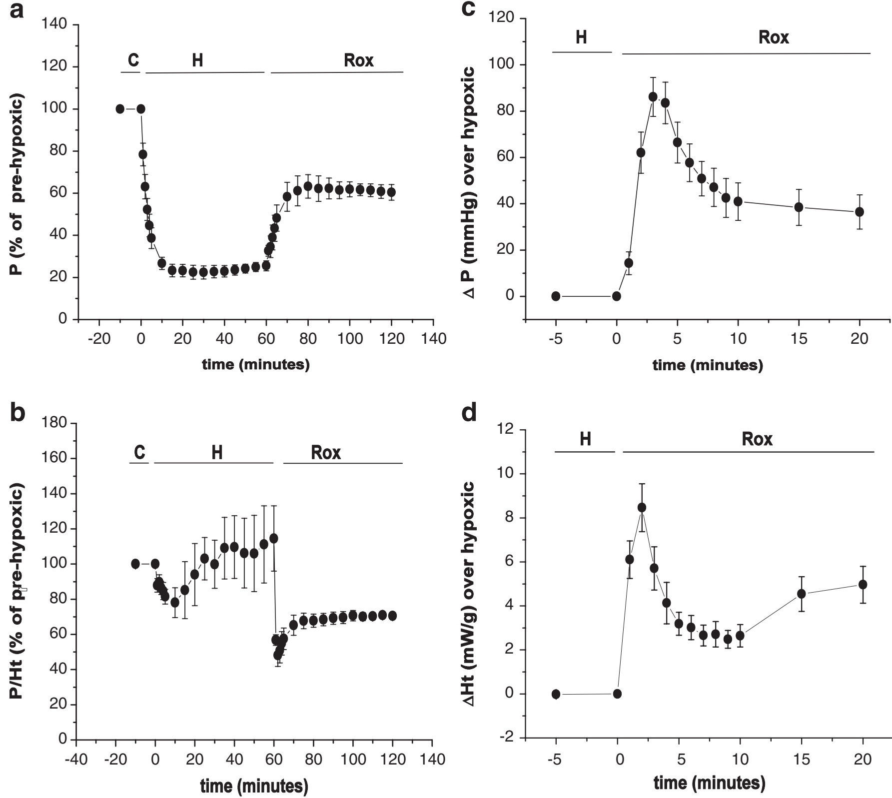


Fig. 8.5 Contractile recovery (**a**) and total muscle economy (P/Ht) (**b**) during hypoxia (*H*) and reoxygenation (*Rox*) of rat hearts. (**c**) Caffeine-low Na induced contracture, and (**d**) total heat production (Ht) over hypoxic value upon reoxygenation with Krebs-10 mM caffeine-36 mM Na^+

Influences of Temperature and Duration of Ischemia on the Mit/SR Functional Interaction

Temperature affects many cellular processes with different rates, more to the primary or secondary active transporters than to channels, binding or passive movements [43]. Obviously, it is possible to obtain two models of moderate stunning with about the same degree of contractile recovery at different temperatures with different periods of ischemia, such us 45 min I at 30 °C and 20 min I at 37 °C in our experiments [20, 25]. Nevertheless, the cellular processes to maintain Ca^{2+} homeostasis are not necessarily functioning at the same degree. Comparing the Mit/RS functional interaction in both models by reperfusing them with Krebs-10 mM caffeine-36 mM Na^+, the model of moderate stunning at 37 °C developed lower peak of LVP and AUC-ΔLVP than the CIC at 30 °C (Fig. 8.6a and Table 8.1). Results suggest that the Ca^{2+} content of SR was reduced at higher and physiological temperature. Thus, temperature increased the rate of cytosolic Ca^{2+} removal by the

cellular transporters still active under caffeine – low Na^+ media (mainly SL-Ca-ATPase and UCam, but much less the SL-NCX). Results agree with some reports on rabbit hearts, in which all Ca^{2+} transporters have Q10 of 2–3 [43]. The low temperature mainly reduces the activity of Na,K-ATPase and increases $[Na^+]i$, consequently reducing Ca^{2+} efflux through the SL-NCX becoming in a higher SR Ca^{2+}content. Evidences of this influence were provided by the magnitude of Ca^{2+} transients and caffeine- and cold-induced contractures [43, 75, 76]. More recently, in rat cardiomyocytes exposed to 10 min of simulated ischemia and reperfusion, it was described the same temperature dependence, since hypothermia to 34 and 30 °C gradually increased Ca^{2+} transients and cell shortening either before or after ischemia [77]. Also, the authors showed that at 30 °C the relationship between contractility and $[Ca^{2+}]o$ was steeper than at 37 °C, and they attributed this effect of hypothermia to the increase in Ca^{2+}-sensitivity of myofilaments. All these effects of temperature remained during reperfusion in spite of the reduction in contractility and Ca^{2+} transient characteristics of the stunning.

The influence of temperature on the rate of Ca^{2+} removal was evidenced by the values of Ht associated to the CIC, which at 37 °C were higher than at 30 °C (compare both models of moderate stunning at the start of CIC in Fig. 8.6b). Consequently, the AUC-Ht was higher in the moderate stunning model at 37 than that at 30 °C, as well as the EC (Table 8.1). The comparison suggests that maintaining the CIC was energetically more expensive at 37 °C than at 30 °C. The difference in Ht may be mostly indicating the increase in Ca^{2+} removal rate, since ΔLVP are not very different especially at the end of CIC. Considering the energetic equivalents from the enthalpic changes of each transporter [78, 79], it is possible to calculate the energy for the futile Ca^{2+} cycling through SERCA induced by caffeine, which moves about 85 nmol $Ca^{2+}.g^{-1}.s^{-1}$ in rat hearts [43]. This cycling would release a heat rate of at least 3.5 mW/g, since SERCA moves 2 Ca^{2+}/ATP and the enthalpic change is 80 kJ/mol ATP. Moreover, the sarcolemmal Ca^{2+}-ATPase would remove Ca^{2+} at maximal rate during the CIC, while the SL-NCX would be slow under the low Na^+-media. The heat release of sarcolemmal Ca^{2+}-pumps can be calculated in 0.3 mW/g (from the described maximal flux of 3.7 nmol $Ca^{2+}g^{-1}$ sec^{-1} [43]). Then, the difference in heat released between both models of moderate stunning at 37 °C vs 30 °C (ΔHt of 7–3.5 mW/g in Fig. 8.6b) is higher than the heat rate calculated as the sum of both pumps, energy and may include the Ca^{2+} uptake to mitochondria. The flows of UCam were estimated under physiological and overload conditions in isolated mitochondria [80], respectively in 32 to 350 nmol $Ca^{2+}.g^{-1}.s^{-1}$. Considering the energetic equivalent of 477 $kJ.mol^{-1}$ O_2 and that the electron chain moves 12 H^+ by O_2 and 2 H^+ by Ca^{2+}, [78]) mitochondria could dissipate from 3 up to 31 $mW.g^{-1}$. Thus the increase in heat release during CIC at 37 as compared with 30 °C is related to the rise in Ca^{2+} fluxes by the pumps and the mitochondrial uptake. In conclusion, when temperature increases the CIC is lower and less economical, because of the increased Ca^{2+} cycling through SERCA, sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uptake.

At 37 °C we also assessed a model of severe stunning (30 min I/45 min R) which produced a PICR of 14.5 % ± 2.4% of initial P, and reduced the pre-ischemic P/Ht

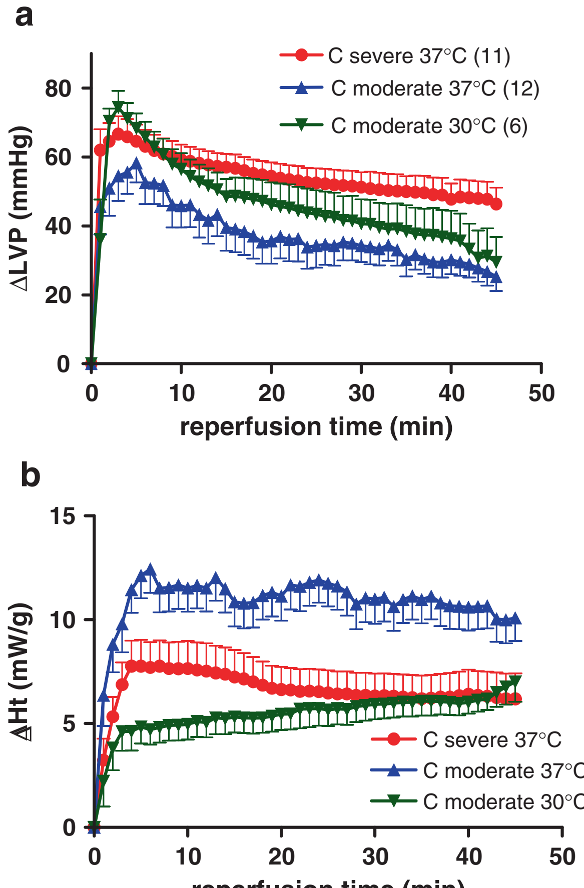


Fig. 8.6 Effects of temperature and degree of stunning on the contracture (Δ*LVP*) (**a**) and the heat rate (Δ*Ht*) (**b**) produced during reperfusion with Krebs-10 mM caffeine-36 mM Na^+ of ischemic rat hearts

to 19.6 % ± 5.5% at the end of R. When we studied the role of SR in this model by reperfusing Krebs-caff-low Na, the peak of the CIC was similar but relaxation was slower than the respective values of the CIC in the model of moderate stunning at the same temperature of 37 °C (see Fig. 8.6). The ΔHt associated to CIC was lower than that of the moderate stunning model, and the respective values of AUC and the EC in Table 8.1 show that the CIC was associated to a lower energy consumption. These results suggest that after a longer period of ischemia mitochondria are in a worse energetic situation, which explains certain difficulty to uptake Ca^{2+}, with a slower relaxation and reduced ΔHt. That is, the energetic consumption (EC) for maintaining the CIC was lower in the severe stunning because of mitochondria have reduced electrochemical gradient, with lower Ca^{2+} uptake and metabolism than in the moderate stunning model.

Effects of Thyroid State on the Ischema/Reperfusion Dysfunction

Hyperthyroidism is considered a risk factor for angor, since when it is severe and untreated it induces tachycardia, high cardiac workload, and risk of atrial fibrillation related to the up-regulation of β-adrenergic receptors [81]. Some patients suffer symptoms of cardiac ischemia because of the high oxygen demand related to the increased cardiac output [82]. Nevertheless, there were described cardiac benefits of levothyroxine (T4) or liothyronine (T3) therapies under critical transient conditions of myocardial stunning consequent to regional or global I/R [83–85]. T4 or T3 were recommended to obtain a rapid restoration of energy stores and myocardial function after organ transplantation [85]. In a previous work we showed that hyperthyroidism (*HpT*), induced in rats by daily injection of 20 $\mu g.kg^{-1}$T3 during 15 days, developed higher PICR and improved total muscle economy (P/Ht) with lower diastolic contracture (ΔLVEDP) than the euthyroid (*EuT*) hearts [25]. To understand mitochondrial bioenergetics and the functional interaction between SR and mitochondria in this pathology, *HpT* hearts exposed to a model of moderate stunning (20 min ischemia at 37 °C) were reperfused with Krebs-caff-low Na^+. Fig. 8.7a shows that *EuT* hearts developed a higher initial contracture than *HpT* hearts and higher AUC-ΔLVP (Table 8.1). This result suggested that *HpT* hearts had a lower Ca^{2+} release from SR than *EuT* hearts. Simultaneously, the ΔHt (Fig. 8.7b) and AUC-ΔHt (Table 8.1) were similar for *HpT* and *EuT*, indicating that Ca-cycling between SERCA and mitochondria was similar in *EuT* and *HpT* hearts. Thus, a lower Ca^{2+} leak from SR could be the responsible for the reduced LVEDP and higher PICR of *HpT* hearts regarding the *EuT* ones.

As in previous conditions, the functional interaction between mitochondria and SR was assessed by blocking the mNCX with 10 μM clonazepam (Clzp) before and during ischemia. In beating *HpT* hearts exposed to 20 min ischemia, Clzp reduced contractile recovery (from 108.8% ± 11.6% to 13.0% ± 3.8% of initial P in the abscence and the presence of Clzp, respectively) while almost improved the PICR in the *EuT* hearts (from 77.5% ± 3.2% to 98% ± 14%, respectively in the absence and presence of Clzp) [25]. Nevertheless, during the reperfusion with Krebs-caff-low Na Clzp reduced the CIC in both conditions, but much more in *EuT* than in *HpT* hearts, as well as AUC-ΔLVP (Fig. 8.7a and Table 8.1). These effects were not followed by significant differences in the AUC-ΔHt, whereby the energetic consumption (EC) during CIC was increased by Clzp (Fig. 8.7b and Table 8.1).

These results suggested that mitochondria regulate the SR Ca^{2+} content by functional interaction from the mNCX to SERCA in both conditions, *EuT* and *HpT*, but the improvement of PICR may be higher in *HpT* because they had attenuated the SR Ca^{2+} leak at the start of R. The rise in EC induced by Clzp in *HpT* and *EuT* clearly shows that mitochondria increased their metabolism when Ca^{2+} extrusion was reduced by Clzp. This is in agreement with the increase in the free mitochondrial Ca^{2+} found during perfusion with Clzp in isolated non-ischemic cardiomyocytes from *HpT* rats [25]. Cells were loaded with Rhod-2 (at 4 °C and washed at 37 °C, as

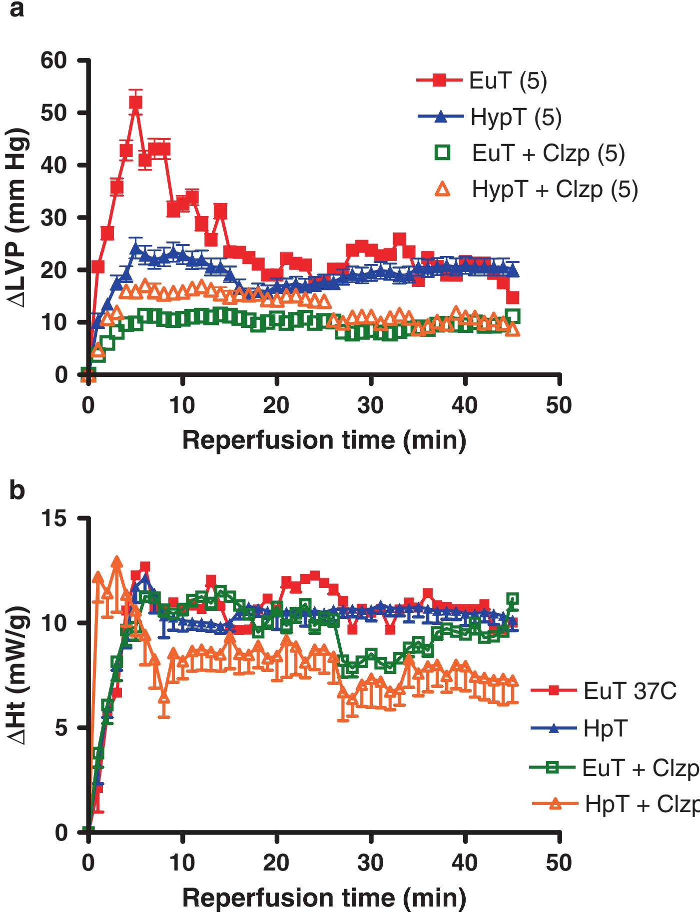


Fig. 8.7 Effects of hyperthyroidism (*HpT*) and blockade of mNCX with 10 μM clonazepam, in comparison with euthyroid rat hearts (*EuT*), on the contracture (Δ*LVP*) (**a**) and the heat rate (Δ*Ht*) (**b**) produced during reperfusion with Krebs-10 mM caffeine-36 mM Na^+ of ischemic rat hearts exposed to moderate stunning (20 min ischemia at 37 °C)

proposed by Trollinger et al. [86]) to measure the fluorometic relative signals (ΔF/Fo) over time under confocal microscopy [25]. Also, either the potentiation of PICR and the rise in the Rhod-2 signal produced by Clzp were partially reversed by addition of ouabain, showing that the high cytosolic Na^+ induced by inhibition of the Na/K-ATPase favored the Ca^{2+} extrusion by mNCX, thus opposing to the inhibition of Clzp [25]. Results show the important role of mitochondrial transporters of Ca^{2+} in the mechano-energetical recovery of the hyperthyroid heart exposed to I/R.

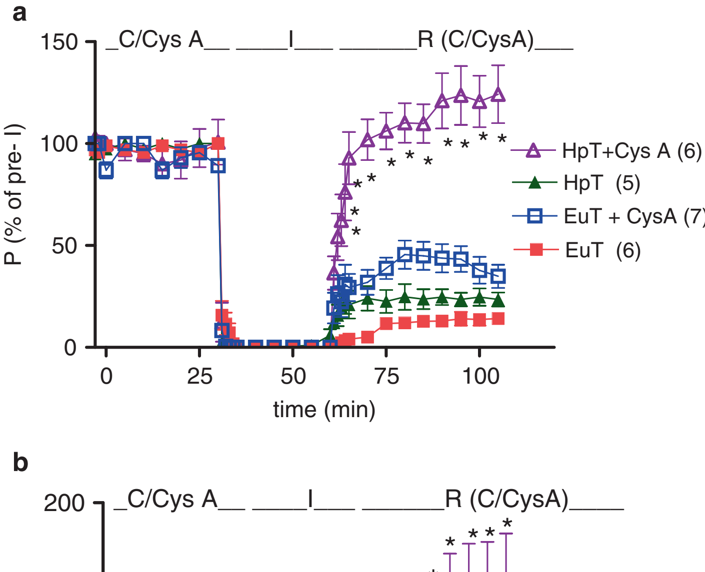
The different responses to Clzp of PICR from *EuT* and *HpT* hearts suggest that there have to be a different threshold of Ca^{2+} for triggering the mitochondrial dysfunction, and this one would be lower in *HpT* than in *EuT* hearts. The possibility

that the opening of the mPTP would participate in the dysfunction induced by Clzp in post-ischemic *HpT* hearts was evaluated by perfusing 0.2 μM cyclosporine-A (Cys-A) during all the I/R protocol. The activation of mPTP causes loss of mitochondrial calcium content, swelling and dysfunction [44], and occurs during the first minutes of R [87]. Effectively, Cys-A improved the PICR of *HpT* hearts treated with Clzp up to 66.4% ± 15.4%, without altering the PICR in the *EuT* analogue group [25]. This result confirmed that a same degree of mNCX blockade induced dysfunction by mPTP opening in *HpT* hearts but not in the *EuT* ones.

Moreover, the role of mKATP channels was explored in *HpT* hearts, by blocking them with 100 μM 5-hydroxidecanoate (5-HD), which reduced PICR up to 66.6% ± 8.3% in *HpT* hearts and 46.9% ± 4.0% in the *EuT* ones [25]. Thus, the hyperpolarizing role of mKATP resulted more critical in *HpT* than in *EuT* hearts during the moderate stunning. It is known that at 100 μM, 5-HD selectively blocks the mK_{ATP} channels [88] and induces mitochondrial energy dissipation to maintain the ΔΨm in beating hearts [89]. Accordingly, we have previously shown that in rat hearts in resting state by perfusing CPG, 5-HD increased Ht before and after I [23]. In *HpT* hearts the treatment with 5HD reduced also the muscle economy (P/Ht) but not in *EuT* ventricles, suggesting again that mitochondria from *HpT* are more sensitive to dysfunction when mKATP are blocked that those of *EuT*. However, 5HD induced diastolic contracture in both, *HpT* and *EuT* hearts, suggesting that the mKATP support part of the mitochondrial Ca^{2+} uptake which contributes to maintain a low diastolic $[Ca^{2+}]i$ in both groups.

The UCam was also essential for the coupling between mitochondrial activity and contractile recovery, without differences between *HpT* and *EuT* hearts, since blocking it selectively with 1 μM Ru-360 [90, 91] reduced PICR to about 17% of initial P and P/Ht to 23% and 13% of the initial P/Ht, respectively [25]. Then, results show that blockade of UCam reduced the post-ischemic contractility as far as muscle economy, suggesting that mitochondrial metabolism was reduced as far as the sarcorreticular Ca^{2+} release, which in turn determines contractility. In this way, Ca^{2+} uptake through UCam regulates $[Ca^{2+}]m$ and metabolism, and consequently the extrusion by the mNCX, which finally influenced the SR Ca^{2+} content and release, and the contractile recovery (Fig. 8.2). Moreover, Ru-360 increased the diastolic contracture during reperfusion in both *HpT* and *EuT* hearts, suggesting again that mitochondria contributes to regulate the diastosolic $[Ca^{2+}]i$ in both groups. A similar potentiation of hypercontracture was described in cardiomyocytes exposed to simulated chemical ischemia, in which blockade of UCam prevented the mitochondrial overload and mPTP opening but developed a hypercontracture associated to high cytosolic $[Ca^{2+}]$ that drove to apoptosis [92].

On the other hand, in a model of severe stunning, obtained with 30 min of I and 45 min R, the cardioprotection of hyperthyroidism was missing. Figure 8.8 shows still not published results of *EuT* and *HpT* hearts which similarly reduced their PICR and muscle economy (P/Ht) to about 15%. The muscle economy (P/Ht) has the same pattern, although the *HpT* hearts developed a lower diastolic contracture than the *EuT* ones. In both thyroid states, the dysfunction can be attributed to the opening of mPTP, since perfusion of Cys-A during the whole I/R period improved



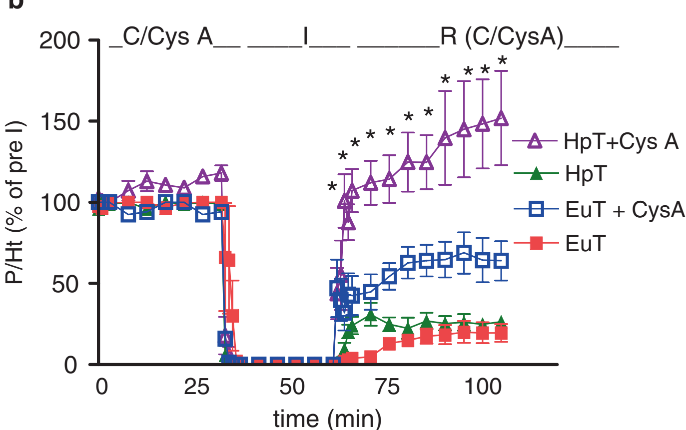


Fig. 8.8 Effects of hyperthyroidism (*HpT*) on the maximal pressure development (*P*, as % of initial) (**a**) and on the total muscle economy (*P/Ht*, as % of initial) of rat hearts exposed to severe stunning (30 min ischemia at 37 °C). Note that the dysfunction was reduced by perfusion of 0.2 μM cyclosporine-A during the I/R in both hyperthyroid (*HpT*) and euthyroid (*EuT*) hearts

PICR and muscle economy, more in *HpT* than in *EuT* hearts (Fig. 8.8). As in previous works, Cys-A did not prevented the diastolic contracture, and this had been previously attributed to non-specific binding of Cys-A [23, 87, 93].

There are different evidences about cardiovascular effects of thyroid hormones, which differ with the type and degree of disease. Low T3 has been considered a beneficial adaptive mechanism activated under stress conditions. Several studies have shown that the low T3 syndrome has adverse prognostic on various cardiac disorders, and T3 is reduced after myocardial infarct. But many of the cardiac alterations observed in subclinical hypothyroidism are reversed once thyroid function has been normalized [94]. The several thyroid effects affect many of the cellular mechanisms for regulating Ca^{2+} and contractility in heart. Because of that, the study

of thyroid alteration on different degrees of ischemia gives new understanding to the influence of thyroid hormones on prevention of angor. Surely, a different situation will be found in myocardial infarct. It is known that thyroid hormones induce genomic and non-genomic effects, which regulate cellular targets, some of them positively (SERCA, Na^+,K^+-ATPase, α-myosin, β receptors, K_V channels, Na^+-channels, troponin I, adenilate cyclase, TRα1, PI3K-Akt) and others negatively (phospholamban, SL-NCX) [42, 95–98]. Thyroid hormones are known as regulators of mitochondrial biogenesis and function, such as opening of the protective mKATP channels due to up-regulation of mitochondrial transcription factor A (mtTFA) and peroxisome proliferator activated receptor gamma coactivator 1 alpha [99]. Moreover, it was reported a correlation between the rate of Ca^{2+} uptake to SR and the PLB/SERCA2a ratio through the hypothyroid, euthyroid, and hyperthyroid hearts, thus determining cardiac contractility [100]. In spite of the expected increase in relaxation rate and energy consumption of SERCA, in our basal conditions of hyperthyroid isolated rat hearts beating at 37 °C, the initial Ht (15.4 ± 0.9 mW/g) was not significantly higher than that of *EuT* rats (17.8 ± 0.9 mW/g) as well as the initial muscle economy (P/Ht) [25]. The effect on SERCA is partially due to the well-known up-regulation of the β-adrenergic receptors [81, 101]. But our protocols in isolated rat hearts did not include adrenergic agonists, in a way that β-adrenergic pathway would not be involved. Also, rat hearts have a more important function and store of SR than rabbits and humans [43], by which it seems less probable to find an increase in SERCA activity by the treatment of 15 days with T3.

Finally, in agreement with the clinical evidences about short periods of **hypothyroidism**, our preliminar results show that under a model of severe stunning (30 min I/45 min R) hearts from rats treated with methimazol 0.02% during 15 days (*HypoT*) had cardioprotection respect to the euthyroid ones (Fig. 8.9). The post-ischemic contractility (PICR) and the muscle economy (P/Ht) were increased and diastolic contracture was reduced in *HypoT* in comparison with *EuT* hearts.

Diminished expression of the SERCA associated to hypothyroidism can be responsible for the lower SR load and consequently a reduced Ca^{2+} leak during the start of R, which reduce diastolic contracture. On this sense, it has been recently reported that the transient and abrupt increase in cytosolic Ca^{2+} which occurs at reperfusion in mice hearts was due to changes in the SR release, in a way that the increased Ca^{2+} sparks in ischemia transform into Ca^{2+} waves during reperfusion, which become in arrhythmia [102]. The high economy (P/Ht) suggests that metabolism would be reduced in *HypoT* rat hearts, resulting in a more efficient post-ischemic heart in comparison to euthyroids. This is in part due to the better diastolic Ca^{2+} removal which avoids diastolic contracture during I/R and improves Ca^{2+} release during the beat. Nevertheless, other experiments will be needed to understand why the *HypoT* hearts are so protected against a severe ischemia and no so much in moderate stunning. Probably other mechanisms could modify the Ca^{2+} homeostasis and signal transduction pathways upon such dysfunction. In other aspects, it was described that hearts from hypothyroid rats resulted more resistant to I/R by a reduced activation of p38 MAPK, in an effect similar but not additive to preconditioning [103].

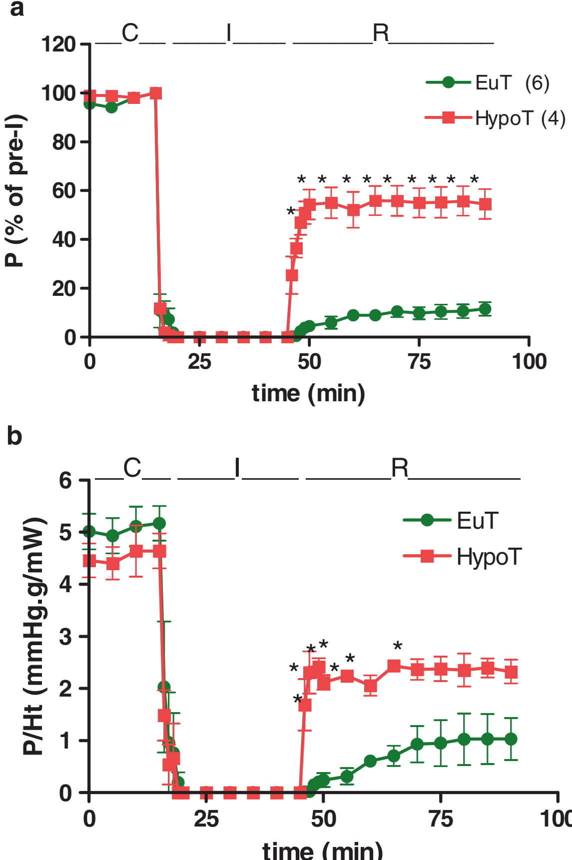


Fig. 8.9 Cardioprotective effects of hypothyroidism (*HypoT*) on the maximal pressure development (*P*, as % of initial) (**a**) and on the total muscle economy (*P/Ht*, as % of initial) of rat hearts exposed to severe stunning (30 min ischemia at 37 °C)

Conclusions

The knowledge of mechanisms and the mitochondrial role during a relatively short period of ischemia/reperfusion is important to find strategies to in order to prevent the dysfunction when a heart suffer a brief episode of coronary obstruction. The energetic approach to study isolated entire hearts provides a tool to evaluate in situ the mitochondrial functions, such as metabolism, resynthesis of ATP and Ca^{2+} fluxes which finally determine the $[Ca^{2+}]$m and metabolism. But calorimetry also permits the evaluation of other exothermic mechanisms that regulate Ca^{2+} homeostasis and contractility, such as sarco-reticular and sarcolemmal transporters and the actomyosin interaction. This review showed several evidences that we experimentally obtained about the functional interaction between mitochondria and SR in models of stunning produced by transient episodes of ischemia and reperfusion. The functional interactions under these conditions differ from that described for the I/R with infarct or H/Rox, and show a regulation of Ca^{2+} from mitochondria towards the SR,

affecting the content and release during reperfusion, which determines the contractile recovery. Moreover, results suggested the existence of a critical level of $[Ca^{2+}]m$ to trigger mPTP opening and dysfunction, that is different upon thyroid diseases or cardioprotective interventions.

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Chapter 9
Mechanistic Role of mPTP in Ischemia-Reperfusion Injury

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Mechanical reperfusion with percutaneous coronary intervention (PCI) represents the gold standard therapy for patients presenting ST-segment elevation myocardial infarction (STEMI) and significantly reduces mortality after acute event. Recent years have seen a significant reduction of 1-year mortality from STEMI, from 15–20% to 5–10% [1, 2]. The "hub and spoke" network for primary PCI and the optimization of techniques, materials, and antithrombotic agents can explain this finding. Unfortunately, improvements in myocardial salvage in some patients have remained small despite successful coronary revascularization.

Reperfusion injury (RI) has significantly hindered the efforts to further optimize STEMI treatment further [3, 4]. RI has been studied for over 30 years and it has been defined as cardiomyocyte damage secondary to myocardial restoration of blood flow [5]. RI is associated with larger infarct size (IS), higher degree of systolic dysfunction, impaired left ventricle ejection fraction (LVEF) and poor prognoses [3]. RI may be responsible for up to 40–50% of the final IS and remains a

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complex phenomenon involving different molecular, cellular and clinical factors that culminate in the genesis of the mitochondrial permeability transition pore (mPTP), which is prompted to open during coronary reperfusion [3, 4]. Reperfusion arrhythmias, myocardial stunning, microvascular obstruction and intramyocardial hemorrhage should be considered different aspects of RI, but we will not discuss them here. In this chapter, we provide an update of recent findings concerning RI. In particular, we focus on mPTP structure and on mPTP involvement in RI genesis. We also summarize the principal aspects of RI in humans, focusing specifically on patients with STEMI and on the mechanistic role played by the mPTP in this pathology, as well as the main pitfalls associated with the application of cardioprotective strategies and therapies in daily clinical practice.

Unmasking Reperfusion Injury: Tools for Quantifying Damage

IS is a well-established independent predictor of poor prognosis after STEMI [6]. Several factors determine IS, and up to 50% of the final IS may be caused by RI if no therapeutic interventions are implemented (Fig. 9.1) [3, 6, 7]. There are currently no efficient therapies for preventing myocardial RI, only promising targets, as discussed later in the chapter.

Cardiomyocytes suffer and die from RI due to mPTP opening at the time of reperfusion in hearts already damaged from sustained ischemia [8]. Quantification of cell death by mPTP opening and identification of damage secondary to RI in daily clinical practice remain complex and misleading. The easiest and most used methods for quantifying the myocardial damage are the evaluation of final LVEF or the area under the curve of the release of specific cardiac markers (e.g., CK-MB and troponin). Thus, they are used to quantify IS reductions. Nevertheless, they are non-specific and cannot be used to differentiate IS due to ischemia from IS due to RI. Otherwise, both electrocardiogram (ST-segment resolution) and coronary artery angiography (myocardial blush grade, MBG) can be used to assess reperfusion outcomes [9, 10]. TIMI (thrombolysis in myocardial infarction) scores at 0–1 flow, ST-segment resolution <30% and/or MBG <2 are markers of poor/absent reperfusion and negative prognoses [9, 10]. The last two indices are significantly more specific than the previous indices but are insufficient for reliably distinguishing among all patients with RI. Majidi and colleagues reported that ventricular arrhythmia bursts (VABs) are associated with larger ISs in patients with similar ST-segment recoveries and MBGs [11]. The authors speculated that VABs reflect myocellular injury in reperfusion settings and that the combination of angiographic and electrocardiogram parameters of epicardial, microvascular and cellular responses may provide a more predictive biosignature of optimal reperfusion [11].

Cardiac magnetic resonance imaging (cMRI) has been used successfully in recent studies to evaluate reperfusion outcomes and to quantify RI, indeed a large and multicenter STEMI population reperfused by primary PCI, cMRI parameters showed an independent and incremental prognostic information in addition to

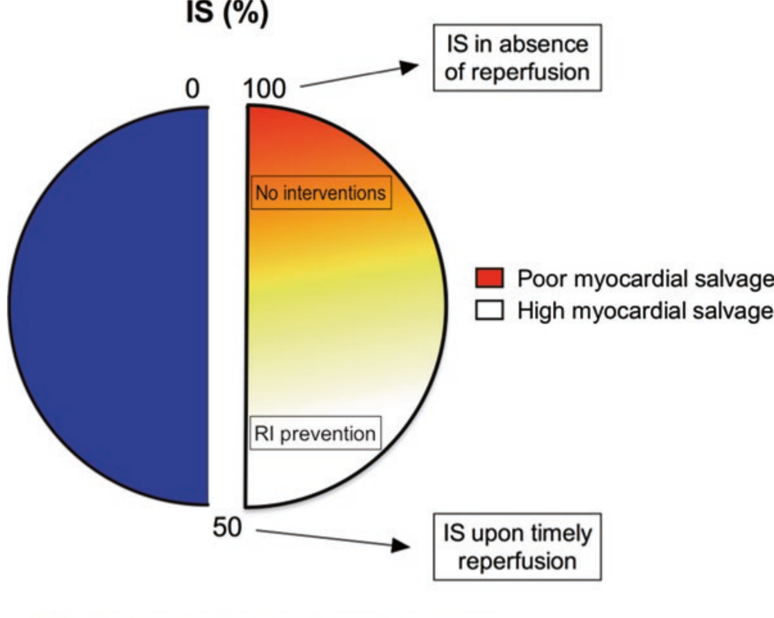

Fig. 9.1 Schematic representation of the final infarct size in percentage (whole graph) in a heart experiencing a myocardial infarction. In the absence of reperfusion, the area at risk represents the entire ischemic area, which has a large final infarct size (100%, whole graph). Successful and timely PCI, which is also responsible for RI, results into 50% of the final IS (*right part*, chromatic scale). Chromatic scale representing the reperfusion-dependent myocardial damage illustrates the possibility of saving the myocardium by preventing and modulating the 50% of the IS caused by clinical intervention-induced RI. The other half of the graph (*blue part*) involves reperfusion-independent mechanisms not reviewed in this chapter. *IS* infarct size, *RI* reperfusion injury

clinical risk scores and LVEF [12]. cMRI is usually performed within the first week after primary PCI [13] and late gadolinium enhancement (LGE) is used to quantify ISs. The latter may be used to calculate the myocardial salvage index (MSI) defined as the difference between the area at risk (AAR) and the normalized LGE divided by the AAR. MSI represents the myocardium that has suffered but survived an ischemic insult and effectively depicts the cardioprotection target [14–16].

Mitochondrial Permeability Transition Pore Contribution in Reperfusion Injury Mechanisms

Oxidative stress, inflammation, calcium (Ca^{2+}) overload, and hypercontracture are significantly implicated in RI genesis (Fig. 9.2). mPTP opening is widely considered a terminal step of RI [17, 18], which begins with an ischemic event. This mPTP

Fig. 9.2 Clinical and cellular determinants of final IS. On the *left*, the angiographic and optical coherence imaging (before and after stent implantation) of two STEMI patients successfully treated with mechanical reperfusion. Cardiac magnetic resonance imaging (on the *right*) reveals the corresponding areas at risk (similar between patients) and the final infarct size (different). *IS* infarct size, *TIMI* thrombolysis in myocardial infarction, *mPTP* mitochondrial permeability transition pore

priming phase was identified by Griffiths and Halestrap in 1995, as these authors proved that mPTP occurs at the time of reperfusion using the mitochondrial "Hot DOG" entrapment technique. They demonstrated that some mitochondria may undergo mPTP opening and closure in ischemic-reperfused hearts [19], as their experimental procedures demonstrated that the extent of 2-deoxy[^{3}H]glucose (DOG) uptake increases until the period of ischemia preceding reperfusion, corresponding to a maximum empirical interval of 30–40 min [19]. During ischemia, which is characterized by the absence of oxygen resulting in progressive ATP depletion, cell metabolism occurs mainly via the anaerobic route, thus lowering the pH. A compensatory mechanism involving the Na^{+}/H^{+} exchanger is activated to counterbalance this condition, leading to a large influx of sodium ions, which reduces the uptake of Ca^{2+} by the endoplasmic reticulum (ER). Thus, cardiomyocytes are subjected to Ca^{2+} overload, reactive oxygen species (ROS) production and long-chain fatty acid accumulation.

These factors increase RI susceptibility but do not cause mPTP opening because a critical value, the pH, remains low. Previous studies have demonstrated that H^{+} inhibits Ca^{2+} binding to the mPTP trigger site [20]. Although the specificity of the

Table 9.1 Schematic subdivision of main intracellular mPTP modulators in negative regulators and inducers

Negative regulators	Refs.	Inducers	Refs.
ADP	[20]	Matrix Ca^{2+} overload	[22]
ATP	[20]	P_i	[23]
Mg^{2+}	[20]	O_2^-	[24]
Low pH	[25]	Oxidized thiols	[26]
High Ψ_M	[27]	Peroxided lipids	[28]
Bcl-2[a]	[29]	High pH	[25]
Bcl-XL[a]	[30]	Low Ψ_M	[27]
HXK I-II[a]	[31]	Bax[b]	[32]
		Bak	[33]
		Bad[c]	[34]
		GSK3-β[d]	[35]
		mtCypD	[36]

GSK3-β glycogen synthase kinase 3 beta, *HXK* hexokinase, *mtCypD* mitochondrial cyclophilin D, P_i inorganic phosphate, *Refs* references, Ψ_M mitochondrial membrane potential
[a]Interaction between the protein and VDAC1 promotes cytoprotection
[b]Required interaction with ANT1 in mPTP opening
[c]Displacement of VDAC1 from Bcl-2,
[d]Modification of HXK-VDAC1 interaction

matrix-localized trigger site for Ca^{2+} is absolute [21], there are many intracellular factors that can modulate the mPTP activity (see Table 9.1 for details). The Ca^{2+} concentration required for mPTP opening is highly dependent on the prevailing conditions within the cell, which can change mPTP Ca^{2+} sensitivity.

The cells that survive ischemic insults die from damage generated by coronary reperfusion. Crompton and co-workers clarified the relationship between the mPTP and RI, demonstrating that oxidative stress and Ca^{2+} overload are critical factors in mPTP opening and RI progression. When reperfusion occurs, the respiratory chain is suddenly exposed to oxygen, leading to oxidative stress. Ca^{2+} accumulates due to rapid mitochondrial membrane potential restoration, and the acidic pH is neutralized. All of these processes induce mPTP opening (Fig. 9.3) [37]. This large pore in the inner mitochondrial membrane (IMM) allows the free passage of all molecules <1.5 kDa into the mitochondrial matrix. The IMM becomes freely permeable to protons, effectively uncoupling oxidative phosphorylation and disrupting ATP production. ATPase reversal also occurs, causing the breakdown of cytosolic ATP generated via glycolysis. Energy metabolism is further impaired, resulting in a continuous cycle of increasing Ca^{2+} deregulation and mPTP opening, which leads to osmotic swelling and damage and mitochondrial disruption. The recent discovery of the mitochondrial Ca^{2+} uniporter (MCU) has highlighted the relationship between Ca^{2+} overload and mPTP opening. The MCU and its associated regulators (see Chaps. 2 and 3) are believed to play key roles in the accumulation of large amounts of Ca^{2+} [38]. In an early study, mitochondria from $MCU^{-/-}$ mice exhibited no Ca^{2+}-induced mPTP opening, suggesting that the MCU is required for this pathway. In

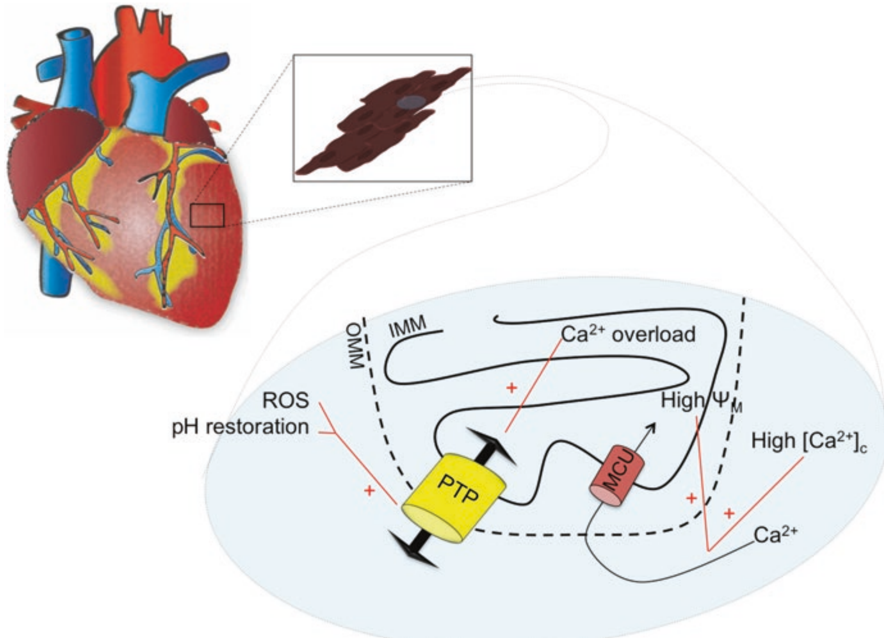

Fig. 9.3 Simplified schematic depicting mPTP involvement in cell death during reperfusion following MI. Restoration of blood flow upon an ischemic event causes RI. Depending on RI severity, cardiomyocytes (*top*) experience oxidative stress, pH increases and matrix Ca^{2+} overload (*bottom*). The diagram depicts all variables that facilitate cell death by mPTP opening. *Red lines* with red plus symbols indicate positive regulation of mPTP opening and Ca^{2+} uptake by the indicated factors. *IMM* inner mitochondrial membrane, *MCU* mitochondrial Ca^{2+} uniporter, *OMM* outer mitochondrial membrane, *PTP* permeability transition pore, *ROS* reactive oxygen species, Ψ_M mitochondrial membrane potential

contrast, recent studies involving $MCU^{-/-}$ mouse hearts subjected to RI revealed that the absence of the MCU does not protect the heart from RI, as these studies noted little difference between hearts of this genotype and wild-type hearts with respect to the incidence of RI [39]. These findings may be explained by the existence of compensatory mechanisms facilitating Ca^{2+}-independent cell death processes involving other molecules that form or regulate the pore when the MCU is absent. However, these aspects require further research.

Mitochondrial permeability transition (MPT) is a critical determinant of RI and is thus responsible for the necrotic and apoptotic cell death processes exhibiting differential contributions to infarct sizes despite their being regulated by many of the same intermediates. Necrosis comprises membrane rupture, cell swelling and cellular debris and intracellular enzyme (lactate dehydrogenase and troponin I) release. Neutrophils migrate to damaged areas and induce an inflammatory response that worsens the pathological condition. In contrast, apoptosis leads to important

morphological and structural changes, including blebbing, cell shrinkage and nuclear fragmentation, without inflammatory response activation [40].

Most of the data regarding these processes have been obtained via studies involving cardiac RI animal models (e.g., rabbit, rat and murine) and have indicated that necrosis occurs at a higher rate during the ischemic phase of MI because of the severity of the insults to which cardiomyocytes are subjected. During ischemic events, bursts of cell death occur in the subendocardium and progress toward the epicardium in a transmural manner [41] peaking approximately 24 h after MI [42]. Necrosis can propagate through enhanced gap junction networks by promoting the spread of contraction band necrosis in the at-risk area [43] and forms the core of the infarct—an area exhibiting irreversible damage—following ischemic insults [44]. Apoptosis, together with autophagy and inflammation, is a reversible process and can be manipulated to allow cardiomyocytes to survive during MI. The duration and kinetics of mPTP opening and the percentage of mitochondria that experience the "open state" in a cell are considered determining factors of the pathological states of reperfused tissues. Prolonged pore opening produces significant cell death waves, whereas short and transient opening allows the cell to either recover completely or initiate the apoptotic pathway via cytochrome c release and caspase 9 and caspase 3 activation. This protease mediates the proteolytic cleavage of a wide range of proteins involved in the rearrangement of the cytoskeleton, the plasma membrane and the nucleus. Numerous reports indicate that apoptosis culminates at the time of reperfusion [44–48] and reaches the periphery of the necrotic core, where the damage inflicted by the insult is less severe. However, only few apoptotic cells are present in non-infarcted myocardial areas [49]. Timely reperfusion is required in clinical practice for obvious reasons but also provides the energy necessary for the completion of the apoptotic process (Fig. 9.3). In addition, several mPTP inhibitors, such as Cyclosporine A (CsA) [50], NIM811 [51], Sanghliferin [52] and Debio-025 [53], reportedly protected the heart from RI-induced cell death in experimental models, indicating that mtCypD (Table 9.1) binding is the most promising target in cardioprotection. Furthermore, genetic mtCypD deletion also provides potent protection against heart IRI, as mtCypD knockout mice have exhibited significant decreases in ISs in previous studies. mPTP opening is not fully dependent on mtCypD, as it can occur even in the setting of mtCypD genetic deletion or CsA-mediated mtCypD inhibition due to higher Ca^{2+} loads or oxidative stress [54]. Thus, targeting other mPTP components may be an attractive strategy for improving cardiac recovery following MI (see the paragraph "Cardioprotection against reperfusion injury: current clinical applications and new attempts at targeting mitochondrial functions and the mPTP"). However, there are many confounding factors that make it difficult to assess if targeted therapies reduce cardiomyocyte death in patients suffering from MI, including age, gender, occlusion localization, TIMI flow, comorbidities and door-to-balloon time. All of these factors may influence apoptosis and necrosis levels; thus, it is important to develop and validate new techniques to better define ISs (see the paragraph "Unmasking reperfusion injury: tools for quantifying damage") in studies utilizing strict inclusion/exclusion criteria with respect to patient enrollment [55].

Mitochondrial Permeability Transition Pore: Molecular Structure

Numerous reports from the late 1980s to the early 2000s indicated that the mPTP was most likely a supramolecular structure assembled in the IMM by proteins representing mitochondrial contact sites between the outer and inner mitochondrial membranes. The list of proteins interacting in the mPTP core is long and includes adenosine nucleotide transporter (ANT), voltage-dependent anion-selective channel (VDAC), mtCypD, hexokinase, creatine kinase, Bak, Bax, Bcl-2, Bcl-x_L, benzodiazepine receptor, glycogen synthase kinase 3 beta (GSK3-β), PKCε, PKG, p53 and complement component 1 Q subcomponent-binding protein (C1QBP) [4, 56]. Accumulating data suggest that the mPTP is not the result of the opening of a pre-existing pore but rather is formed by misfolded mitochondrial membranes proteins modified by oxidative damage [57, 58]. Two distinguished papers published in 2004 and 2007 completely revised our understanding of mPTP architecture. First, Kokoszka et al. presented strong data indicating that mitochondria lacking ANT can still exhibit permeability transition phenomena [59]—indicating that these phenomena are not regulated by ANT ligands—and that cells without ANT can undergo apoptosis initiated by TNF-α and Fas [59]. Second, Baines et al. reported that VDACs (VDAC1, VDAC2 and VDAC3) are not critical for mPTP formation [60], as these authors observed that VDAC-deficient cells can undergo Bax- and Bid-dependent cell death, similar to the ANT-deficient model. Taken together, these data indicate that ANT and VDACs are non-essential mPTP structural components but play roles in mPTP regulation. Interestingly, similar experiments performed with transgenic mice lacking the peptidylprolyl isomerase f (*Ppif*) gene confirmed that mtCypD is the mPTP component responsible for its sensitivity to CsA [54].

However, mtCypD is involved in mPTP regulation (significantly increasing the threshold for Ca^{2+}-induced mPTP opening), not mPTP formation. Based on this evidence, Halestrap et al. proposed that phosphate inorganic carrier (PiC) forms the core of the mPTP [61], a hypothesis supported by other groups [23, 62]. However, later studies by Halestrap's group regarding PiC silencing in HeLa cells revealed that decreasing phosphate carrier levels by up to 70% had no effect on Ca^{2+}-induced mPTP opening [63]. In contrast, Kwong et al. reported that complete PiC genetic deletion desensitizes the mPTP. Thus, PiC involvement in mPTP function cannot be excluded [64]. An important breakthrough regarding mPTP structure occurred at the beginning of 2013, as Pinton et al. observed that the c subunit of mitochondrial ATP synthase plays a critical role in mPTP phenomena (Fig. 9.4) [65, 66]. Detailed studies by Jonas et al. supported the idea that a ring composed of c subunits (c-ring) is the best candidate to form the mPTP core (Fig. 9.4) [67]. These authors demonstrated that purified and reconstituted human c subunit rings can form voltage-sensitive channels. However, neither CsA nor high Ca^{2+} has an effect on purified c subunit ring channel properties, indicating that the c subunit ring alone has no components that regulate mPTP opening and closing. Only when the authors used a complete ATP synthase monomer in the presence of mtCypD did classical mPTP

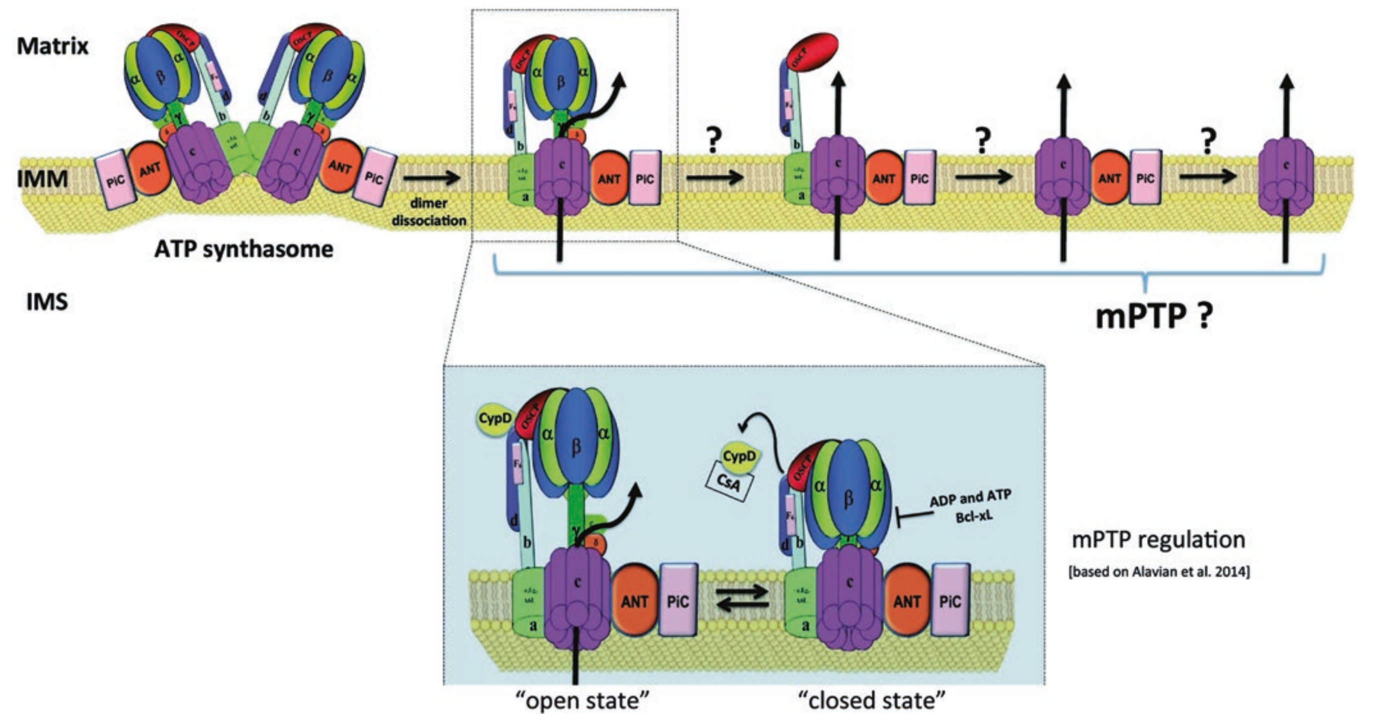

Fig. 9.4 The new-look mitochondrial permeability transition pore. The findings of recent studies indicate that the mPTP is formed via contributions from the mitochondrial ATP synthase c subunit ring under circumstances favorable for mPTP opening. Mitochondrial ATP synthase dimerizes with ANT and PiC to form an ATP synthasome. It is unknown whether the c subunit ring operates as an mPTP alone, in collaboration with other ATP synthase subunits (as a whole monomer or as part of the F_O domain comprising the *a*, *e*, *f*, *g*, and A6L subunits) or with ANT and PiC alone. The lower panel describes ways in which the mPTP may be regulated and thus shift from the open to the closed state (prepared based on [67]). *ATP* adenosine triphosphate, *IMM* inner mitochondrial membrane, *IMS* intermembrane space, *mPTP* mitochondrial permeability transition pore

regulation occur, indicating that Ca^{2+} and CsA (via mtCypD) regulate pore opening/closing by interacting with a peripheral ATP synthase component (probably in combination with the OSCP subunit). Moreover, mutations of highly conserved glycines responsible for optimal c subunit packing within the c-ring cause increases in pore conductance and decreases in sensitivity to CsA, suggesting that impaired c subunit packing may have a large impact on mPTP permeability [67]. Interestingly, adding a purified ATP synthase β-subunit (but not a δ-, ε- or γ-subunit) to the reconstituted c subunit ring decreased pore conductance, indicating that the β-subunit can directly regulate the mPTP. This result explains the well-known inhibitory effects exerted by ADP, as well as the effects exerted by the anti-apoptotic Bcl-xL proteins, on mPTP opening [68]. Contemporaneously, Azarashvili et al. proposed a new mPTP opening/closing regulatory mechanism based on c subunit phosphorylation/dephosphorylation [69]. In their model, PKA-mediated c subunit phosphorylation is responsible for conformational changes in the c subunit that may affect the interactions among the c subunits in the c-ring and may also interfere with the interactions between these c subunits and the stalk subunits of the ATP synthase [69]. These observations suggest that the simplest mPTP model may entail the pore comprising a partially decomposed ATP synthase (e.g., physical decoupling of F_1 from F_O) (see Fig. 9.4 for details). This possibility was confirmed by the existence of free c subunit oligomers that were independent of F_1 components in mitochondria exhibiting Ca^{2+}-induced swelling [67]. However, the presence of free c subunit rings interacting with central and/or lateral stalks has not been confirmed. Therefore, whether physical dissociation of F_1 from F_O is necessary to observe mPTP phenomena, at least in low-conductance mode, in which the inhibitory effects of the β-subunit can be modulated by changing the distance between F_1 and F_O, remains unknown. The latest works of Jonas's and Saris's groups demonstrating that c subunit rings reconstituted in liposomes exhibit channel activity support the original hypothesis that the c subunit is a crucial element responsible for mPTP formation [66–69]. The involvement of ATP synthase in mPTP formation was also proposed by Giorgio et al.; however, their model, in which the mPTP is formed by the dimeric form of ATP synthase [70], seems to fail due to some inconsistencies (e.g., mPTP opening can be detected in Rho0 cells [71]; however, the level of the dimeric form of the ATP synthase in these cells is extremely low [72]). In contrast, Pinton's group developed a model in which the mPTP comprises the monomeric form of ATP synthase, which forms via dimer dissociation (Fig. 9.4). Experimental data tie its activity to a specific c-ring conformation and indicate that variations in the highly conserved glycine zipper domain of the c subunit induced by c subunit-encoding genes have the capacity to influence mPTP activity in various conditions.

The following evidence supports the hypothesis that the mitochondrial ATP synthase is involved in mPTP formation and that the dissociation of the dimeric form causes MPT: Ψ_M and pH influence mPTP opening and also regulate ATP synthesis via ATP synthase, which interacts with ANT and PiC, forming the so-called ATP synthasome. Bcl-2 protein family members physically or functionally interact with ATP synthase (Bcl-x_L inhibits mPTP while enhancing ATP synthesis by ATP synthase), which is sensitive to the oxidation of specific cysteine residues. ATP

synthase binds mtCypD (via the OSCP subunit and subunit d), and the c subunit binds Ca^{2+}. The c subunit has pore-forming properties, and its dephosphorylation and overexpression promote mPTP opening, whereas its transient depletion prevents mPTP opening.

Implications for F_O ATP Synthase c Subunit in Reperfusion Injury: Preliminary Findings Require Further Researches

As mentioned previously, the authors of cell culture studies hypothesized that the long-sought molecular pore of the mPTP is the F_O ATP synthase c subunit [65, 67, 69, 73]. However, the direct involvement of the c subunit in cardiovascular pathology has not been investigated to date, as the c subunit was only recently demonstrated to play a significant role in RI by pre-clinical and preliminary clinical studies.

First, unpublished experiments involving a cardiac animal model of RI demonstrated that targeting the c-ring with selective known inhibitors at the time of reperfusion protected the heart from apoptotic cell death via mPTP inhibition as efficient as that facilitated by the known MPT inhibitor CsA, thus reducing ISs. Second, a very interesting study conducted by Campo G. and co-workers [74] attempted to assess c subunit levels after the STEMI onset in a cohort of 158 patients successfully treated with primary PCI. They observed increases in serum c subunit circulation early after STEMI onset and determined that c subunit protein levels were an independent predictor of all surrogate endpoints of myocardial reperfusion. Elevated c subunit levels were significantly related to a worse prognosis characterized by poor ST-segment resolution values, TIMI myocardial perfusion grades and TIMI frame counts, as well as elevated cardiac marker levels [74]. Although additional studies are necessary to investigate this issue, the collected data clearly indicate that patients with higher F_O ATP synthase c subunit values, indicators of hyper-responsive mPTP activity at the moment of reperfusion, are at higher risk for RI.

Cardioprotection Against Reperfusion Injury: Current Clinical Applications and New Attempts at Targeting Mitochondrial Functions and mPTP

Preventing myocardial RI in STEMI patients is not a simple task. MI is a multifactorial disease influenced by multiple confounding factors and comprising many players, such as cardiomyocytes, microvasculature, inflammation and platelets, that all significantly contribute to its pathology. Lack of knowledge regarding the mechanisms underlying RI and its associated targets has not helped clinicians to achieve the above aim. Several cardioprotective strategies against RI have been developed and tested in STEMI patients. These strategies, including preconditioning,

postconditioning, hypothermia and hyperoxemia, are effective but should be considered mechanical cardioprotection strategies [75, 76]. Mitochondrial function is considered a crucial mediator of RI and cardiomyocyte death (as widely reviewed in [4, 77]), because of the role played by mPTP opening in these processes; thus, increasing numbers of clinical studies regarding mitochondrial targets and pathways have been conducted in recent years [55, 78–84]. Unfortunately, these studies were unable to find evidence that administrating mitochondria-targeting experimental drugs limits ISs. However, these studies observed that therapies with wide ranges of action (e.g., mechanical strategies) contributed to significant improvements in the clinical outcomes of STEMI patients. Taken together, these results indicate that individual key factors in the RI picture have not yet been targeted with respect to establishing adequate and selective therapies. In addition, the failures of some randomized clinical trials may be due to issues related to clinical study design.

Analysis of randomized trials regarding the use of CsA and TRO40303 (MITOCARE study) in humans may help us to understand the importance of adequately identifying correct cardioprotection targets. TRO40303 binds to the mitochondrial translocator protein 18 kDa (TSPO) at its cholesterol site. Although in the past TSPO has been proposed to play a role in the mPTP; a recent study by Bernardi et al. revealed that TSPO plays no role in its structure or regulation. Endogenous and synthetic ligands of TSPO do not regulate mPTP activity, and OMM regulates mPTP activity through an MTP-independent mechanism [85]. In the MITOCARE study, no differences in final ISs or enzyme release were observed between the TRO40303 and placebo groups [78]. CsA inhibits mPTP opening by binding to mtCypD. Past animal model studies and human clinical trials regarding CsA [84] demonstrated that CsA administration at the time of reperfusion was associated with smaller infarct sizes than placebo administration. Cung and colleagues later performed a larger clinical trial (CIRCUS) to confirm the above findings but failed to replicate the expected cardioprotective effects of CsA on MI severity [79] likely due to differences in inclusion criteria between the two studies. The CIRCUS trial enrolled patients presenting within 12 h after symptom onset, by which time most cardiomyocytes are necrotic, and there is not much living myocardium to salvage. Therefore, the strategy of inhibiting mPTP opening to reduce apoptosis was useless for the majority of patients, who experienced longer coronary occlusion and more extensive necrosis than their counterparts in the previous study (see the next paragraph for further details).

Ongoing basic research studies are focusing more on identifying new mPTP constituents (e.g., the F_1F_O ATP synthase c subunit [74]) to develop new cardioprotection strategies than on CsA and its known side effects. Explaining how the dimeric F_1F_O ATP synthase can be converted to the nonspecific mPTP channel (Fig. 9.4) and elucidating the molecular composition of the mPTP are necessary for correctly designing pharmacological approaches based on mPTP targeting. With the assistance of transgenic animals and RI models, we will soon be able to identify, characterize and selectively inhibit each component and/or modulator of the mPTP complex. Discerning the relationship between mPTP activity or its genetic determinants and RI (as assessed by cMRI) in patients undergoing primary PCI for STEMI will be of great importance for achieving that aim.

Major Pitfalls in Clinical Strategies

To conclude this last part of the chapter, we briefly discuss some key points regarding the current major pitfalls associated with attempts to translate the promising therapeutic strategies discovered in basic science laboratories into the clinical setting.

Infarct Size Assessment

Standardizing IS assessments is desirable for minimizing discrepancies and permitting comparisons between studies. cMRI should be considered the gold-standard for evaluating IS. cMRI parameters (LGE, AAR and, in particular, MSI) are well-established and are related to patient prognoses [12, 14]. Furthermore, cMRI reliably identifies cardioprotection targets (cardiomyocytes exhibiting sub-lethal injury and surviving). Unfortunately, cMRI exhibits several limitations. For example, cMRI is time-consuming and expensive and is not available in every cardiovascular center. Moreover, cMRI is not feasible in patients with MI complications, patients who are overweight, or patients with pacemakers and/or implantable cardioverter defibrillators. Finally, cMRI interpretation requires skill and expertise, and several conditions may confound its results (e.g., prior MI and/or prior coronary revascularization).

Potential Confounding Factors of Cardioprotection Success

The major determinant of successful reperfusion therapy is time. Short symptom onset-to-balloon times are associated with smaller ISs, better myocardial viability, higher LVEFs, less severe heart failure and better long-term prognoses than longer symptom-onset-to-balloon times [86]. The majority of previous studies enrolled patients with a median symptom onset-to-balloon time of 6–12 h. We therefore speculate that the benefits of cardioprotection are inversely related to time. More cardiomyocytes suffer death than sub-lethal injury after several hours (>6 h) of sustained ischemia, indicating that sustained ischemia may significantly minimize the benefits of any cardioprotective strategies, especially those targeting mPTP opening. Similarly, baseline and final TIMI flow also affect the success of reperfusion therapy. Spontaneous coronary reperfusion is not rare, and approximately 30% of patients exhibit anterograde coronary flow to ischemic regions [87]. However, administration of cardioprotective agents in patients with baseline TIMI scores of 2–3 may be ineffectual because RI has already started. Similarly, the success of PCI affects the outcomes of cardioprotection strategies. The location of the culprit lesion may play a significant role in patient outcomes since it is directly related to the myocardium at risk. Larger areas at risk may obtain more benefit from cardioprotective strategies than smaller areas at risk, as the probability and extent of RI are greater in larger infarct areas than in smaller infarct areas. However, few studies

have identified specific culprit lesion locations. Finally, patients with cardiovascular histories (prior MI, effort angina, silent ischemia) should be excluded from any studies evaluating cardioprotection success. cMRI interpretations are misleading in these patients and may demonstrate preconditioning-like effects that mask the impact of cardioprotective strategies.

Timing of Cardioprotective Strategy Administration

The ideal time for cardioprotective strategy administration is the time of first medical contact. Early treatment may protect against RI. Unfortunately, early treatment is not always possible in clinical practice. Some mechanical strategies require time and specific instruments that are not available in ambulances or emergency rooms. In addition, the timing of cardioprotective strategies varies significantly between studies. Treatment after the beginning RI (after mPTP opening) may be useless, and the exact moment of mPTP opening, as well as whether it is a reversible process, is unknown. Future research should clarify these points to permit better optimization of study protocols.

Methods

MPT is a fascinating phenomenon and a dangerous intersection in the development of several diseases. It requires a careful and deepen investigation not only in living cells, but also directly in patients. In the ischemic heart diseases, studies involving mPTP in patients are very limited or absent. Thus, if a slice of basic research is studying intensively the molecular composition of mPTP complex with encouraging results [65, 67, 69], simultaneous informative data should be obtained from patients. In this section of the chapter we briefly suggest methods to study mPTP contribution in MI pathology. The investigation of mPTP component(s) behavior in MI and its(their) correlation with standard endpoints of STEMI in patients is a crucial step in understanding how use this(these) potential target(s) in cardioprotection field.

Study Population

Identifying the right cohort of patients is of primary importance. Subjects aged 18–85 years, admitted to the hospital with anterior STEMI which undergo first-time successful PCI (final TIMI flow 3) with an onset to balloon inflation time <4 h and >0.1 mV ST-segment elevation in at least two contiguous precordial leads should be enrolled. Furthermore, the presence of a proximal/mid left anterior descending (LAD) occlusion with TIMI flow 0–1 and no visible evidence of significant coronary collateral flow are essential to study reperfusion damage due to mPTP opening. Major exclusion criteria include a history of prior MI and previous heart failure.

Study Endpoints

The primary endpoint of the studies should be IS and cMRI complete of MVO, LGE and MSI indexes considered the strongest reference points. Second, adverse events in the clinical follow up should be recorded.

mPTP Contribution

Circulating mPTP component(s) and its(their) expression pattern in cells collected from enrolled STEMI patients should be quantified and characterized for mPTP opening kinetics, respectively. Measuring how a putative mPTP component influences the activity of the complex should take into account three different methods: the cobalt-calcein (Co^{2+}–calcein) assay, mitochondrial membrane depolarization, and the swelling technique. The Co^{2+}–calcein assay is the most direct and sensitive experiment for measuring mPTP opening in living cells and can be used in a wide range of cytotype and to study mPTP involvement in many pathological conditions. Since mPTP opening leads to the loss of the proton gradient across membranes, the second suggested assay is the mitochondrial membrane depolarization. The third assay to assess mPTP opening is the measurement of mitochondrial network integrity. Osmotic shock induced by mPTP opening allows for the uptake of solutes into the mitochondrial matrix and concomitant swelling of the inner mitochondrial membrane. This swelling causes mitochondria matrix expansion, which results in rupture of the OMM with loss of mitochondrial network integrity [88].

Statistical Analysis

Biological and clinical data correlation will improve our knowledge on the issue of mPTP contribution in reperfusion injury mechanisms.

Conclusion

Cardioprotection is an area of ongoing active research, especially with the recent discoveries of several components involved in RI, as well as several mechanisms underlying RI occurrence. There remain no effective definitive therapeutic strategies for preventing RI despite numerous failed attempts to devise such strategies; therefore, future studies intended to better characterize mPTP structure, function and regulation and identify agents/strategies that can target its components are mandatory. These studies may improve clinical outcomes in STEMI patients in the future.

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Chapter 10
Functional Role of Mitochondria in Arrhythmogenesis

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A coordinated electrical propagation throughout the heart is necessary for appropriate cardiac function. When such coordination in cardiac action potential (AP) fails, cardiac arrhythmias occur [1]. Several sub-cellular factors contribute to AP heterogeneity, mainly related to sarcolemmal ion channels; however, a growing attention is focused on the action of mitochondria on cell excitability. Indeed, albeit the mitochondrial role in energy production and apoptotic pathways has been determined, the contribution of this highly dynamic organelle in the regulation of excitation–contraction coupling is less clear.

Mitochondria form a functional network within the cardiomyocyte that represents 20–30% of myocardial volume and produces over 95% of cellular ATP. ATP production is made possible by mitochondrial membrane potential ($\Delta\Psi m$) that generate a proton motive force liberating the energy necessary to phosphorylate ADP to ATP [2]. This mechanism is also one of the main sources of reactive oxygen species (ROS) in the cell. In physiological conditions $\Delta\Psi m$ is highly regulated, so that ATP production sufficiently responds to energy demand and ROS does not exceed cell-detoxifying capacity. In response to pathological stimuli, including ischemia and

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structural injury, alterations of ΔΨm cause reduction of ATP generation, and increase of ROS production that once exceeds the detoxifying capacity, leads to oxidative stress.

The involvement of mitochondria in arrhythmogenesis derives from their ability to produce both ATP and ROS; indeed, on one side, mitochondrial dysfunction can affect electrical function of the heart through reduced ATP production, altering sarcolemmal K^+ fluxes via ATP-sensitive potassium channels. On the other side, excessive mitochondrial ROS production can introduce heterogeneity into cardiac action potential since during oxidative stress the oxidation of mitochondrial ion channels can alter their kinetic opening loading to current dispersion and eventually collapse of ΔΨm. The ATP- and ROS- based mechanisms are part of the same cellular response: during mitochondrial dysfunction ΔΨm reduction occurs, resulting in attenuated ATP production and increased ROS level; the latter, in turn, induces further mitochondrial dysfunction, with ΔΨm reduction and consequent reduced ATP generation. This vicious cycle, in which different responses act in synergic manner, causes electrophysiological alterations, thereby conferring a central role to mitochondria in arrhythmogenesis.

Functional Role of Mitochondria in Linking Metabolism and Cell Excitability

Several studies have shown the influence of mitochondrial energetic status on the sarcolemmal action potential and heart excitability. The first experimental evidence derives from simultaneous detection of ΔΨm and AP. ΔΨm oscillations, induced by photo-oxidation, coincide with AP oscillations: in particular, during ΔΨm collapse also AP collapses until a cell non-excitable state, while ΔΨm recovery is mirrored by AP recovery. The impact of mitochondria on cellular excitability is mainly mediated by a class of ion channels abundant in cardiac tissue: energy sensing, ATP sensitive K^+ channels on sarcolemmal membrane (sarcKATP). These channels are heteromultimers inhibited by intracellular ATP and activated by ADP, Pi, Mg^{2+} and extremely sensitive to oxidation. Therefore, sarcKATP channels represent a crucial link between electrical function and metabolism, a dynamic relationship that has been confirmed by independent investigators [3, 4] (Fig. 10.1). During metabolic stress, oscillations of sarcKATP current occur in phase with variation of NADH concentration, with losses of ΔΨm, and that these oscillations are responsible of reduced length of AP [5]. Therefore, metabolic stress like nutrient deprivation can impair mitochondrial function, with reduction of ΔΨm and consequent diminished production of ATP, which in physiological conditions inhibits sarKATP channels. The result is the activation of these channels with K^+ dispersion and reduction of cell excitability, allowing mitochondria to indirectly affect the AP [6]. Such effect has a citoprotective role for myocytes during ischemia, when mitochondria cannot provide adequate ATP to support cardiac energy demand; thus, the sarKATP-mediated reduction in excitability and calcium (Ca^{2+}) transients attenuates cell death during metabolic stress [7–10].

Mounting evidence supports the mechanistic role of sarKATP in arrhythmogenesis as a sensor of metabolic condition of the cell. Importantly, a reduction in ventricular

Fig. 10.1 Role of sarcKATP channels in linking cell metabolic status and cell excitability. Mitochondrial dysfunction lead to increase of ROS and reduction of mitochondrial potential with consequent decrease of ATP production. ATP decrease, ATP and Pi activate sarcKATP channel, with K dissipating current and reduction of AP that predispose to arrhythmia

arrhythmia development after blocking sarcKATP channels using HMR1883 has been reported in studies in both animal and human studies [11–14]. However, other studies have shown that the blocking of these channels with glibenclamide is not enough to rescue an arrhythmic phenotype [15]. Because of these conflicting findings further studies are necessary to elucidate the molecular mechanisms that occur before and after activation of these channels, and to clarify the role of several mitochondrial ion channels that could modify their kinetics in response to metabolic stress. The correct dynamic activity of cellular ion channels is synergistically linked to mitochondria, not only for ATP demand but also for mitochondrial ROS generation.

Role of Mitochondrial ROS in the Pathogenesis of Cardiac Arrhythmias

If reduced ATP production as consequence of mitochondrial dysfunction can directly affect the activity of several ion channels, concomitant ROS generation can activate a complex response which in turn amplifies $\Delta\Psi m$ alterations that further inhibit ATP production and increase ROS generation. Sallott and colleagues demonstrated a ROS-dependent oscillation of $\Delta\Psi m$, with collapse of $\Delta\Psi m$ prevented by

ROS scavenger [16, 17]. As mentioned above, given that ΔΨm oscillations promote alterations of myocyte AP, the ability of ROS to affect mitochondrial membrane potential confers them a pro-arrhythmogenic role. In particular such arrhythmogenic effect of ROS depends on complex and multifactorial response of cardiac mitochondria, named ROS-induced ROS release (RIRR), an autocatalytic process by which high levels of ROS induce further ROS release from mitochondria [17]. Mitochondrial-dependent RIRR regulates electro-chemical equilibrium, through oxidation of several proteins that control current fluxes inside the myocyte; the main consequences of oxidative stress are: activation of sarcKATP channels (that are sensible to oxidation, too), with reduction of Na^+ and K^+ currents [18]; altered kinetic of L-Type Ca^{2+} channels; increase of intracellular Ca^{2+} leak from RyR on the sarcoplasmic reticulum [19]. The functional role of mitochondria-dependent RIRR in arrhythmogenesis has been demonstrated also in whole heart, in ex-vivo experiments; stimulation of the heart with H_2O_2 leads to a two-phase ROS production: the first pick of ROS is directly linked to H_2O_2 action, while the second pick corresponds to ROS of endogenous production derived by mitochondrial RIRR and is associated with arrhythmia [20]. The mechanism of RIRR represents a concerted response mediated by different mitochondrial channels, that are sensitive to ROS concentration, including internal Mitochondria Anion Channel (IMAC), Permeability Transition Pore (PTP), Translocator Protein (TSPO), Mitochondrial Ca^{2+} Uniporter (MCU), and mitochondrial ATP- sensitive K^+ channel (mKATP channel). These channels change their activities in response to crescent levels of ROS and then activate RIRR. Despite the specific contribute of each of these channels to RIRR is not fully understood, the important evidence is that mitochondrial ion channels have a central role in the regulation of electrophysiology equilibrium; indeed through them cell excitability is affected by metabolic status of the cell; therefore, they could represent new and innovative therapeutic target for pathological conditions characterized by electrical alterations, typically arrhythmias, mostly in response to metabolic stress.

Mitochondrial Channels and Arrhythmogenesis

The metabolism-excitation axis is finely regulated by several mitochondrial channels including the inner membrane anion channel (IMAC), the mitochondrial permeability transition pore (mPTP) and the translocator protein (TSPO), and by their crosstalk.

IMAC is a channel deputed to anion efflux from mitochondria [21]. Although its chemical structure is not completely determined, it is known to be one of the channels proposed to participate in energy dissipation in mitochondria, determining ΔΨm collapse and consequent AP reduction [22–24]. In vitro, the blockade of IMAC during metabolic stress stabilizes ΔΨm and determines recovery of AP; on the other hand, activation of IMAC accelerates the shortening of AP through RIRR [10]. These results are confirmed in intact mammalian hearts [10, 25, 26]. Indeed,

in guinea pig hearts IMAC inhibition reduces the ischemia-induced AP shortening and coincides with decreased tachycardia/fibrillation during reperfusion [10]. This evidence opens the possibility to target IMAC as a primary mitochondrial mediator of ROS amplification, ΔΨm reduction, and AP oscillations, for novel anti-arrhythmic therapeutic strategies.

The electrochemical equilibrium in mitochondria is finely orchestrated by mPTP, a large conductance channel located on the inner mitochondrial membrane that plays a crucial role in the regulation of cell death [27]. Although some studies demonstrate that the inhibition of mPTP is protective for cardiac cells during ischemia, there are controversial results about the role of this channel in determining arrhythmia. In isolated cells, the collapse of ΔΨm induced by laser flash is not inhibited by mPTP blocker, cyclosporine A [4, 16, 28]. A non-protective effect of cyclosporine on arrhythmia was confirmed in rat, pig, and rabbit [10, 25, 29]. However, mPTP is directly involved in RIRR; therefore this channel contributes to the effects of metabolism on the cell excitability, but its inhibition is not enough to prevent arrhythmia [30].

Interestingly, IMAC-mediated RIRR and mPTP-mediated RIRR are both regulated by a third protein, located on the outer mitochondrial membrane, TSPO. Altered expression and activity of this translocator protein have been reported in ischemia/reperfusion injury and myocardial infarction, both of which are considered mayor risk factors for arrhythmias. Growing evidence suggests that TSPO can affect arrhythmic response regulating RIRR, directly through IMAC and indirectly through mPTP. TSPO ligands abolish metabolic and electrophysiological oscillations induced by oxidative stress, and reduce ROS levels in cardiomyocytes [31]; moreover, TSPO inhibition abolishes the second pick of ROS production in response to H_2O_2 that represents the RIRR- derived ROS, suppressing ventricular fibrillation and the frequency of arrhythmogenic triggers [20]. This ability of TSPO to promote RIRR with consequent pro-arrhythmogenic effect, is mediated mainly by its action on IMAC. IMAC is tight regulated by several TSPO -acting -ligands, suggesting a direct interaction between pore- forming subunit of IMAC in the inner-mitochondria-membrane, and the regulatory protein TSPO in out mitochondrial membrane [32–34]. Several studies, demonstrate that TSPO is able to regulate mPTP too, indirectly through interaction with VDAC and ANT [35, 36]. Indeed, TSPO block results in inhibition of ΔΨm depolarization after ROS production, but at the same time also in increase of the cell survival in response to oxidative stress, with reduction of cytochrome C release, caspase-3 activation and DNA fragmentation [37]. This evidence suggests a hierarchal activation pattern of mitochondrial ion channels, with TSPO playing a key regulatory role. In response to moderate levels of ROS, TSPO could mediate activation of IMAC with initial energy dissipation that results in RIRR activation, ΔΨm partial depolarization and consequent AP reduction, ensuring a protective status for the cell characterized by reduced excitability to lower energy demand. When metabolic stress is persistent and ROS levels, derived also from RIRR, mediate extreme oxidative stress, TSPO oxidation leads to the activation of the large conductance mPTP and eventually to irreversible mitochondrial membrane potential depolarization, promoting cell dysfunction and death

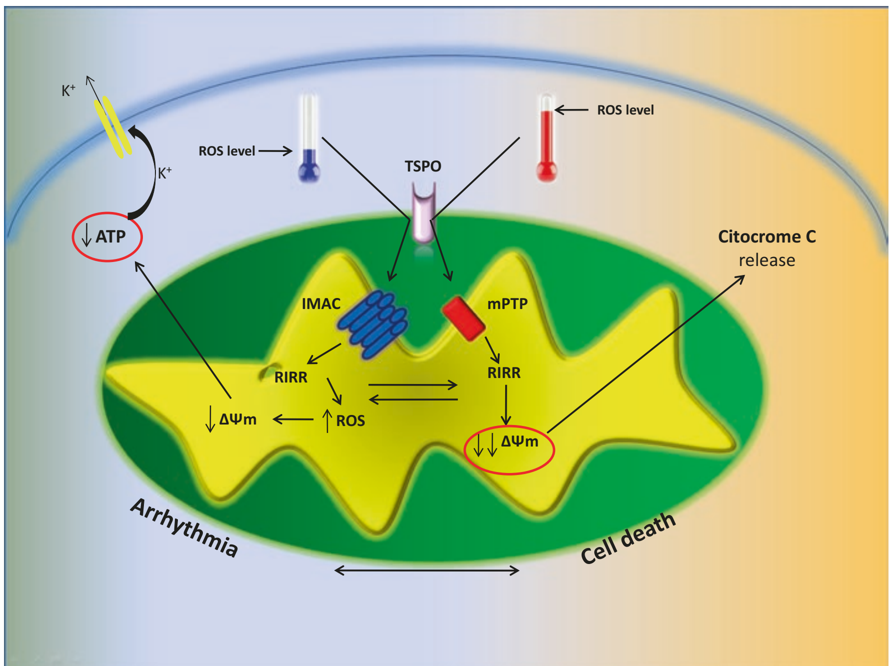

Fig. 10.2 TSPO regulates mitochondrial dissipating energy channel. In presence of low levels of ROS, TSPO is able to activate IMAC with consequent RIRR response that can induce arrhythmia. In presence of high levels of ROS, TSPO becomes able to activate mPTP with terminal collapse of mitochondrial potential and cell death

(Fig. 10.2). This hypothesis confers to mitochondria a central role in determining cell destiny: on one hand this organelle activates cardio-protection at the expense of cell excitability; on the other hand, mitochondrial dysfunction can cause cell death. In this complex response TSPO acts as a sensor of metabolic stress tolerability; not surprisingly, TSPO ligands are promising in preventing ischemia-induced ventricular fibrillation [38].

mitoKATP Channels

A crucial role in arrhythmogenesis is played by a specific class of mitochondrial channels, initially identified in hepatic mitochondria and then found also in the heart: mitochondrial ATP-sensitive potassium channels (mitoKATP) [39]. Little is known about these channels and some studies yielded conflicting results, probably because of the low specificity of the compounds used to target mitoKATP [40–43]. Nevertheless, recent evidence supports a protective role of these channels against

arrhythmia, since their opening before metabolic stress (ischemia) induces partial dissipation of $\Delta\Psi m$, reducing the drive force for mitochondrial Ca^{2+} uptake and improving cellular respiration. The ATP sensibility of these channels allow them to behave as sensors of metabolic stress [40–42].

Mechanistic Role of Mitochondrial Ca^{2+} in Arrhythmogenesis

Ca^{2+} homeostasis in mitochondria is mainly ensured by Ca^{2+} influx through MCU (see Chap. 2), and Ca^{2+} efflux through mitochondrial Na^+/Ca^{2+} exchanger [44]. The administration of MCU blockers reduces the incidence of ventricular fibrillation in anesthetized rats [45]. Most likely, the inhibition of MCU opening plays a protective role keeping mitochondrial Ca^{2+} concentration low. Indeed, mitochondrial Ca^{2+} overload can induce mitochondrial dysfunction with increase of open probability of mPTP [45].

Mitochondrial Ca^{2+} overload might trigger opening of the mPTP, causing uncoupling of oxidative phosphorylation, swelling of the mitochondria, and rupture of the mitochondrial outer membrane. It is not easy to define precisely the role of MCU in arrhythmogenesis given the non-specific effects of the compounds used as blockers (ruthenium red and Ru360); however, mitochondrial dysfunction as result of Ca^{2+} overload suggests that these organelles can contribute also indirectly to arrhythmogenesis, through ROS production and $\Delta\Psi m$ collapse in response to high Ca^{2+} influx. In cardiac myocytes, mitochondria and SR are structurally and functionally related, co-localizing in the so-called mitochondrial microdomain; mitochondrial Ca^{2+} influx through MCU is tightly linked to SR Ca^{2+} release [46]. Alterations of RyR2 on SR, and consequent increase of Ca^{2+} leak in the cytosol, are among the causes of cardiac arrhythmias [47]. Mitochondria, while not representing the primary cause, can contribute strongly to the arrhythmic phenotype, being a key component of the following vicious cycle (Fig. 10.3):

RyR alterations → increased Ca^{2+} leak → mitochondrial Ca^{2+} overload → mitochondrial dysfunction → ROS production → RyR2 oxidation → further increase of Ca^{2+} leak.

We have shown the importance of this vicious cycle, indicating that mitochondrial Ca^{2+} overload plays a key role in atrial fibrillation [48], one of the most common arrhythmias [49, 50]. We used murine models characterized by RyR2 mutations causing SR Ca^{2+} leak, and these mice exhibited mitochondrial dysfunction, RyR2 oxidation, high ROS level and atrial fibrillation. The most important evidence is that not only RyR2 pharmacological block, but also inhibition of mitochondrial ROS production, can prevent arrhythmias in these models [48]. Therefore, Ca^{2+} mediated crosstalk between mitochondria and SR represents an interesting field of investigation to identify new and innovative therapeutic strategies for prevention and treatment of arrhythmia.

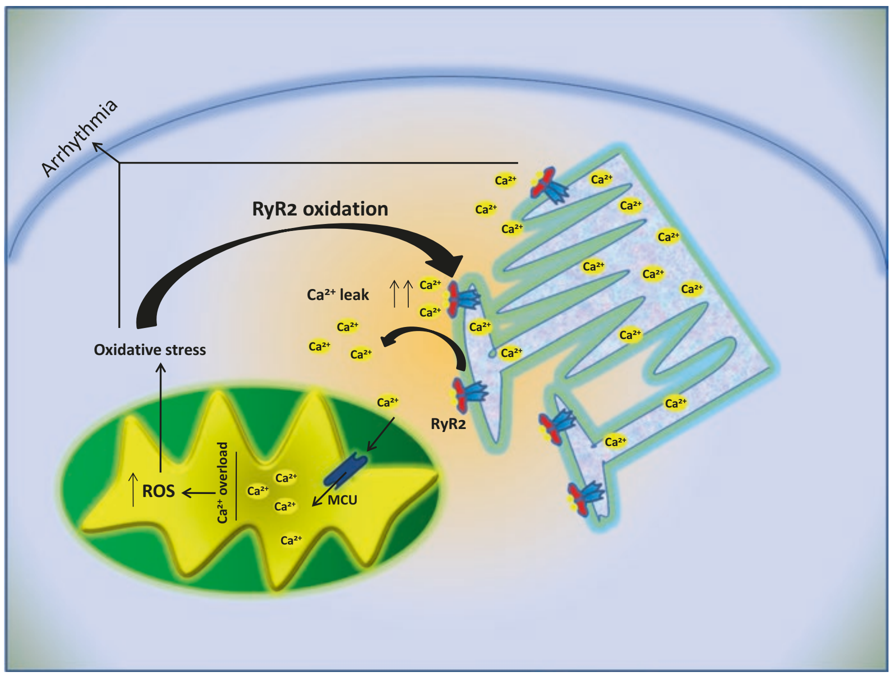

Fig. 10.3 Ca^{2+} dependent pathways and mitochondrial function in arrhythmogenesis. Alterations of Ca^{2+} release from sarcoplasmic reticulum can induce mitochondrial dysfunction as consequence of mitochondrial Ca^{2+} overload. Mitochondrial dysfunction in turn, through means of oxidative stress, can mediate further alterations with increase of Ca^{2+} leak from SR

Mitochondria as Therapeutic Target

As mentioned above, the role of mitochondria in cardiac arrhythmia is the result of a complex network between mitochondrial environment and extramitochondrial environment (cytosol, SR, plasmatic membrane). In particular, these dynamic organelles can contribute to electrical alterations in different circumstances, both when excitability dysregulation is a consequence of metabolic alterations, and when electrical dysfunction is the result of altered Ca^{2+} homeostasis.

Approximately 80% of clinical arrhythmia are consequence of alterations of coronary circulation that induce ischemic events, resulting in mitochondrial metabolic dysfunction [51, 52]. During these events, the mechanisms of RIRR orchestrated by mitochondria are essential, eventually inducing opening of sarcKATP channels with inhomogeneous AP alterations and consequent arrhythmia. After ischemia, restoration of normal blood flow results in additional cardiac damage known as reperfusion injury, with high ROS production that promotes mechano-

electrical dysfunction. In these circumstances, the possibility to modulate mitochondrial response could represent an efficient strategy of intervention to prevent or to treat arrhythmia; for instance, the block of IMAC to inhibit RIRR and prevent AP oscillations, or increasing the anti-oxidant capacity of mitochondria in order to neutralize the first ROS production induced by deprivation of oxygen and metabolite during ischemia, preventing RIRR.

Left ventricular hypertrophy (LVH) is another pathological condition where mitochondrial dysfunction is essential for arrhythmogenesis. During LVH, an increase in $\Delta\Psi m$ occurs, with reduction of apoptosis and bioenergetic alterations since high $\Delta\Psi m$ can interfere with the transport of substrates inside mitochondria with consequent metabolic shift from fatty acid to glucose utilization [53]. The modifications of $\Delta\Psi m$ during LVH, could be attributed to altered expression of mitochondrial uncoupling protein (UCP) that is observed in hypertrophy [54]. Indeed, UCP regulates $\Delta\Psi m$ and ROS production, and mice with low UCP3 develop arrhythmia [55]. Therefore, mitochondrial uncoupling via UCP alterations can induce ventricular fibrillation through AP heterogeneity as consequence of $\Delta\Psi m$ dependent sarcKATP activation [56]. Also in this case, mitochondria can be exploited as therapeutic targets, with possibility to modulate UCP activity to prevent hypertrophy dependent arrhythmia. Moreover, IMAC blockers show to produce beneficial effects in isoproterenol-dependent hypertrophy, most likely via inhibition of RIRR after ROS production, as result of UCP alterations [57].

All these findings indicate that mitochondria could offer several molecular targets for prevention and/or treatment of a broad spectrum of arrhythmic conditions, from ion channels involved in RIRR (IMAC, mPTP), regulatory proteins (TPSO), to channels directly involved in mitochondrial electrical homeostasis and energetic coupling (MCU, UCP). The main problems are represented by difficulties in realizing pharmacological compounds that are specific and efficient ligands of these mitochondrial proteins, since several are able to interact also with other substrates outside mitochondria.

Given the central role of mitochondrial ROS production and RIRR mechanism as primary or secondary cause of cardiac arrhythmias, many investigators are focused on the development of compounds with ROS- scavenger properties, or at least with the ability to increase the anti-oxidant capacity of mitochondria (see Chap. 32). For instance, treatment with superoxide dismutase mimics or mitochondrial-targeted anti-oxidant peptides has been successful in decreasing incidence of arrhythmia [58, 59]. Moreover, administration of N-acetylcysteine to humans after cardiac surgery significantly decreases the probability of arrhythmia development [60].

Further studies are necessary to expand our knowledge on the molecular composition and regulation of mitochondrial targets, in order to develop selective pharmacological compounds able to suppress cardiac arrhythmias.

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Chapter 11
Mitochondria and Cardiac Hypertrophy

Heberty di Tarso Fernandes Facundo, Robert Eli Brainard, Francisco Rodrigo de Lemos Caldas, and Aline Maria Brito Lucas

Cardiac tissue adapts to pressure or volume-overload with an enlargement of the myocardium characterized by growth of individual cardiomyocytes (hypertrophy) rather than an increase in cell number (hyperplasia). Long term cardiac hypertrophy, despite being adaptive against hemodynamic overloads, often progresses to heart failure [1]. During hypertrophy a series of intracellular cascades are activated resulting in changes in intracellular Ca^{2+} levels, contractile protein content, reactivation of fetal gene program [2, 3], O-glycosylation of proteins [4] and, oxidative stress [5–10]. The mitochondrion is the power-house of the cell and its dysfunction has been implicated in the pathology of cardiac hypertrophy, primarily, in the transition from cardiac hypertrophy into heart failure. Mitochondria occupy approximately 30% of the cardiomyocytes' mass and functionally correlate with the heart rate, and the oxygen consumption [11]. Additionally, mitochondria play a role in the regulation of intracellular calcium (Ca^{2+}) homeostasis, intracellular signaling, cell death. Furthermore, mitochondria generate >95% of ATP used by the heart [12]. A normal heart is largely fueled by fatty acid oxidation (60–90%) which occurs in the mitochondrial matrix, while the remaining (10–40%) is supplied by carbohydrates [13]. Cardiomyocytes may use other pathways (ketone bodies and amino acids) for ATP generation as well. However, in response to pathological hypertrophy the cardiac metabolism is reprogrammed to rely more on glucose than on fatty acid as a fuel source [12, 14].

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Even though the hypertrophic phenotype is initially a compensatory mechanism, chronically sustained pressure or volume-overload will deteriorate the heart's pumping capacity. When this occurs, blood flow is not maintained by the heart, which causes a multifactorial clinical syndrome – that is, heart failure, the leading cause of death in the industrialized world [15]. Initially, the heart normalizes the increase in wall tension during systole (in pressure-overload models) and sustains cardiac output by the development of left ventricular concentric hypertrophy. Conversely, volume overload leads to eccentric hypertrophy of the heart. This thickening of the cardiac chambers increases the diffusion distances for oxygen and other sundry substrates, generating a negative impact on the hearts contractile performance [16, 17]. This is partially triggered by anatomical changes including myocardial fibrosis, thickening of the intramyocardial coronary arteries, and perivascular fibrosis [18, 19].

Cardiac mitochondria generate ~95% of the ATP used by the heart, regulate Ca^{2+} homeostasis, participate in signaling, and in some instances trigger cell death [20]. Thus, mitochondria are central organelles responsible for the coordination of energy transduction in cardiomyocytes. Mitochondria, as a consequence of their aerobic metabolism, are the major source of cellular reactive oxygen species (ROS), such as anion superoxide ($O_2^{\cdot-}$) and hydrogen peroxide [21] (H_2O_2). ROS are by-products of the metabolism and are produced by several cellular sources. The electron leak from various sites (mainly complex I and III) of the Electron Transport Chain (ETC) results in reduction of molecular oxygen forming anion superoxide [21–23]. Other sources of intracellular ROS include NADPH oxidases, xanthine oxidase, nitric oxide synthase, lipoxygenases and mieloperoxidases [24]. Their excess generation is counteracted by molecules that eliminate ROS either enzymatically or through the action of antioxidant molecules. The enzymatic system includes superoxide dismutase (converts $O_2^{\cdot-}$ to H_2O_2), catalase and peroxidases (catalyze the breakdown of H_2O_2 to water). The secondary system for ridding the cell of ROS includes vitamins A, E, C and as well as glutathione and thioredoxin, which reduce thiol groups in oxidized proteins [25]. Redox-sensitive signaling pathways play an important role in the pathogenesis of cardiac hypertrophy and heart failure [5–10, 26–28]. For example, H_2O_2 is associated with cardiomyocyte hypertrophy [29], and agonists for G protein-coupled receptors, such as phenylephrine and angiotensin II induce hypertrophy with concomitant increase in ROS formation [7]. Additionally, antagonizing ROS accumulation in heart cells has been found to be beneficial by preventing cardiac hypertrophy [5, 6, 27].

In this chapter we will present and discuss the changes that occur in the mitochondria of the hypertrophic heart, followed by an overview of the potential therapeutic targets and approaches that can possibly reverse these changes and preserve cardiac health. Special attention will be given to adaptations and changes in the mitochondrial electron transport system, substrates metabolism, ROS generation, opening of the mitochondrial permeability transition pore and mitochondrial biogenesis.

Electron Transport Chain in Cardiac Hypertrophy

Mitochondria generate energy by metabolizing dietary carbohydrates, lipids and amino acids. This process is conducted by a series of metabolic pathways that generate energy through oxidative phosphorylation (OXPHOS) in the inner mitochondrial membrane. Mitochondrial energy production is a process in which electrons are passed along four mitochondrial carriers (complexes I, II, III and IV), cytochrome-c and ubiquinone. Once the electrons reach the last carrier, they are utilized by the complex IV for the reduction of oxygen to water. As a result of electron transfer through the carriers, protons are actively pumped into the intermembrane space by the complexes I, III and IV. Protons then flow out of the matrix and into the inter membrane space thereby generating an electrochemical gradient (composed of an electrical potential – $\Delta\psi$ and a protons concentration ratio – ΔpH). This energy is taken by complex V (FO/F1-ATP-synthase) which passively allows protons to flow down their concentration gradient back into the mitochondrial matrix, thus generating energy. This energy is then invested into a bond that joins ADP and P_i which produces ATP. This system will generate the vast majority of cellular ATP that will be either used by the mitochondrion or exported to be used to fuel a multitude of cellular functions [30, 31] (Fig. 11.1).

Maximal mitochondrial oxidative phosphorylation capacity is reduced by cardiac hypertrophy in part due to decreased activity of the respiratory chain complexes [32, 33]. Biochemically, this respiration defect will disturb ATP production by causing a decline in membrane potential and by lowering ATP synthase activity. Additionally, this would lower the substrate oxidation pathways resulting in an accumulation of reducing equivalents. The respiratory chain (complexes I–IV) is the final pathway for the oxidation of reducing equivalents (NADH, $FADH_2$). NADH and $FADH_2$ feed electrons into complex I and II, respectively, which are then transported along the other complexes to eventually reduce O_2 to H_2O. A dysfunctional respiratory chain will lead to an impairment of oxidative phosphorylation resulting not only in decreased ATP production and redox imbalance because of changes in NAD+/NADH, but also leading to increased levels of intracellular Ca^{2+} and increased generation of ROS. Interestingly, up to 2% of the oxygen consumed by the mitochondria may be converted into ROS as byproducts of respiratory chain activity [23]. Mitochondrial complex I contains 45 different subunits and its main role is to transfer electrons from NADH to the electron transport chain. Additionally, this complex has been credited as an important site of electron leakage contributing significantly to ROS formation [34–36]. The transition from compensated LV (left ventricular) hypertrophy to cardiac failure is preceded by complex I and II dysfunction. This is then followed by an increase in ROS and pro-apoptotic markers (Bax/Bcl- 2 ratio) [37]. Other studies have implicated that even at the phase of compensated hypertrophy, the activities of mitochondrial complex I and IV were reduced. This was associated with mitochondrial DNA depletion. These mitochondrial abnormalities were significantly correlated with the degree of cardiac hypertrophy,

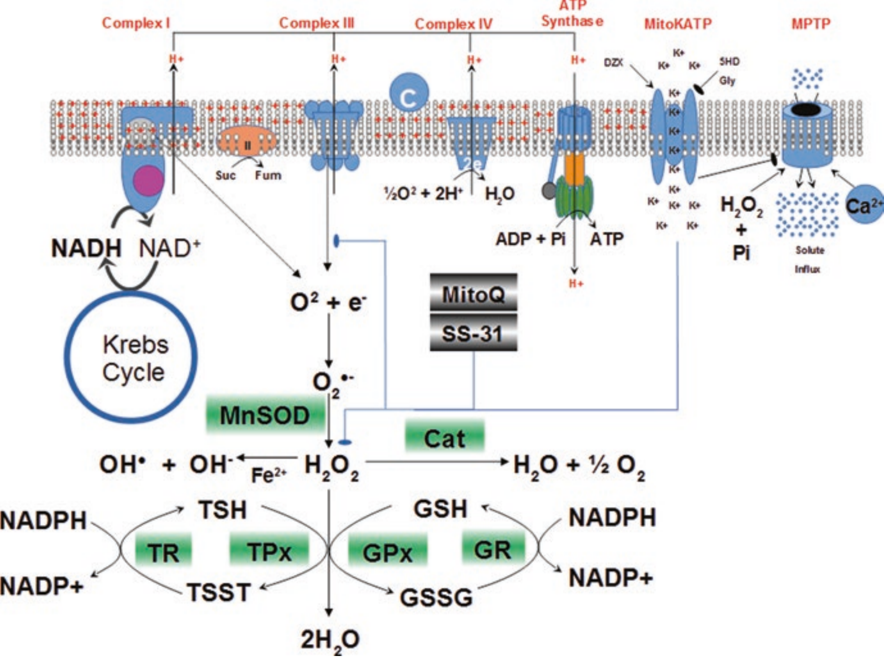

Fig. 11.1 Mitochondrial oxidative phosphorylation and oxidative stress. The enzymes (complex I, II, III, and IV) of the electron transport chain transfer electrons to molecular oxygen (O_2) and pump protons (complex I, III and IV) from the matrix to the intermembrane space. Single electrons may leak to oxygen instead of passing down to complex IV, forming reactive oxygen species (ROS) – anion superoxide ($O_2^{\bullet-}$). Mitochondria possess several enzymatic ROS scavengers: *MnSOD* Manganese Superoxide Dismutase (dismutate $O_2^{\bullet-}$ to H_2O_2), *Cat* Catalase, *Gpx* Glutathione Peroxidase and *Tpx* Thioredoxin Peroxidase that will break H_2O_2 to H_2O. The activities of these two enzymes are coupled to Glutathione Reductase (*GR*) and Thioredoxin Reductase (*TR*). Further, mitochondrial oxidative stress may be interrupted by the Szeto-Schiller peptide (SS-31), mitochondria-targeted ubiquinone (MitoQ10) or by the activity of the mitochondrial ATP-sensitive K^+ channel (mitoKATP). Diazoxide (DZX) opens mitoKATP and 5-hydroxidecanoate (5HD) and Glybenclamide (GLY) close. H_2O_2 together with Ca^{2+}, high phosphate and low adenine nucleotide (not shown on figure) will cause opening of the Mitochondrial Permeability Transition Pore (MPTP)

based on the ratio of heart/body weight [33]. Additionally, one study showed that complex IV activity was decreased by 6 weeks of abdominal aortic banding [32]. Other studies found increased activity of both the nuclear-encoded enzymes of the krebs cycle and the subunits of complex I, III and IV after transverse aortic constriction [38, 39]. However, these studies did not test if the increased complex subunits (by proteomic methods) would impact their individual activity or if they would change the ATP produced by the metabolism of individual substrates.

It is important to highlight that the mitochondrial defect could be at the level of protein import into mitochondria and/or their assembly on complexes in the inner mitochondrial membrane. A direct consequence would be an impaired cardiac oxidative phosphorylation [40]. Mitochondrial proteins (approximately 1,000) are in

majority, encoded by nuclear DNA and imported into the mitochondria. More important, mitochondrial function is maintained by the import of proteins into the organelle [41]. In order to be precisely targeted to the mitochondria, proteins must be recognized by receptors through their mitochondrial-targeting signals (Tom70, Tom20 and Tom22) located on the outer mitochondrial membrane. A more detailed description of the mitochondrial entry gate system can be found elsewhere [42]. Interestingly, Tom 70 is down-regulated during pathological cardiac hypertrophy induced by several stimuli (angiotensin-II, isoproterenol and endothelin-1). Conversely, Tom 70 overexpression abolished pathological hypertrophic growth through modulation of ROS production. Strikingly, this protein is also reduced in human pathological cardiac hypertrophy [43]. Therefore, the disturbances in electron transport chain activities may be related to the poor protein import into mitochondria triggered by cardiac hypertrophy. This and other possibilities need to be systematically tested on future studies.

Mitochondrial Metabolism in Hypertrophy

Maintenance of normal cardiac contractility is correlated with the energy (ATP) generation inside the cardiomyocyte. This cell has a low storage of ATP. Mitochondria normally generate energy by burning fatty acyl-coenzyme-A and pyruvate (fatty acid and carbohydrates metabolism, respectively). Additionally, the majority of the ATP used by the heart [12] is produced into mitochondria. The heart is able to efficiently control ATP production by each substrate, which allows for optimized adaptation to changes in oxygen supply, substrate availability, and cardiac workload [44]. This reprogrammed substrate utilization is often associated with altered ATP yield. For instance, the use of fatty acids produces more ATP per mole than glucose oxidation. Additionally, the adult heart predominately utilizes fatty acids as its main substrate for energy production.

Pathological cardiac hypertrophy, once established, leads to a preferential shift in substrate utilization – from fatty acid oxidation to a less efficient glucose oxidation [45, 46]. The first question here is whether this “substrate switch” is a real phenomenon. Firstly, this is measured by the ratio of fatty acid oxidation to glucose oxidation. Therefore, any decrease in fatty acid oxidation would lead to the so-called “substrate switch” even though glucose utilization stays the same. This shift could be considered beneficial because glucose oxidation improves oxygen efficiency [47] but some glucotoxicity may be in effect if glucose uptake increases [48]. Additionally, there is a higher cardiac glucose uptake in animal models of hypertrophy [49, 50]. The increased glucose uptake will feed the well characterized increased glycolysis flux of the hypertrophic heart [49, 51–53].

Some studies have determined that glucose oxidation may be unchanged during cardiac hypertrophy [51, 54] or even reduced [55, 56], while some others investigations have pointed to higher glucose oxidation [57–59]. As evidenced by the various studies, the intricacies of glucose oxidation during cardiac hypertrophy remain to be definitively described. Taking these findings into account, many have proposed an

uncoupling of glycolysis and glucose oxidation during hypertrophy [51, 52, 54, 56] as one explanation of these results.

One question that is raised by the unchanged or decreased glucose oxidation is how are mitochondrial NADH levels maintained during decreased glucose and fatty acid oxidation (low acetil-coA formation)? The first possibility is that glycolytic flux will increase cytosolic NADH that, under aerobic conditions, will be delivered to the mitochondria through the malate-aspartate shuttle [60]. However, some studies have failed to show enhanced activity or expression of the key shuttle protein oxoglutarate–malate carrier during hypertrophy [61, 62]. A second possibility is through the introduction of carbons in the Krebs cycle by anaplerotic reactions. In fact, hypertrophied hearts displayed increased anaplerotic flux into the Krebs cycle but this flux bypasses the energy-yielding reactions and lead to a reduced energetic efficiency. Additionally, hypertrophied hearts had an increased anaplerotic flux associated with enhanced expression of malic enzyme (the enzyme that carboxylate pyruvate to malate) [63]. Consistent with this idea, hypertrophied rat hearts had increased malate content [54]. Certainly, both possibilities will have to coordinately operate for an improved mitochondrial number, homeostasis, and substrate use. Therefore, the reprogrammed metabolism of the hypertrophied heart will impact overall ATP synthesis by oxidative phosphorylation [64]. For a schematic view of this see Fig. 11.2.

In the adult heart, fatty acid metabolism is more robust when compared to glucose metabolism. As stated above, cardiac tissue undergoes reprogramming, changing its preference for glucose metabolism with a concomitant decrease in fatty acid oxidation. The decreased fatty acid oxidation observed is due, in part, to the inhibition of carnitine-palmitoyl transferase-1 (CPT-1), which is the rate-limiting enzyme for the entry and oxidation of fatty acids into mitochondria [65]. Further, the mitochondrial uptake and oxidation of long chain fatty acid may be reduced by carnitine deficiency during hypertrophy [66]. This will impair the heart's ability to generate energy since the normal adult heart uses the fatty acid oxidation as a primary energetic source. Furthermore, fatty acid oxidation may be reduced by decreased expression of sarcolemmal fatty acid transporters [67, 68]. Therefore, strategies focusing on enhancing the fatty acid oxidation (or preventing its deficiency) seem reasonable. In the same vein, by sustaining fatty acids as the primary fuel source of the heart, one can prevent the detrimental effect of hypertrophy in cardiac energetics and function [69, 70].

Fatty acid oxidation is transcriptionally regulated by peroxisome proliferator-activated receptor-alpha (PPARα) and its coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PPARα activity increasess the activation of the transcriptional program of fatty acid oxidation [71, 72]. Indeed, the activation of PPARα with an agonist during hypertrophy reversed the down regulation of measured PPARα-regulated genes, preventing substrate switching, but contributing to contractile dysfunction [73]. This observed effect could be a direct result of lipotoxicity (increased fatty acid uptake relative to its mitochondrial oxidation) since PPARα activation may induce fatty acid uptake [74]. Others studies using different models have shown mixed results, with some showing improved [75, 76] or relatively modest improvement in cardiac function [77, 78]. It is reasonable to

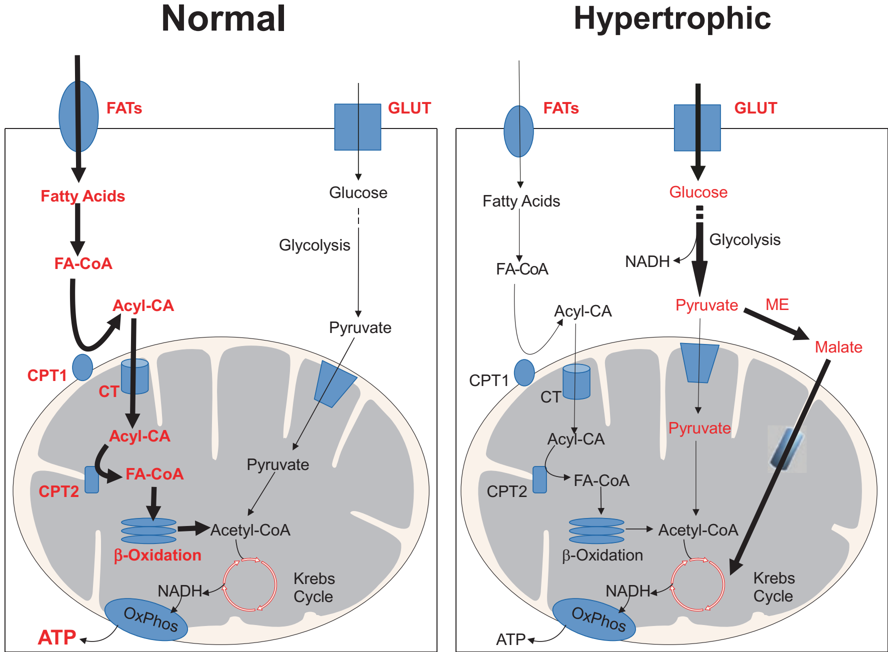

Fig. 11.2 Metabolism in the normal and hypertrophic heart. The metabolic pathways most active in each condition are highlighted in red. Fatty acyl-CoA (FA-CoA – derived from fatty acid) and pyruvate (derived from glucose) are transformed into acetyl coenzyme A (CoA) in the mitochondria. Acetyl-CoA is then fed into the Krebs cycle producing NADH which is then used by the oxidative phosphorylation system (OxPhos) to generate ATP. During hypertrophy, a metabolic shift with up-regulation of glucose utilization and decrease fatty acid metabolism, in part mediated by down-regulation of sarcolemal fatty acid transporters (FATs) and lower carnitine concentrations. This will cause an overall reduction in energy production via OxPhos. The hypertrophied heart has increased anaplerotic flux associated with enhanced expression of malic enzyme, however, this is insufficient to meet the energetic demand of the organ. *CPT* Carnitine palmitoyltransferase 1 and 2, *CT* Carnitine acyltranslocase, *Acyl-CA* Acyl Carnitine, *ME* malic enzyme, *GLUT* Glucose Transporter. *Thick arrows* mean higher flux through that pathway

think that a more direct approach would seek to activate fatty-acid oxidation at the level of the mitochondria.

ATP produced by the mitochondria needs to be transferred from the mitochondria to the cytosol in order to sustain the cardiac excitation-contraction-relaxation cycle. The adenine nucleotide translocator (ANT) is the protein responsible for the transference of ATP from mitochondria to the cytosol. Interestingly, ANT1 knockout mice have enlarged hearts accompanied by mitochondrial proliferation [79]. Additionally, the overexpression of ANT attenuates cardiac dysfunction induced by angiotensin [80]. Therefore, disturbances in ANT activity during hypertrophy would induce a lowering of ATP in the cytosol, thus prompting the “energy-starved heart” condition. This theory links the disturbed mitochondrial metabolism (production of ATP) to the mechanical dysfunction of the heart [81, 82]. In agreement to this theory, ATP (mostly produced by mitochondria) is critical to compensate for the

elevated cardiac demand during hypertrophy. Taking into account the previous findings, it appears that one of the primary contributors to cardiac hypertrophy is the defective ATP production brought about by a low rate of fatty acid oxidation coupled with the low yield of ATP produced via glycolysis.

Mitochondrial Biogenesis in Cardiac Hypertrophy

Mitochondrial biogenesis is a complex process triggered by variations in preexisting mitochondria. These mitochondria will grow and divide with changes in size, number and mass. This biogenesis depends primarily on mitochondrial DNA replication as well as synthesis, import, and incorporation of lipids and proteins into organelle structure. It is easy to understand that all of these processes must be tightly coupled and regulated in order to meet the tissue requirements. The peroxisome proliferator-activated receptor gamma co-activator (PGC-1α) discovered first by Spiegelman and collaborators [83] is a master regulator of mitochondrial biogenesis in mammals. This co-activator lacks DNA-binding sites and activates transcription by binding numerous transcription factors. It is highly expressed in the heart, skeletal muscle and brown adipose; tissues with high oxidative capacity [84, 85]. PGC-1α has two structural homologs one called PGC-1β and another called PRC (PGC-1-related coactivator 1). PGC-1β exhibits practically the same properties including the regulation of mitochondrial activity, thermogenesis and cardiac performance [86, 87]. PGC-1α [88, 89] and PGC-1β [90] are transcriptionally repressed during cardiac hypertrophy that is induced by transverse aortic constriction. The repression of PGC-1α has been suggested as being an important phenomenon responsible for driving the shift from fatty-acid oxidation towards glucose oxidation, which contributes to the impaired ATP production which is seen during pressure overload induced hypertrophy and heart failure [66, 89, 91]. PRC expression is not affected by cardiac hypertrophy/heart failure [88].

The absence of PGC-1α causes serious deficiencies on the energetics and function of the heart [92]. ATP production is insufficient due to abnormalities of the expression of mitochondrial enzymes. Moreover, the ablation of PGC-1α had diminished cardiac function after treadmill running [93]. PGC-1α also suppresses the pro-hypertrophic signaling pathway (calcineurin-nuclear factor of activated T cells c4 – NFATc4) which contributes to the protective effect of PGC-1α on cardiomyocytes during hypertrophy [88, 94]. Likewise, PGC-1β deficiency leads to defects on components of the electron transport chain, which contributes to the poor cardiac performance seen after dobutamine stimulation [87]. It is important to understand that the absence of PGC-1α or PGC-1β alone is insufficient to cause any disturbances in contractile function under non-stressed conditions. However, combined deficiency of PGC-1α and PGC-1β revealed an extensive redundancy between these co-activators. These mice died shortly after birth due to decreased mitochondrial number and size combined with reduced cardiac output [95].

Overexpression studies [96–99] have indicated that PGC-1α activates the fatty acid oxidation program and directs oxidative respiration in cardiac tissue. Cardiac-specific (driven by αMHC – α-myosin heavy chain promoter) knockout of PGC-1α, starting at embryonic day 11.5, causes uncontrolled increases in mitochondria number that disturb the sarcomeric structure [97]. These myofilament derangement contribute to cardiac dysfunction [98]. A pitfall on these studies is that they always utilized high-levels of PGC-1α overexpression. However, it is important to point that overexpression of a protein does not necessarily equate increased activity. More recently the effect of moderate overexpression of PGC-1α was tested, but it did not improve cardiac function during chronic pressure overload [100]. One possibility is that PGC-1α is already highly expressed in the heart and any additional attempts to increase its level will not be satisfactory.

Several studies on PGC-1α suppression are based on transcriptional gene expression. It is important to point that gene expression does not necessarily depict its activity. More specifically, the mRNA for MCAD (medium chain acyl coA dehydrogenase – a gene controlled by PGC-1α) was decreased while MCAD protein and activity was not changed during cardiac hypertrophy [101]. Recent studies showed no decrease in PGC-1α protein levels in samples from human failing heart [102]. Besides no defects on PGC-1α protein various abnormalities in mitochondrial biogenesis may be present due to binding and activity impairments. Future strategies must focus targeting the PGC-1α protein as a therapy.

Hypertrophy and Oxidative Stress

Reactive oxygen species are associated with cardiac hypertrophy [6–8, 10, 26–29, 103, 104]. For example, hypertrophy in isolated cardiomyocytes induced by angiotensin II, endothelin-1, alpha-adrenergic agonists, tumor necrosis factor-alpha, ouabain, or cyclic stretch has been shown to elicit increased ROS production [7–9, 27, 105], while utilizing antioxidants inhibited the development of pressure-overload cardiac hypertrophy in mice [5, 6, 27]. Interestingly, the overexpression of mitochondrial-targeted catalase avoids the age-dependent protein oxidation of mitochondrial heart proteins, and also, reduces the age-dependent left ventricular hypertrophy and diastolic dysfunction [106]. Conversely, H_2O_2 (a cell-permeable ROS) causes, at low levels, the hypertrophic phenotype in isolated cardiomyocytes. On the other hand, at higher levels this molecule promotes cell death by necrosis and apoptosis [29]. Also, superoxide dismutase inhibition increases superoxide anion inside cells and activates growth of cardiac cells [107].

The increased evidence for the involvement of oxidative stress in cardiac hypertrophy and heart failure [5–10, 26–28, 106] highlights the need for a better understanding of the mechanisms involved. A better understanding will ultimately help to elucidate new tools to prevent heart failure. Evidence for the role of oxidative stress in cardiac hypertrophy first emerged from a study reporting that angiotensin II

induced ROS generation in cardiac myocytes [9]. This hypothesis was confirmed by others showing that the antioxidants could abrogate the observed effect [5, 26, 27, 106]. More specifically, the treatment with antioxidant N-2-mercaptopropionylglycine alleviated cardiac hypertrophy in vivo [5]. This apparent decrease in hypertrophy was associated with less oxidized glutathione. Indeed, intracellular ROS generated during hypertrophy could cause thiol oxidation of cysteines [25]. Moreover, the direct administration of H_2O_2 induced hypertrophy in isolated cardiomyocytes [29]. These ROS are also implicated on the expression of the hypertrophic marker beta-myosin heavy chain via MAPK pathway [108]. Our recent studies pointed toward a simultaneous attenuation of the antioxidant enzymes catalase and SOD during hypertrophy [109]. More importantly, the inhibition of SOD [107] or its cardiac specific knockout [110] is sufficient for the induction of cardiac hypertrophy.

Oxidative stress activates the apoptotic cascade and contributes to maladaptive myocardial remodeling. Additionally, augmented ROS production will stimulate the expression and post-translational activation of matrix metalloproteinases and stimulate cardiac fibroblast proliferation, two events that are central to maladaptive extracellular remodeling [111]. Several hypertrophic signaling molecules are changed by increases in ROS production [112–118]. The ROS cascade culminates in the activation of several hypertrophic signaling molecules including: PKC [112], JNK [119], ERK1/2 [113], AKT [115], MAPKs [117], NF-kB [118]. An interesting molecule is the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This protein is central to hypertrophy/heart failure mechanisms [120]. CaMKII may be oxidized in Met residues (281/282) and become activated, altering growth and hypertrophic gene expression [114].

Following CaMKII-mediated phosphorylation, class II HDACs are exported to the cytosol where they can no longer suppress target transcription factors [121, 122]. This lack of suppression will release and activate MEF2/NFAT mediated hypertrophic gene expression [122]. Additionally, HDACs are directly oxidized by ROS that are being exported from the nucleus [123]. Although a direct link between these signaling molecules (specially oxidized CaMKII) and ROS produced by mitochondria during cardiac hypertrophy is still lacking, studies aimed on specifically demonstrating this relationship would be of great value. It is appropriate to affirm that mitochondrial-derived ROS may induce these signaling proteins and regulates the cellular hypertrophic status (Fig. 11.3).

Mitochondrial Oxidative Stress in Cardiac Hypertrophy

ROS are generated inside mitochondria by the leak of electrons from the electron transport chain (mainly complex I and III) to molecular oxygen [21–23] or they can be produced by the activity of NADPH oxidase, [124, 125] among other sources. The leak of electrons to the molecular oxygen will generate the $O_2^{\cdot-}$. The intra-mitochondrial $O_2^{\cdot-}$ it is likely to be very low because of the superoxide dismutase present in the mitochondrial matrix which uses manganese as a cofactor (MnSOD).

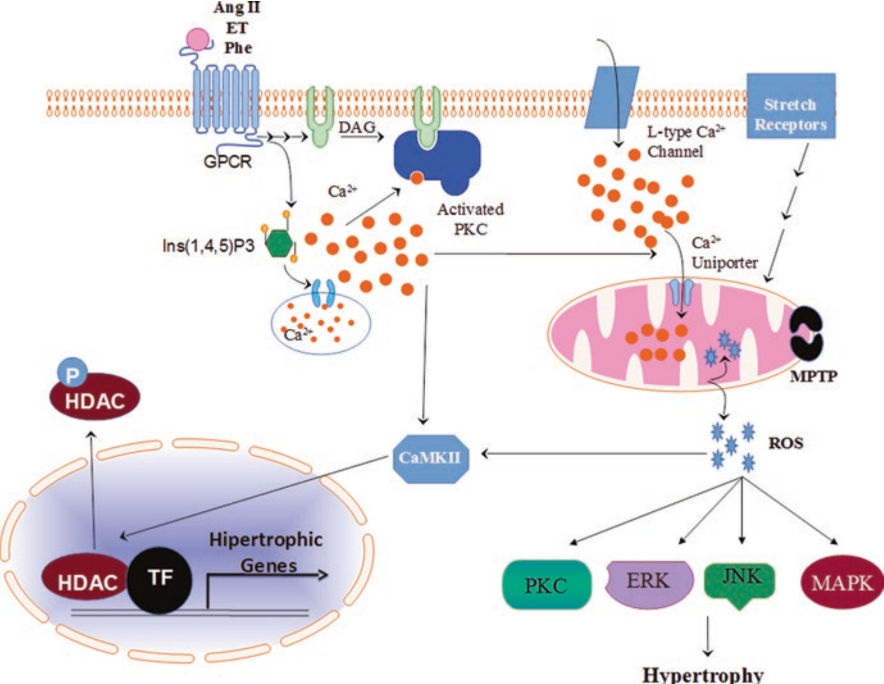

Fig. 11.3 Hypertrophic oxidative signaling. Schematic diagram of key pathways involved in oxidative stress induced by hypertrophy. Pharmacological/neurohumoral signaling or cyclic stretch is sensed by receptors. Receptor activation allows the entrance of Ca^{2+} into the mitochondria and beginning the production of ROS. These ROS will activate direct downstream kinase effectors and together with Ca^{2+} will trigger the opening of the mitochondrial permeability transition pore (MPTP). Additionally, these signals will promote gene transcription (including reactivation of fetal genes) and activation of cellular growth. L-type Ca^{2+} receptors are also activated and contribute to increased intracellular Ca^{2+}. *Ang II* angiotensin II, *Phe* Phenylephrine, *ET* endothelin, *CaMKII* calmodulin-dependent kinase II, *DAG* diacylglycerol, *ERK* extracellular signal regulated kinase, *GPCR* G-protein-coupled receptors, *HDAC* histone deacetylases, *Ins(1,4,5)P3*, inositol-1,4,5-trisphosphate, *JNK* c-Jun N-terminal kinase, *MAPK* mitogen-activated protein kinase, *PDK* phosphoinositide-dependent kinase, *ROS* Reactive Oxygen Species, *TF* Transcription Factor (Part of images from Motifolio drawing toolkits (http://www.motifolio.com/) were utilized in the figure preparation)

This enzyme specifically converts $O_2^{\cdot-}$ to H_2O_2 [126]. This resultant molecule is more stable and may easily diffuse to the inter-membrane space and cytosol, making this a more appropriate compound to be detected in mitochondrial suspensions [127]. On the other hand, not all H_2O_2 produced within the mitochondrial matrix will diffuse out of mitochondria. This molecule can be converted to O_2 and H_20 by catalase [128]. H_2O_2 can be additionally broken down into water by Glutathione peroxidase/glutathione reductase which uses glutathione as a cofactor [129]. Additionally, mitochondria possess thioredoxin peroxidase/redutase that breaks H_2O_2 to H_2O by using thioredoxin as electron donor [130]. If H_2O_2 accumulates its

interaction with Fe^{2+} will drive the formation of the highly reactive hydroxyl radical, of which no specific antioxidant system is known (Fig. 11.1).

Mitochondrial damage seems to play a central role in the transition from hypertrophy to heart failure [13]. The activity of the mitochondrial electron transport chain complex has been shown to be impaired in pressure overload-induced hypertrophy leading, consequently, to heart failure [37]. Mitochondrial protection by cardiac-specific deletion of NADPH oxidase isoform 4 (Nox-4), a known source of mitochondrial anion superoxide, leads to resistance against pressure overload-induced hypertrophy [103]. Consistent with these observations, mitochondrial-specific superoxide dismutase knockout mice present high levels of mitochondrial ROS production and die after several weeks of birth from severe dilated cardiomyopathy [116]. One mechanism for the protection of hearts against oxidative damage is through the administration of synthetic antioxidant molecules. For instance, the mitochondrial targeted antioxidant peptide Szeto-Schiller (SS)-31 [131–137] or the mitochondria-targeted ubiquinone, MitoQ10 [138–141]. Thus, the increased ROS during hypertrophy [7–9, 27] and the attenuation of cardiac hypertrophy with antioxidants [5, 26, 27, 106] is a strong point for the implication of mitochondrial oxidative stress on cardiac hypertrophy/heart failure since mitochondria are the main intracellular source of ROS in cardiomyocytes.

NADPH oxidase is another important source of mitochondrial ROS [124, 125]. In fact, treating cells with isoproterenol induces oxidative stress by upregulation by a NOX isoform 4 [125]. Nox-4 is an important source of mitochondrial ROS in myocytes during hypertrophy [103, 124]. NADPH oxidase is the only enzyme designed for the purpose of ROS production. In general, Nox-4 is localized into the mitochondria and is upregulated during Angiotensin II, phenylephrine, or pressure overload-induced hypertrophy [124]. Furthermore, cardiac-specific knockout of Nox4 in mice attenuated cardiac hypertrophy accompanied with less interstitial fibrosis and mitochondrial ROS. The overall cardiac function of these hearts was improved by Nox4 knockout [103].

Cardiac hypertrophy is significantly reduced by increasing mitochondrial antioxidant defenses. For example, mice overexpressing the mitochondrial-targeted catalase had attenuated hypertrophy after pressure-overload triggered by transverse aortic constriction [38] or Angiotensin II treatment [142]. Furthermore, mice lacking one allele of MnSOD or its knockout had enlarged hearts, which supports the concept that mitochondrial ROS is a cause of cardiac hypertrophy [116, 143]. Although these observations prove the point, they are almost ineffective as a therapeutic strategy for humans. A more reliable therapy would be with drugs that will penetrate and target ROS inside mitochondria.

An attractive pharmacological tool for cardiac hypertrophy/heart failure treatment, is the mitochondrial targeted antioxidant peptide Szeto-Schiller (SS)-31 (Fig. 11.1). Peptides are not typically good candidates for the development of new drugs, not only because of their inability to cross cellular membranes, but also because of stability and solubility issues. On the other hand, SS-31 treated mice were resistant against angiotensin II-induced cardiac hypertrophy [133]. Interestingly, SS peptides rapidly accumulate into mitochondria, independent of

membrane potential, and avoid ROS production from mitochondrial Complex II and III [137] and lipid peroxidation after ischemia/reperfusion in the cardiac tissue [131]. These peptides possess excellent pharmacokinetic properties, low enzymatic degradation, and rapid accumulation in the heart [134]. Additionally, these peptides increased the ATP content of cardiac tissue subjected to ischemia and reperfusion [131] and restored expression of mitochondrial energy metabolism related genes [135]. Although additional studies must be performed to validate such compounds as therapeutic tools, these compounds can be delivered via intraperitoneal, intravenous, or subcutaneous which allows them to be rapidly distributed to the heart, lung and brain – all highly perfused tissues [136]. Furthermore, the protective action of the peptide SS-31 (MTP-131; Bendavia) is being currently being evaluated in four phase-I and a phase-II clinical trials for ischemic cardiomyopathy [132].

A developed mitochondria-targeted ubiquinone, MitoQ10, is another attractive way to overcome the problem of directly delivering an antioxidant to mitochondria [144]. Indeed, this molecule has been shown to be useful in preventing cardiac hypertrophy [138]. Its antioxidant effect is through its direct oxidation by ROS formed inside mitochondria (Fig. 11.1). The action of mitochondrial complex II recycles the molecule back to its active form. Furthermore, MitoQ10 heavily accumulates within the mitochondrial matrix, driven by membrane potential [140]. MitoQ10 also presented a direct anti-hypertrophic effect in vitro and potentiated the anti-hypertensive effect of losartan [139]. One might question the ability of MitoQ10 to act in the mitochondrial matrix of hypertrophic hearts because this drug is taken up by mitochondria depending on membrane potential integrity. In fact, one possibility is that the delivery of MitoQ might be higher in the normal membrane potential [141]. On the other hand, MitoQ10 had anti-hypertrophic effects and significantly improved the disrupted mitochondrial membrane potential stimulated by cyclic stretch in cardiomyocytes or by volume-overload in rats [105]. Taking these findings into account, a great deal of future research will be needed to prove the significant therapeutic potential of this and other molecules.

The pharmacological opening of a mitochondrial ATP sensitive potassium channel (mitoKATP) attenuates cardiac hypertrophy in vitro [145] and in vivo [109]. Notably, we have shown that the mitochondrial ROS production is decreased by pharmacological mitoKATP opening [146, 147], and although this data is still controversial [148], we believe this is an accurate model displaying the effect of mitoKatp on ROS production in the mitochondria. Therefore, we considered two potential mechanisms for mitoKATP protection against cardiac hypertrophy. First, specific opening of mitoKATP might confer cellular survival and/or protective advantages against the mitochondrial oxidative stress. According to this hypothesis, cells treated with openers of the mitoKATP would induce a favorable redox condition by avoiding the leak of electrons from the electron transport chain to molecular oxygen or by upregulating antioxidant enzymes (Fig. 11.1). These two events ultimately may diminish the hypertrophic phenotype. Secondly, the cardiac protection could occur at the level of the MPTP opening, independent of antioxidant enzyme induction, which preserves mitochondrial homeostasis and oxidative phosphorylation. Interestingly, stimulating mitochondrial potassium flux, through mitoKATP

opening, triggers cardioprotection by decreasing mitochondrial [149] and cytosolic [150] Ca^{2+} accumulation. As for other therapeutic drugs, more research must be conducted in order to establish the real impact of the opening of mitoKATP on cardiac hypertrophy and its protective mechanisms.

MPTP Hypertrophy

The transition of hypertrophy to heart failure is accompanied by mitochondrial damage [13]. Perturbed mitochondrial function impairs the ability of this organelle to produce ATP efficiently. It is important to point out that mitochondria produce 90% of all intracellular ATP [151]. Additionally, cardiac Ca^{2+} accumulation may result in increased Ca^{2+}-dependent activation of intracellular hypertrophic factors in cardiac cells [2]. Upregulation of cardiac contractility with isoproterenol induces greater mitochondrial Ca^{2+} load and oxidative stress with consequent mitochondrial permeability transition pore (MPTP) opening [152]. Under normal conditions mitochondria function to balance the cytosolic Ca^{2+} [153]. Ca^{2+} overload during hypertrophy induces cardiac deterioration in part by triggering the opening of the MPTP [154, 155] as a result of increased mitochondrial Ca^{2+} and activation of ROS generation [156]. Mitochondrial Ca^{2+} overload further enhances the generation of ROS by effecting Complex I, complex III, IV of the electrons transport chain [157].

Although the identity of all the proteins of the MPTP is still unknown, the mitochondrial matrix protein Cyclophilin D is the only component of the pore that was genetically verified as present. Cyclophilin D binds to putative pore components on the inner membrane and sensitizes the mitochondria for opening of the MPTP [158, 159]. Interestingly, cardiac hypertrophy leads to MPTP opening with enhanced expression and intramitochondrial translocation of Cyclophilin D [160]. Genetic deletion of cyclophilin D in the mouse protected against massive Ca^{2+} overload and heart failure by overexpression of Beta2a [161]. MPTP opening during cardiac hypertrophy induced by alpha-adrenergic agonists is successfully blocked by Cyclosporin A treatment – a cyclophilin D inhibitor [162]. In addition, mitochondria from volume overload hypertrophied hearts had greater vulnerability to stress-induced opening of the MPTP [155]. Additionally, the net release of Ca^{2+} from the endoplasmic reticulum into the cytosol and its cellular entrance by the L-type Ca^{2+} channel (Fig. 11.3) may lead to respiratory impairment through MPTP opening in mitochondria contributing to cardiac hypertrophy/heart failure.

Some medications used to attenuate the deleterious consequences of cardiac hypertrophy also affect mitochondrial redox and Ca^{2+} levels, which may contribute to their therapeutic efficacy. For example, the blockage of the angiotensin receptor type I (using candesartan) protected mitochondria against swelling [163]. Additionally, beta receptor blocker carvedilol protected heart mitochondria against oxidative stress-induced mitochondrial damage [164], mitochondrial swelling [165] and reverted cardiac hypertrophy [166]. Taken together, these observations suggest that clinically used drugs may improve the overall heart condition by the maintenance of mitochondrial normal function.

Conclusion

Mitochondria are major players in the development of cardiac hypertrophy. Research using cultured cells and animal models has consistently demonstrated that the hypertrophic heart has altered control of mitochondrial function, bioenergetics, and metabolic dysregulations. The induction of hypertrophy (by Angiotensin II (Ang II), activation of alpha (α) and/or beta (β) adrenergic receptors, or pressure/volume overload) has consistently demonstrated mitochondrial dysfunction. The dysfunctional mitochondrial will: (1) generate high levels of ROS with lower antioxidant enzymes. (2) Be more susceptible to Ca^{2+}-induced mitochondrial permeability transition pore (MPTP) and (3) will be energetically impaired producing lower ATP levels. Additionally, hypertrophy impairs mitochondrial biogenesis. Cardiac tissue is highly dependent on mitochondrial homeostasis and number. Some drugs and strategies have been used to target mitochondria (in animal and cellular models) in hopes of avoiding cardiac hypertrophy/heart failure (Fig. 11.4). Future work should be directed on improving their overall cytotoxicity and efficacy. Increased knowledge and improved therapies directed towards mitochondrial function, redox status, biogenesis and energetics will help in understanding and treating the events involved in cardiac hypertrophy.

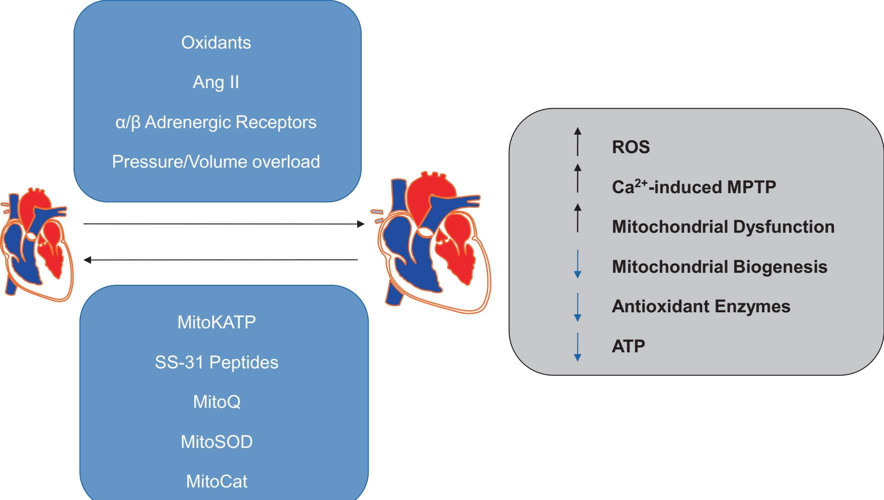

Fig. 11.4 Summary of the mitochondrial dysfunction during cardiac hypertrophy. Oxidants, Angiotensin II (Ang II), activation of alpha (α) and/or beta (β) adrenergic receptors or pressure/volume overload are known inducers of cardiac hypertrophy. The cardiac hypertrophic program includes increases in mitochondrial Reactive Oxygen Species (ROS), Ca^{2+}-induced mitochondrial permeability transition pore (MPTP) and mitochondrial dysfunction. Additionally, there are decreases in mitochondrial biogenesis, antioxidant enzymes and ATP levels. These effects are reversed by mitochondrial ATP-sensitive potassium channel (MitoKATP) opening, antioxidant peptide Szeto-Schiller (SS-31), the mitochondria-targeted ubiquinone (MitoQ) and the overexpression of mitochondrial superoxide dismutase (MitoSOD) or mitochondrial catalase (MitoCat)

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Chapter 12
Connexin 43 and Mitochondria in Cardiovascular Health and Disease

Kerstin Boengler and Rainer Schulz

Connexin (Cx) proteins are widely expressed in different tissues and are classified according to their molecular weight. Twenty one genes have been identified in the human genome which encode Cx proteins with a molecular weight ranging from 26 to 60 kDa [1]. The heart mainly expresses the Cx isoforms Cx40, Cx43, and Cx45, whereby Cx43 is the most abundant isoform and is found in ventricular cardiomyocytes, but also in the atria, in fibroblasts and in endothelial cells [2]. Cx40 is mainly located in the atria and in coronary endothelial cells, which also express Cx37, and the expression of Cx45 is specific for the conduction system under physiological conditions.

Cx are integral membrane proteins with their amino- and carboxyterminus located in the cytosol. The proteins have four transmembrane domains, two extracellular and one intracellular loop. Six Cx monomers assemble in the Golgi/trans-Golgi network into a so-called connexon or hemichannel which is then – mediated by the cytoskeleton – transported to the plasma membrane. Docking of two hemichannels from neighbouring cells results in the formation of gap junction channels [3, 4]. Gap junctions are assemblies of such closely packed gap junction channels and allow the direct connection between the cytosol of two adjacent cells and the passage of molecules with a molecular weight up to 1,000 Da. Thereby, electrical and metabolic cell coupling is mediated [5]. Cx43 is essential for development since embryos with a homozygous deletion of Cx43 die at birth due to blockage of the right ventricular outflow tract [6].

Due to the short half-life of Cx43 (approximately 1.3 h [7]), the protein has to be regulated at short notice, e.g. via phosphorylation or protein-protein interactions. Cx43 interacts with the scaffold protein zonula occludens 1 (ZO-1) via the PDZ

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domain in the carboxyterminus of Cx43 [8]. This protein-protein interaction plays a role in the aggregation of undocked hemichannels into gap junctions, since the inhibition of the Cx43/ZO-1 interaction increases the size of gap junctions [9]. At the intercalated discs, Cx43 interacts with the transmembrane protein 65 (Tmem65), which functionally regulates Cx43 and thereby cellular coupling [10].

Hemichannels not only function as precursors of gap junctions, but also exist as free, non-junctional channels in the plasma membranes of cardiomyocytes [11]. Under physiological conditions, hemichannels are predominantly in a closed state [12], but they open in response to stress conditions, for example changes in calcium (Ca^{2+}) concentrations [11, 13]. Such hemichannel opening leads to the release of adenosine triphosphate (ATP), the uptake of sodium- (Na^{+}) and Ca^{2+} ions, and the efflux of potassium- (K^{+}) ions. The resulting osmotic shifts, energy depletion, and Ca^{2+} overload induce cell swelling and finally cell death [14].

The localization of Cx43 at the plasma membrane is well described and characterized in several models, however, the presence of the protein is not restricted to it, since reports demonstrate the presence of Cx43 within mitochondria [15].

In this chapter, we will review the expression and regulation of Cx43 in the cardiovascular system and focus on the role of the protein – especially mitochondrial Cx43 – in health and disease.

Phosphorylation of Cx43

The extent of cell-cell communication depends on the open-probability of gap junctions, which – upon other factors – is controlled by the phosphorylation status of Cx43. Twenty one phosphorylation sites have been identified within Cx43 [16, 17]. Predominantly the serine residues within the cytoplasmic tail of Cx43 appear to be phosphorylated, although tyrosine phosphorylation at residues 247 and 265 has been reported [18]. The amount of phosphorylated Cx43-residues renders the electrophoretic migration from 41 kDa (unphosphorylated) to about 45 kDa (fully phosphorylated). The phosphorylation of Cx43 regulates e.g. the assembly of Cx43-formed hexamers, trafficking, and turnover of the protein and accordingly electrical and metabolic cell-cell coupling [19, 20]. However, phosphorylation of Cx43 within the cytosolic carboxyterminus is no prerequisite for gap junction formation, since mice with a truncated Cx43 carboxyterminus are principally capable of forming gap junction channels, although with altered electrophysiological properties [21]. Cx43 is the target of multiple kinases e.g. protein kinase A (PKA) [22], protein kinase B (PKB or Akt) [23], protein kinase C (PKC) [24], casein kinase 1 (CK1) [25], Ca^{2+}/calmodulin kinase II (CamKII) [26], mitogen-activated protein kinase (MAPK) [27], $p34^{cdc2}$/cyclin B kinase (p34cdc2) [28], and the pp60src kinase (src) [29]. The phosphorylation of Cx43 by different kinases has consequences for intercellular cell-coupling in the way that Cx43 phosphorylation via src, MAPK, or PKC inhibits, whereas Cx43 phosphorylation via CK1 or Akt enhances cell-cell communication [30] e.g. via regulation of gap junction assembly [31]. The

importance of Cx43 phosphorylation for gap junctional and hemichannel function is reviewed e.g. in [16, 32].

The phosphorylation status of Cx43 is not only controlled by protein kinases but represents a dynamic interplay also with protein phosphatases. Compared to the involvement of protein kinases for Cx43 function, less is known about the role of protein phosphatases (for review see [33]. Recent data show that the overexpression of calcineurin results in dephosphorylation and downregulation of Cx43 and this is associated with the development of fibrosis, hypertrophy, and arrhythmias [34]. In addition to phosphorylation, also S-nitrosation of Cx43 is described [35].

Cx43- More Than a Gap Junction Protein

The importance of Cx43 as a gap junctional protein has been analyzed under several physiological and pathophysiological conditions in various species; however, besides the well described localization of Cx43 at the intercalated discs, the protein is also present at other compartments. At the plasma membrane, Cx43 is localized to the periphery of the gap junction plaque – the so-called perinexus – where it is involved e.g. in intermolecular interactions and sodium currents [9, 36].

Furthermore, the carboxyterminus of Cx43 translocates into the nucleus and inhibits the proliferation of HeLa cells [37]. In human endometrial stromal cells, levonorgestrel induces the expression of Cx43 in combination with a nuclear translocation of phosphorylated Cx43 and finally decreases cell proliferation [38].

A recent study demonstrates the presence of Cx43 within exosomes, extracellular vesicles formed and released by various cell types (including cardiac cell lines), which function in intercellular communication at longer distances. Within exosomes, Cx43 forms hexameric channels and is able to mediate interactions between exosomes and target cells [39].

Mitochondria

Whereas the highest amount of Cx43 is found at the plasma membrane, a certain fraction of the protein also resides within mitochondria from endothelial cells [40–42], hepatocytes [43], H9C2 cells [44], and cardiomyocytes [15]. Cx43 is detected in cardiomyocyte mitochondria from mouse, rat, porcine and human origin using antibody-dependent and –independent techniques [15, 45]. Cardiomyocytes contain at least two functionally different mitochondrial subpopulations: the subsarcolemmal mitochondria (SSM), which are located directly beneath the sarcolemma and the interfibrillar mitochondria (IFM), which are present between the myofibrils. IFM have a higher respiratory capacity and a more pronounced Ca^{2+} retention capacity [46, 47]. The analysis of cardiac SSM and IFM for the presence of Cx43 shows that Cx43 is almost exclusively localized in SSM, whereas IFM are devoid of

Cx43 [48, 49]. Cx43 is encoded in the nuclear genome and therefore has to be imported into the mitochondria. This translocation is achieved by an interaction between Cx43 and the heat-shock protein 90 (Hsp90), which delivers proteins to the general mitochondrial import machinery. Here, Cx43 interacts with the translocase of the outer membrane 20 (Tom20), which directs proteins to be imported into the mitochondria to other components of the import machinery, e.g. translocases of the inner mitochondrial membrane. Finally, Cx43 is directed to the inner mitochondrial membrane, with its carboxyterminus oriented towards the intermembrane space [45, 50]. However, one study also demonstrates that Cx43 is present in the outer mitochondrial membrane [51]. Within mitochondria, Cx43 is suggested to interact with the apoptosis-inducing factor, the beta-subunit of the electron-transfer protein [52], and the ATP-sensitive potassium channel subunit Kir6.1 [44]. The amount of mitochondrial Cx43 declines in cardiomyocytes after activation of the N-methyl-D-aspartate receptor 1 via enhanced mitochondrial translocation of the matrix-metalloproteinase 9 [53].

Taking into account that the main function of Cx43 at the plasma membrane is to contribute to cell-cell communication by the formation of gap junctional channels, the question is, whether or not mitochondrial Cx43 also forms channel-like structures. The chemical cross-linking of isolated mitochondria demonstrates Cx43-dependent protein-complexes at a molecular weight corresponding to that of gap junction-enriched membranes which presumably represent Cx43 hexamers. Further evidence for a Cx43-formed channel within mitochondria comes from experiments where the mitochondrial uptake of the Cx43-channel permeable dye Lucifer Yellow is inhibited by the chemically unrelated hemichannel blockers carbenoxolone and heptanol [45]. Carbenoxolone is also effective in reducing Lucifer yellow uptake in rat SSM, but not in Cx43-deficient IFM [35]. The findings that the administration of the Cx43-blockers 18-alpha glycyrrhetinic acid (18αGA), the Cx43-hemichannel-specific peptide Gap19 as well as the genetic ablation of Cx43 reduce the mitochondrial potassium influx further strengthens the hypothesis that Cx43 channels are present in the inner mitochondrial membrane [45, 54].

Since the main function of mitochondria is to generate ATP via the oxidative phosphorylation, the influence of mitochondrial Cx43 on respiration was studied. Both a pharmacological inhibition and a genetic ablation of Cx43 reduce mitochondrial complex 1-mediated oxygen consumption and ATP production, whereas complex 2-mediated respiration is not affected. In contrast, the overexpression of Cx43 within atrial-derived HL-1 cells increases mitochondrial complex 1-, but not complex 2-mediated respiration [55]. Possibly, Cx43 interacts with proteins of complex 1 of the electron transport chain as recently demonstrated [52, 55].

A putative relationship between mitochondrial Cx43 and the mitochondrial permeability transition pore (MPTP) has been suggested. The MPTP represents a large conductance pore – mainly formed by components of the ATP synthase [56] – which is predominantly closed under physiological conditions and opens under various forms of stress (e.g. enhanced amounts of reactive oxygen species (ROS), inorganic phosphate, elevated matrix Ca^{2+}, or a reduced mitochondrial membrane potential) leading to mitochondrial swelling and membrane rupture and ultimately cell death.

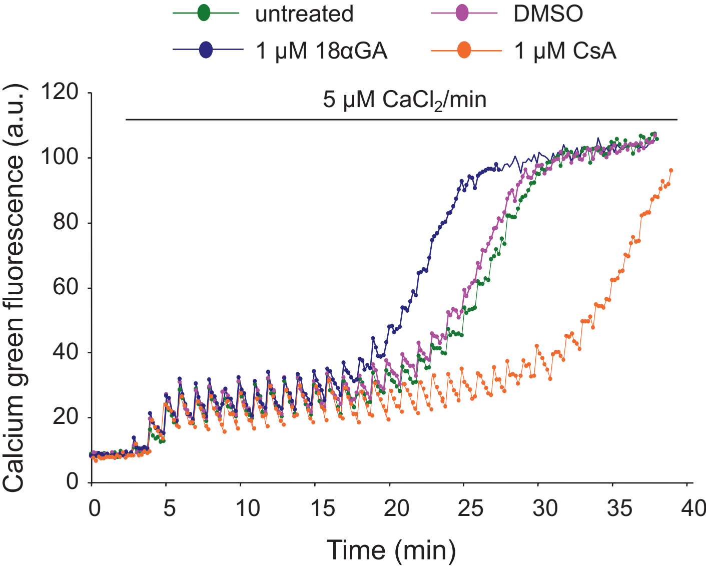

Fig. 12.1 Pharmacological inhibition of Cx43 enhances Ca^{2+}-induced MPTP opening. Original traces showing Ca^{2+} Green 5 N fluorescence (in arbitrary units, a.u.) of 100 μg/ml rat ventricular subsarcolemmal mitochondria (SSM) subjected to increasing $CaCl_2$ concentrations. Five μM $CaCl_2$ was added per minute until MPTP opening (increase in Ca^{2+} Green 5 N fluorescence) occurred. Ca^{2+}-induced MPTP opening was measured in untreated SSM, in the presence of DMSO, the Cx-channel inhibitor 18-alpha glycyrrhetinic acid (18αGA, 1 μM), or the MPTP inhibitor cyclosporin A (CsA, 1 μM)

In SSM isolated from rat hearts, treatment with the Cx43-channel blocking peptide Gap27 reduces the mitochondrial Ca^{2+} retention capacity [57]. Also, treatment of rat SSM with the Cx43 inhibitor 18α-glycyrrhetinic acid (18αGA) induces untimely MPTP opening in response to an extramitochondrial Ca^{2+} stimulus (Fig. 12.1).

Within cardiomyocytes, mitochondria are the predominant source of ROS and a relationship between Cx43 and ROS formation has been established [35, 58], whereby Cx43 inhibition or downregulation decreases the amount of ROS.

The phosphorylation status of mitochondrial Cx43 has not been analyzed in detail yet and therefore, it is not known whether all phosphorylation sites described in gap junctional Cx43 are also phosphorylated in mitochondrial Cx43. However, phosphorylation of mitochondrial Cx43 is found at serine 262 in rat SSM and mitochondria isolated from H9C2 cells [44, 57], whereas Cx43 phosphorylation at serine 368 is detected in mouse, rat and porcine SSM [57, 59–61]. Phosphorylation of Cx43 is not necessarily achieved outside the mitochondria, since PKC is present in mitochondria and the stimulation of such mitochondrial PKC leads to a phosphorylation of Cx43 at serine 262 and serine 368 in rat [57] and mouse SSM (Fig. 12.2).

Activation of mitochondrial PKC renders the organelles more tolerant towards a Ca^{2+} stimulus [57].

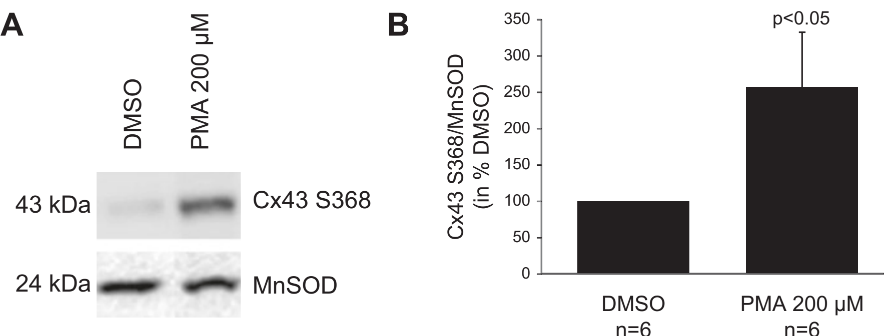

Fig. 12.2 Stimulation of mitochondrial PKC induces Cx43 phosphorylation. (**a**) Subsarcolemmal mitochondria were isolated from the mouse left ventricle and were stimulated for 30 min with 200 µM ATP and 200 µM of the PKC activator PMA or DMSO as solvent. Western blot analysis was performed for Cx43 phosphorylated at serine 368 (Cx43 S368) and the mitochondrial marker protein MnSOD (manganese superoxide dismutase). (**b**) Bar graphs demonstrate the ratio of phosphorylated Cx43 over MnSOD

Cx43 and Risk Factors of Cardiovascular Diseases

Age

The expression of Cx43 is found to decline with age in the hearts of hamsters [62], guinea pigs [63, 64], mouse [65], rats [66, 67] and rabbits [68]. In aged mouse hearts, not only the amount of gap junctional Cx43 but also that of mitochondrial Cx43 is reduced [69]. In addition to such decreased amounts of the protein also enhanced lateralization of the protein is detected in aged rat [70] and rabbit hearts [71] and this correlates with the age-dependent occurrence of arrhythmias in rat hearts [70] and that of atrial fibrillation in patients [72] (see also Chap. 10).

Hypertension and Hypertrophy

The expression of Cx43 in hypertension is mostly investigated in spontaneously hypertensive rats (SHR). Here, Cx43 protein levels either decline [73–75] or increase [76–78] with hypertension. Following abdominal aortic banding, Cx43 is displaced from its usual location at the intercalated discs [79]. In mice, pressure-overload by transverse aortic constriction reduces the expression of Cx43 [80]. The differences between the aforementioned studies may be due to the degree of hypertrophy associated with hypertension, since in porcine myocardium subjected to volume overload Cx43 expression increases in the initial phase, but gradually decreases with the progression of hypertrophy [81]. Also, in the ventricles from pressure-overloaded human hearts with valvular aortic stenosis, Cx43 expression is enhanced

in compensated, but reduced in decompensated hypertrophy [82]. The latter is associated with augmented lateralization of the protein. Interventions known to reduce hypertension and/or hypertrophy such as blockage or deletion of angiotensin II receptor I [78, 83], aldosterone antagonists [84], or renin inhibition [75] prevent the changes in Cx43 expression, phosphorylation or lateralization seen with hypertension and hypertrophy.

The expression of mitochondrial Cx43 in hypertension and hypertrophy has not been studied up to now.

Diabetes and Hypercholesterolemia

Diabetes is associated with abnormal conduction and increased risk of arrhythmias in the heart. To elucidate the underlying pathophysiological mechanisms, the expression and phosphorylation of Cx43 is studied, however, the available data give conflicting results. In a pre-diabetes model in rats, Cx43 expression is unaltered and the fraction of Cx43 localized at the intercalated discs increases [85]. Cx43 mRNA and protein levels are either reduced [86–88], unchanged [89, 90], or elevated [91–94] with diabetes. Enhanced lateralization of Cx43 is observed with diabetes in rat hearts with increased expression of Cx43 [91, 93], but also under conditions where the amount of Cx43 is unaltered [89]. The decreased amount of Cx43 in obese-diabetic mice is restored by moderate exercise [88], and the lateralization of Cx43 is normalized by omega-3 polyunsaturated fatty acids [93]. With diabetes, overall [92] and tyrosine [91] Cx43 phosphorylation are found to decrease, but also increased Cx43 phosphorylation is described [86, 93, 95].

Cx43 is detected at the inner membrane of mitochondria from rat retinal endothelial cells [42]. Here, maintenance of the cells under high glucose conditions decreases the level of mitochondrial Cx43.

In rabbits fed a high-cholesterol diet, the expression of Cx43 is increased in left atria [96], but decreased in ventricular tissue, where also the lateralization of Cx43 is enhanced [97]. Such disturbed distribution of Cx43 is also seen in rats fed a high-fat diet, whereby here the expression of Cx43 is unchanged [98]. In this study, the mitochondrial amount of Cx43 decreases in hyperlipidemic animals.

Post-myocardial Infarction

The amount of Cx43 decreases and the localization of Cx43 is disturbed post-myocardial infarction in the border zone and also in the infarcted area of mouse [99, 100], rat [101, 102], and dog [103] hearts. The downregulation of Cx43 is attributed e.g. to the inducible nitric oxide synthase-mediated availability of nitric oxide (NO) [99]. Moreover, the finding that the Cx43 protein level following myocardial infarction is preserved in matrix-metalloproteinase 7 (MMP7)-deficient mice indicates

that Cx43 may represent an MMP-7 substrate [100]. The lateralization of Cx43 is limited when mice are given a diet enriched with omega-3 fatty acids prior to myocardial infarction [104]. The transplantation of cardiac stem cells elevates the expression of Cx43 following myocardial infarction in rat hearts [105], and also the administration of a combination of hepatocyte growth factor and insulin-like growth factor improves Cx43 expression and intercellular coupling [102].

The expression of mitochondrial Cx43 post-myocardial infarction has again not been studied so far.

Heart Failure

The level of the Cx43 protein is reduced in an arrhythmogenic model in rabbits [106]. Here, the overexpression of Cx43 in cardiomyocytes from failing hearts to protein levels comparable to that of control cells improves cell coupling and reduces arrhythmias [107]. Additionally, the total amount of Cx43 decreases in failing mouse [108], rat [109, 110], and dog [111] hearts. Moreover, a heterogeneous distribution of Cx43 at the plasma membrane is observed in patients with end-stage heart failure due to ischemic or dilated cardiomyopathy [112, 113]. The analysis of the phosphorylation status of Cx43 shows either dephosphorylation [106, 111] or phosphorylation of the protein at serine 255 [114]. The induction of mitophagy and matrix-metalloproteinase activity is suggested to contribute to the decreased expression of Cx43 in heart failure [115], and the inhibition of the proteasome increases Cx43 amounts in adriamycin-induced heart failure in rats [116].

Data on the role of mitochondrial Cx43 in heart failure are limited. One study addresses the function of mitochondrial Cx43 in a model of doxorubicin cardiotoxicity in H9C2 cells [117]. Here, doxorubicin induces mitochondrial translocation of Cx43. Blocking the Hsp90-mediated import of Cx43 into the mitochondria increases ROS formation and mitochondrial Ca^{2+} content, and enhances depolarization of the mitochondrial membrane compared to doxorubin treatment alone, suggesting that mitochondrial Cx43 is important for the protection against doxorubin-induced cardiotoxicity. In a second study, the induction of dilated cardiomyopathy by furazolidone decreases the amount of Cx43 both in the myocardium as well as in isolated mitochondria [61]. Moreover, mitochondrial Cx43 is dephosphorylated at serine 368. The functional importance of altered mitochondrial Cx43 expression remains unknown.

Cx43 in Ischemia/Reperfusion Injury

Cx43-formed hemichannels open upon metabolic inhibition [118] or ischemia Accordingly, hemichannel inhibition by the specific hemichannel inhibitor Gap19 protects cardiomyocytes from volume overload, increases cell viability, and reduces

infarct size following ischemia and reperfusion [14]. The Cx43-hemichannel activity is determined by the phosphorylation status of the protein [119].

The use of less hemichannel-specific inhibitors such as Gap26/Gap27 also reduces myocardial infarction following ischemia/reperfusion [120, 121], and mice in which Cx43 was replaced by Cx32 have smaller infarcts than wildtype mice [122]. The use of heterozygous Cx43-deficient mice in the analysis of myocardial infarction following ischemia/reperfusion demonstrates a reduction of infarct size in one study [123], whereas no effect is seen in other investigations [124, 125]. The further reduction of the Cx43 level in conditional knockout mice results in smaller infarcts after ischemia/reperfusion, whereas non-induced mice (with 50 % Cx43) have similar infarct sizes compared to wildtype mice [126].

Cx43 phosphorylation has been analyzed under conditions of ischemia and reperfusion and seems to be dependent on the cellular ATP content [127]. In particular, ischemia or hypoxia induce dephosphorylation of Cx43 at serine 365 [128] or at serine 325/328/330 [31], but also Cx43 phosphorylation at serine 262, 368 or serine 373 [24, 129, 130]. Serine 365 is suggested to function as a so-called "gatekeeper", since serine 365 phosphorylation prevents serine 368 phosphorylation [128]. The interaction between Cx43 and ZO-1 is regulated by phosphorylation at the Akt target site serine 373, whereby the phosphorylation of Cx43 at serine 373 disturbes the interaction between the two proteins resulting in increased size of gap junctions and enhanced cell-cell communication [129]. The mutation of serine 373 to phosphorylation-insensitive alanine reduces the interaction between Cx43 and 14-3-3- proteins, stabilizes Cx43 at the plasma membrane and thereby avoids Cx43 degradation [130]. During ischemia, a redistribution of Cx43 occurs from the intercalated discs to the lateral sides of the cardiomyocytes [131]. Such lateralized Cx43 is mainly dephosphorylated, whereas phosphorylated Cx43 remains at the gap junctions [2]. Recent data show that ischemia induces Cx43 ubiquitination at the intercalated discs and subsequent autophagic degradation of the protein [132, 133]. Ubiquitination of Cx43 is decreased by the mutation of the phosphorylation site serine 373 to alanine [130]. Additionally, Cx43 is degraded via the proteasome and the lysosome [134].

In H9C2 cells subjected to 12 h hypoxia, the phosphorylation of mitochondrial Cx43 is increased, however, no specific phosphorylation sites are tested [44]. Following a shorter phase of ischemia (30 min ischemia, 5 min reperfusion), an overall dephosphorylation of Cx43 is observed in rat myocardial mitochondria [98]. Also in porcine myocardial mitochondria, ischemia induces a dephosphorylation at the PKC-target site serine 368 [60].

Several pharmacological interventions are known to reduce infarct size after ischemia/reperfusion and some of them impact on the phosphorylation status of Cx43. In canine hearts, administration of 17beta-estradiol decreases myocardial infarction and this effect is associated with an decrease in the overall phosphorylation of Cx43 [135]. Also, inhibition of p38 MAPK induces phosphorylation of Cx43 at serine 368 – eventually via phosphatase inhibition – and reduces infarct size [136]. The sphingosine-1 phosphate (S1P) constituent of high-density lipoptoteins limits cell death following ischemia/reperfusion and this is accompagnied

with an increased Cx43 phosphorylation at serine 368. A causal role for this phosphorylation site in cardioprotection by S1P is shown in mice, in which serine 368 of Cx43 is mutated to alanine; here, the infarct size reduction by S1P is lost [137]. Enhanced phosphorylation of Cx43 (total and mitochondrial) at serine 262 and serine 368 is achieved in isolated rat hearts by diazoxide or fibroblast growth factor 2 (FGF-2) [57, 138]. The overexpression of Cx43 in which serine 262 is mutated to alanine in neonatal cardiomyocytes results in augemented cell death following simulated ischemia [138].

Cx43 in Cardioprotection

The phenomena of endogenous conditioning describe the infarct size reduction following sustained ischemia/reperfusion by brief non-lethal periods of ischemia and reperfusion, which are performed either before (pre-), during (per-) or following (post-) the sustained phase of ischemia followed by reperfusion. The protection by such endogenous conditioning is often lost in the presence of cardiovascular risk factors or diseases [139], where total and mitochondrial Cx43 epxression, localization and/or phosphorylation is altered. Ischemic preconditioning (IPC) suppresses chemical coupling of cardiomyocytes via gap junctions during ischemia. Using a protocol of simulated ischemia and reperfusion in vitro, only wildtype cardiomyocytes were protected by IPC, whereas cardiomyocytes isolated from heterozygous Cx43-deficient cells show no increase in cell viability [140]. In heterozygous Cx43-deficient animals, the infarct size reduction by IPC is abolished [124], whereas non-induced conditional Cx43 knockout mice (also having a 50 % reduction of Cx43) demonstrate cardioprotection by IPC [126]. The further reduction of the Cx43 content in these mice, however, abolishes IPC's cardioprotection [126]. In addition, in mice, in which Cx43 is replaced by Cx32, IPC is not effective [122]. When Cx43-formed channels are inhibited by heptanol during the preconditioning cylces of ischemia and reperfusion infarct size reduction by IPC is lost [141].

The analysis of the phosphorylation status of Cx43 reveals that IPC prevents the ischemia-induced dephosphorylation of Cx43 in rat hearts in vitro [142] in vivo [143], or in pig hearts in vivo [60, 144], however, in one study IPC has no impact on Cx43 phosphorylation [145]. Preserved Cx43 phosphorylation is in part due to increased actvity of PKC, since knockout of PKC not only abrogates the protection, but also the maintained Cx43 phosphorylation by IPC [146]. The interaction of Cx43 with protein phosphatases is not affected by IPC [60].

IPC not only affects gap junctional, but also mitochondrial Cx43. The amount of the protein within mitochondria is increased upon IPC in isolated mouse [49] and rat hearts [15, 35], and pig hearts in vivo [15]. In bone marrow stem cells, the overexpression of specifically mitochondrial Cx43 is sufficient to confer cytoprotection [147], pointing to the important role of mitochondrial Cx43 in cell survival.

Pharmacological preconditioning with diazoxide augments mitochondrial Cx43 in a model of hypothermia [148]. When the mitochondrial import of Cx43 is

blocked by the use of the Hsp90-inhibitor geldanamycin, the infarct size reduction by diazoxide is lost. Since in this model the gap junctional content of Cx43 remains unaffected, the loss of cardioprotection by diazoxide is associated with the reduced content of mitochondrial Cx43 [50]. In addition to Cx43, geldanamycin also inhibits the mitochondrial import of PKCepsilon, which may phosphorylate Cx43 [149]. The analysis of the mechanims by which mitochondrial Cx43 contributes to diazoxide's cardioprotection demonstrates that the generation of ROS, which, if present in low amounts, function as trigger molecules of preconditioning, is reduced in cardiomyocytes isolated from Cx43-deficient mice [125]. Furthermore, IPC induces S-nitrosation, an important posttranslational modification in cardioprotection [150], predominantly of SSM proteins [49]. One protein with enhanced amounts of S-nitrosation following IPC is mitochondrial Cx43, and the S-nitrosation of mitochondrial Cx43 enhances the permeability of SSM towards potassium ions and increases ROS formation [35]. SSM isolated from wildtype mice show increased respiration when the isolated mitochondria undergo IPC, however, in mice, in which Cx43 is replaced by Cx32, such preserved oxygen consumption is lost [151]. Also, mitochondrial Cx43 may contribute to cardioprotection via its effect on MPTP opening (see above), since the inhibition of MPTP opening reduces myocardial infarction [152].

In remote ischemic preconditioning, where the short periods of ischemia/reperfusion occur in organs other than the heart, sustained Cx43 expression and phosphorylation is oberserved [153].

Ischemic postconditioning induces Cx43 phosphorylation in rat hearts in vivo [154, 155], but also in mitochondria isolated from mice which were subjected to a postconditioning protocol [59]. However, one study demonstrates reduced phosphorylation of mitochondrial Cx43 after postconditioning compared to ischemia/reperfusion alone [156]. Nevertheless, altered Cx43 phosphorylation is not important for the cardioprotection by postconditioning, since heterozygous Cx43-deficient mice still have smaller infarcts than wildtype mice upon a postconditioning stimulus [157].

Conclusion

Cx43, which is mainly known as a gap junction protein, is also present within mitochondria from different cell types. The regulation of Cx43 is achieved – among other factors – by the phosphorylation status of the protein. Several cardiovascular diseases alter the expression and/or localization of gap junctional and mitochondrial Cx43, and dysregulated Cx43 is associated with the loss of cardioprotection. More research is needed to clearly identify such changes in Cx43 expression/regulation in cardiovascular diseases and accordingly to develop strategies to prevent such modifications. The maintenance of the Cx43 expression/phosphorylation under pathophysiological conditions – including that of mitochondrial Cx43 – may represent a therapeutic target to overcome the consequences of cardiovascular diseases.

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Chapter 13
Mitochondrial Mechanosensor Microdomains in Cardiovascular Disorders

Michele Miragoli and Aderville Cabassi

The heart is an exquisite electromechanical organ, which synchronizes every contraction with the preceding sinus bioelectrical stimulus. However, the mechanism whereby bioelectrical propagation activates mechanical contraction is not univocal as the parenchymal cardiac cells can generate bioelectricity after mechanical stretch or insult [1]. Cardiomyocytes possess a sort of mechanosensitive related 'cardiac memory', which may adjust their mechanical performance for the subsequent heartbeat (e.g. Frank-Starling phenomenon) [2–4]. It is a common assumption that altered mechanosensitivity initiates electrical instability and arrhythmia in heart failure [5]. While pro-arrhythmic mechanoelectric transduction has been extensively investigated in intact hearts *in-situ*, isolated hearts and in isolated cellular preparations, the initial subcellular mechanisms required for (mechanoelectric) signal transduction and its initiation are a very recent matter of investigation [6–8]. Attention has focused upon different sarcomeric components [9] and, in addition to force generation, several sarcomeric proteins were found to provide mechanosensing and/or signaling functions [10, 11]. Mutations in these sarcomeric or Z-disk complex proteins cause abnormal intracellular Ca^{2+} responses [11]. Cellular remodeling can be both 'structural', thus involving the cell membrane, intracellular organelles, loss of T-tubules [12], the cytoskeleton [13], intercalated disks [14], cellular swelling [15], ryanodine receptors (RyRs) and the sarcoplasmic reticulum (SR) [16] and 'electrical', thus involving ion channel and ion pump redistribution and re-expression [17, 18], adrenergic receptors (de)compartmentation [19],

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altered sequence of electrical activation [20] and complex signalling pathways changes [21].

Mitochondria are individual organelles localized in clusters beneath the sarcolemma (subsarcolemmal mitochondria) and in longitudinal rows within the contractile apparatus (interfibrillar mitochondria) [22]. Cardiac mitochondria are recognized as primary acting subcellular organelles involved in the metabolic changes occurring in ischemic and failing heart, also directly or indirectly participating in the arrhythmogenic mechanisms or altered contractility mainly associated to energy deficiency [22, 23]. Cardiomyocyte in fact houses the highest mitochondrial density (27–35% of cellular volume versus 3–8% in skeletal and smooth muscle cells) of any organ, and the mitochondria exhibit the greatest number of cristae, which enhance oxidative phosphorylation. Mitochondrial dysfunction is of particular importance because of the specific task of these organelles as suppliers of constantly energy demanding cardiomyocytes [24].

Besides, mitochondria change their subcellular location [25, 26] and the interfibrillar mitochondria alignment is altered early following myocardial infarction [27]. Regular alignment of subsarcolemmal mitochondria within the dyad plays a pivotal role in the homeostasis of excitation-contraction coupling [7, 28] together with intracellular calcium (Ca^{2+}) handling [29].

Remodeling includes also the mitochondrial function [30], and metabolism as observed in aging and failing heart but in particular during the development of various cardiovascular diseases when compared to cardiac energetics of a healthy person. In fact, cardiac damages, caused by ischemia, infarction, hypertrophy/dilation due to pressure and volume overloads are characterized by cardiac structural and metabolic remodeling all progressing to the heart failure condition [31, 32].

From the above the following question emerges: does the alteration of microdomain-related mitochondrial alignment play a role in arrhythmogenesis? The answer for this question is thought-provoking as many conventional technologies cannot selectively activate or interrogate mitochondrial mechanosensitivity at the sarcolemmal microdomain level. We started to address this question by employing scanning ion conductance microscopy (SICM) [8, 33] and surface confocal SSICM [34] to resolve cellular topography and subsarcolemmal mitochondria localization in combination with optical recording of intracellular Ca^{2+} transient initiation and propagation.

Mechanical Properties of Cardiac Cell Membrane Interrogated by Scanning Ion Conductance Microscopy

We have applied a hydrojet pressure via the SICM nanopipette with nanometer precision to investigate the subcellular mechanisms underlying mechanically-induced intracellular Ca^{2+} release in heart failure. We scanned the cellular topography of normal and failing rat ventricular cardiomyocytes (mainly from compensatory hypertrophy, derived from the distal zone from the scar) using SICM at different

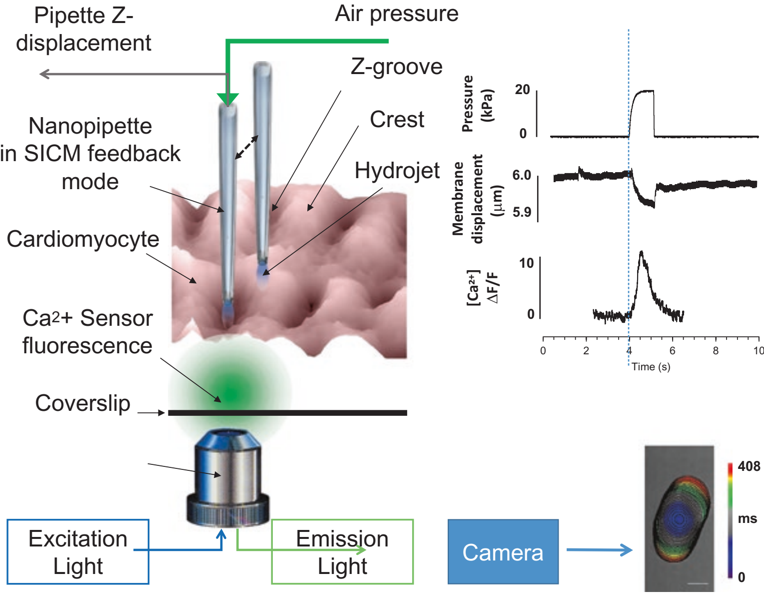

Fig. 13.1 Schematic representation of the experimental approach. After topographical live scan of the cell membrane, the nanopipette was positioned either on the crest or on the grove of a Ca^{2+} -indicator loaded cardiomyocyte cells. Then a hydrojet has been applied at 20 kPa for 2-s and membrane displacement and Ca^{2+} transient initiations and propagations have been recorded (Modified from Miragoli et al. [8], with permission)

time-points following myocardial infarction (MI). Then we positioned the pipette at 200 nm over a precised pre-selected site on the sarcolemma (either a crest or a Z-groove or an area without structure) which was chosen from a previously acquired topographical image (Fig. 13.1).

Then, we applied a localized 20-kPa hydrojet for 2 s. The area indented by the hydrojet is in the range of 0.125 μm². We studied rat ventricular cardiomyocytes following MI, progressing towards heart failure. We first obtained a 10 × 10-μm SICM topographical image of a normal or a failing cardiomyocyte (Fig. 13.1). These scanned topographical images were used to quantify the disruption of surface structural regularity; the images showed that cells progressively change their sarcolemmal regularity. Membrane organization is known to be substantially altered following MI including the disappearance of crests and grooves [12]. Hydrojets delivered on regular Z-grooves in healthy cardiomyocytes caused mechanically-induced intracellular Ca^{2+} release ($MiCa_i$) events, which were spatially confined (Figs. 13.1 and 13.2a -focal). By contrast, $MiCa_i$ propagated throughout the cell in failing cardiomyocytes with irregular Z-grooves (Fig. 13.2a -total). The occurrence of $MiCa_i$ events, transient and amplitude differ compared to AMC where hydrojet had been delivered either on crests or on grooves (Fig. 13.2b, c). Notwithstanding that blocking LTCC does not affect $MiCa_i$ (Fig. 13.2c), such difference cannot be ascribed

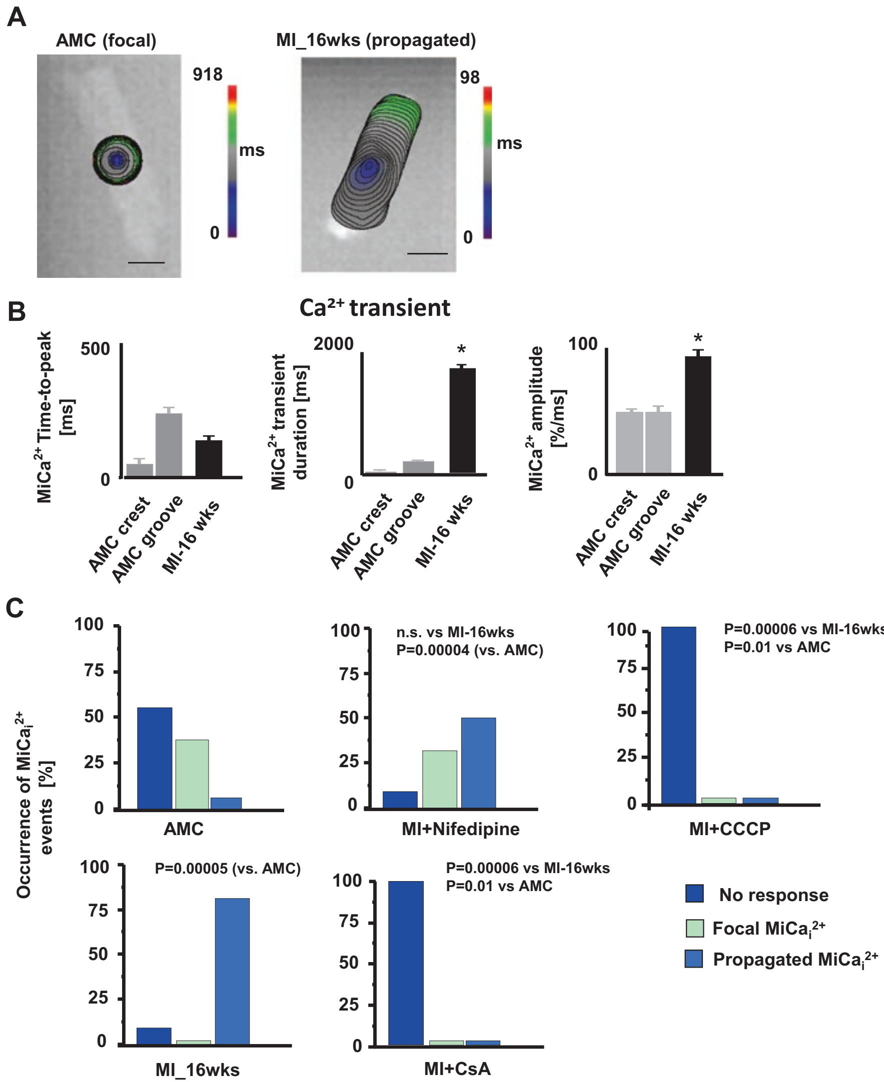

Fig. 13.2 Mechanical induced Ca^{2+} release in failing cardiomyocyte. (**a**) *Left* Hydrojet targeting non-failing structure evoke focal mechanical induced Ca^{2+} release activation (MiCai). (**a**) *Right* Infarcted cardiomyocytes (16-weeks following coronary ligation) response after hydrojet delivering (total MiCai). (**b**) Ca^{2+} transient parameters following crest/groove stimulation (AMC cells) or unstriated regions (MI cells). (**c**) Occurrence of MiCai events (no response, focal and total) in AMC and MI cells (*left*) and in the presence of selective blockers of LTCC (Nifedipine) or mitochondrial function (CCCP and CsA) (Modified from Miragoli et al. [8], with permission)

to LTCC while both (i) uncoupling mitochondrial proton gradient by Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or (ii) attenuating mitochondria permeability transition by Cyclosporin A (CsA) [35], completely abolished the focal and the propagated conditions.

Mitochondrial Displacement in the Failing Cardiomyocytes

It emerges that (mechanically) Ca^{2+}-induced Ca^{2+} release machinery [36] is still preserved but the existence of another intracellular Ca^{2+} source is supported, and having excluded the SR [8], a possible candidate is the mitochondrion. The involvement of mitochondria in pressure-induced intracellular Ca^{2+} release has been demonstrated previously [37], so we employed both confocal microscopy in combination with SICM (SSCM) and transmission electron microscopy (TEM) to investigate the sub-membrane interaction between dyads and mitochondria in failing cells.

In AMC cardiomyocytes, active TMRM-labeled mitochondria align with crests with a periodic arrangement, which reflects regular arrangement of Z-grooves and T-tubule openings (Fig. 13.3 top), as shown in particular on the TEM panel. Infarcted cells do not display the same regularity but show mitochondrial elongation and fusion [38]. These observations suggest a correlation between mitochondria derangement and the occurrence of propagated $MiCa_i$ and indicate a possible active role of remodeled mitochondria microdomains in $MiCa_i$ initiation.

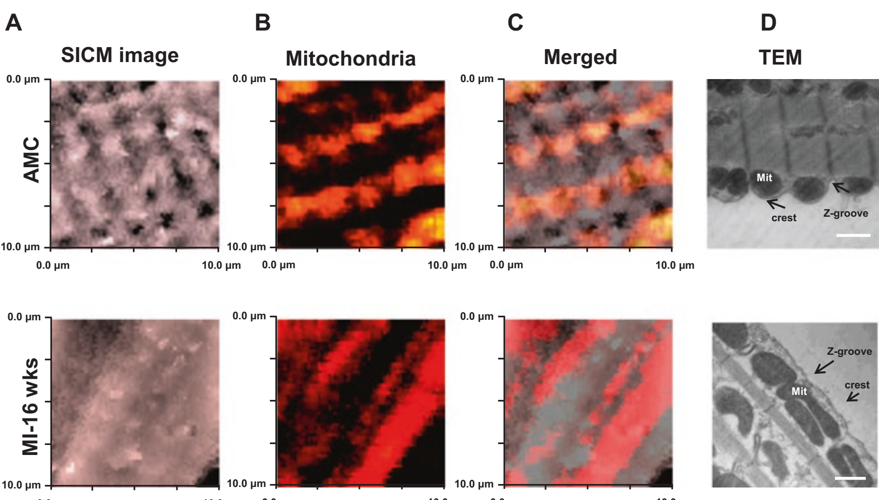

Fig. 13.3 Ultrastructural analysis of mitochondrial displacement in heart failure. (**a**) SICM images of 10 × 10-µm cardiomyocyte region of AMC (*top*) and MI (*bottom*) cells. (**b**) Surface confocal images (obtained by Surface-confocal SICM) of the labelled TMRM mitochondria in (**a**). (**c**) Merged images for SICM topography (**a**) and SICM TMRM-labelled mitochondria (**b**). (**d**) Mitochondrial displacement observed by TEM (Modified from Miragoli et al. [8], with permission)

Microtubules Involvement in Mitochondrial Displacement and MiCai Initiation and Propagation

Recent studies have implicated X-ROS signaling in inducing Ca^{2+} release (independently from mitochondria) by stretching the cell [39].

However, this activation required an intact microtubule network, which is supported by the observation that an increase in the microtubule network compactness (e.g. Duchenne muscular dystrophy) decreases X-ROS signaling [40]. Pharmacologically induced microtubular depolymerization in control cells by colchicine administration produces a functional phenotype which is similar by the one observed in heart failure cells (MI-16 weeks, Fig. 13.4a bottom-b) and increases the likelihood of $MiCa_i$ propagation (Fig. 13.4b), which we see in 69% of the cases. Our data also indicated that colchicine affects the mitochondrial total area (Fig. 13.4c) similar to what we have found in HF cells. We observed a reduction in

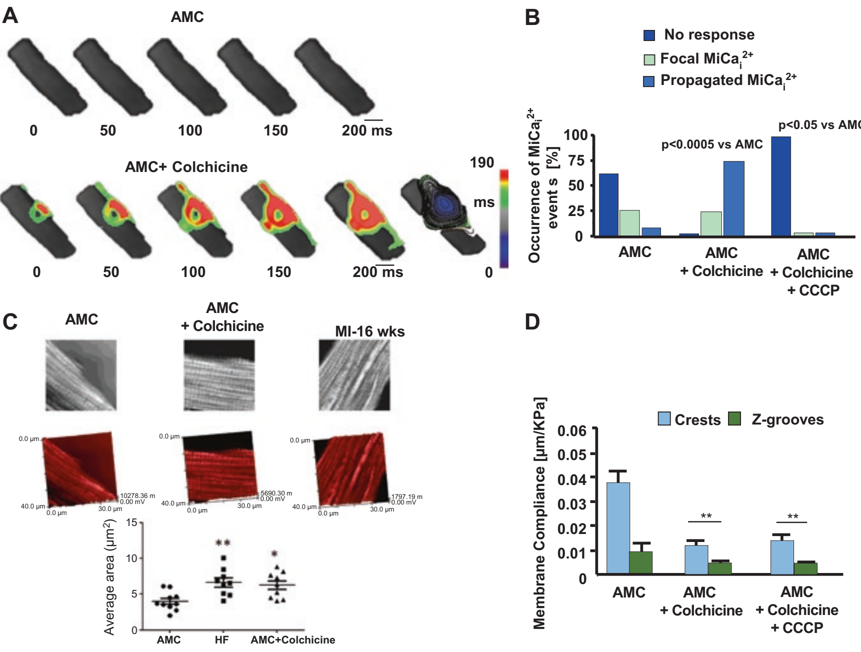

Fig. 13.4 Microtubule network derangement is a prerequisite for MiCai initiation. (**a**) Mechanically induced Ca^{2+} initiation in AMC cell (*top*) and the same cell in the presence of colchicine (*bottom*). (**b**) Occurrence of MiCai events in AMC, AMC+colchicine, AMC+colchcine+CCCP. (**c**) SICM topographical images (*top*) and TMRM-labelled mitochondria position (*middle*) and mitochondria count (*bottom*) in AMC, AMC+colchicine and HF (MI-16 weeks). (**d**) Membrane compliance measured in the three conditions mentioned in (**b**) (Modified from Miragoli et al. [8], with permission)

membrane compliance (measured as Z-displacement during hydrojet delivery, Fig. 13.4d). Moreover, our intracellular findings agree with a large animal study, where colchicine exacerbated chest impact-induced ventricular fibrillation (*commotio cordis*) [1].

Up-regulation of microtubular proteins encountered during cardiac failing [41] is implicated in destabilizing the microtubules network, by affecting T-tubule density and regularity (data not shown here, [8]). Disruption of the microtubule network allowed spreading of the normally constrained $MiCa_i$, mimicking the pattern of $MiCa_i$ propagation observed in failing cardiomyocytes.

Mitochondria and microtubules are in close contact at subsarcolemmal levels since β-tubulin is confined to the perinuclear and interfibrillar spaces and is largely co-localized with the cytoplasmic organelles [42]. In cardiomyocytes the distribution of β-tubulin-2 [43] is restricted to the outer mitochondrial-containing domain which binds to the outer mitochondrial membrane, and this probably also involves microtubular based trans-locators and/or MAPs.

Stretch-Activated Channels: A Secondary Source for Mechanical Induced Ca^{2+} Propagation

Stretch-activated channels from both cardiomyocytes and non-cardiomyocytes cells are well-known to be directly implicated in the mechano-electric transduction [44] and arrhythmias [45]. We sought to investigate their role in the mechanically-induced Ca^{2+} initiation because, in more advanced stages of pathological remodeling following MI. We observed a generation of second ‘ectopic’ Ca^{2+} waves which arise from remote regions of the cell (Fig. 13.5a, arrows).

In the three conditions presented in Fig. 13.5, we measured the intracellular Ca^{2+} propagation velocity and the real total activation time (TAT). We observed a complex pattern with the $MiCa_i$ ‘ripple’ starting underneath the pressure site, but after ~1–2 ms an additional remote $MiCa_i$ signal or signals (binary emergence) from the cell periphery follows the initial wave. The latter double or triplet Ca^{2+} wave fronts collide and propagate more rapidly throughout the cell (Fig. 13.5 left and middle panels). One plausible explanation is related to cross bridge cycling [46, 47]. The local sarcomere contraction underneath the pressure site’s $MiCa_i$ relaxes while more distant sarcomeres contract, shortening against the lower compliance of the relaxing region, mainly related to stretch-activated channels activations [48].

The shortening of these distant sarcomeres release Ca^{2+} from its cycling troponin [49] into the sarcoplasm. This could manifests as the remote peripheral Ca^{2+} signal. A second conceivable mechanism could be related simply to a stiffer sarcolemma, which leads to transmission of pressure to remote SACs. To further elucidate this mechanism we blocked SACs by streptomycin (not shown) or Gadolinium (Gd^{3+}, selective blockers of SACs). Both interventions abolish this peripheral activation (Fig. 13.5a right panel). To note, TAT is reduced in the presence of multiple activation (Fig. 13.5b, c) because of fast intracellular Ca^{2+} activation (Fig. 13.5b, the cell contraction is completely desynchronized).

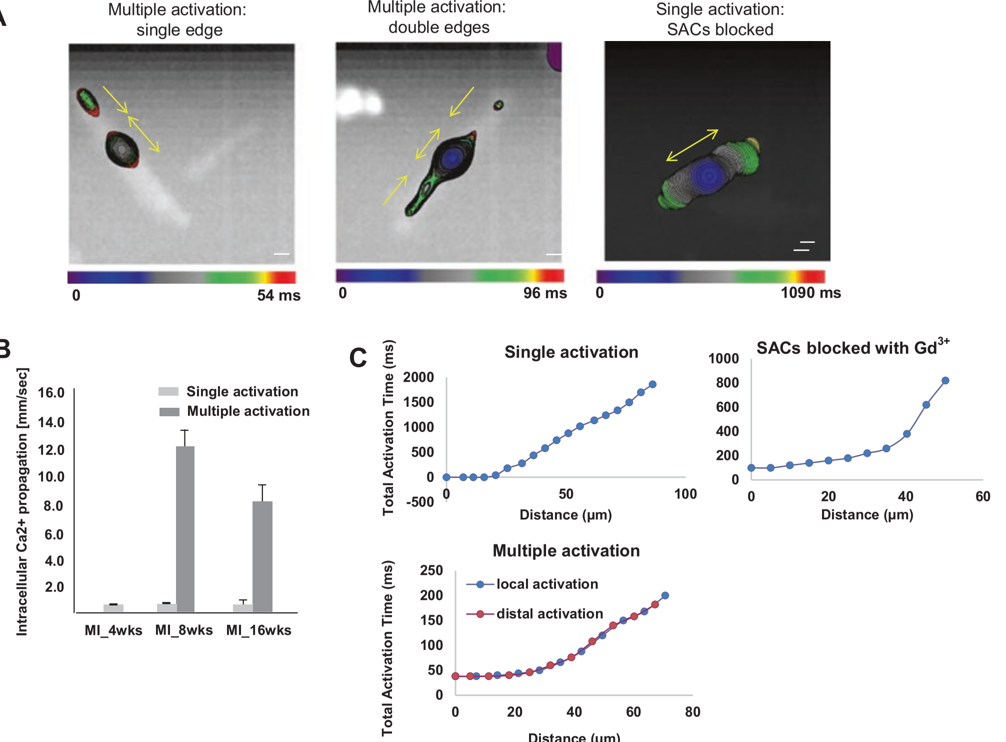

Fig. 13.5 Role of stretch-activated channels (*SACs*) following $MiCa_i$. (**a**) *Left* Secondary remote activation from a single edge (unidirectional *arrow*) following $MiCa_i$ (bidirectional *arrow*). *Middle* Secondary remote activation from the two edges (unidirectional *arrows*) following $MiCa_i$ (bidirectional *arrow*). *Right* Abolishing of multiple activation by selectively blocking of SACs by Gd^{3+}. (**b**) Overall Ca^{2+} intracellular propagation velocity during HF progression. (**c**) Total activation time from normal activation (*top left*), multiple activation (*bottom left*), and in the presence of SACs blocker (*top right*) (Modified from Miragoli et al. [8], with permission)

Mechanosensor Microdomains in Human Failing Cardiomyocytes

We sought to investigate whether $MiCa_i$ phenomena occur in human failing ventricular cardiomyocytes (Fig. 13.6) isolated from patient suffering from dilated cardiomyopathy (DCM). Similar to what has been found in rat cardiomyocytes, SICM imaging revealed a topographical heterogeneity (Fig. 13.6a left) [12]. We observed propagating $MiCa_i$ (Fig. 13.6a right) following pressure application over non-striated areas with a TAT mimicking the rat heart failure model. Mechanically-induced Ca^{2+} initiation occurred in 65% of the cases (Fig. 13.6b); more intriguingly, we measured an upregulation of several microtubules-related proteins. We found that α1C-tubulin (*TUBA1C*), β2A-tubulin (*TUBB2A*), β3-tubulin (*TUBB3),*

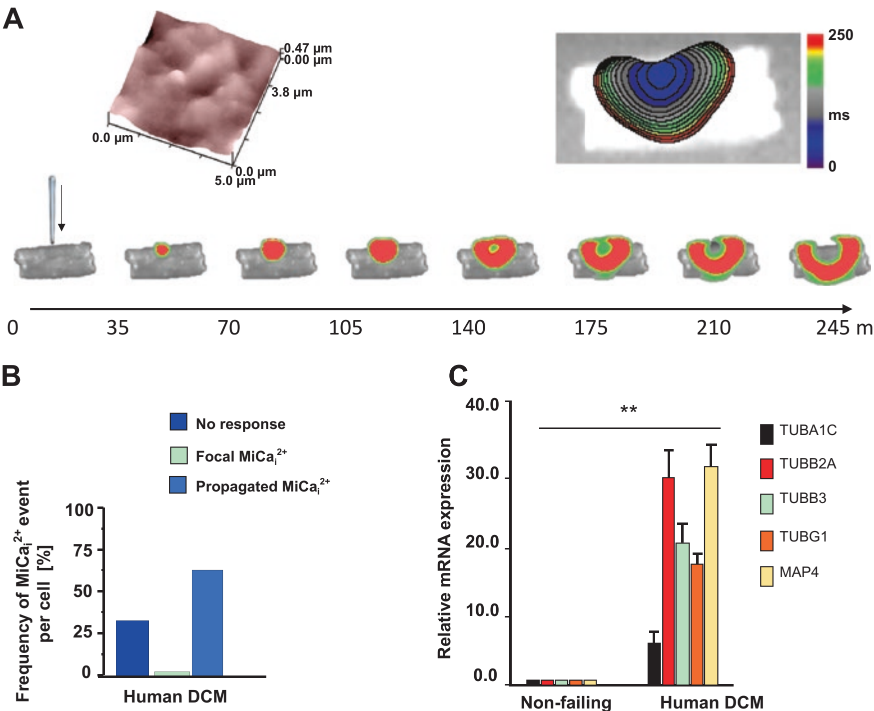

Fig. 13.6 Mechanical induced Calcium initiation in human failing myocytes. (**a**) *Left* Topographical SICM image (5×5 μm) showing partially striated area. *Right* Color-coded isochrones image of Ca^{2+} activation in the same cell. *Bottom* Intracellular $MiCa_i$ initiation and propagation with a TAT of 245 ms. $\Delta F/F_0 = 1\%$. 40× magnification, 0.6 n.a. (**b**) Frequency of $MiCa_i$ events. $n > 12$. (**c**) Relative mRNA expression for microtubules-related proteins. Technical triplicate Normalized to 18 s. mRNA quantities are presented as mean ± SEM (**$p < 0.01$), $n = 7$ (Modified from Miragoli et al. [8], with permission)

γ1-tubulin (*TUBG1)* and microtubule associated protein 4 (*MAP4)* were significantly upregulated as compared to non-failing cardiomyocytes, suggesting a primary role for microtubule disruption, together with mitochondria misalignment, in cellular vulnerability to $MiCa_i$ generation and propagation (Fig. 13.6c).

Ca^{2+} Handling, Mitochondria Metabolism and Function

Among the fundamental factors allowing the adequate integrity of mitochondrial machinery and energy generation fine Ca^{2+} regulation and tuning by cardiomyocyte at the subcellular level is central. Ca^{2+} regulatory mechanisms represent the key elements for a correct functioning of mitochondrial oxidative phosphorylation and energy production and for a controlled reactive oxygen and nitrogen species (ROS and RNS) generation by these organelles both in a healthy as well as in ischemic or failing hearts [50].

The continuous ATP generation from cardiac mitochondria is closely dependent on the oxygen and fuel substrates supplies. ATP generation depends on an efficient oxidative phosphorylation, which generates high amount of ATP. Two-thirds of generated ATP is consumed for cardiac contractions and the rest for the functioning of ion pumps: those linked to intracellular Ca^{2+} handling (e.g. sarcoplasmic reticulum Ca^{2+} -ATPase) of a relevant portion of consumed ATP [51]. In normal heart, fatty acid uptake and utilization is responsible for 70–90% of myocardial ATP produced whereas glucose uptake, utilization and oxidation accounts for remaining 10–30% of cardiac ATP production. Fatty acids generate more ATP than glucose; however, ATP production to oxygen consumption ratio is higher for glucose. Therefore, increased FA oxidation reduces cardiac efficiency (cardiac work/myocardial oxygen consumption). Even in normal hearts, oxidative phosphorylation generates a small amount (1–2%) of electrons leak from electron transfer chain to form reactive oxygen species (ROS). There are several changes observed in mitochondrial function of cardiomyocytes from normal, hypertrophic and failing hearts [24], concerning Ca^{2+} regulation even if a clear and complete picture of the phenomenon is still far. In the early stages of heart failure cardiomyocyte, structural and metabolic remodeling plays compensatory role with a slightly increased fatty acid beta-oxidation. While progressing the heart failure condition, fatty acid beta oxidation and mitochondrial respiratory activity decrease leading to decrease cardiac ATP generation. As a compensation to reduced fatty acid oxidative metabolism, glucose uptake and glycolysis are upregulated, even if absolutely insufficient to compensate the 60% reduction in ATP generation [25].

Noteworthy, mitochondrial Ca^{2+} regulation is fundamental because its signaling is closely implicated in ATP production, NADH generation [52] and activation of metabolic enzymes involved in oxidative phosphorylation [53]. In cardiomyocytes

from healthy hearts, the close proximity of endoplasmic reticulum to mitochondria can determine rapid changes of Ca^{2+} after its release from type-2 ryanodine receptor/Ca^{2+} release channel (RyR2) and type 2 inositol 1,4,5-trisphosphate receptor (IP_3R_2), the most important intracellular Ca^{2+} release channels in the heart [16]. RyR2 is essential for cardiac excitation–contraction coupling [16]; such task needs energy in the form of ATP produced primarily by oxidative phosphorylation in mitochondria [54]. Several are the authors that hypothesized that sarcoplasmic Ca^{2+} release via RyR2 and/or IP_3R_2 channels determines mitochondrial Ca^{2+} accumulation. Both mitochondrial excess and deficiency of Ca^{2+} have been reported as determinants of mitochondrial dysfunction [16, 55]. Intracellular Ca^{2+} accumulation in particular can impair mitochondrial function, leading to reduced ATP production and increased release of reactive oxygen species [55].

It has been reported that other Ca^{2+}-dependent pathways may alter oxidative phosphorylation mitochondria, such as Ca^{2+} -dependent protein kinases and calcineurin [56]. Small physiological levels of Ca^{2+} taken up into the mitochondria can regulate mitochondrial metabolism. An increase in contractility needs more increased availability of ATP; increased contractility is linked to higher cytosolic Ca^{2+} transient whose transmission to the mitochondria via Ca^{2+} uptake activates Ca^{2+} -sensitive mitochondrial dehydrogenases and several complexes of electron transport chain [57]. Mitochondrial Na^+-Ca^{2+} exchange has been shown to regulate mitochondrial Ca^{2+} levels linking mitochondrial Ca^{2+} to intracellular sodium. Sodium intracellular rise observed in hypertrophic and heart failure cardiomyocytes is strictly linked to changes of mitochondrial Ca^{2+} concentration that profoundly alter ROS generation, energy production and metabolism [58].

Under pathological conditions of increased Ca^{2+} cytosolic overload as occurs in heart failure, mitochondria after having taking up higher amounts of Ca^{2+}, displayed an opening of mitochondrial permeability transition pore (mPTP) that if maintained can be a trigger for mitochondrial dysfunction, damage and cell death. In our situation blocking mPTP is necessary to avoid MiCai.

Another factor involved in mitochondrial Ca^{2+} handling and energy and ATP generation, concerns the identification of a mitochondrial Ca^{2+} uniporter (MCU) that transport it across the mitochondrial inner membrane (see Chap. 2). Several groups developed knockout mice or mice without a functional MCU to study the role of the channel in modulating metabolism and cell death (see Chap. 3). In mice with MCU knocked out or mutated before birth, the hearts did not show altered metabolism and a decrease in infarct size after ischemia-reperfusion [59]. In adult mice, when uniporter function was loss because of the administration of tamoxifen, a protective effect was observed with smaller infarcts after ischemia and reperfusion [60]. In our situation, blocking mPTP and uniporter abolish $MiCa_i$ initiation and propagation without altering the normal Ca^{2+} -induced-Ca^{2+} release [8]. However, there are still apparent discrepancies in the relationship between changes in MCU expression and metabolic changes and potential protective effects of its deletion in adult mice and further studies are necessary to clarify these phenomena.

Ca^{2+} Microdomains and Mitochondrial ROS and RNS Generation

The relationship between Ca^{2+} regulation and ROS production is strictly interdependent and recent observations opened the understanding of fine regulation at different subcellular levels in cardiomyocytes [25, 61]. It has been shown that ROS play important roles in reshaping local and global Ca^{2+} signal amplitudes and kinetics at different levels of regulation in both physiology and pathology.

First it is well known that cardiomyocytes can generate ROS from several sources, especially mitochondria but other sources such as NAD(P)H oxidase, xanthine oxidase, and uncoupled nitric oxide synthase enzymes are also important [24]. In addition, cardiomyocytes generate not only reactive oxygen species but also reactive nitrogen species (RNS). Enzymatic production of nitric oxide from L-arginine is catalyzed by one of the three nitric oxide (NO)-synthase isoforms (nNOS, iNOS, and eNOS), all expressed in cardiomyocytes [62]. The activity of a major regulator of Ca^{2+} homeostasis SERCA is markedly affected by specific ONOO–mediated tyrosine nitration or cysteine oxidation linking ROS and RNS to cytoplasmic Ca^{2+} level (SERCA) [63]. An increased generation of RNS and ROS has been demonstrated in different settings of myocardial hypertrophy and heart failure in experimental models of hypertension and in heart failure patients [64, 65]. Such a ROS and RNS-mediated functional regulation of Ca^{2+} homeostasis is bidirectional and is related to the type of ROS generated, the amount of ROS and the location of the ROS and RNS subcellular source.

Failing hearts frequently show signs of increased ROS and RNS including lipid peroxidation, protein nitration as well reduction of antioxidants defenses [66].

Ca^{2+} microdomains at their associated dyads can be influenced by mitochondrial superoxide generation. It has been recently demonstrated that an intermittent, quantal generation of superoxide by the mitochondria, named mitochondrial "superoxide flash", active under physiological conditions, affects Ca^{2+} sparks [67]. While superoxide flashes do not normally propagate, there is also an inter-mitochondrial ROS-induced ROS release occurring during pathological conditions that can burst ROS generation. Mitochondrial Ca^{2+} plays a profound role in regulating the aforementioned modes of mitochondrial ROS production both in constitutively or in pathophysiological settings [68].

ROS generation can affect Ca^{2+} -tuning components at the levels of dyads. Under physiological condition, spontaneous Ca^{2+} sparks activity is finely regulated by basal ROS cellular generation [69].

In heart failure the elevated oxidation of RyR increases sarcoplasmic Ca^{2+} leakage during diastole, depleting sarcoplasmic Ca^{2+} content [55]. The depletion of sarcoplasmic reticulum content is also dependent on SERCA oxidation, which tends to inhibit its activity [70]. Diastolic Ca^{2+} increases due to oxidation of RyRs augments the susceptibility to arrhythmogenic afterdepolarizations. Higher generation of ROS and stretch-activated X-ROS leads to oxidize the RyR that generate a greater Ca^{2+} spark response and trigger more Ca^{2+} waves (see Chap. 10).

The bidirectional influence of ROS regulating Ca^{2+} spark activity and in an opposite manner Ca^{2+} modulating ROS generation, indicate a close interaction of Ca^{2+} and ROS and RNS signaling systems. In particular, mitochondrial ROS generation and ROS-mediated damage are strongly involved in the development and progression of HF. Albeit many are the recent discoveries on the relationship between subcellular Ca^{2+} regulation and oxidative stress a complete picture of ROS involvement in heart diseases is still lacking. Future investigations are warranted to unravel how such cellular remodeling affects both Ca^{2+} regulation and ROS generation at different levels from subcellular microdomains ROS generation in terms of X-ROS and superoxide flashes to ROS constitutive and inducible production under physiological and pathological conditions.

Conclusions

The attention paid to the role and function of mitochondria in the context on cardiovascular diseases is well established and studied [71–73]. In this chapter we focus on another aspect, which seems to jeopardize cardiovascular function in a similar manner: their 'belonging' to structural and organized microdomains [74] and their displacement/relocation and fusion [75] when such organization is lost.

The observations described herein have a potential translational element. The mechanically-induced Ca^{2+} changes have electrophysiological consequences that are potentially pro-arrhythmic. While arrhythmia is a multicellular phenomenon, the mechanisms by which mitochondrial relocation and microtubules derangement transmit abnormal $MiCa_i$ can be included in the 'maladaptive electrical and mechanical remodeling', which is known to ultimately predispose the heart to arrhythmias by, for example, inducing Ca^{2+} overload-related triggered activity [76, 77] or Ca^{2+} alternans [78]. As intracellular Ca^{2+} waves contribute to arrhythmogenesis on multicellular scales, the mechanisms that we describe may represent a novel arrhythmogenic substrate for ectopic initiation and propagation. This not only provides mechanistic insights, it also provides new therapeutic targets.

Outlook on Mitochondria Function

Using novel microscopic and super resolution-based technologies will provide new insight into cardiac microdomain levels [79, 80]. Our unique combination of SICM and optical mapping of mechanically-induced impulse propagation in a single cell allow the identification and localization of functional mechano-sensing with nanometer precision in live cells in general, and in particular, cardiomyocytes. Mitochondria are highly dynamic organelles which, depending on the metabolic demands or pathological conditions, frequently change their shape not only in eukaryotic cells but also in yeast or other unicellular organisms [81]. However, their

role is not merely related to energetic balance maintenance or signaling. A very recent study has shown that mitochondria translocate from astrocytes to neurons cells in the brain after stroke [82], thus working as 'heterocellular messenger' for the stromal-parenchymal crosstalk. In summary, mitochondrial function, structure and location together with the interaction among cytoskeleton and adjacent cells need to be adequately and jointly addressed for gaining a complete understanding of their role in pathological heart.

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Chapter 14
Mechanistic Role of Thioredoxin 2 in Heart Failure

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Heart failure is one of the primary causes of cardiovascular morbidity and mortality, which is predicted to increase dramatically worldwide in the decades ahead [1]. Many diseases including inherited cardiomyopathy, dilated cardiomyopathy, cardiac hypertrophy, myocardial infarction and diabetic cardiomyopathy can all ultimately lead to heart failure. Studies conducted with human and animal models have suggested that heart failure is closely related with increased levels of ROS [2–5]. ROS induced apoptosis of cardiomyocytes has been causally related to the pathogenesis of heart failure [6, 7]. Mitochondria are a major source of ROS, which produce 85–90 % of cellular ROS [8]. Meanwhile, several antioxidants systems are localized in mitochondria and maintain the intracellular redox balance [9]. Of these, the mitochondrial thioredoxin system, consisting of Trx2, peroxiredoxin 3 (Prx3), and thioredoxin reductase 2 (TrxR2) is the major scavenger of ROS and participates in redox-controlled signaling [10, 11]. A better understanding of the role of the

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mitochondrial thioredoxin system in heart failure is required for the development of new therapeutic strategies. Here, we provide an overview of current advances in the understanding of mechanistic role of thioredoxin 2 in heart failure.

ROS Generation and Elimination Mechanisms

Heart failure is a physiological state that occurs when the heart is unable to pump enough blood to meet the body's needs. Mitochondria are the primary place to produce adenosine triphosphate (ATP) that supports the pumping activity of the heart. The insufficient energy supplying in cardiac cells leads to heart failure [12–14]. There are variety supportive productions of energy in heart, including glucose, fatty acids, lactate, ketones, and amino acids. Of these metabolic progresses, Nicotinamide adenine dinucleotide hydrogen (NADH), reduced flavin adenine dinucleotide (FADH2) and acetyl-coenzyme (Acetyl-CoA) are produced. Then acetyl-CoA enters citric acid cycle to generate enough amount of ATP to meet the heart needs. Meanwhile, more NADH and FADH2 are byproducts in this progress. However, when the heart has insufficient energy to bump blood, the resultant NADH and FADH2 enter into electron transport chain, which produces ATP by consuming oxygen through oxidative phosphorylation in the heart mitochondria. During this progress, 0.4–4 % of the consumed oxygen is released in the mitochondria as ROS. Thus, the formation of "electron leakage" phenomenon in electron transport chain leads to ROS generation [15–17]. Besides, there are also other ROS-producing systems including numerous NAD(P)H oxidases, xanthine oxidase, the uncoupling of NO synthase [18–21]. The univalent reduction of molecular oxygen will transform into O_2^- by leaking electrons from the mitochondrial electron transport chain. O_2^- turns into H_2O_2 through mitochondria-specific manganese-dependent superoxide dismutase (MnSOD). H_2O_2 could be converted to oxidant $\cdot OH$ via the Fenton reaction without suitable regulation. So as other ROS members, like O_2^- and $\cdot OH$ [22].

Although physiological levels of ROS are benefit for phagocytes to deal with the bacteria and other metabolism, excessive ROS disrupts mitochondrial structure and function, leading to severe derangement of cells or apoptosis in heart [23]. Overexpression of catalase in mitochondria, but not in other cellular compartments, prolongs the life span of mice [18, 19, 24, 25]. Thus, how to deal with the excessive ROS is essential for the heart and other tissues.

There are several cellular mechanisms to regulate the counterbalance formation of ROS, including enzymatic and nonenzymatic pathways [10]. Both catalase and glutathione peroxidase are the members of well-clarified enzymatic pathways and they can catalysis H_2O_2 into H_2O dependently or cooperatively. The superoxide dismutases (SODs) have the gift to change O_2^- into H_2O_2 [26–29]. Additionally, thioredoxin and thioredoxin reductase coordinately form an additional enzymatic antioxidant and redox regulatory system [10]. The thioredoxin system can catalyze the regeneration of other antioxidant molecules to defense against ROS, such as ubiquinone (Q10), lipoic acid, and ascorbic acid. Calculative data show that the

heart-specific deletion of thioredoxin or thioredoxin reductase in mice results in myocyte apoptosis and heart developmental abnormalities [23, 30].

Nonenzymatic pathways include intracellular antioxidants such as the vitamins E, C, and β-carotene (a precursor to vitamin A), ubiquinone, lipoic acid, and urate.

Trx2 is an essential gene regulating mitochondria-dependent apoptosis, which also play an antioxidative role in scavenging ROS. Since thioredoxin system is essential for maintaining mitochondrial integrity and reducing ROS generation, we will mainly introduce the role Trs2 in heart failure.

The Thioredoxin (Trx) System

The thioredoxin (Trx) system, consisting of Trx, Trx reductase (TrxR), and peroxidase (Prx), plays an important role in regulating the cellular redox state [31–33]. Trxs are small redox proteins, which have proximate cysteines in their conserved CXXC motif. Reduced Trx reduces the disulfide bonds in target proteins through cysteine thiol-disulfide exchanges. In turn, oxidized Trx is reduced by TrxR and NADPH to facilitate its redox activity. Trx-dependent Prx plays a direct role in scavenging ROS (H_2O_2). There are at least three distinct variants of Trxs in human: thioredoxin-1 (Trx1), mitochondrial Trx2, and Sp-trx [11, 34]. In mammalian cells, Cytosolic Trx1 system is composed of Trx1 reductase (TrxR1), Trx1, and Trx1-dependent peroxidase. The mitochondrial-specific Trx2 system consists of Trx2 reductase (TrxR2), Trx2, and peroxiredoxin-3 (Prx3), which is highly expressed in tissues with great metabolic demand, such as the heart, brain, and liver [31–33]. Besides their character in regulating the cellular redox state, both cytosolic Trx1 and mitochondrial Trx2 can form a complex with a redox-sensitive serine/threonine kinase ASK1, which is activated in response to oxidative stress [35]. When the cellular ROS increase, redox-active cysteine residues in Trxs catalytic center become oxidized, and Trxs are dissociated from the Trx-ASK1 complex, which lead to the autoactivation of ASK1 and induct mitochondrial apoptotic cell death pathway [36, 37].

Roles of Thioredoxin System in Heart Failure

Genetic deletion of Trx1, TrxR1, Trx2, or TrxR2 leads to embryonic lethality likely attributable to the increased cellular oxidative stress [30, 38–40]. Mice with Trx1 homozygous mutants die shortly in embryonic period because of the defects of proliferation ability of cell mass cells [41]. The deletion of TrxR1 in vivo causes early embryonic morbidity and homozygous mutation embryos live a short lifespan and fail to survival [40]. Besides, embryonic fibroblasts with TrxR1 knockout can't undergo further proliferation in vitro. A global deletion Trx1 or TrxR1 display similar growth retardation and reduced cell proliferation. Mice with systemic knockout

of TrxR2 show severely anemic, increased apoptosis in the liver, and thinner of the ventricular heart wall, resulting in embryonic lethality at embryonic day 13 (E13) [30]. Trx2 homozygous mutant embryos cannot survive to birth with massive apoptosis and anencephaly [38]. Cells treating with buthionine-sulfoximine in order to delete Trx2 leads to an increased intracellular ROS accumulation, as well as the decreased number of total and mitochondrial glutathione (GSH) level [39]. Heart-specific deletions of TrxR1 or TrxR2 show different phenotypes. Mice with TrxR1 heart-specific deletion are not affected and develop normally and appear healthy [40]. It seems that TrxR1 is not that important for cardiomyocytes formation. On the contrary, the deletion of cardiac cell-specific TrxR2 gene in mice causes fatal dilated cardiomyopathy and died quickly after birth [30]. Of potential clinical relevance, a recent clinical genetic study demonstrates that TrxR2 loss-of-function mutations cause dilated cardiomyopathy [42]. According to these results, it seems that both Trx2/TrxR2 are essential for maintaining cardiac normal function. Since Trx2 has capacities to directly scavenge ROS and catalyze mitochondrial thiol-disulfide exchanges, and this capacity is more obvious than TrxR2, we believe that the expression and activity of Trx2 play a crucial role in maintaining the normal cardiac function. But the mechanistic role of Trx2 in heart development and pathogenesis is not clear. Therefore, we recently have reported the role of Trx2 in human hearts with cardiomyopathy and create a mouse line with cardiac-specific deletion of Trx2 to investigate the intrinsic role in the heart.

Myocardium form patients with severe idiopathic DCM shows significant reduced Trx2 expression compared with that form organ donors with preserved cardiac function [23]. However, TrxR2 and Prx3, the other two components of Trx2 system, are increased in idiopathic DCM. Prx3 is an indicator of oxidative stress, whose function is dependent on the dimerization of Trx2. TrxR2 expression is increased, which possibly results from a compensatory expression for reduced Trx2. Trx2 play an inhibitory effect on ASK1 activity and apoptosis [37]. Consistent with this concept, phosphorylated ASK1 and active caspase-3 are observably increased in idiopathic DCM patients. All these results suggest that Trx2 is important in maintaining myocardial function through inhibiting oxidative stress and ASK1 activity.

Because of embryonic lethality in Trx2 global knockout mice, the function of Trx2 in cardiac function in the post-developmental environment has not been clarified before [30, 38–40]. Therefore, in the cardiac-specific Trx2 knockout (Trx2-cKO) mice model, we also identify that Trx2 is critical to preserving cardiac function through maintaining mitochondrial integrity and function, suppressing ROS production, and preventing ASK1-dependent apoptosis [23]. The Trx2-cKO mice develop a spontaneous DCM, characterized by increased heart size, wall thinning, interstitial fibrosis, and a progressive decline in left ventricular contractile function, resulting in heart failure and death by 4 months of age. At the cellular level, cardiomyocyte shows age-dependent increases of cell size in Trx2-cKO mice hearts. The molecular analysis shows conspicuous expression changes of genes that participate in human heart failure in the Trx-cKO mice. Taken together, these results suggest that Trx2 is essential for maintaining normal cardiac structure and function during early cardiac growth.

Roles of Trx2 in Cardiomyocyte and Heart Failure

Morphological and biochemical analyses of Trx2-cKO hearts indicate that Trx2 deletion results in a progressive loss of mitochondrial integrity and function, leading to mitochondrial swelling, ultrastructural derangement, and reduced respiration and ATP generation. Meanwhile, ROS generation and cellular apoptosis are age-dependent increased in Trx2-cKO mitochondria compared with WT control, revealing that Trx2 deletion directly promotes mitochondrial ROS generation and cellular apoptosis. Both increased ROS and cellular apoptosis have been forcefully associated with the pathological process of hypertrophy and heart failure [2–5, 43, 44]. Besides, Trx2-cKO strongly induces increased ASK1 activity without significant increases in JNK phosphorylation, which is consistent with our previous result that Trx2-ASK1 regulates mitochondrial apoptosis in a JNK-independent way [37]. Consistently, Trx2 deletion cardiomyocytes show a similar cellular phenotype of mitochondrial dysfunction with increased cellular ROS, ASK1 activation, and apoptosis, demonstrating that the cardioprotective function of Trx2 observed in the heart is intrinsic to the cardiomyocyte. Selective ASK1 inhibition reduces ROS generation and apoptosis in cardiomyocytes and retards the progression of DCM and heart failure in Trx2-cKO mice. These results suggest that Trx2 is important for suppressing ROS-induced ASK1 activation and keeping off maladaptive left ventricular (LV) remodeling as a key antioxidant enzyme in the heart.

Both the cytosolic Trx1/TrxR1 and the mitochondrial Trx2/TrxR2 systems play a protective role in the pathogenesis of cardiac dysfunction [7, 45]. Trx1 shows cardioprotective under multiple pathological conditions, which is well established and reviewed previously [46]. Trx1 protects cardiomyocytes mainly through its antioxidative activities and interaction with several critical signaling molecules [47]. Exogenous Trx1 has cardioprotective effects in myocardial ischemia/reperfusion through its antiapoptotic activities [48]. Trx1 gene therapy in infarcted myocardium of diabetic rats reduces ROS generation and cardiomyocytes apoptosis, resulting in enhanced angiogenic signaling and reduced ventricular remodeling [49]. In this model, Trx1 gene therapy significantly increases the expression of anti-apoptotic p38MAPKβ and decreased the expression of proapoptotic p38MAPKα and phosphorylated JNK (p-JNK). Trx1 transgenic mouse hearts overexpressing Trx1 have a protective role against to adriamycin-induced cardiotoxicity by reducing oxidative stress and to ischemia/reperfusion through redox signaling [50, 51]. On the contrary, transgenic mice with cardiac-specific overexpression of a dominant negative mutant (C32S/C35S) of Trx1 exhibit cardiac hypertrophy not DCM or heart failure, in which the activity of endogenous Trx1 is diminished [52]. Trx1 attenuates cardiac hypertrophy through modulating the redox modification and nucleocytoplasmic shuttling of class II histone deacetylases, master regulators of cardiac hypertrophy [53]. However, mitochondrial Trx2 has different functions from the Trx1 in heart. In our study, the cardiac-specific deletion of Trx2 causes severe spontaneous DCM, resulting in heart failure. In agreement with our study, mice with heart-specific ablation of TrxR2 also develop to fatal DCM and

morphological abnormalities of cardiomyocytes. [30] Auranofin (an antirheumatic gold compound) is known as an efficient inhibitor of TrxR2. Administration of auranofin leads to poor cardiac recovery following ischemia/reperfusion and increased cardiac apoptosis [54]. Besides, there is a selenocysteine in the active site of TrR2, whose activity relies on the presence of this amino acid. Removing selenium from the diet of rats downregulates the activity of TrxR2 and also results into poor cardiac recovery following ischemia/reperfusion [54]. Analogously, cardiac-specific deletion of TrxR2 in the adult mice results into an excessive vulnerability to ischemia/reperfusion injury [55]. A recent clinical study has indicated that mutations in the mitochondrial TrxR2 gene cause DCM in patients [42]. Two mutations in the highly conserved helices result in acid substitutions and reduced flavin-adenine dinucleotide (FAD) binding of TrxR2. These mutations likely do harm to the function of cardiomyocyte through a dominant negative mechanism. In addition, TrxR2 plays a critical role during the progression of age-related heart failure [46, 56]. These results show that mitochondrial TrxR2, like Trx2, is indispensable to endogenous cardioprotection. As we reported recently, TrxR2 inhibitor auranofin reduces Trx2 protein level but not its mRNA, suggesting that TrxR2 is important for the stability of Trx2 [57]. In Trx2-cKO mice, the expression of mitochondrial antioxidant proteins TrxR2 and superoxide dismutase 2 (SOD2) are increased by compensatory mechanism, while the cytosolic Trx1 and glutathione peroxidase 1 are not specifically altered.

Cardiomyocyte Apoptosis and Heart Diseases

Cardiomyocyte apoptosis is causally related to the pathogenesis of DCM and heart failure [58, 59]. Several studies demonstrate that reduced apoptosis protects the heart from failing [6, 60]. Cardiac-restricted inducible expression of caspase-8 fusion protein lead to very low levels of cardiomyocyte apoptosis, which is sufficient to cause a lethal DCM within 8–24 weeks [59]. In the Trx2-cKO mice, it takes similar time to cause DCM and lethality [23]. Overexpression of Trx2 can inhibit apoptosis induced by t-butylhydroperoxide and etoposide [61]. However, since by cell number 75 % cells in the heart are non-myocytes, the importance of non-myocyte apoptosis in heart may be overlooked and the apoptosis in non-myocytes is greater than in myocytes in heart failure [62, 63]. The antiapoptotic role of Trx2 in other non-myocyte cells in the heart has not been elucidated.

Mitochondrial Dysfunction and Heart Failure

Since the heart is an energy-demanding organ, destruction of cardiac energy metabolism and mitochondrial function have been inexorably linked to cardiac dysfunction [64]. It is increasingly recognized that mitochondrial dysfunction contributes to damaged myocardial energetics and increased oxidative stress in heart failure. Trx2

is a small mitochondrial antioxidant, which protects heart against excessive mitochondrial ROS and keeps the integrity and function of Mitochondria [23]. In the Trx2-cKO mice, the disrupted structure and reduced function most likely directly result in DCM. Trx2-cKO hearts have a progressive loss of mitochondrial integrity and function, as measured by mitochondrial DNA copy number and mass, ultrastructure, mitochondrial permeability, ATP production and oxygen consumption rate. The well-aligned rows of mitochondria are in close proximity to the longitudinally oriented cardiac myofibrils, offering a quick and constant supply of ATP for contractile function [65]. On the contrary, mitochondrial arrays are disorganized and swollen mitochondria are aggregated with mild lysis of the cristae in Trx2-cKO hearts. Besides, the cytochrome c oxidases (COX1/4) expression has a dramatic reduction, which is an important terminal enzyme complex of in the mitochondrial electron transport chain. All these alterations result in the disruption of the mitochondrial membrane potential, significantly reduced ATP production and ROS production. Overexpression of Trx2 enhances mitochondrial membrane potential [66]. Exome sequencing has found a homozygous stop mutation of Trx2 in a 16-year-old adolescent with early-onset neurodegeneration and severe cerebellar atrophy [67]. Fibroblasts from the patient show absence of Trx2 protein, increased ROS, disrupted oxidative stress and oxidative phosphorylation dysfunction. Interestingly, reconstitution the expression of Trx2 restores all the parameters, demonstrating that Trx2 mutations play a causal role in the disease development. However, a Trx2 gene mutation involved in human DCM or heart failure has not been found. Exome sequencing should be done to find the endogenous Trx2 mutations in patients with heart diseases.

Signaling Pathways of Trx2 in Heart Failure

Many extracellular factors have capacities of inducing heart failure, and many of the various downstream signaling pathways that mediate the heart failure growth response to these factors can be activated directly or indirectly by ROS. These include apoptosis-signaling kinase 1 (ASK1), protein kinase C (PKC); the MAPKs p38, c-Jun N-terminal kinase (JNK), and ERK1/2; phosphatidylinositol 3-kinase (PI3K); Protein kinase B (PKB); several tyrosine kinases (e.g., src and FAK); nuclear factor-κB (NF-κB) and calcineurin [68, 69]. For instance, ASK1 protein, a serine/threonine kinase upstream of p38/JNK, plays an important role in mediating apoptosis and hypertrophy in the heart. The reduced form of Trx2 physically interacts with ASK1 and suppresses its kinase activity. Mice with Trx2 heart specific knockout presents ASK1 kinase dramatically up-regulation in the early stage of DCM disease progression. Besides, the morphological and biochemical changes including mitochondrial integrity damage, cellular apoptosis and cardiac dysfunction will emerge in the Trx2-cKO heart with ASK1 overexpression. Additionally, ASK1 is also detected in normal heart mitochondria. A surprising result shows that GS-444217, a selective, small molecule inhibitor of ASK1, preserves Trx2-KO

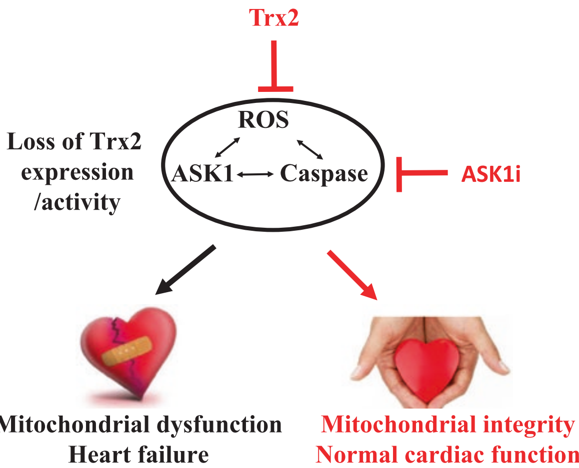

Fig. 14.1 Trx2-cKO Model and therapeutics for the treatment of heart failure. Loss of Trx2 expression or activity in heart induces ASK1 activation, ROS production and caspase-dependent cardiomyocyte apoptosis, leading to mitochondrial dysfunction and heart failure. ASK1 inhibitor could blunt activities of ASK1, ROS and caspases, and rescue heart failure in Trx2-deficient mice

mice from mitochondrial dysfunction, ROS accumulation, and apoptosis in cardiomyocytes. Besides, inhibition of ASK1 maintains mitochondrial integrity and function by blocking ROS production, cellular apoptosis which is induced by Trx2 deletion. It seems that ASK1 activation, mitochondrial dysfunction and ROS production are 3 interdependent issues that may form a vicious positive-feedback cycle, which promotes cardiomyocyte cell apoptosis (Fig. 14.1). Two recent reports have proved that a small molecule inhibitor of ASK1 could also reduce myocardial infarct size in rat and mouse ischemia/reperfusion models [70, 71]. ASK1 inhibition is a potential therapeutic strategy for the treatment of DCM and heart failure.

Conclusion

The heart is a prominent oxygen-consuming organ and the redox states critically affect its function. In this chapter, we have reviewed the main mechanistic role of Trx2 in heart failure through suppressing ROS generation, keeping mitochondria integrity and function, and inhibiting ASK1-dependent cardiomyocytes apoptosis. Although numerous studies have clarified the mechanisms of Trx2 in heart failure, there still remain issues to be solved. Future work in the field needs to better address several key aspects that include (a) the mechanism(s) responsible for Trx2 downregulation in human DCM and heart failure, (b) the role of Trx2 in other nonmyocyte cells in heart, (c) identification and research if genetic mutations or polymorphisms of the Trx2 gene are participated in DCM in humans. A better understanding of the role of the mitochondrial thioredoxin system in the physiological and pathological processes of heart may allow the development of more specific therapeutic strategies for heart diseases.

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Chapter 15
Mitochondria in Structural and Functional Cardiac Remodeling

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The heart is the organ responsible for pumping blood to the whole body, allowing thus the distribution of oxygen and nutrients to peripheral tissues and the elimination of toxic products through excretory organs. Cardiac tissue is composed of multiple cell types, including cardiomyocytes, fibroblasts, endothelial cells, vascular smooth muscle cells, macrophages and mast cells. Cardiomyocytes are the specialized cells responsible for myocardial contraction. Although they correspond to only 30–40% of the total number of cells in the heart, cardiomyocytes represent almost 75% of the heart volume [1]. Contractile function is manifest from the fetal stage forward, however, mitotic capacity of cardiomyocytes diminishes in the later stages of embryogenesis and stops almost completely after birth [2]. Therefore, the ability of the heart to respond to physiological or pathological conditions involves cellular remodeling processes rather than cellular proliferation. Cardiac remodeling is classically defined as changes in the structure of cardiac tissue, evidenced by changes in

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ventricle mass, volume or shape, fibrotic content, vascularization, cellular hypertrophy, and cell death. For instance, when the heart faces sustained work overload, compensatory mechanisms are activated in order to increase the capacity to pump blood. Initial mechanisms involve an increase in the frequency of contraction. When this is not sufficient, cardiac remodeling pathways are triggered. Cardiac hypertrophy can be caused by an increase in the size of individual cardiomyocytes [3] as well as by an increase in fibroblast proliferation (hyperplasia) [4]. It can be classified as adaptive or pathological, depending on the mechanisms activated. In adaptive hypertrophy muscular, vascular, and interstitial compartments maintain normal proportionality and collagen content, thus preserving tissue structure and function. This type of hypertrophy occurs in response to exercise training or during pregnancy, and changes to the heart can revert when the workload normalizes [5]. In contrast, during pathological hypertrophy, intercompartmental proportionality is ultimately lost, leading to structural heterogeneity of the myocardium. Importantly, relative collagen content of the heart increases, leading to myocardial fibrosis and detrimental structural remodeling [6].

Cardiac remodeling also occurs after a myocardial infarction (MI), where a large number of cardiac cells undergo cell death by necrosis, necroptosis, and apoptosis initiated by nutrient and oxygen deprivation. In an attempt to maintain cardiac output surviving cardiomyocytes increase in size, mass and volume. The left ventricle generally undergoes the most pronounced remodeling following MI, because it is the primary chamber responsible for pumping blood to the rest of the body [7]. Similar changes occur in the spared myocardium following damage from ischemia reperfusion (I/R).

Other stimuli leading to cardiac remodeling, and especially to hypertrophy, are elevated blood pressure and volume overload, both regulated primarily through the sympathetic and renin-angiotensin-aldosterone (RAA) systems. The sympathetic system is mediated by the action of norepinephrine and epinephrine on adrenergic receptors, regulating the rate and strength of cardiac contraction, as well as blood pressure in blood vessels [8]. Adrenergic receptors are G protein-coupled receptors that are desensitized upon sustained exposure to agonists, leading to attenuation of receptor responsiveness. Depressed receptor function is strongly associated with ventricular dysfunction, hypertrophy and heart failure [8].

Similarly, the function of the RAA system is to regulate blood pressure. When cardiac function becomes less efficient, secretion of renin by the kidney is increased, leading to an increase in renin- dependent conversion of angiotensinogen into angiotensin I (Ang I). In turn, angiotensin-converting enzyme (ACE) converts Ang I into angiotensin II (Ang II), which binds to angiotensin receptor (AT1) mediating changes in cells of both the vascular system and the heart, primarily involving vasoconstriction of vessels that increases blood pressure. Activation of the RAA is closely associated with the severity of heart failure, dilated cardiomyopathy and left ventricular (LV) hypertrophy [9].

Cardiac fibroblasts are responsible for secreting components of the extracellular matrix (ECM). During cardiac remodeling, fibroblasts can differentiate to myofibroblasts, that express contractile proteins including alpha-smooth muscle actin

(α-SMA), and exhibit increased migratory, proliferative and secretory properties [4]. Cardiac myofibroblasts are activated by proinflammatory cytokines (e.g. tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), transforming growth factor beta (TGF-β)), vasoactive peptides (e.g. Ang II, endothelin-1, natriuretic peptides) and hormones (e.g. noradrenaline). This leads to increased cell proliferation and migration, enhanced secretion of the previously mentioned cytokines and vasoactive peptides, and secretion of additional growth factors such as VEGF. This can further increase fibrosis, and remodeling of the heart in a feed-forward mechanism. Myofibroblasts are also be activated directly by mechanical stretch and in response to I/R [4].

In addition to changes in organ structure, cardiac remodeling includes changes in structure or function on the subcellular level. Although many organelles in cardiomyocytes undergo remodeling, this chapter will focus on those changes directly related to mitochondria. In particular, we will discuss the ability of mitochondria to undergo dynamic changes that affect both their outer membrane structure (fusion/fission) and their inner membrane structure (cristae remodeling), and how these processes contribute to the development or progression of cardiac pathologies. In addition we will address the cardiovascular consequences of changes to mitochondrial function, including loss of mitochondrial respiration, increase in ROS production, diminished ATP synthesis and the release of pro-death factors into the cytosol.

In the human heart, ATP production is carried out primarily by mitochondria, and is estimated to be as much as 6 kg ATP per day [10]. Although, during the fetal stage, lactate and glucose are the major sources for energy production, the heart undergoes a metabolic remodeling at birth, switching substrate oxidation from glucose to fatty acids. Thus, β-oxidation of free fatty acids and oxidative phosphorylation become the primary mechanisms for ATP production, producing approximately 70% of the ATP consumed by the heart [11].

In addition to ATP production, mitochondria participate in processes involving cell proliferation, differentiation, cellular immunity, calcium regulation, iron homeostasis, lipid metabolism, cellular aging, cell death, production of reactive oxygen species (ROS) and ROS scavenging [12].

In neonatal cardiomyocytes, mitochondria are distributed throughout the cytoplasm and around the nucleus. In contrast, in the adult heart they are packaged and aligned [13], located in parallel, longitudinal rows interleaved with the contractile machinery (interfibrillary mitochondria, IFM) or in monolayers immediately underneath sarcolemma (subsarcolemmal mitochondria, SSM) [14]. These differentially distributed mitochondria, also differ in the morphology of their cristae, being mostly tubular in the IFM and mainly lamelliform in SSM [15]. The contrasting morphologies of the two populations imply metabolic differences, as IFM display a higher rate of oxidative phosphorylation and enzymatic activity than SSM [16]. SSM also exhibit a lower capacity for calcium uptake than IFM [17], potentially leading to increased susceptibility to cytochrome c release and initiation of death signals in this population. Thus these two functionally distinct pools of mitochondria may play different roles in the pathologies leading to cardiac remodeling.

Mitochondria have their own genome (mtDNA), which in mammals is a densely packed double- stranded DNA molecule of 16.6 kb, containing 37 genes encoding 11 messenger RNAs (mRNAs; translated to 13 proteins), 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) [18]. The proteins encoded in the mitochondrial genome are core constituents of the mitochondrial respiratory complexes I, III, IV and V that are embedded in the inner mitochondrial membrane (IMM) [19]. Similarly, the tRNAs and rRNAs encoded in mtDNA are essential for mitochondrial ribosome assembly the translation of mitochondrial mRNAs [20].

Although mitochondria are the primary organelles responsible for energy production, the nuclear genome is the primary site for encoding the bulk of mitochondrial proteins. Therefore, the two organelles need to communicate closely. This allows nuclear control of mitochondrial ATP production [21] and conversely, changes in mtDNA or mitochondrial function are capable of modifying nuclear gene expression.

Mitochondria are highly dynamic organelles, continuously undergoing repeated cycles of fusion and fission in response to environmental changes and to the metabolic status of the cell (Fig. 15.1). These processes are strictly controlled by the activity of a group of guanosine triphosphatases (GTPases) related to the dynamin family [13]. Mitochondrial fission creates smaller and more circular mitochondria, through the activity of Dynamin-1-like protein (DRP1) and its adapter proteins mitochondrial fission 1 protein (FIS1) and mitochondrial fission factor (MFF) [22]. This process is required for redistribution of mitochondria in mitosis, release of cytochrome c during cell death by apoptosis and for selective mitochondrial degradation (mitophagy) [22]. On the other hand, mitochondrial fusion generates fewer, larger and more elongated mitochondria, in a process regulated by mitofusin (MFN) 1 and 2, both situated in the outer mitochondrial membrane (OMM), and by optical atrophy protein 1 (OPA1), located in the IMM. This process allows the exchange of material (matrix components, damaged mtDNA) and promotes balance in bioenergetics properties (e.g. mitochondrial membrane potential) [11].

Increased oxidative stress, altered calcium load or cellular pH are each capable of impacting mitochondrial dynamics, disrupting mitochondrial membrane potential, and inducing opening of the mitochondrial permeability transition pore (MPTP). This latter process has been widely associated with remodeling of the mitochondrial cristae, especially during I/R [23].

Existing therapies to prevent the progression of cardiac remodeling primarily act at the cardiac or vascular level. These include drugs for reducing hypertension, such as inhibitors of ACE (enalapril), angiotensin receptor blockers (losartan) and beta-blockers (atenolol); and drugs that reduce cholesterol levels like statins and fibrates [4]. However, no drugs currently in use are targeted directly at the causes of subcellular remodeling of mitochondria, warranting further research aimed at understanding the possible relation between the prevention of functional or metabolic remodeling and the prevention or amelioration of cardiac diseases.

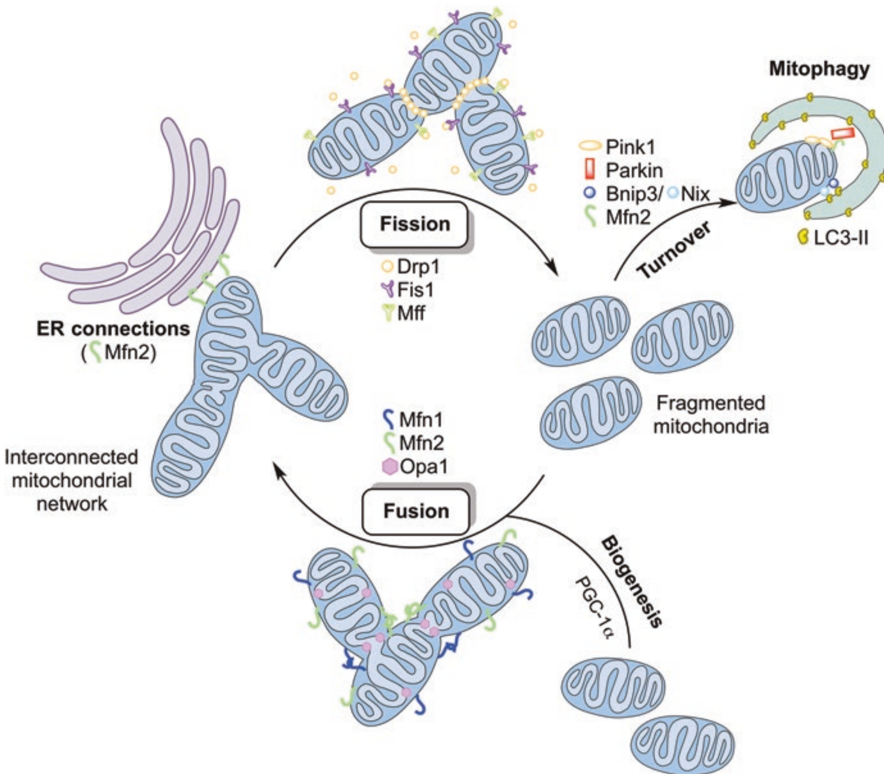

Fig. 15.1 Mitochondrial life cycle. Mitochondrial dynamics enable mitochondria to respond to external stimuli or metabolic signals in a way that maintains proper function. Mitochondrial fusion is regulated by mitofusin (*Mfn*) 1 and 2 and optical atrophy protein 1 (*Opa1*), at the outer and inner mitochondrial membranes, respectively. This process results in a more interconnected mitochondrial network that enhances communication with the endoplasmic reticulum (*ER*) through mitochondria-associated endoplasmic reticulum membranes (*MAM*) and a complex mechanism involving the presence of Mfn2 amongst other proteins, thus enhancing calcium movement from ER to mitochondria and regulating its function. Conversely, Drp1 and its adapter proteins Fis1 and Mff control mitochondrial fission. This process is required for selective mitochondrial turnover (mitophagy), which allows isolation of damaged mitochondria to ensure proper mitochondrial quality control. Here, the mitochondrial kinase Pink1 phosphorylates Mfn2 which allows Parkin E3 ligase bind phosphorylated Mfn2 to catalyze the ubiquitination of several proteins on the OMM that promotes interaction with LC3 at nascent phagophores, initiating mitochondrial autophagy. Nix and Bnip3 are mitophagy regulators that sense myocardial damage and facilitate the elimination of damaged cardiac mitochondria. Peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) is a critical factor that coordinates mitochondrial biogenesis and the expression of metabolic genes and therefore critical to the synthesis aspect of mitochondrial dynamics

Mitochondrial Dynamics

The term mitochondrial dynamics comprises at least three processes. One is the capability of mitochondria to undergo fission and fusion processes, changing its network between punctate and fragmented, and elongated and interconnected mitochondria, respectively [6]. Secondly remodeling of the mitochondrial matrix, which influences metabolite diffusion, and thus, mitochondrial metabolism [24]. And finally, mitochondrial motility within the cell, which is mediated by the interplay of mitochondrial fission and fusion proteins with kinesin 1 and 3 motors and the adaptors Milton and Miro (Miro/Milton/kinesin complex), thus allowing interaction of mitochondria with cytoskeletal components to facilitate their movement along microtubules [25].

In cardiac cells, the most prominent events related to mitochondrial dynamics are fission and fusion and remodeling of the cristae. These dynamic processes occur in response to external stimuli and metabolic signals. Changes in mitochondrial morphology have been implicated in cell division, embryonic development, apoptosis, autophagy, metabolism, development, and differentiation [26]. In the heart, disruption of these mechanisms has been identified in multiple cardiac diseases, including cardiac hypertrophy, heart failure, dilated cardiomyopathy, and ischemic heart disease. In addition, modification of these processes has been shown to protect the heart against injury in specific settings [27].

Mitofusins 1 and 2

Mitofusins are present in all tissues, but in the heart the levels of mRNA encoding these proteins are particularly high, with MFN2 in greater abundance than MFN1 [28]. Both are members of the family of transmembrane GTPase and reside in the OMM. They mediate OMM fusion through GTP hydrolysis. MFN1 has a higher GTPase activity than MFN2, making it more efficient in the fusion process [29]. The C- terminal end of the proteins is oriented towards the cytosol and contains both a coiled-coil domain 2 (also called hepta-repeat domain or HR2) and a transmembrane domain. The N-terminal end contains the GTP binding domain and another coiled-coil domain (HR1) [30]. To facilitate fusion of the OMM, the HR2 domains of MFN1 or MFN2 form dimeric complexes with MFN1 or MFN2 on adjacent mitochondrion, allowing tethering of the two mitochondria.

OPA1

OPA1 is expressed throughout the body, but is present in greater quantity in the retina, brain, testis, heart and skeletal muscle [31]. This protein is a large GTPase located in the IMM, whose function is to promote mitochondrial fusion of the IMM and is also involved in remodeling of mitochondrial cristae [32]. The protein is

comprised of a N-terminal domain containing a mitochondrial import sequence (MIS, which is removed by a mitochondrial matrix protease when the protein is imported to the mitochondria), a transmembrane domain (TM), a region of alternative splicing, a coiled-coil region, a GTPase domain, a middle domain, and a GTPase effector domain (GED or assembly domain) at the C-terminus [26]. In addition to the GTPase domain, OPA1 requires the presence of MFN1 to induce mitochondrial fusion [33]. OPA1 undergoes post-translational modifications by alternative splicing at exons 4, 4b, and 5b, generating eight human isoforms of OPA1. Four of these isoforms (3, 5, 6, and 8) can be cleaved by YME1L, an intermembrane AAA (ATPase associated with diverse cellular activities) protease, generating shorter forms of OPA1 (S-OPA1), which are soluble and reside in the mitochondrial intermembrane space. The other isoforms of OPA1 (1, 2, 4, and 7) do not normally undergo cleavage and constitute the long forms of OPA1 (L-OPA1), which contain the TM domain, and are anchored into the IMM. Both, long and short isoforms are required for mitochondrial fusion [26]. Under conditions of mitochondrial membrane depolarization, ATP deficiency and apoptosis induction, the L-OPA1 isoforms undergo inducible cleavage by OMA1 generating short forms of OPA1. This prevents the pro-fusion activity of OPA1, resulting in mitochondrial fragmentation. Through this process, depolarized fragmented mitochondria can be removed by mitophagy [34]. In addition, short and long forms of OPA1 form oligomers, keeping cristae junctions tightly closed, thereby opposing cytosolic release of cytochrome c that can initiate apoptosis [35].

DRP1

DRP1 is abundant in brain, heart and muscle [36]. It is located in the cytosol and translocates to the OMM to induce fission or division of mitochondria [26]. DRP1 contains a GTPase domain, a central domain and a GTPase effector domain or assembly domain [36], which regulates the GTPase activity of the protein and directs it to the mitochondria [37]. To initiate fission, DRP1 translocates to the OMM where it binds to the receptor, FIS1 or MFF, undergoes oligomerization, and then begins constriction of the mitochondrial scission site using GTP to power the process [37]. DRP1 is regulated by a diversity of post- translational modifications such as phosphorylation and nitrosylation [38]. Particularly important in cardiac remodeling, dephosphorylation of DRP1 by calcineurin initiates fission, whereas phosphorylation of the same site by PKA opposes it.

FIS1

FIS1 is uniformly distributed in the OMM and is comprised of an N-terminal domain, required for protein–protein interactions, FIS1 oligomerization, and mitochondrial fission; a transmembrane domain and a C-terminal domain exposed to the intermembrane space, that are essential for mitochondrial localization and the

induction of apoptosis [39]. It has been shown that, although FIS1 is required for mitochondrial fission, it is not a limiting factor in the process and mitochondrial recruitment of DRP1 can be regulated by other factors [45].

MFF

Mitochondrial fission factor (MFF) mediates both mitochondrial and peroxisome fission and is highly expressed in heart, kidney, liver, brain, muscle, and stomach [40]. It has a C-terminal transmembrane domain essential for its localization to mitochondria, anchoring to the OMM, and the ability to multimerize, exhibiting similar properties to FIS1 [40]. MFF (and specifically its N-terminal cytosolic region) is required for DRP1 recruitment to mitochondria and together MFF and DRP1 are capable of mediating mitochondrial fission independent of FIS1 [41]. This indicates that FIS1 and MFF are independent of one another and may activate fission in response to different stressors. Interestingly, both proteins also mediate fission of peroxisomes [40].

Mitochondrial Elongation Factors

The mitochondrial elongation factor proteins, MIEF1 and MEIF2 are also known as mitochondrial dynamics proteins MiD49 and MiD51 respectively [49]. The levels of MIEF1 mRNA are high in adult human heart, skeletal muscle, pancreas and kidney [42]. The activity of these proteins has not been fully characterized, however, studies indicate that although overexpression of MiD49/MiD51 recruits DRP1 to mitochondria, it actually reduces DRP1's GTP-binding activity, thereby promoting mitochondrial fusion and increasing mitochondrial elongation. Furthermore, knockdown of MiD49/MiD51 increases fragmentation [42] and suggests that these proteins may prevent DRP1's interaction with FIS1, and thus, its fission-related function. In contrast, studies from other investigators [43] indicate that, rather than blocking fission, MiDs might recruit DRP1 to mitochondria and maintain it in an inactive state until a cellular signal triggers fission. Palmer et al. also demonstrated that siRNA knockdown of both MiD49 and MiD51 reduced translocation of DRP1 to mitochondria and resulted in mitochondrial fusion and elongation [44]. Yet, overexpression of MiD49/51 blocked fission by sequestering DRP1 specifically at mitochondria, and promoted MFN1 and MFN2-dependent mitochondrial fusion [45]. Thus, the exact function and effect of these proteins remains to be elucidated.

Pathological Mitochondrial Remodeling

As will be expanded upon later in the chapter, pathological remodeling of mitochondria can be particularly onerous in the heart as it can lead to depletion of ATP and increases in intracellular calcium and ROS, thereby triggering mechanisms

such as mitophagy or apoptosis, outcomes often observed in the failing heart or following cardiac I/R. In this sense, proteins involved in mitochondrial fission and fusion are particularly important, as they can be critical for maintaining or restoring mitochondrial integrity and function. Cellular imbalances such as oxidative stress, damage to mtDNA or loss of mitochondrial membrane potential, among others, affect the integrity of mitochondria, leading to energy insufficiency, and thus, an inability to support cell function that may eventually lead to cell death (Fig. 15.2).

Remodeling of mitochondrial cristae is a necessary prerequisite for apoptosis. Loss of cristae structure allows redistribution of cytochrome c in the intermembrane space and its release into the cytoplasm through pores in the OMM generated by Bax and Bak, initiating activation of caspase cascades leading to cell death [46]. In the IMM, OPA1 oligomerizes and stabilizes the morphology of the cristae, preventing remodeling of such structures and thus opposing the release of cytochrome c and subsequent apoptosis [35]. For cytochrome c release to occur, L-OPA1 isoforms are processed to S-OPA1, allowing the disassembly of oligomers [47]. Therefore, OPA1 acts as an anti-apoptotic protein, preventing remodeling, whereas the cleaved forms are pro-apoptotic.

The combined actions of DRP1 and MFF are also important in this process. Otera and colleagues demonstrated that knockdown of DRP1 and MFF compromised mitochondrial fission and apoptosis induced by exogenous stimuli. In turn, MFF overexpression induced extensive mitochondrial fragmentation concomitant with an increased sensitivity to apoptosis, suggesting that mitochondrial fission facilitates apoptotic cristae remodeling [41]. hFIS1 likewise displays dual functionality, participating in mitochondrial fission as a component of normal mitochondrial dynamics, as well as being capable of trigger caspase-dependent cell death causing the release of cytochrome c from mitochondria, in the setting of calcium release from the endoplasmic reticulum (ER) and opening the MPTP [44].

MFN2 is another key player that carries out multiple functions. In addition to its role in mitochondrial fusion, it also modulates mitochondrial apoptotic pathways. Recent studies have shown that MFN2 is also present on ER where it assists in the tethering of ER to mitochondria. This contact between ER and mitochondria, enables calcium signaling, inositol trisphosphate (IP3) signaling, and calcium-dependent induction of apoptotic signaling [48]. MFN2 protein levels and apoptotic cell death increased in rat cardiomyocytes exposed to hydrogen peroxide. Whereas, siRNA depletion of MFN2 suppressed apoptosis [49]. Activation of apoptosis by oxidative stress is likewise reduced in the hearts of mice with a genetic disruption of MFN2 [50]. Therefore, MFN2 appears to play an active role in cardiomyocyte death mediated by oxidative stress [49]. In contrast, in other contexts, MFN2 may exert an anti-apoptotic effect. When cardiomyocytes are treated with ceramide, DRP1/FIS1-dependent mitochondrial fragmentation and apoptosis occurs [51]. When MFN2 is depleted, ceramide's effects are exacerbated (mitochondrial fragmentation, DRP1 co-localization with FIS1, mitochondrial membrane depolarization, cytochrome c release, and cell death), suggesting that MFN2 may exert an anti-apoptotic effect against ceramide [51]. Therefore, the role of MFN2 in the apoptotic process appears to be mediated by the nature of the apoptotic stimulus.

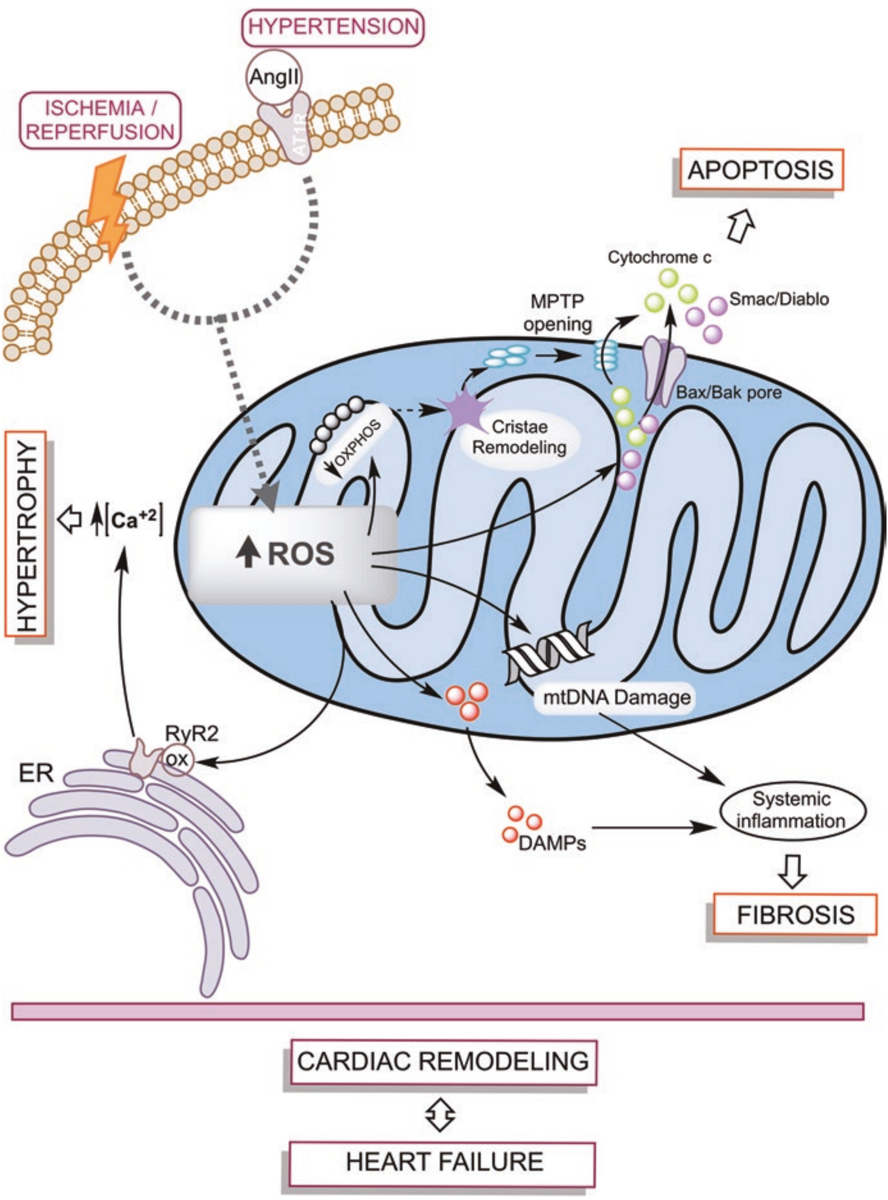

Fig. 15.2 Mitochondrial ROS in cardiac remodeling. Cardiac remodeling is induced by several mitochondrial ROS-related mechanisms. Cardiac pathological conditions like hypertension and ischemia/reperfusion (I/R) both induce an increase in mitochondrial ROS production. This leads to cristae remodeling, which in turn triggers the opening of the mitochondrial permeability transition pore (*MPTP*) and the assembly of the Bax/Bak pore. Both pores allow the release of pro-apoptotic proteins (like cytochrome c and Smac/Diablo), inducing the activation of the intrinsic cell death pathways in cardiac cells. Mitochondrial ROS can also cause the release of intra-mitochondrial damage-associated molecular patterns (*DAMPs*) and damage mitochondrial DNA, leading to systemic inflammation and triggering cardiac fibrosis. ROS generated by mitochondria can increase cytosolic calcium levels through oxidative damage of the ryanodine receptor (*RyR2*), leading to the activation of pro- hypertrophic pathways. Together, the cumulative effects of mitochondrial ROS triggers cardiac remodeling that ultimately leads to chronic heart failure

MARCH5, an E3 ubiquitin ligase associated with the OMM can act as a negative regulator of mitochondrial fission by mediating proteasomal degradation of MiD49 [52]. When cells were subjected to pro-apoptotic stimuli or to stimuli that affects mitochondrial activity, protein levels of MiD49 were decreased in a MARCH5-dependent manner. This data suggests that MARCH5, by increasing MiD49 degradation, inhibits fission and may protect cell from apoptosis induced by stress.

Proteins involved in mitochondrial dynamics are also affected during I/R, a prominent initiator of myocardial remodeling. In this context, Pei and colleagues [53] reported a reduction in MFN2 after I/R. The authors also observed a significant decrease in ATP production and an increase in ROS generation in MFN2 deficient mice subjected to I/R compared to the response of wild type mice. Subsequent suppression of cardiac function and the extent of myocardial fibrosis were likewise greater in the MFN2-deficient mice. They also showed that melatonin can increase MFN2 expression in ischemic myocardium by activating Notch1, thereby preserving mitochondrial integrity, increasing ATP production, reducing ROS production and preventing cardiomyocyte apoptosis after I/R, leading to an improved cardiac function and reduced fibrosis in myocardium, and attenuating cardiac remodeling.

Changes to mitochondrial form and function are a prominent feature of cardiac remodeling is observed in heart failure. Chen et al. [54] documented decreases in OPA1 protein levels in failing hearts, that occurred in conjunction with an increase in smaller and fragmented mitochondria. Mitochondrial fragmentation and reduction in OPA1 protein levels likewise occurs in vitro in H9c2 myoblasts subjected to simulated ischemia [34]. In addition, reduction of OPA1 levels with shRNA increased mitochondrial fragmentation, cytochrome c release, and apoptosis in response to simulated ischemia [54]. All these findings suggest that OPA1 helps limit fission of mitochondria during ischemia, and reduction of OPA1 levels following ischemia may contribute to heart failure progression.

DRP1 plays a central role promoting mitochondrial fragmentation and death during simulated ischemia, which can be prevented by expressing a dominant negative mutant of DRP1 [55, 56]. Treatment with a pharmacological inhibitor of DRP1, mdivi-1, increases the proportion of elongated mitochondria in adult cardiomyocytes, protecting them against simulated I/R in vitro and decreasing the myocardial infarct size in vivo [41]. This suggests that inhibition of DRP1 may be a good therapeutic target to mitigate the damage caused by I/R injury, thus preventing the subsequent pathological remodeling of the heart. Consistent with this, the prohyperthrophic-activation of calcineurin has been shown to recruit DRP1 to mitochondria, thereby stimulating mitochondrial fission, and the depletion of MFN2 to stimulate cardiac hypertrophy [57]. Either calcineruin inhibition or expression of a dominant-negative of Drp1 (K38A) was sufficient to prevent norepinephrine-induced hypertrophy, demonstrating that fission is a requisite step for calcineurin-mediated hypertrophic remodeling [57].

Disatnik and colleagues [58] demonstrated that treatment with p110 (a peptide that selectively inhibits the FIS1/DRP1 interaction, thereby preventing fission) restores mitochondrial functions in different models of cardiac I/R injury, including isolated primary cardiomyocytes, ex vivo heart, and in vivo myocardial infarction

models. The authors showed that inhibition of fission during reperfusion preserves cardiac tissue integrity and cardiac function. This highlights the importance of mitochondrial dynamics in the progression of heart disease and supports the notion that intervening in such processes can help stop its progression.

Mitochondrial ROS, Cell Death in Cardiac Remodeling

Mitochondria are broadly recognized as one of the main sources of oxidative stress and ROS production [59]. This is particularly true in cardiomyocytes, in which several studies have shown that approximately 2% of the oxygen reduced during the normal functioning of the electron transport chain is lost as superoxide anion (O2·-) due to leak of the electron transport chain, mainly at the level of complexes I and III [60]. Thus, it is not surprising that mitochondria are involved in many oxidative stress-related cardiac pathologies, including cardiac hypertrophy [61], fibrosis [62], diabetic [63] or dilated [64] cardiomyopathies, I/R damage [53], and heart failure [65]. ROS can impact many signaling pathways. It is therefore capable of triggering a diversity of signaling cascades relevant to cardiomyocyte hypertrophy [66]. Moreover, ROS generation plays an important role in both Ang II and α-adrenergic-receptor induced hypertrophy [67] as well as in the pro-hypertrophic activation of NFκB [68]. ROS can likewise stimulate proliferation of cardiac fibroblast [69] as well as enhancing expression of matrix metalloproteinases (MMPs) [70] and their activation [71]. Based on the pro-hypertrophic action of ROS, antioxidant compounds such as omega-3 fatty acids have been proposed as protective against pressure overload-induced myocardial hypertrophy [72].

I/R damage to the heart is broadly linked to mitochondrial ROS production [53]. During ischemic events, mitochondrial respiration is lowered to near-zero, and energy production through oxidative phosphorylation is almost stopped. Later, when reperfusion is achieved and oxygen flow through the electron transport chain restored abruptly, the initial burst in ROS production is thought to overwhelm cellular antioxidant mechanisms [73]. This increase in mitochondrial generation ROS is thought to underlie most of the cellular damage associated with I/R. The antioxidant compounds picroside II [74], lycopene [75], all-trans retinoic acid [76] and vitamin C [77] have been shown to diminish ROS production during I/R in cardiomyocytes, thereby reducing mitochondrial damage and cellular death. These studies support the contention that increased ROS production activate cellular death pathways to effect cardiac damage during I/R.

Over the course of time, each of the above-discussed cardiac pathologies can lead to heart failure [78]. Thus, heart failure is considered and end-phase condition, triggered by accumulative damage in both the structure and function of the heart [79]. Given the cumulative, progressive nature of the disease, it is perhaps not surprising that ROS is capable of damaging a range of macromolecules relevant to cardiac excitation-contraction coupling and mitochondrial energy production rather than targeting a single substrate [65]. Additional support to idea that ROS plays an

fundamental role in the development of heart failure comes from the evidence of changes in uncoupling proteins (UCPs) [65]. For instance, mRNA and protein levels of UCP2 and UCP3 are reported to decrease in the heart during the early stages of heart failure in both animal and human models [80, 81]. UCPs are endogenous proteins capable of dissipating the mitochondrial membrane potential and increasing efficient electron flow through the electron transport chain by transporting of protons across the IMM [82]. UCPs increase oxygen consumption but diminish ROS production, by reducing electron leak responsible for O2·– production [83]. Thus, reduced UCP expression may represent an attempt to preserve oxygen and ATP in the early stages of heart failure that could come at the cost of increasing the capacity for generating ROS. In the later stages of heart failure, however, UCP2 and UCP3 protein levels are reported to increase [84, 85] following the activation of PPARα by free fatty acids in plasma. It is unclear whether this late stage increase in UCP ultimately plays a protective or detrimental role in chronic heart failure [65].

Mitochondrial induction of apoptosis has been broadly associated with cardiac remodeling. Indeed, the frequency of apoptotic cardiomyocytes has been shown to be 10–100-fold higher after myocardial infarction than those observed in control hearts [86]. Mitochondria contain several intrinsic apoptosis- inducing factors, such as cytochrome c, the SMAC/Diablo protein and some of the calpain proteases [87]. Upon internal damage signals, such as DNA fragmentation, accumulation of intracellular toxic compounds, or uncontrolled oxidative stress, mitochondrial inner membrane is permeabilized, thereby releasing these pro-apoptotic factors into the cytosol and triggering the activation of the intrinsic cell death pathway [88]. Oxidative stress in particular, when not controlled by the antioxidant systems, is able to rapidly destabilize the structure of the IMM [89]. Therefore, it is likely that many of the cardiac pathologies linked to increased ROS production involve mitochondrial activation of cell death signals.

One of the main avenues by which mitochondrial pro-apoptotic molecules are released into the cytosol is via a pore formed by the pro-apoptotic Bcl-2 family proteins [90]. Upon apoptosis-inducing stimuli, Bcl-2 proteins (such as Bak and Bax) oligomerize on the IMM, opening a pore that allows the release of pro- apoptotic factors. Another important channel in mitochondria-activated apoptosis is the MPTP [90]. Such channel is thought to be formed by several mitochondrial membrane proteins and is activated upon IMM damage, thereby contributing to cell death [90]. The importance of MPTP opening in cardiac damage is supported by several articles reporting on the potential of MPTP inhibition to diminish apoptotic death, offering cardioprotection in diverse cardiac pathological states [91–93].

Mitophagy in Cardiac Remodeling

Autophagy is a catabolic recycling pathway triggered by various intra or extracellular stimuli that is conserved from yeast to mammals. During autophagy diverse cytosolic constituents are enveloped by double-membrane vesicles named

autophagosomes, which later fuse with lysosomes in order to degrade their cargo. Dysregulation in autophagy is associated with a diverse range of pathologies including cardiovascular diseases [94]. Mitochondrial autophagy or mitophagy, which literally means “eating mitochondria,” is the term applied to the cellular mechanism for identifying and selectively eliminating dysfunctional mitochondria as part of the overall mitochondrial quality control process. Increasing lines of evidence indicate that autophagy is intimately involved in the survival and death of cardiomyocytes under stress, however it is likely also involved in maintaining normal cardiac function and metabolism (Fig. 15.3). The beneficial effects of autophagy are partly mediated by the elimination of damaged mitochondria and the consequent prevention of mitochondrial dysfunction, oxidative stress increase, and cell death [95].

The most well studied mechanism of mitophagy in cardiomyocytes is the one mediated by the cytosolic E3 ubiquitin ligase Parkin [96] and the mitochondrial membrane kinase PTEN-induced putative kinase-1 (Pink1) [97]. Pink1-Parkin-mediated autophagy participates in mitochondrial quality control and in the maintenance of cardiac function in the heart at baseline. Cardiomyocytes in which Bnip3 is overexpressed are able to eliminate damaged mitochondria through autophagy via a Parkin-dependent mechanism, indicating that Parkin-mediated mitochondrial autophagy exists in cardiomyocytes [98]. The lack of Parkin-mediated mitophagy in heart-specific MFN2 KO mice is compensated by activation of non-selective autophagy in cardiomyocytes [95], thereby allowing cardiomyocytes to maintain mitochondrial quality.

PINK1−/− mice develop LV dysfunction and pathological cardiac hypertrophy [99], which is mediated by increased oxidative stress and dysfunctional mitochondria in cardiomyocytes. In the same line, PINK1 protein levels are significantly decreased in heart failure [99]. Consistently, mouse hearts lacking MFN2 develop cardiac dysfunction with age [100], suggesting that mitophagy regulated by the PINK1-MFN2 pathway plays an important role in maintaining cardiac function at baseline.

Translocation of Parkin to mitochondria and ubiquitination of mitochondrial proteins occurs during the acute phase of I/R, suggesting that Parkin-mediated mitophagy is stimulated [101]. Parkin-deficient mice exhibit a decrease in mitochondrial autophagy, accumulation of dysfunctional mitochondria and a reduced survival rate after I/R [101]. Taken together, these results suggest that mitophagy is stimulated in the heart during the chronic phase of cardiac remodeling and that it would be protective for the heart.

In cardiomyocytes, mitochondrial dynamics play an important role in mitophagy quality control through the process of DRP1-mediated asymmetric fission [102]. Accordingly, mitochondria in the early stages of senescence or those that have sustained moderate damage, will segregate dysfunctional components into one of the two daughter organelles generated by a fission event. The damaged daughter mitochondrion will be promptly identified as such and removed via Pink1-Parkin mediated mitophagy, whereas the healthy daughter will re-join the cellular mitochondrial pool, likely by fusing with other similarly fit mitochondria [103].

Fig. 15.3 Cardiac remodeling. Cardiac remodeling may occur at tissue, cellular or subcellular levels. At the tissue level, pathological conditions such as myocardial infarction, hypertension or heart failure lead to contractile myocardial dysfunction, cardiac hypertrophy and cardiac fibrosis. At the subcellular level, impaired mitochondrial function, cristae remodeling, altered mitochondrial dynamics and decreased mitophagy, together with increased ROS production, trigger a variety of downstream effects, including increased β-MHC expression, elevated cytosolic calcium, and decreased fatty acid oxidation. Mitochondrial function also impacts collagen-based extracellular matrix homeostasis by increasing the activity and synthesis of matrix metalloproteinases (MMPs) and the levels of TGF-β. Increased TGF-β production leads to differentiation and proliferation of cardiac fibroblasts into myofibroblasts, thus increasing cardiac fibrosis. Similarly, increased mitochondrial ROS production damages mtDNA and leads to the release of DAMPs, triggering an inflammatory response that further enhances cardiac fibrotic remodeling. Finally, damage to mitochondrial function can leads to the activation of cell death pathways, causing further contractile dysfunction, hypertrophy and cardiac fibrosis

The protective effect of mitophagy in the heart generally appears most prominently during stress. During ischemia, autophagy is triggered as an adaptive mechanism providing nutrients and eliminating damaged mitochondria, which could otherwise release damaging ROS and initiate apoptosis [104]. In fact, pharmacological inhibition of autophagy increases cardiomyocyte death by I/R, indicating that autophagy functions as a pro-survival mechanism in such conditions [105]. After reperfusion, triggered cardiac autophagy can be either adaptive or detrimental [94], and the impact of these processes on cardiac remodeling remains to be fully understood. Moreover, in response to myocardial infarction, activation of autophagy in the border zone is increased at an early stage, returning to normal during the chronic phase [106]. The autophagic vacuoles in the border zone are over-sized and contain organelles, including degraded mitochondria, in contrast with normalized autophagosomes which are upregulated in the remote zone during the chronic phase [106]. Pharmacological enhancing of autophagy with rapamycin 2 weeks after coronary ligation, ameliorates cardiac dysfunction and maladaptive remodeling, whereas inhibition of this process with bafilomycin A1 worsens them [106].

In mice, myocardial ischemia induced by permanent coronary artery ligation upregulates p53 and TP53-induced glycolysis and apoptosis regulator (TIGAR). Genetic deletion of p53 or TIGAR in mice stimulates mitophagy and inhibits accumulation of damaged mitochondria and apoptosis, an effect abolished by chloroquine. The authors suggest that the beneficial effects of the p53 or TIGAR downregulation may be mediated by mitophagy [107].

During myocardial reperfusion after a period of myocardial ischemia, mitochondria generate ROS, initiating a feedforward mechanism of oxidative stress, mitochondrial injury and cell death [108]. Selective elimination of damaged mitochondria by mitophagy is predicted to protect cardiomyocytes during reperfusion.

Despite the discussed reports, further investigations are needed in order to introduce modification of autophagy and mitophagy as useful therapeutic targets in the treatment of cardiovascular diseases, and to better understand where and when autophagy is activated or inhibited and how it affects the function of mitochondria in response to a wide variety of cardiovascular stress conditions that leads to heart remodeling.

Mitochondria in Cardiac Pathology

As previously discussed in this chapter, the main causes of cardiac remodeling that lead to heart failure are hypertension and I/R. At a cellular level, the processes that contribute to cardiac remodeling include cardiomyocyte hypertrophy, cell death and increased interstitial fibrosis. In this section, we will discuss the role of mitochondria in this processes and its potential as a therapeutic target.

Mitochondria and Cardiac Hypertrophy

It is generally accepted that mitochondrial dysfunction develops in the failing heart, and in many of the studies the model used was one of pathological cardiac hypertrophy. Cardiac hypertrophy can be divided into pathological or physiological hypertrophy. Thus, if after an initial phase of compensation, the growth response leads to contractile dysfunction, ventricular dilation, and heart failure, hypertrophy is considered pathological [45]. Pathological cardiac hypertrophy is a chronic complex disease that occurs in response to hemodynamic overload and is accompanied by oxidative stress and mitochondrial dysfunction [109]. In most cases of pathological hypertrophy, mitochondrial changes in structure and function have been described. In contrast, physiological cardiac hypertrophy is associated with enhanced mitochondrial function [45].

Myocardial oxidative stress has been implicated in the transition from compensated cardiac hypertrophy to heart failure, and evidence exists to support a role for mitochondrial and non-mitochondrial sources of ROS [45]. Oxidative stress can further impair mitochondrial function by leading to oxidative modifications of mitochondrial proteins, mutations of mtDNA and activation of the MPTP [77].

Pathological cardiac hypertrophy is associated with activation of many signaling pathways, exemplified by the calcineurin/nuclear factor of activated T-cell pathway, histone deacetylases, phosphatidyl inositol 3 kinase (PI3K)/Akt/Forkhead box protein O1 (FoxO1)/ mammalian target of rapamycin (mTOR) signaling networks, the ERK signaling pathway, G-protein-coupled receptor signaling pathways, among others [45, 110]. Change in calcium homeostasis is a critical factor in the deterioration of cardiac hypertrophy. Calcium affects the integrity of cardiomyocyte function in two ways: one is through calcium/calcineurin signaling pathway that directly activates/inactivates calcium/calcineurin dependent kinase [111, 112]. Another is through calcium channels that control the calcium flow in and out of organelles like mitochondria and ER [110]. Under normal conditions, mitochondria play a key role in cytosolic calcium clearance [113].

In vivo studies using pigs with hypertrophic cardiomyopathy revealed swollen cardiac mitochondria with disrupted cristae and substantial mtDNA depletion. Complex I and complex IV activity were also reduced in this model of hypertrophy [54].

Modulation of mitochondrial energy supply may provide means for therapy against cardiac hypertrophy. In cardiomyocytes, mitochondria produce ATP mainly through fatty acid oxidation (FAO) and oxidative phosphorylation. Many groups have described reduced myocardial FAO in pressure overload cardiac hypertrophy and heart failure. However, during the early stages of hypertrophy, which precede heart failure, changes in, or potential mechanisms for reduced FAO are less clear [45].

Currently, there are no effective methods to treat cardiac hypertrophy. It is reasonable to conclude that compensated cardiac hypertrophy is associated with relatively preserved mitochondrial function and that the development of mitochondrial dysfunction occurs in parallel with the development of heart failure. It remains to be

definitively proved if adapting mitochondria to the hypertrophic state and maintaining mitochondrial function can be a promising therapeutic method in preventing the deterioration of cardiac hypertrophy and remodeling.

Mitochondrial Metabolism in Cardiac Fibrosis

Cardiac fibrosis is characterized by accumulation of ECM in the myocardium and it is present in most cardiac pathological conditions [114]. Because the adult mammalian myocardium has negligible regenerative capacity, the most extensive fibrotic remodeling of the ventricle is found in diseases associated with acute cardiomyocyte death. Following acute I/R, the sudden loss of a large number of cardiomyocytes triggers an inflammatory reaction, ultimately leading to replacement of dead myocardium with a collagen- based scar [115]. Similarly, pressure overload induced by hypertension, results in extensive cardiac fibrosis that is initially associated with increased stiffness and diastolic dysfunction. A persistent pressure load may lead to ventricular dilation and further diastolic and systolic heart failure [114]. In addition, hypertrophic cardiomyopathy has been associated with the development of significant cardiac fibrosis [116].

Several cell types are implicated in fibrotic remodeling of the heart, either directly by producing matrix proteins (fibroblasts) or indirectly by secreting fibrogenic mediators (macrophages, mast cells, lymphocytes, cardiomyocytes, and vascular cells). In all conditions associated with cardiac fibrosis, fibroblast transdifferentiation into secretory and contractile cells, termed myofibroblasts, is the key cellular event that drives the fibrotic response [117]. Regardless of the aetiology of cardiac injury, myofibroblasts are prominently involved in both reparative and fibrotic processes. Indeed, myofibroblast accumulation in the cardiac interstitium has been reported, not only in myocardial infarction [118] but also in the pressure and volume overloaded myocardium [119].

Oxidative stress has also been implicated in the pathogenesis of cardiac fibrosis, both through direct actions and through its involvement in cytokine and growth factor signaling. ROS are able to directly regulate the quantity and quality of interstitial ECM by modulating both matrix protein expression and metabolism. Inappropriate production of ROS in mitochondria, overwhelming the antioxidant defense systems, and the resulting oxidative damage to mtDNA and mitochondrial proteins have long been recognized as playing a causative role in the development and progression of cardiac remodeling and dysfunction [120]. Recent data support an essential role for the mitochondrial antioxidant enzyme thioredoxin 2 in preserving cardiac function by suppressing mitochondrial ROS production [64]. Mice with cardiac specific deletion of thioredoxin 2 develop progressive dilated cardiomyopathy with impairment of contractile function, heart chamber dilation, and marked interstitial fibrosis [121].

TGF-β1 is required for fibroblast differentiation into a pro-fibrotic myofibroblast phenotype, and it has been established that NADPH oxidase 4 (NOX)-dependent

$O2\bullet-$ production by TGF-β1 is required for this differentiation [122, 123]. Moreover, ROS generated from mitochondrial electron transport chain complex III are required for TGF-β1–mediated transcription of NOX4 [124] and the initial activation of NOX4 amplifies and sustains TGF-β1–induced oxidative stress. Therefore, prevention of early mitochondrial $O2\bullet-$ formation and ensuing NOX4 up-regulation might be a key mechanism involved in the anti-fibrotic effect of PETN [125]. In conclusion, long-term PETN treatment targeting superoxide generation and NO• bioavailability most likely prevented the changes of mitochondrial scavenging pathways and progressive fibrotic remodeling, leading to improved cardiac functional performance in congestive heart failure.

Alterations in the balance of MMPs and tissue inhibitors of metalloproteinases (TIMPs) are also involved in myocardial matrix remodeling. MMPs are expressed at very low levels in normal myocardium but markedly increased expression and activity of MMPs have been shown in human and animal hearts during the remodeling process after I/R [126, 127]. Increased oxidative stress activates MMPs and decreases fibrillar collagen synthesis in cardiac fibroblasts [62]. On the other hand, the TGF-β activating effects of ROS may enhance ECM deposition in the cardiac interstitium [128]. In adult rat cardiac fibroblasts, Ang II-stimulated collagen production is mediated through ROS generation [129]. When the mitochondrial bioenergetics and ROS production is pharmacologically restored, up-regulation of MMP9 is completely prevented, while maintaining TIMP1 gene expression, suggesting a reduction of MMP activity and less pronounced ventricular remodeling in this condition [130].

Injured mitochondria possess inflammatory properties, including damage-associated molecular patterns (DAMPs), which might produce cardiomyocyte injury [58]. Cellular injury releases mitochondrial DAMPs into the circulation eliciting a sepsis-like neutrophil mediated organ injury [131]. Furthermore, mitochondrial DAMPs activate the inflammasome, a group of intracellular multiprotein complexes that activates caspase-1 and proinflammatory cytokines [132]. Alternatively, mtDNA that escapes from autophagy leads to inflammatory responses in cardiomyocytes, inducing myocarditis and dilated cardiomyopathy [43]. Hypertension-induced inflammatory mediators may also compromise mitochondrial integrity and function. Progressive increase in interleukin (IL)-1 levels in diabetic rats is associated with decreased activity of the cardiac mitochondrial aldehyde dehydrogenase-2 [76]. Similarly, TNF-α treatment in vitro magnifies morphological changes in mitochondria and decreases membrane potential and ATP production in adipocytes [133], implicating mitochondria as targets of systemic inflammation that can lead to immunologic and fibrotic response. Taken together, these observations implicate mitochondria in the genesis of chronic inflammation in failing hearts.

Mitochondrial dysfunction also alters intracellular calcium homeostasis, which in smooth muscle cells regulates peripheral vascular resistance, influencing the risk of hypertension. Mitochondrial calcium additionally promotes biogenesis by upregulating PGC-1α expression [134]. In addition, increased myocardial expression of mitophagy markers is associated with changes in calcium cycling proteins,

contributing to LV interstitial fibrosis and diastolic dysfunction [135]. Hence, mitochondrial abnormalities might also provoke myocardial fibrotic remodeling and dysfunction through alterations in intracellular calcium signaling.

Mitochondria During Hypertension

Myocardial mitochondria are vulnerable to the effects of hypertension, which most commonly impairs mitochondrial structure, bioenergetics, or homeostasis. Although antihypertensive drugs have the capacity to attenuate mitochondrial injury secondary to hypertension, drugs that specifically target the mitochondria may prove more efficacious in ameliorating hypertensive mitochondrial dysfunction or end-organ damage. Structural mitochondrial alterations secondary to hypertension may include decreased mass and density, swelling, as well as cristae remodeling, fragmentation, or loss. Decreased mitochondrial mass and density have ramifications for mitochondrial function, as fewer and smaller-sized mitochondria compromise their oxidative capacity and energy production. Hypertension has been prominently associated with damage and loss of cardiolipin, a phospholipid uniquely found in the IMM and necessary for proper cristae formation [136]. Cardiolipin regulates mitochondrial dynamics and prevents the formation and opening of the MPTP, and release of cytochrome c from into the cytosol where it triggers apoptosis [64].

Several studies provide evidence of hypertension-induced mitochondrial structural and functional abnormalities, including alterations of biogenesis and dynamics, RAAS-induced mitochondrial damage (ATP synthase, and creatine kinase activity are reduced in hypertensive heart mitochondria, while cytochrome c release and caspase-3 expression are upregulated, implying stimulated apoptosis), ROS overproduction, apoptosis, and mtDNA mutations. For example, despite similar volume, cardiac mitochondria from hypertensive rats are disorganized and show reduced number of cristae [74]. Cardiac mitochondria are normally in continuous interaction with the sarcoplasmic reticulum and sarcomeric structures forming ‘intracellular energetic units’ to allow optimal energy transfer. Thus, alterations in mitochondrial arrangement might impair muscle power and contractile function [137]. Mitochondrial structural changes are also accompanied by reduced mitochondrial respiration, triggered by decreased complex-I activity. The notion that mitochondrial damage compromises cardiomyocyte contractility suggests a possible role of mitochondria in the development of hypertension induced cardiac dysfunction. Conversely, ECM expansion and fibrosis secondary to hypertension may directly contribute to structural mitochondrial abnormalities and contractile dysfunction [75]. Therefore, despite the link between damaged mitochondria and hypertensive heart disease, a cause effect relationship remains to be established.

In hypertensive rats, mRNA levels of the fusion proteins MFN1 and MFN2, and OPA1 were decreased, suggesting a shift towards increased mitochondrial fragmentation. Furthermore, hypertensive rats exhibited reduced enzyme activities and

expression of ETC subunits, indicating that mitochondrial function and remodeling are compromised in hypertension-induced cardiac hypertrophy. However, whether mitochondrial alterations are the primary cause or downstream consequence of hypertensive myocardial injury remains obscure.

Mitochondria-targeted antioxidants are novel cell-permeable compounds that may attenuate hypertensive injury by protecting mitochondria from oxidative damage. MitoQ is a ubiquinone derivative that prevents lipid peroxidation and mitochondrial damage in several experimental models. MitoQ also blunts hypertension, improves endothelial function, and reduces cardiac hypertrophy in spontaneously hypertensive rats [69]. Furthermore, its combination with low-dose losartan provides additive therapeutic benefit, by attenuating hypertension and reducing LV hypertrophy in spontaneously hypertensive rats, and by a direct anti-hypertrophic effect on rat cardiomyocytes in vitro [71]. Hence, MitoQ provides both hemodynamic and mitochondria-specific beneficial effects.

Mitochondria During Cardiac I/R Injury

I/R injury occurs when the blood supply to a tissue is blocked for minutes to hours (ischemia) and then restored (reperfusion). This important pathological mechanism underlies a range of disorders, including heart attack and stroke, where the prevention of blood flow to the tissue, for example by a blood clot, is followed by reperfusion when the blockage is removed mechanically or pharmacologically [138].

The current consensus is that a period of ischemia primes the tissue for subsequent damage upon reperfusion. Ischemic cells will eventually die if blood flow is not restored, but it is during reperfusion itself that most I/R damage is initiated. Thus, paradoxically, the essential therapeutic intervention to treat ischemia drives tissue pathophysiology [139]. The first damaging event upon reperfusion is a burst of ROS production from mitochondria [136]. Mitochondrial ROS not only drive acute damage but also initiate the pathology that develops over the minutes, days, and weeks following reperfusion [140]. The initial burst of ROS production upon reperfusion directly causes oxidative damage to mitochondria, thereby disrupting ATP production [141]. The mitochondria are recognized as a key player in cardiomyocyte death after myocardial infarction and cardiomyopathies. The events occurred during cardiac I/R significantly alter mitochondrial structure and function: reduce membrane potential, impair oxidative phosphorylation and decrease high energy phosphate synthesis, dysregulate calcium homeostasis, increase ROS production, matrix swelling and membrane permeability, and release of cytochrome c and other apoptotic factors leading to cell death. These events are initiated during ischemia and extend throughout reperfusion to severely compromise cardiac post-ischemic functional recovery and cell viability [142, 143].

During reperfusion, the reoxygenation of ischemic tissue results in mitochondrial calcium overload and renormalization of intracellular and matrix pH which are

accompanied by the prodigious generation of ROS that synergistically induce the opening of the MPTP [144–146]. Together, this mitochondrial disruptions are the principal mechanism of apoptotic/necrotic cardiomyocyte death accounting for the majority of I/R injury [136, 139, 147, 148].

ROS-mediated mitochondrial damage also releases DAMPs, such as mtDNA, which can initiate the sterile inflammatory response [149, 150]. This activation of the innate immune system causes inflammation that contributes to I/R injury and can continue for days after the initial damage [149]. Therefore, during the weeks and months following the initial I/R injury, fibrotic scar tissue will form replacing dead cells along with extensive tissue remodeling [115]. The end result is often an organ that is structurally altered and functions poorly, frequently leading to persistent long-term pathology such as chronic heart disease [117].

Improvement in the clinical management of ischemic heart disease remains elusive despite the discovery of many molecular and cellular mechanisms that may be valuable targets against I/R injury. The importance of mitochondrial bioenergetics and function in contributing not only to cardiac injury but also to reducing cardiac injury is now well recognized. There are several cardioprotective strategies or treatments against I/R injury directed to mitochondria. For instance, cyclosporine administered at the time of reperfusion seems to have a sustained beneficial effect on infarct size reduction, which might improve the post-infarction remodeling [151]. Pharmacological modulation with the mitochondria-targeting peptide bendavia leads to sustainment of $\Delta\Psi m$ in cardiomyocytes during I/R, suggesting that bendavia might act as a direct MPTP blocker [152]. Moreover, mitochondria can be delivered to the ischemic heart through the vasculature and transplantation of these organelles affords an important functional benefit [153].

Conclusions

- Cardiac remodeling not only occurs at the organ or tissue levels, but can be also evidenced at the level of organelles like mitochondria.
- Mitochondrial dynamics remodeling has shown to be a cause or consequence of the development of several cardiac pathologies.
- Increased ROS production derived from mitochondrial metabolism has been associated with the development of cardiac pathologies.
- Mitophagy is stimulated in the heart during the chronic phase of cardiac remodeling and would be protective for the heart.
- The mitochondria is a therapeutic target in cardiac remodeling that leads to heart failure, hypertension and I/R.

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Chapter 16
Functional Role of Nox4 in Autophagy

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NADPH oxidase (Nox) are transmembrane proteins dedicated to the production of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [1, 2]. Importantly, Nox are the only enzymes that generate ROS as their primary function, and not as a byproduct like other enzymes [1, 3]. To date, seven isoforms belonging to NADPH oxidase family have been identified and characterized: Nox1, Nox2 (also known as gp91^{phox}), Nox3, Nox4, Nox5, Dual oxidase (Duox) 1 and Duox2 [1, 2, 4]. A large body of evidence suggests the involvement of Nox in a plethora of cellular processes, such as proliferation, migration, apoptosis, as well as in inflammatory responses and in cellular senescence [1, 2, 5]. Several stimuli have been shown to activate Nox, such as cytokines, growth factors, high glucose, angiotensin II (Ang II), as well as different types of stress [6–10].

It is well known that high levels of ROS cause oxidative stress, which in turn contribute to the oxidation of proteins, nucleic acids and lipids, culminating in cellular damage and death [11–15]. For example, in the cardiovascular system,

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ROS promote endothelial dysfunction, vascular inflammation, proliferation and migration of vascular smooth muscle cells (VSMCs). These factors ultimately lead to vascular remodeling and dysfunction, a common feature in several cardiovascular diseases (CVDs) [16–18]. These processes are in part mediated by the reduction of nitric oxide (NO). O_2^- chemically reacts and oxidizes NO, producing peroxynitrite ($ONOO^-$). Peroxynitrite not only enhances pre-existing oxidative stress, but it is also responsible for endothelial nitric oxide synthase (eNOS) uncoupling through the reduction of tetrahydro-L-biopterin (BH4), an essential cofactor for eNOS catalysis [18, 19]. When eNOS becomes uncoupled, it produces mainly $ONOO^-$ and O_2^- rather than NO, creating a vicious cycle that exacerbates oxidative stress [18, 20, 21].

In contrast, when levels of ROS are low or compartmentalized (a condition termed "redox state"), they act as signaling molecules in different pathways, thereby mediating physiological responses through the activation or inhibition of a cascade of downstream molecules involved in several cellular functions, such as proliferation, differentiation and cytoskeleton rearrangement [22–24]. In this regard, ROS act as second messengers downstream of specific ligands and may enhance the activity of transcription factors, or may be involved in post-transcriptional modifications of proteins [22]. Moreover, insufficient or absent generation of ROS may have detrimental consequences since phagocytic ROS are involved in host defense. This is illustrated in patients suffering from X-linked chronic granulomatous disease (CGD); these patients have an increased susceptibility to infections due to their inability to eliminate invading pathogens [25–27].

Autophagy is one of the functions modulated by ROS during cellular stress [28–30]. Autophagy is an evolutionarily-conserved, intracellular degradation process found in all mammalian cells, necessary for the elimination of damaged proteins or long-lived organelles [31, 32]. Autophagy represents a survival mechanism since it reduces the accumulation of misfolded proteins or damaged organelles during cellular stress, and recycles the indispensable substrates necessary for fundamental cellular processes such as ATP and protein synthesis [33, 34]. Studies suggest that ROS activate autophagy whereas reactive nitrogen species (RNS) may inhibit autophagy and autophagic flux [28–30, 35, 36]. The mechanisms through which ROS activate autophagy are not completely understood and remain to be elucidated. For instance, ROS can induce autophagy by directly oxidizing components of the autophagic machinery. However, cells also activate autophagy after increased generation of ROS as a compensatory and survival mechanism to reduce cellular damage and death that excessive oxidative stress may otherwise promote [28]. Recent evidence suggests that Nox may serve as a gatekeeper in regulating the activation of autophagy based on cell type and cellular conditions [37–40]. Furthermore, studies show that ROS are responsible for the induction of autophagy through Nox.

In the next several paragraphs, we will discuss the importance of Nox in the modulation of autophagy, with particular emphasis on the Nox4 isoform in the cardiovascular system. Studies providing evidence of the involvement of Nox4 in other cells will also be highlighted. A description of NADPH oxidase and autophagy biology will be also be briefly described.

NADPH Biology

The first NADPH oxidase was described in phagocytic cells and to date seven different isoforms of NADPH have been identified: Nox1, Nox2 (or gp91^{phox}), Nox3, Nox4, Nox5, Duox 1 and Duox2. All Nox isoforms transfer electrons to molecular oxygen (O_2) to generate O_2^-, using NADPH as an electron donor. Subsequently, O_2^- can be rapidly dismutated into hydrogen peroxide (H_2O_2) [1, 41]. Structurally, Nox isoforms are characterized by at least six transmembrane domains containing a NADPH binding domain in their cytosolic c- terminus. This binding domain represents the catalytic core of Nox. In order to exert its function, Nox isoforms are associated with another membrane bound subunit, called p22^{phox} for Nox1-4 and DuoxA1 and DuoxA2, for Duox1 and Duox2, respectively [4, 42]. p22^{phox} is necessary for the stabilization and the activation of Nox enzymes. Nox5 and Duox present an aminoterminal calcium-binding domain on the cytosolic side of the membrane, whereas Duox alone additionally presents a peroxidase domain on the opposite side of the membrane. Cytosolic subunit partners are also required for Nox activation: p47^{phox}, p67^{phox} and p40^{phox} for Nox2, and NOXA1 for Nox1 and Nox3. In response to various stimuli, these subunits associate with the catalytic subunit then translocate onto the membrane, thereby activating Nox [43, 44]. In contrast, Nox4 appears to be mainly transcriptionally-regulated, although it contains a polymerase δ-interacting protein 2 (POLDIP2) domain that acts as a positive regulator of Nox4 mediating its association with p22^{phox}, as reported in VSMCs [45].

Nox2 activation has been widely studied. Specifically, p47^{phox} (called the “organizer subunit”) is initially activated by phosphorylation by protein kinase C (PKC). Once activated, it translocates onto the membrane and physically interacts with p22^{phox} along with p67^{phox} and p40^{phox}. For its final activation, Nox2 requires the involvement of the small cytosolic GTPase Ras-related C3 botulinum toxin substrate (Rac). Different isoforms of Rac have been described, such as Rac2, expressed predominantly in phagocytic cells, and Rac1, found in non-phagocytic cells [46]. Rac has also been shown to be associated with Nox1 and Nox3 [47]. Interestingly, Nox1-3 and Nox5 release O_2^- while Nox4 generates both O_2^- and H_2O_2 [48]. Duoxs generate mainly H_2O_2 [49].

Among the Nox isoforms, Nox2 and Nox4 share 35 % of sequence similarity and also share a similar hydrophobicity profile [1, 2, 50]. However, while Nox2 is mainly expressed on the plasma membrane, Nox4 displays different subcellular localizations [1, 45, 51].

Both Nox2 and Nox4 are widely distributed in the cardiovascular system, such as in endothelial cells, cadiomyocytes, fibroblasts and VSMCs. Nox2 and Nox4 have been demonstrated to play a prominent role in vascular physiology during different stress conditions [6, 52–56]. In this regard, recent evidence suggests the involvement of Nox2 in hypertension, atherosclerosis, as well as cardiac hypertrophy, fibrosis and remodeling. Conversely, the systemic deletion of Nox2, or the inhibition of Nox2 by means of selective inhibitors has been reported to improve vascular physiology, for example, reducing blood pressure and improving endothelial and cardiac function [57–61].

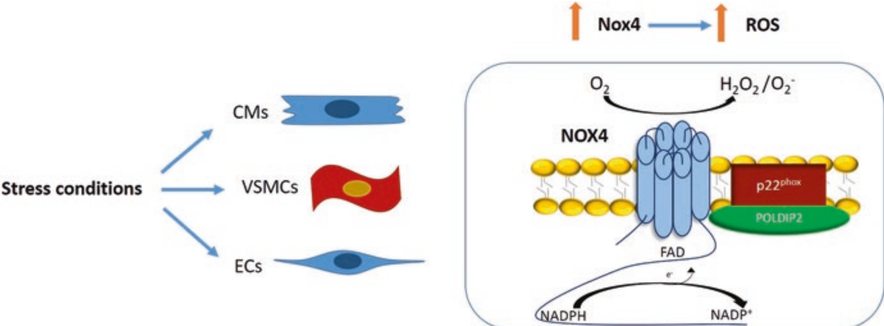

Fig. 16.1 Schematic representation of Nox4 and its associated membrane bound subunits (p22phox and POLDIP2). Nox4 displays different subcellular localizations and it is constitutively expressed in cardiomyocytes (*CMs*), vascular smooth muscle cells (*VSMCs*) and endothelial cells (*ECs*). Different stressors are able to enhance Nox4 expression and activity, leading to increased production of ROS

Nox4

Nox4 (Fig. 16.1), first identified as a renal-specific Nox, has also been found to be abundantly expressed in the cardiovascular system [2]. In particular, Nox4 represents the most expressed NADPH oxidase in endothelial and VSMCs [1, 62]. Depending on the cell type and the cell content, Nox4 is variably expressed in different organelles such as mitochondria, the nucleus, and the endoplasmic reticulum (ER). The distinct localizations of Nox4 are due to differences in the signal sequences of its primary structure [63–65]. Alternative splicing, either in the coding sequence of Nox4 or in its mRNA untranslated regions (UTRs), may contribute to the differences in intracellular localization of Nox4. Additionally, post-translational modifications in the forming protein (for example myristoylation and glycosylation) may also change localization [66–68]. In general, Nox4 activity is thought be transcriptionally-regulated: however, recent evidence suggests that POLDIP2 may be also considered a specific regulator of Nox4 [45].

Several stimuli have been demonstrated to activate both Nox2 and Nox4 in the cardiovascular system: G-protein coupled receptor agonists, such as Ang-II and thrombin, growth factors (vascular endothelial growth factor – VEGF; platelet-derived growth factor, PDGF), cytokines (tumor necrosis factor – TNFα), different cellular conditions such as high levels of glucose, glycated products and oxidized low density lipoproteins (LDL), or hypoxia-regeneration and nutrient starvation [7, 69]. TGF-β and E2F have been reported to enhance the expression of Nox4 in cadiomyocytes and in vascular cells under unstressed conditions [70–72], whereas NF-kB enhances expression of Nox4 in vascular cells under conditions of hypoxia [73, 74]. In contrast, thrombin, PDGF and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands were found to reduce Nox4 expression in VSMCs and in endothelial cells [53, 75, 76].

Similar to Nox2, Nox4 also is activated during stress conditions in the vascular system, and it is believed to be the major source of ROS in cadiomyocytes [77]. Nox4 displays dichotomous effects in cardiac and vascular physiology, being either detrimental or protective.

It has been shown that Nox4 inhibition prevents cardiac hypertrophy under pressure overload [77]. While Nox4 overexpression contributes to diabetes and vascular abnormalities [78–80]. Systemic Nox4 gene deletion increases brain injury in response to both transient and permanent cerebral ischemia [81]. Moreover, aged mice with cardiac specific overexpression of Nox4 showed enhanced cardiac hypertrophy and increased production of O_2^- [63]. Moreover, Nox4 was found to promote neointimal hyperplasia in VSMCs [82, 83]. On the other hand, systemic knockout mice for Nox4 were shown to develop cardiac hypertrophy and dilatation during chronic pressure overload whereas mice with overexpression of Nox4 in cardiomyocytes were protected [70]. Previous work showed that Nox4 deletion increases myocardial ischemic injury and induces cardiac dysfunction in response to starvation [40, 84]. Similarly, overexpression of a dominant-negative form of Nox4 exacerbates ischemic injury in ex vivo experiments [85]. A combined deletion of Nox2 and Nox4 exacerbates reperfusion injury, although a single deletion of these genes was found to be beneficial [86]. In addition, downregulation of Nox4 in the aortas of Ang-II hypertensive mice models was found to increase inflammation and endothelial dysfunction compared to wild type [87]. Similarly, pulmonary arterial hypertension and endothelial dysfunction were reported in knockout Nox4 mice. Nox4 has also been shown to prevent atherosclerosis progression in Nox4 and ApoE double knockout animals [88, 89].

The downstream pathways that mediate the protective functions exerted by Nox4 have been investigated. For example, in cardiomyocytes and in VSMCs, Nox4 activates the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathways, regulating the anti-oxidant responses to stress [87, 90]. In this regard, in endothelial cells under ischemic and inflammatory stressors, loss of Nox4 has been reported to impair heme oxygenase-1 (HO-1) expression, a protein strictly associated with inflammation and apoptosis [87]. Moreover, Nox4 was demonstrated to activate eNOS in endothelial cells [91] and to promote vascular cell survival through the activation of the RAS/extracellular signal-regulated kinases (ERK) pathway in response to accumulation of misfolded proteins or oxidized lipoproteins [39]. In addition, Nox4 was seen to promote cardiomyocyte survival in response to stress by inhibiting PP1, thereby promoting the activation of eIF2α signaling [84]. Nox4 induces vascular cell proliferation and migration in response to hypoxia through the stabilization of hypoxia inducible factor (HIF)-α levels [73, 92, 93]. Nox4 was found to promote angiogenesis during pressure overload and autophagy in the heart [40, 70]. Previous work also showed that the beneficial role of Nox4 in promoting angiogenesis in the heart under chronic stress involves increased expression of HIF-α and vascular endothelial growth factor (VEGF) [70].

This dual role of Nox4 is likely to be dependent on the stress condition, on the level and duration of its activation and on the subcellular compartment in which it produces ROS. The pathophysiological function of Nox4 during cardiac stress may

also be determined by the cell-type in which Nox4 is activated. For example, global deletion versus cellular-specific deletion of Nox4 leads to different outcomes in response to pressure overload [70, 77]. This is probably dependent on the fact that Nox4-dependent angiogenesis is critical in response to mechanical stress, but it is not affected in the cardiomyocyte-specific knockout model.

Autophagy Biology

Autophagy (from the Greek, "auto" self, "phagein" to eat) is a cellular process in which cytoplasmic elements, such as proteins and organelles, are delivered to and digested in lysosomes/vacuoles (Fig. 16.2). The resulting molecular components – amino acids, lipids, nucleic acids, and carbohydrates – are recycled and reintroduced for cellular metabolism [31, 32]. In this way, autophagy ensures the homeostasis of cells, especially during stress and nutrient starvation. It is an

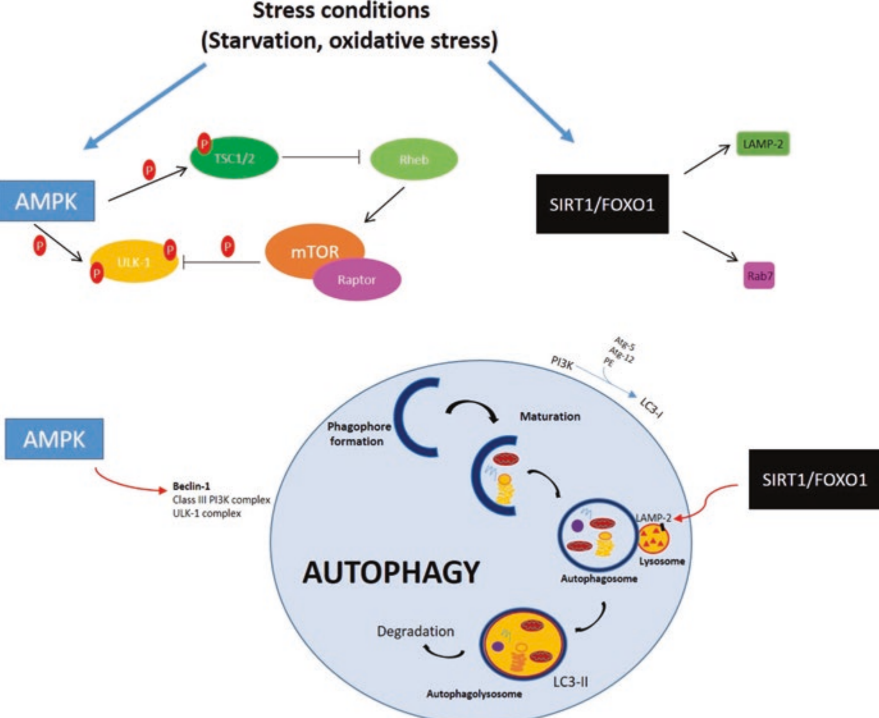

Fig. 16.2 Schematic representation of autophagy. AMPK/Rheb/mammalian target of rapamycin (*mTOR*) and SIRT1/FOXO1 are major signaling regulators of autophagy (*upper*). Several molecules are involved in the autophagic flux, from phagophore to autophagolysosome formation (see text for details)

evolutionarily conserved mechanism, first discovered in yeast and now found to occur in all eukaryotic cells [33].

Three primary forms of autophagy have been characterized: microautophagy, chaperone- mediated autophagy and macroautophagy. Chaperone- mediated autophagy involves the degradation of cytoplasmic proteins with a specific amino acid motif (KFERQ). That amino acid motif is recognized by chaperones which facilitate delivery of the protein into lysosomes through an interaction with the lysosomal membrane protein LAMP2A [94]. In microautophagy the cargoes are internalized directly through the lysosomal membrane [95]. Finally, in macroautophagy cellular components are internalized by double-membrane vesicles called autophagosomes that then deliver them to lysosomes. Functionally, both microautophagy and macroautophagy (hereafter the terms macroautophagy and autophagy will be used synonymously) can be non-selective or selective; non-selective autophagy is a process in which cargoes are not specific and it is required for the turnover of bulk cytoplasm [96]. In contrast, selective autophagy is finely regulated by several mechanisms and refers to the delivery and the digestion of specific cargoes such as mitochondria (mitophagy) and ribosomes (ribophagy). In the past decade, the complex signaling network that regulated the different stages of autophagy has been extensively studied and characterized. However, new molecules involved in autophagy are continuously identified.

The different stages of autophagy can be divided and summarized as it follows: (a) phagophore formation and sequestration of damaged cargoes, (b) autophagosome maturation and (c) autolysosome formation. In the initial step of autophagy, an isolation double-membrane, derived from ER or mitochondria, is formed. Next, it elongates (paghophore), surrounds, and invaginates the cargo, ultimately closing to generate an autophagosome. The recently formed autophagosome then transports the cargo and fuses with a lysosome, generating the autolysosome [97, 98]. Autophagy is a mechanism that is highly dynamic and finely regulated, under a state of constant intracellular flux termed "autophagic flux" [99, 100].

AMPK/Rheb/mammalian target of rapamycin (mTOR), together with SIRT1/FOXO1, represent important pathways involved in the promotion of stress-induced autophagy [33, 101]. Specifically, AMPK induces autophagy through the direct phosphorylation of Unc-51 like kinase (ULK) 1. Additionally, the inhibition of mTOR through the phosphorylation of tuberous sclerosis factor (TSC)-2 or Raptor and Rheb inhibition also induces autophagy. The inhibition of autophagy by mTOR occurs via the phosphorylation of Ulk-1, Ulk-2, Atg13 and focal adhesion kinase family interacting protein of 200 kD (FIP200). Ulk phosphorylates and activates Beclin-1 which is part of a complex containing the autophagy-specific class III phosphatidylinositol 3-kinase (PI3K) which is needed for phagophore elongation. PI3K recruits two additional ubiquitin-like complexes, Atg5-Atg12, as well as phosphatidylethanolamine (PE)-LC3-I for the formation of the autophagosome. PE-LC3-I forms LC3-II, which represents the only autophagy protein associated with the complete autophagosome. LC3-II is then cleaved from PE by Atg4 and returns to the cytoplasm. LAMP-2 and the small GTPase Rab7 regulate autophagolysosome formation, and they are activated by the SIRT1/FOXO1 pathway and by the transcription factor EB [33, 34, 101, 102].

Several findings suggest a crucial role of autophagy in the cardiovascular system. For example, mice with cardiac specific deletion of Atg5 were shown to develop contractile dysfunction and left ventricular dilatation, together with an increased number of dysfunctional mitochondria [103]. Moderate activation of autophagy has also been shown to be protective during pressure overload and chronic myocardial infarction [103, 104]. Interestingly, in aged mice, cardiac autophagy was shown to be reduced, which corresponded to an increased propensity to develop cardiac abnormalities [33, 34]. Moreover, the inhibition of autophagy through cardiac specific downregulation of AMPK or through Rheb overexpression has been found to significantly increase ischemic injury [31, 102, 105]. Mitochondrial autophagy has been reported to protect the heart from ischemia-reperfusion injury in a pathway mediated by the GTPase dynamin-related protein 1 (Drp1) [106]. In contrast, several studies also report that exaggerated activation of autophagy may be the cause of myocardial injury and cardiac remodeling [31, 107, 108].

Overall, the findings may suggest that autophagy at physiologic levels may be considered an adaptive and stress-protective mechanism for cardiovascular pathophysiology; conversely, high levels of autophagy may be the cause of several pathological conditions in response to specific types of stress, likely by inducing autosis [109].

ROS and Autophagy

The role of ROS and radical nitrogen species (RNS) as key signaling molecules is now well recognized. Owing to their ability to oxidize target molecules, ROS and RNS can modulate molecular pathways, for example, by switching on and off proteins by means of post-translational modifications [22–24].

Compelling evidence showed that oxidative stress modulates autophagy under cellular stress conditions [28–30].

The mechanisms through which ROS activate autophagy are still under investigation. Some data suggest that in most cases autophagy is activated as a compensatory and death-limiting mechanism under elevated level of ROS. Other findings suggest instead that ROS activate the SIRT1/FOXO1 pathway [110] or induce autophagy through the upregulation of Beclin1 and Atg5 [111, 112]. Other signaling pathways are involved in the activation of autophagy by ROS; for example, in cardiomyocytes during ischemia-reperfusion, ROS enhance BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip-3) expression that mediates Drp-1 translocation to the mitochondria, upregulating autophagy [113, 114]. Moreover, in the heart it has been demonstrated that ROS induce autophagy via NF-kB signaling pathways [115], as well as through the translocation of Nrf2 in the nucleus [116]. Importantly, activation of autophagy in the heart via Nrf2 prevents cardiac dysfunction and necrosis under pressure overload and proteotoxic stress [117]. Nrf2 was also reported to enhance the expression of NDP52, an adaptor protein of autophagic machinery. In addition, it has also been demonstrated that ROS are able

to stimulate autophagy by the direct oxidation of the proteins involved in autophagy. In this regard, in ovarian cells H_2O_2 was reported to inactivate Atg4 by oxidation of cysteine residue 81 at its C-terminal portion [118]. The inactivation of Atg4, as mentioned above, prevents the dissociation of LC3-II from the autophagosome membrane.

NADPH Oxidase and Autophagy

Several studies reported that Nox derived-ROS are able to regulate autophagy under different cellular conditions, both in the cardiovascular system, and in other tissues.

For example, in human macrophages and dendritic cells, Nox2 was found to stimulate autophagy, leading to pathogen recognition and phagocytosis regulation. In particular, Nox2 derived ROS were able to increase levels of Atg8/LC3-II bound to phagosomes in a pathway mediated by the Toll-like receptor (TLR). Interestingly, no other proteins of autophagic machinery were found to be associated with phagosomes. Moreover, the Atg8/LC3 coated phagosomes display prolonged antigen presentation by MHC class II molecules, retaining the antigen for a longer period of time [119]. The same findings were also reported in murine cells [38].

Cancer cells also modulate autophagy via Nox activation. In HeLa cells under starvation, autophagy was found to be enhanced concomitantly with Nox activity and ROS generation, which in turn contribute to the activation of the JAK2-STAT3 pathway. JAK-STAT is a well characterized cytokine signaling pathway in which JAK tyrosine kinase phosphorylates STAT, which in turn forms dimers and moves to the cell nucleus leading to transcription activation. Precisely, in HeLa cells under starvation, STAT3 was found to be activated by tyrosine phosphorylation and to localize in cell nuclei, leading to an increased LC3-II formation. Once activated by Nox derived-ROS, STAT3 enhances the expression of interleukin (IL)-6. IL-6 expression contributes to cell survival and inhibition of cell death [120]. However, in this study, authors did not identify the specific Nox isoform responsible for autophagy activation, since in the experiments nonspecific inhibitors of Nox were used. Additionally, the deletion of $p22^{phox}$, the membrane bound subunit, is common in more than one Nox isoform.

Nox2 activation was also found to inhibit autophagy. Abel's group found that high fat diet-induced Nox2 activation inhibits autophagic flux in cardiomyoytes [121]. In skeletal muscles of a mouse model of Duchenne muscular dystrophy (DMD), Nox2 activation was found to inhibit autophagy, contributing to the pathogenesis of DMD. In particular, Nox2-derived ROS activate Src kinase. Src kinase in turn enhances mTOR activity via the PI3K/Akt pathway leading to inhibition of autophagosome formation and impairment of lysosome biogenesis. Additionally, inhibition of Nox2 upregulates autophagy and improves the pathophysiological phenotype of DMD in skeletal muscles with corresponding decrease in serum creatine kinase levels [122]. Authors of this study suggest that the impairment of

autophagy via Nox2/Src is based on the particular location of the caveolae within the skeletal muscle sarcolemma [123, 124]. Inhibition of autophagy via Nox2 has been also reported in neuronal cells treated with rotenone, a toxic environmental agent that causes a sporadic form of Parkinson's disease due to its oxidative properties [125]. Interestingly, when cells were treated with rotenone for a short time, autophagic flux was inhibited through the Nox2/Src/PI3K/Akt pathway, independently of the mTOR pathway, thereby leading to a decreased Beclin1-Phosphatidylinositol 3-kinase catalytic subunit type 3 (VPS34) complex formation. Furthermore, exposure time with rotenone was found to decrease lysosomal function and to promote protein accumulation, ultimately promoting cell death [126]. The effect of rotenone with regard to autophagy modulation via Nox2 was also demonstrated in lung cancer cells. Acute exposure to rotenone impairs the autophagic flux via an mTOR-dependent manner whereas chronic exposure increases autophagy [127]. Recently, in a mouse model of epilepsy induced by pentylenetetrazole (PTZ), Nox-derived ROS were found to modulate autophagy in the hippocampal regions. It was further shown that inhibition of autophagy increased the deleterious effects of PTZ [128].

In endothelial cells, Nox2 has also been shown to induce autophagy, exerting both protective and maladaptive functions. For example, in pulmonary artery endothelial cells with persistent pulmonary hypertension, autophagy stimulation via Nox2 derived- ROS impairs angiogenesis and enhances apoptosis. Inhibition of autophagy has been found to lead to a decreased association between Nox2 and $p47^{phox}$ resulting in the improvement of angiogenesis (see Chap. 21). These findings suggest a crosstalk between Nox2 and autophagy in hypertensive pulmonary endothelial cells [129]. In contrast, autophagy induced by Nox2 was found to improve endothelial function in transgenic mice with endothelial overexpression of Nox2. Specifically, the increase in autophagy via Nox2-derived ROS enhances coronary vaso-relaxation and promotes endothelial cell survival in a pathway mediated by CaMKKβ kinase-AMPK. AMPK enhances autophagy through inhibition of mTOR while also increasing eNOS activity and NO bioavailability [37].

Nox4 and Autophagy

As shown for Nox2, Nox4 has also been demonstrated to exert a functional role in autophagy in different cell types. All these studies suggested that Nox4 induces autophagy. Nox4-induced autophagy was reported to enhance cell resistance to chemotherapy. Specifically, epidermal growth factor receptor (EGFR) is upregulated in many types of cancers [130] and the therapeutic strategies for cancer are often to target EGFR with specific EGFR inhibitors. However, cancer cells frequently develop resistance to cytotoxic effects exerted by EGFR inhibitors. For example, in head and neck squamous cancer cells, erlotinib, an EGFR inhibitor, was shown to promote cell survival through activation of autophagy via Nox4-derived H_2O_2. Interestingly, erlotinib was also reported to increase the expression of Nox4 by

enhancing its promoter activity and mRNA stability. This suggests that autophagy is often used by cancer cells as a survival mechanism under stress conditions, causing some therapeutic strategies to fail to promote cell death [131]. Conversely, in breast cancer cells (MCF-7), psoralidin, a natural polyphenol coumarin exerting anti-cancer effects, was found to arrest cell proliferation and to induce DNA damage through the activation of Nox4-dependent ROS production. Interestingly, Nox4 was also found to induce autophagy as a compensatory mechanism [132].

Nox4 also promotes autophagy in the cardiovascular system. In human umbilical vein endothelial cells (HUVEC) the signaling pathway through which Nox4 activates autophagy has been reported. In response to misfolded protein accumulation, Nox4-derived H_2O_2 in the ER activates local RAS. RAS phosphorylates ERK promoting autophagy in an mTOR-independent pathway, which in turn contributes to increased endothelial cell survival [133]. 7-ketocholesterol (7-KC), an oxidized lipoprotein, was found to enhance autophagy in aortic smooth muscle cells, promoting cell survival. Specifically, H_2O_2 produced by Nox4 increased the autophagic flux via inhibition of Atg4. Interestingly, in the same study, the inhibition of autophagy was shown to increase ER stress and to enhance cell death [39]. In a rat model of sepsis, Nox4 was found to activate autophagy in response to high doses of lipopolysaccharides (LPS). The natural anti- inflammatory molecule cinnamaldehyde (CA) was found to attenuate Nox4-derived ROS, autophagy and alleviate LPS-induced cardiac dysfunction [134].

Nox4 also appears to elicit a critical pro-autophagic function in response to cardiomyocyte energy deprivation (Fig. 16.3). We previously demonstrated that protein levels of Nox4 are upregulated in the ER in glucose-deprived cardiomyocytes and this effect was paralleled by an increased production of ROS in this organelle. Nox4 appeared to be required for glucose deprivation-induced autophagy through the protein kinase RNA-activated-like ER kinase (PERK)/eukaryotic initiation factor 2α (eIF-2α)/activating transcription factor 4 (ATF4) pathway, a pathway modulating autophagy also in cancer cells [135]. Importantly, Nox4-induced autophagy promoted cardiomyocyte survival, since Nox4 knockdown increased cardiomyocyte cell death in response to glucose deprivation and this effect was rescued by autophagy reactivation. Nox4 also activated autophagy in vivo leading to cardioprotection in response to fasting and prolonged ischemia in the mouse heart [40]. This latter result was recently corroborated by a work from Shah's group, which demonstrated that Nox4 induces cardioprotective effects, also in response to I/R injury, through the activation of the eIF-2α/ATF4 signaling pathway [84].

A recent paper from Sadoshima's group also provided new insights into the mechanisms regulating Nox4 activity and autophagy in response to energy deprivation. FYN, a Src family tyrosine kinase, was shown to co-localize with Nox4 in the mitochondria, nucleus and ER and to phosphorylate it on the tyrosine 566 residue in the C-terminal NADPH binding domain. Phosphorylation on tyrosine 566 impaired the interaction between Nox4 and its membrane domain $p22^{phox}$, leading to enzyme inactivation. FYN appeared to regulate both maladaptive and physiological functions of Nox4. It was found to limit oxidative stress and cardiac remodeling in response to pressure overload by inhibiting Nox4. FYN knockout mice displayed increased

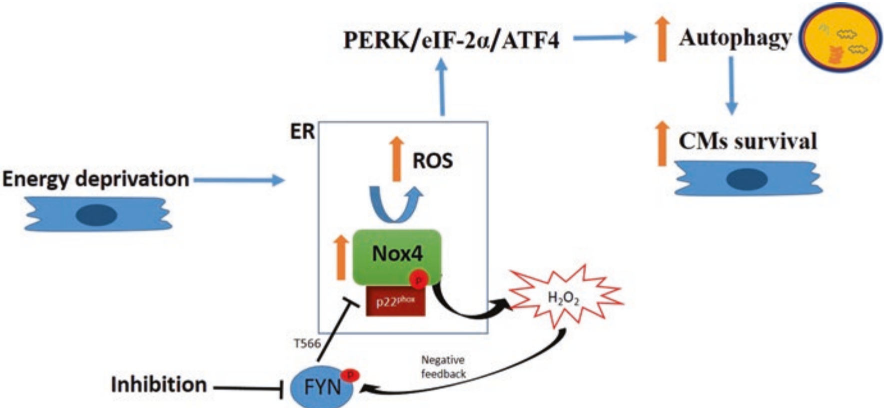

Fig. 16.3 Functional role of Nox4 in autophagy in cardiomyocytes (*CMs*) under energy deprivation. Nox4 is activated in the endoplasmic reticulum (*ER*) of CMs, where it enhances the production of ROS that leads to autophagy activation through the PERK/eIF2α/ATF4 pathway. Autophagy promotes CM survival under energy deprivation. Tyrosine kinase FYN inhibits Nox4 through the phosphorylation of Tyrosine 566 that impairs Nox4 association with its subunit partner $p22^{phox}$. Furthermore, Nox4 modulates FYN activity with a negative feedback mechanism. Inhibition of FYN contributes to CM survival under energy deprivation through autophagy activation (see text for details)

apoptosis in the heart during cardiac remodeling, which was attenuated by the concomitant deletion of Nox4. On the other hand, inhibition of FYN was shown to be beneficial in cardiomyocytes in response to glucose deprivation through the activation of Nox4. Precisely, in cardiomyocytes under glucose deprivation, overexpression of FYN led to decreased levels of autophagy and decreased cell viability whereas downregulation of FYN resulted in autophagy upregulation and cytoprotective effects in a Nox4-dependent manner. These results indicate that FYN activation may attenuate autophagy in cardiomyocytes under glucose deprivation, thereby inhibiting the adaptive response that Nox4 promotes in order to preserve cardiomyocyte survival during energy deprivation [136].

Conclusions

The evidence described in this chapter underlines the functional role of NADPH oxidase in the promotion of autophagy. We discussed experimental data in which Nox-derived ROS play a pivotal role as intracellular messengers, activating or inhibiting several downstream targets, such as autophagy.

Interestingly, the effects of Nox2 and Nox4 in autophagy regulation do not appear to be redundant. In fact, in specific cell types and stress conditions Nox2 was shown to inhibit autophagy, whereas Nox4 was consistently shown to activate the

autophagic process. Several reasons may underline this differential role of Nox2 and Nox4 in autophagy regulation. Nox2 produces O_2^- whereas Nox4 mainly produces H_2O_2. It is possible that in specific conditions and cell types O_2^- inhibits autophagy, whereas H_2O_2 mainly exerts pro-autophagic effects. The amount of ROS produced by the two Nox isoforms may also determine their ability to activate or inhibit autophagy. Finally, the different subcellular localization and signaling pathways that are modulated by the two isoforms could be another reason. Future studies are warranted to elucidate this issue. It will also be interesting to investigate in the future the role of Nox2 and Nox4 in the regulation of mitophagy and mitochondrial dynamics in response to stress, particularly in the cardiovascular system.

Autophagy and Nox4 also have a dual role in the regulation of cardiomyocyte survival, promoting either beneficial or deleterious effects. It is possible that the dichotomous effect of autophagy in the regulation of survival or death might in turn determine when Nox4 activation is beneficial and when it is detrimental, since Nox4 appears to activate autophagy in response to stress. Of course, also other mechanisms may explain the dual role of Nox4 in the regulation of cell survival and death. The recent discovery of FYN as a negative regulator of Nox4 in mediating autophagy may open new scenarios on Nox4 biology and ROS-mediated cell signaling and regulation of cell survival.

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Chapter 17
Mitochondrial Ubiquitin Ligase in Cardiovascular Disorders

Tao Yu, Yinfeng Zhang, and Pei-feng Li

The mitochondrion is a double-membrane organelle found in almost all eukaryotic organisms that retain its own small genome, and it is normally in a size between 0.75 and 3 μm in diameter. The word mitochondrion comes from the Greek *μίτος*, *mitos*, i.e. “thread”, and *χονδρίον*, *chondrion*, i.e. “granule” or “grain-like”. It is considered as the center of cellular energy production, which is commonly called “powerhouse” and supply adenosine triphosphate (ATP) to most of the cells [1]. Mitochondrion is a remarkably dynamic organelle, which undergoes constant migration and morphological changes during physiological as well as pathological processes. Particularly, mitochondrial fission and fusion machinery plays an essential role in the dynamics, division, distribution and morphology of the organelle, while these activities are independent of the cell cycle activity in most of the time [2–5]. Moreover, mitochondria are critically involved in regulating apoptosis, calcium homeostasis, lipid metabolism, aging, and the production of reactive oxygen species, which contribute to series of physiological abnormalities, especially heart diseases, including cardiac hypertrophy, heart failure, dilated cardiomyopathy and ischemia [6–9].

Ubiquitination is a post-translational modification in which a small conserved peptide, ubiquitin (Ub), is appended to target proteins in the cell through a series of complex enzymatic reactions [8]. Ub is a small protein with 76 amino acids, which prefers to join to an amino group on a substrate, either at the free amino terminus or on an internal lysine residue, and that results in protein modification [10]. Further, this tagging activity can influence the target protein’s biological half-life, cellular localization and the recognition with its binding partners [11]. Numerous studies demonstrated that this ubiquitin proteasome system (UPS) is an essential regulator

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of protein quality control in all cells, including cardiomyocytes. UPS participates in protein trafficking, protein degradation, cellular signal transduction, cell cycle progression, cell transformation and immune recognition [12].

Recently researches focus on the functions of ubiquitin ligase in mitochondrial system and its significant role and mechanism in regulating mitochondrial dynamics attracts more and more attention. The mitochondrial ubiquitin ligase (MITOL) is also known as MARCH5/RNF153, which is localized in the mitochondrial outer membrane reported to play a functional role in mitochondria and apoptosis. It can potentially modulate mitochondrial fission as well as mitochondrial morphology [13–15]. Mitochondrial ubiquitin ligase contains four transmembrane spanning segments for the binding of mitochondrial fission proteins, namely, human mitochondrial Fission 1 (hFis1), dynamin-related protein 1 (Drp1) and mitofusin 2 [16–18]. MITOL possesses a Plant Homeo-Domain (PHD) motif responsible for E3 ubiquitin ligase activity, which is rapidly degraded by auto-ubiquitination activity in a PHD-dependent manner. MITOL has been shown to control mitochondrial dynamics by regulating mitochondrial fission factors such as mitochondrial fission factor Drp1, microtubule-associated protein 1B and mitofusin2 [13, 16, 18]. However, the regulatory mechanism of MITOL in the mitochondrial dynamics remains unclear. In addition, there are other kinds of ubiquitin ligases, such as MULAN (also known as GIDE) and RNF185 also found in mitochondria.

Critical Role of Mitochondrial Ubiquitin Ligases in the Pathophysiology of Cardiovascular Diseases

As mitochondrial ubiquitin ligases maintain protein quality control and regulating many critical cellular processes, they play an important role in maintaining the health and diseases. A increasing number of evidence proved that mitochondrial ubiquitin ligases play fundamental role in the development of cardiovascular diseases.

Regulation of Mitochondrial Dynamics by MITOL/MARCH5 via Controlling the Balance Between Mitochondrial Fission and Fusion Proteins

Apoptosis plays a vital role in the cardiac and vascular structures during both the early morphogenesis and later developmental stages [19]. However, excessive apoptosis could trigger many cardiovascular diseases such as myocardial infarction, cardiomyopathy, cardiac hypertrophy and anthracycline-induced cardiotoxicity [20]. Cardiomyocytes are enriched with mitochondria, which constantly undergo fusion and fission to maintain organelle health. However, abnormal fusion or fission

participates in the regulation of cellular apoptosis [21, 22]. The mitochondrial ubiquitin ligase (MITOL), a E3 Ub ligase, can ubiquitinate a variety of mitochondria-associated proteins [13, 21]. The well-known substrates of MITOL are two mitochondrial fission factors (Fis1 and Drp1), which are essential components of the mitochondrial membrane fission machinery. Drp1 accumulation induces reactive oxygen species (ROS) production and mitochondrial dysfunction [13, 16]. The denatured and aggregated proteins accumulate in mitochondria and disrupt normal mitochondrial function, and that could be degraded by MITOL [23]. For example, overexpression of MITOL ubiquitinate mutant superoxide dismutase1 (mSOD1) and promotes its degradation. This can suppress both accumulation of mSOD1 and mSOD1-induced ROS generation in mitochondria, which indicates that MITOL protects against mitochondrial dysfunction caused by the accumulation of mSOD1 [24]. Wang and colleagues recently found that MITOL plays a protective role against apoptosis in cardiomyocytes by weakening mitochondrial fission through suppression of H2O2 induced accumulation of Drp1 in mitochondria [15]. However, there is a contrary report that MITOL is required for Drp1 dependent mitochondrial division, and the inhibition of MITOL caused mitochondrial fusion. A study by Nagashima and colleagues explained that MITOL-dependent Drp1 regulation could vary depends on the circumstances, such as cell cycle phase or nutritional status [16].

Mitochondrial fusion is mainly regulated by membrane bounded GTPases, mitofusin 1 (MFN1) and mitofusin 2 (MFN2), which mainly control outer membrane fusion [18, 24]. MFN2 is also localized in the endoplasmic reticulum (ER). However, MITOL only interacts with and ubiquitinates mitochondrial MFN2, but not ER associated MFN2 [25]. In addition, researchers found that the C-terminal domain of MITOL is specifically interact with the HR1 domain of MFN2, and MITOL regulates ER tethering to mitochondria by stimulating MFN2 via the ubiquitination of the GTPase domain K192. Thus, knockdown of MITOL can suppress MFN2 complex formation and triggers MFN2 mislocalization and ER membrane dysfunction [25]. Additionally, HDAC6, a cytosolic ubiquitin binding deacetylase, can irritate adaptive mitochondrial fusion under glucose starvation condition [26]. Recently, Kim and colleagues discovered that MITOL is responsible for hypoxia-induced MFN2 degradation in HDAC6 deficient cells, and genetic abolition of HDAC6 in amyotrophic lateral sclerosis model mice suppressed MFN2 degradation even MARCH5 induction is high, which illustrates that HDAC6 is a vital regulator of MFN2 degradation by MITOL [27, 28]. However, the relationship of this pathway and hypoxia induced cardiovascular diseases hasn't been evaluated, thus further studies need to be conducted.

Under mitochondrial stress conditions, the level of MFN1 is significantly increased and its depletion can induce cell death. Interestingly, overexpression of MFN1 also sensitizes cells to apoptotic death, which indicates that a fine tuning of MFN1 levels is necessary for cell survival [29]. Moreover, acetylated MFN1 is elevated under starvation induced mitochondrial stress conditions, thus the balancing of MFN1 levels is maintained by MITOL-mediated control on acetylation of MFN1, which is crucial for cell survival under mitochondria stress conditions [29].

MULAN Has an Important Role in Cell Growth, Cell Death, and Mitophagy

MULAN (for Mitochondrial Ubiquitin Ligase Activator of NF-κB) is another RING-finger E3 Ub ligase anchoring to mitochondrial outer membrane, which can also influence mitochondrial dynamics [27]. NF-κB plays a pivotal role in the regulation of the cell proliferation and survival in cardiovascular systems in addition to its typical role in promoting inflammation in response to pathogens [30]. Tacchi and colleagues cloned and analyzed the expression of MULAN in Atlantic salmon (*Salmo salar*) to confirm its role in the activation of NF-κB pathway [31]. The capability of MULAN to stimulate NF-κB depends on RING domain of MULAN and E3 ubiquitin ligase binding TRAF2. During stress-induced mitochondrial hyperfusion (SIMH), MULAN forms a complex with TRAF2 and modulates its ubiquitylation, signifying that TRAF2 may serve as an ubiquitylated transmitter of NF-κB signaling in this pathway [32, 33]. The endoplasmic reticulum (ER) is the major site of cellular homeostasis regulation, in which the accumulation of misfolded protein could cause stress [31]. Fujita and colleagues discovered that MULAN could activate NF-κB to protect cells from ER stress-induced apoptosis [34]. Furthermore, the serine/threonine kinase Akt (protein kinase B) is known to be involved in the cellular response to various stimuli. The activation of Akt controls multiple biological responses in cardiovascular systems, including protein synthesis, metabolism, cell proliferation and growth, cell-cycle progression, and the suppression of apoptosis [35, 36]. MULAN can degrade Akt and thus inhibits Akt dependent cell proliferation and viability [34].

Interestingly, MULAN is involved in numerous biological pathways including cell growth, cell death, and more recently in mitophagy. In addition, the interaction of Gamma-aminobutyric acid receptor-associated protein (GABARAP) with MULAN–Ube2E3 confirms that MULAN is a vital regulator of mitophagy and it provides a credible mechanism for its function [37]. The nuclear encoded mitochondrial serine protease Omi/HtrA2 is a dual function protein, whose function depends entirely on its subcellular localization. During apoptosis, Omi/HtrA2 is released into the cytoplasm to participate in cell death [38]. Under oxidative stress condition, Omi/HtrA2 degrades MULAN, but inactivation of Omi/HtrA2 protease leads to the deregulation of MULAN and increases mitophagy [39] .

Other Important Mitochondrial Ubiquitin E3 Ligase

Currently, there is no direct evidence for a relationship between other mitochondrial ubiquitin ligase, such as GIDE and RNF185, and cardiovascular disorders. Nevertheless, several studies found the effect of these ligases on cell growth, apoptosis and autophagy in other systems or cell types.

GIDE is a mitochondrial E3 ubiquitin ligase, which was identified by Zhang and colleagues in 2008 [40]. GIDE contains a conserved C-terminal RING finger domain, which is required for E3 ligase activity [41]. JNK is a stress responsive mitogen-activated protein kinase (MAPK), which acts as a pro-apoptotic factor under some circumstances in several cell types as well as in the cardiovascular system [42]. GIDE can activate JNK, and overexpression of GIDE induces apoptosis via a pathway involving activation of caspases. On the contrary, GIDE accelerates cell growth when its expression is low by unknown mechanisms.

RNF185 is another novel mitochondrial ubiquitin E3 ligase, which selectively regulates mitochondrial autophagy in cultured cells [40]. RNF185 promotes LC3II to accumulate in human cell lines as well as the formation of autophagolysosomes. In addition, the Bcl-2 family protein BNIP1 is a substrate of RNF185. The polyubiquitinated BNIP1 recruits autophagy receptor p62, which attaches both ubiquitin and LC3 to link ubiquitination and autophagy [40]. In the ER, misfolded or improperly assembled proteins are exported to the cytoplasm and degraded by the ubiquitin-proteasome pathway through a process called ER-associated degradation (ERAD). RNF18 can manipulate the stability of Cystic fibrosis transmembrane conductance regulator (CFTR), a substrate of ERAD, in a RING and proteasome-dependent manner [43].

Conclusion

The protein quality control is fundamental for the maintenance of normal cellular function. When this equilibrium is collapsed, a variety of diseases, including cardiovascular disorders, will emerge. Accumulating evidence indicates that mitochondrial ubiquitin ligases are prominent in the maintenance of protein quality control. In particular, many studies have illustrated the critical role of mitochondrial ubiquitin ligases in the heart. Despite the regulatory functions of mitochondrial ubiquitin ligases such as MULAN, GIDE, and RNF185 are reported in different cell systems, there is yet no direct indication proving their regulatory role in cardiovascular diseases. The available evidence indicates the crucial role of MITOL in cardiomyocytes and heart disease. Thus, attention to the research on understanding of the relationship between mitochondrial ubiquitin ligase and cardiovascular diseases could offer a potential therapeutic target for cardiac disorders in the future.

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Chapter 18
Nitrite-Nitric Oxide Signaling and Cardioprotection

Matthias Totzeck, Ulrike B. Hendgen-Cotta, and Tienush Rassaf

An acute myocardial infarction remains the leading single cause of death worldwide. Although reperfusion strategies have significantly improved overall mortality rates, reduction of the I/R-associated cardiomyocyte damage in patients remains an unmet goal [1]. Mitochondria as central energy suppliers in cardiac cells are key players in I/R-driven lethal injury. Protection of mitochondrial integrity and function is therefore a pivotal target in cardioprotection. Therapies that modify NO levels protect the heart from lethal myocardial I/R injury but both the resolution of the downstream signaling as well as the translation into clinical practice have not been achieved so far [2, 3]. This pertains both to the exact nature of how the response to the NO donors are transferred to the heart and which exact signaling cascades are activated within the cardiomyocyte and in cardiac mitochondria. Transfer of NO to the cardiomyocyte can be achieved by direct application of so-called NO-donors – compounds that continuously release the free radical NO. Recent experimental evidence also points to a potentially mitochondria-selective NO donor (mito-*S*NO) [4]. NO metabolizes to nitrite and nitrate. These chemically more stable forms distribute throughout the circulation and tissues but may be recycled to NO under hypoxic and ischemic conditions – an activation mechanism, which is canalized by metalloproteins, e.g. myoglobin in the heart [5–12]. Exogenous application of nitrate or nitrite can significantly enhance the bodily provision of these compounds and has been shown to contribute to cardioprotection in experimental studies. Furthermore, endogenous NO production can be enhanced by mechanical maneuvers, particularly rIPC – short alternating phases of extremity ischemia by cuff occlusion followed by reperfusion [3, 5]. Here, we briefly recapitulate the events that characterize myocardial I/R injury. This is followed by an outline of the NO-related signaling in

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I/R with particular emphasis on mitochondrial functions. Finally, we discuss how NO signaling can be effectively enhanced and how this could translate into cardioprotection.

Myocardial I/R Injury: Underlying Mechanisms

The events leading to myocardial I/R injury are complex and still under intense debate. The three major modifiers of I/R injury are ROS, a deranged Ca^{2+} signaling and the destruction of mitochondrial integrity and function.

Reperfusion therapy by percutaneous coronary intervention or pharmacological thrombolysis rapidly increases intracellular oxygen levels. A subsequent insufficient electron transport over mitochondrial membranes can result in the formation of ROS. This may overcome the availability of endogenous antioxidant mechanisms. The relative imbalance between the generation and the decomposition of ROS is one of the key initiators of I/R injury with consecutive organelle membrane and protein damage and the induction of apoptosis and necrosis [13]. Paralleled by increased ROS concentrations, the levels of bioactive NO appear to decrease during the early reperfusion phase. NO fulfills numerous functions in the heart including scavenging of radicals, modulating immune response, improvement of blood flow and left ventricular function [14–16]. As a consequence, these functions are impaired.

Evidence from experimental studies also implicates that a rapid burst-like increase in Ca^{2+} concentration contributes to the lethal injury of the reperfused myocardium [17–20]. Elevated Ca^{2+} levels can trigger arrhythmias, hypercontracture [21] and the activation of deleterious signaling cascades (see Chap. 10). In addition, hypercontracture and necrosis is not limited to one cell but also disseminated by directed contracture from cell to cell and by cell-to-cell progression through intercellular gap junctions [22].

A third central mediator/target of I/R injury are the mitochondria and specifically the mitochondrial membranes [23–25]. Mitochondria are not only the main source of energy production but can also signal cell death and cardiomyocyte disintegration [26]. However, modification of mitochondrial function is regarded a promising therapeutic option given to modulate functional recovery, reduce infarct size, improve cardiac function and prevent heart failure progression after ischemic injuries in experimental approaches [27]. Results from our studies implicate that administration of exogenous nitrite during myocardial I/R injury yields bioactive NO leading in particular to an *S*-nitrosation modification of mitochondrial complex I. This in turn regulates mitochondrial respiration and limits formation of mitochondria- derived ROS. *S*-nitrosation of complex I is furthermore associated with an adaption of myocardial functions to a reduced oxygen supply — a mechanism which is known as hibernation. Finally, nitrite reduction to NO contributes to a decrease in myocardial necrosis and apoptosis [8, 12, 28, 29].

In addition to the regulation of mitochondrial function by hypoxic nitrite signaling, mitochondrial integrity is maintained by the intraorganellar proteolytic system and the dynamic nature of the mitochondrial population in the cell. Membrane fusion and fission allow mitochondrial content adaption for cell integrity, and damaged mitochondria are selectively removed by a process, termed mitophagy, which protects against cell death [27, 30]. When mitochondria are damaged thus losing membrane potential, the kinase PTEN-induced putative kinase protein 1 (PINK1) accumulates and recruits the E3 ubiquitin ligase parkin, which then ubiquitylates mitochondrial proteins and causes mitochondria to become enclosed by membranes. BNIP3 as well as NIX, both related to the BH3-only family, also contribute to mitophagy in response to hypoxia. The current knowledge of the cellular mechanism behind mitochondrial quality control promotes interest in associated interventions in physiology and diseases such as aging, cancer, degenerative disorders and coronary heart disease. Notably, initial experimental evidence suggests that mitophagy is involved in the protection from ischaemic preconditioning (IPC) applied locally to a myocardial region. This mechanism leads to cardioprotection of the reperfused areas [27, 31]. It is speculated that the inhibition of the formation of mitochondrial membrane pores, e.g. the so-called mitochondrial permeability transition pore (mPTP), are involved in protection from ischemic preconditioning applied to the heart before index ischemia as well as in the regulation of mitophagy to reduce the numbers of dysfunctional organelles [27, 30, 31] (see Chap. 9). Less is known about the relative contribution to cardioprotection when the protective stimulus is applied to a heart-distant organ – rIPC.

Cardiomyocytes die primarily by apoptosis or necrosis with mitochondria at the center of regulation [32]. Apoptosis causes cell shrinkage, cellular and compartment fragmentation, and phagocytosis. The general characteristics of necrosis include cellular swelling and rupture, marked depletion of energy carriers, and an inflammatory response. Newer studies argue that both apoptosis and necrosis are regulated by a complex signaling machinery with overlapping processes. The critical step for apoptosis is the permeabilization of the MOM pore (MOMP). This should occur by oligomerization of BAX and other pro-apoptotic BH3 proteins. Subsequently, a release of apoptogenic factors, e.g. of apoptosis-inducing factor (AIF) which is also cleaved and activated by calpains, and endonuclease G, cause DNA damage [33, 34]. By contrast, the key characteristic of necrotic cell death is the permeabilization of the mitochondrial inner membrane. This may be further substantiated by a destruction of mitochondrial complex I. However, also BAX substantially contributes to necrotic cell death and *Bax* deficient mice show a remarkable reduction in necrosis. Interestingly, BAX-signaling is not only involved as critical step in apoptosis and necrosis, but also furthermore involved in mitochondrial structural dynamism [35].

Mitochondrial morphology and structural dynamism is regulated by a fine equilibrium between fission and fusion, repeated cycles of which re-distribute mitochondrial constituents by separation and fusion of mitochondria [36]. Fission is mediated by dynamin-related protein 1 (DRP1), a GTPase that transits from cytosol to mitochondria. Fusion is controlled by three dynamin-related GTPases: mitofusin

(Mfn1) and Mfn2 in the MOM and optic atrophy (OPA1) in the mitochondrial inner membrane. Interestingly, *Bax* knockout mice (formerly examined to have reduced apoptosis and necrosis) also show smaller, more fragmented mitochondria, which are less susceptible to cell death induction in I/R. Comparable characteristics were found in *Mfn2* knockout mice and cell lines. BAX reconstitution has been shown to cause an increase in mitochondrial fusion with mitochondria much more sensible to I/R related cellular stress. Although BAX is principally involved in both mitochondria-driven cell death and structural dynamism, the relationship between mitochondrial dynamics and cell death is poorly understood [37–42].

NO in Acute I/R Injury

As demonstrated in numerous experimental studies, myocardial I/R injury can be modulated. However, although numerous animal model-based studies showed much-reduced I/R injury, the respective translational clinical trials failed to demonstrate the same benefit in humans. Several of these experimental studies point to a protective role of the NO pool during myocardial I/R (Fig. 18.1) [28–31]. Increase of the circulating and the tissue (cardiac) NO pool exerts protective tissue effects in a mouse model of I/R injury in vivo [28, 30, 32]. The protective effects can derive from a hemeprotein-dependent reduction of nitrite to NO, as demonstrated in mice without myoglobin [30].

NO derived from nitrite reduction via cardiac myoglobin reversibly modulates mitochondrial electron transport, thus decreasing reperfusion-derived oxidative stress and inhibiting cellular apoptosis leading to a smaller final infarct size. In details, under hypoxic conditions the non-enzymatically formed NO binds to the aa_3 side of cytochrome oxidase (complex IV in the mitochondria) and thus inhibits respiration. As a result of this, a reduction in the energy status occurs, which results in an attenuation of the myocardial pump function and consequently, in reduced oxygen consumption. If the myocardium is again adequately supplied with oxygen, the non-enzymatic NO formation via myoglobin ceases and concurrently the inhibition of energy production as well as the restriction (hibernation) of the myocardial pump function also ends. Myoglobin thus appears to assume the role of an oxygen sensor in the myocardium via NO adjusts the myocardial energetics to the diminished oxygen supply [28].

Posttranslational modifications of relevant cardiomyocyte proteins are the second major aspect of hypoxic NO signaling. *S*-nitrosation is the addition of an NO moiety to a protein sulfhydryls. This can effectively change proteins conformation and function [8]. We and others have demonstrated that within the cardiomyocyte, NO reacts with numerous yet incompletely identified proteins [43]. Macrophage-migration inhibitory factor (MIF) plays a very important role in myocardial I/R injury. *S*-nitrosation of MIF was able to enhance the ROS decomposition properties of this molecule resulting in less necrosis and apoptosis in the reperfused myocardium [8]. *S*-nitrosation may further affect mitochondrial elements. It has recently

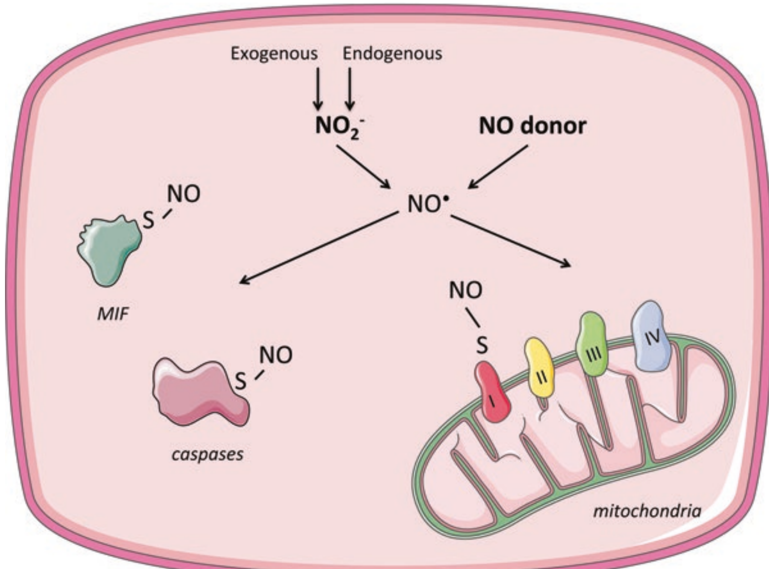

Fig. 18.1 Hypoxic NO signaling in myocardial I/R. Hypoxic NO signaling can be enhanced by e.g. increasing the levels of cardiomyocyte nitrite either by direct intravenous injection of by intake of dietary nitrate (exogenous) or be increase of endogenous NO/nitrite generation through mechanical endothelium stimulation (e.g. by rIPC). Alternatively, NO donors directly release NO which then causes an S-nitrosation modification of cytoplasmatic proteins involved in apoptosis (e.g. caspases) or related to ROS decomposition (e.g. MIF). NO can furthermore modifiy mitochondrial respiratory chain elements by S-nitrosation of complex I

been forwarded that elements of the fusion and fission machinery were significantly altered by NO-dependent modification [44]. While this may relate to a preservation of mitochondrial structure, *S*-nitrosation of mitochondrial complex I, has been significantly associated with a decrease in mitochondria-derived ROS through a decrease in misguided respiration [12, 45]. Taken together, multiple effects of NO have been characterized during I/R. The relative contribution of each of these signaling aspects remains obscure. However, the majority was in support of the notion that NO is cardioprotective in the scope of myocardial I/R.

Delivery of NO

Novel therapeutic strategies are required to alleviate the burden of I/R injury. As demonstrated in numerous infarction studies in animals and a small number of clinical proof-of-concept investigations, I/R injury can be effectively reduced by mechanical and pharmacological interventions [1].

Several pathways have been characterized that may affect the levels of NO in the reperfused myocardium. This ranges from chemical compounds that release NO on a continuous basis (NO donors) to inorganic nitrate and nitrite and mechanical maneuvers in particularly rIPC. While the next section provides and overview of these three entities, the reader is kindly referred to previous reviews discussing the use and benefits of organic nitrates which are characterized by a completely different mechanism of NO release [2].

NO Donors

A wide range of NO-releasing molecules has been forwarded with some interesting pharmacological agents among them. The reader is kindly referred to recent relevant reviews of this topic [2]. However, some very interesting aspects must be mentioned. Recent studies have evaluated compounds that combine standard medications in ischemic heart disease with NO-releasing agents. Among these were NO releasing pravastatin and nitro-aspirin. For both substances preclinical data implicate a therapeutical benefit for the reduction of myocardial I/R injury. Clinical data have to be awaited for.

While these donors cause a more or less continuous release of NO regardless of the localization (circulation, cytoplasma, mitochondria), the mitochondria-selective S-nitrosating agent mito-SNO potentially avoids these caveats by selective activation when crossing mitochondrial borders only [4]. This caused a significant S-nitrosation of mitochondrial complex I, which was reversible and protected the heart in the first few minutes of reperfusion. It remains to be evaluated whether this pertains to the clinical setting.

The Nitrate-Nitrite-NO Pathway

Nitrate and nitrite are now regarded as source for NO under hypoxic, ischemic, or injury conditions [11, 46]. Nitrite is reduced significantly to bioactive NO with decreased oxygen levels and pH by a variety of mechanisms, including reaction with deoxygenated myoglobin, in the heart as well as other heme proteins in solid organs and blood cells [28, 47–49]. Nitrite levels result from the reduction of dietary inorganic nitrate intake, which is reduced to nitrite by oral cavitiy bacteria [50, 51]. A dose of 1.2 nmoles of nitrite was sufficient in reducting myocardial and liver injury in I/R also these cause a moderate increase of 10% of the circulating nitrite levels [52, 53]. Furthermore, mice with decreased endgenous circulating nitrite concentrations are more susceptible to IR injury, an effect that is attenuated by exogenous nitrate [54]. These data suggest that a diet rich in nitrate and nitrite may have profound cytoprotective effects. In addition, eldery participants given dietary nitrate supplementation showed a marked incresase in endothelial function, which is a hallmark of arteriosclerosis, which, in turn, precedes myocardial infaction [55].

rIPC

Mechanical strategies that reduce I/R injury have been named IPC and postcondioning (PostC) [56, 57] They are applied as brief non-deleterious cycles of I/R before (IPC) or after (PostC) the main ischemic event. It is widely accepted that these mechanical interventions condition the heart and activate endogenous, protective modalities [58, 59]. Interestingly, the conditioning stimulus can also be applied to an organ distant (remote) to the heart such as the limb, kidney or intestine (rIPC) [60] and rPostC [61] respectively. The protective assets of IPC and rIPC rely on a distinct cellular signaling cascade, whose individual members can be subdivided into triggers, effectors, mediators and end-effectors [21, 62–66]. Endogenous triggering molecules are e.g. opioids, bradykinin, prostaglandins and adenosine and effects are mostly exerted by G-protein coupled receptors (GPCR) [67–70]. The rIPC downstream signaling cascade is structurally complex [65, 71, 72]. Activation of GPCR leads to an activation of phosphoinositide 3-kinase (PI3K) and further downstream targets [73–78]. Mitochondria are generally regarded to be the end-effector of the IPC signaling cascade [79–81]. Disruption may lead to cell swelling and disintegration of mitochondrial membranes.

rIPC is one of the most effective techniques in rendering the myocardium capable of protecting itself against I/R injury. rIPC is induced via short non-deleterious phases of I/R prior to an index ischemic event. While the cardioprotective effects of rIPC are generally acknowledged, the underlying mechanism and specifically a role for NO remain under intense debate. The signaling initiated by the rIPC stimulus involves a trigger, and a distinct cardiac signal transduction mechanism finally protecting the cardiomyocyte from I/R injury. As triggering pathways both humoral/blood borne factors [82–84] and nerval transmission [85] have been proposed. Given that the cardioprotective effects can be transferred when transfusing blood from preconditioned donor animals to unconditioned recipients in both in vivo and ex vivo preparations [82, 84, 86], a contribution of a blood borne factor appears rather presumable. Using a mouse model of warm liver I/R, it was recently demonstrated that ablation of endothelial NO Snythase (eNOS) abrogates the protective effects seen with rIPC on microscopic liver damage [87], eNOS generates NO which can modulate cardiovascular functions either locally or at a distance when transported as nitrite or nitroso species [88]. Changes in shear stress, e.g. due to an increase in blood flow, are the strongest physiological stimulus of eNOS activity, which is mirrored in higher circulating NO metabolites. In a recent investigation in humans, we determined that a 5 min phase of forearm occlusion massively increased postischemic blood flow in the proximal conduit arteries. This was paralleled by an increase in nitrite in the plasma of these individuals [89]. This rIPC maneuver causes an increase of circulating endogenous nitrite both in an experimental and clinical setting, enhances nitrite levels in the myocardium and thus protects from myocardial I/R injury [5]. The relative importance of this pathway remains to be elucidated.

Conclusion

Mitochondria remain a central target of cardioprotection strategies. This relates to the preservation of mitochondrial structure and function. Experimental evidence points to a benficial role NO in mediating protection during I/R. Therefore an increase of NO in the reperfused myocardium is a desired approach to protect the heart. The strategies to achiev this range from NO donors, inorganic nitrate and nitrite to an endogenouse enhancement of NO/nitrite production, e.g. by rIPC: the relative importance and a potential clinical application must be evaluated in future clinical trials.

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Chapter 19
Peripheral Blood Mitochondrial DNA and Myocardial Function

Tatiana Kuznetsova and Judita Knez

A major burden of modern society is the progressive increase in age-associated disorders due to the continuously increasing life expectancy and high prevalence of cardiovascular risk factors such as hypertension, diabetes and obesity. Symptomatic heart failure (HF) is one of the most common age-related diseases, currently affecting an estimated 15 million Europeans and 10,000 people are newly diagnosed every day [1]. In the US, HF contributes to an economic burden close to $40 billion per year and affects 1–2% of the US population [2]. Thus, with the increasing burden of symptomatic HF there is a need for a better early diagnostic and prevention strategies, and for a better treatment regimen for HF patients.

While traditionally associated with the concept of pump failure or reduced left ventricular ejection fraction, it has become widely recognized that HF can occur even when ejection fraction is preserved, constituting the syndrome of HF with preserved ejection fraction or so called diastolic HF [3] which is characterized by impaired left ventricular relaxation and increased left ventricular stiffness. It is important to note that HF is a progressive disorder that begins with cardiovascular risk factors, proceeds to asymptomatic maladaptive left ventricular remodeling and dysfunction, and then evolves into clinically overt HF and disability. Recent population studies revealed a high prevalence (up to 25%) of asymptomatic left ventricular systolic and/or diastolic dysfunction using echocardiographic imaging [4, 5]. Understanding to what extent genetic along with environmental factors influence

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myocardial function is an important issue in view of the relation of left ventricular systolic and diastolic dysfunction with outcome [5].

The progression of left ventricular dysfunction to HF is associated with changes in cardiac energy metabolism [6]. Mitochondria play a central role in a variety of cell functions, including oxidative energy production, storage of calcium ions, and programmed cell death [7]. Cardiomyocyte function depends on mitochondrial oxidative respiration for energy production almost entirely [8]. Each mitochondrion contains two to ten copies of its own mitochondrial genome (Fig. 19.1) [9]. The number of mitochondrial DNA (mtDNA) copies in cells correlates with the size and number of mitochondria, which change not only under different energy demands but also due to increased oxidative stress and in different pathological conditions [9]. The major difference which makes mtDNA more vulnerable to damage as compare to nuclear DNA is that mtDNA lacks protective histones, chromatin structure, introns and its DNA repair mechanisms work less efficiently.

Accumulation of mtDNA damages can perturb its replication mechanisms and result in decreased overall cellular mitochondrial-to-nuclear DNA ratio (mtDNA content) [10]. The process of depletion of mtDNA content might be caused by both mtDNA production disturbances as well as its degradation. Experimental studies showed that mitochondria carrying mutant mtDNA could be removed by mitophagy [11]. On the other hand, increased reactive oxygen species (ROS) impair mitochondrial function, damage mtDNA replication mechanisms, and result in decreased mtDNA production [12]. Notably, excessive oxidative stress is recognized as an important contributor to the pathophysiology of heart failure [13]. The precise molecular mechanisms and the contribution of each of the processes to changes in mtDNA content remain to be unveiled. Depletion of mtDNA and mitochondrial malfunction has been implicated in pathogenesis of different diseases such as cancer, neurodegeneration, diabetes, and HF [9].

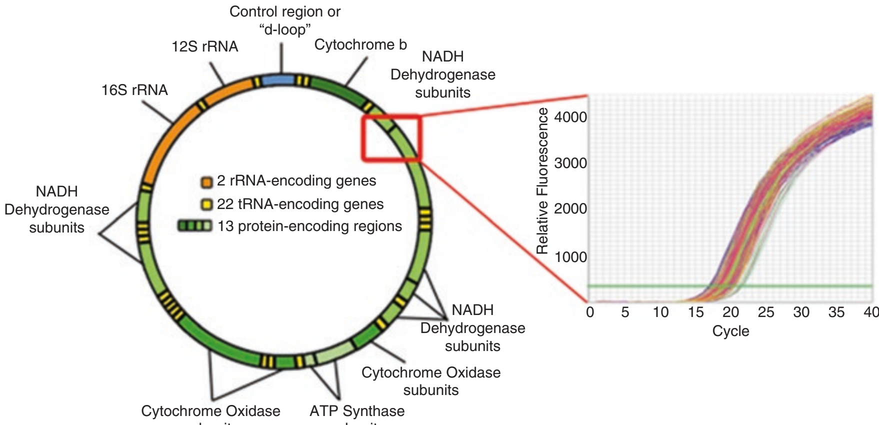

Fig. 19.1 Real time qPCR amplification of stable mitochondrial sequences. The graph shows real time qPCR amplification curves for a single 386 well plate. qPCR, quantitative polymerase chain reaction

Measurements of mtDNA Content in Peripheral Blood Cells

Mitochondrial function in various diseases and conditions could be estimated by assessment of the mtDNA content in specific tissues or in easily accessible peripheral blood cells. The mtDNA content could be measured by determining the ratio of several mitochondrial sequences (for instance, MT-ND1 and MTF3212/R3319) to one or two single-copy housekeeping nuclear genes (for instance, RPLP0) using real time PCR [14, 15]. Both mtDNA primers should not be located around the D-loop since this region is thought to be hypervariable and more prone to deletions or mutations than other mitochondrial genes (Fig. 19.1). The relative mtDNA content is calculated by normalizing cycle threshold (Ct) values of the mitochondrial DNA sequences relative to the nuclear genes (Fig. 19.1).

Recent epidemiological studies including ours described the distribution and determinants of the peripheral blood mtDNA content measured in a general population [16, 17]. Overall, the distribution of blood mtDNA content measured in general population follows a normal distribution curve with range from 0.39 to 3.06 (Fig. 19.2).

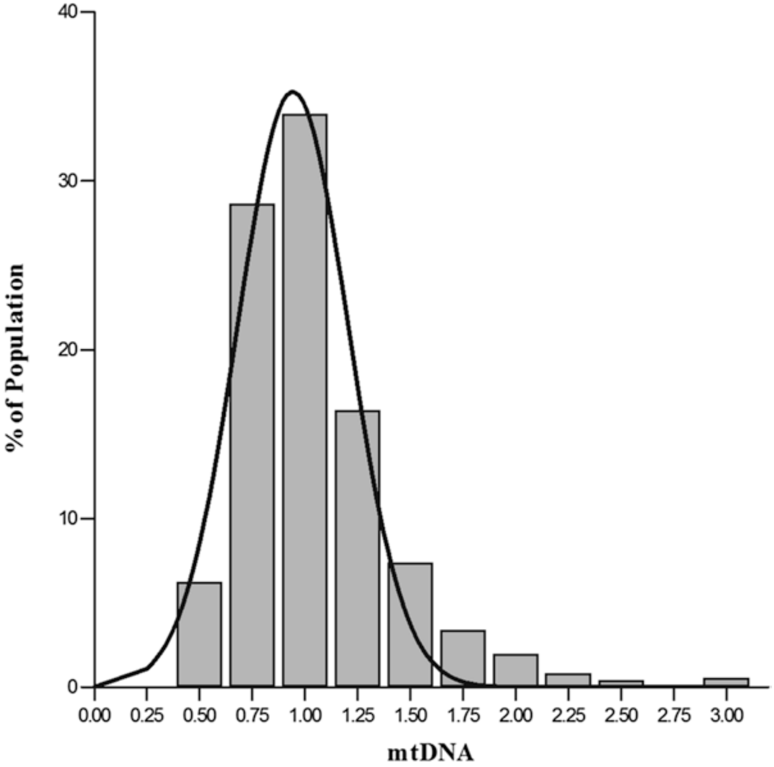

Fig. 19.2 Distribution of the mitochondrial DNA (mtDNA) content in 710 participants recruited from the general population (Reproduced from Knez J. et al. [34] with permission)

Previous studies reported a curvilinear relationship between the relative mtDNA content and age with a significant decline in blood mtDNA content in older subjects [16–19]. Importantly, higher mtDNA content in the elderly subjects was associated with better cognitive function, physical performance [18], and longevity [16, 19]. Women had higher mtDNA content as compared to men [16, 17, 20]. The mtDNA content once it measured in the whole pool of peripheral blood cells was significantly increased with platelet count, whereas it was inversely associated with white blood cell count [17]. Thus, measurement of the mtDNA content in peripheral blood cells could serve as a reliable marker to assess mitochondrial function. Studies involving measurements of the mtDNA content in peripheral blood should take into account these covariates.

Heart Maladaptation in Rodents with mtDNA Depletion

There is strong evidence that mitochondrial DNA depletion may play a key role in the progression to cardiac remodeling and dysfunction. Indeed, previous experimental studies demonstrated that a decrease in myocardial mtDNA content could trigger development of dilated cardiomyopathy in mice [21–27]. Genetic manipulation of key proteins involved in the maintenance, replication and transcription of mtDNA such as mitochondrial transcription factor A (Tfam) or DNA polymerase γ (Pol γ) can trigger the development of HF. Indeed, in the tissue-specific Tfam knockout mice, the decline of respiratory chain function following depletion of mtDNA resulted in mitochondrial cardiomyopathy, characterized by left ventricular dilation and increased cardiac mass [22, 23]. On the other hand, overexpression of Tfam in mice after myocardial infarction ameliorated post-infarct remodeling including reduced left ventricular cavity size, myocytes hypertrophy, interstitial fibrosis, and apoptosis of non-ischemic cardiomyocytes [28]. These beneficial effects of Tfam overexpression were associated with an attenuation of mitochondrial oxidative stress, mtDNA decline, and dysfunction [28].

Transgenic cardiac targeting of human Pol γ harboring the pathogenic mutation was also resulted in depletion of mtDNA, oxidative stress, dilated cardiomyopathy with left ventricular dysfunction, bradycardia, and premature death [26]. In addition, Sahin et al. [27] demonstrated that impaired mitochondrial biogenesis including decline in mtDNA content in the telomere-deficient mice causes maladaptive changes in heart such as an increase of left ventricular diameter, thinning of left ventricular walls, and a decrease of fractional shortening.

Overexpression of mitochondrial antioxidant Prx-3 protein in transgenic mice with myocardial infarction caused alleviation of mitochondrial oxidative stress and, therefore, preservation of mtDNA copy number as compared to wild-type mice [29]. In these transgenic mice left ventricular cavity dilatation and dysfunction were also attenuated compared to wild-type mice, with no significant differences in infarct and aortic pressure between groups [29]. Thus, experimental studies provided solid evidences that mtDNA depletion and mitochondrial dysfunction lead left ventricular cavity size dilatation and dysfunction in rodents.

mtDNA Content in Patients with Symptomatic HF

Recent clinical studies in patients with advanced stages of HF suggested an impaired mitochondrial biogenesis in biopsies obtained from human heart [30–32]. Karamanlidis et al. showed that mtDNA content in cardiomyocytes was decreased by 40% in 23 human end-stages failing hearts as compared to 19 control hearts [30]. Moreover, progressive depletion of mtDNA was observed in cardiomyocytes derived from the right ventricle of patients with congenital heart disease during the transition from hypertrophy to HF [31]. Of notice, in this study decrease in mtDNA content preceded any changes in mitochondrial enzyme activity or protein levels in cardiomyocytes [31]. On the other hand, Ahuja et al. observed divergent mtDNA content in tissues obtained from hearts of 16 patients with dilated and ischemic cardiomyopathy [32]. In patients with dilated cardiomyopathy, the authors observed an increase amount of defective mtDNA. In contrast, end-stage ischemic hearts show a depletion of mtDNA [32].

Huang et al. showed that mtDNA content measured in peripheral blood cells is significantly lower in patients with advanced HF in particular of ischemic origin compared with age-matched controls [33]. In this study, HF patients with lower blood mtDNA had a higher risk for cardiovascular death (adjusted hazard ratio [HR] for 1 standard deviation decrease in mtDNA content = 1.25; $p = 0.023$) and for HF hospitalization (HR = 1.32; $p < 0.001$). Thus, increasing evidences suggest that imbalances in mitochondrial function and associated oxidative stress play an important role in the pathophysiology of congestive HF.

Association of Subclinical Left Ventricular Remodeling and Dysfunction with Peripheral mtDNA Content

Hence, mtDNA content might be an important biomarker in heart disease prediction and to date studies addressing the relationship of mtDNA content with parameters reflecting cardiac structure and function analyzed continuously are scarce. Previously, we investigated in a general population in cross-sectional and longitudinal studies whether echocardiographic indexes of left ventricular structure and function are associated with mtDNA content measured in peripheral blood cells [34]. *First*, in the cross-sectional analysis, we demonstrated that participants with higher blood mtDNA content had smaller left ventricular volumes. *Second*, we observed that baseline mtDNA content was also a significant predictor of longitudinal changes of left ventricular diastolic cavity volume and size. As shown in Fig. 19.3, left ventricular diastolic volume, which overall decreased during the follow-up period, was less likely to decrease in participants with low mtDNA content.

In the Framingham heart study, the presence of certain risk factors in midlife, including hypertension and diabetes mellitus, was also associated with a lesser decrease in left ventricular dimensions with older age [35]. Furthermore, individuals with symptomatic HF are more likely to have greater left ventricular end-diastolic

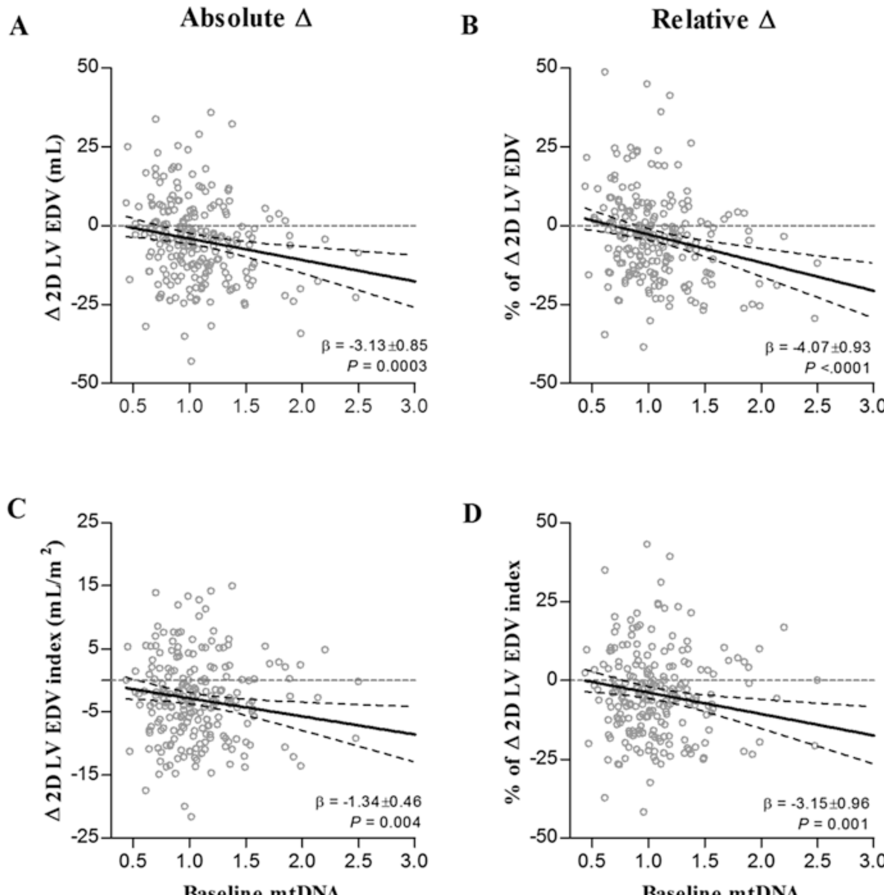

Fig. 19.3 Scatterplots of absolute (panels **a** and **c**) and relative (panels **b** and **d**) changes in 2D left ventricular diastolic volume by baseline mtDNA content in multivariable adjusted analyses. Within-subject changes were calculated by subtracting baseline from follow-up values. Percent changes were calculated by dividing the within-subject changes by the baseline echocardiographic indexes. Model for left ventricular diastolic volume was adjusted for sex, age, body weight and height, systolic blood pressure, use of different classes of antihypertensive drugs, heart rate and changes in heart rate, while accounting for family clusters and follow-up duration. For left ventricular diastolic volume index, model did not include anthropometric covariables. β (±SE) represents the effect size associated with 1-SD increase in baseline mtDNA (+0.37). *mtDNA* mitochondrial DNA content, 2D, two-dimensional; *LV* left ventricle; *EDV* end-diastolic volume (Reproduced from Knez J et al. [34] with permission)

volume than subjects of similar age range but without HF [36]. Therefore, mtDNA content along with other risk factors might contribute to alterations in the typical course of left ventricle remodeling with age [35] and may, in turn, contribute to the risk for overt HF in older age. *Third*, in our study, higher baseline blood mtDNA content predicted less increase in left ventricular mass during the follow-up period (Fig. 19.4). Based on these findings, we might speculate that having a higher level of blood mtDNA content might delay adverse left ventricular remodeling over time

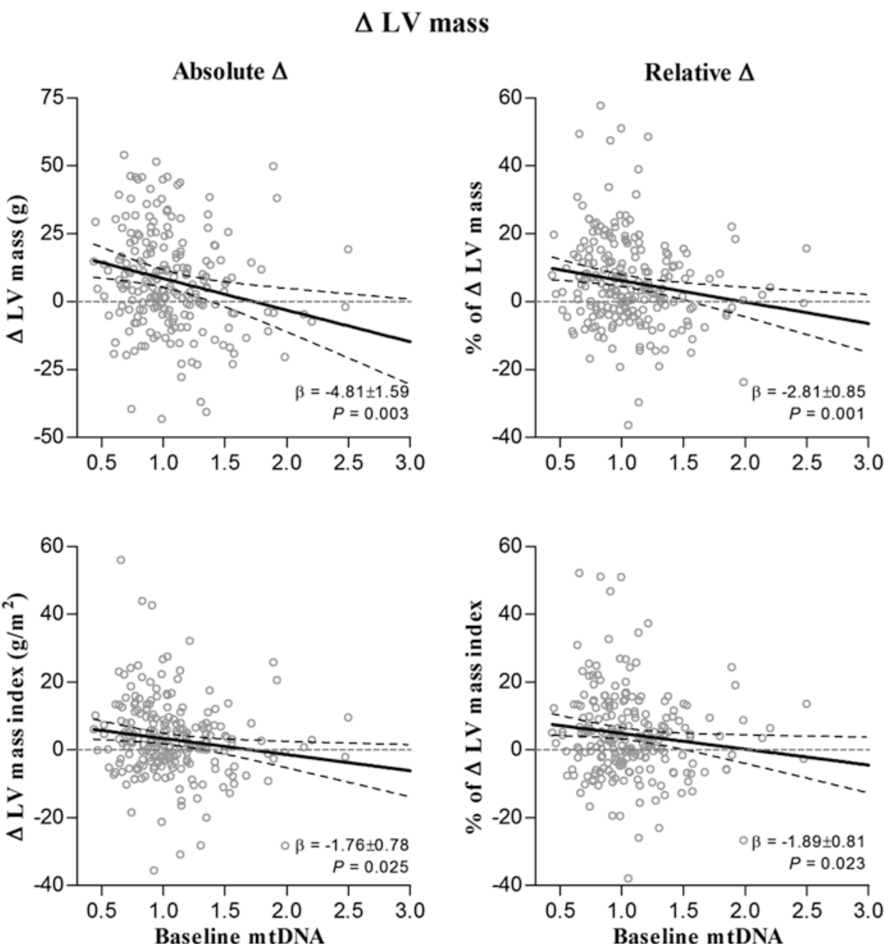

Fig. 19.4 Scatterplots of absolute and relative changes in left ventricular mass index by baseline mtDNA content in multivariable adjusted analyses. Models for left ventricular mass were adjusted for sex, age, systolic blood pressure, use of different classes of antihypertensive drugs, heart rate and changes in heart rate, while accounting for family clusters and follow-up duration. β (±SE) represents the effect size associated with 1-SD increase in baseline mtDNA (+0.37). *mtDNA* mitochondrial DNA content, *2D* two-dimensional, *LV* left ventricle (Reproduced from Knez J et al. [34] with permission)

and, therefore, future cardiovascular events. In line with these findings, several previous population studies found that higher blood mtDNA content in elderly was associated with better outcome and healthy aging [16, 18, 19].

We also found that higher mtDNA content was associated with better left ventricular systolic and diastolic function as reflected by an increase in longitudinal mitral annulus movement during systole (s′ peak) and a decrease in ratio of transmitral flow velocity to early diastolic mitral annular velocity (E/e′ ratio) [34]. Low longitudinal systolic s′ velocity [37] has independent prognostic value in patients with HF [38] and in asymptomatic subjects with cardiovascular risk factors [39]. Combining transmitral flow velocity with early diastolic mitral annular velocity (E/e′ ratio) is frequently used for assessing the LV diastolic filling pressure [40]. High E/e′ independently predicted cardiac mortality in HF patients [41]. In our study, higher blood mtDNA content was associated with better left ventricular systolic function characterized by higher s' peak and better left ventricular diastolic function characterized by lower E/e′.

These findings could have a biological basis related to the role of mitochondrial biogenesis in determining cardiac function and size. Whether these associations can aid in risk stratification for early stages of cardiac remodeling and dysfunction and whether mechanisms governing mtDNA content can be a target for pharmacological interventions require further investigation.

Correlation Between mtDNA Measured in Tissues and Peripheral Blood Cells

As we previously described the mtDNA content could be measured in tissues as well as in easily accessible peripheral blood buffy coat, which contains leukocytes and platelets. Some previous experimental and clinical studies compared mtDNA content in blood cells and in tissue samples. Sahin et al. reported a similar parallel decrease in mtDNA content in myocardial and haematopoietic cells in rapidly ageing mice [27]. In patients with coronary artery disease, Chen et al. found the significant positive association between mtDNA measured in peripheral blood cells and in tissue obtained from atherosclerotic plaque [42]. Moreover, the authors reported that lower blood mtDNA content was associated with higher cardiovascular risk [42]. To our knowledge, so far, only one study reported significant correlation (Pearson $r = 0.72$; $p = 0.019$) between mtDNA contents measured in both peripheral blood cells and cardiomyocytes of 10 end-stages HF patients who received heart transplantation [33]. Overall, these findings suggested that mtDNA measured in peripheral blood cells might in some degree reflect the overall oxidative status and, therefore, might be served as a marker useful for prognostication in patients with HF. However, the assumption that mtDNA content measured in peripheral blood cells reflects in some degree mtDNA content of cardiomyocytes should be further confirmed in clinical studies.

Future Directions

Future experimental and clinical studies are necessary to further elucidate the role of mtDNA in the progression of left ventricular remodeling and dysfunction to symptomatic HF. Because disturbances in mitochondrial biogenesis could lead to the development of HF, mitochondria represent targets for therapeutic interventions [43–45]. Current treatment strategies for HF employ strategies to decreasing workload on heart by lowering volume load, blood pressure and heart rate. An alternative approach to the treatment of HF might be related to targeting mitochondrial plasticity which lead to improving ATP supply and therefore, restoring energy to the failing heart. A recent experimental study in rats showed that administration of a novel mitoprotective agent after ischemic injury can prevent adverse cardiac remodeling and decrease left ventricular dilation [46]. Few clinical trials are currently on-going to evaluate safety, tolerability and efficacy of this agent to treat HF patients. Therefore, pharmacologic substances targeting mechanisms that maintain sufficient level and quality of mtDNA might stabilize mitochondrial function and, therefore, prevent progression of maladaptive left ventricular remodeling and dysfunction.

Conclusion

There is increasing evidence from experimental and clinical studies that mtDNA depletion play a key role in the progression to cardiomyopathy. Recent epidemiological study also demonstrated that higher mtDNA content measured in peripheral blood cells was associated with smaller left ventricular volume and size, better systolic and diastolic function and more favorable trend in longitudinal changes in left heart structure. These findings could have a biological basis related to the role of mitochondrial biogenesis in determining myocardial function. Whether these associations can aid in risk stratification of patients with early and advanced stages of HF and whether mechanisms governing mtDNA content can be a target for pharmacological interventions require further investigation.

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Chapter 20
Mitochondrial Proton Leak Plays a Critical Role in Pathogenesis of Cardiovascular Diseases

Jiali Cheng, Gayani Nanayakkara, Ying Shao, Ramon Cueto, Luqiao Wang, William Y. Yang, Ye Tian, Hong Wang, and Xiaofeng Yang

Historically considered as merely cellular "powerhouse" that manufactures ATP and other metabolites, the mitochondrion is increasingly being recognized as a "sentinel" organelle capable of both detecting cellular insults and orchestrating inflammatory responses [1]. Mitochondria are complex organelles, containing their own DNA and composed of a double membrane: outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM). This double membrane gives rise to two compartments; (1) the intermembrane space (IMS) located between OMM and IMM, and (2) the matrix (M) located in the internal space created by the IMM itself.

During oxidative phosphorylation, the electrons removed from biological fuels such as glucose and fatty acids goes through a series of electron carrier system

Gayani Nanayakkara, Ying Shao, Ramon Cueto, Luqiao Wang, contributed to this work equally.

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located in IMM, which is widely known as mitochondrial electron transport chain (ETC). The passage of electrons through ETC generate energy; and ultimately these electrons reduce molecular oxygen in to water [2]. According to the chemiosmotic theory introduced by Peter Mitchell in 1961, the energy produced by electron transfer through ETC is used to establish a proton gradient across the IMM that drives mitochondrial ATP production [3]. Simply, chemiosmotic theory explains that the mechanism which propels oxidative phosphorylation is the proton gradient formed across the IMM, further coupling respiratory oxygen to ADP phosphorylation/ATP generation.

The proton gradient is established by pumping protons against their electrochemical gradient across the IMM. This process simultaneously induce a proton gradient (chemical) across the membranes which is known as a protonmotive force (ΔP) and electrical gradient known as mitochondrial membrane potential ($\Delta\Psi_m$) [4].

Mitochondrial Proton Leak Is the Principal, but Not the Only, Mechanism That Incompletely Couples Substrate Oxygen to ATP Generation

The mitochondrial ETC is comprised of a series of redox carriers named: Complex I(NADH: ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome c reductase) and complex IV (cytochrome c oxidase). The exergonic process of electron transport through complex I–IV create ΔP, which will drive the protons back to matrix from IMS via ATP synthase (also known as complex V), during which the ATP are generated with the aid of adenine nucleotide and phosphate carriers. However, protons can migrate to the matrix independent of ATP synthase, a process known as “proton leak”. Proton leak can also be defined as the dissipation of ΔP in the presence of ATP synthase inhibitor Oligomycin in both isolated mitochondria and intact cells [5]. As proton leak depicts the protons that migrate in to the matrix without producing ATP, it makes the coupling of substrate oxygen and ATP generation incomplete. Proton leak is the principal, but not the only, mechanism that incompletely couples substrate oxygen to ATP generation (Fig. 20.1). Even though the contribution is insignificant, a phenomenon called “electron slip” is also attributed to incomplete coupling of ATP generation and substrate oxygen as well. Electron slip refers to the process where electrons are transported via ETC without pumping protons to IMS. Therefore, electron slip results in disproportionate increase in oxygen consumption at high ΔP [6, 7].

The exact mechanism of how proton leak takes place is not fully known. The physiological regulation of proton leak is categorized in to two; (1) basal/constitutive proton leak, and (2) regulated/inducible proton leak.

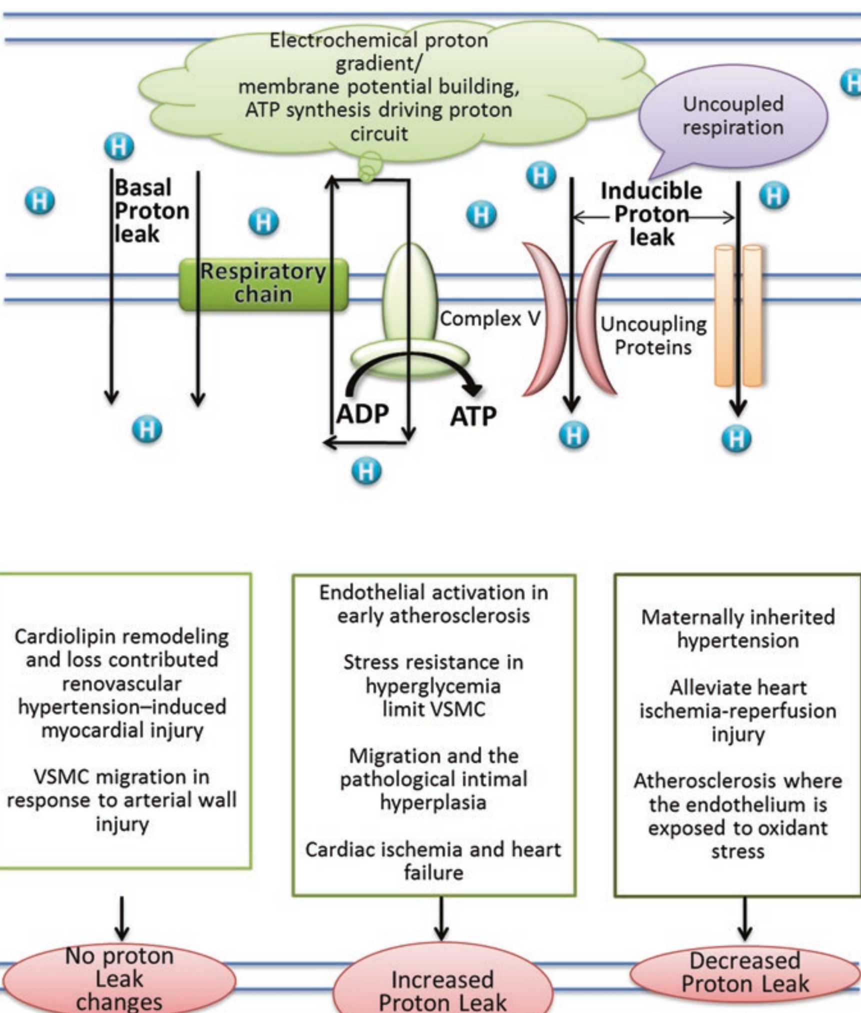

Fig. 20.1 Schematic representation of proton-leak and of the proposed role of UCPs in pathogenesis of cardiovascular disease

Basal Proton Conductance Accounts for Only 5% of Proton Leak

Basal proton conductance is generally unregulated, and largely depends on the fatty acyl composition of the inner membrane phospholipids [8, 9]. However, the proton conductance through the lipid bilayer of the inner membrane accounts for only 5%

of the proton leak. Therefore, this suggests that majority of basal conductance is regulated via mitochondrial inner membrane proteins. Approximately, two-third of the basal proton conductance is correlated to the abundance of ANT (adenine nucleotide translocase), which is a type of mitochondrial anion carrier protein found in the mitochondrial inner membrane [10].

Inducible Proton Leak Is Catalyzed by Specific Mitochondrial Inner Membrane Proteins Such as Uncoupling Proteins (UCPs)

Inducible proton leak is catalyzed by specific mitochondrial inner membrane proteins such as uncoupling proteins (UCPs) [11]. UCPs are a subfamily of the mitochondrial solute carrier family proteins that mediate transport of various metabolites across the IMM. There are five types of UCPs found in mammals, named as UCP1-5. Out of the five UCPs, UCP1 was the first to be identified in brown adipose tissue and had been extensively studied. It had been implicated in converting mitochondrial energy potential to heat, thus mainly regulate adaptive thermogenesis. Even though UCP2 and UCP3 reduce the mitochondrial coupling efficiency, it is evident that their role is largely focused on regulating reactive oxygen species (ROS) levels rather that regulating thermogenesis [12, 13]. UCPs-mediated proton conductance is regulated at molecular, transcriptional, translational and proteolytic levels [14].

Electron Leak Is the Major Causative Factor for Production of Mitochondrial Superoxide

Electron leak refers to exit of electrons from the ETC before they get reduced to water at Complex IV, and is the major causative factor for production of mitochondrial superoxide. Generally, superoxide generation is attributed to Complexes I and III, however, other mitochondrial proteins such as flavin adenine dinucleotide (FAD)-linked pyruvate and α-ketoglutarate dehydrogenase complexes are also reported to generate mitochondrial superoxide [15]. Superoxide is the primary reactive oxygen species (ROS) generated by ETC. Increased level of ROS can be detrimental to overall viability of the cells, therefore, superoxide once generated get rapidly dismutated by superoxide dismutase (SOD) to hydrogen peroxide as a protective mechanism [16]. However, at low concentrations, ROS are involved as signaling molecules in various biological pathways. Under non-pathological conditions, mitochondria are the most significant ROS producers in the cells. Other than mitochondrial ROS, cell membrane bound NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase enzyme complex also contribute to total cellular ROS production during certain pathological conditions [17].

ROS Generation and Proton Leak

There are contradictory reports that link proton leak to ROS generation. Some reports provide evidence that the dissipation of ΔP [18–20] or presence of ADP decreases the mtROS production, while few claims that uncoupling enhances the ROS production [21, 22]. These contradictory evidence might be due to the differences in biological and experimental settings. However, the view that uncoupling decreases mitochondrial ROS production prevails. Therefore, uncoupling may play a protective role by mitigating ROS production in cells. This cyto-protective role of uncoupling was specifically observed in heart under conditions of oxidative stress such as ischemia reperfusion (IR) injury [23], aging [24] and diabetes [25]. Most prominently, UCP1 and UCP2, and also chemical uncouplers offers protection against IR injury. Similarly, it was observed that uncoupling can exert a profound protective effect against toxicity produced in the presence of hyperglycemia in endothelial cells. In addition, protective role of uncoupling against atherosclerosis was reported.

On the other hand, increased level of ROS seems to cause an increase proton leak [18]. It was observed that peroxynitrite, which is a potent inducer of lipid oxidation, increased the proton leak in isolated brain mitochondria [26]. Additionally, superoxide can also enhance the electron leak to a similar extent as peroxynitrite, and was shown to activate UCPs [27]. The UCPs and ANTs are attributed to enhanced proton conductance in the presence of ROS. Therefore, the presence of a protective feedback loop had been suggested, where increased ROS generation activates mechanisms that promote proton leak; and the enhanced proton leak in turn reduce the ROS production limiting further damage to mitochondrial function [18, 27].

"Uncoupling" in Pathogenesis of Cardiovascular Disease

The Magnitude of the Proton Leak and the Mechanism Involved in Mediating the Proton Leak Determine Whether There Is a Protective Effect in IR Injury

IR injury, atherosclerosis and diabetes-mediated cardiovascular complications are known to induce oxidative stress by elevating ROS generation including peroxynitrite, superoxide and hydrogen peroxide that adversely affect the viability of cardiomyocytes. Increased levels of ROS produced during various cardiac pathologies were shown to mediate breakdown of plasma membrane of cardiomyocytes, increased the potential for arrhythmogenesis during a post-ischemic events and also led to apoptosis of myocytes [28, 29] (see Chap. 10). Recent evidence suggests that UCPs exert a protective role against oxidative stress by suppressing ROS.

Diseased heart has a strong association with increased oxidative stress and an impairment of reserve respiratory capacity. During physiological conditions,

mitochondria does not use its full bioenergetic capacity unless there is an increase in energy demand during situations where cellular repair and detoxifying ROS is required to maintain cellular function. Mitochondrial reserve respiratory capacity refers to the difference between maximum respiratory capacity and basal respiratory capacity [30]. Therefore, depletion of reserve respiratory capacity indicate the inability of the cells to meet the increased energy demand in response to cellular stress that subsequently affect adversely to the cell viability. Intact rat neonatal ventricular myocytes exhibit exhaustion of the reserve capacity with an increased proton leak when exposed to pathologically relevant concentrations of HNE (4-hydroxynonenal), which is a reactive lipid species accumulated in the heart during ischemia and heart failure [31].

Higher rate of proton leak is associated in mitochondria isolated from hearts subjected to IR [32, 33]. Furthermore, few research groups have shown that using uncoupling agents such as FCCP [carbonyl cyanide p-(tri-fluromethoxy) phenylhydrzone] or DNP (2,4-Dinitrophenol) protects mitochondrial and cardiomyocyte function from IR injury [34, 35]. However, this observation was reversed in mitochondria subjected to ischemic preconditioning (IPC), which is a cardioprotective strategy consisting of brief cycles of IR (3–5 min) to protect the myocardium from the damaging effects of subsequent longer episodes of IR. Surprisingly, mitochondria subjected to protective IPC had a smaller increment in proton conductance after IR compared to non-preconditioned mitochondria with higher proton conductance [32, 33]. This is contradictory to the observation that increased proton conductance exert cardioprotective against IR injury. This observation was explained by differential mechanisms that are responsible for promoting mild proton leak and intensive proton leak. It was observed that generally mild-moderate proton leak during IR injury is regulated by UCPs [33], whereas a larger proton leak is mediated by ANT, which was shown to be a part of mitochondrial permeability transition pore (MPTP) [36]. Opening of MPTP under pathological conditions rapidly dissipate $\Delta\Psi_m$ leading mitochondrial swelling, and mediate cellular apoptosis or necrosis. The larger proton leak mediated by opening of MPTP is detrimental to mitochondria and adversely impact to the cell viability [36]. Therefore, these observations suggest that magnitude of the proton leak and the mechanism involved in mediating the proton leak determine whether there is a protective effect against IR injury.

Uncoupling by UCP2 Preserves Vascular Function in Diet-Induced Obesity Mice as Well as In Diabetes

Oxidative stress plays a central role in development of diabetes-mediated macrovascular and microvascular complications. In bovine aortic endothelial cells, overexpressed UCP1 blocked hyperglycemia-induced ROS production, indicating that uncoupling may play a role in reducing the progression of diabetes induced vascular

complications [37]. Further, endothelial UCP2 also function as a sensor and negatively regulate mitochondrial ROS production in response to hyperglycemia, further validating importance of uncoupling in attenuating diabetes induced vascular diseases [38].

Interestingly, it was observed that UCP2 expression was gradually reduced with increased lipid deposition in atherosclerotic lesion in aorta in hyperlipidemic apolipoprotein E deficient (ApoE$^{-/-}$) mice. Furthermore, it was observed that pro-inflammatory tumor necrosis factor-α (TNF-α) can downregulate expression of UCP2 in vasculature and accelerate vascular damage. Therefore, this suggests that lowering the TNF-α expression within the aorta during exposure to vascular stress factors such as hyperinsulinemia may prevent vascular damage [39]. UCP2 preserves endothelial function through increasing endothelial nitric oxide synthase (eNOS) phosphorylation secondary to the inhibition of ROS production in the endothelium of obese diabetic mice. Silencing UCP_2 impairs endothelium-dependent relaxation in aorta and mesenteric arteries from diet-induced obesity mice as well as aortic rings exposed to high glucose [40].

UCP2 in lung endothelial cells was recently reported to play a significant role in progression of pulmonary hypertension. UCP2 ablation in mice causes high right ventricular pressure and right ventricular hypertrophy when subjected to intermittent hypoxia induced pulmonary hypertension. Loss of UCP2 in lung endothelial cells increases mitophagy leading to progression of pulmonary hypertension. Mitophagy refers to selective mitochondrial autophagy, a process initiated by changes in the $\Delta\Psi_m$. Therefore, loss of UCP2 was implicated in deficiency of mitochondria and increased apoptosis in lung endothelial cells that contribute to exacerbation of pulmonary hypertension [41].

Also, in maternally inherited hypertension, it was shown that a specific mutation in mitochondrial $tRNA^{Ala}$ (A5665G) significantly reduced the proton leak. The changes in the mitochondrial proton conductance and bioenergetics led to increased ROS and subsequently elevated blood pressure [42].

However, *Eirin and colleagues* demonstrated that the use of organic peroxide tert-Butyl hydrogen peroxide (tBHP) in isolated rat cardiomyocytes can suppress the basal respiration, maximal respiration and ATP production without altering the proton conductance in mitochondria. Also, the same research group demonstrated that use of MTP (mitochondrial peptide), which protects cardiolipin from peroxidation, ameliorated this effects while keeping the proton leak still intact. Cardiolipin is an inner membrane mitochondrial protein, which tethers cytochrome C to the inner membrane, thus facilitating the electron transport from complex III to complex IV. This protein is highly vulnerable to oxidative damage and subsequent loss, therefore leading to mitochondrial loss and contributing to development of cardiovascular disorders. The authors also demonstrate that restoration of cardiolipin improves cardiac function of pigs subjected to renovascular hypertension [43]. Therefore, all these observations indicate that the response of altering proton conductance depends on the etiology of hypertension.

Proton Leak Regulates ATP Synthesis-Uncoupled Mitochondrial ROS Generation, Which Determines Pathological Activation of Endothelial Cells for Recruitment of Inflammatory Cells

Most interestingly, alterations in mitochondrial morphology were reported to alter the proton conductance as well. Mitochondria are dynamic organelles which undergo constant shape changes by fission and fusion. As the name implies, fission results in smaller mitochondria while fusion results in larger mitochondria. In addition, variations in mitochondrial morphology have been found to contribute to vascular smooth muscle cell migration in response to arterial wall injury. Silencing Drp1 (mitochondrial fission protein dynamin-like protein 1), which is the protein that regulates mitochondrial fission, limits VSMC migration through a markedly increase in proton leak, which suppresses not only ATP synthesis but also ROS production. Similarly, respiration coupling efficiency reduction was also validated in vivo with mitochondrial fission suppression, proposing an effective strategy to inhibit intimal hyperplasia in restenosis and the progression of atherosclerotic lesions [44].

Furthermore, UCP2 was shown to mitigate the atherosclerotic burden as well. *Blanc and colleagues* demonstrated that UCP2 is a protective mediator against early stage of atherosclerosis. The same group had shown that depletion of UCP2 in blood cells significantly increased the lesion size in thoracic aorta with more macrophage infiltration in hyperlipidemic mice. This finding was attributed to increased ROS production and inflammatory responsiveness in UCP2 deficient macrophages [45]. Similarly, another study demonstrated that UCP2 prevents progression of atherosclerosis by exerting antioxidant effect presumably by suppressing NF-κB activation in human aortic endothelial cells (HAECs). Moreover, UCP2 can attenuate apoptotic death of HAECs mediated by pro-atherogenic LPC (lysophosphatidylcholine) [46].

We recently demonstrated that LPC promotes HAEC activation, which is one of the primary steps in disease progression of atherosclerosis [47]. We demonstrated the LPC specifically induces mitochondrial ROS rather than cytosolic ROS generation. Interestingly, we observed that LPC-mediated ROS generation was accompanied by increased proton leak without affecting the overall ATP production. Albeit the proton conductance was increased in LPC treated HAECs, we could not detect any change in the net proton gradient (ΔP) in mitochondria. The fact that ΔP and ATP production was not significantly compromised in LPC-mediated endothelial cell activation indicates that proton conductance through complex V was not impaired. Also, intact ΔP in the presence of an increased proton leak implies that pro-atherogenic stimuli such as LPC may promote ETC activity. Furthermore, we demonstrated that LPC-mediated mitochondrial ROS was responsible for increasing the expression of adhesion molecules such as ICAM-1 in endothelial cells, which initiates atherosclerosis process in vasculature [48]. We have further validated this observation by using mitoTempo, which is a specific mitochondrial ROS inhibitor. Our data implies that inhibition of mitochondrial ROS generation can sig-

nificantly reduce the atherosclerotic burden in aortas of hyperlipidemic mice. Therefore, the major purpose for LPC-mediated increase in proton leak may be to modulate the mitochondrial ROS generation for signal transduction that ultimately lead to endothelial cell activation, without inducing mitochondrial damage or endothelial cell death [47, 49].

Conclusions

Mitochondria account for one third of the volume of cardiomyocytes and regulate ATP production that is essential to maintain cardiomyocyte health and survival [50]. Therefore, changes in mitochondrial biogenesis, morphology and function may contribute significantly to the development of cardiovascular diseases. Increased ROS can enhance proton leak, and in turn increased proton leak reduces the ROS generation, indicating the existence of a protective feedback loop that helps to ameliorate the detrimental effects caused by ROS on biological systems. The magnitude of proton current and the mechanism of proton conductance determine the level of ROS production, extent of mitochondrial damage and cell viability during cardiac pathologies. Moreover, cellular stresses such as pro-atherogenic stimuli can enhance proton leak without affecting the overall energy efficiency in endothelial cells. This finding indicates that proton leak acts as an indirect mediator of signal transduction by fine tuning ROS production, which acts as a signaling molecule to enhance endothelial activation during early atherogenesis, and contribute to the progression of the disease. Therefore, regulation of proton leak can be a potential therapeutic target for the treatment of many cardiovascular disorders.

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Chapter 21
Mitochondria and Angiogenesis

Raluca Marcu, Ying Zheng, and Brian J. Hawkins

During prenatal development, de novo formation of the primitive vascular network to support tissue growth occurs through vasculogenesis by differentiation from mesoderm-derived angioblasts [1]. Angiogenesis ensures expansion and fine remodeling of this vascular network through sprouting of new blood vessels from preexisting structures, anastomosis and pruning [2]. In the postnatal organism, angiogenesis is limited primarily to the physiologic repair of tissue wounding, for the proper functioning of female reproductive organs, and as an adaptive response to exercise training [3, 4]. Nevertheless, abnormal angiogenic vascular growth is frequently associated with the pathogenesis of cancer, inflammation, atherosclerosis, retinopathy, endometriosis and arthritis, while impaired angiogenesis is a common deleterious consequence of ischemic injury, hypertension, and preeclampsia [5]. Pharmacological interventions that target angiogenesis are therefore of great clinical interest for disease treatment where obstructing angiogenesis or promoting revascularization are essential for recovering tissue functions.

Angiogenesis engages a series of highly coordinated events that resolve with the formation of functional, perfusable blood vessels [6]. In the presence of pro-angiogenic and guidance cues (VEGF, netrins, semaphorins) that outperform anti-angiogenic signals, a quiescent endothelial cell known as tip cell is selected and activated based on regulatory VEGF-Dll4-Notch signaling [7]. Following vascular basement membrane degradation, the tip cell guides the migration and proliferation of neighbouring endothelial stalk cells that elongate the vascular sprout under Nrarp/Notch and Wnt signaling control [8]. Subsequent lumen formation,

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recruitment of pericytes, deposition of extracellular matrix and reestablishment of endotheial quiescence contribute to blood vessel maturation [9, 10].

Due to relatively low energetic requirements, baseline performance of quiescent endothelial cells is supported mainly through oxygen-independent glycolysis. It is estimated that endothelial cells acquire 85 % of their total intracellular ATP supply from glycolysis, and that the endothelial glycolytic flux is more than 200-fold higher than the mitochondrial oxidation of glucose, fatty acids or glutamine [11]. In agreement with a predominatly glycolityc metabolism, mitochondrial oxygen utilization in endothelial cells is marginal compared to neurons, hepatocytes, and cardiomyocytes [11]. Accordingly, the mitochondrial content of endothelial cells is low with respect to metabolically active cells: in rat capillary endothelial cells from the hypothalamus, kidney, skin, lung, heart and skeletal muscle, the mitochondrial volumes represent merely 2–5 % of the endothelial cytoplasmic volumes [12] compared to 32 % volume density of mitochondria in rat cardiomiocytes [13]. During angiogenesis, motile, invasive and proliferative behaviour requires increased energetic support, which endothelial cells also accomplish by upregulating glycolysis. Indeed, impairment of glycolysis due to pharmacological or genetic inhibition of phosphofructokinase-2/fructose-2,6-bisphosphatase-3 (PFKB3) compromises vessel sprouting and hyperbranching and reduces pathological angiogenesis in models of age-related macular degeneration, retinopaty of prematurity, and inflammation [11, 14].

However, despite low oxygen metabolism, endothelial mitochondria maintain a considerable bioenergetic reserve capacity that represents an important resource to lessen oxidative stress [15]. Moreover, in conditions of reduced glycolytic rates, endothelial cells retain the ability to switch to oxidative metabolism utilizing glucose, fatty acids, and amino acids [16]. The requirement for functionally intact mitochondria during angiogenesis is clearly demonstrated by impaired neovascularization in aged POLGA mutator mice, a model for mitochondrial dysfunction and aging due to defective mitochondrial DNA polymerase [17]. In POLGA mice, growth factor induced angiogenesis is severely impaired with no adverse effects on established vascular networks, consistent with a role for mitochondrial function specifically during neovessel formation. Growth factors also stimulate production of nitric oxide (NO), which is a potent angiogenic stimulus [18]. While NO can influence multiple angiogenic signaling pathways, a major target is the terminal enzyme of the mitochondrial respiratory chain, mitochondrial cytochrome c oxidase [19]. Both under basal and stimulated conditions, and depending on the calcium influx, NO adjusts the capacity of this enzyme to utilize oxygen and minimizes respiration [20]. Indeed, it is possible that a major role of endothelial NO production may be to inhibit mitochondrial respiration and prompt the increase in glycolysis required to fuel angiogenic vessel growth.

While not preferentially used for bioenergetic purposes, endothelial mitochondria alternatively serve as signaling organelles that are both a source and target of reactive oxygen species (ROS). For example, under NO control, mitochondria contribute to mitochondrial ROS-dependent AMPK activation at low oxygen concentrations, which may play a protective role in endothelial cells [21]. However, the signaling function of mitochondria is not limited to NO targeting and ROS production, but also involves modulation of cytosolic calcium signals, the release of

mitochondrial metabolic intermediates and the intrinsic activation of apoptosis [22], all of which can impact the proliferative properties of endothelial cells prerequisite for angiogenesis.

Mitochondrial Functions and Mitochondrial Proteins Involved in Angiogenesis

Despite a repressed mitochondrial energetic function in endothelial cells, other mitochondrial functions are highly involved in maintaining vascular homeostasis and regulating angiogenesis. Among them, mitochondrial ROS production, regulation of intracellular calcium, metabolic regulation and apoptosis are essential in defining the proper balance necessary to ensure the endothelial switch towards a proliferative phenotype required for angiogenesis (Fig. 21.1).

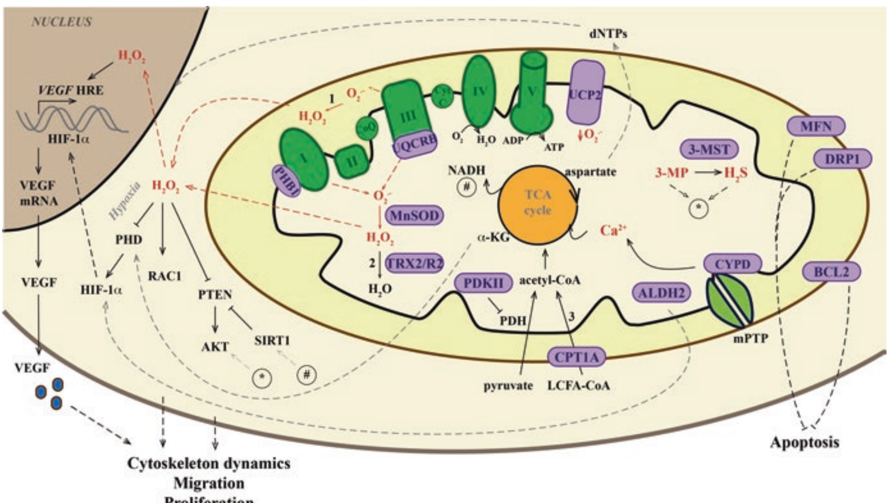

Fig. 21.1 Mitochondrial proteins and angiogenesis. Several mitochondrial proteins (*purple*) and mitochondrial signaling agents (*red*) control angiogenesis by modulating endothelial migration, proliferation and apoptosis. *UQCRB* Ubiquinol-cytochrome c reductase binding protein, *PHB1* Prohibitin 1, *MnSOD* Manganese superoxide dismutase, *TRX2* Thioredoxin 2, *TRXR2* Thioredoxin reductase 2, *UCP2* Uncoupling protein 2, *CPT1A* Carnitine palmitoyltransferase 1A, *PDKII* Pyruvate dehydrogenase kinase II, *PDH* Pyruvate dehydrogenase, *ALDH2* Aldehyde dehydrogenase 2, *3-MP* 3-Mercaptopyruvate, *3-MST* 3-mercaptopyruvate sulfurtransferase, *H_2S* Hydrogen sulfide, *CYPD* Cyclophilin D, *mPTP* Mitochondrial permeability transition pore, *MFN* Mitofusins, *DRP1* Dynamin-related protein 1, *BCL2* B cell lymphoma 2, *1* Copper-zinc superoxide dismutase, *2* Glutaredoxin, *3* β-oxidation; # mitochondrial matrix Ca^{2+} accumulation stimulates TCA cycle activity and NADH production, concomitant with decreased mitochondrial and cytosolic NAD^+/NADH ratio, * 3-MP and H_2S promote AKT activation

Mitochondrial ROS Production

Mitochondrial ROS are products of incomplete oxygen metabolism and despite a longstanding causative involvement in cellular oxidative damage and toxicity, ROS are emerging as physiological regulators of cellular functions [23]. In blood vessels, ROS control shear stress-induced vasodilation, non-vasomotor response to mechanical stimuli and the hypoxia-reoxygenation response, and excessive ROS ensuing mitochondrial dysfunction are responsible for inflammatory vascular reactions leading to cardiovascular disease [24, 25]. The dual character of ROS as both physiologic and pathologic mediators of cellular outcome is determined by the intensity of the pro-oxidant challenge, and as such the balance between mitochondrial ROS production and detoxification is tightly regulated.

Mitochondrial ROS arise from the leak of electrons along the mitochondrial electron transport chain, mainly at complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome c oxidoreductase), followed by partial reduction of molecular oxygen. The proximal reactive species produced is the superoxide anion ($O_2^{\bullet}-$), short-lived and nondiffusible, which is released on both sides of the mitochondrial inner membrane [26]. $O_2^{\bullet}-$ is quickly dismutated into hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutases (manganese-SOD, MnSOD, in the mitochondrial matrix and copper-zinc-SOD, CuZnSOD, in the intermembrane space), for subsequent removal by mitochondrial peroxidases (glutaredoxin-2, peroxiredoxin-3 and thioredoxin-2). H_2O_2 can also generate the short-lived but highly reactive hydroxyl radical, responsible for most deleterious reactions with lipids, proteins and nucleic acids [27].

Among oxidants, H_2O_2 is the most stable and longest-lived species that is both membrane permeable and diffusible, and therefore suitable to act as signaling messenger. At physiologic concentrations, H_2O_2 can impact signal transduction pathways and activate redox-sensitive transcription factors that drive cellular responses via the reversible posttranslational modification of cysteine residues [23]. With respect to angiogenesis, mitochondrial-derived H_2O_2 proved essential for endothelial migration and proliferation during angiogenesis and wound healing. Specifically, pro-angiogenic stimulation with VEGF drove an upregulation of mitochondrial metabolism and mitochondrial H_2O_2 release in endothelial cells, which targeted the Rho-related small GTPase Rac1, a regulator of cytoskeleton dynamics, and triggered downstream activation of cell survival, migratory and proliferative signals via PAK, Akt, p38MAP and ERK [28]. Conversely, mitochondrial ROS scavenging abrogated VEGF-mediated Rac1 activation and endothelial cell migration, and attenuated carotid artery reendothelialization upon injury in vivo. Mitochondrial ROS are also key in regulating VEGF expression and secretion to initiate vascular sprouting by directly stabilizing nuclear VEGF transcription through oxidative modifications of the VEGF hypoxia response element (VEGF HRE) [29], and indirectly, by ensuring HIF-1α stabilization upon hypoxia to promote VEGF transcription [30].

Due to the difficulty in selectively quantifying minute quantities of discrete oxidant species within the mitochondrial compartment, the involvement of mitochondrial ROS signaling in angiogenesis is often defined by genetic manipulation

of mitochondrial antioxidant defenses or by employing mitochondria-targeted antioxidants. Below we provide an overview of the mitochondrial proteins with proven angiogenic functions that are associated with both mitochondrial ROS detoxification (MnSOD, TRX2, TRXR2, and UCP2) and ROS production (UQCRB and PHB1).

Manganese Superoxide Dismutase (MnSOD) The existence of a mitochondrial H_2O_2-directed, redox sensitive, angiogenic pathway is strongly supported by genetic manipulation of MnSOD, the enzyme responsible for superoxide to H_2O_2 dismutation in the mitochondrial matrix. MnSOD is required for normal biological function of tissues, and global MnSOD deletion is lethal in mice because of dilated cardiomyopathy [31]. The endothelium expresses high levels of MnSOD and MnSOD expression is further enhanced upon VEGF via an NADPH oxidase-dependent mechanism [32], and upon inflammatory cytokine challenge (tumor necrosis factor, interleukin-1, lipopolysaccharide) via protein kinase C [33], to protect against oxidative damage. Indeed, targeted scavenging of mitochondrial superoxide by MnSOD overexpression improved vascular function and was protective against hypertension [34], prevented oxidation of low density lipoprotein (LDL) involved in atherosclerosis [35], and ameliorated the development of diabetic retinopathy by counteracting high glucose-induced oxidative mitochondrial DNA damage [36]. Oppositely, decreased MnSOD expression amplified oxidative stress and endothelial dysfunction in the persistent pulmonary hypertension observed in newborns [37].

With respect to vessel growth, enhanced MnSOD expression stimulates angiogenic functions. Explicitly, in lung microvessel cells, overexpression of MnSOD boosted mitochondrial H_2O_2 production, which led to PTEN oxidation and inactivation in favor of PI3K and Akt signaling [38]. Phenotypically, MnSOD increased sprouting angiogenesis and blood vessel development in the chorioallantoic membrane based upon enhanced mitochondrial ROS-dependent VEGF secretion. The pro-angiogenic effect of increased MnSOD expression was counteracted by catalase coexpression that removed excess H_2O_2 [38]. Similarly, the MnSOD mimetic MnTBAP (manganese (III) tetrakis (4-benzoic acid) porphirin) showed a strong pro-angiogenic effect substantiated by increased endothelial migration, tube formation and capillary sprouting [39]. Restored MnSOD expression also accelerated wound healing in diabetic mice in which endothelial progenitor cells were transplanted at the wound site [40]. Impaired wound healing due to defective angiogenesis is a severe complication of diabetes, and a compromised number and functionality of circulating endothelial progenitor cells contribute to defective neovascularization. Endothelial progenitor cells isolated from type 2 diabetic (*db/db*) mice displayed elevated mitochondrial superoxide levels and reduced expression and activity of MnSOD; adenoviral MnSOD gene transfer in these cells hindered mitochondrial superoxide, improved in vitro tube formation and cell adhesion, and enhanced capillary formation and wound closure in vivo [40]. In a similar manner, enhanced MnSOD expression via pharmacologic AMPK activation rescued impaired angiogenic functions of endothelial progenitor cells from streptozotocin-induced type 1 diabetic mice [41]. Conversely, siRNA-mediated knockdown of MnSOD in normal endothelial progenitor cells reduced their angiogenic activity in

diabetic wound healing assays [40]. Together, these experiments support a protective role against endothelial oxidative damage and a pro-angiogenic function for MnSOD, which might have important implications for therapeutic angiogenesis when restoring defective vascularization is required to recover organ functions.

Thioredoxin 2 (Trx2) Similar to MnSOD, the mitochondrial antioxidant enzyme Trx2 removes H_2O_2 and, as a result, exerts vaso-protective and pro-angiogenic properties. Cells deficient in Trx2 accumulate endogenous ROS and are highly sensitive to exogenous oxygen radicals, as evidenced by the embryonic lethality of Trx2-deficient mice early in development [42]. In contrast, genetic targeting of Trx2 using the endothelial-specific VE-cadherin promoter (Trx2 TG mice) produced mice that were both viable and fertile [43]. Trx2 TG mice phenotypically exhibited elevated antioxidant levels and reduced ROS (both H_2O_2 and $O_2^{\bullet-}$) and oxidative stress, as well as increased NO levels that enhanced aortic vasodilation. Pathologically, endothelial Trx2 expression also prevented the atherosclerotic plaque development in apolipoprotin E-deficient mice [43]. The pro-angiogenic role of Trx2 was substantiated during post-ischemic recovery in a femoral artery ligation model in Trx2 TG mice: the enhanced perfusion of the ischemic tissue was due to pericyte recruitment that increased capillary formation and vessel maturation [44]. Trx2 counteracted the deleterious effects of ischemia by reducing superoxide stress and blocking ASK1-JNK/p38 signaling and apoptosis to promote cell survival, while also increasing NO bioavailability to promote endothelial migration and proliferation. Noteworthy, while H_2O_2 itself is pro-angiogenic [28, 38], H_2O_2 and $O_2^{\bullet-}$ removal can lead to a similar angiogenic phenotype by increasing the concentration of pro-angiogenic NO [18, 43, 45]. Thus, stimulated expression of the antioxidant defense can facilitate normalization of injured tissue by promoting either ROS-dependent or NO-dependent endothelial proliferation and neovascularization.

Thioredoxin reductase 2 (TrxR2) The positive angiogenic regulatory function of the mitochondrial antioxidant defense on angiogenesis is reinforced by genetic inactivation of the mitochondrial TrxR2. TrxR2 is a selenoenzyme that plays an important role in the redox homeostasis by serving as an electron donor for the peroxiredoxin/thioredoxin system during H_2O_2 removal. Global knockout of TrxR2 in mice is embryonically lethal and cardiac specific TrxR2 inactivation produces offspring that perish shortly after birth due to dilated cardiomyopathy [46], which demonstrates the importance of TrxR2 for H_2O_2 detoxification during development. In endothelial cells, TrxR2 deletion increased H_2O_2 levels, inhibited endothelial nitric oxide synthase activity and suppressed VEGF-induced NO production [47]. With regard to angiogenesis, the loss of TrxR2 and the resultant abnormal redox balance impaired tumor growth and tumor-related angiogenesis by hindering Hif-1α signaling pathway. Immortalized fibroblasts lacking TrxR2 exhibited increased total cellular ROS levels that led to reduced tumor vascularization and smaller tumor mass when implanted subcutaneously into mice [48]. While the effect of TrxR2 on vascularization may at first appear counter to the ability of ROS stabilize Hif-1α [30], TrxR2-deficient tumor cells fail to activate Hif-1α signaling, and increased ROS in turn activate JNK by inhibiting JNK phosphatases that leads to

PHD2 accumulation and Hif-1α degradation [48]. Failure to stabilize Hif-1α decreased VEGF levels which resulted in a delayed angiogenic switch, reduced tumor vascularization and reduced tumor growth. Therefore, inhibition of mitochondrial ROS scavenging leads to deleterious ROS accumulation that interferes with pro-angiogenic Hif-1α and VEGF signaling and limits neovascularization.

Uncoupling Protein 2 (UCP2) Although UCP2 is not a traditional antioxidant defense protein, recent evidence supports a role for UCP2 in depressing mitochondrial ROS production [49]. Uncoupling proteins are located in the inner mitochondrial membrane and translocate protons from the intermembrane space into the mitochondrial matrix, uncoupling ATP synthesis from proton flux through the mitochondrial membrane. The physiologic functions of UCP2 include the regulation of ROS production, prevention of inflammation, and inhibition of cell death, and thus UCP2 plays central roles in cardiovascular and neurodegenerative diseases. Indeed, increasing UCP2 expression and activity protects neurons from damage following traumatic brain injury and during Parkinson's disease, as well as in atherosclerosis and myocardial ischemia [50]. However, it is the regulation of mitochondrial ROS generation in particular that has received recent attention as mild uncoupling caused by UCP2 can lower mitochondrial ROS production and afford protection against oxidative stress damage [49]. From this perspective, impaired UCP2 expression has been causally linked to defective angiogenesis while UCP2 reconstitution rescued the phenotype [51, 52]. Indeed, upregulation of UCP2 in proliferating and angiogenic endothelial cells reduced membrane potential and limited mitochondrial superoxide production that would otherwise have triggered cellular senescence [51]. In vitro, UCP2-null endothelial cells exhibited impaired proliferation and migration, as well as reduced capillary sprouting. The underlying mechanism for defective angiogenesis involved excess superoxide-dependent mitochondrial fragmentation and p53-dependent cellular senescence that limited proliferation. In agreement, UCP2 knockout mice exhibited lower blood flow and recovery during hind limb ischemia-induced angiogenesis, which could be restored by adenoviral reintroduction of UCP2 [51]. Subsequently, targeting UCP2 may constitute an important means for limiting tumor angiogenesis, and promoting mitochondrial uncoupling could enhance the angiogenic response to ischemia.

The pro-angiogenic properties of UCP2 were also noted in response to ischemia-induced AMPK activation, resulting in enhanced UCP2 expression, which decreased mitochondrial ROS production and increased NO availability [52]. AMPK signaling is essential to support neovascularization in response to hypoxic stress based on pro-angiogenic Akt activation and NO release [53]. As mentioned previously, NO promotes new vessel formation by stimulating VEGF to support endothelial migration and proliferation [18]. In excess, mitochondrial $O_2^{\cdot-}$ can rapidly interact with NO to form the peroxynitrite radical ($ONOO^{\cdot-}$), which effectively reduces NO bioavailability and resultant angiogenic signaling [45]. In agreement with AMPK involvement in angiogenesis, endothelial cells isolated from AMPKα1−/− and AMPKα2−/− mice exhibited reduced UCP2 expression and increased oxidative

stress that resulted in impaired vascular tube formation, similar to the angiogenic phenotype of UCP2-null cells [52]. AMPK-dependent upregulation of UCP2 expression and angiogenesis were confirmed in ischemic thigh adductor muscles from control mice, but were absent in AMPKα1 −/− and AMPKα2 −/− mice [52]. Together, this evidence demonstrates a positive role for UCP2 in angiogenesis similar to bona fide antioxidant enzymes (MnSOD, Trx2, TrxR2), in that UCP2 protects the vasculature by limiting deleterious ROS accumulation and stimulates vascular growth by increasing NO availability.

Ubiquinol-cytochrome c reductase binding protein (UQCRB) In addition to proteins involved in ROS detoxification, proteins that exert regulatory effects on mitochondrial ROS generation are similarly implicated in angiogenesis. UQCRB is a 13.4 kDa subunit of the mitochondrial respiratory chain complex III (ubiquinol-cytochrome c oxidoreductase) with a role in ensuring complex III assembly and maintenance. A deletion in the nuclear gene encoding UQCRB resulted in reduced respiratory function and decreased complex III enzyme activity and manifested as hypoglycemia and lactic acidosis in patients [54]. In vitro and in vivo data specify a central role for UQCRB in endothelial angiogenesis: UQCRB modulates hypoxia-induced mitochondrial ROS and HIF-1α-mediated signaling in cancer cells, UQCRB controls mitochondrial ROS-mediated VEGFR2 signaling in endothelial cells, and UQCRB is a target of the anti-angiogenic compound terpestacin [55].

In endothelial cells, UQCRB knockdown inhibited VEGF-stimulated mitochondrial superoxide production, which downregulated the activation of VEGFR2 and the downstream mediators ERK and Akt. The net result was a decrease in VEGF-induced endothelial invasiveness [56]. Similarly, pharmacological inhibition of UQCRB with the fungal metabolite terpestacin suppressed mitochondrial ROS generation and VEGF-induced angiogenesis, and potentiated the anti-angiogenic activity of the anti-VEGF monoclonal antibody bevacizumab to inhibit glioblastoma xenograft tumor growth in mice [56]. In vivo UQCRB knockdown in zebrafish also lead to the suppression of VEGF expression and inhibition of angiogeneic sprouting, without affecting vasculogenesis [57], supporting the role of UQCRB in vessel outgrowth versus neovascularization.

Mutations in the UQCRB protein also enhanced mitochondrial ROS generation and increased migration, proliferation and angiogenic activity in HEK293 cells that were attributed to HIF-1α stabilization and increased VEGF secretion. Moreover, treatment of endothelial cells with conditioned media from UQCRB mutant HEK293 cells enhanced endothelial invasiveness [58]. Overexpression of UQCRB also presented with pro-angiogenic activity via the fusion of UQCRB with a protein transduction domain (PTD) resulting in the generation of a potent cell-permeable in vitro and in vivo pro-angiogenic agent (PTD-UQCRB) [59]. PTD-UQCRB localized to the mitochondrial inner membrane and enhanced angiogenesis by stimulating mitochondrial ROS production, increasing HIF-1α stability and VEGF protein levels. PTD-UQCRB significantly increased angiogenesis within Matrigel plugs even in

the absence of VEGF and topical application enhanced wound healing by promoting increased microvessel density in the regenerating wounds.

Taken as a whole, the evidence suggests UQCRB expression positively influences angiogenesis by stabilizing HIF-1α and increasing VEGF secretion through an oxidant-dependent mechanism. However, despite the known interaction with complex III, a major site for mitochondrial ROS generation [60], the precise means by which UQCRB influences oxidant generation is not fully understood and warrants further study.

Prohibitin 1 (PHB1) Prohibitins (PHB1 and PHB2) are evolutionarily conserved proteins that localize mainly to the mitochondria, where they form ring-shaped complexes on the inner membrane [61]. They have been functionally linked to a wide range of processes that include transcriptional control, senescence, apoptosis and development. In mitochondria, prohibitins have been associated with mitochondrial biogenesis, mitochondrial dynamics, maintenance of mitochondrial membrane and cristae structure, and the assembly and stabilization of mitochondrial respiratory complexes [62]. While not a constitutive component of the electron transport chain, prohibitin 1 (PHB1) reportedly associates with the mitochondrial Complex I and contributes to angiogenesis by amending ROS levels.

In endothelial cells PHB1 is involved in cellular proliferation, migration and angiogenesis by modulating ROS production at mitochondrial complex I. Specifically, knockdown of PHB1 resulted in mitochondrial depolarization and complex I inhibition that triggered ROS generation and endothelial senescence [63]. Mechanistically, these phenotypic consequences were attributed to a sustained activation of Akt and downstream Rac1, leading to cytoskeletal rearrangements that decreased endothelial migration and the ability to form vascular tubes. A similar effect was observed in an in vivo Matrigel angiogenesis assay, where silencing of PHB1 blocked the formation of functional blood vessels [63]. Taken together, it appears that PHB1 prevents ROS-induced senescence and maintains the angiogenic capacity of endothelial cells. The mechanism by which PHB1 controls ROS production at complex I is unclear. However, PHB1 was reported to associate with complex I subunits, suggesting a possible role for PHB1 in complex I assembly or degradation [64]. Due to its chaperone-like function in stabilization of newly synthesized mitochondrial translation products and respiratory-chain complexes assembly [65] lack of PHB1 might destabilize or misassemble complex I and result in inefficient electron flow and ROS generation. In view of the above evidence, it is clear that the ROS-dependent angiogenic outcome strictly depends on the intensity of the pro-oxidant challenge, with moderate ROS signaling supporting vascular growth via activation of pro-angiogenic pathways, and excessive ROS restricting vascularization via cellular senescence or apoptosis that limit endothelial proliferation. Accordingly, a fine-tuned, complex machinery ensures ROS generation and removal, and therapeutic interventions aimed at regulating the oxidant balance (reviewed in Sect. 2.1 of this chapter) are challenging to achieve.

Mitochondrial Metabolism

As research has expanded beyond the concept of mitochondria as largely ROS generators, recent work has brought endothelial metabolism to the forefront as a viable alternative to growth factor signaling anti-angiogenic strategies [11]. As endothelial cells show a predilection towards glycolysis for endothelial growth, migration and proliferation, pharmacological interventions aimed at limiting the glycolytic pathway to influence neovascularization have been the focus of intense study [14, 66]. In contrast to glycolysis, the contribution of endothelial mitochondrial energetics to angiogenesis has been largely disregarded. This lack of study is despite the fact that the mitochondrial oxidation of glucose and other substrates is a major function of mitochondria and a significantly more efficient means of energy production compared to glycolysis. Mitochondria in endothelial cells are also the site for numerous non-energetic functions. Aside from a role in energy production, mitochondrial metabolism is the primary source for the biological building blocks of proliferating cells, regulates the metabolic flow of substrates, detoxifies many harmful byproducts of cellular processes and generates non-ROS signaling molecules. Each of these processes can impact endothelial functions and may play important primary or secondary roles in angiogenic vessel growth.

Carnitine palmitoyltransferase 1A (CPT1A) To date, fatty acid metabolism appears to be the only mitochondrial energetic pathway to support angiogenic proliferation by providing carbons for de novo nucleotide synthesis required for DNA replication [67]. Mitochondrial fatty acid oxidation (β-oxidation) requires the import of long chain fatty acids (LCFA) in the form of long-chain fatty acyl-CoAs (LCFA-CoA) into the mitochondrial matrix via the carnitine palmitoyltransferase system (CPT) [68]. CPT1A is the most abundant endothelial isoform of CPT1, the mitochondrial outer membrane enzymatic constituent of the CPT transport system, and represents the rate-limiting step in mitochondrial β-oxidation [69]. Genetic and pharmacologic inactivation of CPT1A in the vascular endothelium associated with compromised in vitro and in vivo angiogenesis due to impaired endothelial proliferation [70]. CPT1A silencing decreased vessel sprouting in cultured endothelial cells, and endothelial-specific CPT1A conditional knockout mice presented with reduced vascular branching and radial expansion of the retinal vascular plexus. Similar vascular abnormalities were noted upon pharmacological blockade of CPT1 with etomoxir, which reduced pathological angiogenesis in a model of retinopathy of prematurity. CPT1A-mediated fatty acid transport contributes to endothelial proliferation by providing TCA cycle intermediates (aspartate) required for deoxyribonucleotide synthesis during DNA replication independent of ATP generation [70]. This mechanism of deoxyribonucleotide synthesis appears to be primarily restricted to endothelial cells and fibroblasts, and to a lesser extent select lung and breast cancer cell lines. As such, fatty acid metabolism may constitute a particularly promising target to manipulate angiogenesis in the clinic.

Pyruvate dehydrogenase kinase (PDKII) Unlike fatty acid oxidation, mitochondrial glucose metabolism exerts an anti-angiogenic influence on vessel growth by preventing HIF-1α stabilization. Dichloroacetate (DCA) is an inhibitor of mitochondrial pyruvate dehydrogenase kinase (PDK) that activates pyruvate dehydrogenase (PDH) and increases mitochondrial glucose oxidation. Indeed, DCA-mediated activation of mitochondrial oxidative metabolism in glycolytic cancer cells can induce mitochondrial-directed apoptosis and limit tumor growth [71]. Inhibition of PDK by DCA in cancer cells negatively regulated angiogenesis via the inhibition of HIF-1α and downstream expression of the pro-angiogenic genes VEGF and stromal-derived factor 1 (SDF1) [72]. The effect was demonstrated both in vitro, by treating endothelial cells with tumor cell conditioned media, and in vivo, in rat xenotransplant models of non-small cell lung cancer and breast cancer. Mechanistically, DCA inhibited endothelial tube formation and tumor perfusion both by α-ketoglutarate-dependent activation of prolyl-hydroxylases, which stimulated the degradation HIF-1α, and by increasing mitochondrial H_2O_2 production to inhibit HIF-1α transcriptional activity through p53 [72].

Aldehyde dehydrogenase 2 (ALDH2) Independent of energy metabolism, mitochondrial aldehyde detoxification metabolism is another promising target for angiogenic control due to protective effects against cellular oxidative damage. Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial matrix enzyme that detoxifies numerous intermediate metabolites containing aldehyde functional groups, including acetaldehyde produced during alcohol metabolism [73]. As such, ALDH2 exerts a protective effect on cells by oxidizing both endogenous aldehydic products arising from lipid peroxidation due to oxidative stress, such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehide (MDA), as well as environmental aldehides, such as acrolein [74]. In animal models, the activation of ALDH2 reduces oxidative stress-induced cardiac following ischemic injury [75, 76].

In endothelial cells, exposure to Alzheimer-associated amyloid Aβ protein evoked a decline in ALDH2 enzymatic activity along with marked mitochondrial dysfunction that included membrane potential loss, cytochrome c release, increased superoxide generation, and the accumulation of 4-HNE adducts [77]. Aβ treatment further impaired cell proliferation and the ability to form pseudocapillary networks on Matrigel, compromised barrier function due to disorganization of VE cadherin adherens junctions and tight junction protein ZO-1, and reduced endogenous FGF-2 expression. Pharmacological ALDH2 activation with Alda-1 reversed mitochondrial dysfunction and restored the endothelial angiogenic phenotype [77]. In contrast, deletion of ALDH2 promoted the accumulation of aldehydic adducts and decreased perfusion recovery in a murine ischemic limb model [78]. The suspended tissue repair and regeneration was due to compromised revascularization and perfusion which the investigators attributed to a HIF-1α/VEGF-dependent mechanism. ALDH2 was found to regulate HIF-1α and the accumulation of its downstream angiogenic protein VEGF. ALDH2 deficiency led to unfavorable vascular sequelae including worsened blood supply after ligation of femoral artery, fewer numbers of capillary and small arteries, overt muscular atrophy, as well as reduced endothelial

cell proliferation, migration and tube formation in vitro [78]. Should these findings translate to humans, ALDH2 activation may constitute a particularly promising avenue for the treatment of age-related vasculopathies, including those associated with dementia.

3-mercaptopyruvate sulfurtransferase/H_2S pathway Stimulation of the mitochondrial hydrogen sulfide (H_2S) synthesis pathway is also supportive of endothelial angiogenic proliferation. H_2S is an endogenous gas which can induce vasodilation and angiogenesis via the VEGF pathway [79]. H_2S is mainly generated in the mitochondria from 3-mercaptopyruvate (3MP) by 3-mercaptopyruvate sulfurtransferase (3-MST), and the 3-MP/3-MST/H_2S pathway acts as a physiological stimulator of angiogenesis [80]. Specifically, 3-MP application facilitated the migration and proliferation of microvascular endothelial cells by increasing Akt activation, whereas silencing of 3-MST inhibited the proliferative phenotype. 3-MP also elicited a concentration-dependent increase in microvessel sprouting in rat aortic rings and exerted a pro-angiogenic effect in vivo by promoting the neovascularization of Matrigel plug implants and wound closure in rats. Besides activation of Akt-dependent cellular proliferation, H_2S fueled cellular proliferation by ATP generation through stimulation of mitochondrial bioenergetics at complex II of the mitochondrial electron transfer chain [80]. Thus activation of the mitochondrial pathway for endogenous H_2S synthesis can provide an additional control point for therapeutic angiogenesis in wound-healing and post-ischemic therapy.

Mitochondrial Calcium

Calcium is a ubiquitous second messenger that is an absolute requirement for most vascular endothelium functions, including the synthesis of nitric oxide and vWF, control of vascular tone, vascular permeability, initiation of the inflammatory response, endothelial motility and proliferation, and angiogenesis [81]. Specifically, extracellular mitogens and pro-angiogenic factors bind to endothelial surface receptors, triggering an increase in cytosolic calcium originating from both intracellular stores and the extracellular milieu. Mitochondria modulate calcium signaling by buffering excess cytosolic calcium while at the same time responding metabolically to these calcium signals to fine-tune their functions and elicit a cellular response [82]. Mitochondrial calcium trafficking occurs by uptake mainly through the mitochondrial calcium uniporter and release via the Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers and the mitochondrial permeability transition pore (mPTP) [83] (see Chap. 2 for details).

Cyclophilin D (CypD) Cyclophilin D (CypD) is a peptidyl-prolyl *cis-trans* isomerase localized to the mitochondrial matrix, and a regulatory element of calcium-induced mitochondrial permeability transition pore opening [84]. In vivo, CypD deletion coincides with elevated mitochondrial matrix calcium levels and causes reduced cardiac metabolic flexibility and propensity for heart failure, but is

protective against ischemia/reperfusion-induced cell death [85, 86]. Our laboratories were the first to demonstrate a role for CypD in angiogenesis through a complex mechanism involving mitochondrial calcium dependent changes in NAD^+/NADH homeostasis and a SIRT1/Akt signaling axis [87]. We identified a similar mitochondrial calcium-directed and NAD^+/NADH-dependent SIRT1 regulatory mechanism that coordinated endothelial inflammation and leukocyte adhesion [88]. NAD^+/NADH balance regulates intracellular redox state and controls enzyme and transcription factor activities in discrete cellular compartments, due to a substantial mitochondrial localization for NAD^+/NADH and mitochondrial membrane impermeability towards NAD^+ and NADH [89]. We showed in cultured primary endothelial cells that genetic inactivation of CypD was accompanied by an increase in mitochondrial matrix calcium and bioenergetics without net ATP generation, which led to mitochondrial accumulation of NADH and decreased NAD^+/NADH ratios in the mitochondrial and cytosolic/nuclear compartments [87]. These changes commanded a reduction in SIRT1 expression and SIRT1-dependent PTEN inactivation, ultimately leading to Akt phosphorylation. This translated as increased endothelial proliferation and sprouting angiogenesis that sped revascularization and wound closure in CypD-deficient mice [87]. While genetic SIRT1 deletion inhibits sprouting angiogenesis and neovessel formation [90] our results demonstrate a negative relationship between SIRT1 and angiogenesis when SIRT1 levels and activity are only diminished by the absence of CypD. Thus this study proposes a new level of angiogenic control involving mitochondrial calcium and NAD^+/NADH metabolism that does not engage mitochondrial ROS signaling.

Mitochondrial Dynamics

For many years considered static structures, mitochondria are now appreciated for their dynamic ability to move within a cell, as well as divide and fuse with neighboring mitochondria. This dynamic nature of mitochondria allows them to adapt to changing intracellular and environmental demands, translocate to areas of energetic need, alter mitochondrial metabolism, and remove damaged or dysfunctional mitochondria [91]. Disruption in these mitochondrial dynamics impairs cell function and has been linked with neurodegenerative diseases [92]. Physiologically, mitochondrial movement along microtubules allows recruitment and clustering of mitochondria at specific subcellular locations, such as in close contact with the endoplasmic reticulum to ensure efficient calcium release and transfer (see Chap. 10). In endothelial cells mitochondrial mobility plays an important role in hypoxia-directed VEGF transcriptional activation and thus might have important consequences for angiogenic initiation. During hypoxia, endothelial mitochondria clustered around the nucleus through a dynein motor-driven and microtubule-dependent mechanism that increases ROS levels within the nucleus [29]. Disruption of mitochondrial clustering suppressed this nuclear ROS increase without

affecting HIF-1α accumulation. However, despite accumulation within the nucleus, HIF-1α failed to associate with the hypoxia response element sequences required for VEGF transcription. The observation that antioxidant ROS suppression attenuated mitochondrial nuclear clustering supports the idea that mitochondrial ROS are responsible for initiating mitochondrial movements around the nucleus (see Chap. 1). As such, mitochondrial motility ensures localized ROS delivery and a targeted cellular response.

Mitochondrial fusion and fission, besides defining mitochondria morphology, are important regulators of mitochondrial apoptosis, with fission facilitating apoptosis and fusion playing a protective role against it, possibly by modulating mitochondrial outer membrane permeabilization [93]. Mitochondrial fusion and fission events also serve physiological roles in regulating mitochondrial respiration and cell growth, as perturbations in both fusion and fission impact cell division and therefore are important modulators of angiogenic processes.

Mitofusins (Mfn1 and Mfn2) Mfn1 and Mfn2 are mitochondrial membrane GTPases that mediate fusion events in outer membrane. Experimentally, deletion of either of the two isoforms of Mfn (Mfn1 and Mfn2) impairs mitochondrial fusion and results in exaggerated fission events that present as small, fragmented mitochondria throughout the cell. Global deletion of either Mfn1 or Mfn2 is embryonically lethal in mice [94]. However, in cultured cells and select tissues, mitochondrial fusion defects negatively influence electron transport and respiration, resulting in impaired cell growth [95]. In endothelial cells, the silencing of Mfn1 or Mfn2 decreased VEGF-mediated endothelial migration and the ability of endothelial cells to form vascular network structures on Matrigel. This angiogenic defect was attributed to decreased cellular viability and augmented apoptosis, and was supported by impaired mitochondrial functions and fragmentation of mitochondrial networks. Despite similar angiogenic phenotypes, the two mitofusins rely on different signaling mechanisms: Mfn1 ablation reduces VEGF-stimulated Akt-eNOS signaling, while Mfn2 knockdown significantly decreased ROS generation and reduced mitochondrial respiratory chain and oxidative metabolism gene expression [96]. This data supports an important role for mitochondrial dynamics in endothelial cell viability and angiogenic potential.

Dynamin-related protein 1 (Drp1) In contrast to mitochondrial fission, deletion of the master regulator of mitochondrial fission dynamin-related protein 1 (Drp1) results in a highly interconnected and reticulated mitochondrial network [97]. Drp1 is essential for brain and embryonic development, and like the Mitofusins, Drp1 deletion is embryonically lethal in mice [98]. At the mitochondrial level, Drp1 mutants exhibit defects in endoplasmic reticulum calcium signaling that sensitizes cells to mitochondrial calcium-dependent apoptosis [99], with important implications for cellular proliferation. In cultured pulmonary artery endothelial cells, hypoxia increased Drp1 expression and mitochondrial fragmentation, which promoted migration and proliferation of endothelial cells while inhibiting apoptosis in response to mitochondrial calcium overload [100]. Conversely, Drp1 silencing

inhibited hypoxic-mediated angiogenesis and manifested as reduced tube formation and migration, decreased cellular proliferation, and increased apoptosis due to mitochondrial calcium accumulation. Proliferation of endothelial cells plays a key role in excessive pulmonary angiogenesis and endothelial dysfunction. In a murine model of pulmonary arterial hypertension, treatment with the Drp1 inhibitor Midivi-1 reversed the elevated right ventricular systemic pressure and reduced the increase in microvessel density associated with this disease [100]. Hence targeting mitochondrial fragmentation could become a viable alternative for the inhibition of pathologic angiogenesis.

Mitochondrial Apoptosis

The balance between endothelial angiogenesis and vascular regression is important for the development, maintenance and remodeling of the circulatory system. In endothelial cells, apoptosis is a physiological necessity for vessel regression and can significantly limit angiogenesis by counteracting proliferation. As a result, the selective stimulation of endothelial apoptosis may constitute a promising candidate for anti-angiogenic therapy [101]. Mitochondria actively participate in the apoptotic program through the intrinsic apoptotic pathway by releasing pro-apoptotic molecules (cytochrome c, Smac/DIABLO, Omi/HtrA2) that activate downstream caspases and initiate cellular degradation [102].

B cell lymphoma-2 (Bcl-2) Bcl-2 is an anti-apoptotic member of the Bcl-2 family (together with BCL-X_L, BCL-W, MCL1, BCL-B, and pro-apoptotic BAX, BAK, BOK) that localizes to the outer mitochondrial membrane and regulates apoptosis by controlling cytochrome c release [103]. In agreement with an anti-apoptotic role, pharmacological inhibition and siRNA knockdown of Bcl-2 reduced migration and tube formation in endothelial cells and enhanced growth-factor deprivation-induced apoptosis, while Bcl-2 overexpression had the opposite effect [104]. Similarly, Bcl-2 transfection in senescent endothelial cells that displayed markedly diminished Bcl-2 levels reduced mitochondrial oxidative stress by stimulating glutathione translocation into mitochondria, which improved mitochondrial function and amended their impaired angiogenic capacity. Thus, Bcl-2 plays a crucial role in the regulation of the mitochondrial redox state and Bcl-2 loss exacerbates the impaired angiogenesis by augmenting oxidative stress. In vivo, Bcl-2 adenoviral gene transfer significantly improved angiogenesis in Matrigel plugs implanted into aged mice, whereas the Bcl-2 inhibition reduced angiogenesis in Matrigel plugs implanted into young animals [104]. Accordingly, pharmacological control of mitochondrial apoptotic balance is a viable strategy to manipulate angiogenesis, by ensuring either pro-apoptotic or anti-apoptotic outcomes. An overview of the mitochondrial-targeting compounds with pro-apoptotic and anti-angiogenic properties will be presented in a subsequent section.

Mitochondrial Biogenesis

The dynamic regulation of mitochondrial mass to meet changes in cellular energetic demand is under the transcriptional control of DNA-binding factors that include ERRα, ERRβ, NRF-1, NRF-2, CREB, c-MYC, and PPARγ, and several coregulators modulate transcription factor activity, such as PGC-1α, PGC-1β, PRC and RIP140 [105]. Given that mitochondrial energy production is not required for angiogenic growth, whether the increased glycolytic rate in proliferating endothelial cells is influenced by mitochondrial content is not explicit. Nonetheless, the limited evidence available does support a role for mitochondrial biogenesis during angiogenesis. Firstly, the master regulator of mitochondrial biogenesis, PGC-1α was found to be a strong regulator of VEGF expression and angiogenesis via an ERR-α dependent but HIF-1α-independent pathway [106]. Additionally, PGC-1α played a protective role against oxidative stress in the vascular endothelium via positive regulation of the endothelial mitochondrial antioxidant defense system [107]. Secondly, gene array analysis revealed that VEGF stimulation facilitated the upregulation of several nuclear-encoded mitochondrial genes [108]. Mechanistically, the upregulation of mitochondrial genes was dependent upon the expression of Akt3, silencing of which reduced mitochondrial content as detected by decreased mitochondrial DNA levels, gene expression, and respiratory function. Akt3 further directed mitochondrial biogenesis by modulating the subcellular localization of PGC-1α through control of the major nuclear export receptor CRM-1 [109]. Finally, the angiogenic phenotype of Akt3-null and heterozygous mice supports a role for mitochondrial biogenesis in angiogenesis. Akt3 null animals failed to launch an angiogenic response to growth factor challenge and exhibited a dose-dependent decrease in angiogenesis [109]. In addition, Akt3 knockout animals were unable to form mature, stabilized vasculature compared to WT littermates as assessed by Matrigel plug assay, and cells within Matrigel plugs had reduced mitochondrial content during angiogenesis. Similarly, Akt3 deletion evoked larger, less narrow tubules in Matrigel plugs in vitro, suggesting defects in the cell-cell interactions required for angiogenesis [109]. Taken together, these studies demonstrate that the Akt3/ PGC-1α pathway provides a means to coordinate mitochondrial gene expression and function with growth factor-induced angiogenesis. Whether the increase in mitochondrial mass is necessary to support a surge in cellular energy demand, or to provide enhanced oxidative or metabolic signaling to support angiogenic growth requires further investigation.

Mitochondrial-Targeted Compounds with Pro-angiogenic and Anti-angiogenic Properties

Current therapies aimed at manipulating angiogenesis in cancer patients target growth factors and their receptors, but over time are often met with compensatory cellular adaptations and therapeutic resistance [110]. In theory, endothelial mitochondria would be attractive therapeutic targets for modulating angiogenesis to

counter drug resistance because of their ability to regulate both endothelial proliferation and death. For instance, mitochondrial agents that block endothelial proliferation or stimulate apoptosis would be desirable therapies to limit cancer progression. On the other hand, mitochondrial-targeted compounds that stimulate endothelial growth could be employed to facilitate new vessel growth to counteract hypertension or support the recovery of organs damaged by ischemia/reperfusion injury. As discussed earlier in this chapter, endothelial mitochondrial function can be manipulated to activate both anti- and pro-angiogenic signaling mechanisms. Below we present an overview of compounds with known effect on mitochondrial functions and implications in angiogenesis control, based on their ability to target the generation/scavenging of mitochondrial ROS, activate/inhibit apoptosis, increase mitochondrial membrane permeability, or disrupt the flow of electrons through the respiratory chain (Table 21.1).

Table 21.1 Mitochondrial-targeted compounds with pro-angiogenic and anti-angiogenic properties

Compound	Target/mechanism	Effect on angiogenesis	Ref.
MitoQ	Antioxidant	Anti-angiogenic	[111, 114]
		Anti-apoptotic	
SKQ1	Antioxidant	Anti-angiogenic	[115, 116–118]
		Pro-angiogenic	
MitoVit-E	Antioxidant	Anti-apoptotic	[114]
Cyclosporin A	Pro-oxidant	Pro-angiogenic	[119]
Matairesinol	Antioxidant	Anti-angiogenic	[112]
Terpestacin	UQCRB	Anti-angiogenic	[55]
HDNT	UQCRB	Anti-angiogenic	[113, 120]
Red wine polyphenols		Pro-angiogenic (low dose)	[121]
Auraptene	Complex I	Anti-angiogenic	[122]
Embelin		Anti-angiogenic	[17]
GSAO	mPTP/ANT	Anti-angiogenic, pro-apoptotic	[123, 124]
PENAO	mPTP	Anti-angiogenic, pro-apoptotic	[124]
α-TOS	Complex II	Anti-angiogenic, pro-apoptotic	[125]
α-TEA	Complex II	Anti-angiogenic, pro-apoptotic	[125]
MitoVES	Complex II	Anti-angiogenic, pro-apoptotic	[126]
Canstatin	$\alpha_v\beta_3$ and $\alpha_v\beta_5$	Anti-angiogenic, pro-apoptotic	[127]
Endostatin	HK2/VDAC1	Anti-angiogenic, pro-apoptotic	[128]
Survivin inhibitor	IAP	Anti-angiogenic, pro-apoptotic	[129]
Paclitaxel	Microtubules	Anti-angiogenic, pro-apoptotic	[130]
Silibilin		Anti-angiogenic, pro-apoptotic	[131]
Neovastat		Anti-angiogenic, pro-apoptotic	[132]
Angiostatin	MDH2 , ATP synthase	Anti-angiogenic, pro-apoptotic	[133, 134, 134, 135]
		Anti-proliferative	
Magnolol		Anti-angiogenic, pro-apoptotic	[136]
Bet-CA		Anti-angiogenic, pro-apoptotic	[137]

Modulators of Mitochondrial ROS Scavenging and ROS Production

The potential therapeutic benefits of enhancing mitochondrial ROS scavenging capacity were evident from the deleterious effects exerted by augmented ROS generation in genetic models lacking mitochondrial antioxidant proteins [31, 42, 46]. In experimental models, compounds that suppress mitochondrial ROS production effectively destabilize HIF-1α and inhibit the downstream expression of pro-angiogenic genes such as VEGF, resulting in strong anti-angiogenic and anti-proliferative effects not only on endothelial cells, but also on the tumor cells that secrete angiogenic growth factors (MitoQ, Matairesinol, Terpestacin, HDNT) [55, 111–113].

On the other hand, compounds that enhance mitochondrial pro-oxidant load facilitate endothelial apoptosis and suppress angiogenesis (α-TOS, α-TEA, MitoVES) [125, 126], and will be reviewed in the section dedicated to pro-apoptotic mitochondrial compounds. However, initial efforts aimed at manipulating/scavenging mitochondrial ROS to prevent and treat degenerative diseases have been largely disappointing, possibly due to poor uptake and delivery of antioxidants specifically to mitochondria [138]. Next generation mitochondrial-targeted antioxidants more effectively target mitochondria by conjugating the lipophilic triphenylphosphonium cation to an antioxidant moiety. Owing to their net positive electrical charge, these compounds permeate through biological membranes and accumulate in high concentrations within the negatively charged mitochondrial matrix [139]. However, despite proven antioxidant properties, mitochondrial-targeted compounds can participate in redox cycling at high concentrations, resulting in electron leaks and increased ROS generation. This pro-oxidant behavior, in particular at high compound concentrations, has detrimental effects on mitochondrial functions and triggers apoptosis, limiting practical in vivo utilization [140, 141]. As a result, despite proven benefits in scavenging mitochondrial ROS, the effects of these compounds are highly dependent upon the overall oxidant load within the endothelium.

MitoQ and MitoVit-E MitoQ is a mitochondrial-targeted antioxidant synthesized by covalent attachment of ubiquinone to a lipophilic triphenylphosphonium cation. The compound accumulates inside energized mitochondria in a membrane-potential dependent manner and shows recyclable antioxidant activity, being regenerated by the respiratory chain [142]. In cultured cells MitoQ prevented lipid oxidation and protected mitochondria against oxidative damage and hydrogen peroxide-induced apoptosis [142]. The antioxidant properties resulted from repressed ROS production during reverse electron transport at mitochondrial complex I, but a pro-oxidant effect was also noted corresponding to forward electron transport [143]. However, the antioxidant effect was prevalent in porcine aorta endothelial cells where MitoQ treatment scavenged the increase in mitochondrial ROS stimulated during hypoxia, and also inhibited the hypoxic- and ROS-dependent endothelial proliferation via MEK/ERK inactivation [111]. MitoQ efficiently buffered glucose/glucose oxidase-induced oxidative stress in bovine aortic endothelial cells and prevented oxidative

stress-induced apoptosis by restraining cytochrome-c release, caspase 3 activation and DNA fragmentation [114]. A similar antioxidant and anti-apoptotic effect in endothelial cells was provided through enhanced scavenging of mitochondrial ROS by supplementation with **MitoVit-E** [114], the mitochondrial compound obtained by conjugating the active antioxidant moiety of vitamin E to the lipophilic triphenylphosphonium cation [144].

SkQ1 SkQ1 (10-(6′-plastoquinonyl) decyltriphenylphosphonium) is a potent, membrane permeable, mitochondria-targeted antioxidant containing a plastoquinone attached by a decane linker to positively charged phosphonium. This derivative exerted enhanced antioxidant activity compared to MitoQ along with much lower pro-oxidant tendencies [145]. SkQ1 showed a strong inhibitory effect on tumor growth with a concomitant anti-angiogenic activity on tumor blood vessels [115]. Dietary supplementation of SkQ1 was found to suppress spontaneous tumor development in p53−/− mice and to inhibit growth of human colon carcinoma xenografts by restraining tumor cell proliferation. In vivo angiogenesis in subcutaneous Matrigel implants was also reduced by SkQ1 that decreased the content of small blood vessels in the implants. The anti-angiogenic and anti-tumor properties of SkQ1 possibly involved scavenging of mitochondrial ROS, which stabilize HIF-1α and stimulate of Ras signaling to promote endothelial growth and phenotypic transformation. However, while these results appear promising, SkQ1 does not provide universal benefit at retarding tumor growth in all cancer models. Specifically, SkQ1 proved ineffective at reducing tumor growth and improving survival in a murine pancreatic ductal adenocarcinoma that manifest with increased levels of pro-angiogenic factors both at tumor level (b-cellulin, VEGF) and systemically (angiopoietin, endolgin) [116]. Aside from cancer, SkQ1 increased transcription and translation of VEGF in a rat model of age-related macular degeneration [117]. A pro-angiogenic effect for SkQ1 is also supported by improved dermal wound healing in old mice that consistently showed poor wound recovery [118]. Mechanistically, dietary SkQ1 supplementation resolved inflammation and increased vessel content in the wound to speed dermal healing. In vitro, while SkQ1 did not have a direct effect on endothelial migration in vitro, treatment of endothelial cells with conditioned media from SkQ1-treated fibroblasts promoted migration and tubulogenesis on Matrigel due to elevated TGFb1 [118]. SkQ1 also prevented TNF-induced disorganization of the actin cytoskeleton and VE-Cadherin contacts in the endothelial monolayer, thus preventing the excessive endothelial response to pro-inflammatory cytokines [118].

UQCRB inhibitors Compounds binding the UQCRB subunit of mitochondrial complex III, including terpestacin and HDNT, show strong anti-angiogenic properties based on suppression of hypoxia-induced VEGF signaling [55, 113]. During hypoxia, mitochondria act as cellular oxygen sensors and generate H_2O_2 at complex III to stabilize HIF-1α [146]. UQCRB inhibitors block hypoxia-induced ROS generation at complex III and prevent HIF-1α stabilization, hindering tumor secretion of VEGF and vascular growth. **Terpestacin**, a small molecule identified in a screen of microbial extracts, functions as an angiogenic inhibitor by reducing in vitro and

in vivo angiogenesis, as evidenced by compromised vascular tube formation and decreased blood vessel density in tumor xenografts [55]. Mechanistically, terpestacin disrupted mitochondrial membrane potential and decreased ROS generation without affecting mitochondrial respiration [55]. The small molecule **HDNT** (6-((1-hydroxynaphthalen-4-ylamino)dioxysulfone)-2H–naphtho[1,8-bc]thiophen-2-one) was identified by pharmacological screenings as a synthetic inhibitor of UQCRB with anti-angiogenic properties [113]. HDNT selectively inhibited endothelial cell growth and suppressed VEGF-induced endothelial invasion and tube formation without affecting cell viability. Similar to terpestacin, the mechanism involved a dose-dependent decrease in mitochondrial ROS production, which subsequently inhibited HIF-1α accumulation and VEGF expression during hypoxia [113]. Derivatives of HDNT obtained by functionalization with a sulfonylamide backbone were non-toxic and suppressed mitochondrial ROS-mediated hypoxic signaling and inhibited angiogenesis without toxicity [120]. These compounds effectively hindered neovascularization of the chorioallantoic membrane and during xenograft tumor growth by reductions in HIF-1α and VEGF. The antitumor and anti-angiogenic activity of these derivatives was several-fold more pronounced than of the natural UQCRB inhibitor terpestacin [120].

Matairesinol The lignan matairesinol ((αR,βR)-α,β-bis (4-hydroxy-3-methoxybenzyl) butyrolactone) is a natural antioxidant molecule extracted from *Cedrus deodara* [147]. The anti-angiogenic properties of matairesinol are based on in vitro and in vivo suppression of angiogenesis via disruption of hypoxic mitochondrial ROS production [112]. At non-toxic concentrations, matairesinol inhibited endothelial proliferation, chemoinvasion, tube formation, and angiogenesis of the chorioallantoic membrane, which were mechanistically linked to decreased hypoxia-induced HIF-1α expression and VEGF secretion [112].

Cyclosporin A (CsA) CsA is an immunosuppressant drug used for organ transplantation that binds to cyclophilin A and inhibits calcineurin-dependent NFAT activation and immune response. However, long term use of CsA increases cancer incidence, which cannot be fully explained based on the calcineurin-dependent mechanism [148] CsA also binds the mitochondrial cyclophilin D, a modulator of mPTP opening, and protects against calcium- and oxidative stress-induced necrotic cell death [149]. Recent evidence identified a pro-angiogenic role for CsA, independent of calcineurin signaling, which promoted tumor growth due to mitochondrial ROS accumulation [119]. In mice, CsA treatment increased xenograft and carcinogen-induced tumor growth with pronounced microvessel density. Mechanistically, CsA-directed endothelial migration and proliferation were supported by ERK1/2 activation due to increased mitochondrial ROS [119], which may be due to the inhibitory effect of CsA on mPTP opening and resultant ROS accumulation within mitochondria. Both endothelial proliferation and tumor growth were blocked by antioxidant supplementation, further confirming the involvement of mitochondrial ROS in CsA-directed angiogenesis. Alternatively, independent of ROS generation, the pro-angiogenic effect of mPTP inhibition can be mechanistically linked to mitochondrial calcium accumulation and NAD^+/NADH metabolism-directed Akt activation and endothelial proliferation [87].

Compounds Targeting Mitochondrial Respiratory Capacity

Based on the preferential utilization of glycolysis, and despite a close proximity to oxygenated blood, mitochondrial respiration is lower in endothelial cells than most other oxidative cell types [11] where oxidative phosphorylation subsidizes the cellular energetic demand. Nevertheless, reduced endothelial oxygen utilization is advantageous to preserve oxygen for the underlying tissue, minimize endothelial oxidative damage, and sustain angiogenic ability during hypoxia. Interestingly, endothelial mitochondria retain a significant respiratory reserve that can be accessed to cope with oxidative stress [15]. Targeting mitochondrial respiration and respiratory reserve support a key role for mitochondrial function in establishing angiogenic phenotypes. Compounds that fuel endothelial respiratory capacity are pro-angiogenic (red wine polyphenols) [121] while compounds with inhibitory effects on either endothelial respiration or respiratory reserve prevent neovascularization (embelin, auraptene) [17, 122].

Red wine polyphenols Red wine polyphenolic compounds (RWPC) are red wine extracts that exert protective effects on the cardiovascular system and modulate angiogenesis. However, the effects of RWPC on vascular growth are often paradoxal and concentration-dependent: a low dose (0.2 mg/kg body weight) was pro-angiogenic while a high dose (20 mg/kg body weight) was anti-angiogenic in a rat model of post-ischemic neovascularization [150]. When probing the underlying mechanisms of these findings, it is likely that the beneficial effects of low RWPC doses increased mitochondrial respiratory capacity via an upregulated expression of mitochondrial biogenesis-related genes and increased mitochondrial DNA content [121]. Phenotypically, low doses of RWPC further promoted in vitro capillary tube formation and elongation. High doses of RWPC had no effect on mitochondrial capacity, and inhibition of mitochondrial protein synthesis abolished the pro-angiogenic phenotype observed at low RWPC doses [121]. This data advocates the requirement for increased mitochondrial mass and respiratory function during vessel growth to support the synthesis of either metabolic or oxidative intermediates to promote endothelial proliferation.

Embelin The importance of endothelial respiratory reserve for angiogenic growth is demonstrated through the mitochondrial inhibition by embelin. Embelin is a naturally occurring hydrobenzoquinone from the fruit of Embelia ribes, with anti-tumor and anti-inflammatory properties [151]. Embelin acts as an anti-angiogenic agent that impairs neovascularization associated with tumor growth and wound healing without adversely effecting pre-existent blood vessels [17]. Specifically, embelin treatment reduced tumor microvessel density, which limited tumor growth in xenograft mouse models and attenuated wound closure in mice. Mechanistically, embelin preferentially induced cell death in proliferating but not quiescent endothelial cells independent of ROS production by uncoupling mitochondria and depleting the low respiratory reserve of proliferating cells functioning close to their bioenergetic limit [17]. Unlike quiescent cells, proliferating endothelial cells engage in processes

that require increased energy demand and metabolic adaptation and therefore they are more susceptible to energetic impairment. Consequently, depletion of the respiratory reserve would make these cells more susceptible to secondary stresses and cell death [15], a feature that can be exploited therapeutically to inhibit pathologic angiogenesis.

Auraptene Suppression of mitochondrial respiration by auraptene is also inhibitory for pathologic angiogenesis. Auraptene is a natural coumarin derivative derived from citrus fruits that negatively targets mitochondrial complex I to limit tumor cell proliferation [152]. The anti-proliferative effect of auraptene on tumor cells was complemented by an inhibitory effect on tumor neovascularization, as witnessed by reduced density and blood vessels infiltration in Matrigel plugs and impaired vascularization of tumor xenografts [122]. Mechanistically, the anti-angiogenic effect of auraptene correlated with reduced VEGF levels due to inhibition of HIF-1α translation [122], although a direct link between suppressed respiratory function and HIF-1α was not established and requires further study.

Pro-apoptotic Mitochondrial Compounds

Several groups of compounds initiate apoptosis either by targeting directly mitochondria (arsenical compounds, vitamin E analogs) or indirectly by engaging cytosolic signaling pathways that terminate at mitochondrial apoptosis (survivin inhibitors, paclitaxel) [153]. Anti-cancer agents that target and destabilize mitochondria have been termed “mitocans” [154], and negatively influence mitochondrial function through multiple mechanisms. Induction of endothelial apoptosis is an efficient way to tamper angiogenesis by counteracting proliferation and preventing vessel growth, to limit tumor progression and metastasis. Both cytotoxic and anti-angiogenic cancer therapies depend on endothelial apoptosis, the first because endothelial apoptosis precedes cancer cell death due to immediate contact with the chemotherapeutic drugs, and the second because direct targeting of the endothelium triggers endothelial apoptosis and reduces tumor vascularization and growth [155]. The mechanisms of endothelial apoptosis include mPTP activation by targeting of regulatory pore components, increased generation of mitochondrial ROS, and suppression of anti-apoptotic protein functions.

Arsenical derivative compounds The trivalent arsenical compound phenylarsine oxide (PAO) is a potent activator of the mPTP but presents high cellular toxicity [156]. In response, a membrane-impermeable, hydrophilic derivative of PAO, **4-(N-(S- glutathionylacetyl)amino) phenylarsonous acid** (**GSAO**), was formulated by conjugation with a cysteine thiol of reduced glutathione, to facilitate less toxic metabolism at the cell surface [123]. The product resulting from the extracellular metabolism of GSAO contains the arsenical moiety and concentrates in the mitochondria to trigger cell death [123, 157]. GSAO has a high selectivity towards proliferating endothelial cells compared to both quiescent endothelial and tumor cells,

and strongly inhibits tumor angiogenesis via mPTP-dependent cell death [123]. The anti-angiogenic effect of GSAO was shown in the chick chorioallantoic membrane and solid subcutaneous tumors that presented with reduced microvessel density and tumor growth without organ toxicity [123]. Mechanistically, GSAO targeted the mPTP modulator adenine nucleotide translocator (ANT) to impair mitochondrial and cellular function via superoxide accumulation, loss of mitochondrial membrane potential, mPTP opening, ATP depletion, caspase activation and surface presentation of phosphatidylserine [123]. A GSAO analog in which the arsenical moiety was localized at the *ortho* position on the phenyl ring (***o*-GSAO**) was found to be a more potent inhibitor of endothelial proliferation and tumor growth, but showed toxic side effects due increased cellular accumulation [124]. Similarly, a cysteine mimetic analog of GSAO, **4-(N-(S-penicillaminylacetyl)amino) phenylarsonous acid** (**PENAO**), exhibited faster endothelial accumulation and stronger anti-proliferative and antitumor efficacy following the same apoptotic mechanism of GSAO without obvious signs of cellular and organ toxicity [158].

Vitamin E analogs Vitamin E analogs with pro-apoptotic function are potent anti-cancer agents that selectively trigger apoptosis in cancer cells. These compounds do not possess the antioxidant activity of other vitamin E derivatives and are considered "redox-silent" vitamin E analogs [159]. Whether the derivative will display pro-apoptotic rather then antioxidant behavior depends upon modifications both within the structural moieties of the vitamin E functional domain together with conformational requirements that modulate compound activity [160]. **α-Tocopheryl-succinate (α-TOS)** and **α-tocopheryloxyacetic acid (α-TEA)** are redox-silent, pro-apoptotic vitamin E analogs. In addition to a strong anti-tumor activity, α-TOS and α-TEA exhibit markedly anti-angiogenic properties by triggering apoptosis specifically in proliferating endothelial cells [125]. The apoptotic activity of α-TOS and α-TEA revolves around the ability to inhibit the binding site of ubiquinone at complex II, resulting in the accumulation of mitochondrial ROS [125]. Phenotypically, endothelial apoptosis was responsible for decreased vascular tube formation in vitro, and compromised wound healing and reduced tumor volume and vascularization in vivo [125]. **Mitochondrial-targeted vitamin E succinate** (**MitoVES**) is a potent pro-apoptotic agent obtained upon conjugation of vitamin E succinate with the positively charged triphenylphosphonium group. MitoVES blocks mitochondrial electron transfer from complex II to complex III and interacts with the UbQ-binding site of complex II, enhancing ROS production and triggering ROS-dependent apoptosis [161]. MitoVES showed a strong anti-angiogenic activity via inhibition of endothelial migration, tube formation and neovascularization in models of breast carcinoma and chronic ischemic wound [126]. Noteworthy, the pro-apoptotic effect was specific for proliferating endothelial cells, since contact-arrested cells exhibited limited mitochondrial uptake of MitoVES due to reduced mitochondrial membrane potential.

Collagen-derived compounds Remodeling of the extracellular matrix (ECM) facilitates endothelial migration and proliferation, and proteolytic fragments of ECM known as matrikines are notorious anti-cancer agents and inhibitors of

angiogenesis [162]. Collagen-derived matrikines inhibit tumor growth by targeting both tumor and endothelial cells, restraining their migration and proliferation, while triggering apoptosis [163]. **Canstatin** is a 24-kDa peptide derived from the NC1 domain of the α2 chain of type IV collagen. Canstatin inhibited vascular proliferation both in vitro and in vivo by triggering endothelial death via the combined activation of extrinsic (non-mitochondrial) and intrinsic mitochondrial apoptotic pathways [164]. Mechanistically, extrinsic apoptosis was initiated through membrane death receptors and involved Akt-dependent FLIP downregulation, induction of Fas-ligand expression and caspase-8 activation. Intrinsic apoptosis was triggered by canstatin binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell membrane of both endothelial and tumor cells, followed by disruption of mitochondrial membrane potential and caspase-9 activation [127]. **Endostatin** is a 20 kDa C-terminal fragment of collagen XVIII and one of the most potent inhibitors of angiogenesis. First reports on the pro-apoptotic activity of endostatin showed a marked reduction in the expression of anti-apoptotic proteins Bcl-2 and Bcl-X_L that favored endothelial apoptosis [165]. Another mechanism involved mPTP-dependent apoptosis facilitated by upregulation of voltage-dependent anion channel 1 (VDAC1) as a result of endostatin-directed decrease in the glycolytic enzyme hexokinase 2 (HK2) expression [128]. mPTP activation by endostatin triggered endothelial cell death via loss of mitochondrial membrane potential, mitochondrial membrane permeabilization, cytochrome c release and caspase 9 activation [128].

Survivin inhibitors Survivin is a member of the inhibitor of apoptosis (IAPs) family of proteins. While survivin is largely absent in adult tissues its expression is markedly upregulated in most cancers and angiogenic endothelial cells [166]. Because of this cancer- and angiogenic endothelial-selective expression, the survivin pathway is a viable target for anti-tumor and anti-angiogenic treatments. In tumor cells specifically, survivin expression positively regulates angiogenesis by stimulating the synthesis and secretion of pro-angiogenic VEGF [167]. Thus targeting survivin may have a dual anti-cancer effect by inducing tumor cell apoptosis and suppressing angiogenesis via the localized release of angiogenic growth factors. Indeed, inhibition of the survivin pathway by adenoviral expression of a phosphorylation-defective protein mutant exhibited strong anti-cancer activity by triggering both cancer and endothelial cell apoptosis that subsequently blocked tumor growth and reduced tumor blood vessel density in human breast cancer xenograft models [129]. A DNA vaccine targeting survivin delivered against non-small cell lung carcinoma and deriving metastasis induced a strong T-cell mediated antitumor response that triggered tumor cell apoptosis and suppression of tumor neovasculature [168]. On the other hand, endothelial survivin expression was pro-angiogenic and induced capillary network formation, supporting a role for survivin in the angiogenic sustenance of tumor growth [129].

Paclitaxel (Taxol) Paclitaxel is a strong chemotherapeutic drug used for the treatment of breast, ovarian and lung cancer, based on its ability to induce mitochondrial apoptosis upon stabilization of microtubules, mitotic check-point activation and mitotic arrest [169]. Paclitaxel also exhibits anti-angiogenic activity that is similarly

mediated by mitochondrial apoptotic signaling. Dependent upon concentration, paclitaxel elicited either a cytotoxic effect or a cytostatic effect on endothelial cells by activating apoptosis or inhibiting endothelial proliferation, respectively, that reduced capillary tube formation [130]. The anti-angiogenic activity was exhibited at both cytotoxic and cytostatic concentrations, involving reduced capillary tubes formation. Mechanistically, cytotoxic concentrations involved destabilization of microtubules, G_2-M arrest, mitochondrial membrane potential collapse, Bcl-2 downregulation and apoptosis. Cytostatic concentrations on the other hand, slowed the cell cycle with an early initiation, but overall suppression of apoptosis upstream of mitochondrial permeabilization [130].

Silibilin Silibinin is a flavonolignan isolated form the seeds of *Silybum marianum* (milk thistle). Silibinin has broad anti-cancer efficacy by targeting tumor cell proliferation and apoptosis, and anti-angiogenic activity by targeting of VEGF, VEGF receptors and iNOS [170]. Explicitly, silibinin compromised endothelial capillary tube formation and disrupted capillary networks due to inhibition of endothelial invasion and migration, and decreased matrix metalloproteinase-2 secretion [131]. The mechanism of endothelial growth inhibition involved cell cycle arrest and apoptosis through mitochondrial membrane potential loss, cytochrome c release, increased Bax expression, and the resultant activation of caspase 3/7 [131].

Neovastat (Æ-941) Neovastat is an anti-angiogenic drug derived from marine cartilages. Neovastat inhibits vascular growth by interfering with multiple signaling pathways involved in angiogenesis, including endothelial apoptosis, matrix metalloproteinases signaling and VEGF-mediated signaling [171]. The pro-apoptotic function of neovastat appeared to be specific to endothelial cells and involved mitochondrial cytochrome c release, activation of caspase-3, caspase-8, and caspase-9, chromatin condensation and DNA fragmentation [132]. The activation of both caspase-8 and caspase-9 indicate a possible crosstalk between the extrinsic and intrinsic pathways of apoptosis.

Angiostatin Angiostatin is a 38 kDa proteolytic degradation fragment of plasminogen with strong anti-angiogenic properties. The anti-angiogenic effect of angiostatin results from suppression of tumor cell VEGF secretion [172], and inhibition of endothelial proliferation and tumor neovascularization [173]. Apoptosis contributes significantly to the anti-angiogenic effects of angiostatin with activation of both mitochondrial and surface receptor apoptotic pathways [133]. Downregulation of anti-apoptotic Bcl-2 also provided to decreased viability of angiostatin-treated endothelial cells and tumors [134]. Interestingly, the targeting of endothelial energetic metabolism by angiostatin contributed to its anti-proliferative effect. On the cell surface, angiostatin bound to the plasma membrane-associated α/β-subunits of ATP synthase [135], and upon internalization, localized to mitochondrial malate dehydrogenase (MDH2) and ATP synthase enzymes, reducing intracellular ATP content [134] and limiting the energetic support for angiogenic functions.

Magnolol Magnolol is a bioactive lignan from bark of *Magnolia officinalis* that exerts anti-tumor activity by inhibiting tumor cell proliferation and activating

apoptosis [174]. Mechanistically, magnolol activated ROS-mediated mitochondrial apoptosis via enhanced expression of pro-apoptotic Bax, and suppressed the PI3K/AKT/mTOR pro-survival signaling [136]. Magnolol also negatively influences angiogenesis by inhibiting the endothelial expression of PECAM, which is a cell surface protein required for vascular tube formation and sprouting [136].

Bet-CA Bet-CA is a codrug synthetized by the conjugation of the potent anticancer compounds betulinic acid (3β, hydroxyl-lup-20(29)-en-28-oic acid) and dichloroacetate (DCA). The codrug showed synergistic antitumor properties by selectively triggering mitochondrial apoptosis in cancer cells without apparent cytotoxic manifestations on normal tissues in vitro and in vivo [175]. The antitumor mechanism of Bet-CA involved increased ROS production, mitochondrial membrane potential collapse, mPTP opening and mitochondrial swelling, followed by cytochrome c release, caspase activation and apoptosis. The anti-tumor effect of Bet-CA also suppressed angiogenesis, which significantly attenuated tumor growth and metastatic dissemination in a breast cancer model [137]. Diminished tumor vascular development was associated with arrested endothelial proliferation and migration, which also manifested as decreased angiogenic sprouting in mouse aortic rings and restrictive tube formation in the presence of cancerous effluent [137]. Mechanistically, the anti-angiogenic effect of Bet-CA involved the downregulation of MMP-2 and MMP-9 production by tumor cells and inhibition of VEGFR2 expression and VEGF secretion [137].

Conclusion

Proper mitochondrial functioning and enhanced mitochondrial biogenesis are prerequisites for the accurate execution of endothelial angiogenesis. With reduced energetic support towards vascular growth, mitochondria provide the signaling network required to integrate environmental cues such as growth factors, oxygen and substrate availability with endothelial proliferative, migratory and invasive traits. Mitochondrial functions and mitochondrial proteins are actively involved in coordinating endothelial angiogenesis in multiple ways. Mitochondrial proteins involved in ROS homeostasis ensure the proper balance of pro-oxidants and antioxidants required to support endothelial proliferation and migration, and mitochondrial calcium is employed as a proliferative signal. Metabolic intermediates from mitochondrial fatty acids oxidation fuel DNA synthesis during proliferation, while glucose oxidation and aldehyde metabolism intermediates modulate HIF-1α pro-angiogenic signaling. Mitochondrial dynamics are indispensable to angiogenesis by maintaining the appropriate equilibrium between mitochondrial ROS and calcium-dependent endothelial proliferation and apoptosis, and mitochondrial pro-apoptotic and anti-apoptotic machinery set the threshold for endothelial survival. As such, compounds that target or destabilize mitochondrial functions, particularly with respect to mitochondrial ROS and apoptotic signaling, are attractive opportunities for therapeutic control of angiogenesis, both as positive and negative regulators of vascular growth.

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Chapter 22
High-Density Lipoprotein Regulation of Mitochondrial Function

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HDL serves a prominent anti-atherogenic function by mediating RCT. RCT is initiated by the association of apoA-I in lipid-poor HDL particles with the ATP-binding cassette transporter A1 (ABCA1) on macrophages and other target cells [1]. This interaction allows HDL to act as an acceptor for cholesterol. Lipid-poor HDL can subsequently be converted to a "mature" HDL particle via the action of the HDL-associated enzyme lecithin-cholesterol acyltransferase (LCAT). LCAT activation converts HDL from a nascent discoidal to a mature spherical form and thus increases the cholesterol carrying capacity of the particle. Mature HDL is thought to mediate cholesterol efflux via an interaction with the ABCG1 transporter [1]. The subsequent binding of apoA-I containing HDL particles to the scavenger receptor B1 (SRB1) on hepatocytes permits the unloading of cholesterol which is ultimately secreted as bile [2, 3]. By this mechanism, HDL is thought to attenuate inflammatory injury and reduce atheroma formation [4].

In addition to apoA-I, HDL also serves as a carrier for other exchangeable apolipoproteins, regulatory proteins and anti-oxidant enzymes. Recent proteomic analyses have revealed that more than 85 proteins may associate with HDL particles [5]. This observation suggests that HDL subspecies exist that subserve a variety of cellular functions [5]. Indeed, it is now appreciated that HDL possesses prominent anti-inflammatory and anti-oxidant properties that are independent of its ability to efflux cholesterol [6, 7]. Paraoxonase 1 (PON1) and platelet-activating factor acetylhydrolase (PAF-AH) are esterases that bind to helical regions of apoA-I [8]. These enzymes are important anti-oxidant enzymes that catalyze the hydrolysis of oxidized phospholipids [9]. Data also suggest that the HDL-associated lysosphingolipid sphingosine 1-phosphate exerts anti-inflammatory effects. Thus, both protein

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and lipid components of HDL may help to maintain cellular homeostasis. The observation that circulating levels of HDL inversely correlate with mitochondrial DNA damage in humans suggest a specific role for HDL in maintaining mitochondrial integrity [10].

Hypercholesterolemia is a major, cardiovascular risk factor which contributes to the pathogenesis of atherosclerosis, metabolic syndrome and diabetes. Chylomicrons, VLDL and LDL are apoB-containing lipoproteins that serve an important function by delivering cholesterol and triglycerides to peripheral cells. These particles, however, are susceptible to oxidative modification and may adopt pro-atherogenic properties. The uptake of oxidized LDL (oxLDL) by intimal macrophages is a well-characterized component of atherosclerotic plaque formation [11, 12]. Mitochondria are principal sites of ROS formation in the cell and are also targets for redox injury [10, 13–16]. An increase in plasma cholesterol and triglycerides has been implicated in the development of mitochondrial dysfunction. Lysophosphatidylcholine (lysoPC), a pro-inflammatory lipid associated with oxLDL, induces mitochondrial ROS formation and increases permeability in intact cells and isolated mitochondria [17, 18]. These responses are also induced by I-R injury. An increase in oxidant formation thus damages mitochondrial structural elements and impairs respiration by reducing oxidative phosphorylation and ATP formation [19]. Irreversible damage occurs upon dissipation of mitochondrial membrane potential ($\Delta\Psi$m) and opening of the mitochondrial permeability transition pore (mPTP). HDL possesses anti-inflammatory and anti-oxidant properties that protect mitochondria from injury. The goal of this chapter will be to discuss mechanisms by which HDL-associated proteins and lipids modulate mitochondrial function and improve cell survival.

Mitochondrial Structure and Function

The mitochondrion is an energy producing organelle that generates ROS and heat as byproducts of oxidative phosphorylation [20]. Mitochondria are found in all tissues but are most abundant in those with high metabolic requirements, such as cardiac and skeletal muscle [21]. The mitochondrion is a double membraned organelle that contains its own maternally-inherited DNA [22, 23]. The oxidative phosphorylation complexes; Complex I *(NADH: ubiquinone oxidoreductase)*, Complex II *(succinate dehydrogenase)*, Complex III *(CoQH2-cytochrome c reductase)*, Complex IV *(cytochrome c oxidase)*, and Complex V *(ATP synthase)*, are encoded by both the nuclear and mitochondrial genomes. Mitochondria utilize both electron and proton gradients in order to produce energy, in the form of ATP, while consuming oxygen. Electrons enter the electron transport chain at Complexes I and II and are then passed to Complex III via the Q cycle on coenzyme Q [24]. Electrons are shuttled to Complex IV where cytochrome c is reduced. As oxygen is consumed at Complex IV, cytochrome c is reoxidized, and water is formed. As the electrons are shuttled through the complexes, hydrogen ions are pumped from the mitochondrial matrix

by Complexes I, III, and IV into the intramembrane space, thus establishing a protomotive force. As the protons flow down the gradient from the intermembrane space back into the mitochondrial matrix, ATP is produced at Complex V [23, 25].

The measurement of mitochondrial respiration can be performed using an oxygen electrode in isolated organelles. Chance and Williams defined the different states of mitochondrial respiration by measuring oxygen consumption in the presence of various mitochondrial substrates [26]. Under control conditions, mitochondria remain in state 3.5 respiration, where there is a generous supply of substrates for the electron transport chain and ADP for ATP production. Using the oxygen electrode, State 3 respiration was defined as the amount of oxygen consumed in the presence of pyruvate, succinate and ADP. State 4 respiration represents oxygen consumption after ADP has been fully converted to ATP. Currently, extracellular flux analysis represents state-of-the-art technology for the measurement of mitochondrial function in whole cells rather than isolated mitochondria. Endogenous substrates required for respiration are found within the cell. Under these conditions, inhibitors of the mitochondrial complexes can be administered to cells in order to determine different indices of mitochondrial function including: basal and maximal mitochondrial respiration; oxygen consumption required for ATP production; proton leak; and non-mitochondrial oxygen consumption [27]. Development of this technology has revolutionized the field of mitochondrial biology [27, 28].

Under normal physiological conditions, some electrons may spill off of the electron transport chain and react with oxygen to form superoxide anion. Complexes I and III are the principal sources of ROS during oxidative phosphorylation [29]. At low levels of production, ROS can act as signaling molecules. When produced in excess, however, ROS induce peroxidation of cellular lipids and proteins, damage mitochondrial and nuclear DNA and impair cell division. It follows that mitochondrial oxidative phosphorylation is significantly impaired. Bioenergetic dysfunction ensues and is associated with dissipation of $\Delta\Psi m$ and opening of the mPTP [30]. mPTP induction subsequently leads to mitochondrial swelling and cell death [20, 29]. The antioxidant enzyme manganese superoxide dismutase is present in mitochondria and reduces superoxide to hydrogen peroxide, thus minimizing injury. The ability of the mitochondria to clear ROS thus increases cell survival. Schriner and colleagues reported that overexpression of mitochondrially targeted catalase in cardiac and skeletal muscle resulted in an increase in murine longevity [31]. Mitochondrial DNA damage in myocytes, as assessed by 8-hydroxyguanosine (8-OhdG) formation, increased with age but was attenuated by catalase overexpression in these mice [31]. Atherosclerosis and I-R injury are common cardiovascular disorders that are associated with the development of mitochondrial dysfunction. Defects in electron transport and oxidative phosphorylation enhance ROS formation resulting in mitochondrial injury and the induction of apoptosis via the cleavage of caspase 9. In a mouse model of myocardial infarction, ROS production damages both mitochondrial DNA and proteins, resulting in decreased enzymatic activities of mitochondrial Complexes I, III and IV. Further, coronary I-R injury is associated with opening of the mPTP leading to cell death. Ongoing studies are assessing the effects of mitochondrially-targeted anti-oxidant therapies on the development and progression of cardiovascular disease.

Mitochondrial Clearance

Cells utilize a mitochondrial clearance mechanism termed autophagy in order to avoid cell death secondary to injurious stimuli. The term autophagy was coined by Christian De Duve in the 1960s and is derived from the Greek words auto (self) and phagy (eating) [20, 32, 33]. Today, we know that there are three main types of autophagy: (1) macroautophagy (usually termed autophagy); (2) chaperone mediated autophagy; and (3) microautophagy [34]. Autophagy was first characterized as a response to nutrient starvation and was found to be associated with inactivation of the mammalian target of rapamycin (mTOR) [20, 32, 33]. Autophagy breaks down macromolecules and recycles their components not only to preserve cellular energy but also to clear damaged proteins and mitochondria [20, 32, 33]. More than 30 AuTophagy-related Genes (ATG) are conserved from yeast to mammals and participate in autophagy at different steps throughout the process [35, 36].

Mitochondria can be cleared through macroautophagy, but have also been shown to utilize different ATG proteins to selectively remove damaged mitochondria via mitophagy. As noted above, mitochondria contain their own genome which lacks compact chromatin structure and, compared to nuclear DNA, have a less effective replication and repair system [37]. Further, since mitochondria are the primary cellular producer of ROS, and the mitochondria contains highly reactive iron sulfur clusters, mitochondria are 10–20 times more likely to accumulate DNA damage compared to nuclear DNA. Mitophagy is controlled either in conjunction with general macroautophagy or selectively through specific mitophagy genes. AMPK is activated in response to decreased intracellular ATP, and then phosphorylates the Atg1 homologs ULK1 and ULK2 to activate both general macroautophagy and mitophagy [38]. AMPK or ULK1 deficient hepatocytes exhibit accumulation of p62, ubiquitin aggregates and abnormal mitochondria [38]. In the context of atherosclerosis and I-R injury, mitochondria are damaged and are more likely to produce ROS and less ATP. Mitophagy plays an important role in attenuating apoptosis or necrosis by clearing damaged mitochondria. This prevents the release of cytochrome c, apoptosis-inducing factor (AIF) and other apoptotic factors that can cause the cell to swell and burst. Mitochondrial turnover is thus essential for cell survival.

Mitochondria isolated from rodent hearts have an average half-life of 16–18 days [39]. In rodents exposed to hypoxic conditions, it was shown that mitochondrial half-life is reduced and is associated with a decrease in cardiac cytochrome c content. Further, there was an increase in mitochondrial biogenesis suggesting that formation of new mitochondria is an important response to I-R injury [40]. It is well known that starvation can induce both macroautophagy and mitophagy, but more recent data suggest that autophagy and mitophagy can be initiated by ischemia [41]. The induction of autophagy is essential for the protective effects of ischemic pre- and post- conditioning in the heart [42, 43]. Parkin, pink1 and related proteins play an important role in mitophagy and mitochondrial turnover [44]. In parkin- and pink1-deficient mice, there is an exacerbation of I-R injury, suggesting that the clearance of damaged mitochondria is an essential component of ischemic pre-

conditioning [45]. Since mitochondrial biogenesis and turnover are tightly regulated and have major effects on outcomes, further treatments for heart disease should utilize these pathways in drug discovery.

Mitochondrial and Cell Survival Mechanisms

Cardiac ischemia arises in response to unstable angina and acute myocardial infarction. Upon reperfusion, significant injury occurs at the level of the mitochondrion [46]. Due to the high energy demands of the heart, I-R injury significantly impairs mitochondrial respiration. This is associated with increased ROS formation, uncoupling of oxidative phosphorylation and opening of the mPTP [47]. mPTP opening is associated with dissipation of the $\Delta\Psi m$, calcium influx and the release of pro-apoptotic factors [46]. Ischemic pre-conditioning and post-conditioning have been shown to preserve mitochondrial function thus reducing myocardial reperfusion injury [48, 49]. These conditioning protocols are characterized by brief disruption of blood flow prior to sustained ischemia and reperfusion and result in the activation of two major cell survival pathways [48, 49]. The Reperfusion Injury Salvage Kinase (RISK) pathway is comprised of the pro-survival kinases phosphatidylinositol 3-kinase (PI3K), protein kinase B (*Akt*) and extracellular regulated kinase 1/2 (ERK1/2) [50, 51]. These enzymes phosphorylate multiple substrates in the cell that converge to inhibit opening of mPTP [52–54]. Glycogen synthase kinase 3 beta (GSK3β) appears to be an important target for RISK-dependent cardiomyocyte survival [55]. Phosphorylation of GSK3β reduces its enzymatic activity. Inactive GSK3β attenuates mitochondrial injury by increasing Bcl-2 anti-apoptotic activity, inhibiting the translocation of pro-apoptotic Bax to the outer mitochondrial membrane and preventing mPTP induction via stabilization of the mPTP regulatory protein cyclophilin D [52, 56]. The Survivor Activating Factor Enhancement (SAFE) cascade is an alternate pathway for mitochondrial preservation [57]. TNFα activates the SAFE pathway by binding to TNF receptor type 2 (TNFR2) [58]. TNFR2 engagement results in the activation of Janus kinase (JAK) which phosphorylates the signal transducer and activator of transcription 3 (STAT3). The SAFE pathway has been shown to reduce infarct size in animals undergoing I-R injury by a mechanism involving STAT3 phosphorylation and inhibition of mPTP opening [59]. While the RISK and SAFE pathways preserve mitochondrial function by increasing the activity of specific signaling intermediates, data suggest that crosstalk between these pathways occurs, with stabilization of the mPTP as a final common outcome [48, 57].

HDL and ApoA-I Preserve Mitochondrial Function

Lipid peroxides present in oxLDL have been shown to stimulate ROS formation and impair oxygen consumption at Complexes I, II/III, and IV of the respiratory chain, resulting in mitochondrial dysfunction [60, 61]. The HDL-associated protein PON1

performs an important antioxidant function by hydrolyzing cholesteryl esters and phospholipids in oxidized lipoproteins [61]. PON1 may thus preserve mitochondrial function due to its ability to degrade oxidized lipid species. In contrast to this indirect effect of HDL-associated PON1, a direct role for apoA-I and the lysosphingolipid sphingosine 1-phosphate (S1P) in mediating cardiomyocyte survival has been reported [60–62]. Infarct size in apoA-I$^{-/-}$ mice undergoing coronary artery ligation/reperfusion is significantly increased compared to lesions in wildtype mice [63]. This correlated with a reduction in Coenzyme Q (CoQ) in mitochondria isolated from apoA-I$^{-/-}$ mice. CoQ deficiency was associated with a significant reduction in electron transfer from Complex II to Complex III [63]. In related studies, it was shown that exogenous administration of CoQ restored mitochondrial CoQ levels and attenuated infarct size in apoA-I$^{-/-}$ mice [63]. These results suggested that apoA-I plays a key role in maintaining the coupling of electron transport proteins.

Data suggest that HDL, similar to ischemic pre-conditioning and post-conditioning, prevents mitochondrial injury via activation of RISK and SAFE survival cascades. The SAFE pathway influences mitochondrial function in several ways. First, STAT3-mediated nuclear transcription results in up-regulation of anti-apoptotic Bcl-2 and the antioxidant genes manganese superoxide dismutase and metallothionein while inhibiting pro-apoptotic Bax/Bad expression [48, 64]. STAT3 has also been shown to regulate the electron transport chain and mitochondrial respiration [64, 65]. STAT3 gains access to the mitochondrion with the assistance of the chaperone protein GRIM-19 [66]. At this locus, STAT3 inhibits Complex I and II respiration and the release of cytochrome c [64]. It is proposed that STAT3 ultimately protects mitochondria by reversibly uncoupling electron flow between respiratory complexes, reducing ROS formation and preventing mPTP induction [64, 67–69]. Administration of apoA-I to rodents prior to coronary artery occlusion attenuates morphologic changes associated with ischemic injury and reduces infarct size. Consistent with known targets of the RISK and SAFE signaling cascades, apoA-I treatment increased the phosphorylation of *Akt* and GSK3β. The infarct-sparing response to apoA-I was significantly reduced in animals treated with inhibitors of *Akt*, ERK1/2 and JAK/STAT. These data suggested that the inhibitory effect of apoA-I on myocardial infarct size was due to activation of both RISK and SAFE survival pathways, resulting in attenuation of mitochondrial injury.

HDL-Associated Sphingosine 1-Phosphate: Influences Mitochondrial Function

The lipid composition of HDL plays an important role in determining the function of the lipoprotein particle [70, 71]. Lipid species maintain the structural integrity of HDL and regulate the activities of HDL-associated proteins [72]. Data suggest that the lysosphingolipid S1P of HDL plays an important role in cardioprotection. While S1P is synthesized in hematopoietic and endothelial cells, HDL serves as its principal carrier in plasma [73, 74]. S1P has been shown to act as an inducer of both the

RISK and SAFE pathways [75, 76]. Cardiomyocyte responses to S1P actions are mediated by multiple receptor isoforms (S1P1, S1P2 and S1P3) [77–79]. Addition of HDL or purified S1P to neonatal rat cardiac cardiomyocytes activates S1P2 receptors resulting in STAT3 phosphorylation. In contrast, HDL that is depleted of S1P fails to support STAT3 phosphorylation [75]. HDL treatment was shown to activate *Akt* and ERK1/2 pathways via distinct S1P receptors in mouse cardiomyoctyes exposed to hypoxia-reoxygenation [77]. S1P1 binding resulted in activation of ERK1/2, while S1P3 induced *Akt* activation. Under these conditions, levels of phosphorylated GSK3β, a known inhibitor of mPTP opening, were increased [77]. This response was inhibited by S1P receptor blockers and the PI3K inhibitor wortmannin, suggesting that S1P reduces cardiomyocyte injury by activating the RISK pathway [77]. Numerous in vivo studies support a role for S1P in the activation of RISK and SAFE signaling cascades in the context of I-R injury and heart failure [77, 80–82].

Cardioprotective responses to HDL and S1P are ultimately mediated at the level of the mitochondrion. Administration of HDL to mice undergoing I-R injury reduces infarct size in a concentration-dependent manner [83]. This response was associated with STAT3 phosphorylation and inhibition of mPTP opening in isolated cardiomyocytes. Protective effects at the level of the mitochondrion were abolished in TNF$\alpha^{-/-}$ and cardiomyocyte-specific STAT3$^{-/-}$ mice, suggesting that STAT3 mediates the activation of the SAFE pathway [84]. Forkhead box O-1 (FOXO-1) is a transcription factor that is known to increase ROS formation and apoptosis in the non-phosphorylated state [85]. While the RISK pathway and PI3K/*Akt* activation are associated with the inactivation of FOXO-1, data suggest that the SAFE survival pathway also modulates FOXO-1 activity [79, 85]. S1P stimulated the nuclear phosphorylation/inactivation of FOXO-1 in a manner that was blocked by both a JAK/STAT3 and PI3K inhibitor [79]. These observations suggest that the S1P-dependent phosphorylation of FOXO-1 may represent a point of convergence for the RISK and SAFE survival cascades.

Finally, data show that S1P regulates Complex IV assembly and cellular respiration in mitochondria through an interaction with mitochondrial prohibitin-2 (PHB2) [86, 87]. PHB2 is a scaffold protein which functions to stabilize the structure of the inner mitochondrial membrane [88]. Disruption of S1P-PHB2 binding abolishes the cardioprotective response of cardiomyocytes to ischemic pre-conditioning. Under this condition, a reduction in oxidative phosphorylation was associated with opening of mPTP and mitochondrial injury [46, 54]. These data, therefore, suggest that S1P and PHB2 stabilize Complex IV and reduce ROS formation while also supporting oxidative phosphorylation [86].

HDL and Autophagy Induction

Mitochondrial damage induced by I-R injury releases apoptotic factors that damage neighboring mitochondria. It has been proposed that autophagy serves a cytoprotective role by clearing damaged mitochondria and limiting potentially deleterious

effects on neighboring organelles [89]. Ischemia initiates autophagy by inducing the de-phosphorylation and inactivation of mTOR which normally acts as a suppressor of autophagy [90]. An increase in the ratio of AMP/ATP concurrently induces AMP-activated protein kinase (AMPK) and stimulates autophagy via multiple mechanisms. The vacuolar protein sorting-34 (Vps34) is a class III PI-3 kinase that initiates phagophore formation through its association with beclin1 [90]. This pre-autophagosomal structure engulfs cytoplasmic components, including damaged mitochondria. Phosphatidylethanolamine (PE) and microtubule-associated protein light chain-3 (LC3 I) interact to form the conjugated product LC3 II. LC3 II and the adaptor protein p62 are recruited to yield the mature autophagosome. The autophagosome then fuses with lysosomes where ingested products are digested. Inhibitors of autophagy promote ROS formation and aggravate mitochondrial injury in response to I-R [91]. They have also been shown to negate cell survival mechanisms associated with ischemic pre-conditioning. It follows that autophagy contributes to the cytoprotection associated with pre-conditioning [42, 92]. A role for S1P in the inhibition of mTOR and activation of autophagy has been reviewed [93, 94]. It follows that inhibitors of S1P formation also prevent the induction of autophagy [95]. As the principal carrier of S1P, HDL may induce autophagy as a cell survival mechanism. In support of this, it was shown that HDL inhibits mTOR activity, stimulates the expression of LC3 II and stimulates the formation of autophagosomes [96].

Alternate Apolipoproteins and Mitochondrial Function

In addition to apoA-I and apoB, other apolipoproteins (i.e., apoJ, apoM, apoC and apoO) serve as regulatory molecules for cholesterol homeostasis [97]. Recent data also suggest a role for these apolipoproteins in the regulation of mitochondrial function. ApoJ, also known as clusterin, is a glycoprotein expressed ubiquitously during development and in adults and is associated with small, dense HDL3 particles [98–100]. Due to the presence of disulfide bonds, apoJ possesses antioxidant properties that inhibit ROS-dependent injury and preserve mitochondrial function [20]. In the H9c2 cardiomyocyte cell line, it was shown that apoJ protects against ROS-induced apoptosis by activating *Akt* and GSK-3β, thus suggesting a role in the activation of the RISK survival cascade [101].

ApoM is apolipoprotein that accounts for approximately 5 % of the protein content of HDL. A major function of apoM is to attenuate atherosclerosis by stimulating the formation of small, dense preβ-HDL particles that play an important role in RCT [102–105]. ApoM protects mitochondrial function by virtue of its ability to bind to S1P and facilitate its incorporation into HDL [80, 104, 106, 107]. The relationship between apoM and S1P-mediated cardioprotection has been evaluated in a murine model of I-R injury [108]. Over-expression of apoM in mice was accompanied by a significant reduction in myocardial infarct size and leukocyte accumulation [108]. The underlying mechanism of apoM-S1P-induced cardioprotection was due to inhibition of cardiomyocyte cell-cell coupling [108]. The passage of death

signals through gap junctions was reduced, resulting in attenuation of I-R-induced cardiomyocyte injury [108].

ApoC is an exchangeable apolipoprotein associated with HDL as well as apoB-containing lipoproteins. Three structurally distinct isoforms (apoC-I, apoC-II and apoC-III) have been identified that exert differential effects on lipid metabolism, ROS formation, mitochondrial function and cell death. [109]. Panin and colleagues have tested effects of these apoC isoforms on oxidative phosphorylation in rat liver mitochondria [110]. Using palmitoyl carnitine as a substrate for oxidative phosphorylation, it was shown that apoC-III, but neither apoC-I nor apoC-II, inhibited State 3 respiration [110]. At higher concentrations of apoC-III, the rate of oxygen consumption was reduced ~70 %, and oxidative phosphorylation was completely blocked [110]. These data were the first to show an inhibitory effect of ApoC-III on mitochondrial function. Other data support a role for apoC-I in the development of mitochondrial injury in human aortic smooth muscle cells [111]. Enrichment of HDL with apoC-I was shown to induce cell death in a neutral sphingomyelinase (N-SMAS) dependent manner, with the production of ceramide from N-SMAS stimulating the release of cytochrome c from the mitochondria, the cleavage of caspase 3 and apoptosis. The increased cell death of the aortic smooth muscle cells incubated with ApoC-I may account for the unstable plaque formation seen in patients with hypercholesterolemia [111]. Interestingly, apoC-I enrichment of HDL was associated with a reduction in HDL-associated apoA-I, suggesting that loss of apoA-I and its cytoprotective effects is a component of apoC-I mediated apoptosis [111].

Apoliprotein O is a 198 amino acid protein which is found in association with HDL, LDL and VLDL particles [112]. ApoO is up-regulated by metabolic stress in the diabetic heart where it is thought to play a role in reducing myocardial injury by preventing macrophage lipid accumulation [112]. ApoO and the related apoO-like protein have also been shown to stabilize the inner mitochondrial membrane and the cristea [113–115]. In contrast to this report, over-expression of apoO was shown to impair cardiac function and degrade mitochondrial structure in cardiomyocytes of hypercholesteroleic mice [116]. This observation suggested a pathogenic role for apoO in obesity. To further understand the pathogenic mechanism of apoO, Turkeih and colleagues developed a stable cardiac myoblast cell line that overexpressed apoO. These cells were characterized by an increase in mitochondrial respiration, fatty acid uptake and metabolism, ROS formation and apoptosis compared to control cells [116]. It was suggested that an increase in apoO expression promotes the transition from mitochondrial dysfunction to the development of overt cardiomyopathy [116]. Further research is required to delineate the role of apoO in diverse cardiovascular pathologies.

Conclusion

While HDL plays an important anti-atherogenic role by mediating RCT, it is also an active signaling particle that possesses anti-inflammatory, anti-oxidant and anti-apoptotic properties. HDL-associated proteins and lipids play an important role in

the preservation of mitochondrial function. The anti-oxidant enzyme PON1 prevents damage to respiratory complexes by degrading oxidized lipids such as lysoPC [117]. ApoA-I also protects mitochondria by multiple mechanisms. Through an interaction with CoQ, apoA-I stabilizes complex II and inhibits ROS-mediated damage to respiratory complexes [63]. ApoA-I and the HDL-associated lipid S1P also protect mitochondria via the activation of RISK and SAFE survival pathways [118]. *Akt*, ERK1/2 and JAK/STAT3 are critical mediators of these cell survival cascades. The cytoprotective response to S1P is due to an interaction with cellular S1P receptors, resulting in activation of JAK/STAT3 signaling. Binding of apoA-I to ABCA1 is also reported to activate STAT3 signaling and attenuate inflammatory injury [119]. It has been proposed that phosphorylated FOXO-1 and GSK3β are final, common effectors of these pathways and improve cell survival by attenuating mitochondrial ROS formation, mPTP induction and apoptosis [79, 120]. Autophagy represents an additional survival mechanism activated by apoA-I. Under these conditions, ROS-dependent injury may be reduced via the effective clearance of damaged mitochondria.

The ability of HDL to preserve mitochondrial function may be attenuated under pathological conditions. A reduction in circulating HDL concentration is associated with a number of inflammatory disorders [121, 122]. Under these conditions, depletion of apoA-I, PON1, apoM and apoJ from HDL particles may result in a loss of function [121, 123–126]. A decrease in apoM may ablate the protective effects of S1P on mitochondrial function. This response may augment the vascular leakage observed in patients with sepsis [127]. Lipoprotein oxidation is also associated with a reduction in S1P levels and accumulation of pro-inflammatory lysoPC in HDL particles [128]. With respect to apoJ, a decrease in its association with HDL has been implicated in the development of insulin resistance and an increase in apoptosis [129]. Finally, an increase in the incorporation of apoC in the HDL particle may increase mitochondrial injury and apoptosis. Under these conditions, HDL function may be further degraded by the displacement of apoA-I by apoC. These observations suggest that raising circulating HDL concentration as well as its functional properties represents an important therapeutic strategy to minimize mitochondrial injury.

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Chapter 23
MitomiRs Keep the Heart Beating

Samarjit Das, Hannah R. Vasanthi, and Ramesh Parjapath

Introduction

The vital importance of the heart is obvious and diseases of the heart are soaring. Cardiovascular disease (CVD), the leading cause of death globally, can be precipitated by a series of cardiometabolic risks. Several initiatives are in progress to identify the disease at an early stage and manage the disease with utmost care. In this process several scientists are working on microRNAs (miRNAs) as a novel tool in diagnosis, prognosis, and therapeutics.

MiRNAs, a group of non-coding RNAs constituted by 19–25 nucleotides, bind to protein-coding regions of the 3′-untranslated region (UTR) of mRNAs and inhibit the stability and/or translation of that mRNA. They have been used as biomarkers [1] for different kinds of heart disease [2], metabolic disorders [3], and neurodegenerative diseases [4]. Circulating miRNAs can also be used as therapeutic agents [5]. miRNAs that are present in heart tissue have been found to play a powerful role in various cardiovascular diseases [2]. In cases where a certain cardiovascular disease is characterized by the over-expression or loss of a particular gene, the miRNA(s) that targets this gene can contribute to, if not driving, disease progression.

MiRNAs are encoded by the genome of all eukaryotic organisms, and some DNA viruses. In mammalian cells non-coding intronic regions are the major encoding regions for most miRNAs, but some are encoded within the exonic regions. By acting on specific complementary target sequences of mRNA (formation of the RNA induced silencing complex, RISC), miRNAs silence a particular gene [6, 7].

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Several miRNA loci are in close proximity to each other, constituting a polycistonic transcript. Thus, single promoter activation can transcribe the clustered miRNAs together as a single unit. The 5′-end of miRNAs that spans from the 2 to 9 nucleotide position is crucial for target recognition (3′-UTR end of mRNA) and has been termed the "seed"-sequence. The downstream nucleotides of miRNA contain an additional sequence that controls their post-transcriptional properties, including their subcellular translocation patterns [8]. miRNA sequences are highly conserved across organisms. Approximately 60 % of human mRNAs contain at least one conserved miRNA binding site [9]. The expression levels of these miRNAs vary among individuals, and in conditions ranging from normal to diseased.

The present chapter will examine in detail the basics of cardiovascular system with emphasis on the mitochondria as a key organelle in cardiac, anatomy, physiology, biochemistry and pathology which off shoots to cardiovascular medicine.

Development of the Functional Heart

The heart is one of the earliest differentiating and functioning organs [10]. This emphasizes the critical nature of the heart in distributing blood through the vessels and the exchange of nutrients, oxygen, and wastes both to and from the developing baby. It grows into a functional beating heart in the seventh week of gestation which beats throughout life. Cessation of its activity for more than few minutes is detrimental to life.

The fetal heart, which functions in a relatively hypoxic environment, uses glucose and lactate as the predominant fuel substrates. Endogenous glycogen in the fetal and, to a lesser extent, in the neonatal heart is a significant source of glucose and myocardial ATP. However, in conditions of oxygen deprivation, glycogenolysis may be important and may allow the fetal heart to be more resistant to the effects of hypoxia and ischemia as compared to the adult heart [11].

Likewise, fetal hearts have lower mitochondrial content and therefore lower levels of both respiratory complex and TCA cycle activities. Catabolism of fatty acids by beta oxidation provides a small part of the overall ATP production because of low levels of circulating fatty acids and also due to the inhibition of fatty acid oxidation by high lactate levels present in the fetal heart.

The miRNAs which are nothing but non coded RNAs which are less than 22 nucleotides have been identified in cardiac tissue at all stages of development and those highly expressed in the fetal heart include miR-21, miR-29a, miR-129, miR-210, miR-211, miR-320, miR-423, and let-7c [12]. These cardiac miRNAs play a central role in cardiogenesis, heart function and pathology. miR-1 and miR-133a predominantly control early stages of cardiogenesis supporting commitment of cardiac-specific muscle lineage from embryonic stem cells and mesodermal precursors whereas, miR-208 and miR-499 are involved in the late cardiogenic stages mediating differentiation of cardioblasts to cardiomyocytes and fast/slow muscle fiber specification [13]. In the healthy adult heart, based on a large sequencing

project, along with other studies, a number of miRNAs that are highly expressed in non-diseased cardiac tissue have been identified and thus likely to play a key role in both normal cardiac maintenance and disease. These include miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, miR-208 and the let-7 family [14].

Cellular Function of the Beating Heart

The four-chambered heart is made up of different types of cells which contributes to its structural, biochemical, mechanical and electrical properties of the functional heart. The myocardium is made up of the atrial and ventricular cardiomyocytes which forms the muscular walls of the heart aiding in the rhythmic pumping of the blood. Other cell types are fibroblasts, which, by number, represent more than 50 % of cardiac cells, endothelial cells, which form the endocardium, the inner lining of blood vessels and cardiac valves, and smooth muscle cells, which contribute to the coronary arteries aiding the inflow and outflow vasculature [15]. Specialized cardiomyocytes namely pacemaker cells and Purkinje fibers in the conduction system generate and conduct electrical impulses whereas the epicardium gives rise to the precursors of the coronary vasculature and fibroblasts. It is interesting to note that the human heart contains approximately two to three billion cardiac muscle cells however, they account for fewer than a third of the total number of cells in the heart which includes the smooth muscle cells, endothelial cells, fibroblast cells, connective tissue cells and mast cells. These distinct cell pools are not isolated from one another within the heart but instead interact physically and via a variety of soluble paracrine, autocrine, and endocrine factors [16, 17].

The rhythmic contraction of the heart assures constant oxygenation of the various organs and their cells. The central dogma of cardiac contraction-relaxation cycle is orchestrated by the increase and decrease of Ca^{2+} release in the cytoplasm. Further, the sarcoplasmic reticulum, a specialized myocyte organelle is critical for Ca^{2+} ion concentrations, which is an on-off switch for contraction. Intracellular Ca^{2+} handling is regulated by at least three other second messengers: cAMP, cGMP and inositol 1,4,5 trisphosphate in association with SR [18]. The contraction-relaxation cycle starts when an electrical action potential is initiated at the cell membrane of myocyte. Subsequently, an inward current flows depolarizing the cell membrane which in turn opens the voltage-gated Na^+ channels and causes influx of Na^+. Concomitantly, the membrane potential reaches to a permissive level for opening voltage-gated Ca^{2+} channels. These Ca^{2+} ions trigger the release of more Ca^{2+} from the sarcoplasmic reticulum by opening ryanodine receptors (RyRs). This Ca^{2+} induced Ca^{2+} release increases intracellular Ca^{2+} concentration which initiates the contraction cycle by activating contractile protein apparatus myosin and actin [18]. The Ca^{2+} released from sarcoplasmic reticulum diffuses to adjacent myofibril and binds to troponin C of troponin-tropomyosin complex of actin containing thin filament in sarcomeres, which displaces tropomyosin facilitating cross-bridge formation between actin and myosin to enable contraction of the myocardium. Each cycle

of the cross-bridge consumes one molecule of ATP which is provided at the myosin ATPase catalytic domain, which is the major site of ATP consumption in the beating heart. Relaxation occurs when intracellular Ca^{2+} is restored to sarcoplasmic reticulum and voltage-gated K^{+} channels opens to permit action potential repolarization, making suitable conditions for relaxation. Withdrawal of intracellular Ca^{2+} takes place via four mechanisms consisting of SR Ca^{2+}-ATPase, sarcolemmal Na^{+}/Ca^{2+} exchanger, sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uptake [18]. Thus ATP, the energy currency of the cell, is the key player for the beating heart. However, other signaling mechanisms aid in the cross talk and help the myocytes to be functional.

Mitochondria the Key Organelle for a Functional Heart

Effective myocardial function depends primarily on oxidative energy production. In humans, at a heart rate of 60–70 beats per minute, the oxygen consumption normalized per gram of myocardium is 20-fold higher than that of skeletal muscle at rest. The human heart consumes 30 kg of ATP per day, fueling basal metabolism and normal contraction that is essential to sustain systemic and pulmonary blood pressure. It is interesting to note that almost all ATP is produced in cardiomyocyte mitochondria.

Mitochondria are abundant in energy-demanding cardiac tissue constituting 20–40 % of cellular volume. The beating heart demands continuous supply of chemical energy not only to perform its mechanical pumping of blood to various organs but also to modulate intracellular and trans-sarcolemmal ionic movements and concentration gradients. Most of the energy required for these laborious processes is provided by mitochondrial bioenergetics. Many interacting bioenergetic pathways add to mitochondrial energetics including pyruvate oxidation, the tricarboxylic acid (TCA) cycle, β-oxidation of fatty acids and the final common pathway oxidative phosphorylation (OXPHOS). About 80–90 % of myocyte energy comes from OXPHOS and to a lesser extent from oxidation of glucose, lactose and other substrates [19]. In addition to energy from the mitochondria, cardiomyocytes also maintain high-energy stores in the form of phosphocreatine (pC) produced by mitochondrial creatine kinase using ATP.

Post-natally, a switch occurs from the carbohydrate metabolism, so that fatty acids become the primary energy substrate in the heart [20]. Likewise, the level of cardiac mitochondrial creatine kinase (mtCK) which is undetectable in early fetal heart, is upregulated during neonatal development. The increased expression of mtCK within the first several weeks after birth is coupled to both the availability of ADP to mitochondria as well as to the structural re-organization of cardiac mitochondria from a random arrangement at day 1 to an organized network by 3 weeks postnatally [21]. Subsequently, the maturation of the phosphocreatine shuttle is coordinated with the development of the contractile properties of the myocardium [22].

Reactive Oxygen Species (ROS), including superoxide and hydroxyl radicals and hydrogen peroxide, are by-products of mitochondrial bioenergetic side reac-

tions of the mitochondrial ETC with molecular oxygen directly generate the superoxide anion radical. The main sites for mitochondrial ROS generation are complex I and III activities of the respiratory chain [23]; either excessive or diminished electron flux at these sites can stimulate the auto-oxidation of flavins and quinones (including coenzyme Q) producing superoxide radicals. The superoxide radicals can be converted to H_2O_2 (in the presence of the enzyme superoxide dismutase) which can further react to form the hydroxyl radical [19]. Generally these cell-damaging oxidants are neutralized by anti-oxidant enzymes, some of which are located in the mitochondria while others are cytosolic. Imbalance in the oxidant and the antioxidant status due to increased ROS generation results in oxidative stress. This may cause profound effects on cardiac cells including increased lipid peroxidation effecting primarily membrane phospholipids and proteins. Oxidative damage also occurs with nucleic acids (particularly targeting mtDNA) including induction of strand breaks, a high incidence of base modification (including the formation of 8-oxoguanosine) and subsequent point mutations and deletions [24].

Mitochondria are the primary site of ROS generation and therefore the critical target of their damaging effects; the mitochondrial respiratory chain located in the inner membrane is often damaged resulting in a further increase in ROS generation leading to a vicious cycle of diminished mitochondrial function. In addition to the well characterized role of ROS in cell damage, recent evidence has provided its role as an important regulatory event [25]. Oxidative species (e.g. H_2O_2) can also function as a potent signal sent from mitochondria to other cellular sites rapidly, and reversibly eliciting an array of intracellular cascades leading to different physiological end-points for the cardiomyocyte (e.g. apoptosis, necrosis, cardio-protection, cell proliferation).

Abnormalities in the mitochondrial organelle structure and function have been found with increasing frequency in association with cardiovascular diseases such as dilated (DCM) and hypertrophic cardiomyopathy (HCM), cardiac conduction defects and sudden death, ischemic and alcoholic cardiomyopathy, as well as myocarditis. Some of the mitochondrial abnormalities may have a genetic basis (e.g. mitochondrial DNA [mtDNA] changes leading to oxidative phosphorylation [OXPHOS] dysfunction, fatty acid oxidation defects due to specific nuclear DNA mutations) while other abnormalities appear to be due to a more sporadic or environmental cardiotoxic insult [19]. Figure 23.1 depicts the influence of various risk factors on the cardiomyocyte mitochondria which lead to biochemical and molecular changes resulting in different cardiovascular diseases.

Mitochondrial Dynamics

Mitochondria are highly dynamic, which allows them to constantly change their morphology. Mitochondria constantly divide (fission) and fuse (fusion); an unsuccessful mitochondrial repair process by fission–fusion activates mitophagy to eliminate the dysfunctional mitochondria from the cell. Recent findings, mainly in

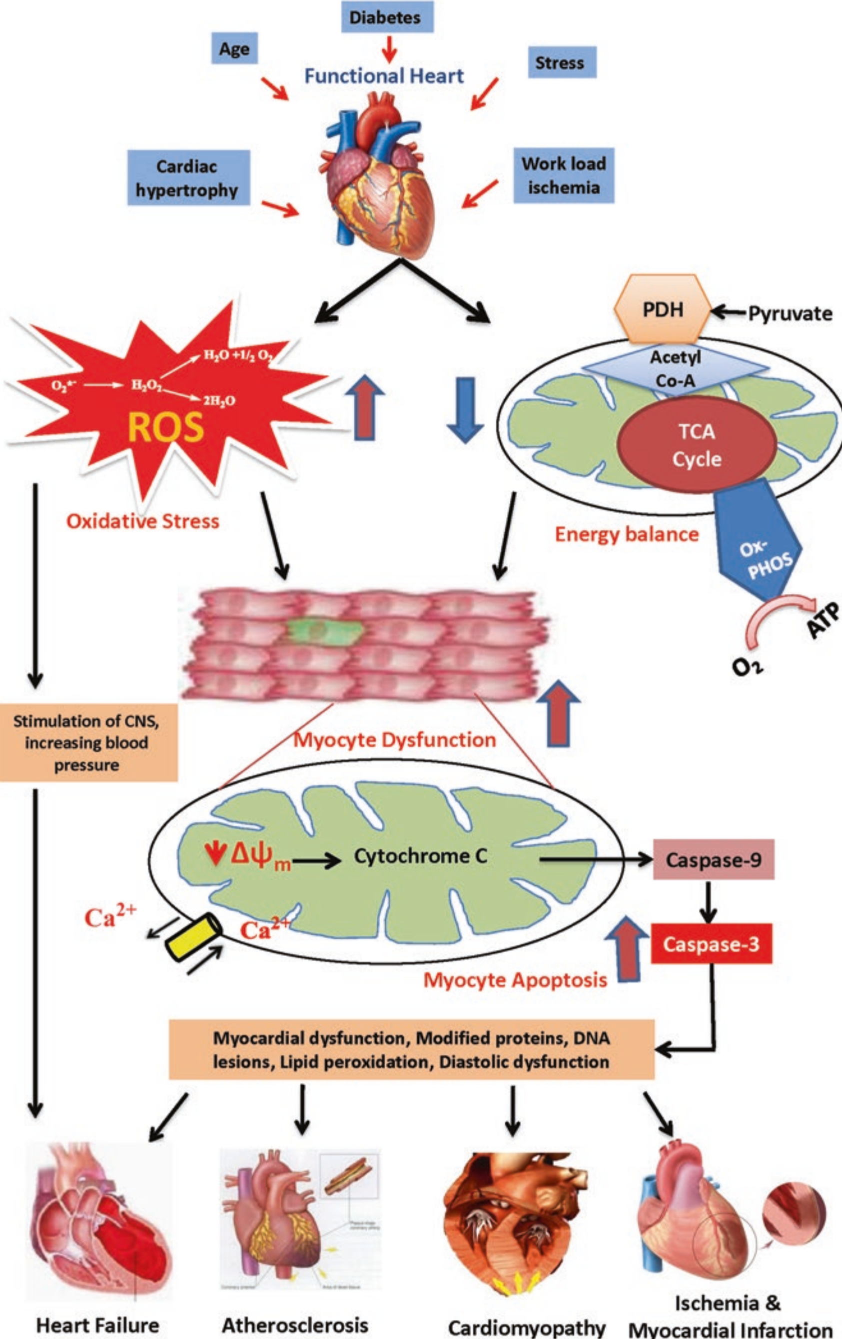


Fig. 23.1 The pivotal role of mitochondria in cardiovascular health

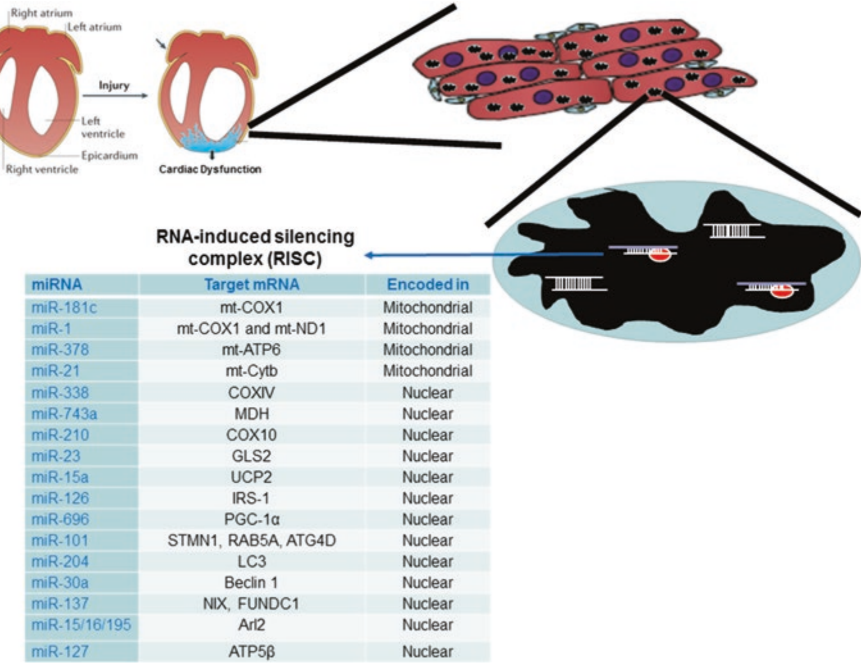
drosophila and mouse models, have been focusing on important role of mitochondrial fission and fusion in cardiac myocytes. The imbalance between mitochondrial fission and fusion affects cellular homeostasis, leading to several disease states, including cancer, neurodegenerative diseases, impaired cardiac development, and cardiovascular diseases. Mitochondrial dynamics also play a critical role in apoptosis, autophagy, and contractile dysfunction [26].

Recent studies focus on the role of miRNAs in the alteration of myocyte function through mitochondrial fission-fusion mechanism. The fission machinery within the mitochondrion is regulated by miR-484, which targets Fis1, a necessary protein for mitochondrial fission and apoptosis [26]. The expression of p53 and dynamin-related protein-1 (Drp1) are down-regulated by miR-30, and thus inhibit mitochondrial fission [27]. Interestingly, dephosphorylation of Drp-1 is suppressed by miR-499, resulting in the inhibition of apoptosis in cardiomyocytes [26]. miR-761 can suppress mitochondrial fission by down-regulating mitochondrial fission factor (MFF) [28]. Subsequent studies have provided additional evidence between mitochondrial morphology and miRNAs; for instance, miR-140 reduce mitochondrial fission by targeting mitofusin-1 [29].

Mitochondrial miRNAs

The mode of action of miRNAs within the cytoplasm has been well studied [6, 7]; however, evidence suggests that miRNAs and Ago2 play an important role also within the mitochondrial compartment [30]. There are studies that suggest the presence of miRNAs in the mitochondrial fraction [30–36], but their functional consequences were largely unknown. In 2012, we demonstrated that miR-181c, a product of the nuclear genome, translocates to the mitochondria, and regulates mitochondrial gene expression. In turn, this alters mitochondrial function by binding to the 3′-end of cytochrome c oxidase subunit 1 (mt-COX1). miR-378 has been shown to target and bind to the mitochondrial transcriptome at the ATP6 locus, causing down-regulation of the protein in the type 1 diabetic heart [34]. ATP6 is a subunit of the F0 complex of the ATP synthase, and its repression impacts ATP generating capacity. In cardiac mitochondria from spontaneous hypertensive rats (SHR) miR-21 has been found to be overexpressed; miR-21 can target mitochondrial genomic product, Cytb [37]. During myogenesis miR-1 enters the mitochondria where it alter the translation of specific mitochondrial genome-encoded transcripts, mt-COX1 and ND-1 [36].

Zhang and colleagues showed that the muscle-specific miR-1 stimulates mitochondrial translation by Ago2-mediated translocation into the mitochondria. The cytoplasmic functions of Ago2 are dependent on its binding partner GW182. In contrast, GW182 has not been detected in mitochondria [36]. While a number of studies (Fig. 23.2) have shown that miRNAs can translocate into the mitochondria when bound to Ago2 (either at pre-RISC or mature-RISC conformation), the underlying mechanism is not well understood. It has been shown that Ago2 acts as a carrier



miRNA	Target mRNA	Encoded in
miR-181c	mt-COX1	Mitochondrial
miR-1	mt-COX1 and mt-ND1	Mitochondrial
miR-378	mt-ATP6	Mitochondrial
miR-21	mt-Cytb	Mitochondrial
miR-338	COXIV	Nuclear
miR-743a	MDH	Nuclear
miR-210	COX10	Nuclear
miR-23	GLS2	Nuclear
miR-15a	UCP2	Nuclear
miR-126	IRS-1	Nuclear
miR-696	PGC-1α	Nuclear
miR-101	STMN1, RAB5A, ATG4D	Nuclear
miR-204	LC3	Nuclear
miR-30a	Beclin 1	Nuclear
miR-137	NIX, FUNDC1	Nuclear
miR-15/16/195	Arl2	Nuclear
miR-127	ATP5β	Nuclear

Fig. 23.2 The mechanistic insights of MitomiRs in the pathophysiology of various heart defects

protein, as it can shuttle mitochondrial proteins (heat-shock proteins) [38], ribosomal proteins [39], and mitochondrial genomic products (a non-coding RNA, tRNAMet) [40] into the cytoplasm. This evidence suggests a pivotal role for Ago2 in transporting proteins or non-coding RNAs into and out from the mitochondrial compartment to the cytosolic fraction via mitochondrial membrane channels: TOM at the outer mitochondrial membrane, and TIM situated within the inner membrane of the mitochondrion. In addition, Barrey and colleagues have suggested that both miRNAs and pre-miRNAs are encoded within the mitochondrial genome [31]. However, the biogenesis and the functional aspects of these mitochondrial miRNAs have not been fully elucidated.

Heart Disease

Mitochondria play an important role in the pathophysiology of multiple cardiovascular disease conditions. Thus, preservation of mitochondrial function during the progression of heart defects is one of the primary focuses in cardiovascular therapeutics. MitomiRs can influence various mitochondrial pathways, such as the electron transport chain (TCA), lipid metabolism, and amino acid metabolism. Below we are discussing the role of miRNA in the pathological cardiac phenotypes involving mitochondria.

Congenital Anomalies

The heart is one of the notable organs which is susceptible to congenital defects, but whether miRNAs play a direct role to these defects is unknown. However, a number of reports describing the alteration of miRNAs expression in cardiogenesis is documented. A miRNA cluster, miR-17–92, has been reported to play an important role in septal defects [41]. Human chromosome21-derived miRNAs were overexpressed in the heart with trisomy 21 (Down syndrome), the most common genetic cause of congenital heart defects [42]. Mice lacking of miR-1-2 have thickened walls due to persistent proliferation; septal defects were common and often fatal in this mouse model [43]. Interestingly, miR-1 has been shown to translocate into the mitochondria during muscle development where targets mitochondrial genes, such as ND-1 and mt-COX1 [36]. It has also been reported that in infants with hypoglycemia, progressive lactic acidosis, an increased serum lactate/pyruvate ratio, and elevated plasma alanine are due to defects in the complex I of the mitochondrial respiratory chain [44]. ND-1 is one of the mitochondrial gene derived complex I subunits which plays an important role in the formation of mitochondrial iron-sulfur clusters. Thus, it is an open question that mitochondrial miRNA can play an important role in congenital anomalies of the heart.

Atherosclerosis

Atherosclerosis along with its associated complications are one of the major causes of morbidity and mortality in the United States of America. The inflammatory mediators and shear stress are the two important biochemical and biomechanical stimuli which lead to atherosclerotic lesions. Recently, miRNAs have served as important regulators of multiple pathophysiological cellular effects and molecular signaling pathways involved in atherosclerosis [45, 46]. miR-33a/b regulates HDL synthesis and reverse cholesterol transport by directly binding to the 3′-UTR of ATP-binding cassette transporter A1 (ABCA1). Inhibition of miR-33 in mice on a western-type diet results in elevated plasma HDL. Thus, miR-33 has been suggested to represent a therapeutic target for ameliorating cardiometabolic diseases [47]. ABCA1 can promote foam cell formation by inhibiting macrophage cholesterol efflux. Among other miRNAs, such as miR-758 [48], miR-106b [49], miR-144 [50, 51], miR-128-1 [52], miR-130b [52], miR-148a [52], miR-301b [52], miR-302a [53], miR-33 has been shown to modulate macrophage cholesterol efflux to maintain cholesterol homeostasis [47, 54, 55]. The mitochondrial role of miR-33 has been well established. miR-33 has been found to target both CROT and CPT1A, which eventually affects fatty acid β oxidation (FAO) [56]. miR-33a-5p and miR-33b-5p is encoded from the intronic region of SREBP2 [57], and SREBP1 [58] genes, respectively. By targeting important enzymes like CROT, CPT1A, and 3-Ketoacyl-CoA thiolase, the two family members, miR-33a and miR-33b, affect fatty acid metabolism [1].

Interestingly, the complementary strand of miR- 33a-5p is miR-33a-3p, which has been found to play an important role in FAO by targeting the same targets as miR-33a: CROT and CPT1A [59]. Additionally, miR-148a/b has been shown to regulate mitochondrial function by regulating TCA cycle by directly binding to the 3′-UTR of citrate synthase mRNA [60].

Ischemia and Myocardial Infarction

In 1986, Murry and colleagues described a phenomenon that produces an energy-sparing effect known as ischemic preconditioning during a sustained ischemic insult of the heart [61]. Later, this group and others [62, 63] recognized that ATP utilization of the preconditioned myocardium was much slower than non-preconditioned myocardium. Slower ATP utilization translates into lower stimulation of anaerobic glycolysis, attenuated fall of intracellular pH, and minimization of the ionic alterations that are typical of ischemia. Heart-specific miR-1 and miR-133a may play an important role in myocardial ischemic post-conditioning protection by regulating pro-apoptotic genes, such as CASP9 [64]. Furthermore, miR-1 protects the myocardium from ischemia-reperfusion injury by targeting Hsp90aa1 [65]. As discussed above miR-1 can regulate mitochondrial function by regulating mitochondrial genes [36]. Mitochondrial role in cardiac-ischemia reperfusion injury is well established [63, 66, 67].

An up-regulation of miR-210 expression in the placenta has been reported earlier [68]. Moreover, miR-210 modulates endothelial cell response to hypoxia [69]. Apart from hypoxia, acute myocardial infarction could also upregulate miR-210 [70]. miR-210 also elicits effects on cardiomyocytes and the vasculature. Overexpression of miR-210 in vitro or by intramyocardial injection (in vivo) reduced cell death and improved cardiac function and angiogenesis after myocardial infarction [70]. Importantly, miR-210 regulates electron transport chain constituents by interacting with iron–sulfur cluster scaffold homolog (ISCU), determining the levels of ROS in the mitochondria [71]. miR-210 has also been reported to modulate complex IV function of the ETC by targeting the nuclear encoded mRNA COX10 [72, 73]. Although the specific isoform/component of the complexes has not been validated, it has been reported that miR-210 and miR-141 target complex III and complex V of the ETC, respectively [74].

In cerebral ischemic-injury models, a down-regulation of miR-30a expression has been identified. It has been proposed that the overexpression of beclin1, direct target of miR-30a in the brain, induces autophagy due to the loss of miR-30a during ischemia/reperfusion injury [75]. Furthermore, the expression of p53 and dynamin-related protein-1 (Drp1) are down-regulated by miR-30, resulting in the inhibition of mitochondrial fission [27]. Li and colleagues showed that miR-137 is another hypoxia-responsive miRNA in the brain, which inhibit mitophagy through targeting two mitophagy receptors, FUNDC1 and NIX [76]. Ischemia-induced up-regulation of miR-140 expression in cardiomyocytes [29]. In an in vivo mouse ischemia-reperfusion

model, it has been shown that the knock-down of miR-140 attenuates myocardial infarction by inhibition of mitochondrial fission mechanism [29].

A miRNA cluster, miR-23/24/27, has been shown to offer cardiomyocyte-protective effects in vitro by targeting the proapoptotic Bcl-2-like protein 11 (also known as Bim) [77, 78]. Additionally, overexpression of miR-24 has been shown to attenuate infarct size and improve cardiac function in rodents following myocardial infarction [78]. Conversely, it has been reported that the cardiomyocyte-specific overexpression of miR-24 is embryonically lethal in mice [79]. Additionally, on one side it has been shown that miR-24 is downregulated in cardiomyocytes and fibroblasts after myocardial infarction [78], but on the other hand in cultured neonatal ventricular cardiomyocytes (NRVM) miR-24 expression was increased by hypoxia [80]. It has also been demonstrated that miR-23 enhances mitochondrial glutaminase expression and glutamine metabolism by targeting c-Myc [81]. To make the matter more complicated, miR-24 exhibited antiangiogenic effects [82, 83], and impaired excitation–contraction coupling [77], after myocardial infarction. Through these mechanisms, miR-23/24/27 inhibition improved neovascularization and cardiac function [77, 83], thereby raising some serious concerns about the use of this miRNA-cluster overexpression as a therapeutic strategy against ischemia-reperfusion injury.

Arrhythmias

Mitochondria are essential to provide the energy demand of the continuous electrical activity and contractile function of cardiac muscle. Emerging evidence indicates that mitochondrial dysfunction can deteriorate cardiac electrical functioning by impairing the intracellular ion homeostasis, reduced ATP, and excess ROS production, resulting in increased tendency to cardiac arrhythmias [84].

The electron transport chain (ETC) is a redox pathway carried out by five complexes – I, II, III, IV and V (ATP synthase) – which play a pivotal role in ATP and ROS production. The ETC complexes I, III and IV generate a proton gradient by oxidizing NADH/NADPH. This proton gradient is then utilized by ATP synthase to produce ATP.

Oxidative stress has been implicated in the etiology of diabetic cardiomyopathy. Mitochondrial ETC complexes I and III generate ROS as a by-product of their respiratory function. Generation of ROS has been found to have deleterious effects on mitochondria and eventually leads to mitochondrial dysfunction [30, 32].

Iron-sulfur clusters (Fe-S) are essential cofactors for the transfer of electrons in oxidative phosphorylation (OXPHOS) [85]. Thus, the function of Complex I and Complex IV is highly dependent on (Fe-S). The Fe-S assembly enzyme (ISCU) plays an important role in the synthesis of these Fe-S clusters [85]. Under hypoxic conditions, it has been demonstrated that miR-210-5p can directly target ISCU [72, 86]. Succinate dehydrogenase subunit D (SDHD), a subunit of Complex II, has been identified as a target of miR-210 [87]. The authors concluded that miR-210 can

ultimately alter complex II activity [87]. COX10, another nuclear encoded subunit of complex IV has been reported as a target of miR-210-5p [72]. Cytochrome c oxidase subunit IV (COXIV), one of the complex IV subunits, plays a vital role during the assembling process of complex IV and in its respiratory function [88]. In vitro studies on neuronal cells showed miR-338-5p alters complex IV activity by targeting the 3′-UTR of COXIV mRNA [89]. In independent studies, it has been shown that the mitochondrial genomic subunit of complex IV, mt-COX1, can be targeted by two different miRNAs, miR-181c [30, 32], and miR-1 [36]. Mitochondrial transcripts are polycistronic in nature, and thus, the effects of miRNA binding to the 3′-UTR of mt-mRNA are still not fully understood. On the one hand, it has been shown that by binding to a miRNA, mt-mRNA expression is downregulated [30, 32, 34]. Conversely, miRNA acts as an activator of post-translational processes when it binds to a mitochondrial encoded-mRNA [36, 37]. Chronic overexpression of a miRNA that targets mitochondrial mRNA confirmed this observation [32, 37].

Zheng and colleagues reported that miR-101-3p negatively regulates the expression of ATP synthase subunit β (ATP5B) [90]. In another study, miR-127-5p was also shown to target the 3′-UTR of the ATP5B transcript, and alters protein content [91]. Additionally, miR-338-5p has been found to target ATP5G1, which ultimately augments ATP synthase activity [89]. Finally, miR-378 has been shown to target and bind to the mitochondrial transcriptome at the ATP6 locus in the heart [34]. ATP6 is a subunit of the F0 complex of the ATP synthase, and its repression impacts ATP generating capacity.

Hypertension

Patients with high blood pressure frequently show abnormalities in cardiac morphology and function, including left ventricular hypertrophy, systolic/diastolic dysfunction, which ultimately leads to heart failure. Once again, excessive ROS production in mitochondria can also be associated with hypertensive cardiomyopathy. The ETC complexes are well known to be the major source of mitochondrial ROS production [32, 66]. Several studies have focused on nuclear-encoded subunits of the ETC and their contributions to mitochondrial ROS production in the spontaneous hypertensive rats (SHR) model. Meng and colleagues proposed that downregulation of the two mitochondrial enzymes, trifunctional enzyme alpha subunit (Hadha) and NADH dehydrogenase 1 alpha subcomplex 10 (Ndufa10), is associated with the development of cardiac hypertrophy in SHR [92]. Furthermore, in SHR cardiac mitochondria the upregulation of multiple subunits of the ETC complexes, such as complexes I, III and IV, and down-regulation of complexes II and V have been reported [93]. Emerging data suggest that mutations in mitochondrial DNA (mtDNA) can play a role in essential hypertension [94]. Recently, it has been shown that downregulation of mtDNA-encoded cytochrome b (mt-Cytb) in SHR can activate mitochondrial ROS production [37]. The authors also demonstrated that miR-21, a key miRNA induced in SHR, can regulate mitochondrial ROS

production by directly binding to the 3′-END of mt-Cytb mRNA [37]. Besides, exogenous miR-21 delivered by recombinant adeno-associated virus (rAAV) was sufficient to lower blood pressure in the SHR model, suggesting a new therapeutic strategy against hypertension [37].

Cardiomyopathy

There are multiple types of cardiomyopathies, including dilated cardiomyopathy (DCM), restrictive cardiomyopathy, hypertrophic cardiomyopathy (HCM), arrhythmogenic right ventricular dysplasia, and unclassified cardiomyopathy. Cardiomyopathy can be acquired or inherited. mtDNA mutation can cause mitochondrial cardiomyopathy, which is estimated about 1 in 5,000 births [95]. Mitochondrial cardiomyopathy can only be inherited from the mother.

Mutations in mitochondrial-encoded genes like Cytb, mt-COX1, mt-COX2 and mt-COX3 all result in the development of DCM [96, 97]. DCM represents the leading cause of mortality in patients with muscular dystrophy. Cardio-specific overexpression of miR-30c mice developed DCM after 6 week of age [98]. These mice showed a significant downregulation of mitochondrial complexes III and IV protein content, altering mitochondrial function in the heart [98]. The expression of miR-30c in the heart during DCM is controversial: some studies have found up-regulation of miR-30c, others have found down-regulation [99–101]. Notably, miR-30c is found in cardiomyocytes as well as in fibroblasts [99]. In vitro, miR-30c has been demonstrated in the regulation of cardiac fibrosis and cardiomyocyte hypertrophy through the alteration of mitochondrial function, apoptosis, and cell proliferation [99]. On the other hand, miR-30c has been proposed to have therapeutic potential for the treatment of hyperlipidemia, as miR-30c can decrease lipid synthesis and lipoprotein secretion [102].

HCM is an autosomal dominant disease characterized by variable expressivity, age penetrance, and a high heterogeneity. The transcriptional profile – miRNAs expression, epigenetic modifications, and posttranslational modifications – seems to be highly relevant for the onset of the disease. Once again, energy deficiency due to HCM ultimately compromises the relaxation capacity of the cardiomyocyte [103, 104]. Thus, mitochondrial role is pivotal in the pathophysiology of HCM. There are several miRNAs that have been found to alter mitochondrial function in HCM. Both in human [105] and mouse models [106], miR-21 has been shown to be upregulated during HCM. On the other hand, miR-1 is down-regulated during HCM. As we have discussed in this chapter both miR-1 and miR-21 can modulate mitochondrial function in the muscle cells by targeting mitochondrial-encoded genes [36, 37]. miR-132 [106] in murine heart and miR-184 in human heart have been found to be up-regulated in HCM. Both miR-132 and miR-184 have been shown to alter mitochondrial transport by targeting CACT for miR-132 [107] and Slc25a22 for miR-184 [108].

Dysfunctional mitochondria are central in the pathogenesis of diabetic cardiomyopathy. MitomiRs can influence diabetic cardiomyopathy by altering multiple pathways: influencing the mitochondrial ETC, altering FAO, amino acid metabolism, nucleotide metabolism, altering mitochondrial transport [109]. Barath and colleagues summarized the possible mechanisms of all the MitomiRs which play an important role in diabetic cardiomyopathy [109].

Conclusions

In conclusion, several data suggest that MitomiRs critically control the pathophysiology of multiple heart diseases. Several MitomiRs might be attractive candidates or targets to improve cardiac health. However, it is evident that further studies are required to identify the mechanisms and binding proteins responsible for the import of miRNAs into mitochondria. More studies are also needed to address the ongoing controversy that when a miRNA binds to mitochondrial-encoded genes, it either increases mt-mRNA activation or mt-mRNA repression. It is clear that by targeting mt-mRNAs, miRNAs are able to influence various aspects of mitochondrial metabolism, resulting in the alteration of mitochondrial function. This alteration ultimately leads to mitochondrial dysfunction, which plays an important role in the development of various cardiac disorders. The use of MitomiRs can offer new therapeutic opportunities for cardiovascular diseases.

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Chapter 24
Mitochondrial Dysfunction in Cardiovascular Aging

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Mitochondria are the primary source of energy metabolism in the heart [1]. Previous studies have shown that as a concomitant of the aging process cardiac mitochondria undergo detrimental changes [2]. It turns out that these changes do not occur through the entire mitochondrial population in given cardiomyocytes, but occur predominantly

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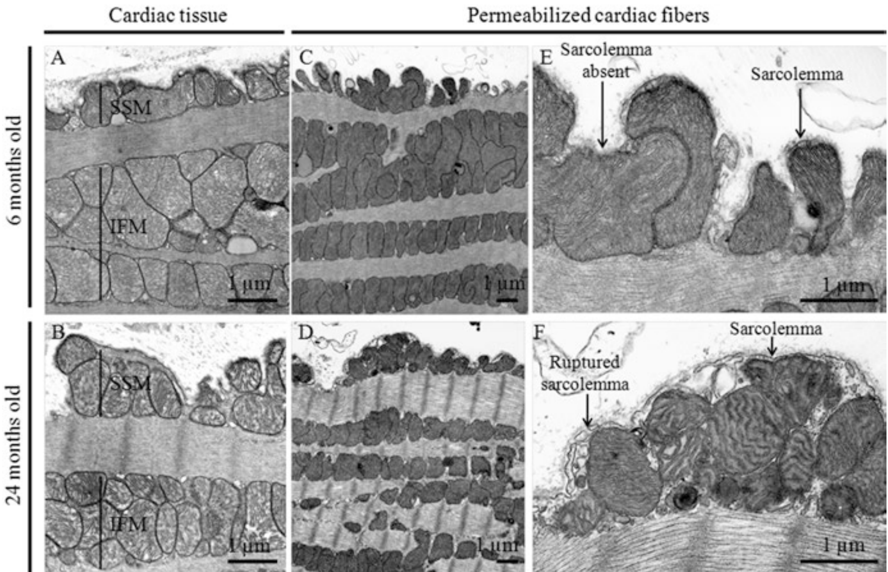

Fig. 24.1 Electron micrographs of the cardiac tissue and permeabilized fibers from young and old Fisher 344 rats: (**a**) 6-month, (**b**) 24-month. The subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria are identified. The ultrastructure of the permeabilized fibers is shown in (**c**) 6-month at low magnification, (**d**) 24-month at low magnification, (**e**) 6-month at high magnification, (**f**) 24-month at high magnification. In the higher magnification micrographs, some segments of the sarcolemma are intact, but other are fragmented; examples are identified with *arrows*. Dimensional bar = 1 µm (Adapted from Lemieux et al. [11] with permission from the Journals of Gerontology, Part A)

in those mitochondria situated between the myofibrils (interfibrillar mitochondria = IFM) [3–5]. In contrast, the subsarcolemmal (SSM) mitochondria are unaffected [3]. These observations are based on isolated mitochondria derived by a technique that separates the two populations.

Ultrastructure of cardiac mitochondria utilizing transmission electron microscopy (TEM) has not revealed obvious differences in structure between the two populations of mitochondria [3, 6, 7]. However, examination by high-resolution scanning electron microscopy (HRSEM) of osmium-extracted, otherwise intact heart tissue revealed differences in the organization of cristae in the two subsets of organelles [8, 9]. This being the case, we proceeded to examine cardiomyocytes that had their plasma membrane disrupted, so-called permeabilized fibers (Fig. 24.1). TEM showed that although remnants of the plasma membrane were still present, there was extensive discontinuities [10].

These permeabilized fibers proved to be excellent simulacra of intact cardiomyocytes in that they use oxygen and elaborate ATP at a level that matches isolated mitochondria [11, 12]. Using such cells we were able to compare the metabolic performance of fibers isolated from three different rat strains that have significantly different life spans [11]. Of the three rat strains used in this study, the Fischer 344 strain has the

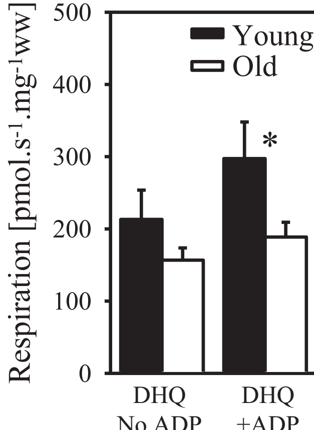

Fig. 24.2 Duroquinol (DHQ) + rotenone oxidation in permeabilized cardiac fibers from young and old Fisher 344. Resting respiration is measured in the presence of DHQ + rotenone without ADP (DHQ, no ADP). Coupled respiration is measured after the addition of saturating ADP to the DHQ (DHQ, +ADP). Data for the different age groups. The *asterisk* indicates significant differences between age groups for the same state and strain (Adapted from Lemieux et al. [11] with permission from the Journals of Gerontology, Part A)

shortest life and exhibits the greatest decrease in mitochondrial function. Brown Norway rats have a longer life span and show less severe alterations in key mitochondrial functions. Hybrids of the two rat strains live significantly longer lives and avert the mitochondrial alterations that occur in their parent strains [11]. From these observations, it can be concluded that cardiac mitochondrial performance is correlated with life span; in other words, the more profound the mitochondrial damage in the heart, the shorter the lifespan, probably because of production of reactive oxygen species and activation of mitochondria-based programs leading to cell death [13–18]. Figure 24.2 shows the decrease in oxidative phosphorylation starting at complex III in cardiac fibers from aged Fischer 334 rats Lemieux et al. [11].

The use of permeabilized isolated cardiac muscle fibers has been touted to be superior to the use of isolated mitochondria [11, 12]. However, although permeabilized fibers do in fact reveal mitochondrial abnormalities it is impossible to determine if these changes involve the entire content of mitochondria or are restricted to a particular subset. In contrast, by separating SSM and IFM it is possible to determine if the damage is ubiquitous or affects only one, thus providing more precise information than the fiber preparations are capable of showing [3]. For this reason, we rely principally on the use of cell fractions to obtain separate sets of mitochondria that are situated in the two discrete venues. The result of these studies is that we determined that mitochondrial damage in the short-lived animals is not ubiquitous for the entire mitochondrial content of these cells, but is restricted to the IFM, whereas the SSM remained unaffected [2, 3, 5]. Integrated mitochondrial function, measured as oxidative phosphorylation, is decreased in IFM because of a defect in

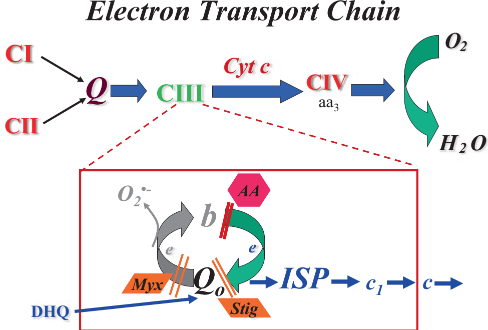

Fig. 24.3 A cartoon of the electron transport chain (*upper portion*) showing the convergence of reducing equivalents from complexes I and II to coenzyme Q. Reduced coenzyme Q is oxidized by complex III to reduce cytochrome c, which is oxidized by complex IV with formation of water. The *lower portion* of the cartoon below the dashed lines shows the Q_o site on cytochrome b with reduction by the coenzyme Q analog duroquinol, the myxothiazol-binding site (Myx) and the stigmatellin-binding site (Stig) in the Q_o site. Superoxide from leakage at the Q_o site is shown. Antimycin A is a traditional complex III inhibitor. The Reiske iron-sulfur protein (ISP) and cytochrome c_1 (c_1) with cytochrome b are the active sites for electron transfer in complex III

oxidation coupled with an increase in net ROS production, again only in the IFM [3, 16]. We showed that the oxidative defect is localized to complexes III and IV, an observation subsequently confirmed by Hagen and colleagues [3, 16, 17, 19]. Further, the site of ROS production was determined to be complex III, locating the defect in complex III at the hub of mitochondrial damage in aging [16]. This defect can be ameliorated by administration of acetylcarnitine to the aged rat [3, 16].

To return to the case of the permeabilized fibers, although the plasmalemma was disrupted, the mitochondria had a normal appearance, both SSM and IFM (Fig. 24.1). Mitochondrial morphology was not altered by age, in that the organelles of both 6- and 24-month old rats were structurally identical by TEM [3, 6, 7]. Thus biochemical alterations that have occurred in the 18-month interval are unrelated to morphology of these mitochondria. It should be noted that the differences in crista architecture between the SSM and IFM that we observed by HRSEM in adult, 6-month rat hearts are effaced by 24 months [10].

As mentioned previously, complexes III and IV are the sites of oxidative defects in IFM in the aged rat heart [3, 4, 16]. Under normal circumstances, complex III oxidizes reduced coenzyme Q and through a series of steps ultimately reduces cytochrome *c* [1, 2] (Fig. 24.3). In the aged rat, the binding site for reduced coenzyme Q (Qo of cytochrome *b*) is altered, resulting in decreased activity of complex III with increased ROS production at that site [3, 4, 16]. Complex IV activity lessens in the IFM of the 24-month rat because of a structural rearrangement of the complex that can be restored by phospholipids [3]. Taken together, the alterations in complexes III and IV lead to an increased susceptibility of the aged cardiomyocyte to stress, including infarction, so that contraction of the cardiomyocyte is interfered with.

This susceptibility in brought about largely by the ROS production at the Qo site in complex III [16]. ROS production is influenced by infarction, which causes a loss of iron from the iron-sulfur protein (ISP) [20] that constitutes a catalyst in complex III, adding a further defect to the aging effect. The defect in the ISP leads to a further increase in ROS production as a retrograde effect, exacerbating the production of ROS, thus worsening the problem [20].

Defects in Electron Transport Induced by Age

Supercomplexes (respirasomes) are assemblies of complex I, the dimer of complex III, and one to four copies of complex IV that facilitate the interaction between components in terms of reducing equivalents [21–28]. In aged animals, changes in the subsidiary complexes III and IV of supercomplexes result in a diminution of the content of the host supercomplex in IFM, leading to a decrease in oxidation [29]. In contrast, complex I activity is unaffected by age in murine hearts [30].

Cytochrome c *Oxidase (Complex IV)*

Cytochrome *c* oxidase plays a significant role in respiration. With age, respiration through cytochrome c oxidase decreases in IFM because of an altered inner membrane, which depends in part on biophysical properties [3, 31, 32]. However, the addition of phospholipid liposomes restored the cytochrome c oxidase respiration and enzyme activity [3, 32]. In IFM (both in situ and isolated) there is a 25% reduction in subunit VIIa, as shown by immunoelectron microscopy [19]. Because analytical immunoblotting fails to reveal such a decrement [19], it may be concluded that the subunit is actually present but is not readily demonstrable by the aforementioned structural study because of morphological alterations in the inner membrane.

The same decrease in complex IV activity and content is present in the heart of aged humans [33]. Decreased cytochrome oxidase activity has an upstream effect on complex III, enhancing the production of mitochondrial ROS as previously noted in a variety of cardiac models [34, 35], eventually favoring activation of cell death programs [36].

Complex III

As in the case for complex IV, complex III activity is decreased only in IFM in aging [3]. It has been found that the Qo site of cytochrome *b* is the critical recipient of the aging effect [16]. This observation is based on the use of two inhibitors, each of which binds to different domains of the Qo site. Stigmatellin inhibits the reduction

of cytochrome b in IFM from the aged heart in the same fashion as it does in young hearts. In contrast, myxothiazol only partially inhibits the reduction of cytochrome *b*, resulting in a leak of electrons from the Qo site of IFM from the aged heart (Fig. 24.3). These observations support the notion that the aging defect in complex III is specifically localized to the myxothiazol-binding site of the Qo site of cytochrome b [16].

It has been shown that the major site of ROS production is complex III and that the Qo site is the principal source of ROS [34, 37]. The superoxide so produced is in the intermembrane space and can either pass directly into the cytosol through the voltage-dependent anion channel [38] or be converted to H_2O_2 by Cu, Zn-superoxide dismutase in the intermembrane space [34]. The H_2O_2 subsequently can leave the mitochondria. In both cases, the released H_2O_2 can damage all extra-mitochondrial structures within the myocyte [39, 40]. The ROS in the intermembrane space activates the permeability transition pores [41] leading to cytochrome *c* release into the cytosol triggering programmed cell death. The inner membrane permeability transition pores may consist in part of complex V [42]. The decrease in complex V activity with aging combined with the presence of ROS might increase the propensity of the inner membrane to elaborate pores that could contribute to mitochondrial damage [14].

Cardiolipin

The phospholipids of the mitochondrial inner membrane include a unique lipid, namely, cardiolipin (CL) [43, 44]. It is essential for the activity of complex I, III, IV, and V as well as translocases and transporters of the inner membrane [44]. Albeit others have attributed aging effects in cardiac mitochondria to a reduction in their content of CL [32], in our hands the content of CL in SSM or IFM in aged Fischer 344 rats remains unaltered [45]. The discrepancies that have been noted might be due to variations in analytical techniques used in these studies [32, 45]. As a result of our findings, we conclude that CL is unrelated to aged cardiac mitochondrial defects.

However in ischemia of the aged heart, a change in composition of CL takes place [46, 47]; this change does not occur in ischemic young hearts [48]. It is paralleled by the increased susceptibility to damage that occurs in the elderly [49]. Some have held that the changes in CL during ischemia are due to insertion in CL of a higher molecular weight fatty acid [50, 51], whereas it also has been reported that oxidation of CL elevates its molecular weight [46, 47]. These differences in interpretation have not be reconciled.

Mitochondrial DNA, Aging, and the Heart

Mitochondria contain DNA (mtDNA) that differs from that in the nucleus in that mtDNA is arranged in circles and utilizes a different coding language to direct synthesis of specific mitochondrial proteins, all of which are components of the

electron transport chain and are hydrophobic. The mtDNA is susceptible to mutation, which may be brought about by exposure to ROS [52, 53]. An increase in mtDNA mutations in aged heart has been reported [52, 53]. Despite the occurrence of mutations, the copy number of mtDNA is unaffected in the aged heart, neither in SSM nor IFM [54, 55], in contrast to what occurs in skeletal muscle and hepatic mitochondria where the copy number decreases [54].

Mitochondria contain an enzyme – 8-oxodeoxyguandine glycosylase – that repairs damaged mtDNA [56, 57]. When this enzyme is knocked down, mitochondrial mutations increase, an enhancement that rises dramatically with age [55]. This observation supports the idea that aging per se is a cause of mtDNA mutations, again implicating ROS [52, 53]. Not only do the latter affect the production of new proteins they also damage already existing mitochondrial proteins, both enzymatic and structural interfering with the function of these proteins [2].

Dynamic Mitochondria Remodeling in the Aged Heart

Mitochondria in the heart have a limited half-life, but the number of mitochondria per myocyte remains fairly constant. This being the case, there must be a source for the generation of replacement organelles. All evidence indicates that new mitochondria are generated by division of already existing mitochondria [58]. TEM studies have revealed that cardiac mitochondria can follow two distinctly different morphological pathways for division [59]. The first involves the formation of a partition that spans the inner compartment. Ingrowth of the outer membrane in a fashion that resembles a closing iris diaphragm ultimately leads to the separation of the two mitochondrial moieties. Little is known of the biochemical events related to this modality of division. The second pathway, one that has been intensely studied by a spectrum of cell biological methods, involves the formation of a belt-like band around the organelle; its constriction ultimately leads to the fission of the host mitochondrion [59]. These belts consist of dynamin-related protein 1 (DRP-1), which has been attracted to specific outer membrane proteins to form a “noose” around the entire organelle [59]. It is the tightening of this noose that carries out the scission process. As the outer membrane is contracting it impinges on the inner membrane, thus involving the entire covering of the mitochondria. [59].

Based on cell fractionation, it has been reported that a concomitant of aging is that the yield of mitochondria is reduced [3]. In fact, it is only one of the two populations of cardiac mitochondria, namely, the IFM that undergo this reduction, whereas the SSM yield remains unaltered [3]. In order for this reduction in mitochondrial yield to be brought about, either there is a slowing of IFM division or an increase in autophagy (mitophagy) or a combination of the two. In our EM studies we failed to note increased evidence of mitophagy [9, 59], bolstering the notion that it is most likely fission that has been slowed. Even though mitophagy does not seem to increase, its steady occurrence will help to shrink the mitochondrial population. Mitophagy probably involves the elimination of those defective organelles that were set free by organelle division. Even after the two processes (fission and mitophagy)

have taken place, the residual mitochondria in aged animals are unable to engage in oxidative phosphorylation at the same rate as mitochondria in younger animals are able [3]. The possibility of enhanced removal of mitochondria in specific regions of the aged myocyte is a provocative notion. Depolarization of mitochondrial membrane potential activates mitophagy [60]. Perhaps IFM, with their defects in oxidative phosphorylation and enhanced susceptibility to permeability transition pore opening, both risks for depolarization of mitochondrial membrane potential, may undergo enhanced rates of removal by mitophagy in comparison to the age-unaltered SSM population [2].

Therapy

Mitochondrial alterations appear to be a major culprit in damage to the aging heart [1, 2]. Age-induced decrements in mitochondrial function are superimposed upon disease-related mitochondrial impairments [20]. Ischemia-reperfusion [61, 62] and congestive heart failure [63] each induce mitochondrial damage that is superimposed upon pre-existing aging defects. Therefore any therapy that is directed towards improving the performance of these organelles should have salubrious effects in hearts that have been exposed to stress, including ischemia and aging [2]. The concept to attenuate disease-induced impairment of the aged heart by addressing pre-existing age-related defects is an appealing therapeutic approach. Using experimental animals, such therapies include behavior modifications such as caloric restriction [64] and exercise [65], nutriceuticals – coenzyme Q10 plus PUFA [66] and acetylcarnitine [67] – and an assortment of pharmacological agents.

Behavioral Modifications

Although caloric restriction in young rats does not improve cardiac mitochondrial function, in aged rats it ameliorates oxidative phosphorylation as noted in permeabilized cardiac fibers derived from these animals [64]. Calorie restriction further improves mitochondrial function related to activation of sirtuins 1 and 3 [68–70]. Cardiac mitochondrial function also improves in elderly rats that have been exercised. This is accompanied by a reduction in H_2O_2 production and MnSOD activity in both SSM and IFM [71].

Nutraceuticals

Among the nutraceuticals administered to elderly animals are resveratrol, Coenzyme Q10 plus poly-unsaturated fatty acids (PUFA), and acetylcarnitine. Resveratrol delays aged-mediated cardiovascular dysfunction by activation of sirtuins 1 and 3 [72–75].

The latter affects mitochondrial function by deacetylation of acetyllysines. In hearts from untreated elderly rats the activity of sirtuin 1 is decreased. This negative effect is exaggerated by the fact that NAD+, an activator of sirtuins, also decreases with age. The result of these changes is decreased activity of complex I-IV and increased content of NADH+ in elderly female Wistar rats [73]; in other words, the consequence is a weakening of mitochondrial performance. A therapy that increases NAD+ content in skeletal muscle and brain, but its impact on heart has not been studied [76–78].

Administration of CoQ10 in PUFA to 24 months old rats extends their life span. This supplementation enhances the activity of cytochrome *c* oxidase in cardiac mitochondria in 24-month old mice, but CoQ10 alone does not affect either ETC activities or ROS production in these mice [79].

Acetylcarnitine is another nutraceutical that has positive effects on cardiac mitochondria in aged rats [80] by increasing mitochondrial oxidative phosphorylation in IFM to values comparable to those of adults, raising complex III and IV activity, and increasing mitochondrial content of cytochromes *b* and aa_3 [67]. It has been noted that elderly hearts do not respond well to stress such as ischemia-reperfusion [49]. The renaissance in mitochondrial activity brought about by acetylcarnitine provides the elderly heart with the ability to counter this type of stress.

Pharmacological agents

Two chemical agents have been studied in terms of their effect on the hearts of experimental animals. SS-31, a cell permeable tetrapeptide, has been shown to protect against the ravages of ischemia-reperfusion in the heart and improves mitochondrial function in heart failure [81, 82]. It is proposed that SS-31 acts through binding to cardiolipin, protecting cytochrome *c*. Clearly these studies need to be extended to the aged heart especially because it has been shown to improve mitochondrial function in aged skeletal muscle [83].

A second chemical agent that has been studied in the context of life span is rapamycin. When administered in late life to mice it extends their life span, probably by slowing metabolism [84] by affecting gene and protein expression in heart and improved cardiac function [85]. Administration of rapamycin to aged female mice for a 10-week period increases mitochondrial biogenesis and energy metabolism, altering the myocardial metabolome. Admittedly studies of the sort have been limited in scope and need to be pursued in the context of cardiac function, pathology, and cardiac mitochondrial function, especially in aging.

Conclusions

Overall, cardiac mitochondrial dysfunction accompanies aging. Rather than affecting the mitochondrial population in toto in the cardiac myocyte, this dysfunction is confined solely to the interfibrillar mitochondria. Integrated mitochondrial function

measured as oxidative phosphorylation is decreased in the latter because of a defect in complexes III and IV. At the same time the number of IFM per myocyte decreases, compounding the loss of mitochondrial activity. The fact that aged cardiomyocytes can be restored to near adult levels of resistance to stress by the simple expedient of improving mitochondrial function is compelling evidence that it is the mitochondria that are the leading players in the pathophysiology of the aging heart.

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Chapter 25
Insulin Resistance and Mitochondrial Dysfunction

Alba Gonzalez-Franquesa and Mary-Elizabeth Patti

The Epidemic of Diabetes

Diabetes is now recognized as a worldwide epidemic. The International Diabetes Federation (www.idf.org) estimated that diabetes was responsible for 5.1 million deaths in 2013, and projected that diabetes will be the seventh leading cause of death in 2030 [1]. The costs for health care related to diabetes are also enormous. American and Caribbean nations spent around 263 billion USD in 2013 (an amount equivalent to that spent by half of the world), while Europe spent 147 million USD. While diabetes is skyrocketing in South-East Asia and Africa, less than 1% of the global healthcare diabetes budget is spent in these regions.

Among the millions of people worldwide suffering from diabetes, type 2 diabetes (T2D) accounts for ~90% of the cases, with type 1 diabetes accounting for about 10%. Monogenic diabetes and other rare forms make up a small percentage [2]. In this review, we will focus exclusively on T2D and its relationship to mitochondrial function.

Pathogenesis of Type 2 Diabetes

T2D is characterized by elevations in plasma glucose. Once diabetes is diagnosed, the key defects which can be observed include (1) ***insulin resistance***, or reduced responsiveness of a tissue to insulin, and (2) ***abnormal insulin secretion*** by

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pancreatic islet β-cells. In this chapter, we will consider these two defects as largely independent, although there is likely a reciprocal relationship. For example, sustained insulin resistance in multiple tissues (including the β-cell) may contribute to loss of insulin secretion, while altered insulin secretion may also increase glucose levels and induce further insulin resistance. Additional defects contributing to the complexity of diabetes risk include altered intestinal incretin hormone secretion and/or responsiveness, altered glucagon secretion by pancreatic α-cells, impaired oxidative metabolism at rest and during exercise, altered insulin-independent glucose disposal, perturbed lipid and amino acid metabolism, dysregulation of neural control of appetite in the brain, and reduced renal glucose excretion [3].

Which of These Defects Are Primary to Pathogenesis of T2D in Humans?

Elegant studies by DeFronzo and Groop demonstrated that insulin resistance was not only a feature of established T2D, but could also be detected in individuals at risk for T2D. For example, cross-sectional analysis demonstrated reduced insulin-stimulated glucose uptake and glycogen synthesis in normal glucose tolerant humans with diabetes family history [4]. Such insulin resistance was subclinical, as plasma glucose levels were maintained within the normal range by increased insulin secretion by pancreatic β-cells.

DeFronzo and colleagues proposed the concept of the Starling's curve of the pancreas [5], with an inverted U-shaped curve showing the initial increase and subsequent decrease in insulin secretion over time with sustained insulin resistance. Time-dependent loss of insulin secretion would ultimately lead to increased glucose levels and the clinical onset of impaired glucose tolerance (rise in glucose after eating) or overt T2D [6, 7]. These concepts were subsequently extended by the work of Bergman and colleagues, who described a hyperbolic relationship between insulin sensitivity and insulin secretion, with their product defined as disposition index [8]. In this context, reductions in insulin sensitivity are compensated by increased insulin secretion by healthy β-cells, maintaining glucose homeostasis. Once the disposition index is reduced, glucose values rise. From these concepts, it is not surpising that β-cell function may be already reduced by as much as 80% once T2D is diagnosed [9–12], with similar reductions in β-cell mass [13, 14].

Longitudinal studies have also underscored the importance of insulin resistance as not only a feature of diabetes risk, but a predictor of T2D development. For example, metabolic analysis of normal glucose tolerant individuals with T2D family history (both parents with T2D) revealed that insulin resistance at baseline, but not insulin secretion, predicted subsequent T2D development over a mean of 25 years [15]. Moreover, longitudinal population analysis over 13 years demonstrated that defects in insulin secretion emerged relatively late in those who ultimately developed T2D [16].

Molecular Etiology of Insulin Resistance

The burden of insulin resistance and T2D is closely linked to the epidemics of obesity and physical inactivity [17], as both obesity and physical inactivity are typically associated with insulin resistance.

As with many phenotypes in humans, there is substantial interindividual variability in the susceptibility to insulin resistance and T2D, likely related in part to genetic background [18, 19]. For example, rare genetic mutations can result in monogenic forms of T2D [20–22]. Genome-wide association studies (GWAS) in individuals with common forms of T2D have identified many polymorphisms associated with disease risk [23–25]. Interestingly, these effects are modest in size, and collectively explain only ≈10% of disease heritability [26]. Genes proposed to be related to these SNPs have largely been assumed to mediate effects via insulin secretion. However, it remains uncertain whether such SNPs are mediating risk via a nearby or more distant gene or noncoding RNA, and many SNP-related genes are expressed rather broadly, making functional assessment of SNP-mediated mechanisms difficult.

More recent GWAS studies and meta-analysis [27] have identified *loci* associated with diabetes-related continuous metabolic variables (i.e., HOMA-IR, HOMA-B, fasting glucose, fasting insulin). For example, a SNP adjacent to insulin receptor substrate 1 (IRS1) was reported as the first T2D risk *locus* associated with insulin resistance and hyperinsulinemia [28]. Moreover, SNPs within the 1-Mb region flanking the gene MTNR1B are associated with fasting glucose, independently of body mass index (BMI) [29–31]. Moreover, T2D, fasting glucose, and glycated hemoglobin [30] are also associated with a variant near MTNR1B. Recently, two large consortia have performed whole-genome and exome sequencing in a large population, and conclude that lower-frequency variants are common, but do not have a major impact on T2D predisposition [32].

Collectively these data suggest that substantial T2D risk is related to non-genetic factors. These may include epigenetic factors, developmental history and exposures, and postnatal environmental factors such as personal or family lifestyle, dietary patterns, physical activity, and the microbiome.

Tissue-Specific Insulin Resistance

While insulin resistance can be defined by reduced responsiveness to insulin, the metabolic manifestations of insulin differ according to tissue and cell type.

Skeletal muscle accounts for over 80% of insulin-stimulated glucose uptake. Thus, insulin signaling in skeletal muscle is essential for normal insulin-stimulated whole-body glucose uptake. Indeed, analysis of human skeletal muscle biopsy samples demonstrated that insulin stimulation activates not only insulin signaling [33], but also downstream metabolic endpoints such as glycogen synthesis [4] and potent

transcriptional responses [34]. In insulin resistant individuals, insulin signaling is disrupted, with increased serine (Ser) phosphorylation of IRS1, decreased AKT phosphorylation, and reduced translocation of the glucose transporter GLUT4 to the sarcolemmal membrane, thus impairing glucose uptake [35]. These signaling defects are also pathway-specific, with preserved activity of the ERK-MAPK pathway despite defects in the AKT pathway [33].

In the ***liver***, insulin resistance results in failure to suppress gluconeogenesis, promoting increased hepatic glucose production (HGP). Indeed, insulin inhibits the transcription of two key gluconeogenic enzymes (phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6 phosphatase (G6Pase) [36]), through AKT and FOXO-dependent mechanisms [37]. Moreover, insulin resistance is often accompanied by hepatic steatosis and nonalcoholic fatty liver disease, reflecting at least in part, increased lipogenesis. In turn, lipid accumulation may also reduce insulin signaling, via activation of PKC-ε and JNK1-dependent pathways [38].

Insulin resistance in ***white adipose tissue*** (WAT) results in not only reduced glucose uptake but also failure to suppress lipolysis, leading to increased release of free fatty acids (FFA) and glycerol from adipose stores, further increasing plasma FFA. This can result in excessive delivery and so-called "ectopic" accumulation of lipids in non-adipose tissues such as skeletal muscle and liver [39]. Furthermore, obesity-related insulin resistance is also associated with altered secretion of adipokines, both pro- and anti-inflammatory, and low-grade systemic inflammation [40].

Since the discovery of human ***brown adipose tissue*** (BAT) [41–43], this tissue has been established as an important endocrine organ which can modulate whole-body glucose metabolism, insulin sensitivity, and thermogenesis [44]. This was demonstrated experimentally in animal models, in which high fat diet (HFD)-fed mice receiving a BAT transplant had reduced body weight, increased insulin sensitivity, and improved glucose tolerance [45]. Whether improvements in systemic metabolism result from BAT secretory products (e.g., circulating norepinephrine, IL6, FGF21), from increased glucose and lipid uptake by brown fat depots, or from other mechanisms, remains uncertain [45]. Metabolically active adipocytes residing in WAT, due to a process called *browning*, may also modulate thermogenesis and whole-body energy expenditure.

Less traditionally insulin-responsive tissues also play key roles in metabolic defects associated with insulin resistance. For example, insulin resistance in the ***brain***, specifically in the satiety centres of the hypothalamus (e.g., arcuate nucleus) [46], can promote hyperphagia and reduce energy expenditure, thus increasing adiposity. Furthermore, brain insulin resistance may also directly regulate metabolism in other organs, e.g., increasing hepatic glucose production and reducing muscle glucose uptake in rodents [47, 48].

The ***gastrointestinal system*** also plays a critical role in regulation of metabolism via secretion of incretin hormones such as GLP-1 and GIP in response to oral intake. These hormones not only increase insulin secretion, but also regulate intestinal motility, appetite, and systemic metabolism [49–51]. GLP-1 and GIP secretion in response to a meal are impaired in normoglycemic insulin resistant humans,

in parallel with the degree of insulin resistance [52]. Thus, incretin deficiency is a likely contributor to progressive decreases in insulin secretion in insulin resistant individuals.

Mitochondrial Function and Human Insulin Resistance

Mitochondria are essential for maintaining cellular energy homeostasis and viability. They are often called the cellular powerhouses given their important role in the generation of adenosine triphosphate (ATP). However, mitochondria also have important roles in reactive oxygen species (ROS) generation, detoxification and scavenging, ROS-dependent intracellular signaling [53, 54], apoptosis and autophagy control [55], and regulation of cytoplasmatic calcium [56]. We will first outline key mitochondrial processes, and then consider the evidence supporting links between insulin resistance, T2D risk, and disruption in mitochondrial content or functional capacity in tissues relevant for metabolic regulation.

Mitochondrial Bioenergetics

Mitochondria produce ATP mainly through oxidative phosphorylation (OxPhos) located in the mitochondrial inner membrane *cristae*, foldings which expand the membrane surface area. Electrons generated from nutrient oxidation are carried by the electron donors NADH and $FADH_2$ and enter the electron transport system (ETS) at complex I (CI) and II (CII), respectively. The flow of electrons results in pumping of protons from the mitochondrial matrix to the intermembrane space, creating both a pH gradient and a membrane potential. When protons return to the matrix via complex V (CV or ATP synthase), ATP is synthesized from adenosine diphosphate (ADP) and inorganic phosphate [57]. Protons can also leak across the inner membrane through uncoupler proteins, thus bypassing ATP synthesis and releasing energy as heat instead, the so-called non-shivering thermogenesis.

Mitochondrial Biogenesis

The content, volume and mass of mitochondria are tightly regulated in response to coordinated signals from both nuclear- and mitochondrial-encoded proteins. One key regulator of this process is the PGC1 family of coactivators [58]. PGC1α and β coactivate transcription factors such as PPAR nuclear receptors, NRF, and ERR, promoting coordinated increases in transcription of mitochondrial genes. This response, which ultimately increases mitochondrial DNA (mtDNA) content, mitochondrial mass, and activity, can be initiated by a wide range of stimuli, including exercise, cellular stress, temperature, nutrients, and nitric oxide (NO) [59].

Mitochondrial Dynamics

Tight regulation of mitochondrial dynamics, including mitochondrial fusion, fission, and movement, is important for cellular bioenergetics, senescence and survival, quality control, and intracellular signaling [60, 61]. Fusion results in elongated, connected mitochondria, a response often seen with nutrient restriction; conversely, fission, promoting fragmented, separated mitochondria, is more common in a nutrient-rich environment [62, 63]. Indeed, repression, ablation, or loss-of-function mutation of fusion genes (i.e., MFN1, MFN2 and OPA1) leads to mitochondrial fragmentation or reduced mitochondrial filaments [64–67], while re-expression normalizes length [68–70]. By contrast, experimental reduction of mitochondrial fission protein (i.e., DRP1, FIS1, MTP18) content or activity elongates the mitochondrial network [71–75], while overexpression promotes fragmentation [73, 74, 76].

Collectively, fusion and fission are both normal parts of the mitochondrial life cycle which promote not only a healthy rearrangement of mitochondrial components but also the release of dysfunctional components, thereby helping to maintaining a healthy pool of mitochondria. Ultimately, damaged mitochondria can be eliminated via the process of mitophagy.

Disruptions in T2D and Insulin Resistance

Obesity, insulin resistance, and established T2D have all been shown to alter mitochondrial number and/or function in different tissues, resulting in the presence of the overly broad term “mitochondrial dysfunction” as a key phenotype linked to T2D pathophysiology. Despite abundant data, it remains uncertain whether perturbations in mitochondrial functional capacity are a cause, consequence, or key contributor to insulin resistance and T2D risk [77–79]. Indeed, this controversy has been the source of much debate in the scientific community [80, 81] (Table 25.1). In the sections below, we will examine data from studies assessing mitochondrial function in different tissues in order to dissect this key question for insulin resistance and T2D pathophysiology.

Skeletal Muscle

Skeletal muscle is a major metabolic tissue of the body. While many consider muscle to be relatively homogeneous, it is important to recognize its heterogeneity from a metabolic perspective. Classification by fiber type, based on immunohistochemical or gene expression patterns, can be useful to illustrate differences in metabolism in discrete muscle groups. For example, type I fibers have higher mitochondrial density and oxidative capacity, while type II fibers have high glycogen content and fewer capillaries [105] and can be further subdivided into types IIa, IIb, and IIx. As

Table 25.1 Main studies supporting links between mitochondrial dysfunction and insulin resistance

Authors	Year	Journal	Sample	Observations
Insulin resistance is associated with impaired mitochondrial function (decreased FA oxidation and increased DAGs)				
Vondra et al. [82]	1977	Diabetologia	T2D subjects	Decreased mitochondrial activity
Kelley et al. [83]	1999	Am J Physiol	T2D patients	30–40% lower mitochondrial enzyme activity
Simoneau et al. [84]	1999	FASEB J		
He et al. [85]	2001	Diabetes		
Kelley et al. [86]	2002	Diabetes		
Kelley et al. [86]	2002	Diabetes	Obese individuals	Smaller mitochondrial size, reduced mitochondrial content, and altered mitochondrial performance
Petersen et al. [87]	2003	Science	Healthy lean elderly	Severe insulin resistance in skeletal muscle, higher levels of TG (in muscle and liver), decreased mitochondrial oxidative capapcity, and decreased ATP synthesis
Mootha et al. [88]	2003	Nat Genet	Obese Caucasians with IGT and T2D	Decreased expression of nuclear-encoded genes regulating mito biogenesis (i.e. PGC1α-responsive genes)
Patti et al. [89]	2003	PNAS	Obese and overweight non-diabetic Mexican-Americans	Decreased expression of PGC1α and β and nuclear OxPhos genes
Petersen et al. [90]	2004	N Engl J Med	Young-insulin resistant offspring of parents with T2D	Decreased mitochondrial activity, increased intramyocellular FA, and lower ratio of type 1 (oxidative) to type 2 (glycolytic) fibers in muscle
Morino et al. [91]	2005	J Clin Invest	T2D and insulin resistant individuals	30% reduction in mitochondrial density
Mitochondrial dysfunction is not in causal relationship to insulin resistance				
Miller et al. [92]	1984	J Appl Physiol Respir Environ Exerc Physiol	Rodents with HFD	Increased levels of mitochondrial marker enzymes (beta-hydroxybutyrate DH and CS)
McAinch et al. [93]	2003	Obes Res		

(continued)

Table 25.1 (continued)

Authors	Year	Journal	Sample	Observations
Han et al. [94]	2004	Am J Physiol Endocrinol Metab	Transgenic rodents with mito dysfunction	Low substrate oxidative capacity, increased AMP, activated AMPK, increased GLUT4 (i.e., improving insulin responsiveness)
Wredenberg et al. [95]	2006	Biochem Bioph Res Co		
Han et al. [96]	2011	PLoS ONE		
Han et al. [94]	2004	Am J Physiol Endocrinol Metab	Transgenic rodents with muscle mitochondrial deficiency/ dysfunction	Increased glucose tolerance, increased insulin sensitivity, and increased insulin-stimulated glucose uptake
Wredenberg et al. [95]	2006	Biochem Bioph Res Co		
Pospisilik et al. [97]	2007	Cell		
Zechner et al. [98]	2010	Cell Metab		
Garcia-Roves et al. [99]	2007	PNAS	Rodents with intermittently elevating plasma FA (with insulin resistance)	Increase in mitochondrial biogenesis
Turner et al. [100]	2007	Diabetes	Rodents fed with HFD (with insulin resistance)	Increase in mitochondrial biogenesis (increased marker enzymes, FA oxidation, and mtDNA copy number)
Hancock et al. [101]	2008	PNAS		
Toledo et al. [102]	2008	Diabetes	Diet-induced weight loss in humans	Improved insulin resistance without increasing mitochondrial capacity
Nair et al. [103]	2008	Diabetes	Insulin resistant Asian Indians with T2D	Muscle mitochondrial capacity similar to nondiabetic Indians and higher than Caucasians
Jin et al. [104]	2011	J Clin Invest	Caucasians with insulin resistance	No change in PGC1 or OXPHOS expression in isolated IR in individuals matched for BMI to insulin sensitive individuals

previously noted, skeletal muscle is responsible for an estimated 80% of glucose uptake during intravenous insulin infusion in healthy individuals [106–108]. Reduced muscle glucose uptake contributes to reduced glycogen synthesis, reduced glucose oxidation, whole-body insulin resistance and postprandial hyperglycemia in T2D [109–112]. These defects are also observed in high-risk individuals prior to the onset of T2D, as indicated by 70% reduction in muscle glycogen synthesis in young lean and glucose tolerant offspring of parents with T2D [113]. Notably, these defects are not observed in other tissues such as brain, fat and splanchnic tissue, which maintain their glucose utilization in T2D [5] (Fig. 25.1). Collectively, these data have stimulated focus on skeletal muscle as a dominant contributor to insulin resistance and diabetes risk.

The molecular mechanisms mediating muscle insulin signaling to glucose uptake have been the focus of intense study for many years. Insulin binds and activates the receptor tyrosine (Tyr) kinase, resulting in increased phosphorylation of Tyr residues in the β-subunit of the insulin receptor [114–117] and subsequent recruitment and phosphorylation of IRS1. IRS1 further activates downstream targets such as PI3K and AKT, ultimately resulting in translocation of GLUT4 to the plasma membrane. Additional effects of insulin include activation of NO synthase, leading to arterial vasodilatation [118, 119].

In patients with T2D, multiple steps of insulin signaling are disrupted. Reduced phosphorylation of the insulin receptor and IRS1 has been demonstrated in T2D [33, 114], in insulin resistant offspring of T2D parents [120, 121], and in obese

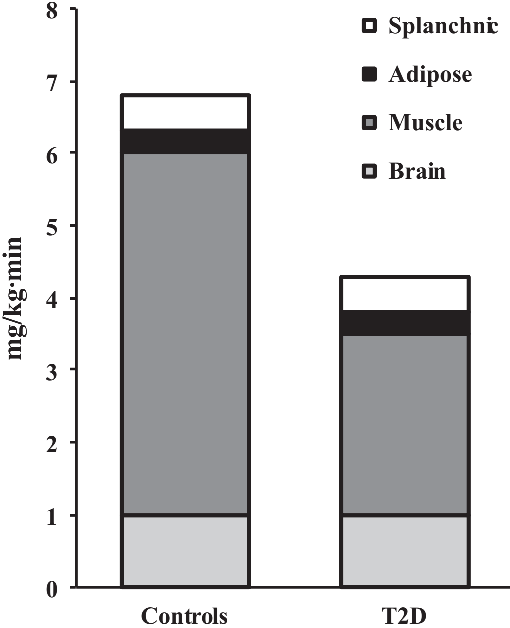

Fig. 25.1 Glucose uptake during euglycemic hyperinsulinemic clamp (100 μU/ml) in healthy control and T2D individuals (Adapted from DeFronzo [5])

individuals with normal glucose tolerance [33]. While IRS1 Tyr phosphorylation is reduced, inhibitory serine (Ser) phosphorylation (Ser307 in humans, Ser302 in rodents) [122] is increased in insulin resistance and T2D. This results in reduced interaction of IRS1 with the insulin receptor [123] and reduced downstream signaling [124].

Mitochondrial Function and Insulin Resistance in Skeletal Muscle

Many studies using a variety of methods have demonstrated abnormalities in mitochondrial function and/or number in muscle from humans with insulin resistance and T2D [86, 90, 125, 126] (Table 25.1). In vivo nuclear magnetic resonance (NMR) spectroscopy studies have revealed decreased mitochondrial ATP production and phosphocreatine recovery in both T2D and lean insulin resistant offspring of individuals with T2D [90, 127]. Reduced ATP production has also been observed in response to exercise in healthy young offspring of parents with T2D, suggesting this phenotype may be consistently related to insulin resistance [128].

Beyond NMR, more traditional assays of mitochondrial enzymatic activity in muscle biopsy samples evaluated ex vivo have also been used to probe functional defects in insulin resistance and T2D. Analysis of OxPhos enzymatic activity has yielded varying results, depending on the methodology and the population under study. Initial studies of Simoneau and colleagues reported decreased activity of citrate synthase (CS), a surrogate for mitochondrial content and activity, in both obesity and T2D [84]. Subsequent analysis of OxPhos activity, as measured by NADH:O_2 oxidoreductase activity (CI activity), demonstrated a 40% reduction in T2D patients [86]. Similarly, lower muscle OxPhos capacity in T2D was demonstrated in studies analyzing either muscle biopsy samples [86, 129–131] or isolated mitochondria [132]. These differences remained even after matching for age, sex and body mass [127, 129]. Moreover, analysis of lean insulin resistant but normoglycemic offspring also revealed a 50% lower cytochrome C oxidase activity` [91]. Elderly insulin resistant subjects have also decreased mitochondrial OxPhos activity [87]. By contrast, other studies have reported normal mitochondrial activity in both T2D [133, 134] and in obese and insulin resistant individuals [135]. Interestingly, studies in South Asian Indians demonstrated increased OxPhos activity despite insulin resistance [103].

The impact of insulin resistance and T2D on mitochondrial content and subcellular localization has also been evaluated using electron microscopy. In general, reduced mitochondrial content is observed in either T2D [86, 91, 136, 137] or insulin resistance [91, 137]. These differences are particularly prominent in the subsarcolemmal compartment, with a threefold reduction in patients with T2D [126]. Collectively, these data suggest that differences in mitochondrial energetics may result, at least in part, from reduced mitochondrial number.

Transcriptional Regulation of Mitochondrial Biogenesis and Function in Skeletal Muscle

Reduced mitochondrial biogenesis may contribute to observed alterations in mitochondrial number and function. Transcriptomic studies revealed a coordinated reduction in expression of genes encoded by PGC1α in patients with T2D in both the fasting [89] and insulin-stimulated states [138]. In addition, expression of PGC1α and β was reduced in obese insulin resistant individuals [89]. However, this reduction has not been consistently observed in lean, insulin resistant offspring of parents with T2D or non-diabetic relatives [91, 104, 139], highlighting the potential impact of obesity as driver of insulin resistance-related phenotypes.

Perturbations in mitochondrial biogenesis can also be associated with altered mitochondrial dynamics contributing to functional derangements. Obese insulin resistant individuals have reduced expression of the mitochondrial fusion gene MFN2 [140], while mutations in the fission protein OPA1 reduce muscle ATP synthesis [141]. Mice with either genetic (*ob/ob*) or diet-induced obesity have increased expression of DRP1 and FIS1 in parallel with smaller, shorter mitochondria [142]. Surprisingly, protein content of MFN2, DRP1, and OPA1 are increased in obesity with hyperglycemia (*db/db* mice) [143]. Conversely, inhibition of mitochondrial fission improves both muscle insulin signaling and whole-body insulin sensitivity [142], suggesting that fission might underlie both mitochondrial derangements and insulin resistance in muscle.

Mitochondrial Alterations and Insulin Resistance in Skeletal Muscle: Interpretation

The variability in results of analysis of mitochondrial function in insulin resistance and T2D suggests not only differences in methodology, but also genotypic and phenotypic differences in populations under study. These include age, BMI, diet, activity and physical fitness, muscle fiber type, early-life environmental exposures, and magnitude of insulin resistance. Among these, obesity appears to be a potential cofounding factor in many studies: a reduction in mitochondrial functional capacity might be related to obesity itself rather than insulin resistance, given that obese patients show small mitochondria. Careful matching for BMI reduces differences in mitochondrial content and activity in some studies [129, 130, 132] but not in others [127, 129]. Moreover, muscle mitochondrial function appears to be dissociated from insulin resistance given that mitochondrial content, activity, and OxPhos capacity do not correlate with insulin sensitivity in some insulin resistant cohorts [103, 135, 144].

Defects in function can be dissociated from mitochondrial mass, a parameter less precisely measured, especially in skeletal muscle. When oxygen consumption measurements in muscle fibers are normalized by mtDNA content or CS activity,

differences between T2D and controls are reduced or completely abolished in some studies [130]. In other studies, ADP-stimulated and maximal respiration is reduced in T2D, despite unchanged mitochondrial content [129]. Similarly, reductions in OxPhos activity can be observed even in isolated subsarcolemmal mitochondria [126]. In this setting, differences in fission/fusion/tethering to other organelles, or to local metabolite gradients potentially disrupted during the isolation and assay process, could contribute to variable functional capacity.

Given the variability inherent to human studies, rodent models have also been used to decipher the relationship between insulin resistance, T2D, and mitochondrial dysfunction. Mitochondrial respiratory capacity is increased in glycolytic muscle from *db/db* [143] and *ob/ob* mice [145], but is unaltered [145] or reduced [143] in oxidative muscle, in parallel with decreased mtDNA content and expression of mitochondrial biogenesis regulators [143]. Mitochondrial function in rats is also variable, with increases in Zucker diabetic fatty (ZDF) rats [146] and HFD-fed animals [100, 147], but unchanged capacity in other studies using ZDF rats [148].

Rodent models have also demonstrated that experimentally-induced alterations in mitochondrial function are not consistently linked to insulin resistance. For example, rats made iron deficient have reduced protein content of cytochrome C and cytochrome C oxidase subunits I and IV, but unchanged insulin-stimulated glucose uptake [96]. Furthermore, experimental disruption of OxPhos CI (e.g., muscle- and liver-specific apoptosis-induced factor (AIF)-deficient mice) increases insulin sensitivity [97]. Similarly, deficiency of TFAM [95], the PGC1 family of transcriptional activators [98], or cytochrome C oxidase subunit IV peptide 2a (COX6A2) [149] yields protection against insulin resistance. Thus, rodent studies support an inconsistent relationship, and in some cases, full dissociation between insulin resistance and mitochondrial function.

Potential Factors Beyond Insulin Resistance Contributing to Mitochondrial Functional Defects Associated with Insulin Resistance and T2D

The data above from both humans and rodent models suggest that insulin resistance is often, but not always, associated with disruption in some components of mitochondrial metabolic function. Moreover, experimental disruption of mitochondrial OxPhos, sufficient to reduce energy metabolism, does not produce insulin resistance, at least in animal models. What additional factors contribute to mitochondrial dysfunction but not directly linked to insulin resistance might account for this? Potential mediators (Fig. 25.2) include ectopic lipid accumulation, oxidative stress (e.g., ROS), imbalance between fatty acid oxidation (FAO) and TCA cycle/OxPhos capacity, incomplete FAO, all of which may in turn be linked to physical inactivity.

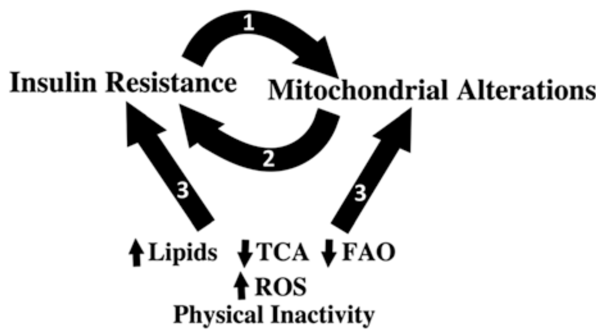

Fig. 25.2 Main hypotheses linking insulin resistance and mitochondrial dysfunction. (*1*) Insulin resistance causes alterations in multiple facets of mitochondrial function. (*2*) Mitochondrial alterations lead to insulin resistance. (*3*) Other factors lead to both insulin resistance and mitochondrial alterations concomitantly, including increased tissue lipid supply, imbalance between tricarboxylic acid cycle and OxPhos capacity, incomplete lipid oxidation, and increased ROS

Ectopic Lipid Accumulation

One phenomenon characteristic of insulin resistance and T2D in skeletal muscle is inappropriate or "ectopic" intramyocellular lipid accumulation [150], reviewed in [151, 152], and in [153]. Tissue accumulation of triglycerides (TG) can also predict insulin resistance in both adults and children [154–157]. Moreover, inhibition or activation of genes regulating muscle lipid uptake (i.e., LPL, FATP, CD36) in rodents yields protection or susceptibility to insulin resistance, respectively [158–162], confirming the important role of ectopic lipid accumulation. One mechanism by which accumulation of excess lipid yields insulin resistance is via disruption of insulin signaling. Experimentally increasing plasma fatty acids (FA) in both humans [163] and rodents [164, 165] blunts insulin activation of IRS1-associated PI3K activity in muscle. The delayed time course of insulin resistance in this protocol (>3 h after lipid infusion) suggests that tissue lipid uptake and metabolism are required to reduce glucose uptake [163].

It remains unclear which molecular lipid species are the dominant mediators of insulin resistance. While initial studies focused on TG accumulation as a common signature, these species are also elevated in muscle of highly trained and insulin-sensitive athletes, and thus not likely to play the dominant role in the pathophysiology [166, 167]. Rather, additional lipids such as diacylglycerols (DAGs) may be more closely linked to insulin resistance [122, 168–170]. DAGs disrupt insulin signaling by activating serine/threonine (Ser/Thr) kinases such as PKC, inhibitory IKKβ and NFκB, reducing post-receptor insulin action [171]. Moreover, FFA may directly activate Toll-like receptors, mediating inflammatory signals, IKK and JNK activation, and downregulation of insulin signaling [172–174]. Ceramides have also been linked to insulin resistance in obese humans [175, 176].

Different studies report increased FAO in insulin resistance and T2D [177–180]. Furthermore, when lipid oxidation is increased as a result of increased lipid delivery, but not matched by appropriate increases in oxidative capacity, products of incomplete FAO may accumulate; this may also contribute to oxidative stress, activation of Ser/Thr kinases, and additional stimulus for insulin resistance [100]. Hence, lipid accumulation can parallel reduced mitochondrial oxidative capacity [87, 90, 181]. Whether lipid excess could either cause or result from mitochondrial dysfunction remains unclear [86, 182, 183]. Experimental lipid infusion in healthy individuals reduces insulin-stimulated ATP synthesis [184]. However, OxPhos activity is not reduced in obesity in the fasting state (when FA substrate utilization is maximal) [185, 186], despite insulin resistance [187, 188]. Not surprisingly, lipid oxidation and mitochondrial content are not consistently correlated in individuals with obesity or T2D, potentially due to reduced expression of PGC1α and dysregulated FA activation of PPAR [189, 190].

Rodent studies have also provided conflicting data. In obese ZDF rats, intramyocellular lipids are increased without altered mitochondrial function [191]. In other studies, elevations in circulating FFA accompanying HFD-induced obesity are associated with increased mitochondrial OxPhos capacity and biogenesis [99, 101, 192], together with increased FAO and expression of mitochondrial genes [100]. Increased lipid supply may actually promote lipid oxidative capacity via activation of PPARδ [99]. Similarly, experimental increases in FAO induced by HFD in mice with reduced malonyl CoA levels (acetyl-CoA carboxylase 2 KO mice) yields protection against diet-induced insulin resistance [193].

The relationship between lipid acccumulation, insulin sensitivity, and mitochondrial function has also been tested during intervention studies. Weight loss consistently increases insulin sensitivity, but alterations in intramyocellular TG content and mitochondrial respiration or enzymatic activity are not always observed [102, 194]. Exercise training in T2D improves both insulin sensitivity and mitochondrial content, but does not consistently decrease intramyocellular lipids [195]. Strategies to pharmacologically alter lipid content have also been used to study this relationship: administration of acipimox, a drug that reduces lipolysis, modestly increases insulin sensitivity and decreases ROS in muscle, but does not improve basal or insulin-stimulated OxPhos capacity in obese individuals with or without T2D [196, 197].

Oxidative Stress

Another potential mechanism contributing to insulin resistance and mitochondrial dysfunction could be oxidative stress (i.e., increased levels of ROS), since mitochondria are one of the major sites of ROS production in the cell [198]. Normal ROS production and signaling are essential for cellular and mitochondrial functionality, a phenomenon known as *mitohormesis* [199, 200]. However, chronic sustained increases in ROS may be deleterious, damaging DNA, protein, and lipids, and altering transcriptional regulation. With chronic nutrient oversupply, excessive reduction of NADH and $FADH_2$ can lead to increased mitochondrial membrane potential; if

demand for ATP or uncoupling is not increased in parallel, this may increase mitochondrial ROS production [201] and could contribute to lower intrinsic mitochondrial activity in T2D individuals [126, 129, 132].

Several experimental paradigms support the possibility that excessive ROS could contribute to insulin resistance. Treatment of cells with glucocorticoids or the proinflammatory hormone TNFα leads to insulin resistance and a parallel increase in ROS and related transcriptional effectors [202]. Moreover, increased ROS has been associated with insulin resistance in animal models [203]. For example, hypercaloric feeding in rodents increases plasma levels of hydrogen peroxide (H_2O_2) [204], while HFD feeding increases mitochondrial H_2O_2 production, decreasing redox-buffering capacity [201], and decreases oxidative capacity and ATP levels [205]. Studies in T2D individuals failed to show an increase in mitochondrial H_2O_2 [206, 207], while antioxidant capacity was reduced in skeletal muscle [208].

Mechanisms linking ROS to insulin resistance remain uncertain. Mitochondrial ROS have been linked to activation of pro-inflammatory molecules such as IKKs [209] or PKCs [91], both of which can yield disruption of insulin signaling. These and other oxidative stress species activate Ser kinases, ultimately disrupting insulin signaling [115, 201, 210]. Conversely, ROS have been shown to oxidize and eversibly inhibit protein tyrosine phosphatases, increasing insulin signaling in some studies [211].

Since association between mitochondrial-derived oxidative stress and insulin resistance cannot establish causality, several studies have attempted to reduce oxidative stress by treatment with antioxidants or overexpression of antioxidant genes. For example, mitochondrial-targeted antioxidant treatment and overexpression of mitochondrial catalase in HFD-fed rodents preserves insulin sensitivity [201], and reduces DAG concentration [212]. Similarly, overexpression of manganese superoxide dismutase ameliorates insulin resistance [213]. By contrast, antioxidant treatment in another study normalized muscle mitochondrial function but did not normalize insulin sensitivity or glucose tolerance [205].

Physical inactivity is also linked to mitochondrial dysfunction, independent of insulin sensitivity. Exercise training increases mitochondrial biogenesis in skeletal muscle [214], and restores oxidative capacity even in T2D [207, 215]; in some studies, insulin sensitivity and metabolic flexibility also improve in parallel [216]. However, insulin sensitivity and mitochondrial function do not always respond concordantly. For example, insulin sensitivity is improved with weight loss achieved through diet and exercise without measurable changes in mitochondrial capacity [102]. Furthermore, the effectiveness of physical training may differ from individual to individual. For example, offspring of mothers with T2D are not able to improve insulin sensitivity as much as non-diabetic controls after 9 days of intensive training, despite similar skeletal muscle OxPhos capacity [217].

Taken together, these data support two principal hypotheses for mechanisms mediating the link between mitochondrial function and insulin resistance (Fig. 25.3). Firstly, increases in intracellular lipids due to increased delivery and/or altered metabolism can disrupt insulin signaling. Secondly, increased mitochondrial ROS generation, due to imbalance between oxidative demand and mitochondrial capacity,

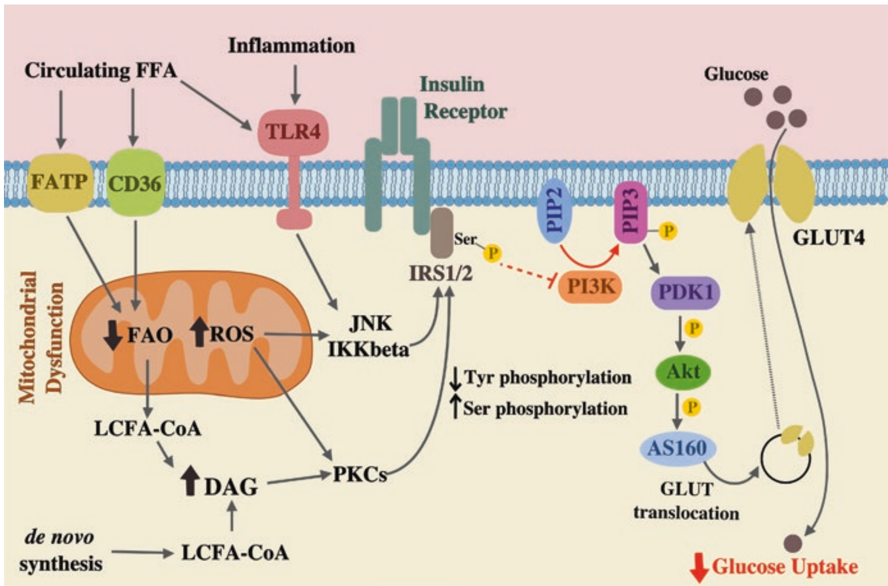

Fig. 25.3 Main hypotheses linking mitochondrial dysfunction to insulin resistance. Low mitochondrial fatty acid oxidation (*FAO*) relative to supply may contribute to incomplete oxidation and/or accumulation of species such as DAG that activate PKCs and disrupt insulin signaling. ROS overproduction within mitochondrial can activate inflammatory pathways ultimately disrupting insulin signaling

can also perturb insulin signaling. Both of these mechanisms may mediate, at least in part, the impact of obesity and physical inactivity on both insulin sensitivity and mitochondrial function.

Liver

The liver plays a key role in maintenance of whole body energy metabolism via functions such as storage of glucose-derived carbons, synthesis of plasma proteins, and hormone production and detoxification. The liver can store glucose as glycogen (glycogenesis) after a meal, while producing glucose in the fasting state and during exercise by depolymerizing glycogen (glycogenolysis), or synthesizing glucose from other sugars, amino acids and glycerol (gluconeogenesis). Apart from carbohydrate metabolism, the liver also has an important role in lipid metabolism, via both oxidation of TG and de novo lipogenesis from excess sugar and amino acids, and in the synthesis of cholesterol and bile acids. The liver can also deaminate amino acids and produce plasma proteins and urea.

Obesity and insulin resistance are associated with abnormalities in nuclear-encoded genes regulating OxPhos in the liver, in parallel with reduced PGC1α expression and increased hepatic lipid accumulation [218]. By contrast, OxPhos complexes genes are increased in parallel with BMI and insulin resistance in Japanese obese T2D individuals [219]. It remains uncertain whether these differences are related to ethnic differences or degree of obesity in the population under study. Given the difficulty in accessing liver tissue sample for analysis in humans, rodent experimental models are required, but have yielded discordant conclusions. Analysis of isolated mitochondria after HFD-feeding in rodents [220–222] revealed reduced respiratory capacity and increased oxidative stress. Similarly, mice with genetic obesity (*db/db* and *ob/ob*) have decreased activity of mitochondrial complexes CI to CV in both isolated mitochondria [223] or liver homogenates [143, 145]. By contrast, other studies revealed unchanged [224] or increased liver mitochondrial oxidative capacity in adult *ob/ob* mice [225] and insulin resistant diabetic GK rats [226]. The reasons for these differences remain unclear, but may be related to different diets, magnitude of obesity, and experimental methods used to assess mitochondrial function (i.e., mitochondrial respiration, mtDNA analysis, etc). Regarding lipid accumulation in the liver, genetic defects in FAO (i.e., lack of LCAD) predispose animals to hepatic steatosis and insulin resistance [148].

White Adipose Tissue

Adipose tissue is now recognized as a key endocrine tissue that participates in the pathogenesis of insulin resistance and T2D. While lipid-storing adipocytes represent the main component of adipose tissue, the stromal vascular fraction (SVF) also contains pre-adipocytes, endothelial cells, fibroblasts and resident macrophages. Full differentiation is critical for lipid accumulation and the metabolic function of adipose tissue. Differentiated WAT acts as a nutrient and hormonal sensor coordinating either FA or glucose oxidation or storage [227]. Beyond lipid storage, adipose tissue can also regulate systemic metabolism by secreting adipokines (i.e., cytokines, chemokines, hormone-like factors, other mediators) that regulate appetite, inflammation, insulin action, and glucose and lipid metabolism [228, 229].

There are two major types of WAT: visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). VAT has been considered the *harmful* fat, as compared with *beneficial* SAT [230]. VAT is associated with insulin resistance, increased T2D risk, dyslipidemia, atherosclerosis and mortality [231–233]. Indeed, VAT secretes less adiponectin [234] and more pro-inflammatory cytokines [235] while accumulating more macrophages.

With sustained obesity, insulin resistance, and T2D, adipose tissue is characterized by low-grade chronic inflammation [236], with macrophage infiltration and secretion of pro-inflammatory cytokines such as TNFα, IL6 and MCP1 among others contributing to obesity-associated insulin resistance and hyperglycemia [236–247]. In fact, antibody-mediated neutralization of TNFα improves insulin sensitivity [237].

Obesity-related inflammation has been linked to mitochondria since these organelles can activate the inflammasome, a multiprotein complex that both initiates and controls the inflammatory response to stress conditions, such as oxidative stress [248, 249]. This complex can also be activated by mtDNA [250] and lipids such as ceramides [251]. Interestingly, ceramides have been associated themselves with mitochondrial dysfunction [252].

Mitochondrial Health and Adipocyte Differentiation

Mitochondrial health is intimately linked to adipocyte differentiation. Indeed, some studies reported an increase in mitochondrial biogenesis and mitochondrial protein levels during differentiation [253–256]. During the course of differentiation, there is also a 20 to 30-fold increase in mitochondrial content, together with increases in β-oxidation [254, 255], mtDNA copy number, and transcription of both nuclear and mitochondrial-encoded genes [257]. Conversely, adequate mitochondria are required for regulation of adipocyte differentiation [258]. For example, reduction in expression of the mitochondrial transcription factor TFAM, a highly expressed gene during differentiation, reduces ETS capacity and disrupted GLUT4 translocation in 3T3-L1 adipocytes despite promoting insulin-stimulated AKT phosphorylation [259]; these data suggest that glucose transport in adipocytes might be disrupted by mitochondrial dysfunction via a mechanism downstream of AKT-dependent signaling. Similarly, rotenone-induced inhibition of mitochondrial respiration altered adipocyte differentiation [253]. Moreover, obesity-induced alterations in mitochondrial biogenesis also disrupt differentiation [254, 260], while in mature adipocytes, obesity, age and T2D are associated with reduced mitochondrial content [260].

Mitochondrial Dysfunction in WAT in Obesity

Metabolic function of adipose mitochondria is also altered in obesity. Adipocytes facing nutritional overload have increased lipid storage, increased glycolysis, and reduced mitochondrial biogenesis [261] and function [262–264]. Mitochondrial content and number are reduced in subcutaneous WAT biopsies from humans with obesity and T2D [265]. Mitochondrial number correlated with lipogenesis but not with BMI or insulin sensitivity [266]. Moreover, reduced abundance of mitochondria and expression of mitochondrial genes was reported in patients with insulin resistance, T2D and severe obesity [267]. In these patients, functional studies in WAT indicated a decrease in adipocyte oxygen consumption and ATP production [268], with similar findings in both VAT and SAT from obese individuals [269]. In studies of monozygotic twins, the more obese co-twin was more insulin resistant, in association with reduced expression of mitochondrial OxPhos-encoding genes in abdominal SAT [270]. Surprisingly, VAT adipocytes have higher numbers of

mitochondria [271] and higher mitochondrial respiration compared to SAT [272]. However, T2D has been associated with decreased expression of mitochondrial ETS genes in VAT regardless of obesity [273], with potential links to mTORC1-dependent mechanisms [274].

Rodent models of obesity, both genetic (*ob/ob*) and dietary (HFD feeding) obesity, and T2D (*db/db*) have also been used to assess derangements in adipose tissue. WAT from *ob/ob* and from *db/db* mice showed reduced expression of markers of mitochondrial capacity [275–277], with downregulation of PPARα, ERRα and PGC1α in *db/db* and obese HFD-fed mice [278]. At a protein level, mitochondrial proteins are markedly decreased in the adipocytes of *db/db* mice, but not in *ob/ob* mice [276]. Moreover, protein levels of ETS components (i.e., NADH dehydrogenase NDUFB8, succinate dehydrogenase SDHB and COX4I1) are reduced in HFD-fed mice [279]. Electron microscopy also demonstrates autophagosomes near mitochondria in HFD-fed mice; parallele increases in autophagy related proteins such as PINK1 and PARKIN also suggests autophagy-related metabolic remodeling [279].

Oxidative Stress in WAT

As in many tissues, optimal levels of ROS are required for normal function, including differentiation, in adipocytes [258]. Indeed, the adipocyte can tolerate high levels of ROS under basal conditions without damaging the cell or leading to apoptosis [202, 280]. However, most studies indicate that oxidative stress is chronically increased at high levels in WAT from models of dietary and genetic obesity [203, 212, 281]. This stress is a likely consequence of increased ROS production in adipose mitochondria [201] that may be related to chronic increases in non-esterified FA (NEFA) and TNFα [282, 283]. Excessive ROS may have several deleterious consequences, including reduced mitochondrial biogenesis [203], reduced proliferation of adipogenesis precursors [284], substrate and TG accumulation due to incomplete oxidation, and carbonylation of key insulin signaling intermediates such as IRS1 and 2, reducing insulin-stimulated Tyr phosphorylation [124]. Collectively, this can lead to hypertrophic, dysfunctional, and large adipocytes. Conversely, antioxidants can restore mitochondrial function and promote adipogenesis in parallel [285].

Brown and Beige Adipose Tissue

The presence of brown adipose tissue (BAT) in human adults was discovered less than a decade ago [41, 43, 286]. BAT is now considered a key tissue potentially contributing to modulation of whole-body metabolism and thermogenesis [44]. Brown adipocytes have many mitochondria with high oxidative capacity that also contain UCP1 uncoupling protein along the inner membrane [287]. In parallel, white adipose tissue has been shown to be capable of so-called *browning* or *beiging*,

increasing mitochondrial number and expression of UCP1, and conferring increased metabolic activity [288, 289]. Indeed, cold exposure can potently increase UCP1 protein levels in WAT undergoing *browning*, with levels similar to those in constitutive BAT; the relative contribution of these tissues to thermogenesis remains uncertain [290, 291]. These data have stimulated an intensive search for pharmacologic agents that could either increase activity of BAT or promote *beiging* of WAT, in hopes that stimulation of systemic energy expenditure could decrease obesity [292].

Mitochondrial Uncoupling and Glucose Metabolism in BAT

The capacity for uncoupling mitochondrial respiration is a defining feature of BAT and beige WAT; in turn, the ability to fine-tune respiration may play an important regulatory role in whole-body glucose and lipid homeostasis. Data suggest that activated BAT may contribute to as much as 75% of glucose clearance in mice; whether BAT similarly contributes to glucose metabolism in humans remains uncertain [293, 294]. Studies of BAT in humans rely on fluorodeoxyglucose uptake during PET scans to assess activity [293]. Indeed, BAT glucose consumption is increased during active thermogenesis, potentially via both insulin-dependent (GLUT4) and insulin-independent (GLUT1) uptake [293, 295], and could contribute to oxidative metabolism of glucose [296, 297] or to glucose-derived lipogenesis [295, 298]. BAT may be also a major site of triglyceride clearance from the circulation [299].

Conversely, loss of uncoupling blunts the increase in glucose uptake and oxidation [300]. Whether this contributes to diabetes risk remains uncertain, but it is interesting that human studies have associated polymorphisms at the UCP1 *locus* with T2D [301, 302]. UCP1 deficiency in mice also increases FGF21 expression and release in this tissue [303]. Interestingly, FGF21 is released from BAT during active thermogenesis [304], promotes BAT uncoupling and glucose oxidation [305], and reduces glucose in rodent models of T2D [306]. Thus, FGF21 may play a key role as an effector maintaining activity of BAT.

Central Nervous System Regulation

Central nervous system regulation is an essential element of control of appetite and energy expenditure, and thus whole-body energy homeostasis. Substantial progress has been made in the last decade in identifying the neural pathways controlling food intake. The dominant circuits regulating metabolism are located in the hypothalamus [307], with the arcuate nucleus (in the mediobasal hypothalamus) having a particularly important role in energy intake and feeding [308]. Neuronal populations in this region express orexigenic (agouti-related protein, AGRP; and neuropeptide Y, NPY) or anorexigenic neuropeptides (pro-opiomelanocortin, POMC; and cocaine and amphetamine-related transcript, CART) [309].

Insulin action in the hypothalamus suppresses appetite in rodents [310–312] via modulation of neuropeptides [313]. Indeed, insulin increases POMC and reduces NPY and AGRP expression, leading to activation of second-order neurons ultimately reducing food intake and increasing energy expenditure. Central action of insulin also regulates systemic glucose metabolism, including inhibition of HGP [314, 315] and glycogen synthesis in skeletal muscle [316], and stimulates lipogenesis in adipose tissue [317]. Leptin can also regulate central insulin signaling and action [317] through a PI3K-dependent mechanism [318]. Thus, both insulin and leptin signaling [319, 320] are essential regulators of hypothalamic control of whole-body energy homeostasis.

Hypothalamic regulation is perturbed in obesity and insulin resistance. For example, obese, insulin resistant individuals have reduced appetite inhibition upon glucose intake [3], despite higher insulin levels, indicating hypothalamic insulin resistance. Similarly, studies of obese rodents indicate central insulin resistance, contributing to increased HGP and reduced muscle glucose uptake [47, 48]. Lipotoxicity and increased ceramides [321–323], inflammation [319, 324] and endoplasmatic reticulum (ER) stress [325] have all been implicated as contributors to pathogenesis of central insulin and leptin resistance [312, 326, 327].

Mitochondrial Function in the Hypothalamus

Analysis of mitochondrial function in the hypothalamus is challenging, given the presence of both orexigenic and anorexigenic neuronal populations. In permeabilized hypothalamus from obese Zucker rats, no differences in respiratory control ratio were observed in comparison to lean rats [328]; by contrast, increased mitochondrial respiratory capacity has been observed in isolated mitochondria from obese rats [329]. Moreover, mitochondrial function in the hypothalamus may be compromised by altered mitochondrial dynamics. For example, ablation of mitofusin2 (Mfn2) in POMC neurons leads to disrupted POMC processing, leptin resistance, reduced energy expenditure and obesity [309], with similar impact in AGRP neurons [309, 330].

Oxidative Stress in the Hypothalamus

ROS have been shown to modulate hypothalamic neuronal function, thus potentially linking perturbed mitochondrial function and central regulation. ROS can acutely activate POMC firing; conversely, suppression of ROS inhibits POMC neuronal activity [331]. Effects of ROS are more modest in NPY/AGRP neuronal populations [332], in which ROS production is decreased with activation of UCP2 [333]. In T2D, increased ROS due to hyperglycemia can inactivate nicotinic acetylcholine receptors on autonomic neurons, suggesting oxidative stress may act as a second messenger to disrupt synaptic transmission [334].

Stem Cell Populations

Stem cells are undifferentiated cells that can both divide into other stem cells or differentiate into specialized cells required for normal development, differentiation, function and repair of tissues. Hence, alterations in stem cell function may impact developmental trajectories and even tissue function, thus potentially contributing to risk of metabolic disease emerging during adult life (e.g., insulin resistance and T2D) [335].

Mitochondrial Function in Insulin Resistant Stem Cells

Analysis of metabolic function in stem cells has been used to decipher relationship between insulin resistance and mitochondrial function, in the absence of complicating differentiation-dependent phenotypes. For example, stem cells obtained from patients with monogenic insulin resistance (i.e., mutations in the insulin receptor) [336, 337] have robust metabolic phenotypes, with decreased spare respiratory capacity, decreased endogenous CS activity, and a 34% increase in ADP/ATP ratio, suggesting reduced energy availability [337]. Moreover, insulin resistant cells have a 22% increase in mitochondrial number and a 16% decrease in mitochondrial area, along with increased expression of the mitochondrial fission genes MFF and INF2 [337]. Given that mitochondrial mass, mtDNA and CS protein remained unchanged, these data collectively support that insulin resistance may promote a switch to mitochondrial fission and energetic stress. Although these studies indicate that insulin resistance in stem cell populations can promote mitochondrial alterations, it remains unclear whether more common forms of insulin resistance or T2D, which typically arise in a polygenic background, can also be a primary driver of mitochondrial dysfunction.

Alterations in mitochondrial metabolism can also affect the function of germ cells, including both oocytes and sperm. This may instigate a vicious cycle whereby alterations in parental metabolism can promote metabolic disease in offspring (reviewed in [338, 339].

Mitochondrion as a Therapeutic Target

Collectively, the data presented above underscore the strong relationships between mitochondrial dysfunction and metabolic diseases such as insulin resistance and T2D. Whether these relationships are essential for the pathogenesis of disease, or are secondary phenomena, remains uncertain. Thus, testing whether improvement in mitochondrial function can improve metabolism is a key element. In this section, we will review the impact of therapeutic strategies to modify, regulate and control mitochondrial bioenergetics, biogenesis and overall functional capacity (Fig. 25.4).

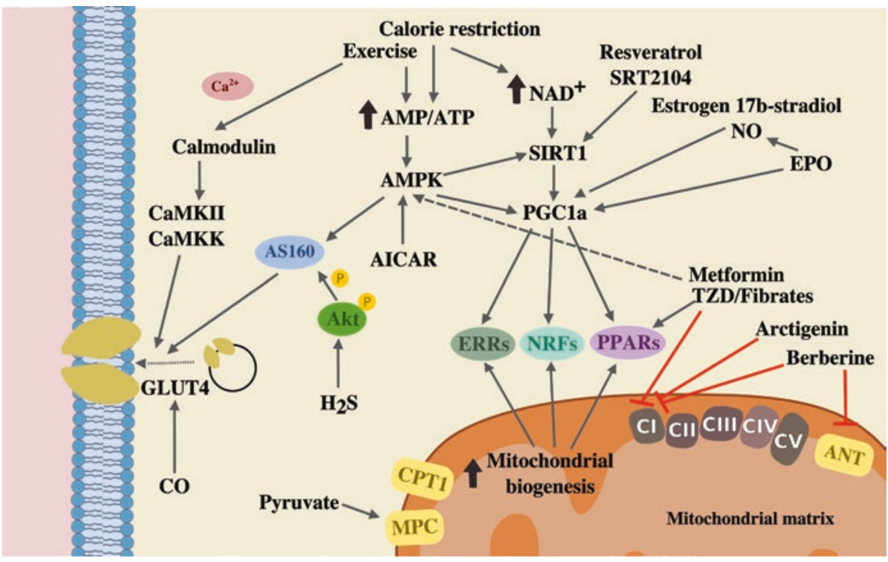

Fig. 25.4 Cellular mechanisms by which drugs, compounds or other interventions may modify, regulate and control mitochondrial health

Non-pharmacological Interventions: Exercise and Weight Loss

Lifestyle interventions such as physical activity and calorie restriction improve insulin sensitivity and glucose tolerance in patients with insulin resistance and T2D, and in parallel, improve mitochondrial health. Multiple studies have reported the benefits of exercise and muscle contraction on insulin sensitivity and glucose homeostasis [340–343], largely via insulin-independent AMPK-dependent pathways (lund 1995 [344, 345] [342]. Exercise also promotes anti-inflammatory responses [346], with release of IL-6 and other myokines [347, 348] as well as cytokines (i.e., IL10, sTNFR) that inhibit the production of pro-inflammatory cytokines (i.e., IL1β, TNFα) [349, 350].

Exercise training can robustly increase OxPhos capacity in rat skeletal muscle [351], in parallel with an increase in mitochondrial number and widening of *cristae*. Endurance training is particularly potent to increase muscle mitochondrial content [352], CS activity [207, 353], cardiolipin [216], and protein content of OxPhos complexes [195]. Muscle mitochondrial biogenesis (i.e., PPARγ, NRF1 and TFAM genes) is also stimulated after endurance exercise [354]. Exercise training also induces neovascularization through pro-angiogenic factors (i.e., VEGF) in an ERRα- and PGC1α-dependent manner [355], thus coordinately regulating oxygen delivery and oxygen consumption. Mitochondrial ROS production is also decreased after physical activity, possibly due to an increase in CI and CIII capacity, the sites of ROS production, or in muscle UCP3 [207] that may limit excessive mitochondrial ROS [356]. However, some but not all studies indicate that responses to exercise

training are reduced in individuals with T2D, with no increase in either mitochondrial-related proteins (i.e., PDK4, COX1 and COX4) or mitochondrial content [195, 207] even with improved insulin sensitivity after strength training [357].

Exercise interventions in humans can also impact the liver, yielding reduced hepatic lipids [358]; in rodents, exercise results in increased liver insulin sensitivity and AKT phosphorylation [359], even during continued HFD feeding [360]. In this setting, endurance training reverses the decrease in maximal FCCP-mediated uncoupled respiration observed in sedentary, HFD-fed rats [361]. Interestingly, maximal effects were observed in response to voluntary wheel exercise, irrespective of diet.

Adipose tissue also responds potently to exercise training. Individuals with or without a family history of T2D (first-degree relative) have improvements in VO_{2max} and broad changes in gene expression, including enrichment of the OxPhos pathway [362]. Moreover, exercise decreases expression of inflammatory markers [363–366]. Similar effects have been observed in rodent adipose tissue in response to exercise. Mitochondrial activity, including cytochrome C oxidase and malate dehydrogenase activity, increases in VAT after swimming [367], in parallel with increased mitochondrial biogenesis, mtDNA content and glucose uptake in SAT [368]. In rodent models of insulin resistance and T2D, physical activity normalized VAT mitochondrial content and improved glucose homeostasis [263]. The effects of exercise are often associated with *browning* in SAT, with increased basal oxygen consumption reflecting increased mitochondrial biogenesis and upregulation of PGC1α, PRDM16, CIDEA, and UCP1 [369–375], and increased blood vessel number potentially linked to upregulation of the pro-angiogenic gene VEGFA [374, 375]. NO synthesis may also be required for exercise-dependent effects [368]. Interestingly, the effect of exercise on adipose tissue persists after transplantation of adipose from an exercised animal to a sedentary animal, suggesting that secreted factors may play a role in this response [374].

Calorie restriction sufficient to induce weight loss also improves insulin sensitivity, and in parallel, normalizes respiratory exchange ratio (RER) in both the basal [194] and insulin-stimulated states [376]. Moreover, this dietary intervention in humans (i.e., calorie restriction with weight loss) improves insulin resistance and fasting hyperglycemia, and decreases hepatocellular lipid accumulation [377]. The combination of both exercise and diet can normalize many phenotypes (i.e., body weight, glucose and insulin tolerance, fasting glucose, plasma insulin, TG, cholesterol, NEFA, insulin action) alongside with normalized expression of genes regulating mitochondrial function and biogenesis, decreased ROS, and improved ATP synthesis [378]. By contrast, many studies show that weight loss alone does not increase mtDNA content or maximal aerobic capacity [379], with either no increase [102, 190] or even a decrease in mitochondrial function [194]. Furthermore, weight loss without increased physical activity improved insulin resistance without increased muscle mitochondrial function [102], thus indicating that restoration of insulin sensitivity in muscle is independent of mitochondrial function, at least in some paradigms.

More directed dietary interventions in humans, such as omega-3 FA feeding, can increase PPARα-dependent FAO [380, 381] and reduce steatosis [381]. Omega-3

FA feeding also increases plasma adiponectin [380] and promote a shift to mitochondrial fusion, potentially contributing to reduced oxidative stress [382].

Thus, both exercise and calorie restriction improve aspects of mitochondrial function, largely in parallel with improved insulin sensitivity; both of these interventions often converge on key regulatory points of energy metabolism, such as AMPK or PGC1α [383, 384].

Pharmacological Interventions

Beyond lifestyle interventions, targeting mitochondrial dysfunction has been considered a potential approach to reduce insulin resistance. The goal of such pharmacological interventions could include (a) uncoupling mitochondrial respiration to increase energy expenditure and FAO [385, 386], (b) increasing mitochondrial biogenesis, and (c) inhibiting or decreasing OxPhos-generated oxidative stress.

Inducing Mitochondrial Uncoupling

Uncoupling has been considered as one strategy to increase energy expenditure and promote weight loss. Late in the 1930s the uncoupler 2,4-dinitrophenol (DNP) was used for treatment of obesity [387], but its narrow therapeutic window and serious side effects ultimately resulted in its withdrawal from the market in 1938. Additional uncouplers with reduced activity and improved safety profiles (e.g., Rhodamine 19) have been proposed over the ensuing years [386, 388].

Inducing Mitochondrial Biogenesis and Activity

Different metabolic challenges affect cellular energetics (i.e., exercise, calorie restriction), increasing AMP and NAD^+ and activating the metabolic sensors AMPK and SIRT1, respectively. In turn, AMPK and SIRT1 can stimulate PGC1α phosphorylation and deacetylation, ultimately increasing its activity [389].

Natural antioxidants that are able to scavenge carbon and lipid radicals and ROS have been used in attempt to increase mitochondrial biogenesis and function. The best studied compound is the polyphenol resveratrol (3,5,4′-trihydroxystillbene), a compound found in red wine that mimics the effects of calorie restriction through activation of SIRT1 and AMPK [390, 391], and increases mitochondrial biogenesis via upregulation of PGC1α, NRF1 and TFAM [392]. Of note, resveratrol has been also proposed to exert antiaging effects and extend survival [390, 393, 394]. In rodents, resveratrol protects against diet-induced obesity, activates PGC1α and increases aerobic capacity [395]. Interestingly, resveratrol may also act via SIRT1-independent mechanisms [396], potentially via increased mitochondrial content [390, 395, 397]. However, mitochondrial content increases are not observed in all

studies [398, 399]. These relatively modest and inconsistent effects may also explain in part the lack of impact on body weight in humans [400–402].

Other small molecules and vitamins which increase NAD^+, activate SIRT1, or induce antioxidant responses have also been studied [403–405], some specifically targeting mitochondria [406]. The SIRT1-activating compounds, SRT1720 and SRT2104 improve lipid profiles and induce weight loss in obese individuals [407]. Furthermore, SRT2104 increases oxidative capacity in elderly lean individuals, without affecting body weight [408]. Moreover, treatment with NAD^+ or the NAD^+ precursor nicotinamide riboside could also stimulate SIRT1 activity. Indeed, NAD^+ supplementation in HFD-fed mice protects against weight gain, potentially via increased energy expenditure [409] and increased BAT mitochondrial biogenesis and thermogenesis.

Multiple other pharmacologic agents have been evaluated for their impact on mitochondrial biogenesis. **Nitric oxide** (NO) and its derivates can activate mitochondrial biogenesis at the transcriptional level [410] through activation of cGMP and PGC1α [411, 412]. **Hydrogen sulfide** (H_2S) preserves mitochondrial function in injured heart muscle [413, 414] via increased AKT phosphorylation, increased nuclear localization of NRF1/2, and increased mitochondrial biogenesis [414]. Inhalation of low doses of **carbon monoxide** (CO) improves muscle mitochondrial density, capillary density, and translocation of GLUT4 [415]. The estrogen 17beta-estradiol can also activate NRF1 and 2, PGC1α and TFAM [416], while **erythropoietin** activates mitochondrial biogenesis via NRF1, PGC1α and NO [417]. **Adiponectin** increases mitochondrial content via AMPK and SIRT1 [418, 419], and can also activate PGC1α and p38 MAPK via MAPK phosphatase 1 suppression [420]. **Fibrates** activate the nuclear receptor PPARα and have been used to treat dyslipidemias [421], but may also have an effect on mitochondrial function via PGC1α action [422]. **Thiazolidinediones** (TZD) can improve mitochondrial function in human SAT [265] in parallel with stimulation of FA uptake and oxidation [276, 277, 423]. Natural compounds such as the alkaloid **berberine** increase energy expenditure [424] potentially due to increased BAT mass and *browning* in WAT, via AMPK- and PGC1α-dependent mechanisms [425]. Furthermore, animal studies indicate that this compound delays obesity onset [424–428].

AMPK activators have also been studied as potential inducers of mitochondrial biogenesis. 5-Aminoimizadole-4-Carboxamide Ribonucleotide (AICAR) administration to sedentary mice increases endurace performance [429], potentially through PGC1α-induced mitochondrial biogenesis [430] and/or direct modulation of gluconeogenesis and glycogenolysis [431, 432]. Pharmacological manipulation of AMPK [433] may also enhance glucose and lipid oxidation [434, 435].

Ameliorating Insulin Resistance by Inhibiting Mitochondrial Respiration

While mitochondrial dysfunction has been associated with insulin resistance and T2D in many studies, inhibition of specific enzymes within mitochondrial OxPhos can ameliorate insulin resistance and improve overall metabolism. For example,

inhibition of certain OxPhos complexes (i.e., CI) could alter mitochondrial substrate load and potentially reduce deleterious levels of ROS production. This concept has also been supported by animal studies demonstrating that disruption of OxPhos in multiple tissues is associated with improved insulin sensitivity [97].

Metformin and TZD are best recognized for their efficacy to improve insulin resistance and T2D; and their effects may be mediated by their impact to reduce mitochondrial function. **Metformin** decreases plasma glucose by decreasing HGP, increasing insulin-stimulated glucose uptake, and in parallel, inhibiting CI of OxPhos in cells, isolated mitochondria and skeletal muscle [436], similar to effects of other biguanides [437, 438]. Metformin inhibition of CI may reduce ATP production and content, thus activating AMPK and stimulating mitochondrial biogenesis and lipid oxidation via PGC1-dependent mechanisms [430, 439] and reducing ROS production [438, 440–442]. A novel anti-diabetic drug that also inhibits CI, activates AMPK, and increases muscle glucose uptake has been shown to be even more potent than metformin [443], with fewer off-target effects [444, 445]. Whether this drug will be useful clinically remains unknown.

Like metformin**, TZD** also inhibit CI. TZD are best known as activating ligands for the PPAR family of nuclear receptors (i.e., PPARγ in adipose, PPARα and PPARδ in muscle), increasing expression of genes related to differentiation, and yielding adipocytes with increased lipogenic capacity, mitochondrial number, and insulin action [257, 446]. Increased capacity for adipose lipid storage ultimately decreases ectopic lipid accumulation in muscle and liver, reduces circulating lipids [447, 448], and thus improves whole-body insulin sensitivity. Whether enhanced mitochondrial biogenesis in this context is simply a feature of increased adipose differentiation, or also contributes independently to metabolic effects in either adipose [265, 449] or muscle [450–452] remains uncertain. TZD can also directly inhibit CI [453], promoting shifts in cellular energetics toward anaerobic fuel utilization [454].

Inhibition of CI may be a common mechanism by which many compounds improve insulin resistance. **Fibrates**, the family of PPARα ligands [455], reduce plasma lipids by stimulating PPARα-dependent FAO in the liver [456]. Interestingly, fibrates also inhibit CI activity [453] and stimulate CS and CIV activity [457]. Other compounds which inhibit CI also improve insulin resistance and reduce plasma glucose levels; these include **berberine** [458] and **arctigenin** [459]. Berberine also inhibits adenine nucleotide translocate (ANT) [460], while its metabolite demethyleneberberine decreases ROS levels [461].

Inhibition of the mitochondrial pyruvate carrier (MPC) may also be a therapeutic approach. While first described for TZD [462] even specific inhibitors of the MPC can increase glucose uptake in both human and rodent myotubes [462]. Similarly, inhibition of carnitine-palmitoyl-transferase 1 (CPT1), another mitochondrial transporter, improves insulin resistance in experimental models and humans [463].

Collectively, these data suggest that limiting nutrient oxidation, ATP production, and/or ROS generation in the setting of chronic nutrient excess may have beneficial metabolic effects in obesity and diabetes. Reducing ATP and NADH levels may stimulate activation of nutrient sensing effector pathways such as AMPK/SIRT1/

PGC1, increasing glucose uptake and mitochondrial biogenesis in parallel. Since there are likely to be important tissue-specific effects of each of these strategies in vivo, additional studies will be required to fully validate these approaches.

Conclusion

Mitochondrial dysfunction is a consistent phenotype associated with insulin resistance in multiple tissues. Deciphering whether this is a critical causal link in the pathogenesis of human insulin resistance and type 2 diabetes remains a challenging goal for several reasons. Firstly, mitochondrial function cannot be defined by a single phenotype, as mitochondria have such a broad impact on multiple aspects of cellular function. Unfortunately, few studies comprehensively and simultaneously analyze multiple phenotypes. Secondly, it is likely that oxidative dysfunction is not simply an "on-off" phenotype but rather a continuous spectrum which progresses over time during the life course of disease risk. By the time clinical disease is recognized, the phenotype represents a mixture of primary effects as well as chronic secondary effects of perturbed metabolism, altered ROS, obesity, and hyperglycemia on regulation of transcriptional and epigenetic mechanisms. Even insulin resistant but non-diabetic individuals at risk for disease have likely had subclinical perturbations for years, as demonstrated by the studies of Tabak and colleagues [16]. Thus, dissecting the primary effects from secondary effects is challenging, especially in human disease.

Recent studies in undifferentiated human stem cells indicate that primary insulin resistance due to monogenic mutations in the insulin receptor, even in the absence of differentiation, induces changes in mitochondrial size, number, and functional capacity. Thus, chronic "primary" insulin resistance can yield secondary changes in mitochondrial metabolism. Whether these results are generalizable to more common forms of diabetes risk resulting from the combined impact of polygenic genetic, epigenetic, and environmental influences, remains an unanswered question currently under study. It remains possible that tissue-specific primary defects which alter mitochondrial metabolism could underlie additional diabetes-related risk patterns, influencing regulation of insulin secretion, exercise capacity, appetite, and sensitivity to calorie excess and inactivity.

Regardless of whether defects in mitochondrial metabolism are primary or secondary culprits in disease pathogenesis, it is likely that these defects can initiate a vicious cycle of reducing energetic capacity, increasing oxidative stress, and further reducing mitochondrial biogenesis and dynamics. Reduced mitochondrial capacity in the setting of chronic nutrient excess and inactivity may further impair exercise capacity, promote weight gain in an obesogenic environment, and contribute to metabolic instability. Thus, both lifestyle and pharmacologic strategies focused on interrupting this vicious cycle to improve mitochondrial metabolism may be an effective approach for disease prevention and treatment.

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Chapter 26
Mechanistic Role of Kinases in the Regulation of Mitochondrial Fitness

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Mounting evidence indicates that mitochondria contain multiple phosphorylation substrates and that protein kinases translocate into mitochondria, suggesting that protein phosphorylation in this organelle could be fundamental for the regulation of its own function. Here we examine the mechanistic role of cellular kinases in the fine regulation of key mitochondrial activities, including mitochondrial quality control, fission/fusion processes, metabolism, and mitophagy.

Mitochondrial Respiration

Mitochondria are cytoplasmic organelles that are involved in oxidative energy metabolism, producing the most of our cellular energy by oxidative phosphorylation [1, 2], through the metabolization of nutrients and production of ATP. Mitochondrial ATP production relies on the electron transport chain (ETC), composed of respiratory chain complexes I–IV.

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Table 26.1 Substrates of SRC in the ETC e the effects of substrate phosphorylation

SRC substrates	Phosphorylation sites	Effect
ANT1	Tyr[190] and Tyr[194]	Protection of cardiac mitochondria against ischemic-reperfusion injury
Cytochrome c oxidase	unknown	Increase of the enzymatic activity of complex IV
NDUFV2	Tyr[193]	Increase of NADH dehydrogenase activity
SDHA	Tyr[215]	Regulation of reactive oxygen species production

The Regulation of Proteins in the ETC

SRC family kinases (SFKs) are the most involved in tyrosine phosphorylation of mitochondrial proteins to regulate mitochondrial respiration. Src kinases affect the enzymatic activities of complexes I, III–V; importantly, SRC phosphorylates different substrates in the ETC (Table 26.1). Indeed, Src is present inside mitochondria where it phosphorylates the subunit II of cytochrome c oxidase [3], increasing the enzymatic activity of complex IV. Moreover, adenine nucleotide translocase 1 (ANT1), which is able to transport ADP from cytosol within mitochondria in exchange of ATP, is known to be phosphorylated on Tyr[190] and Tyr[194] by c-Src kinase [4–6].

Src is also known to phosphorylate NDUFV2 (NADH dehydrogenase [ubiquinone] flavoprotein 2) of complex I at Tyr[193], and SDHA (succinate dehydrogenase A) of complex II at Tyr[215] [7]. NDUFV2 phosphorylation is required for NADH dehydrogenase activity, affecting respiration activity and cellular ATP content, while SDHA phosphorylation has no effect on enzyme activity, but affects reactive oxygen species production. Furthermore, the tyrosine protein kinase Fgr phosphorylates flavoprotein of succinate dehydrogenase at Tyr[535] and Tyr[596] and aconitase at Tyr[71], Tyr[544], and Tyr[665] [8, 9].

Mitochondrial Biogenesis

Mitochondrial biogenesis can be defined as the growth and division of pre-existing mitochondria leading to a variation in number, size, and mass. It is dependent on different signaling cascades and transcriptional complexes that promote the formation and assembly of mitochondria.

The Regulation of PCG1α

The peroxisome proliferator-activated receptor γ coactivator 1 (PGC1) family of transcriptional coactivators has recently emerged as central regulator of metabolism being a positive modulator of mitochondrial biogenesis and respiration [10]. PGC1α is a co-transcriptional regulation factor that induces mitochondrial biogenesis by activating different transcription factors, including NRF-1 and NRF-2. These latters

on turn activate Tfam to increase replication of mtDNA and to induce the transcription of key mitochondrial enzymes [11]. AMPK, p38 MAPK, and GSK3β are the best-characterized protein kinases known to target PGC1α. It has been shown that PGC1α is increased in response to activation of AMPK and is reduced in both AMPK null [12] and dominant negative mice [13]. Indeed, AMPK directly phosphorylates PGC1α on Thr^{177} and Ser^{538} [14] thereby enhancing its co-transcriptional activity and consequently mitochondrial gene expression. p38 MAPK phosphorylates PGC1α at Thr^{262}, Ser^{265}, and Thr^{298} in response to cytokine stimulation in muscle cells [15]. Moreover, it enhances the activity of PGC1α by increasing its stability and by disrupting the inactivating interaction between PGC1α and the co-repressor p160MBP in myoblasts [16]. Finally, PGC-1α is also phosphorylated by glycogen synthase kinase 3β (GSK3β), which inhibits PGC1α by enhancing its proteasomal degradation in the nucleus during oxidative stress [17].

Mitochondrial Quality Control

Mitochondria have an important role in the regulation of cell survival, cell death and metabolic homeostasis. They continuously fuse or divide to maintain their functions and damaged mitochondria after fission are removed through mitophagy. Thus, several mechanisms are involved in the regulation of mitochondrial quality control including mitochondrial fission and fusion [18], Parkin-dependent pathways [19] and degradation of damaged mitochondria by lysosomes [20] and autophagosomes [21].

The Regulation of Mitochondrial Fission

Mitochondria fission and fusion are mainly mediated by highly conserved guanosine triphosphatases (GTPases) [22, 23]. Among them, dynamin-related protein 1 (DRP1) is the GTPase that regulates mitochondrial fission [24, 25]. It is a cytosolic protein that once activated translocates to the outer mitochondrial membrane where multimerizes in order to create a ring-like structure that constricts and divides the organelle [26, 27]. Drp1 activity is mainly regulated through phosphorylation in different sites by several protein kinases (Table 26.2). However, data on the effects of DRP1 phosphorylation are rather puzzling since different kinases phosphorylates the same site in DRP1 resulting in opposing effects, as described below.

Table 26.2 Protein kinases that phosphorylate DRP1 and their effects on DRP1 activation

Protein kinases	DRP1 phosphorylation sites	Effect on DRP1
PKA	Ser^{656}	Inhibition
	Ser^{637}	Inhibition
CaMK1α	Ser^{637}	Activation
ERK	Ser^{616}	Activation
CDK	Ser^{616}	Inhibition

PKA has a key role in the regulation of DRP1 activity by preventing its translocation to the mitochondria and inhibiting the process of mitochondrial fission. Indeed, Cribbs & Strack demonstrated that PKA phosphorylates Drp1 at Ser^{656} and this attenuates the GTPase activity of Drp1 promoting cell survival [28]. Chang & Blackstone discovered that PKA phosphorylates DRP1 also at Ser^{637} inhibiting its GTPase activity [29]. Accordingly, the phospho-mimetic substitution Ser637Asp blocks mitochondrial fission and apoptotic cell death [29]. Thus, PKA exerts an inhibitory effect on DRP1 activation by phosphorylation of both Ser^{656} and Ser^{637}, even if no data are available to understand whether phosphorylation at these sites could have different physiological implications. On the contrary, phosphorylation of Ser^{637}, which is inhibitory in PKA signaling, induced mitochondrial fission when is due to Ca^{2+}/calmodulin-dependent protein kinase Iα (CaMKIα) [30]. Similarly, phosphorylation of DRP1 at Ser^{616} by ERK2 activates DRP1 and promotes mitochondrial fission [31], whereas phosphorylation at the same residue by CDK5 exerts opposite effects [32]. Thus, the effects of DRP1 phosphorylation on mitochondrial fission depend on both the type of kinase and the specific phosphorylated residues. However, further studies are needed to clarify such effects.

Regulation of Mitochondrial Fusion

Mitofusins (MFNs) 1 and 2 are a class of conserved GTPases of the mitochondrial outer membrane that are essential for mitochondrial fusion and consequently to maintain normal mitochondrial morphology. MFNs are essential for normal cardiac function. Indeed, the combined deletion of MFN1 and MFN2 in murine hearts induces mitochondrial and cardiomyocyte dysfunction which rapidly leads to progressive and lethal dilated cardiomyopathy. MFN1 and 2 can be phosphorylated and such phosphorylation affects their ability to modulate mitochondrial fusion (Table 26.3).

Indeed, MFN2 was found to be phosphorylated by PINK1 at Thr^{111} and S^{442} to become a mitochondrial receptor for Parkin and eventually promote mitophagy. Moreover, MFN2 can be also phosphorylated by JNK at Ser^{27} causing its degradation through the ubiquitin-proteasome pathway which in turn affects both mitochondrial dynamics and apoptosis [33]. Also MFN1 can be phosphorylated at Thr^{562} by ERK to modulate apoptotic responses [34]. Indeed, this inhibitory phosphorylation of MFN1 induces its association with BAK facilitating its oligomerization and inducing cytochrome *c* release and cell death [34].

Table 26.3 Mitofusins phosphorylation sites and effects of phosphorylation on mitophagy

Protein kinases	MFN phosphorylation sites	Effect on mitophagy
PINK1	T^{111} of MFN2	Activation
PINK1	S^{442} of MFN2	Activation
JNK	Ser^{27} of MFN2	Inhibition
ERK	T^{562} of MFN1	Inhibition

Parkin-Dependent Mechanisms

PINK1 is a kinase associated with mitochondria: the loss of this kinase expression causes mitochondrial dysfunction and mitophagy [35–37]. Parkin is an E3 ubiquitin ligase suggested to be downstream of PINK1 to regulate the removal of damaged mitochondria. Indeed, PINK1 is activated by mitochondria membrane potential depolarization and is imported into mitochondria to activate Parkin [38–41]. This latter causes proteasomal degradation of outer mitochondrial membrane proteins [42, 43] and selective autophagy of damaged mitochondria [44], suggesting that PINK1 and Parkin mediate a mitochondrial quality control pathway. The removal of damaged mitochondria was thought to be mainly attributable to the activation of PINK1-Parkin-Ubiquitin cascade: PINK1 directly phosphorylates Parkin at Ser^{65} which on turn activates Ubiquitin [45]. Actually, recent findings changed this view: PINK1 has been shown to recruit Parkin to mitochondria also in presence of mutation of Ser^{65} to Alanine suggesting the ability of PINK1 to regulate Parkin in a phosphorylation independent manner. Moreover, PINK1 directly phosphorylates Ubiquitin at Ser^{65} which on turn activates Parkin [46]. Thus, PINK1 phosphorylates at Ser^{65} both Parkin and Ubiquitin to induce the full activation of Parkin, as summarized in Fig. 26.1.

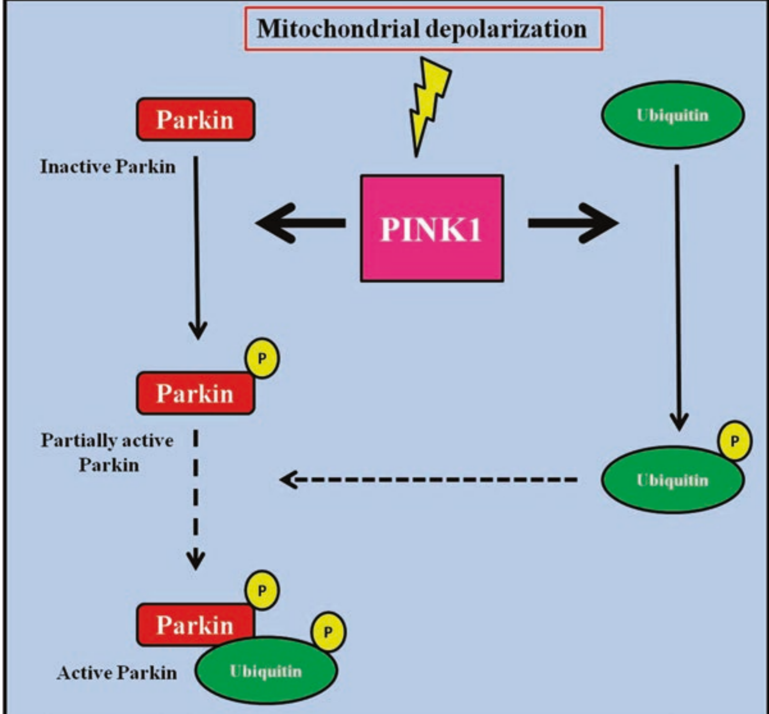

Fig. 26.1 The full activation of Parkin is dependent on PINK1-mediated phosphorylation of both Parkin and ubiquitin at Ser^{65}

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Chapter 27
Mitochondria Damage and Kidney Disease

Pu Duann and Pei-Hui Lin

Abbreviations

AKI	Acute kidney injury
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
CKD	Chronic kidney disease
coQ	Coenzyme Q
CsA	Cyclosporin A
cyt C	Cytochrome C
DN	Diabetic nephropathy
dNTPs	Deoxynucleotides triphosphates
DRP1	Dynamin related protein 1
ESRD	End-stage renal disease
ETC	Electron transport chain
FSGS	Focal and segmental glomerulosclerosis
GN	Glomerulonephritis
GSK	Glycogen synthase kinase
H^+	Proton
I/R injury or IRI	Ischemic reperfusion injury

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IMM	Inner mitochondrial membrane
KLF-6	Krüppel-like factor 6
MELAS	Myopathy encephalopathy lactic acidosis and stroke-like episodes
Mfn1 and 2	Mitofusins 1 and 2
MPT	Mitochondrial permeability transition
mPTP	Mitochondrial permeability transition pore
mtDNA	Mitochondrial DNA
MA-5	Mitochonic acid 5
NAD^+	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NRF	Nuclear respiratory factors
OMM	Outer mitochondrial membrane
OPA1	Optical atrophy 1
OXPHOS	Oxidative phosphorylation
PGC-1α	PPARγ-coactivator -1α
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SIRT	Sirtuin
TLR	Toll-like receptor
$\Delta\Psi m$	Mitochondrial inner membrane potential

In human body, the kidney receives 20% of cardiac output and consumes 10% of the body's oxygen to perform its functions. The kidney is composed of multiple cell populations which are involved in several vital functions that maintain body's homeostasis such as acid-base and electrolytes balance, blood pressure regulation, nutrients reabsorption and hormone secretion [1]. The human adult kidney is composed with about 3 million functional units, or "nephrons", and their primary functions and structures are subdivided into a glomerular filtration unit and several tubular segments. The glomerular filtration is a process of filtering blood circulation, retaining circulating cells and useful macromolecules. The tubular segments are involved in active transport processes to reabsorb water, electrolytes, and nutrients to maintain fluid and osmolarity homeostasis. The coordination of both processes maintains body homeostasis and results in metabolic waste excretion with only trace amount of proteins in the urine output [2]. Hence, kidney dysfunction often results in kidney disease with systemic complications.

Kidney diseases can be classified into two categories- acute kidney injury (AKI) or chronic kidney disease (CKD). Renal disease and complications are the current global health concerns due to the vital roles of kidney in body homeostasis [3–5]. AKI is characterized by rapid renal function decline along with accompanying electrolytes abnormalities, fluid overload, severe acidosis, hematologic abnormalities (e.g. anemia, uremic platelet dysfunction), and possibly multi-organs failure and poor clinical outcomes [6]. CKD is linked to gradual loss of renal function over time. CKD could derive from defects in glomerular filtration units or chronic tubular injuries which could lead to multiple complications including cardiovascular disease,

high blood pressure, bone loss, and malnutrition. Both AKI and CKD are closely integrated and could serve as a risk factor for one another which often linked with increase cardiovascular risk and uprising mortality and morbidity rates [7, 8]. Additionally, kidney functions are susceptible to certain inherited genetic diseases and aging processes [9, 10]. All different forms of renal injuries could ultimately progress into severe end-stage renal disease (ESRD) with renal replacement as the only therapeutic option. Therefore, therapeutic strategies are in dire need to prevent renal diseases progression.

Mitochondria is the cell's energy-producing organelle that maintains cellular redox and energy homeostasis, and therefore a major source of intracellular oxidative stress [11]. In addition to its role in adenosine triphosphate (ATP) generation through oxidative phosphorylation (OXPHOS), the mitochondria also play an essential role in metabolic signaling such as pyrimidine, heme biosynthesis, TCA cycle and fatty acid β-oxidation pathways, in calcium ion (Ca^{2+}) homeostasis, thermogenesis, proliferation and regulating intrinsic apoptotic pathway. Mitochondria are heterogeneous and dynamic organelles. The mitochondria populations can be different in size, mass, metabolic activity, and membrane potential within a cell. Additionally, mitochondria constantly change shape, dynamics and turnover to maintain cellular homeostasis. Cellular stress can induce compromised mitochondria membrane integrity or dysfunctions, leading to cell death via different mechanisms. This could involve the release of apoptotic molecules such as cytochrome C (cyt C), and apoptosomes from mitochondrial inter-membrane spaces. Alternatively, activation of mitochondrial permeability transition (MPT) can trigger mitochondrial inner membrane potential ($\Delta\Psi m$) dissipations and subsequent loss of energy production [12].

Mitochodrial damages and dysfunction are recognized as a leading factor to many chronic and acute renal diseases [11]. Therefore, it is remarkably important to understand mitochondrial biology and pathophysiology for effective therapeutics discoveries in renal diseases. In this chapter, we discuss evidence supporting mitochondrial dysfunction in the pathogenesis of kidney disease, and summarize the recent development of mitochondria-targeted therapies which hold high promise alleviating renal injury.

Mitochondria in Kidney Health

Mitochondria are especially important in metabolically active organs such as brain, heart, kidney and muscle. Kidney consumes roughly 7% of the body's daily ATP energy expenditure [13, 14]. Due to various energy demands, different nephron segments have different mitochondria densities and distributions. It is generally accepted that renal tubule cells are rich in mitochondria, with the S1 segment containing the highest mitochondria density. These renal tubule cells require this large amount of mitochondria due to their high-energy demand for reabsorption and secretion against chemical gradients, which heavily rely on normal mitochondrial oxidative phosphorylation to supply ATP as an energy source [15]. Nevertheless, the

high-energy requirement of the podocytes was only recently highlighted for possible mechanisms in structural stability, organization of cytoskeletal and extracellular matrix proteins, motility, remodeling of foot process, uptake of filtered proteins, and some other more obscure mechanisms [16]. Bioenergetic profiles studies of mitochondrial function confirmed that podocytes are very susceptible to dysfunction in energy supply during stress condtions [17]. Several factors such as mitochondrial biogenesis and turnover, bioenergetics, dynamics, and autophagy (mitophagy) regulate the conditions of mitochondria.

Mitochondrial Biogenesis

Mitochondrial turnover is an exquisitely coordinated process to maintain mitochondrial homeostasis. In this regard, dysfunctional mitochondria are selectively eliminated and replaced through biogenesis, a coordinated process between nuclear and mitochondrial genomes, to increase the mass and number of functional mitochondria in compensation for the lost or damaged mitochondria [18]. The mitochondrial DNA (mtDNA) encodes 37 genes which include 13 structural subunits of the mitochondrial respiratory chain. The molecular mechanism of mitochondrial biogenesis during tissue injury and repair are actively studied topics [19, 20] (Fig. 27.1a, b). The mitochondrial biogenesis program involves mito-nuclear communication and several key regulators have been identified such as: peroxisome proliferator-activated receptor (PPAR), PPARγ coactivator 1α (PGC-1α), sirtuin-1 and 3 (SIRT 1/3) family deacetylase, AMP-activated protein kinase (AMPK), and nuclear respiratory factors 1 and 2 (NRF1 and NRF2) [21]. AMPK and SIRT1 positively regulate PGC-1α, the master regulator of mitochondria biogenesis, through post-translational modifications with phosphorylation or deacetylation respectively. In addition, AMPK and SIRT1 are important modulators of energy metabolism. In kidney, the PGC-1α transcriptional coactivator is predominantly expressed in proximal tubules, and therefore is pivotal to tubular homeostasis. PGC-1α regulates the expression of NRF1 and NRF2 and increases the biosynthesis of nicotinamide adenine dinucleotide (NAD^+), a central metabolic coenzyme/cosubstrate involved in cellular energy metabolism, to link oxidative metabolism to renal protection [22–24]. Both NRF1 and NRF2 are nuclear DNA (nDNA)-encoded transcription factors that activate genes coding for the OXPHOS system and genes involved in mtDNA transcription and replication [25, 26].

Mitochondrial Bioenergetics

The mitochondrial OXPHOS respiratory chain is composed of four protein complexes which are known as complexes I–IV. These complexes transfer electrons and protons (H^+) across inner mitochondrial membrane (IMM) to generate an electrochemical gradient for ATP synthesis in a fifth protein complex, known as

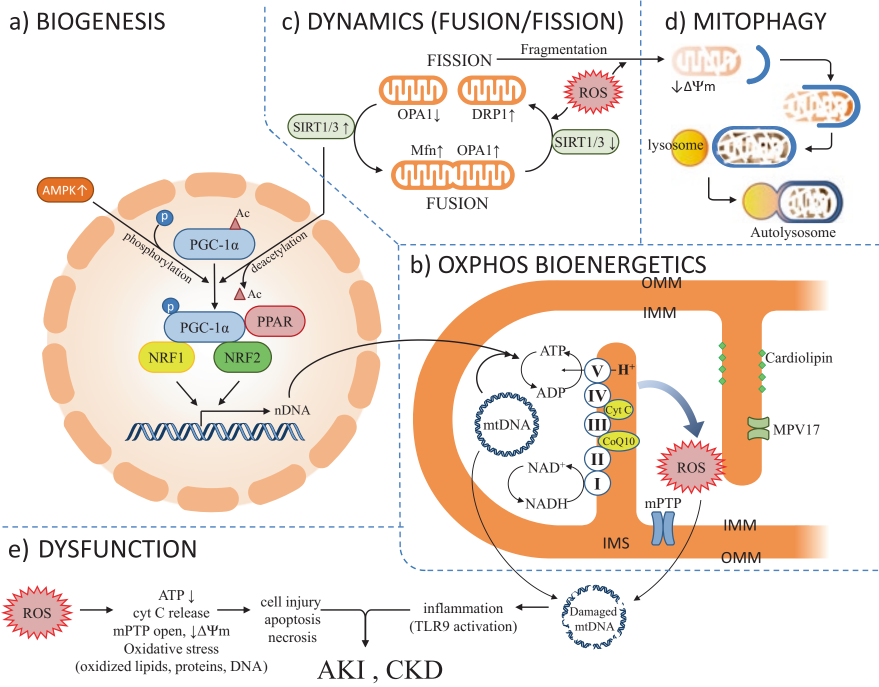

Fig. 27.1 The health and disease of kidney are regulated by the pathophysiological conditions of mitochondria. Several processes regulate the health and disease states of mitochondria which include (panel **a**) mitochondrial biogenesis – a process requires coordinated expression of both nDNA and mtDNA and several transcriptional factors and co-activators; (panel **b**) OXPHOS bioenergetics – an electrochemical gradient and ATP synthesis process which depends on respiratory electron transfer chain (complexes I–V), cofactors (MPV17 and others), and cardiolipin to maintain normal oxidative phosphorylation; (panel **c**) mitochondrial dynamics – a process requires functional balance between fusion and fission; and (panel **d**) mitochondrial turnover by autophagy (or mitophagy) – an important process to clear the dysfunctional mitochondria. (Panel **e**) Disturbance in any steps of the processes in panels **a**–**d** will cause ROS overproduction, decrease in ATP generation, loss of IMM potential (ΔΨm), mPTP opening and cyt C release. The oxidative stress could lead to cell apoptosis or cell injury and the damaged mtDNA, nDNA could stimulate innate immune response through activation of Toll-like receptor (TLR) and inflammation which, all together, lead to AKI or CKD. See Text and Abbreviations for details

complex V (ATP synthase) (Fig. 27.1b). Some important factors were identified in this process. For example, NAD concentrations determine the rate-limiting process [27, 28] and Coenzyme Q10 (coQ10) shuttles electrons in the respiratory chain [29]. Additionally, depending on its interaction with cardiolipin, the IMM resident protein cytochrome C (cyt C) regulates an intricate balance between mitochondrial respiration and apoptosis. Cardiolipin was identified as a phospholipid that was exclusively expressed on the IMM, where it forms microdomains and plays a central structural role in cristae formation, a property of cardiolipin on membrane

curvature, organization of the electron transport chain (ETC) complexes into supra-complex for OXPHOS activity, and as a platform for initiation of apoptosis [30, 31]. The interaction of cyt C and cardiolipin determines whether or not cyt C functions as an electron carrier or a peroxidase.

During normal electron transfer, some (<4%) of the consumed oxygen is converted into superoxide radicals such as reactive oxygen free radical species (ROS) and reactive nitrogen free radical species (RNS) via electron leakage, which constitutes different forms of oxidative stress [32]. However, under ischemic conditions, Ca^{2+} overflows into mitochondria during rapid loss of ATP and aggravates ROS production. These reactive radicals cause modification of biomolecules of DNAs (nDNAs and mtDNAs) and proteins, lead to lipid peroxidation, and may impair their bioactivities which eventually lead to the opening of the mitochondrial permeability transition pore (mPTP) and loss of mitochondrial membrane potential ($\Delta\Psi$m) [12].

The importance of the IMM-resident MPV17 protein in mitochondrial physiology has come to light recently. MPV17 is encoded by nDNA and functions as a $\Delta\Psi$m-modulating channel that contributes to mitochondrial homeostasis under different conditions. MPV17 forms weak cation-selective channesl and has shown several subconductance states in vitro [33]. Interestingly, MPV17 channel protein allows for the passage of small molecules such as deoxynucleotides triphosphates (dNTPs). It is possible that MPV17 maintains mitochondrial dNTP homeostasis, and therefore the perturbation of dNTP pools is a recognized cause of mitochondrial genomic instability [34]. Mutations of MPV17 are associated with mtDNA depletion syndrome, an inherited autosomal recessive disease in humans. Additionally, MPV-like proteins are identified as epithelial and neuronal restricted protein and implicated in ROS metabolism and apoptosis regulation through its binding and functional interaction with mitochondrial serine protease, HTRA2 [35]. Altogether, excess oxidative stress accumulation, mtDNA instability, and imbalance of bioenergetics will lead to mitochondrial dysfunction which could be important in the pathogenesis of kidney diseases [36].

Mitochondrial Dynamics

Mitochondria are highly dynamic organelles – their number, size and locations change constantly in response to energy demands. They constantly move, fuse and divide – switching between elongated interconnected networks or fragmented discrete morphologies via coordinated fusion and fission. These dynamics are essential to their size, morphology, energy biogenesis, function, and maintenance of cellular homeostasis and viability [37–39]. Perturbation of mitochondrial dynamics results in mitochondrial dysfunction and is linked to aging, end organ injury, and human diseases [40, 41]. Mitochondrial dynamics are regulated by a complex relationship between fission proteins (the large GTPase, dynamin related protein 1 (DRP1), and mitochondrial fission 1 (Fis1)) and fusion proteins (mitofusins 1 and 2 (Mfn1, Mfn2)

and optical atrophy (OPA1)) [5, 42] (Fig. 27.1c). The NAD^+-dependent SIRT family deacetylases, especially the mitochondrial matrix-resided SIRT3 protein, play an important role in regulating mitochondrial dynamics and function. In this role, the SIRT3 deacetylates and activates the mitochondrial fusion protein OPA1 to improve mitochondrial function under stress conditions [43] or acute kidney injury [44].

Mitochondrial Turnover: Autophagy and Mitophagy

Autophagy is a non-selective process where cytoplasmic contents, e.g. damaged organelles and aged protein aggregates, are sequestered into autophagosomes for lysosome delivery and bulk degradation within the cell. Autophagy occurs during nutrient stress for ATP preservation, amino acid recycling, and anabolic protein synthesis. In certain stress conditions, selective autophagy occurs to efficiently remove toxic cellular materials such as damaged organelles. Mitophagy is one of the selective autophagy processes to remove accumulated dysfunctional mitochondria. Mitophagy clearance encompasses several steps – the damaged mitochondria are first marked by a loss of mitochondrial inner membrane potential ($\Delta\Psi m$) via intricate protein interactions involving kinases, E3 ubiquitin ligase, and proteins regulating mitochondrial dynamics and transportation [45] (Fig. 27.1d). The damaged mitochondria are eventually encapsulated into autophagosomes and degraded in autolysosomes. Autophagy (and mitophagy) is ROS-dependent, which is heavily regulated by cellular redox activity. Growing evidences support the notion that disturbance of autophagy/mitophagy is associated with the pathogenesis of renal diseases such as AKI [5], diabetic nephropathy [45] and glomerulosclerosis [46].

Mitochondria Damage in Kidney Diseases

Renal diseases encompassing both acute and chronic conditions of kidneys injury with declined renal functions are current global health concerns with tremendous medical burdens [3–5]. A common link between all forms of acute and chronic kidney injuries is the generation of toxic ROS and RNS when the disease manifests. This oxidative stress injury could be derived from ischemia/reperfusion, energy shortage from impaired mitochondrial biogenesis or ATP energetics, or defective clearance of damaged mitochondria (mitophagy). All of the listed conditions could link to recruitment of immune cells, inflammatory cytokines accumulation, apoptosis, and tissue injury. The renal phenotypes of mitochondrial dysfunction may manifest as proximal tubular dysfunction, tubularinterstitial disease, cystic kidney disease, podocytopathy, and nephrotic syndromes. All different forms of renal injuries might increase cardiovascular risk with uprising mortality and morbidity rates.

Acute Kidney Injury (AKI)

AKI has been a global concern with worldwide prevalence of 13 million people and death toll roughly 1.7 million annually [47]. AKI is a clinical condition that is commonly related to an acute episode of systemic injuries that occur in cases such as: kidney transplantation and septic and trauma patients. In the USA, AKI is the source of 1–2% of all hospital admissions and the mortality rates in hospitalized ICU patients with AKI could reach 50–70% [48]. Several etiologies such as ischemic, toxic, septic, and hypertensive injuries cause AKI with symptoms of abrupt reduction in kidney functions. AKI symptoms are manifested as acute decline in glomerular filtration rate, concomitant decreased urinary output, tubular necrosis, tubular interstitial inflammation and vascular permeability changes [5, 49].

Interestingly, in a polymicrobial septic AKI model, Tsuji and colleagues recently identified circulating mtDNA that stimulate systemic inflammation via *Toll*-like receptor 9 (TLR9) signaling as a novel pathogenic mechanism for AKI (Fig. 27.1e). The source of circulating mtDNA has been speculated to be derived from immune cells upon septic clearance or from spleen immune response [50, 51]. This study suggests that timely protection and removal of damaged mitochondria would reduce inflammation and could be an effective therapeutic strategy against AKI.

Ischemic-Reperfusion (I/R) Induced AKI

Renal ischemia-reperfusion injury (IRI) is a common cause of AKI and post-transplantation kidney allograft dysfunction resulting from an acute decline in general or localized renal oxygen and nutrients supplies to the affected tissue, and impairment of timely removal of metabolite wastes in kidney cells. Extensive oxidative stress generated during this process injures the tubular epithelial cells, causing histologically characteristic inflammation and necrotic cell death (necrosis) which was referred as acute tubule necrosis previously [52]. Emerging evidence supports the notion that multiple signaling pathways are involved in the pathobiology of AKI [52, 53]. Of these, damaged mitochondria with disrupted matrix cristae and mitochondrial permeability-mediated necrosis are crucial to ischemic AKI. Proper functional autophagy (mitophagy) is reno-protective during renal tissue regeneration if the injury is under a certain threshold, whereas defective mitophagy impedes tissue regeneration [5].

Nephrotoxic AKI

Exposure to nephrotoxins, such as some chemotherapy drugs, medications, intravascular contrast media, trace heavy metals, drug abuse, or certain chemicals/drugs combinations could induce AKI, especially in the elderly, young children, and high-risk patients [54, 55]. Drug nephrotoxicity was found to be responsible for 19% of

all AKI cases on critically ill patients. The kidney is susceptible to intrinsic renal toxin injury which could result in various clinical syndromes ranging from minimal changes in tubular function to fulminant kidney failure. Injury may also occur to altered hemodynamic AKI. Some examples include: NSAIDs and renin-angiotensin system (RAS) antagonism, tubular epithelium direct cell injury from chemotherapy drugs like cisplatin, tubular obstruction of urinary flow due to precipitates of toxins or their metabolites, and angiopathogenesis from vascular injury and interstitial inflammation. Several research efforts have been dedicated to identify biomarkers of nephrotoxic AKI [56]. Cisplatin-induced renal mitochondrial structural, functional and homeostatic changes include swollen mitochondria, ultra-structurally disrupted IMM cristae, reduced mitochondrial mass, mtDNA depletion, and diminished cyt C oxidase activity in animal model [57]. Permeability of mitochondrial OMM upregulates the expression of mitochondrial dynamin related protein 1 (DRP1), an OMM resident fission protein, which induces mitochondrial fragmentation, cyt C release and apoptosis [58]. Reduction of mitochondria biogenesis regulators such as PGC-1α and SIRT3 were also documented [44]. Moreover, kidney specific overexpression of SIRT1 was protective against cisplatin-induced AKI [59]. The nephrotoxic effect and its pathophysiology of cisplatin-induced AKI had been a seminary topic of a recent review [60].

Septic AKI

Septic shock is one of the most frequent injury which could account for nearly half of the cause of AKI in critical ill patients [61]. Mitochondrial damage is an important contributing factor in the pathogenesis of septic AKI [62]. Clinical biopsies obtained from intensive care of human sepsis subjects also support this notion [63]. Apparent mitochondrial ultrastructure changes and biochemical studies revealed mitochondrial pathogenesis includes loss of mitochondrial mass, swelling, fragmentation due to aberrant mitochondrial dynamics, PGC-1α reduction, extensive mitochondrial cristae remodeling, and cyt C release during apoptosis. As aforementioned, mitochondrial damage and the release of mtDNA is linked to systemic inflammation and tissue injury. The intricate interplays between mitochondrial energy precursor NAD/NADH, mitochondrial health and kidney function were shown in several studies of septic AKI animal model [23, 24]. In summary, mitochondrial NAD concentration regulates the rate-limiting process of mitochondrial energy production and, hence, PGC-1α causes an increase of NAD biogenesis, mitochondrial numbers and subsequent renoprotection.

Chronic Kidney Disease (CKD)

CKD is characterized by persistent renal dysfunction for more than 3 months. The causes of CKD have been commonly associated with a number of comorbid conditions such as type 2 diabetes and hypertension [64]. The prevalence of CKD is

estimated to be roughly 8–16% globally. According to the United States Renal Data System report, more than 13% of adults develop CKD and 30% of the population at the age of 65 and above experience some form of kidney failure in the USA [65]. CKD is staged based on estimated glomerular filtration rate (eGFR) and albuminuria with conditions ranging from asymptomatic stage to end-stage renal disease (ESRD) [66]. Different from AKI, CKD is recognized as a separate, irreversible and progressive pathologic condition which often inevitably leads to ESRD [7, 8, 49]. Individuals with diabetes or hypertension have high risk to develop CKD. Recent findings support the close relation between AKI and CKD and confirm, clinically, that one could serve as the risk factor to the other in maladaptive recovery conditions [7, 8].

Diabetic Nephropathy (DN)

Diabetic Nephropathy (DN), a progressive microvascular complication that arises from diabetes mellitus (DM) which affects individuals with both type 1 or type 2 DM, is the leading cause of CKD. DN is a chronic disease characterized by decreased glomerular filtration, proteinuria, glomerular hypertrophy, and renal fibrosis with gradual decline in renal function which contributes to 40% kidney failure and eventually requires renal replacement therapy [67–69].

The pathogenesis of DN is complex due to all four renal compartments-glomeruli, tubules, interstitium and blood vessel being involved. Moreover, etiologies of DN originated from hyperglycemia mediated microvasculate abnormalities which affect several signaling pathways. These include: increased glucose metabolite flux, advanced glycation end-products formation, endoplasmic reticulum stress, ROS overproduction, pro-inflammation and apoptotoc cell death in podocytes [68, 69]. It is well documented that aberrant mitochondrial homeostasis plays pivotal roles in DN progression [11, 68–73]. Hyperglycemia induced increased oxidative stress derived from renal ROS over-production in conditions like impaired mito-glutathione transport [74], increased mitochondrial activation of an ubiquitous NAD(P)H oxidase (Nox4) in respiratory chain [75], decreased mtDNA stability to against ROS production [76] and podocytes apoptosis [77] all contribute to cell damage and lethal effects linked to diabetic complications. Interestingly, hyperglycemia triggers mitochondrial fission and is mediated by Rho-associated coiled coil-containing protein kinase 1 (ROCK1)-dependent phosphorylation of dynamin related protein 1 (DRP1) and its recruitment to mitochondria [78]. These results suggest that mitochondrial dynamics play an important role in the pathogenesis of DN. Additionally, defects in mitochondrial biogenesis with downregulated expression or activity of PGC-1α is recognized to contribute to DN [71, 72]. It appears that the protective role of PGC-1α is associated with the inhibition of DRP1-mediated mitochondrial dynamics remodeling and ROS production [79]. Growing evidence also suggests that autophagy or mitophagy is altered in DN [45]. It is plausible that

impairment in autophagic flux would lead to the DN complication associated with extracellular matrix deposition and fibrosis.

Glomerulonephritis (GN)

Chronic glomerulonephritis (GN), characterized by dysfunction in the glomerular filtration barrier, is a major feature of CKD. GN accounts for roughly 10% of CKD. GN is the inflammation of the glomeruli with clinical symptoms which may include hematuria, proteinuria, edema, and hypertension. Congenital GN can occur as genetic mutations on mitochondrial proteins (see section on "Genetic Mitochondrial Disease Affecting Kidney Functions"). Immune diseases may also cause chronic GN. Abnormal-shaped mitochondria are often found before the disease progress into secondary (or acquired) focal segmental glomerulosclerosis (FSGS), a severe form of GN [46]. Therefore, these evidence support the notion that impaired autophagic mitochondrial turnover is responsible for FSGS phenotype in mice.

Recently, Kruppel-like factors 6 (KLF6), a subfamily of DNA-binding zinc finger protein, was identified as an inducible early injury response gene that encodes a crucial transcriptional regulator of mitochondrial function in podocytes under stress response [80–83]. Podocyte-specific loss of *Klf6* reduced mitochondrial function and increased the susceptibility to FSGS in mice. Likewise, podocyte-specific KLF6 expression was significantly reduced in FSGS patients when compared to healthy individuals. Mechanistic studies indicated KLF6 prevents mitochondrial injury via enhancing cyt C assembly [82].

The function roles of MPV17 were explored further as discussed earlier. Growing body of evidence indicated that the glomerulosclerosis gene *mpv17* and its encoded MPV17 protein as important factors in regulating peroxisomal metabolism of ROS [84]. MPV17 was identified as an IMM- localized protein in podocyte. Loss of *Mpv17* was found to be associated with glomerulosclerosis and knockout mice developed renal failure later on in their lifespan (9–12 months). The glomerular lesions were caused by toxic oxidative stress and lipid peroxidation adducts. Interestingly, expression of MPV17 is diminished in several glomerular injury models and also in human FSGS subjects [85]. Altogether, the evidence further confirmed that MPV17 is important for mitochondrial homeostasis in podocytes.

Genetic Mitochondrial Disease Affecting Kidney Functions

Genetic disorders that affect mitochondrial function can be mutations related to either the nuclear or mitochondrial genomes which encode mitochondrial proteins. They represent genetically and clinically heterogeneous disorders which affect mostly metabolic-active organs. Genetic mitochondrial mutations could be derived

from primary (inherited) or secondary to environmental predisposition to drugs or oxidative stress injury. Kidney phenotypes are often manifested as tubular dysfunction, interstitial nephritis, glomerular dysfunction, and renal tumors [9, 86].

MELAS and tRNA-Leu (m.A3243G) Transition

MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke like symptoms) is a mitochondrial syndrome manifested in patients that are 40-years old or younger with symptoms like seizure and stroke-like episodes, muscle myopathy, lactic acidosis, and maternally inherited diabetes with deafness. A base transition (A3243G) in mitochondrial gene tRNA-Leu accounts for 80% of MELAS cases with occasional renal phenotypes of FSGS, or less commonly with tubular interstitial nephritis, which results in progressive renal insufficiency [87, 88]. Electron microscopic images from renal biopsies of MELAS patient revealed abnormal mitochondria in podocytes and tubular cells, indicating dysfunctional mitochondria is central to disease pathogenesis.

Mitochondrial DNA Deletion

Different from the mtDNA point mutations, few mitochondrial disease syndromes which are often associated with large mtDNA deletion at different genome locations and affect tubular pathologies, are Fanconi [89], Kearns-Sayre, and Pearson syndromes [90]. The clinical features of Pearson syndrome are manifested with pancreatic fibrosis with insulin-dependent diabetes, whereas the kidney phenotype of Kearns-Sayre syndrome includes renal tubular acidosis (proximal or distal) which occasionally progress to ESRD. Although occurrences may be rare, these syndromes involve renal tubulopathy (proximal or distal) manifested as electrolyte abnormalities and fibrosis. Renal biopsies of patients with Kearns-Sayre syndrome revealed impaired cyt C oxidase activity, defect in energy metabolism, and mitochondrial ultrastructures alterations [90].

Mutations in Nuclear-Encoded Genes for Mitochondrial Proteins

The mitochondria proteome is composed with about 1,000 proteins which are mostly encoded by nDNA. nDNA mutations with prominent tubular dysfunction include mutations that affect energy metabolism, often affecting complex III and IV, and mutations that affect fatty acid metabolism. Podocyte dysfunction is often correlated with multi-mutations in coenzyme Q10 biosynthesis pathways and the

pathogenic mechanism was confirmed in *pdss2* gene encoding prenyl diphosphate synthase subunit 2, the first enzyme of coQ10 pathway [29, 91].

Aging in Kidney Disease

Older age is associated with an elevated risk of kidney disease and increased population of older renal transplant recipients and poor graft survival. The renal aging phenotype includes profound functional alterations leading to increased renal vascular resistance and reduced repair capacity. Aging kidney is often associated with histological changes such as tubular atrophy, interstitial fibrosis and glomerulosclerosis. Clinically, renal aging is associated with an increased susceptibility to AKI [92], and CKD may share accelerated vascular disease phenotype similar to the condition of premature aging [93]. The kidney suffers from increasing oxidative stress and disturbance in autophagy with aging [94, 95]. The molecular mechanism of renal cell senescence has been explored to certain extent. Downregulation of PGC-1α may link telomere-dysfunction to compromised renal mitochondrial function through p53 activation and alteration of transcription [96]. The renin–angiotensin–aldosterone system (RAAS) is a key regulator of blood pressure, fluid homeostasis and cardiovascular physiology. Angiotensin II and the age-dependent switch of mitochondrial expression of the two types of cell surface angiotensin receptors (AT1R *vs* AT2R) are important factor in determining respiratory activity during aging [97]. Further studies are needed to explore the role of the telomere–mitochondrial axis in renal physiology, aging and disease.

Mitochondria-Targeted Therapeutics

As protection of mitochondria could serve as a potential effective therapeutic strategy, it has been research endeavors focused on this development toward kidney diseases [49, 98–101]. These agents include antagonizing mitochondria oxidants, promoting mitochondrial biogenesis and ATP synthesis, regulating ROS metabolism, cardiolipin protection, inhibitors of mPTP, or inhibitors of mitochondrial fragmentation as detailed below (Table 27.1).

Mitochondria-Targeted Antioxidants

Mitochondria-targeted antioxidants (e.g. MitoQ, MitoTEMPO, MitoE, Mito-CP, SkQ1 and SkQR1) have been a part of the efforts to reduce mitochondrial oxidative stress. These agents are based on the mechanism of delivery of known redox agents to mitochondrial matrix through conjugation with TPP+ (triphenylalkylphosphonium

Table 27.1 Mitochondria-targeted therapeutics

Therapeutics	Chemicals	Action mechanisms	Experimental model
Antioxidants			
MitoQ[a]	Mitoquinone	Anti-oxidant concentrate at matrix in a ΔΨm-dependent manner; ROS scavenger	IRI-AKI, cisplatin-AKI, sepsis-AKI
Biogenesis activator			
Resveratrol (SRT501)	Small peptides	AMPK / SIRT-1 / PGC-1α axis activator	Hemorrhagic shock, sepsis-AKI
AICAR		SIRT3 activator, AMPK agonist	Cisplatin-AKI, IRI-AKI
Formoterol		β2-adrenoceptor agonist, increase PGC-1α synthesis	IRI-AKI
ROS metabolism and bioenergetics			
Mitochonic acid (MA-5)	Synthetic compound	OXPHOS-independent increase of ATP synthesis, reduce ROS	Sepsis-AKI
Cardiolipin protection			
Bendavia (SS-31)[a]	Szeto-Schiller tetrapeptide	Protect cardiolipin from peroxidation; increase ATP and reduce ROS	AKI and CKD
		Prevent cyt C transformation into peroxidase	
mPTP inhibitor			
cyclosporin A (CsA)[a]	Small molecule	Interact with cyclophilin D (a mPTP structural protein)	Adriamycin-induced podocyte injury
TDZD-8		mPTP inhibitors, GSK3β inhibitor	Podocyte injury or drug induced AKI
Fission inhibitor			
Mdivi1	Small molecule	Inhibitor of mitochondrial division; induce mitochondria fusion	IRI-AKI
		Block DRP1 assembly and translocation	
K(ATP) channel opener			
Simdax (Levosimendan)[a]	Small molecule	Calcium sensitizer, K(ATP) channel opener	IRI-AKI
cyt C assembly			
KLF6	DNA-binding zinc finger transcriptional regulator	enhance cyt C assembly	Podocytes FSGS (animal study)

[a]Clinical trials. https://clinicaltrials.gov

cation) moiety [100]. These ROS scavenger compounds could cross the membrane lipid bilayer and concentrate at the matrix in a membrane potential ($\Delta\Psi m$) – dependent manner. MitoQ was shown to be safe in clinical trial with Parkinson's disease, fatty acid disease, and is currently under clinical trial (NCT02364648) for CKD. In summary, several animal models have confirmed that mitochondrial-targeted antioxidants represent an effective strategy to prevent or attenuate different forms of kidney diseases.

Activator of Mitochondria Biogenesis

Activation of Mitochondria biogenesis is required for the increased metabolic and energy demands during the recovery phase of acute organ injury. The AMPK/SIRT/PGC-1α axis plays crucial roles in mitochondrial biogenesis. PGC-1α is a pivotal factor that coordinately regulates NAD biogenesiss, reduces oxidative stress, and facilitates recovery from AKI [24]. SIRT1 is a NAD-dependent deacetylase that positively regulates PGC-1α expression and activity. Agents in this category include resveratrol, AICAR, and formoterol. Treatment with resveratrol, a natural plant phytoalexin, in a rat hemorrhagic shock model increases mitochondria mass, restores mitochondrial respiratory capacity, and reduces oxidative stress [102]. Moreover, resveratrol modulates immune response by suppressing inflammation driven by macrophage in a lipopolysaccharide -induced septic AKI model [103]. Pretreatment with AICAR, an AMPK activator, attenuated injury and tubular necrosis, decreased nitrosative stress, and ameliorated renal function in a rat I/R induced AKI [104]. Formoterol, a potent agonist of β2-adrenoreceptor, induces mitochondrial biogenesis by increasing mtDNA copy numbers, oxygen consumption rate, and PGC-1α synthesis. In doing so it restores renal function, rescues renal tubules from injury, and diminishes necrosis in an I/R-induced AKI animal model [105]. In summary, agents affect mitochondrial biogenesis and NAD modulation hold great promise in treating kidney disease, but their clinical translation still awaits further investigation.

Modulation of ROS Metabolism and ATP Synthesis

Mitochonic acid 5 (MA-5), a synthetic derivative of indole acetic acid plant hormone, was identified as a compound to enhance ATP production. MA-5 was found to improve the survival of fibroblasts collected from patients with mitochondrial diseases, including Leigh syndrome and MELAS [106]. Evidence supports that MA-5 enhances ATP production by promoting assembly and oligomerization of complex V at mitochondrial crista junction, and therefore preserving the mitochondria dynamics and preventing its fragmentation. These new findings provide new mechanisms in renal and cardiac protection [106, 107].

Cardiolipin Protections

As cardiolipin regulates IMM structural and functional plasticity, peroxidation or loss of cardiolipin has been associated with aging and several forms of AKI and CKD. Development of cardiolipin-target compound to optimize efficiency of the ETC and thereby restore cellular bioenergetics has been an innovative discovery. SS-31 peptide (Szeto-Schiller peptide, also named MTP-131 or Bendavia) is a cardiolipin-targeted tetrapeptide (D-Arg-dimethylTyr-Lys-Phe-NH2) that stabilizes cardiolipin, scavenges mitochondrial ROS, regulates cyt C activity, and inhibits the mitochondrial permeability transition (MPT) pore in AKI and CKD models [108–110]. SS-31 treatment demonstrated mitochondrial protective effects on swine stenotic-kidney injury and improved renal hemodynamics and function [111]. SS-31 has entered several clinical trials for treatment with AKI renal microvascular dysfunction in hypertension, and I/R injury of myocardial infarction [108].

mPTP Inhibitors

Opening of the IMM localized mitochondrial permeability transition pore (mPTP), a Ca^{2+}-dependent nonselective pore, under certain pathological conditions such as Ca^{2+} overload or oxidative stress leads to the IMM permeability to small molecules (<1,500 Da in molecular weight), loss of proton motive force, uncoupling of OXPHOS, and mitochondrial swelling which often followed by cell death. Opening of mPTP appears to be a pivotal event in AKI [112]. Cyclosporin A (CsA) is a potent inhibitor of mPTP through interaction with cyclophilin D, a structural component and mediator of mPTP which critically regulates Ca^{2+}- and ROS-dependent opening. Low dose of CsA (at submicromolar concentration) suppress mPTP opening and mitochondria swelling, and is currently under clinical trial for its effect on reperfusion injury on acute myocardial infarction (NCT01595958). CsA reduced podocyte damage through mitochondrial protection in an adriamycin-induced podocyte injury animal model [113]. A clinical trial (NCT02397213) is underway to test CsA renal protection in cardiac surgery (potential AKI condition). High dose of CsA is renotoxic and thus limits its clinical use.

Evidence suggests that glycogen synthase kinase (GSK)-3β resides at the nexus of multiple signaling pathways implicated in the regulation of mitochondrial permeability transition (MPT). The selective GSK-3β inhibitor TDZD-8, a thiadiazolidinone derivative, works as a non-ATP competitive inhibitor to prevent MPT and improve cell viability during AKI [112, 114].

Fission Inhibitors

Mitochondrial fusion is required for homogenous distribution of mtDNA, matrix proteins, and lipids across all fused mitochondria. However, mitochondria fission is important in mitochondrial proliferation following mitosis and is also involved in

damaged (fragmented) mitochondrial removal by mitophagy. Mdivi-1 (mitochondrial fission inhibitor-1) was identified as a small molecule that selectively and reversibly inhibits the assembly and the GTPase activity of fission protein DRP1 [115]. Mdivi-1 induces rapid and reversible formation of fused mitochondria in wide varieties of cells and several animal disease models including I/R-injury in the heart, liver, kidney, and brain [116]. Mdivi-1 ameliorates rhabdomyolysis-induced AKI in rat by reducing ROS stress and tubular epithelial cell apoposis with simultaneous increase of ATP production [117] and protection from mPTP opening in acute cardiorenal syndrome [118]. Despite its therapeutic potential, Mdivi-1 exhibits divergent functions which could be cytoprotective or cytotoxic and the renoprotective effect of Mdivi-1 in humans is still lacking [116].

K(ATP) Channel Opener

Levosimendan is a Ca^{2+} sensitizing smooth muscle vasodilator clinically used for the treatment of heart failure. Levosimendan exerts pleiotropic beneficial effects including mitochondria protection in cardiomyocytes during ischemic heart disease [119]. Possible molecular mechanisms of Levosimendan might involve its function as a mitochondrial ATP-sensitive potassium channel to favorably conserve mitochondrial energy in cardiomyocytes. Pre-clinical and clinical data also indicated positive circulatory effects in the brain, lung, and renoprotection under potentially lethal stress conditions. Currently, a clinical trial (NCT01720030) with Levosimendan in an AKI study is ongoing.

Concluding Remarks and Future Perspectives

Mitochondria are the “power house” of the high-energy demanding kidney cells. The mitochondrial networks also affect the cross-talk with other cellular organelles such as the endoplasmic reticulum, nucleus, peroxisomes, and thus impact many cellular functions. Renal mitochondrial dysfunction could derive from defects on mitochondrial biogenesis, bioenergetics, dynamics, turnover and genetic mutations which may contribute to respiratory chain-derived oxidative stress, abnormal ultrastructures, increased sensitivity to apoptosis, and accumulation of damaged mitochondria with unstable mitochondrial DNA. Disturbance of renal mitochondrial homeostasis could lead to damages to microvasculatures, inflammation, fibrosis, and kidney failure. Although kidney mitochondrial dysfunctions have been exquisitely demonstrated to be tightly linked to pathobiology in many preclinical animal studies of CKD, AKI, aging and genetic defected mitochondrial disease, many direct cause-effect mechanisms remain elusive before development of clinical translations.

The mitochondria-targeting therapeutics as effective interventions to preserve mitochondria structures and functions have been demonstrated in several animal models of kidney injuries. Among these, MitoQ and SS-31 have shown promising

potential in clinical trials towards certain kidney injuries and diseases. With better understandings of mitochondrial genome and its physiological functions, advances in in vivo animal studies, and human clinical trials to confirm efficacy and safety of mitochondria-targeting therapeutics, we have high hope to translate these discoveries into therapeutic strategies to ameliorate kidney injuries and diseases in the future.

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Conflict of Interest No competing interest.

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Chapter 28
Mitochondrial Dysfunction in the Diabetic Kidney

Kumar Sharma

The pathobiology of diabetic kidney disease has primarily focused on a variety of metabolic pathways linked to glucose metabolism and inflammatory mediators [1]. However, many of the pathways implicated including the poly-ol pathway, hexosamine formation, glycosylation, protein kinase C have not translated to interventions showing clinical utility [1–3]. Beginning with the work from Brownlee et al., the concept that mitochondrial superoxide production played an upstream role in regulating a variety of pathways has held sway [4, 5]. Unfortunately, clinical approaches to reduce superoxide production have not had promising results [6, 7]. However, recent work has not found evidence of an increase in mitochondrial superoxide production in the diabetic kidney [8, 9]. In contrast, an opposing theory stating that reduced mitochondrial function (or dysfunction) is a major upstream consequence of hyperglycemia and diabetes has been gaining traction [10]. In support of this newer view, an approach to augment mitochondrial function may provide novel therapeutics to delay and arrest diabetic complications.

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Mitochondrial Function in the Kidney

Apart from the heart, the kidney is considered to contain the greatest density of mitochondria per mass of tissue [11]. An explanation for this observation may be that apart from the heart, the kidney is constantly required to be functioning to maintain adequate homeostasis of water, electrolytes, minerals and many other organic and inorganic ions within the organism. Therefore, a loss of mitochondrial function or content could be the basis or consequence of reduced organ function. The role of mitochondria in organ dysfunction may be best understood in the setting of ischemia-reperfusion syndrome in the heart and kidney. The normal heart has to continuously perform a coordinated function linking electrical activity to diastolic and systolic activity of the right and left ventricles. The energy demands amount to about 30 kg of ATP production on a daily basis and much of the ATP generation is considered to be derived from ox-phos activity. However, it is exceedingly difficult to quantitate the amount and function of mitochondria in an organ. One approach is to quantify the supramolecular complexes or respirasomes [12]. Recently, respirasomes were found to be reduced in heart failure [12] setting up a paradigm that reduced mitochondrial energy output could contribute to the progressive failure of heart function in CHF [13].

Similarly, the kidney tubular cells are considered to require ATP for the enormous requirements of the proximal tubular cell to retain water, sodium and electrolytes. The TAL cell also requires a large amount of ATP to pump sodium, potassium and chloride into the interstitium and generate the counter-current gradient. In models of ischemia-reperfusion injury and septic kidney disease there appears to be a marked depletion of mitochondrial function [11, 14], which may underlie the basis of reduced renal function despite normal appearing glomeruli and restoration of renal blood flow. Whether mitochondrial dysfunction plays a role in human diabetic kidney disease was not comprehensively evaluated until quite recently.

Metabolomic Studies Implicating Mitochondrial Dysfunction in Diabetes

Recently, several groups have applied both urine and serum metabolomics to characterize biochemical disturbances in diabetic kidney disease. Blood studies revealed a role for pseudouridine as a biomarker for diabetic kidney disease [15, 16], however the role of pseudouridine remains unclear. Other metabolites that have been identified include indoxylsulfate [17] which may be a marker of renal dysfunction and has been recognized as a uremic metabolite and partly controlled by gut bacteria [16]. Kynurenine has also been found to be elevated in patients and may predispose to kidney disease [18]. Additionally, plasma sphingomyelin has been found to be elevated in patients with diabetes and may pre-date kidney disease [19].

Urine metabolomics studies have been plagued by lack of quality control in collecting timed samples of urine [3]. A well conducted study using both GC-MS and

LC-MS based analysis of urine [20] identified that acyl-carnitines, acyl-glycines and tryptophan metabolites discriminated patients with DKD. In our study of subjects with diabetes and evidence of kidney disease, characterized by reduced eGFR (<75 ml/min/1.73 m^2) using 24 h urine collections, there was a consistent reduction in a panel of 13 metabolites [21]. These 13 metabolites were found to be reduced in subjects from many parts of the USA and Europe with diabetes and CKD. Using a network analysis approach it was found that the 13 metabolites were all linked in a large network and that 12/13 metabolites were controlled by enzymes localized to mitochondria. As there was a significant reduction of the metabolites it was concluded that overall mitochondrial function was reduced in patients with diabetic kidney disease. Support from this conclusion came from studies of kidney biopsies which indicated a reduction in mitochondrial protein (complex IV) and reduction in PGC1α the master regulator of biogenesis.

A reduction in mitochondria in patients with diabetic kidney disease was also demonstrated by a reduction of urine exosomal mtDNA [21] and a reduction in blood levels of mtDNA [22]. These studies demonstrate from a variety of approaches that diabetic kidney disease is marked by reduced mitochondrial content and function. Of major interest is that non-diabetic kidney disease is also marked by reduction of circulating mtDNA [23] and reduced PGC1α in kidney tissues [24].

Mitochondrial Content and Function in Experimental DKD

Mitochondrial function has been considered to be at the heart of understanding diabetic complications as glucose oxidation fundamentally involves the mitochondrial TCA cycle and oxidative phosphorylation. However, the response of mitochondria to excess glucose in many cells and organs still remain unclear. Using a targeted metabolomics approach, Mootha's group studied the metabolic response to glucose [25]. In healthy volunteers the ingestion of an oral glucose challenge resulted in a remarkable increase in glycolytic intermediates and an increase in bile acids in the blood. Surprisingly, there was a marked reduction in many amino acids, including the branched chain amino acids, leucine, isoleucine and valine. There was also a marked reduction in the ketone body, β-OH butyrate. The overall response was considered a switch from catabolism to anabolism. Once mitochondrial function analysis was developed initial studies focused on the skeletal muscle response in patients with diabetes. As skeletal muscle is considered to contain the greatest content of mitochondria, it is logical to assess the skeletal muscle mitochondrial response in the diabetic milieu. A series of studies found that mitochondrial content and function were reduced in the skeletal muscle [26, 27]. However, therapeutic approaches to improve mitochondrial function in patients with diabetes have not been pursued until very recently.

The kidney is considered to have the greatest amount of mitochondria per tissue mass, and is only second to the heart [28]. With hyperglycemia from type 1 diabetes, the kidney response was found to reduce the content of mitochondria per mass

of tissue [8]. Electron chain complex activity was measured with classical methods and found to be generally reduced across all of the complexes [8]. There may be a reduction of pyruvate entry into the mitochondria as the pyruvate dehydrogenase complex was phosphorylated [8]. Despite the reduction in mitochondrial electron chain complex activity and PDH, several studies have found an increase in TCA metabolites in the urine of mouse models of type 1 diabetes. Recent studies have also addressed mitochondrial function and content with type 1 diabetic animal models. With STZ-induced diabetes in the rat, mitochondrial fragmentation was found at 4 weeks of diabetes in association with reduced ATP content and these disturbances preceded albuminuria and elevation in urinary KIM1 levels [9]. Reduced mitochondrial electron chain complex III activity was found in the diabetic rat kidney by a separate group [29]. Mitochondrial DNA content was found to be increased with diabetes in this model. Mitochondrial DNA was found to be elevated in mesangial cells exposed to high glucose in the first 4 days before reduction was noted with longer durations [22].

Several groups have also found marked differences in mitochondria with type 2 diabetes. In the *db/db* mouse model, there is a fragmentation and reduced function of mitochondria in the podocytes [30]. There was an increase in mitochondrial fission which was mediated via Rho-associated coiled coil-containing protein kinase 1 (ROCK1) activation. The role of dynamin related protein (Drp1) was recently implicated as mice with podocyte specific inducible reduction of Drp1 had improved mitochondrial structure and function [31]. Rap1b, a small GTPase, was also found to improve mitochondrial function in renal tubular cells treated with high glucose and Rap1b was recently found to be reduced in tubular cells in biopsy tissue from patients with diabetic nephropathy [32]. Using electron microscopy, there was evidence of reduction of elongated mitochondria in tubular cells of diabetic nephropathy specimens [32].

In patients with diabetic kidney disease our group initially found a reduction in mitochondrial DNA content in urine exosomes [21]. As urine exosomes are largely derived from podocytes and tubular epithelial cells, the reduction in urine exosomal mtDNA was considered to reflect a reduction in renal epithelial mitochondrial content. Support for this hypothesis was found with reduction of mitochondrial complex protein levels in kidney biopsies from patients with diabetic nephropathy. The reduction of mitochondria is likely due in large part due to reduction of PGC1α, the master regulator of mitochondrial biogenesis.

Role of PGC1α in CKD and DKD

The dependence of mitochondrial function for recovery of kidney function may be exemplified by the role of PGC1α. As the master regulator of mitochondrial biogenesis, PGC1α has been recognized to be critically important in a variety of tissues including muscle tissue [33]. With diabetes, it was recognized that PGC1α was

reduced in the skeletal muscle of patients with type 2 diabetes. However, the role of PGC1α in the muscle remains unclear as overexpression of PGC1α leads to insulin resistance (see Chap. 25). A recent study found that upregulation of PGC1α was associated with stimulation of a specific metabolite, 3-hydroxyisobutyrate (3-HIB), a catabolic intermediate of valine [34]. 3-HIB mediated enhanced trans-endothelial fatty acid transport and may play a role in the link with branched chain amino acids and development of insulin resistance and type 2 diabetes [34]. Interestingly, one of the 13 metabolites that were reduced in the urine of patients with DKD is 3-HIB and we were the first to recognize that PGC1α was reduced in kidney disease [21].

The role of PGC1α was found to play a major role initially in acute kidney injury. In studies by Parikh and colleagues, tubular reduction of PGC1α made mice susceptible to ischemic and sepsis related injury [35] whereas over expression of PGC1α in tubular cells conferred protection [14]. The benefit of upregulating PGC1α in ischemic renal injury may partly be due to regulation of NAD. In a model of chronic kidney disease and renal fibrosis with folic acid-induced nephropathy, tubular upregulation of PGC1α was found to be remarkably protective [24].

In animal models of diabetic kidney disease, PGC1α has been found to be reduced and with stimulation of AMPK, PGC1α protein levels were restored along with markers of mitochondrial content and electron transport chain activity [8]. Several groups have now demonstrated a similar relationship in diabetic kidney disease [36, 37]. The regulation of PGC1α and mitochondrial biogenesis in DKD appears to be primarily be regulated by AMPK.

AMPK, Mitochondrial Function and ROS in DKD

PGC1α can be affected by numerous signaling pathways as well as potentially by epigenetic regulation of the PGC1α promoter. In the context of diabetes, a major upstream pathway appears to be AMPK, the master energy sensor. 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase that is a key player in maintaining cellular energy homeostasis. AMPK is highly regulated by the AMP/ATP ratio and is activated under conditions of low ATP levels. In this context activation of AMPK serves to improve energy efficiency by reducing protein synthesis and enhancing glucose entry into cells and stimulation of PGC1α and mitochondrial biogenesis. AMPK is also now recognized to have a variety of functional roles and is also regulated by a variety of kinases such as liver kinase B1 (LKB1), calcium−/calmodulin-dependent kinase kinase 2 (CAMKK2), TGF-β activated kinase 1 (TAK1) as well as a variety of phosphatases, such as protein phosphatase 2A (PP2A), protein phosphatase 2C (PP2C) and Mg^{2+}/Mn^{2+} dependent protein phosphatase 1E (PPM1E) [38]. Several groups have identified that AMPK is reduced in models of type 1 and type 2 diabetic kidney disease as well as in the diet-induced obesity model of kidney disease [8, 39–41].

The regulation of AMPK in diabetic kidney disease remains unclear. Studies by Kasinath's group identified that LKB1 activity was reduced by high glucose due to acetylation of LKB1 and contributed to reduced AMPK activity [42]. Similar results were found by Susztak's group as deletion of LKB1 in tubular cells led to AMPK reduction and apoptosis whereas stimulation of AMPK was able to rescue the phenotype [43]. Recently, our group evaluated the regulation of ATP, ADP and AMP in the diabetic kidney using mass spec imaging [44]. As ATP is easily metabolized in harvested tissue and AMP is of very low levels, accurate measurements are difficult to perform. With the advent of mass spec imaging on frozen tissues, the aspect of tissue processing is limited and the likelihood that ATP levels are similar to the in vivo condition is higher. Using MALDI-TOF approach with targeted mass spec imaging, we found that glomerular levels of ATP/AMP or ATP/ADP were elevated in diabetic kidneys. Thus, it is likely that at the glomerular level, the reduction of AMPK is at least in part due to an elevation in the ATP/AMP ratio.

Stimulation of AMPK in diabetic kidney disease was found to have a remarkable effect to reduce albuminuria, mesangial matrix expansion and the stimulation of matrix molecules and TGF-β. AMPK activation also reduced a key producer of reactive oxygen species in the kidney, NADPH oxidase. The benefit of AMPK activation with AICAR was found to be dependent on AMPK, as mice deficient in AMPKα2 had increased albuminuria but no beneficial effect with AICAR. AMPK activation also markedly reduced mTOR activation in the diabetic kidney and restored PGC1α levels in the diabetic kidney independent of lowering blood glucose or changes in body weights in type 1 models of diabetic kidney disease.

A key insight was the effect of AMPK activation on markers of mitochondrial biogenesis and electron chain activity. The diabetic kidney was found to have a reduction of mitochondrial content and reduced activity of the electron chain complexes. This was completely restored with AMPK activation, likely via stimulation of PGC1α. Therefore, this study provided compelling evidence that stimulation of mitochondrial biogenesis and mitochondrial electron transport chain activity led to beneficial outcomes in diabetic kidney disease. This study also provided new insight into the role of mitochondrial ROS in the context of diabetic kidney disease.

It has been widely believed that overproduction of superoxide from mitochondria played a key role in causing diabetic complications and diabetic kidney disease. However, this theory had not been supported by direct in vivo measurement of mitochondrial superoxide. We developed a DHE-based in vivo assessment of superoxide in mice and found that the normal kidney had a high level of superoxide production [8]. Surprisingly, with type 1 diabetes there was a marked reduction of renal superoxide production in association with reduced mitochondrial electron transport chain activity. Therefore, we concluded that the reduction of mitochondrial electron transport chain activity was reflected by reduced production of superoxide. As the mitochondrial contribution of superoxide in the baseline state is widely believed to be 80% of basal superoxide production, our DHE-based studies support the idea that mitochondrial superoxide is reduced in the diabetic kidney. Additional studies using electron paramagnetic resonance with kidney extracts also found a reduced level of

superoxide from the diabetic kidney. Finally, isolated mitochondria from the diabetic kidney also exhibited reduced levels of ROS production.

To further evaluate the role of mitochondrial superoxide, studies were also performed in the SOD2$^{+/-}$ mouse. The reduced levels of mitochondrial SOD were associated with a high level of DHE-based superoxide production in the kidney. However, despite this high level of superoxide the SOD2$^{+/-}$ mouse has no evidence of kidney disease [8]. Even with diabetes, this mouse does not develop any worsening of diabetic kidney disease. These results indicate that mitochondrial superoxide are neither necessary nor sufficient for development of diabetic kidney disease. The major detrimental source of ROS in the diabetic kidney is likely to be non-mitochondrial and possibly arising from NADPH oxidase. Indeed, stimulation of mitochondrial electron transport chain activity is associated with an enhancement of superoxide production and amelioration of renal inflammation and fibrosis. This concept is captured in the theory of mitochondrial hormesis in the context of diabetic complications. At present, several pharmaceutical companies are pursuing strategies to improve mitochondrial function for many chronic conditions, including diabetic nephropathy.

New Concepts Linking Reduced Mitochondrial Function to Diabetic Kidney Disease

A major impediment to advancing novel treatments for diabetic kidney disease is the lack of insightful biomarkers. Recently an inhibitor of Nox4 was found to benefit diabetic kidney disease by several groups, however the downstream target of Nox4 activity has not been identified. Using urine metabolomics, we found that inhibition of Nox4 was found to dose-dependently reduce levels of urine fumarate in type 1 diabetic micei [45]. Tissue levels of fumarate were also increased in the diabetic kidney and reduced with the Nox4 inhibitor.

Fumarate is a key metabolite within the TCA cycle and arises from conversion of succinate to fumarate by succinate dehydrogenase (SDH). Fumarate itself is converted to malate by fumarate hydratase. As fumarate was increased in the diabetic tissue, we hypothesized that FH activity would be reduced in the diabetic kidney. Indeed there was a marked reduction of FH activity and FH protein in the diabetic kidney. FH appears to be a key target of Nox4, as overexpression of Nox4 led to marked reduction of FH levels and Nox4 inhibition restored FH levels. Importantly, FH was also found to be reduced in the human diabetic kidney.

Thus, there is now supportive data that a key mitochondrial enzyme, FH, is affected in diabetic kidney disease and likely contributes to an upregulation of tissue and urine fumarate levels. A recent study using patients with diabetes found that urine levels of fumarate were increased in patients with diabetes who had progressive renal disease. Further studies are ongoing with larger prospective cohorts to determine the role of urine fumarate as a biomarker of progressive diabetic kidney disease.

Fumarate itself has been identified as a "fibrogenic metabolite". Addition of fumarate to renal cells leads to stimulation of TGF-β and gene expression of extracellular matrix molecules. Fumarate is also a potent stimulator of HIF1α accumulation by inhibiting prolyl-hydroxylase activity. The combination of increased HIF1α and TGF-β may work in concert to suppress mitochondrial ox-phos activity, suppress mitochondrial biogenesis and promote matrix accumulation and fibrosis.

Another major insight has been the recognition that reduced fatty acid oxidation and impaired cholesterol export could contribute to progressive kidney disease, including diabetic kidney disease. With reduced mitochondrial biogenesis, there could be reduced fatty acid oxidation leading to accumulation of fatty acids. In addition, reduced mitochondrial biogenesis may contribute to reduced cholesterol export pathways and lipotoxicity. Stimulation of fatty acid oxidation, possibly via PPARα stimulation is an attractive target for future therapeutics.

Concluding Remarks

In the past 5 years there has been a remarkable resurgence of interest in the role of mitochondrial function in diabetic kidney disease. In contrast to the prevailing theory, accumulating evidence suggests that mitochondrial dysfunction and suppression are consequence of diabetes and not causative for disease progression. By restoring mitochondrial biogenesis, reducing mitochondrial fragmentation and improving mitochondrial activity there is marked improvement in disease parameters. Future studies to specifically target pathways of mitochondrial biogenesis via ROCK1 inhibitors, AMPK activation and PGC1α stimulation would provide novel approaches. Newer targets such as fumarate hydratase activators and pathways to enhance fatty acid oxidation may also provide potent therapeutic approaches. With the parallel improvements in metabolomics, a coordinated companion diagnostic approach would be of added value to provide informative biomarkers to assure that disease pathways are being engaged by the therapeutic and efficacy will hopefully be predicted.

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Chapter 29
Prohibitin Signaling at the Kidney Filtration Barrier

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Podocytes and Their Importance for the Kidney Filtration Barrier

Each kidney comprises about a million small vascular filtration units termed glomeruli where approximately 160 l of urine are filtered and cleared per day. The kidney filter is characterized by its size and charge selectivity. The glomerular ultrafiltrate, i.e. the primary urine is almost completely free of proteins. The ability of the kidney to retain plasma proteins is essential for life and a defective filtration barrier often becomes apparent due to the increased loss of proteins into the urine and its associated side effects, namely edema [1]. More than 5% of all human beings worldwide suffer from chronic kidney diseases (CKD) with great impact on quality of life and rising socio-economical burdens on our societies.

Anatomically, the glomerular filtration barrier consists of three layers: a fenestrated endothelial cell layer with a highly negatively charged glycocalyx, the glomerular basement membrane and as third and outer layer the glomerular epithelial cells, the podocytes (Fig. 29.1).

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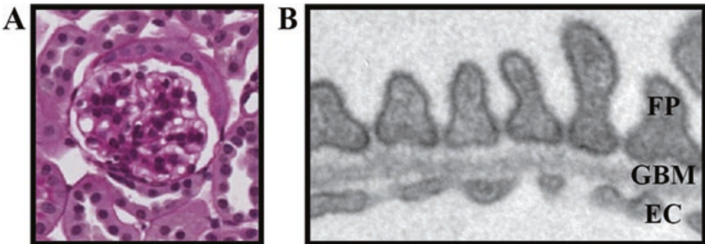

Fig. 29.1 The glomerular filtration barrier, (**a**) PAS staining of a kidney showing a glomerulus with the surrounding tubular system. (**b**) Electron microscopy image depicting the three layers of the glomerular filtration barrier. (*FP* podocyte foot process, *GBM* glomerular basement membrane, *EC* endothelial cell layer)

In the past several years, accumulating evidence suggested that glomerular podocytes are crucial for the function of the kidney filter and critically involved in the development of proteinuria [2, 3]. Podocytes are terminally differentiated and post-mitotic cells resting within the G0 phase of the cell cycle. They elaborate primary and secondary processes with long, regularly spaced foot processes that completely enwrap the glomerular capillaries [4]. The foot processes of neighboring podocytes interdigitate and form a specialized intercellular junction, the slit diaphragm, which contributes to the selective filtration barrier and regulates podocyte function [5–7]. Podocyte function is critical for the filtration barrier. They not only seal the filtration barrier and prevent the loss of albumin into the urine; they also maintain the GBM and secret growth factors that influence endothelial cell function.

Glomerular disorders, with diabetic nephropathy being the leading cause, account for the majority of cases of chronic kidney disease. Although multiple pathways contribute to the glomerular injury, the severity of glomerular diseases of any kind is defined by the degree of podocyte damage. The stereotypical response of podocytes to injury is shape change (effacement), dysfunction, and eventually cell death and/or detachment from the glomerular basement membrane [8]. Given their post-mitotic nature, podocyte damage and eventually loss cannot easily be compensated by proliferation of neighboring cells, subsequently leading to the development of proteinuria and irreversible scarring, i.e. glomerulosclerosis [2, 3, 6, 9, 10]. Importantly, proteinuria and chronic kidney disease are independently associated with an increased risk for end stage renal failure and cardiovascular disease [11–13]. Accordingly, one of the principal goals of kidney research is to understand the pathobiology of proteinuria.

The Slit Diaphragm Controls Signaling Networks for Podocyte Function and Survival

The slit diaphragm is a unique structure in the mammalian organism that consists of a well-defined set of proteins including the IgG-domain proteins NEPH and nephrin, which recruit cytoplasmic adaptor proteins to initiate signal transduction events

that regulate podocyte function [14]. Among these proteins is podocin, a stomatin/prohibitin/flotillin/HflK/C (SPFH) domain-containing protein, which orchestrates the lipid environment at the slit diaphragm and by doing so regulates the TRPC6 ion-channel [15]. Podocin (NPHS2) gene mutations lead to childhood onset of proteinuria and steroid-resistant forms of focal-segmental glomerulosclerosis (FSGS) [16]. Interestingly, the podocin homolog MEC-2 in *C. elegans* regulates the activity of the ion-channel MEC-4/-10, which mediates mechanosensation in touch receptor neurons [17]. There is a strong similarity of the mechanosensory machinery in *C. elegans* and the complex organization of the slit diaphragm, including a tight anchoring to the cytoskeleton. This led to the hypothesis that podocin might be involved in glomerular mechanosensation. Although this is still speculative, podocin could play an important role as part of a protein complex that regulates glomerular mechanotransduction by linking the slit diaphragm to the cytoskeleton and regulating membrane bilayer stiffness [15].

The Mitochondrial Contribution to Renal Disease

Mitochondria are the primary cellular source of ATP and essential for all eukaryotic cells [18]. They are highly dynamic structures that constantly divide and fuse to build an intracellular network that assures the distribution within the cells and is required to execute their pleiotropic activities beyond aerobic ATP production. These include intrinsic apoptosis pathways, participation in RNA and DNA production, biosynthesis of heme, certain amino acids, lipids and others [19, 20]. In addition, the inner mitochondrial membrane potential participates in the uptake of cytosolic Ca^{2+} and potentially serves as buffer for locally increased intracellular Ca^{2+} levels [21].

Mitochondrial diseases might occur as a consequence of mutations in either the nuclear or the mitochondrial genome (for review see O'Toole et al. [22] and Che et al. [23]). In general, there is a poor relationship between the mitochondrial gene defect and the clinical presentation challenging clinicians to establish a definitive diagnosis and to understand the pathobiology underlying the clinical symptoms. Presentations during childhood and adolescence are often associated with tubular dysfunction.

Podocytes have the most abundant mitochondrial network among glomerular cells [24]. Several lines of evidence suggest a critical role for a mitochondrial contribution in glomerulopathies. Mutations in enzymes responsible for coenzyme Q generation, i.e. COQ2 [25] and PDSS2 [26], and in the mitochondrial $tRNA^{Leu}$(UUR) [27, 28] are associated with FSGS (Table 29.1).

In addition, the susceptibility gene for renal disease in kd/kd mice, a model for collapsing glomerulopathy, was mapped to encode a prenyltransferase-like mitochondrial protein (PMLP) [30]. In the puromycin aminonucleoside nephrosis (PAN) model of FSGS there is not only a strong correlation between mitochondrial reactive oxygen species (ROS) production and glomerular disease but also reduced Cox

Table 29.1 Glomerular manifestations of genetic mitochondrial disease

Gene	Locus	Inheritance	Protein	Function	Phenotype or syndrome
COQ2	4q21–q22	AR	Polyprenyl-transferase	CoQ10 biosynthesis, which transfers electrons from the mitochondrial respiratory chain	COQ10 deficiency, early-onset SRNS, with or without encephalo-myopathy
PDSS2	6q21	AR	Decaprenyl diphosphate synthase-2	CoQ10 biosynthesis, which transfers electrons from the mitochondrial respiratory chain	COQ10 deficiency, Leigh syndrome and SRNS
MTTL1	mtDNA		tRNA-LEU	Mitochondrial tRNA for leucine	MELAS syndrome. Mitochondrial diabetes, deafness and FSGS, with or without nephrotic syndrome

According to Machuca et al. [29]

I levels, reduced copy numbers of total mtDNA and decreased TFAM/NRF-1 levels [31]. Diabetic rats following STZ administration show accumulation of oxidative damage of mtDNA [32]. Holthofer et al. reported that the expression of the mitochondrially encoded respiratory chain complex is decreased in glomeruli from patients suffering from congenital nephrotic syndrome of the Finnish type [33]. Moreover, activated protein C controls expression of the partially mitochondrial localized redox-regulating protein p66Shc, thus linking the extracellular protease APC to mitochondrial function in the diabetic milieu [34]. Additionally, work performed in a transformed podocyte cell line and primary cells suggested that podocytes primarily depend on mitochondria for energy homeostasis using a range of substrates including glucose, pyruvate, palmitate and lactate [35]. Inhibition of glycolysis or oxidative phosphorylation each reduced ATP levels and may explain the susceptibility of podocytes to oxidative stress as observed in patients with diabetic nephropathy. Additional work by Ozawa et al. [36] further evaluated the roles of mitochondrial OXPHOS and glycolysis in podocytes. While blocking mitochondrial function had only minor effects on cell shape and migratory ability, inhibiting glycolysis significantly reduced the formation of lamellipodia, decreased the cell migratory ability and induced the onset of apoptosis. Consistently, the local ATP production in lamellipodia was predominantly regulated by glycolysis. These data suggest that mitochondria and glycolysis play parallel but distinct roles in podocytes. Last, podocytes employ a number of cellular pathways to inhibit intrinsic apoptotic pathways. Among these is Cdk5 that governs podocyte survival by regulating levels of the pro-survival protein Bcl-2 through the ERK1/2 pathway [37–40].

The Effect of Prohibitin Depletion on Mitochondrial Function in Podocytes

Maintaining a physiological balance between mitochondrial fusion and fission events is essential to prevent neurodegeneration due to damaged post-mitotic neurons. Mitochondrial dynamics are needed as a quality control, as they help to alleviate effects of damaged mitochondria. Fusion leads to redistribution of content from damaged to healthy mitochondria, whereas fission ensures elimination of dysfunctional mitochondria by mitophagy. These processes are necessary not only to maintain a healthy mitochondrial network, but also to protect from mitochondria-induced apoptosis [41]. In this regard, disturbed processing of the central regulator of fusion, OPA1, leads to severe mitochondrial fragmentation and a higher susceptibility to cell death [42–45]. Interestingly, loss of the mitochondrial membrane proteins prohibitins induces selective cleavage of OPA1 to short over long isoforms leading to disorganized and swollen mitochondrial cristae structures [45].

Two prohibitins – Prohibitin-1 and Prohibitin-2 (PHB1 and PHB2) – are involved in this process. Loss of either one leads to loss of the other, as they stabilize each other [46, 47]. This relationship has also been seen in glomerular podocytes, where loss of PHB2 destabilizes PHB1 [48]. In line with results obtained in neurons, a podocyte-specific PHB2-knockout mouse model revealed fragmented mitochondrial cristae structures within podocytes (Fig. 29.2). These mice presented with fast progressing glomerulosclerosis, severe albuminuria and eventually premature death within 4–5 weeks postnatally. Interestingly, there are reports on disturbed mitochondrial cristae structures in podocytes in some cases of FSGS in patients as well as in disease models [31, 49–53].

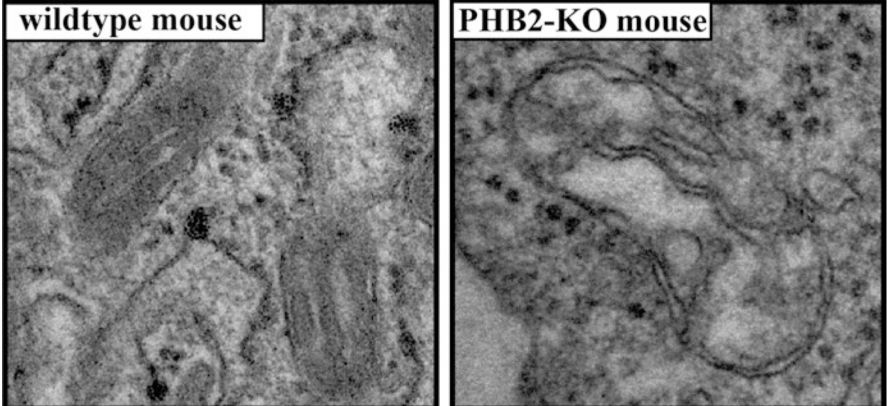

Fig. 29.2 Mitochondrial appearance in podocytes. Podocytes of wildtype mice have healthy mitochondria with normal cristae structures (*left*), whereas podocytes of PHB2 knockout mice contain mitochondria with disorganized and swollen cristae structures (*right*)

Fragmented cristae structures could lead to impaired respiratory function, as the respiratory chain proteins are localized within cristae structures. However, studies investigating the effect of prohibitin loss on ATP levels and the formation of reactive oxygen species (ROS) over the last years revealed conflicting results, with some in vitro studies showing increased ROS production [54–56] and other in vitro and in vivo studies finding no effect on respiratory chain activity [45, 57, 58]. Investigation of the respiratory chain function as well as oxygen consumption specifically in PHB2-knockdown podocytes revealed no impairments in these cells [48]. However, these results did not address the question whether the disruption of the mitochondrial network plays a role in the development of glomerulosclerosis or if it is a secondary effect of podocyte damage.

Prohibitins and Their Relationship to the Insulin Signaling Pathway

In a landmark paper in 2009, Artal-Sanz and Tavernarakis described a so far unknown link between prohibitins and insulin signaling. They were able to show that already long-lived *daf-2* mutant worms – *daf-2* being the *C elegans* homolog of the mammalian insulin receptor - had an even longer lifespan upon loss of *phb-2*. Additionally, *phb-2*-deficiency promoted longevity under dietary restriction in *C. elegans* [59]. In contrast to the nematode, in mammals two receptors mediate the action of growth factors on cell metabolism: the insulin receptor (IR) and the Insulin-like Growth Factor 1 Receptor (IGF1R). Both receptors have been shown to be expressed in podocytes [60, 61].

Intriguingly, by using an IR/IGF1R/PHB2-triple podocyte-specific knockout mouse line, the loss of insulin/IGF-1 signaling in podocytes alleviated not only renal disease and improved kidney function as compared to single PHB2-podocyte knockout mice, but also delayed the onset of kidney failure and thereby prolonged survival of the PHB2-podocyte knockout mice for several weeks [48]. Further analysis of the podocyte-specific knockout mice revealed increased mTORC1 activation in glomeruli of these mice, which is a downstream target of the insulin signaling pathway. This is of particular interest as hyperactivation of mTORC1 can cause glomerular diseases in mice and humans [62]. However, several studies since then have identified a phenotype more closely resembling diabetic nephropathy with thickening of the glomerular basement membrane and mesangial expansion than showing features of FSGS [62–65]. Furthermore, mTORC1 activation in podocytes induced by loss of PHB2 revealed some striking differences between this model and other mouse models that more directly induced mTORC1 hyperactivation. For example, PHB2-knockout mice present with an aggravated course of disease and shortened life span as compared to mice with a podocyte-specific knockout of the negative regulator of mTOR signaling, TSC1 [63]. While treatment with the mTORC1-inhibitor rapamycin alleviated albuminuria in TSC1 knockout mice, it had no effect on levels of

albuminuria in PHB2 knockout mice even though it prolonged survival in this model [48, 63]. Of note, hyperactivity of mTORC1, but also suppression of mTORC1 may cause podocyte damage and ultimately glomerular diseases [62, 66, 67]. These reports emphasize the importance of a balanced mTORC1 activation in podocyte biology, which is disturbed upon loss of PHB2 [48].

Prohibitin-2 at the Slit Diaphragm

While prohibitin complexes are mainly considered as an integral part of a functional mitochondrial network, several studies suggest that prohibitins might also localize to other parts of a cell. For example, prohibitins have been shown to be important for the regulation of apoptosis [45, 68–70], to localize to the nucleus where they modulate transcriptional activity [68, 70–73] or modulate signaling at the plasma membrane [74, 75]. With regard to localization in podocytes PHB2 can be found not only in mitochondria but also at the slit diaphragm of podocytes [76] (Fig. 29.3).

Initially, podocyte-specific PHB2 knockout mice develop normal slit diaphragm structures between their foot processes, which are then lost again after a short period of time [76]. The localization of PHB2 to the slit diaphragm could contribute to the onset of albuminuria observed in these mice, as loss of the slit diaphragm structure severely impairs the filtration barrier [77–80]. Intriguingly, PHB2 knockout mice with an additional knockout of the insulin and IGF1 receptor or treated with the mTOR inhibitor rapamycin remained albuminuric despite a delayed onset of kidney failure and prolonged survival [48], further arguing into the direction of distinct roles of prohibitins in mitochondria and at the slit diaphragm in podocytes.

Proteins at the slit diaphragm have also been implicated in signaling processes in healthy podocytes [14]. Given the strong similarity of the mechanosensory machin-

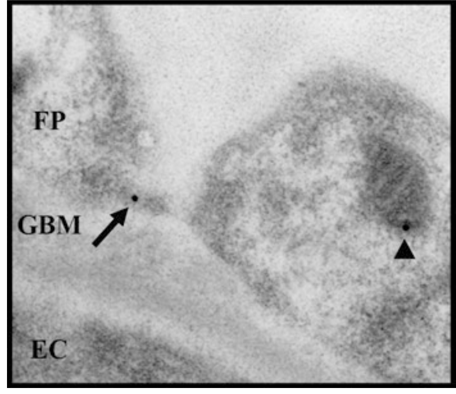

Fig. 29.3 Immunogold labeling for PHB2 in podocytes. Immunogold labeling of kidney sections showed PHB2 not only in mitochondria (*arrowhead*), but also close to the slit diaphragm in podocytes (*arrow*)

ery of the nematode *C. elegans* and the complex organization of the slit diaphragm in mammals, *C. elegans* has gained much attention as model organism to study signaling events at the slit diaphragm [15, 81, 82]. Interestingly, the mechanosensory protein complex in *C. elegans* contains MEC-2 [83, 84] – the homolog of the well-known slit diaphragm protein podocin – but also the worm homolog of PHB2 [76]. The mechanosensory complex converts mechanical forces into electrical signals, measurable by a touch response. This response is reduced upon depletion of either PHB-1 or PHB-2 specifically from the touch neurons, where PHB-2 was shown to co-localize with MEC-2 [76]. Even though there is only little experimental evidence for localization of PHB1 to the slit diaphragm and/or mechanosensory complex, it seems very likely that both, PHB1 and PHB2, localize to these protein complexes as they are usually stabilizing each other [76].

Prohibitins: A Dual Function Within Podocytes

Work on the role of prohibitins in the kidney revealed an unexpected impact of these proteins on podocyte health. On the one hand, PHB2 and possibly PHB1 can be found at the slit diaphragm, where their loss may contribute to the development of albuminuria [76] (Fig. 29.4). However, it remains to be elucidated what role prohibitins play with regard to signaling at this specialized cell-cell contact. On the other hand, loss of prohibitins leads to activation of the insulin/IGF1 signaling

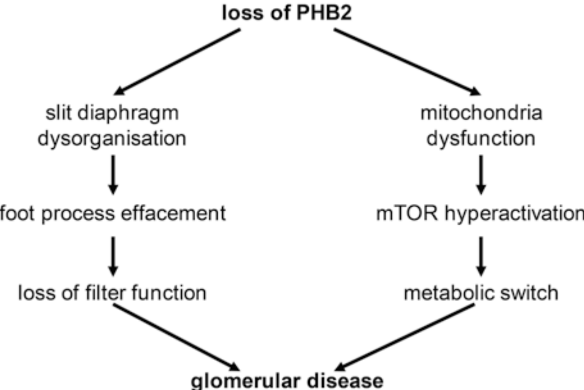

Fig. 29.4 Suggested model for why loss of PHB2 leads to glomerular disease [76]. Loss of PHB2 ultimately leads to glomerular disease potentially via two distinct pathways. On the one hand, PHB2 depletion results in foot process effacement because of its localization to the slit diaphragm, which results in loss of filter function and the onset of albuminuria. On the other hand, PHB2 deficiency leads to mitochondrial dysfunction that enhances mTOR activation, resulting in a detrimental metabolic switch in podocytes leading to podocyte loss. These two pathways together underlie the severe glomerular disease seen in PHB2 knockout mice. (*mTOR* mammalian target of rapamycin, *PHB* prohibitin)

pathway, resulting in downstream mTORC1 activation. Interestingly, treatment with the mTORC1 inhibitor rapamycin can improve kidney function and prolong survival [48], which renders the FDA-approved drug a potential treatment strategy for patients presenting with glomerular diseases involving mitochondrial structural changes.

In 2009, a direct link between insulin signaling and prohibitins has been proposed for the first time [75, 85]. Recently, metabolomic profiling in *C. elegans* has revealed changes in the fatty acid composition after PHB depletion. These modulations lead to a shortened lifespan in wild-type worms, but an increased lifespan of already long-lived *daf*2 mutants [86]. Intriguingly, recent work in cardiomyocytes unraveled a metabolic shift from lipid utilization to carbohydrate utilization in a mitochondrial-fusion deficient model system in vitro and in vivo [87]. These findings taken together might be transferable to podocytes, where loss of mitochondrially localized prohibitins leads to podocyte loss, ultimately leading to kidney failure [48]. However, it remains to be studied if the metabolomic changes as reported in other model systems have a similar impact on the phenotype observed in podocyte-specific PHB2 knockout mice.

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Chapter 30
Mitochondrial Heteroplasmy

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Intriguingly, a small subset of heritable polymorphisms in the haploid mtDNA genome is associated with the phenotypic characteristics of severe genetic disorders, originating from markedly compromised mitochondrial function [1–4]. This observation is supported by a relatively recent body of literature, demonstrating interactive effects of heritable mtDNA polymorphisms on altered expression of select nuclear genes that are associated with several human disease states [5–7]. Arguably, a therapeutically more attractive approach than attempting to target the more elusive heritable genetic polymorphisms that, in concert with accumulated somatic mutations and epigenetic factors, influence the etiology and progression of cardiovascular [8] metastatic [9, 10], autoimmune [11, 12], and neurodegenerative [10, 13, 14] disorders.

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Biological Significance of Mitochondrial Heteroplasmy

To successfully translate human genetic aberrations to effective therapeutic approaches/therapies, we must seek critical foci to probe the multiple biological roles of mitochondrial heteroplasmy. For example, the dynamically determined co-expression of wildtype (WT) inherited polymorphisms and somatic mutations in varying ratios within individual mtDNA genomes distributed throughout the intra-organelle compartments of individual cells are noteworthy (Fig. 30.1). Human mtDNA is a 16.6-kilo base circular genome, corresponding to subunits ND4 and ND4L including ND1-6, of respiratory complex I, catalytic units CO1-3 of respiratory complex IV, subunits ATP8 and ATP6 of F1F0 ATPase, and CYTB of respiratory complex III. The remaining genes encode 22 tRNAs and 12 and 16S rRNAs [15]. Since the number of mitochondrial genomes, normalized against each diploid nuclear genome, varies according to cell type and total mtDNA copy number, estimates in the biomedical literature range from 100 to 10,000 [2, 15, 16]. Considering the complex sequence heterogeneity within mixed intra-mitochondrial populations of several thousand individual heteroplasmic mtDNA genomes within individual cells; it may be technically too challenging to sort empirically sort presumed biological activities of distinct patterns of mtDNA heteroplasmy based on molecular biological, biochemical, physiological, and bioinformatic criteria [17, 18].

The existence of highly regulated and the habitual presence of heteroplasmic mtDNA genomes, in mitochondrial compartments of separate human cell types which complements normative mitochondrial function, can be postulated based on findings of distinct and repeatable patterns of mtDNA heteroplasmy, varying across different cell types from the same individual [17, 19]. This observation is in concert with an earlier study monitoring intra-mitochondrial organization of heterologous heteroplasmic mtDNA genomes into DNA-protein complexes termed nucleoids [20]. Observing heterologous mtDNAs stably maintained in distinct nucleoid populations while trans-complementation of heteroplasmic nucleoids appeared to be achieved by the diffusion of mtDNA-derived transcripts within the mitochondrial matrix, the authors speculated on the presence of a restorative mechanism of trans-complementation to operationally increase homoplasmic WT mtDNA and mitochondrial bioenergetics. The patterns of tissue- and cell-specific heteroplasmic mtDNA appear mechanistically maintained via intra-mitochondrial trans-complementation of heteroplasmic nucleoids and mtDNA-derived transcripts [20] as does the intercellular exchange of mtDNA [17]. Further, polycistronic nature heteroplasmic mtDNA-encoded transcripts introduce an additional level of intricacy which can be employed to evaluate theoretical facilitative roles of preserved patterns of mtDNA heteroplasmy on homeostatic metabolic processes [15].

A Drosophila genetic model, utilizing a temperature-sensitive lethal mtDNA mutation in functional linkage to the cytochrome c oxidase subunit I (CO1) locus [21], validates of the potential existential role of cell-specific patterns of mtDNA heteroplasmy on normative mitochondrial functions. Importantly, expressing an alternative CO1 oxidase, which specifically rendered restorative mtDNA hetero-

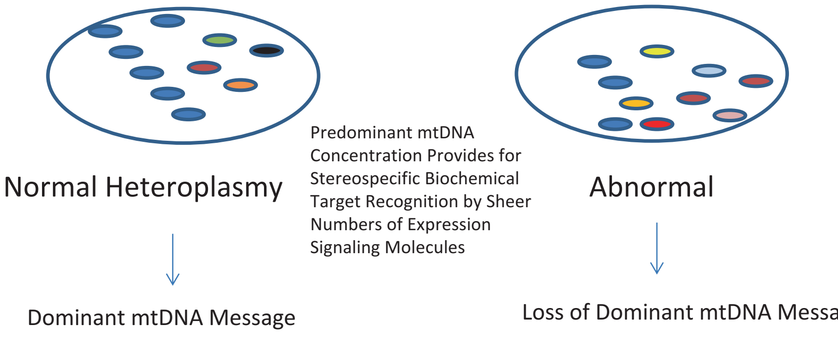

Fig. 30.1 Heteroplasmy: Mitochondrial diversity. Alterations of mitochondrial DNA (mtDNA) are largely associated with post-mitotic neural- and muscular-cell disorders. The pathology may emerge from primary mtDNA mutations and alterations in nuclear-encoded mtDNA maintenance proteins. Our understanding of these phenomena is lacking. However, answers may be present from our understanding of mitochondrial origin from large populations of bacteria. Indeed, vesicular and tunneling nanotubular phenomena may represent a process whereby the vast mtDNA variations are summated, shared, and the dominant ratio emerges. Alterations occur when various modified genomes have micro-environmental survival mechanisms (affinities) or the immediate microbiome (Gut Phenomena) selects a special microbe genome to emerge (as a comparison), bringing together intrinsic selective alteration and those imposed by environmental idiosyncrasies. Under these circumstances, an abnormally expressed genome may become the 'norm', until the conditions or physiological environment changes. Thus, this dynamic mixing system provides functional mitochondria that are dynamically suited for the prevailing circumstances by providing genetic diversity when called upon. In this regard, pathological ratio expression emerges when the chemical signals are misinterpreted or a totally novel "stress" appears. However, under normal circumstances, abundant and normal ratios emerge to meet an appropriate and specific demand. The regulation comes from the population numbers of the functioning mitochondria, much like a summation process. Because a mitochondrion environment is so hazardous, e.g., ROS, they must rely on numbers to survive by buffering the potential hazards

plasmy associated with fully viable and tissue-specific phenotypes, maintained the viability of homoplasmic flies despite restrictive temperatures. On the contrary, restoration of mtDNA homoplasmy in the eye of heteroplasmic flies, using a genetic replacement model to induce tissue-specific mutant CO1 homoplasmy, resulted in severe neurodegeneration at restrictive temperatures.

Findings in this Drosophila model further show a decrease in the frequency of the mutant allele in heteroplasmic flies in the germ line and over multiple generations [22]. We surmise, in reviewing the studies noted above, that selection against potentially disadvantageous mtDNA heteroplasmic mutations in oogenesis may be in contrast to developmentally determined, cell-specific patterns of mtDNA

heteroplasmy generated during various stages of tissue differentiation (non-random gene flow rather than drift). Concurrently, cellular processes that regulate physiologically compatible patterns of heteroplasmic mtDNA may undergo state-dependent dysregulation, resulting in the altered metabolically-compromised phenotype characteristic of congestive heart failure [23] and other pathophysiologically altered states [24]. Of further relevance is a recent study linking allele-specific expression of nuclear DNA and mtDNA heteroplasmy with functional impairment in bioenergetics and a favorable selection for impaired mitochondrial function [25].

The mtDNA genome represents a self-contained genetic system, preserved through evolution, encoding vital catalytic and regulatory subunits of the respiratory complexes noted earlier [26–28]. Ultimately, the maintenance of redox potential by electron transport through respiratory complexes represents a major mechanism responsible for reciprocal regulation of energy production. Thus, the transformation of cell-specific mitochondria into powerful bio-engines shows dependency on the accuracy of ongoing gene expression within insular metabolic or physiological demands. A recent, pivotal review depicts a binary regulatory system responsible for selective expression of genes contained within mitochondrial and chloroplast genomes [27–29]. Functional mitochondria are crucial in maintaining, differentiating and reprogramming induced pluripotent stem cells (iPSCs) [30]. The critical regulatory roles of cell-specific patterns of mtDNA heteroplasmy are fundamental in maintaining essential metabolic capacity during habitual aging processes [17].

For instance, in Barth syndrome, an inherited dilated cardiomyopathy stemming from a mutation in TAZ, the gene encoding the mitochondrial protein tafazzin, decreased basal mitochondrial oxygen consumption associated with structural changes in mitochondrial respiratory chain super complexes, results in increased output of putatively harmful ROS. Consequently, human iPSCs from Barth syndrome patients demonstrate structural and functional abnormalities, including increased ROS, which intriguingly reverse by the reintroduction of WT TAZ gene, indicating a therapeutic potential of human stem cell-derived cardiomyocytes [8, 31–33].

Furthermore, hypoxia-inducible factor one alpha (HIF1α) signaling pathways, may strongly regulate the transition from oxidative to glycolytic metabolic processes in cardiomyocytes providing a novel insight into survival in a hypoxic microenvironment [34, 35]. A shift from somatic mitochondrial oxidative metabolism to glycolytic metabolism, highly reminiscent of cancer cells, is requisite for successful reprogramming of iPSCs [35]. Consequently, somatic mitochondria and affiliated oxidative bioenergetics are profoundly altered with the induction of an iPSC-like phenotype. The authors conclude that early induction of HIF1α target genes may be required for iPSC derivation via the activation of a glycolytic program highly reminiscent of undifferentiated cancer cells. This process in all likelihood may occur in cardiomyocytes given the vulnerabilities of cells to hypoxia.

Examples of Heteroplasmy in Cardiomyopathy

Cardiomyopathies refer to a heterogeneous group of diseases characterized by the impaired function of heart muscle. Although the causes remain largely unclear, recent studies have recognized a significant role for genetic factors [36]. Although the true prevalence of mtDNA-related cardiomyopathy is unknown, at least 1 in 10,000–15,000 of the general population will be affected based on the prevalence of mtDNA disease and also the frequency of cardiac involvement.

Large-Scale Rearrangements of mtDNA

Large-scale rearrangements of mtDNA including single deletions and duplications are always heteroplasmic. A variety of clinical presentations exists, such as the sporadic chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), and other rare syndromes [36]. KSS is commonly caused by single deletions in mtDNA and probably the most characteristic clinical syndrome with an onset before 20 years of age, pigmentary retinopathy, and external ophthalmoplegia. Cardiomyopathy is rarely observed in this syndrome and if present, has a late clinical onset [36].

Point Mutations (PM) of mtDNA

tRNA$^{Leu(UUR)}$ Gene

A3243G Mutation

This point mutation in the mitochondrial DNA is the most prevalent mtDNA mutation and results in the nucleotide substitution of Guanine for Adenine at nucleotide 3243 in the leucine tRNA gene. The A3243G mutation is associated with 80% of patients with MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) [37], even though this syndrome is also associated with at least 12 other mtDNA gene mutations reported in the literature [37]. MELAS syndrome is clinically characterized by abnormal development, lactic acidosis, intermittent vomiting, seizures, stroke-like episodes and hemiparesis or cortical blindness [36]. About 20–38% patients with MELAS may show cardiac involvement [36, 37]. In a study of 17 patients with mitochondrial disease and cardiac involvement, Anan et al. [38] found that five patients with MELAS all had the A3243G point mutation, and two of them showed left ventricular hypertrophy on echocardiogram. Likewise, Majamaa-Voltti et al. [39] found that the risk of left ventricular hypertrophy was significantly increased in patients with the A3243G

mutation. Recently, Lindroos et al. [40] reported that myocardial glucose uptake is decreased in patients with the A3243G mutation, adding to the impaired cardiac energetics and likely contributing to the progression of the mitochondrial cardiomyopathy. The A3243G mutation may be a part of a multisystem involvement but rarely present as the sole clinical manifestation of cardiomyopathy [41]. Other phenotypes as CPEO, deafness, and maternally inherited diabetes mellitus, have also been described [41].

A3260G Mutation

Zeviani et al. first described a large pedigree with a clinical presentations including proximal muscle weakness, increased blood lactate, exercise intolerance, both during exercise and at rest, and reduced cardiac ejection fraction. Hypertrophic cardiomyopathy was recognized in three of the probands by echocardiography. Affected individuals were heteroplasmic for the A3260G mutation. This syndrome was categorized under the category "Maternally Inherited Myopathy and Cardiomyopathies" by Zeviani et al. [42].

C3303T Mutation

This mutation in the tRNA$^{Leu(UUR)}$ gene was first reported by Silvestri et al. [43]. Then, Bruno et al. [44] described the same mutation in eight patients from different families and tissues from the individuals appeared to be homoplasmic or near homoplasmic for the C3303T mutation in all families. Finally, Ueki et al. [45] studied 44 patients with lactic acidosis. Among them, one patient had the clinical manifestation of isolated cardiomyopathy and then tested positive for the C3303T mutation. It is interesting to note that the A3302G mutation, adjacent to the C3303T mutation, has also been described. In a study discussing two case reports with the A3302G mutation [46], one of them died of cardiorespiratory failure. Since the two genes are adjacent, we raise the question of whether the A3302G mutation could also lead to cardiomyopathy, with a higher level of mutant mtDNA resulting in a more severe manifestation of the heart [46].

tRNA$^{Leu(CUN)}$ Gene

T12297C Mutation

The mutation was initially reported by Tessa et al. in 1999 [47]. They studied ten children of eight unrelated families with dilated cardiomyopathy. The T12297C mutation was identified in two siblings of unrelated parents with dilated cardiomyopathy and endocardial fibroelastosis, and the novel mutation was abundant and

heteroplasmic in both patients. The same mutation was also reported in a 36-year-old man who presented with congestive heart failure and dilated ventricles by echocardiography. The ultrastructural studies of tissues showed proliferation of mitochondria, with a wide range of changes in shape and size. The mutation was heteroplasmic, and 88% mutant was in the heart DNA [48]. Recently, Wang et al. [49] reported a family with multiple members who had the T12297C mutation and T14487C mutation, which is found in Leigh syndrome. All subjects tested were homoplasmic for the T12297C mutation, but no individuals had dilated cardiomyopathy.

tRNA^{-Ile} Gene

A4317 Mutation

The mutation was first reported in a 1-year-old boy with metabolic acidosis and general weakness. The boy developed hypertrophic cardiomyopathy and then died of heart failure on the seventh day of his presentation due to fatal infantile cardiomyopathy [50]. Tanaka et al. [50] and subsequently Tomari et al. suggested that the A4317G mutation caused structural rearrangement of the T-stem of the tRNA^{Ile}, leading to the instability in the mitochondria, and hence may result in mitochondrial dysfunction [51].

A4269G Mutation

The first patient with this mutation was an 18-year-old boy with short stature, who finally died as a result of heart failure. The boy initially presented at the age of 4, epilepsy, multiorgan dysfunction, and later developed dilated cardiomyopathy. In the study, the A4269G mutation was found in the index case with no mutation found in the controls [52]. Then, it was found that the A4269G point mutation inhibits protein synthesis completely in mitochondria, and therefore results in a reduction of the respiratory activity [53, 54]. Hino et al. studied the molecular mechanism by which the A4269Gmutation affects the translational activity of the mutant tRNA^{Ile}. Interestingly, they found that the point mutation destabilizes the T-stem of the tRNA and also decreases the affinity of binding of the elongation factor EF-Tu, and finally can't protect the mutant tRNA^{Ile} from degradation [53].

A4295G Mutation

This mutation was initially reported in a 7-month-old girl. The child had a sudden onset of cyanotic spells and finally died of complications of hypertrophic cardiomyopathy [55]. Ultrastructural findings showed massive mitochondrial proliferation in

heart and liver but not in skeletal muscle fibers. The brother of the patient was also found to have left ventricular hypertrophic cardiomyopathy and underwent heart transplantation because of a sudden decline in cardiac function.

A4300G Mutation

The mutation was detected in a large Italian family with maternally inherited cardiomyopathy. The proband and ten other family members all developed isolated hypertrophic cardiomyopathy. Importantly, the heart appeared to be the only organ affected, unlike other point mutations in the tRNA (Ile) gene [36].

m.4322dupC Mutation

Mahjoub et al. [56] identified a novel heteroplasmic mtDNA (m.4322dupC) mutation in the $tRNA^{Ile}$ gene associated with isolated dilated cardiomyopathy as a maternal trait. This new mutation was heteroplasmic in muscle and blood, was not present in healthy controls, and was accompanied by slightly reduced the level of activity of respiratory chain enzyme.

$tRNA^{Lys}$ Gene

A8344G Mutation

The A8344G mutation in the $tRNA^{Lys}$ gene has typically been associated with MERRF syndrome (Myoclonic Epilepsy with Ragged Red Fibers). The multisystem disorder is characterized by myoclonus, generalized epilepsy, weakness, ataxia, and ragged red fibers. Patients diagnosed with MERRF usually present in childhood or early adulthood. Recently, a review of [53, 57] patients showed that 22% of them developed cardiomyopathy. The A8344G mutation is also found in other clinical phenotypes, such as Leigh's syndrome, myoclonus or myopathy with truncal lipomas, and proximal myopathy [58].

Vallance et al. [59] reported a case of infantile histiocytoid cardiomyopathy caused by the A8344G (MERRF) mutation, which is another rare cardiomyopathy characterized by pathognomonic histiocyte-like cells within the subendocardium.

G8363A Mutation

This mutation was first described in two families with maternally inherited hypertrophic cardiomyopathy [60]. The proband in the first family was a boy from Spain who presented with heart failure and cognitive regression at the age of 8 and died of

cardiorespiratory arrest at 17 years old. The patient's sibling presented with mild developmental delay, weakness of pelvic girdle, and hypertrophic cardiomyopathy at the age of 4 years. In the second family, the proband was a 44-year-old American, who developed progressive hearing loss at the age of 35 years. Later, there were a variety of clinical features, which included gait difficulties, slurred speech, shortness of breath, and chest pain. Echocardiography suggested cardiomyopathy. The proband's daughter had congenital hypertrophic cardiomyopathy and severe mental retardation [60]. A comprehensive systematic review regarding the clinical and molecular data of G8363A mutation in 6 studies showed that 4 of 41 patients with a varying level of the mutational load in the blood developed cardiomyopathy [61].

tRNA^{Val} Gene

G1644A Mutation

The novel pathological mtDNA mutation in the tRNA^{Val} was found in a family. The proband was diagnosed with MELAS and hypertrophic cardiomyopathy. Meanwhile, her mother was diagnosed with mild hypertrophy and both mother and grandmother manifested sensorineural deafness [62].

tRNA^{glycine} Gene

T9997C Mutation

Merante et al. [63] reported a unique heteroplasmic T-to-C transition at nucleotide 9997 in the mitochondrial tRNA^{glycine} gene in a multiplex family who showed non-obstructive cardiomyopathy and the severity of the symptoms correlated with the degree of mtDNA heteroplasmy.

12S rRNA Gene

A1555G Mutation

This mutation in the 12S rRNA gene was reported in a family with maternally inherited cardiomyopathy and was associated with restrictive cardiomyopathy, unlike other mtDNA mutations associated with cardiomyopathy. The mutation was found to be heteroplasmic in several tissues including the heart muscle [64].

Polypeptide-encoding mtDNA

Leigh Syndrome

Leigh syndrome, a necrotizing encephalomyelopathy, is characterized by progressive neurodegenerative disorder, affecting infants and adults. One of the main diagnostic criteria is the presence of brainstem degeneration with or without basal ganglia disease [65]. It constitutes one of the progressive neurodegenerative diseases associated with an abnormal generation of mitochondrial energy. The T8993G and T8993C mtDNA mutations account for 10–20% of Leigh syndrome mutations and other mtDNA mutations account for another 10–20% of Leigh syndrome mutations [66]. Extraneurologic symptoms might include cardiac involvement and particularly hypertrophic cardiomyopathy [67].

T8528C Mutation

Four unrelated infants with infantile hypertrophic cardiomyopathy and multisystem disease were reported by Ware et al. [68]. Sequencing of the entire mitochondrial genome revealed the novel T8528C mitochondrial mutation. This mutation leads to alteration of both ATPases 6 and 8 and results in cardiomyopathy when nearly homoplasmic. Position 8528 is located at the overlapping region encoding the ATPase 6 and ATPase 8 subunits, and the nucleotide alteration leads to the change of the initiation of methionine to threonine in the ATPase 6 subunit, abrogating the start of translation. Recently, Imai et al. [69] report a case of a Japanese patient with the T8528C mutation who died of a rapidly progressive cardiomyopathy within 5.5 months of age, demonstrating that the mutation is highly heteroplasmic in the heart (90%), and protein synthesis of both ATPase 6 and 8 was impaired in the heart. They further confirm the T8528C mutation in blood using Sanger Sequencing, and quantitative PCR showed a high heteroplasmy rate in blood DNA (88%), demonstrating that a high heteroplasmy rate of a causative mitochondrial DNA mutation in blood DNA extracted from a Guthrie card is similar to that in heart tissue in severe infantile cardiomyopathy [70].

A8701G Mutation

Zhu et al. [71] report a Han Chinese family in which several members were diagnosed with maternally transmitted hypertension and dilated cardiomyopathy. The study found a significantly associated mitochondrial variant, i.e., A8701G mutation, which was enriched in this family, may be due to the consanguineous marriage in the first generation. This mutation was heteroplasmic in blood and was accompanied by slightly reduced deficiency of mitochondrial respiratory chain enzymes.

Mitochondrial ND1 *Gene*

G3337A Mutation

Recently, Zifa et al. [72] reported a novel G3337A mutation in mitochondrial NADH dehydrogenase-1 gene (*ND1*) in two unrelated patients. The first patient was a newborn female who required resuscitation at birth. She had respiratory dysfunction and was hypotonic. Cardiac findings included aneurysm of the intra-auricular septum, and narrowing of the pulmonary artery, and some features suggestive of cardiomyopathy. The second patient was a 65-year old woman with diabetes mellitus, presenting with chest pain and dyspnea. The echocardiogram showed left ventricular hypertrophy. In the study, the mutation was 100% homoplasmic in both of the patients' blood, and in the blood of the mother and grandmother of the first patient. Surprisingly, the first patient's mother only complained of mild muscle hypotonia, exercise intolerance and weakness. Her maternal grandmother was asymptomatic at 65-years-old, as well as her father. The G3337A mutation was not detected in 150 control individuals [73].

T3398C Mutation

A recent study reported an association between an m.3398T.C MTND1 variant and left ventricular non-compaction [73]. Left ventricular non-compaction is caused by abnormal compaction of myofibrils during cardiac development and leads to progressive ventricular dilatation, and systolic and has been recognized as a cardiac symptom of mtDNA disease, particularly in pediatric populations, and most commonly as part of multisystem disease [74].

Mitochondrial DNA D-Loop

Boles et al. [75] demonstrated that mtDNA control region point heteroplasmy, particularly of the D-loop, is associated with a protean clinical disorder in children "at risk" for mitochondrial disease, being found in 15 of 75 patients. Four among the 15 children were noted to show severe cardiomyopathy with congestive heart failure in infancy, which completely or essentially resolved with supportive therapy only.

mtDNA Lesions Transmitted in a Mendelian Manner

This group of mitochondrial diseases is associated with mtDNA abnormalities due to nuclear DNA (nDNA) mutations and are inherited as Mendelian genetic traits. Then the nDNA mutation secondarily affects mtDNA leading to multiple mtDNA deletions or mtDNA depletion syndrome [36, 76].

Multiple mtDNA Deletions

An Italian family with adult-onset mitochondrial encephalomyopathy was described by Zeviani et al. [76]. The disease is characterized by progressive external ophthalmoplegia (PEO) and inherited as an autosomal dominant (AD) trait. There were different mtDNA deletions in the proband's muscle mtDNA by Southern blot analysis. Maternal inheritance was excluded as the disease was inherited through both maternal and paternal lineages in the subsequent generations. Multiple mtDNA deletions in muscle specimens from two siblings with optic atrophy, PEO, peripheral neuropathy and muscle weakness have been reported. The siblings were born to consanguineous healthy parents [44, 66, 76]. Then, an autosomal recessive syndrome associated with multiple mtDNA deletions in muscle was shown in six patients from two unrelated families. The patients presented with childhood-onset, PEO and severe cardiomyopathy requiring cardiac transplantation. The study has found that cardiac abnormalities are found in 37% of patients with AD-PEO with multiple mtDNA deletions [76–78].

mtDNA Depletion Syndrome

Mitochondrial DNA depletion syndrome represents an autosomal recessive disorder characterized by decreased mitochondrial DNA copy number in affected tissues, which has been reported in hepatic failure and skeletal muscle myopathies [79]. Thus, mtDNA depletion is tissue-specific occurring in either liver or muscle. The first report of depleted mtDNA with hypertrophic cardiomyopathy was a 5-month-old child with a history of loss of muscle tone, progressive weakness, and developmental delay from 3 months of age. Left ventricular hypertrophy without outflow obstruction was shown by echocardiogram. The patient died from circulatory shock and respiratory distress [79].

In sum, mitochondrial heteroplasmy plays a critical role in contributing to the pathogenesis of some cardiomyopathy, in which it functions as an etiological aspect. As among the above-mentioned mtDNA-associated cardiomyopathies, heteroplasmy resulted from congenital, or acquired mutation determines the eventual outcomes. Of them, some are from hereditary, and some are from sporadic. For this emerging field, a lot of questions must be examined. We speculate that, in addition to the mutation-related heteroplasmy, most have congenital characteristic. The common cardiac diseases like idiopathic hypertension, hypertrophic cardiomyopathy, and congestive heart failure etc. have a predilection in particular populations within whom the cardiac mtDNAs are prone to acquire mutation under chronic mutation-evoking stresses (Fig. 30.2). If this could be verified, corresponding novel therapeutic treatments can be implemented to conquer, or at least to delay the progression, of the disease through focusing on the heteroplasmy.

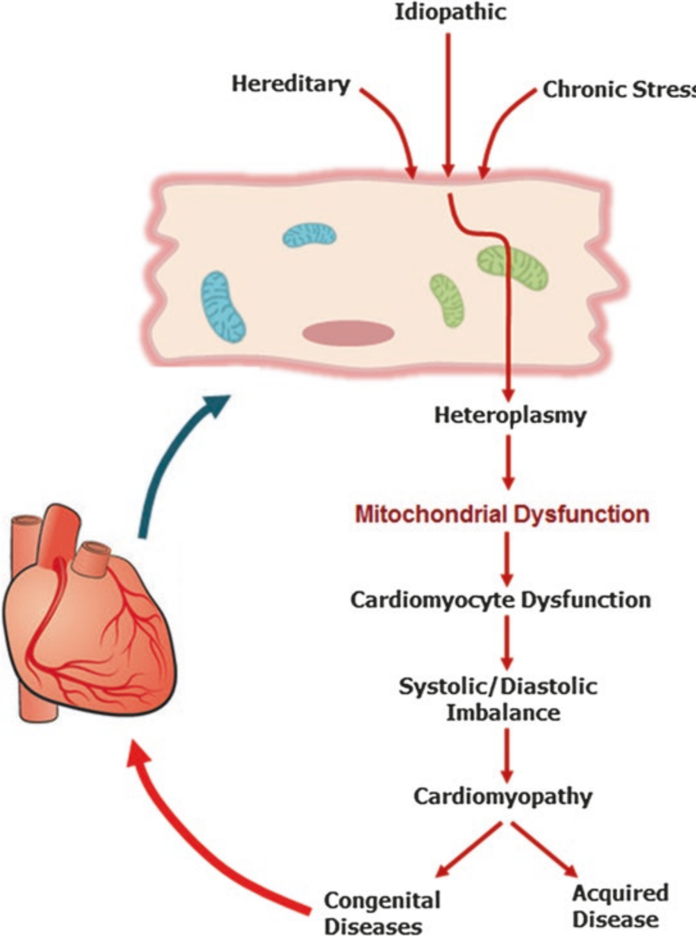

Fig. 30.2 Proposed cardiomyopathy related to mitochondrial heteroplasmy. Mitochondrial genetic materials of cardiomyocytes undergoing hereditary, idiopathic, and chronic stress (hypertension, hypoxia, homeostatic imbalance, etc.) respond correspondingly, and the heteroplasmy now initiated results in mitochondrial dysfunction. Subsequently, cardiomyocyte function is undermined and the overall systolic/diastolic balance is overturned, and then the eventual outcome like congenital or acquired diseases occur. This proposed etiological contribution of heteroplasmy to various common or uncommon cardiac diseases needs to be tested further, and corresponding therapeutic targets implemented

Conclusions

We postulate that biological significance of mtDNA heteroplasmy is demonstrated by the ability of cellular mitochondria to effectively modulate energy state-dependent changes by concerted transcriptional and translational mechanisms [17, 80, 81]. Altered homeostatic regulation of mtDNA heteroplasmy patterns can be

amplified in the initiation and progression of biomedical pathophysiological processes linked with human disease states [16, 17, 82–85]. Moreover, bidirectional intra-cellular communication involving mitochondrial signaling pathways allow for co-ordinate regulation of nuclear DNA- and mtDNA-derived gene expression. This phenomenon occurs within the dynamic of a constantly changing physiological environment, which is designed to promote molecular switching of the metabolic machinery, e.g., anabolic to catabolic demands [27, 86]. Since technological transplantation of functionally viable mitochondria envisages a significant restoration of normative cellular function, a comprehensive understanding and mapping of cell-specific mosaic patterns of heteroplasmic mtDNA expression are essential to the therapeutic potential of future translational studies.

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Chapter 31
Mitochondrial Transplantation in Myocardial Ischemia and Reperfusion Injury

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Ischemic heart disease remains the leading cause of death worldwide and ischemia and reperfusion injury hallmark the central pathophysiology that determines clinical outcomes [1]. Robust investigations have elucidated the mechanisms of myocardial ischemia [2–4] and reperfusion injury [5] as well as the intrinsic adaptive mechanisms of myocardial stunning [6], hibernation [7], preconditioning [8, 9] and postconditioning [10–12]. They enabled the current knowledge of cardiac metabolism, unraveling the complex biochemical and cellular changes resulting from myocardial ischemia and reperfusion including disruptions of cellular energy, ionic homeostasis and oxidative stress which all converge on mitochondrial dysfunction to culminate in cellular apoptosis and necrosis [13–16].

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The functional and structural derangements governing the pathophysiology of ischemia-reperfusion injury as well as changes in myocardial survival pathways inspired many pharmacological, genetic and procedural therapies in a variety of models including energy substrates, antioxidants, inhibitors of calcium (Ca^{2+}) and sodium accumulation, mitochondrial components, immunomodulation as well as procedural techniques. However, a major dichotomy exists between positive results of preclinical studies and equivocal or negative results of the same therapies on limiting infarct size in human clinical trials [17]. Meanwhile, multi-disciplinary approaches in both experimental and clinical research targeting myocardial ischemia continue to highlight mitochondrial malfunction as the common denominator of cardiomyocyte's ischemic response, emphasizing that the preservation of mitochondrial function and energy production may be the essential gateway to cardioprotection.

Rather than ameliorating one mediator or a step within the vast array of mitochondrial responses to ischemia and reperfusion, a newly emerging method of replacing damaged cardiomyocyte mitochondria with healthy mitochondria isolated from non-ischemic tissue of the same patient is being explored in various in vitro and in vivo models with promising results. In this chapter, we will explore (1) the basic physiology of myocardial ischemia and reperfusion injury, (2) the key features of mitochondrial metabolism and changes in ischemia and reperfusion injury, (3) current methodologies of mitochondrial transplantation, and (4) potential mechanisms of cardioprotection conferred by mitochondrial transplantation based on the most current experimental developments.

Mitochondria in Cardiac Tissue

The heart is a syncytial, muscular network of cardiomyocytes specialized to propagate electrical impulses across communicating cells allowing the varied components of the heart to act as a single contractile apparatus. The incessant contractility of the myocardium and cellular homeostasis is almost exclusively powered by the production of ATP by the mitochondria which constitute about 30% of the myocardial cell volume and provide for the daily turnover rate of ATP up to 15–20 times the weight of the heart in humans [18]. Thus, tight coupling of metabolic proficiency and myocardial contraction is essential for normal cardiac function and govern the intricate set of metabolic pathways resulting in both ATP producing and non-ATP producing endpoints for different classes of substrates under normal physiological conditions and during severe energy deprivation.

ATP is produced by the process of oxidative phosphorylation carried out by a series of four multi-subunit complexes embedded in the inner mitochondrial membrane as the Electron Transport Chain (ETC). The components of the electron transport chain include: Complex I (NADH dehydrogenase), II (succinate ubiquinone oxidoreductase), III (ubiquinol: cytochrome c oxidoreductase), IV (cytochrome c oxidase), V (ATP synthase), and two main electron carriers ubiquinone and

cytochrome *c*. Thirteen of these 76 protein subunits making up the five complexes of the electron transport chain are encoded by the mitochondrial DNA and the remaining are encoded by the nuclear DNA. The various catabolic pathways of energy-yielding substrates (fatty acid, glucose, amino acids and ketones) converge on Acetyl-CoA production which mainly enter the tricarboxylic acid cycle (TCA) in the mitochondrial matrix to generate reducing equivalents NADH and $FADH_{2.}$ As electrons from NADH and $FADH_2$ flow through the ETC to reduce oxygen to water (by complex IV), the resultant release of potential energy is used to pump protons into the mitochondrial intermembrane space (by complex I, III and IV) that generates an electrochemical gradient (−200 mV) across the mitochondrial inner membrane which is harnessed by complex V (ATP synthase) to couple P_i and ADP to ATP. The ATP synthesized in the matrix is transported into the intermembrane space by an inner membrane channel adenine nucleotide translocase (ANT), where mitochondrial creatine kinase (miCK) transfers the high energy phosphate of ATP to create phosphocreatine which is then stored in the cytosol as energy reserves. Additional ATP is transported out to the cytosol via a complex formed by ANT, miCK and the voltage-dependent anion channel (VDAC) located in the outer membrane, where miCK bridges the space between ANT and VDAC, allowing for low conductance transfer of ATP out into the cytosol. In addition to the maintenance of cellular homeostasis, ATP consuming reactions vital to cardiomyocytes are exemplified by three main reactions which include (1) Myosin-ATPase which provides energy for myosin filament sliding for muscle contraction, (2) Ca^{2+}/Mg^{2+}-ATPase which stores Ca^{2+} in the sarcoplasmic reticulum for activation of myocardial contraction upon release, and (3) Na^+/K^+-ATPase used to maintain cellular excitability and Ca^{2+} efflux [19].

Cardiac mitochondria consist of two spatially distinct subpopulations, namely subsarcolemmal mitochondria (SSM), located directly beneath the cellular membrane, and interfibrillar mitochondria (IFM) which reside in rows between the myofibrils [20, 21]. Scanning and transmission electron microscopy of left ventricular papillary muscle sections of Japanese Monkeys and humans have revealed many structural differences between SSM and IFM [22, 23]. IFM are generally elongated in shape (1.5–2.0 μm), and occupy the space between Z-lines flanked by the junctional sarcoplasmic reticulum, and have predominantly tubular cristae. SSM located beneath the sarcolemma have more variable lengths and sizes (0.4–3.0 μm), possessing more lamelliform and closely-packed cristae [23, 24]. Studies using three dimensional modeling approach coupled with MitoTracker Red staining also revealed that IFM were arranged in a more orderly pattern in contrast to SSM which had a more random arrangement [25, 26]. Numerous reports have also indicated that SSM and IFM possess distinct functional differences such as substrate utilizations [20, 27, 28], enzymatic rates [21] and ATP synthesis [29, 30] which have been found to be generally higher in IFM.

Mitochondrial subpopulations appear to be differentially influenced during different pathological insults. However, many inconsistencies exist across different animal models. For example, cardiac ischemia has been reported to primarily effect SSM in rats [31] and rabbit models [32, 33] but both SSM and IFM populations in

canines [21, 34]. In contrast to some of the ischemic models, ischemia and reperfusion injury have been reported to effect both populations in the mouse, rat and rabbit models [35–37]. The clinical significance of spatial distinctions of cardiac mitochondria with regards to pathological conditions and therapeutic interventions thereof, remain to be investigated.

Mitochondria in Myocardial Ischemia and Reperfusion

Ischemia-reperfusion injury occurs as a result of attenuation or cessation of coronary blood flow such that oxygen delivery to the myocardium is insufficient to meet the myocardial oxygen requirements to preserve cellular function and contraction. The energy stores generated by the mitochondria are only sufficient to sustain myocardial contractions for a few seconds, and this explains the need for the robustness of the cardiac metabolism, consuming the highest rate of oxygen per unit weight basis. Under normal conditions, 75% of the oxygen supplied by the coronary vasculature is extracted by the myocardium during a single passage through the heart. Therefore, the heart is highly susceptible to alterations in oxygen delivery where an increase in myocardial oxygen consumption (MVO_2) can only be met by an increase in coronary blood flow, a phenomena unique to the cardiac muscle. The loss of myocardial oxygen delivery has been shown to exhaust myocardial oxygen reserves within 8 seconds after the onset of normothermic global ischemia with subsequent decrease in energy reserves [38]. In addition, studies measuring energy content by phosphorous-31 NMR spectroscopy have demonstrated that decreases in myocardial energy reserves are directly associated with decreases in myocardial function and viability which in turn are associated with detrimental clinical pathologies [39–43].

Reversible Myocardial Ischemia

With the onset of coronary occlusion, myocardial injury begins in approximately 20 min, being first evident in the subendocardium and papillary muscle, then extending into the mid-myocardial bed-at-risk by about 60–90 min, such that the wavefront of irreversible transmural infarction is complete in 3–4 hrs [44–47]. The infarcted myocardium subsequently exhibits features of inflammation and necrosis. Depending on the extent and duration of the ischemic event, myocardial injury may be reversible where the myocardium activates physiologic adaptations of survival manifested by myocardial stunning, hibernation and pre- and post-ischemic conditioning. These conditions result from changes in gene expressions that sustain cell survival under oxygen deprivation and during the stresses of reperfusion.

Myocardial stunning, first described by Heyndrickx and colleagues in 1975, refers to a state of contractile dysfunction which persists after reperfusion despite the absence

of irreversible myocellular damage and return of normal or near-normal perfusion. Heyndrickx demonstrated the reversibility of ischemic myocardium following a 5 min or 15 min episode of ischemia and reperfusion in dogs, which resulted in a prolonged regional contractile deficit that required 6 h to recover from a 5 min ischemic episode and greater than 24 h to recover from a 15 min ischemic episode [48]. Stress of reperfusion can be exemplified in cardiac surgery patients where, despite modern methods of cardioprotection, the heart weaning from cardiopulmonary bypass often exhibit varying degrees of myocardial stunning and requires inotropes hours to days after surgery without objective evidence of myocardial infarction [49, 50].

In contrast to the stunned myocardium, the hibernating myocardium refers to a myocardium with chronically reduced contractility associated with local reduction in myocardial perfusion [51, 52]. Although the hypo-perfused cardiomyocytes can develop degenerative changes over time, the contractile function of the hibernating myocardium can be restored by reperfusion [53–55], allowing the myocardium to be called dysfunctional but 'viable'. As in line with the 'smart heart hypothesis', hibernation is considered an adaptive mechanism of myocardial metabolism in which myocardial contractility and cellular activity is deliberately reduced to minimize metabolic demands and recruit coronary flow reserves in the face of severely depressed local perfusion [51]. However, hibernating myocardium cannot maintain viability indefinitely and delayed revascularization is associated with worse outcomes [56, 57]. It is now widely believed that repetitive episodes of stunning (i.e. ischemic dysfunction) create a sustained depression of contractile function as in hibernation and that stunning and hibernation are likely to be part of a continuous disease spectrum [58, 59]. In a swine model where the heart was subjected to repetitive episodes of 90 min of coronary occlusion followed by 12 h of reperfusion cycled up to six times, the regional myocardial function was depressed despite normalization of perfusion, reflecting myocardial stunning. However, additional episodes of coronary occlusion did not result in further decreases in ventricular function, reflecting the mismatch between perfusion and contractile function which characterizes hibernation [59]. This model along with several older studies demonstrated the hallmarks of chronic dysfunction in the human hibernating myocardium including loss of myofibrils, higher dependence on glucose and glycogen accumulation [60–62].

Irreversible Myocardial Ischemia and Mitochondrial Dysfunction

Despite regional differences in myocardial hypoxic tolerance and intrinsic adaptive mechanisms, irreversible myocardial injury inevitably follows prolonged ischemia. Countless studies have identified the pathological modulators of irreversible myocardial ischemic injury and the intricate linkages that converge on mitochondrial damage as the central culprit of cascade to myocardial cell death and tissue damage.

With the cessation of blood flow and oxygen supply, declines in the rate of oxygen consumption by oxidative phosphorylation and the synthesis of high energy phosphate products are immediately detectable. Mitochondrial function assessed by oximetry in many animal models have shown that ischemia significantly decreases state three oxygen consumption and respiratory control index in malate (complex I substrate) and succinate (complex III substrate) in energized mitochondria [63, 64]. Similar findings were measured for complex III and cytochrome oxidase in buffer-perfused rat hearts [32] and rabbit hearts [65] where global ischemia decreases their Vmax, mRNA levels as well as the content of cytochrome *c*. These declines were also shown to be paralleled with significant decreases in phosphocreatine, inorganic phosphate, ATP, and high energy stores, up to 36 ± 3% following 30 min of ischemia which remained decreased at 15 min post reperfusion via ^{31}P nuclear magnetic resonance (NMR) [43].

With ischemic depletion of ATP synthesis, multiple biochemical and ultrastructural changes occur in the mitochondria that are hallmarked by several key cellular events which are cellular acidosis and accumulation of intracellular, mitochondrial and even nuclear Ca^{2+} ($[Ca^{+2}]_n$). With depletion of oxygen, the heart switches from fatty acid oxidation to anaerobic glycolysis resulting in a build-up of lactate and a decrease in the cellular pH. This rapid metabolic acidosis can be quantified by the measurement of tissue PCO_2 and ^{21}P NMR spectroscopy in the lab as well as with a pH probe in the operating room [66, 67]. Accumulation of intracellular hydrogen ion (H^+) opens the sodium-hydrogen (Na^+/H^+) exchanger (NHE), resulting in the transport of H^+ into the extracellular space and the movement of Na^+ into the cytosol [68]. The excess Na^+ is then extruded in exchange for the intake of Ca^{2+} through the reverse action of the plasma membrane sodium-Ca^{2+} (Na^+/Ca^{2+}) exchanger (NCX), increasing the cytosolic Ca^{2+}concentration ($[Ca^{2+}])_i$. The increase in $[Ca^{2+}]_i$ is further augmented by depolarization of the plasma membrane potential, which leads to the opening of the L-type Ca^{2+}channels causing further Ca^{2+} influx, as well as the cessation of uptake of Ca^{2+} into the sarcoplasmic reticulum by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) due to the decline in ATP [69]. Cellular Ca^{2+}-dependent phospholipases and proteases are in turn activated inducing membrane injury and further Ca^{2+} entry into the cell.

The increase in cytosolic Ca^{2+} leads to the augmented mitochondrial Ca^{2+} that has deleterious effects on their structure and function [70]. In a normal state, mitochondrial inner membrane pump H^+ out to the cytosol creating a voltage gradient that provides the passive energy for Ca^{2+} influx into the matrix by the Ca^{2+}-uniporter. During ischemia, increases in the intracellular Ca^{2+} ($[Ca^{2+}]_i$) lead to increased Ca^{2+} uptake by the mitochondria which destabilizes the inner membrane potential. As Ca^{2+} accumulates, mitochondria use ATP to transport excess Ca^{2+} out of the mitochondria against the electrochemical gradient which wastes more ATP required to maintain cellular viability, a mechanism called 'futile Ca^{2+} cycling' [70–72]. As Ca^{2+} overloads in the mitochondria, water passively follow and the mitochondria swell. This has been visualized via ECM and light scattering technique on isolated perfused heart and in vivo pig and sheep models, where greater than 88% of myocardial mitochondria are electron translucent and swollen following 20–30 min of

global ischemia and exhibit increased intermembrane space, enlarged cristae and disrupted matrix [63, 64, 73, 74]. These alterations in mitochondrial structure are noted to be damaging to ATP transfer from the mitochondria via the adenine nucleotide translocase (ANT)-mitochondrial creatine kinase (miCK)-voltage-dependent anion carrier (VDAC) complex. It has been proposed that mitochondrial swelling increases intermembrane distance and dissociate the functional interaction between ANT, miCK and VDAC that compromise efficient energy transfer during reperfusion and decrease high energy synthesis in the myocardium post reperfusion [75].

The role of Ca^{2+} overload has been demonstrated by studies that showed warm global ischemia results in rapid accumulation of intracellular and mitochondrial Ca^{2+} which is associated with the depletion of ATP, and precedes the onset of ischemic contracture in various animal models [76–78]. Conversely, amelioration of this Ca^{2+} overload has been shown to correlate with maintenance of high energy phosphates in the myocardium, results of which formed the conceptual basis for optimization of many cardioplegic solutions in cardiac surgery. For example, Hearse et al. and many subsequent investigators showed that magnesium, when added to potassium cardioplegic solutions, significantly decreases cytosolic and nuclear Ca^{2+} accumulation during global ischemia [79, 80], enhances preservation and re-synthesis of high-energy phosphates, and significantly decreases myocardial injury upon reperfusion in perfused rabbit heart [43, 78, 81] and in situ blood-perfused sheep and pig heart models [47, 82, 83]. Furthermore, the addition of pharmacological agents such as diazoxide to the K^+/Mg^{2+} cardioplegia solution, was shown to ameliorate mitochondrial Ca^{2+} accumulation by opening of the mitochondrial ATP-sensitive K^+ channels which correlated with prevention of mitochondrial swelling, decrease in apoptosis and improved post-ischemic function [82, 84–87].

Myocardial Reperfusion and Mitochondrial Injury

While restoration of blood flow is the absolute therapeutic aim to salvage ischemic cell death, it is proposed that reperfusion itself independently activates a cascade of cellular injuries which inflate the damages in excess of those produced by ischemia alone. First described by Jennings et al. in 1960 in their description of histological features of reperfused ischemic canine myocardium, they reported that features of cellular injury after 3 h of ischemia followed by 1 h of reperfusion were far worse than changes observed after 4 h of ischemia alone; the reperfused myocardium had significantly more cell swelling, contracture of myofibrils and mitochondrial and sarcolemmal disruption [88]. Clinically, reperfusion injury leads to arrhythmias, conversion of myocardial necrosis into a hemorrhagic infarct with prominent contraction band necrosis, disruption of the microvasculature leading to the 'no-reflow' phenomenon, which refers to impairment of blood flow to an ischemic region in spite of opening the infarct-related artery [17, 89–91]. In addition, it is associated with activation of inflammatory reactions which can be extensive enough to cause inflammatory responses on distant, non-ischemic organs [92].

Extensive experimental efforts have identified the potential mechanisms of reperfusion injury that build on the existing elements of the damages sustained from ischemia, described by distinct biochemical changes that occur from reperfusion in the mitochondria. These changes include (1) rapid normalization of pH, (2) exacerbation of existing mitochondrial and intracellular Ca^{2+} overload, and (3) generation of reactive oxygen species.

Reperfusion and re-oxygenation results in rapid restoration of intracellular pH by lactic acid washout, clearance of H^+ by the Na^+/H^+ exchanger (NHE) and Na^+/HCO_3^- exchanger, and the restoration of the mitochondrial membrane potential. As the mitochondrial membrane potential normalizes, the mitochondrial Ca^{2+}-uniporter (MCU) opens causing an even more influx of Ca^{2+} into an already Ca^{2+}-overloaded mitochondria [93]. Cytosolic Ca^{2+} is also proposed to be further increased by the damaged sarcolemmal membrane and oxidative stress-induced dysfunction of the sarcoplasmic reticulum, all of which can overwhelm the cardiomyocyte's mechanisms for ionic homeostasis.

Many experimental studies have also implicated Reactive Oxygen Species (ROS) as partial mediators of reperfusion injury and the damaged mitochondria as the potential primary source of ROS production. Various modalities including electron spin resonance spectrometer [94] have detected bursts of ROS during myocardial reperfusion in different animal models and patients undergoing coronary revascularization [95]. Examples include H_2O_2, HO^-, HOCl, and O_2^-, the superoxide anion radial which is considered to be the most toxic ROS generated primarily by the mitochondria. In addition, it has been recently proposed that mitochondrial alterations at complex I of the electron transport chain and the accumulation of succinate during ischemia may be related to mitochondrial oxidative injury during reperfusion. Succinate is the substrate of complex II, and the sole mitochondrial component to the three metabolites that accumulate during ischemia: succinate, xanthine and hypoxanthine [96]. Accumulation succinate is detected in the mitochondria when the electron transport chain comes to a halt during ischemia. It is proposed that upon reperfusion, succinate is abruptly hydrolyzed by complex II [97, 98] at a maximal rate that overwhelms the speed of ATP synthesis generating an excess pool of electrons that start to flow backwards by a process termed Reverse Electron Transport (RET) [99]. As the excess electrons are forced back through complex I, the reverse reaction of NADH dehydrogenase generates large amounts of superoxide. This has been demonstrated by various animal experiments which showed that inhibition of complex I slows reactivation of mitochondria during the early phase of reperfusion, reduce ROS and protects against ischemic-reperfusion injury [100–103]. Excessive ROS generated during reperfusion cause tissue damage by a myriad of mechanisms including peroxidation of lipids, inactivation of proteins/enzymes, nuclear and mitochondrial DNA strand breaks, oxidation of Cardiolipin, SER dysfunction and activate inflammation, all of which serve to activate apoptotic and necrotic signaling pathways. ROS also reduce the viability of the vasodilator nitric oxide (NO), removing its cardioprotective effects such as inactivation of superoxide radicals, inhibition of neutrophil accumulation and improvement of coronary flow [104].

Multitude of the acute fluctuations described above are proposed to act in concert to lead to the opening of the voltage dependent, nonspecific pore in the inner mitochondrial membrane known as the Mitochondrial Permeability Transition Pore (mTPT), which is thought to be the most noxious step marking the transition from reversible to irreversible reperfusion injury. The mTPT is closed during normal conditions and ischemia. Opening of mTPT pore at reperfusion allows the free passage of any molecule smaller than 1.5 kDa, causing a buildup of colloidal osmotic pressure leading to severe mitochondrial swelling, complete collapse of the mitochondrial membrane potential, uncoupling of oxidative phosphorylation and disruption of the mitochondrial membrane and eventual rupture. This process leads to release of the cytochrome c from the ETC, which is a potent activator of apoptotic signaling cascades leading to cardiomyocyte death [105, 106]. Systemically, activation of corresponding immune responses leads to leukocyte aggregation, leukocyte-mediated tissue destruction and endothelial dysfunction leading to platelet aggregation causing damage to the surrounding microvasculature.

Ischemia Versus Reperfusion Injury

The concept of reperfusion injury as an independent mediator of cardiomyocyte death distinct from ischemic injury has been controversial. The uncertainty relates to the inability to accurately assess the exact progression of necrosis during the transition from myocardial ischemia to reperfusion in situ [17]. Moreover, understanding the sequential evolution of mitochondrial damage in relation to the timeframe along myocardial ischemia and reperfusion is not straightforward. In spite of these challenges, studies demonstrate that critical events of mitochondrial damage occur at the time of ischemia and persist through reperfusion.

Transcriptomic and proteomic enrichment analyses in both Langendorff-perfused and in situ blood-perfused rabbit hearts have demonstrated that global ischemia downregulates genes/proteins associated with mitochondrial structure, function, energy production, cofactor metabolism, generation of precursor metabolites of energy, carbohydrate metabolism and regulation of biosynthesis and transcription [107]. In contrast, the same analyses in globally ischemic rabbit hearts treated with cardioplegia showed that cardioprotection was associated with significant increases in differentially expressed transcripts/proteins associated with mitochondrial function, energy production, fatty acid beta oxidation, cellular lipid metabolism as well as processes of muscle contraction [107]. These results indicate that (1) the mitochondrion plays a significant role in both global ischemia and cardioprotective pathways, (2) the mechanisms of mitochondrial damage resulting from global ischemia as well as pathways of cardioprotection are modulated, at least in part, by RNA- and protein-dependent mechanisms, and (3) these critical events occur at the time of ischemia rather than reperfusion.

The challenges of elucidating reperfusion injury are also demonstrated by the equivocal results of studies targeting the accepted mediators of reperfusion injury.

While attractive targets of intervention such as the accumulation of Ca^{2+}, reactive oxygen species, components of the electron transport chain and inflammation have yielded promising preclinical results, their clinical translations have been largely disappointing. For example, efforts to decrease Ca^{2+} overload with antagonists of the sarcolemmal Ca^{2+} ion channels, mitochondrial Ca^{2+} uniporter, or the Na^{+}/H^{+} exchanger have shown to decrease myocardial infarct sizes up to 50% in animal models [108–110], but have been unsuccessful in the corresponding clinical studies [111–113]. The same trend is present in animal studies where these interventions were applied just at reperfusion to yield reductions in the final infarct sizes up to 50%, their applications in patients have been largely negative or inconclusive [17]. A host of antioxidants such as superoxide dismutase, trimetazimide, vitamin C and E with positive results in animal models failed to show meaningful improvement in infarct size, post-ischemic cardiac function nor mortality benefit in human clinical trials [114–116]. Similarly, attempts at targeting neutrophils such as leukocyte depleted blood [117], antibodies against the cell adhesion molecule P selection [118] as well as inhibitors of complement activation [119] have shown significant cardioprotection in animals with negative results in their corresponding clinical studies [120–124].

Several reasons may exist for the discrepancy between the experimental animal studies and human trials. First, although the existence of ischemia and reperfusion injury and the essential role of the mitochondria is well established in humans, variabilities may exist in the detailed biochemical mechanisms underlying cell death pathways amongst different species. Second, heterogeneities in a clinical setting in the varying degrees of ischemia as well as timing and context of intervention may contribute to the inconclusive results of clinical studies. Third, the interventions examined so far may have been of questionable benefit in preclinical studies or lacking in adequate knowledge of their pharmacological actions and kinetics in clinical subjects. Although the unequivocal role of mitochondrial dysfunction in myocardial ischemia and reperfusion is increasingly evident, the low rate of clinical success in the vast number of therapeutic targets investigated thus far calls for a need to evaluate our current understanding of the sequential mechanism of mitochondrial dysfunction during ischemia and reperfusion, as well as the way we devise our remedies.

Mitochondrial Transplantation

Mitochondrial dysfunction is the prominent feature of myocardial ischemic injury which are compounded by the process of reperfusion to have lasting effects on post-ischemic function and cellular viability. A myriad of experimental studies confirms that loss of mitochondrial function during ischemia significantly decreases cardiomyocyte viability and post-ischemic recovery. Despite this clear trend, there is currently no meaningful therapy for mitochondrial rescue from ischemia-reperfusion injury with growing discrepancies between preclinical and clinical studies. This

calls for a review and a shift in the current paradigm and exploration of new approaches in therapeutic design. Instead of targeting a single step or one mediator of a complex, interconnected reactions of mitochondrial injury, a new therapy of mitochondrial transplantation is being investigated with the hypothesis that replacement of damaged mitochondria with viable, respiration-competent mitochondria isolated from non-ischemic tissue would overcome a host of deleterious effects of native mitochondrial dysfunction resulting in cardioprotection.

Mitochondrial Transplantation for Cardioprotection

Based on the hypothesis that myocardial mitochondrial injury occurs during ischemia and persists during reperfusion to significantly compromise post-ischemic myocardial function and viability, the concept of mitochondrial transplantation at the time of reperfusion was proposed in the recent decade. The first validation of mitochondrial transplantation in the ischemic heart model was demonstrated in the Langendorff-perfused ischemic rabbit heart in 2009 [125]. In this study, viable, respiration-competent mitochondria isolated from healthy left ventricular tissue of donor rabbits were directly injected into the ischemic zone of isolated hearts after 30 min of regional ischemia just prior to 120 min of reperfusion. Ischemic hearts that were transplanted with healthy mitochondria were found to have significantly enhanced cardioprotection by increased ATP content, reduction in infarct size, decreased cardiomyocyte loss, and improved post-ischemic myocardial function. Confocal microscopy showed that injected mitochondria were present and viable after 120 min of reperfusion and were distributed from the epicardium to the subendocardium. These findings heightened new interests in mitochondrial therapy calling for a deeper understanding of the mechanisms behind mitochondrial transfer, uptake, cardioprotection as well as the optimization of methods for enhancing clinical applicability.

Mitochondrial Source and Viability

In efforts to enhance clinical practicality and reduce immunogenic complications, rather than transplanting mitochondria from an allogenic source such as a donor organism, an approach of isolating mitochondria from a remote, nonischemic tissue in the same organism (autologous) to be transplanted into an ischemic region of the myocardium have been explored with promising results [126, 127]. A recent study in an in-vivo rabbit model of regional myocardial ischemia and reperfusion, autologous mitochondria from the pectoralis major muscle of the same organism were isolated via the newest isolation technique of standard homogenization and filtration, and injected in the myocardium following 29 min of ischemia immediately prior to reperfusion [126]. Results showed that transplantation of mitochondria

isolated this way significantly decreased infarct size, improved cardiomyocyte viability and myocardial function at 4 weeks of recovery. Furthermore, additional studies showed that transplantation of autogenic mitochondria did not induce any autoimmunity via multiplex and ELISA assays which did not show any increases in inflammatory cytokines associated with immune responses seen in patients with heart transplant rejection such as C-Reactive Protein (CRP), Tumor Necrosis Factor-alpha (TNFα), Interleukins -1, -4, -6, -12, -18, Macrophage Inflammatory Protein -1 alpha and -1 beta (MIP-1α, -1β) [126].

In addition to the benefits of the autologous origin of mitochondria, efficacy of mitochondrial isolation from various sources of tissues has been investigated. As expected, different sources of tissues provide different absolute number of mitochondria, with liver being the highest, followed by skeletal muscle followed by cardiac muscle [127]. However, no differences were detected in the cardioprotective effects of mitochondria derived from different tissue types [125, 126], nor their subpopulations (subsarcolemmal or interfibrillar) [125]. On the other hand, mitochondrial viability is found to be a prime determinant of efficacy of mitochondrial transplantation for cardioprotection. Studies have shown that Nonviable mitochondria isolated from frozen tissue with confirmed absence of oxygen consumption failed to provide cardioprotection [125]. Similarly, mitochondrial proteins, mitochondrial complexes and mitochondrial RNA and DNA have not been found to provide cardioprotection [125]. Injection of exogenous ATP, ATP synthesis promoters, and exogenous ADP have also not shown to result in any protection against myocardial ischemia [125, 128]. These findings together indicate the imperative requirement of transplanted mitochondria to be viable, freshly-isolated and respiration-competent to be efficacious.

Mitochondrial Isolation and Purification

To assure the viability of the transplanted mitochondria and the clinical practicality of mitochondrial transplantation, it is critical that the isolation process of mitochondria is simple, cost-effective and feasible within the clinical timeframe of intervention for myocardial ischemia. For example, in the case of ST-elevation myocardial infarction (STEMI), the goal time from the onset of evaluation to revascularization via PCI (door-to-balloon time) is less than 90 min. For cardiac surgery, most operations last from 40 min to several hours in duration. However, the time from the onset of actual incision to revascularization or to the time to wean from bypass are often less than 1 h. This makes the standard technique of mitochondrial isolation which takes over 90 min at best, impractical and unreliable for yielding viable mitochondria. In 2014, a new method of rapid isolation and purification of mitochondria was developed by Preble and colleagues, who employ standardized tissue dissociator and differential filtration rather than centrifugation, cutting the isolation time to less than 30 min [129]. The purity and viability of the mitochondria isolated from this method have been validated by many different quality control parameters involving

oxygen consumption rates via the Clarke-type electrode, ATP assays, fluorescent probe analysis, as well as light microscopy using fluorescent mitochondrial labels (MitoTracker CMXros) and transmission electron microscopy [125, 126, 129, 130].

Mitochondrial Delivery, Mode of Uptake and Distribution

To further enhance the therapeutic potential of mitochondrial transplantation, different modes of delivery of exogenous mitochondria have been investigated which include direct myocardial injection and intracoronary vascular infusion. From initial studies in both Langendorff-perfused and in situ-blood perfused rabbit hearts, respiration competent, autologous mitochondria were injected directly into eight to ten sites in the ischemic left ventricular free wall which showed significant improvement in infarct size and myocardial function. In a recent study using Langendorff-perfused rabbit heart model of both regional and global ischemia, the mode of vascular delivery via intracoronary infusion was validated and compared to the method of direct injection with regards to myocardial distribution using ^{18}F-rhodamine 6G and iron oxide nanoparticles labeling followed by visualization with positron emission tomography, microcomputed tomography and magnetic resonance imaging [127]. Intracoronary delivery of mitochondria was shown to result in a rapid and a more widespread distribution throughout the myocardium as compared to direct injection, which permitted higher concentrations of mitochondria in the targeted region. The efficacy of cardioprotection via intracoronary delivery of autologous mitochondria paralleled that of direct injection in another experiment using Langendorff-perfused rabbit hearts of regional ischemia which showed similar decrease in myocardial infarct size and improvement in both global and regional myocardial function [127]. While additional in vivo studies are needed, the ability to deliver mitochondria by vascular infusion expands the therapeutic potential such as global delivery to the ischemic heart just prior to reperfusion after cardiopulmonary bypass or delivery into the coronary artery at time of percutaneous coronary interventions (PCI) for revascularization.

On a histological level, both in situ and in isolated heart models, transplanted mitochondria have been shown to be initially present in the interstitial spaces surrounding cardiomyocytes coinciding with the time of increase in myocardial function, approximately 10 min post injection. Within 1–2 h post-delivery, the transplanted mitochondria were detectable within cardiomyocytes residing near the sarcolemma between Z-lines of the sarcomeres, in clusters around endogenous damaged mitochondria as well as near the nucleus. This internalization has been confirmed using human mitochondria isolated from HeLA cells in a rabbit heart. The use of human mitochondria in the rabbit model allows for the differentiation between native rabbit mitochondria and transplanted human mitochondria based on immune reactivity to a monoclonal anti-human mitochondrion, and internalization of the mitochondria into cardiomyocytes were visualized via transmission electron microscopy using immune-gold staining [125, 126]. These studies along with other

in vitro studies demonstrated that the uptake of mitochondria is linear and time dependent, and remain viable for at least 24 h, significantly enhancing rate of oxygen consumption and high-energy synthesis in both neonatal and adult cardiomyocytes [125, 126, 131].

Many hypotheses have been proposed as to the mechanism of mitochondrial internalization by cardiomyocytes such as actin-meditated endocytosis, Caveolae-dependent-clathrin dependent endocytosis [132], tunneling nanotubes [133] and macro-pinocytosis [134] based on prior experiments in cell types other than cardiomyocytes. A recent in vitro study of cardiomyocytes using specific inhibitors of these mechanisms showed that only Cyctochalasin D, a specific inhibitor of actin polymerization, decreased internalization of mitochondria into cardiomyocytes and deceased ATP content, suggesting actin-mediated endocytosis as a potential mechanism of mitochondrial internalization by cardiomyocytes [131]. Additionally, the internalized mitochondrial did not co-localize with lysosomal or autophagocytosis markers raising another mechanism by which the internalized mitochondria escape endosomes. Actin-mediated endocytosis of mitochondria is in agreement with the widely accepted theory of the endosymbiotic origin of mitochondria. While externally presented mitochondria may be internalized via actin-mediated endocytosis in cardiomyocytes, intercellular transfer of mitochondria between cells may involve tunneling nanotubes within the syncytial architecture of cardiomyocytes in vivo [135].

Mechanisms of Cardioprotection

The mechanism through which transplanted mitochondria provide cardioprotection reflect the mediators of mitochondrial damage accrued during ischemic injury. Based on the established observations, at the least, cardioprotection via autologous mitochondrial transplantation are considered to involve (1) increased myocardial ATP content and metabolic recovery (2) change in proteomic and transcriptomics (3) upregulations of cytokine and chemokines that enhance post-infarct myocardial function and (4) replacement of damaged mitochondrial DNA.

To date, all studies of mitochondrial transplantation in cardiac tissue have shown that transplanted mitochondria increase cellular ATP content and oxygen consumption. In the in vivo rabbit model of regional ischemia/reperfusion injury, total tissue ATP content in the area of risk in mitochondria-transplanted hearts were significantly increased starting as early as 15 min of mitochondrial delivery and present even at 21 days of recovery compared to their controls. On the other hand, the use of exogenous ATP or ADP have not shown to restore high-energy phosphate stores nor have beneficial effects on post-ischemic functional recovery [126, 128, 136]. This is believed to be due, in part, to the lability of ATP and its short half-life in vivo. In line with this observation, cardioprotection by mitochondrial transplantation requires mitochondria that are intact, viable and respiration competent. Use of non-viable mitochondria or mitochondrial components such as mitochondrial RNA

and DNA have not shown to confer improvement in postischemic function or cellular viability, although some mitochondrial complexes such as cytochrome c [137] and coenzyme Q_{10} have been shown to have modest benefit at very high doses [138].

The increases in the energy level and metabolic recovery is also reflected by the changes in myocardial proteomics and increases in differentially expressed proteins post mitochondrial transplantation. Study using proteomic analysis and functional annotation clustering in post-ischemic rabbit myocardial tissue have shown that proteome clusters of the mitochondrion, the generations of precursor metabolites for energy, and cellular respiration were significantly increased in hearts treated with mitochondrial transplantation compared with their controls [126].

Mitochondrial transplantation has also been shown to result in downregulation of inflammatory cytokines and upregulation of chemokines that play key roles in angiogenesis, ateriogenesis, progenitor cell migration, prevention and protection against cardiomyocyte apoptosis and enhanced cardiac functional recovery. Rabbit hearts with regional ischemia treated with autologous mitochondrial transplantation were shown in vivo to have significantly decreased inflammatory markers when compared to untreated regional ischemic hearts, including tumor necrosis factor alpha (TNFα), interleukin -6 and -10 (IL-6, -10), monocyte chemoattractant protein-1 (MCP-1) and high-sensitivity C-reactive protein (hsCRP). Multiplex analysis indicated that autologous mitochondria significantly upregulated expression of epidermal growth factor (EGF), growth-related oncogene (GRO), interleukin-6 (IL-6) and monocyte chemoattractant protein-3 (MCP-3). EGF been shown to play a key protective role in myocardial ischemic injury by stimulating cell growth, proliferation and migration [139, 140]. GRO and IL-6 have been implicated in reconstitution of cardiac tissue mass post myocardial infarction by acting as chemoattractants for vascularization and protection against cardiomyocyte apoptosis [141]. These chemokines all act with MCP-3 to enhance post-infarct myocardial function and improve myocardial remodeling. It has been proposed that together with increased tissue ATP levels in the area of ischemic injury, induction of cardioprotective cytokines augment the role of differentially expressed proteins and mitochondrial pathways to enhance metabolic recovery and post ischemic cardiac function.

The effect on reactive oxygen species (ROS) by mitochondrial transplantation remains to be delineated, although results are gearing more towards the idea that ROS may not to play a major role in cardioprotection provided by mitochondrial transplantation. In Langendorff-perfused rabbit hearts of regional ischemia, ROS in form of thiobarbituric acid-reactive substances (TBARS) were found to be significantly decreased in hearts that received mitochondrial transplantation when compared to untreated hearts. However, ROS scavenger 2-Mercaptopropionylglycine (MPG) failed to block cardioprotection afforded by mitochondrial mitochondria when used throughout reperfusion, or added to the transplanted mitochondria, suggesting that cardioprotection by transplanted mitochondria occurs through mechanism that are not significantly modified by ROS [126]. Whether these observations represent primary or secondary effects along with more clear role of ROS in the spectrum of mitochondrial injury and recue remain open to continued investigation.

Consistent with studies in many other fields, recent studies have also shown that mitochondrial transplantation not only rescues cellular energy and function, but also replaces damaged mitochondrial DNA [131]. In vitro studies using human HeLa cells depleted of mitochondrial DNA (HeLa p^0) incapable of oxygen consumption were rescued by co-incubation with mitochondria containing intact mtDNA with significantly increased ATP content and oxygen consumption rates detected even at 2 weeks post co-incubation. Quantitative real-time RT-PCR analysis confirmed replacement of mtDNA in HeLa p^0 cells following mitochondrial transplantation. Whether the mechanism of this type of mitochondrial rescue is related to the replication of mtDNA in the transplanted mitochondria or expansion of transplanted mitochondrial population requires further characterization.

Mitochondrial Transplantation in Human Subjects

First clinical application of mitochondrial transplantation therapy has been done in 2016 in pediatric patients who suffered myocardial ischemia-reperfusion injury at our institution. Five pediatric patients in critical condition who were unable to be weaned off extracorporeal membrane oxygenation (ECMO) support due to myocardial dysfunction related to ischemia and reperfusion were treated with autologous mitochondria isolated from the patients' rectus abdominis muscle and were injected at ten different places in the most hypokinetic regions of the myocardium [142]. All five patients had significant improvement in their myocardial systolic function and all but one patient were successfully weaned off ECMO support by the second day post mitochondrial transplantation. The single patient who was unable to wean off ECMO support suffered irreversible multi-organ failure despite the recovery of myocardial function following mitochondrial transplantation. As shown in animal models, mitochondrial auto-transplantation successfully improved post-ischemic myocardial function and was not associated with adverse short-term complications related to mitochondrial delivery such as arrhythmia, intramyocardial hematoma or scarring. This case demonstrates the potential role of the novel therapy of mitochondrial transplantation to improve ventricular dysfunction following ischemia-reperfusion injury in humans. Additional experimental investigations and larger scale trials are necessary to evaluate details of safety, efficacy, dosing and modes of transplantation with paralleled improvements in our understanding of ischemia and reperfusion injury and the role of mitochondria.

Conclusion

Mitochondrial dysfunction is the principal feature of myocardial ischemic injury which prevails through reperfusion to severely compromise cardiomyocyte survival and post-ischemic ventricular function. These include alterations in mitochondrial structure, function, ionic accumulations, mitochondrial enzyme and complex

activity, high energy synthesis, mitochondrial DNA structure, transcriptomics, proteomics, and mitochondria-mediated apoptotic pathways. Mitochondrial auto-transplantation involves replacing damaged mitochondria in the ischemic heart with fresh, viable, and respiration competent mitochondria isolated from a nonischemic area of the patient's own body to circumvent damaged mitochondrial function accrued during ischemia and rescue myocardial function. This novel method has been shown to improve myocardial infarct severity, cellular viability, and ventricular function in many different animal models and preliminarily, in humans. The known mechanisms of cardioprotection by mitochondrial transplantation include increase in myocardial ATP content, changes in mitochondrial proteomics and transcriptomics, upregulation of cytokine and chemokines that enhance post-infarct myocardial function, as well as replacement of damaged mitochondrial DNA. Moreover, the advent of mitochondrial isolation to a simple, rapid and highly pure technique, permits application of this method within the clinical timeframe of most medical and surgical procedures.

Possible applications of mitochondrial transplantation abound, as mitochondrial damage underlie a vast number of disorders including Alzheimer's disease, Parkinson's disease, ischemia-reperfusion injury of the liver, kidney, brain and muscle compartments, as well as rare mitochondrial genetic disorders. Recently, mitochondrial transplantation has been shown to restore damaged mitochondrial function in Parkinson's' disease brain in rats [143], decrease cell death from ischemia-reperfusion injury in rat liver [144] and attenuate hypoxic pulmonary hypertension in rats [145]. A recent study by Hayakawa et al. described the transfer of healthy mitochondria from astrocytes to surrounding neuronal cells during transient focal cerebral ischemia to induce cell survival pathways [146].

From natural phenomena to constructed therapies, the diversified transmissibility of mitochondria and their exertive roles on cellular transformation and survival are difficult to underestimate. Much remains in question with regards to the mechanism of mitochondrial influence on cell survival as well as their specific roles in different disease models; and optimizations of safety and long-term outcomes provoke future experimental tasks and innovation. Along with many varied fields of mitochondrial disorders, mitochondrial transplantation offers a valuable strategy for cardioprotection in ischemic heart disease and opens the gateway to deeper understanding of mitochondrial function and therapeutic potential.

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Chapter 32
Mitochondria-Targeted Antioxidants for the Treatment of Cardiovascular Disorders

Hyoung Kyu Kim and Jin Han

Oxidative Stress and Antioxidant Systems

Diatomic oxygen (O_2) is essential for the survival of most species that live in aerobic environments. Biologic oxidations inevitably produce metabolites known as reactive oxygen species (ROS), examples of which include superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Mammalian tissues and cells produce ROS at various sites including mitochondrial electron-transfer complexes (ETCs), the endoplasmic reticulum, and peroxisomes [1]. The mitochondrial ETCs are the sites where oxidative phosphorylation occurs, producing large amounts of the energy-containing molecule adenosine triphosphate (ATP). Consequently, the mitochondrial ETCs are also the sites that produce the most ROS. ROS play roles as signaling molecules in various biological processes including cell differentiation, cell growth, anti-inflammation, and intercellular signaling under physiological conditions. Imbalanced ROS levels cause the detrimental oxidation of various biomolecules including protein, lipid, and DNA, resulting in dysfunction from the cellular level to the organ level, which is known as oxidative stress [1]. Oxidative stress is a

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fundamental factor in a variety of diseases including inflammatory diseases, neurodegenerative diseases, metabolic syndromes, many cancers, and cardiovascular diseases (CVDs) [1–6].

The human heart is one of the most energy-dependent organs, producing and spending approximately 30 kg ATP/day to continuously pump blood throughout the body [7]. Mitochondria make up more than 30% of its mass [8]. Mitochondrial ETCs I and III have long been known as major sites of ROS generation. A recent study demonstrated that complexes II and IV also produce ROS in disease conditions [9]. Cardiac myocytes can easily be exposed to mitochondrial oxidative stress, resulting in irreversible oxidative damage and cell death. Normal heart function cannot be maintained in the presence of widespread apoptotic or necrotic cardiac-cell death and decreased mitochondrial function in surviving cardiomyocytes, resulting in heart failure and death as a final consequence.

To prevent irreversible oxidative damage, human cells have an effective primary antioxidant system consisting of endogenous antioxidant enzymes such as manganese or copper and zinc superoxide dismutase (Enzyme Commission Number, EC 1.15.1.1), catalase (EC 1.11.1.6), glutathione reductase (EC 1.8.1.7), and multiple isoforms of glutathione peroxidase (GPx 1–8, EC 1.11.1.9) and peroxiredoxin (Prx 1–6, EC 1.11.1.15) [1, 6]. As a second line of defense, the human body has endogenous, non-enzymatic antioxidant systems, which include thiols (glutathione, lipoic acid, and N-acetyl cysteine), L-arginine, NADPH and NADH, ubiquinone (coenzyme Q10), melatonin, uric acid, bilirubin, and metal-binding proteins (ferritin, myoglobin, transferrin, albumin, ceruloplasmin, and metallothionein) [6]. In addition, exogenous antioxidants provided by food or supplements can complement the action of the endogenous antioxidants or can scavenge ROS directly. The exogenous antioxidants include some vitamins and minerals, carotenoids, polyphenols, flavonoids, and omega-3 and omega-6 fatty acids [6] (Fig. 32.1).

Antioxidant Therapy

Various antioxidant therapies have been tested to treat a variety of CVDs [10–16]. Despite some preclinical successes, most global antioxidants failed to improve, or even worsened, the targeted disease states in clinical trials [14–18] (Table 32.1). Several reasons were suggested to explain those results [17, 18].

First, the selected antioxidants were chosen not for their efficiency but instead for their availability. For example, some clinical studies used vitamins E or C, because those vitamins are relatively easy to obtain commercially. Second, there is a lack of biochemical markers to directly evaluate the antioxidant potential of the drugs. Third, the relatively low doses and short durations of the therapies were not enough to effectively treat the tissue damage caused by more than 40 years of oxidative stress in adult patients. Furthermore, the follow-up periods might not have been long enough to realize the benefits of the therapies. Fourth, the trials might not have included patients whose disease was actually related to an antioxidant deficiency. If

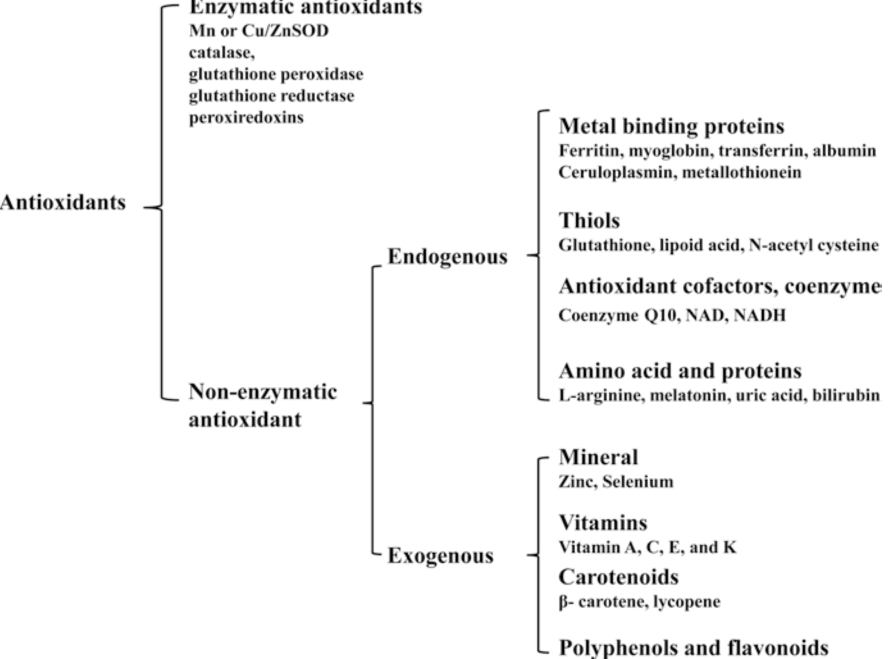

Fig. 32.1 Antioxidant systems

a patient's antioxidant levels are normal or at least sufficient to balance ROS production and removal, then antioxidant therapy would not likely provide any benefit. Finally, the antioxidants tested might not have been able to efficiently reach the mitochondria, where the major ROS generation and oxidative stress take place [32].

Despite the failures of antioxidant therapies in previous clinical trials, oxidative stress remains a focus of research on many diseases. Clinical researchers and pharmaceutical companies therefore continue to develop new strategies to provide effective antioxidant therapy.

Mitochondria-Targeted Antioxidants

In light of the unmet need for effective antioxidant therapy, mitochondria-targeted antioxidants that are selectively taken into the mitochondria in vivo have been developed over the last decade. There is accumulating evidence that the targeted antioxidant therapies are better than their nonspecific predecessors at protecting the heart against oxidative stress and damage [33, 34]. Antioxidants that selectively accumulate in the mitochondria have had beneficial effects in animal models of diseases involving oxidative damage in the mitochondria [34].

Table 32.1 Selected clinical trials in which antioxidants had no effect or had negative effects on events related to cardiovascular disorders

Study name (year) (reference)	No of patients	Subjects	Agent and dose	Study duration (years)	Outcome
Vitamin E					
ATBC (1994) [19]	29,133	Smokers with no medical problems	50 mg vitamin E	6.1	Increase in hemorrhagic stroke
GISSI (1999) [20]	11,324	Post-myocardial infarction adults	300 mg (synthetic) vitamin E	3.5	No beneficial effect
HOPE (2000) [21]	9,541	Patients with high CVD risk	400 IU (natural) vitamin E	4.5	No beneficial effect
PPP (2001) [22]	4,495	Patients with high CVD risk	300 mg (synthetic) vitamin E	3.6	No beneficial effect
MICRO-HOPE (2002) [23]	3,654	Patients with diabetes	400 IU (natural) vitamin E	4.5	No beneficial effect
VEAPS (2002) [24]	353	Patients with elevated LDL-C	400 IU dl-α-tocopherol	3	No beneficial effect
β-carotene					
ATBC (1994) [19]	29,133	Smokers with no medical problems	20 mg β-carotene	6.1	Increase in overall mortality and mortality due to ischemic heart disease, hemorrhagic stroke, and ischemic stroke
ATBC (1998) [25]	27,271	Smokers with no history of myocardial infarction	20 mg β-carotene	6.1	No beneficial effect
SCPS (1996) [26]	1,805	Patients with skin cancer	50 mg β-carotene	8.2	No beneficial effect
PHS (1996) [27]	22,071	Healthy individuals	50 mg β-carotene	12	No beneficial effect

(continued)

Table 32.1 (continued)

Antioxidant cocktails					
CARET (1996) [28]	4,060	Asbestos workers	30 mg β-carotene, 2,500 IU retinol	5.5	Increase in all-cause mortality
	14,254	Current/former smokers			No beneficial effect
ATBC (1998) [25]	27,271	Smokers with no history of myocardial infarction	50 mg vitamin E, 20 mg β-carotene	6.1	No beneficial effect
HATS (2001) [29]	160	Patients high CVD	800 IU vitamin E (as d-α-tocopherol), 1,000 mg vitamin C, 25 mg (natural) β-carotene, 100 μg selenium, simvastatin (10 mg/day)+ niacin (250–1,000 mg twice/day)	3.5	Simvastatin/niacin induced atheroregression; antioxidant cocktail resulted in stenosis progression, cardiovascular disease-related mortality, nonfatal infarction, or revascularization
HPS (2002) [30]	20,536	Patients with high CVD risk	600 mg vitamin E, 250 mg vitamin C, 20 mg β-carotene	5	No beneficial effect
WAVE (2002) [31]	423	Postmenopausal women with CVD	800 IU vitamin E, 1,000 mg vitamin C plus HRT	2.8	All-cause mortality (hazard ratio) was higher in the treatment group vs. that in the placebo group

Adapted from Kris-Etheron's review [17]
MI myocardial infarction, *HRT* hormone replacement therapy, *CVD* cardiovascular disease

There are two major issues in the development of successful mitochondria-targeted antioxidant therapies. The first is the selection of optimal antioxidants with high ROS-scavenging potential. The second is the establishment of methods to target specific tissues and mitochondria. Here, we discuss the current status of the mitochondria-targeted antioxidants (Table 32.2) [35].

Table 32.2 Mitochondria-targeted (or membrane permeable) antioxidants

Agent	Drug property	Related disease	Clinical status
MitoQ	Coenzyme Q10 derivative	NASH	Phase II (NASH)
	+TPP	Parkinson's disease	Phase II
		Aging related dysfunction	Phase I
MitoVit E (TPPB)	Vitamin E derivative + TPP	Friederich Ataxia, alcohol syndrome, ischemic neural injury, ocular disease	Animal study only
MitoPBN LPBNAH	Nitrone radical trap alpha-phenyl-tert-butylnitrone + TPP	Neuroprotection	Not yet tested
MitoPeroxidase (Ebselen analog) 1 and 2	Peroxidase derivative (ebselen) + TPP	Radiomitigative effect	Animal study only
SkQ	Plastoquinone + TPP	Aging induced dry eye	Phase II
		IR heart injury, hypertrophy	Animal study only
		Myocardial fibrosis	
MitoGSH	Glutathione + choline ester	Cardiac oxidative stress	Not yet tested
MitoNAC	N-acetyl-l-cysteine +choline ester,	Not yet tested	Not yet tested
SS (Szeto-Schiller) peptides (Bendavia)	Peptide antioxidants targeted to the inner mitochondrial membrane	IR heart injury,	Phase II (STEMI)
		Mitochondrial myopathy	Phase II
		Hypertensive cardiomyopathy	Animal study
Edaravone	Synthetic antioxidant	Brian IR injury	Used in Japan
		Cardiac IR injury,	Phase IV (AMI)
		Diabetic cardiomyopathy, cardiac stem cell therapy	Animal study
Idebenone	Synthetic antioxidant	Friedereich's Ataxia-CVD	Phase III
		MELAS	Phase II
		Duchenne muscular dystrophy	Phase III
		LHON	Drug approved
NecroX	Synthetic antioxidant, mitochondria Ca^{2+} uniporter blocker	IR heart injury	Phase II (STEMI)
		Myocardial fibrosis	Animal study
		Live injury	
		Allergic inflammation	
		IR kidney injury	
		Atherosclerosis	

(continued)

Table 32.2 (continued)

Agent	Drug property	Related disease	Clinical status
Astaxanthin	Dietary carotenoid	Hyperlipidemia	Phase IV
		Atherosclerosis, coronary heart disease and ischemic brain damage, age-related macular degeneration, acute pain, inflammation, cancer, and cardiovascular diseases	Animal study

I/R ischemia/reperfusion, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *STEMI* ST-segment elevation in myocardial infarction, *AMI* acute myocardial infarction, *NASH* nonalcoholic steatohepatitis, *MELAS* Mitochondrial Encephalopathy Lactic Acidosis & Stroke-like Episodes, *LHON* Leber's hereditary optic neuropathy

MitoQ

MitoQ (or mitoquinone mesylate) is one of the most well-known mitochondria-targeted synthetic antioxidants. MitoQ was developed in the late 1990s to penetrate the mitochondrial membrane and accumulate within the mitochondria to increase the local antioxidant capacity [36]. They used antioxidative ubiquinone, a mimetic structure of coenzyme Q10, to incorporate the lipophilic cation triphenylphosphonium (TPP), which made it easy for the positively charged molecule to target the negatively charged mitochondrial membrane and subsequently accumulate within the mitochondrial compartment (Fig. 32.2a). Within the mitochondria, ETC II, also known as succinate dehydrogenase, converts the ubiquinone of MitoQ to the active antioxidant ubiquinol, which then scavenges excess ROS in the mitochondrial matrix. After reducing the ROS, the ubiquinol is oxidized to ubiquinone and then recycled by ETC II [37] (Fig. 32.2b).

MitoQ was tested in various animal models of diseases including Alzheimer Disease [38], Parkinson's Disease [39], type I diabetes [40], fatty-liver disease [41, 42], metabolic syndrome [43, 44], and alcohol-induced steatohepatitis [42]. The ability of MitoQ to preserve organs for transplantation was also tested [45]. MitoQ showed remarkable therapeutic potential against a wide range of CVDs including ischemic heart disease [46, 47], hypertrophy [48, 49], hypertension [49], and vascular endothelial dysfunction [50]. It also demonstrated cardioprotection against endotoxin-induced heart failure [51], doxorubicin [52], and cocaine-induced cardiotoxicity [53].

Those findings strongly support MitoQ as a promising clinical therapy for CVDs. Antipodean Pharmaceuticals Inc. completed a successful Phase I clinical trial that demonstrated the safety of oral MitoQ tablets in healthy individuals. Two Phase II clinical trials were subsequently conducted in patients with Parkinson's disease (clinicaltrials.gov as NCT00329056) and hepatitis (clinicaltrials.gov as NCT00433108), respectively. In the former, patients were treated with a daily oral dose of 40 or 80 mg MitoQ for 1 year and compared with patients that received a

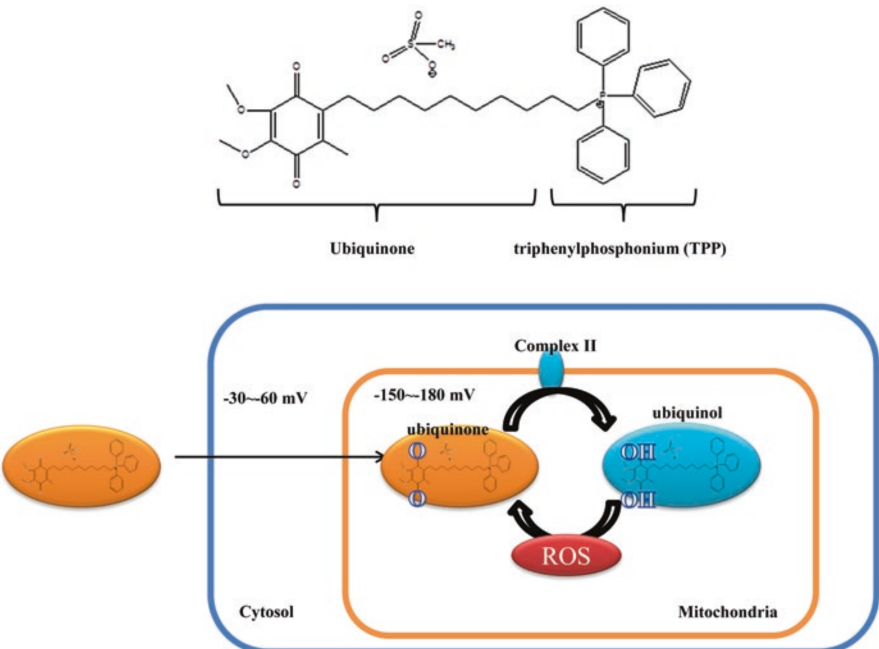

Fig. 32.2 Structure and mechanism of action of MitoQ

placebo. The trial was verified in July 2010. The results were negative, showing no benefit of MitoQ for patients with Parkinson's disease. The negative result might be explained by the relatively short period of the treatment, which was not sufficient to reverse the prolonged neuronal damage suffered by the patients prior to their diagnosis. The study did, however, provide a year's worth of safety data [54]. In the other Phase II clinical trial of MitoQ, 36 patients with chronic hepatitis C infection who were unresponsive to antiviral treatments were treated with MitoQ (40 or 80 mg) over 28 days to prevent liver inflammation, which was determined by serum alanine transaminase (ALT), aminotransferases, and viral RNA. The trial was verified in August 2008. The patients that were treated with MitoQ showed lower serum ALT and aminotransferase levels, demonstrating the therapeutic potential of MitoQ in chronic liver diseases [55].

The two clinical trials showed that it was safe to target human mitochondria with antioxidants over a prolonged period and that the efficacy of the treatment is dependent on the time course of the disease. In 2015, a new clinical trial to study the effects of MitoQ to improve physiological functions (vascular, motor, and cognitive) in middle-aged and older adults was registered on clinicaltrials.gov (NCT02597023) and began recruiting participants.

Mitochondria-Targeted Vitamin E

Together with the successful development of MitoQ, the same group produced a mitochondria-targeted vitamin E (MitoVit E) by binding the active phenolic moiety of vitamin E (α-tocopherol) with a lipophilic TPP cation [56] (Fig. 32.3). The major limitation of vitamin E, or N-acetyl cysteine, is its global distribution in cell, which renders it ineffective for the removal of mitochondrial ROS. MitoVit E showed much greater accumulation in the mitochondria with 5,000–6,000: 1 (accumulated in mitochondria: remained outside) in the incubated mitochondria with 1–20 μM MitoVit E. The accumulated MitoVit E protected the mitochondria against oxidative damage [56]. Although MitoVit E showed therapeutic potential in various models of diseases including Friedreich ataxia (FRDA) [57], aortic endothelial dysfunction [58], fetal alcohol syndrome [59], ischemic neuronal injury [60], obesity and liver disease [61], cadmium toxicity [62], and ocular diseases [63, 64], its therapeutic potential in CVDs has not yet been tested.

MitoPBN

MitoPBN is a derivative form of α-phenyl-*N*-*tert*-butylnitrone (PBN) (Fig. 32.3). Unlike other mitochondria-targeted antioxidants, MitoPBN specifically reacts with carbon-centered radicals but not with superoxide [65]. It was developed to counter the effects of ROS on the activity of uncoupling proteins, which readily accumulate at 2.2–4.0 mM concentrations in mitochondria [65, 66]. The therapeutic effects of MitoPBN have not yet been tested in any disease condition. Another derivative of PBN, *N*-[4-(octa-*O*-acetyllactobionamidomethylene) benzylidene]-*N*-[1,1-dimethyl-2-(*N*-octanoyl) amido]-ethylamine *N*-oxide (LPBNAH), has shown antioxidative and neuroprotective activity in preclinical experiments [67]. The cardioprotective effects of mitochondria-targeted PBN derivatives need to be investigated further.

MitoVit E

Mito PBN

Fig. 32.3 Structure of MitoVit E and Mito PBN

MitoPeroxidase (Ebselen Analog)

MitoPeroxidase is a conjugated compound consisting of the peroxidase mimetic ebselen and a TPP cation [68]. Ebselen, first synthesized in 1924, effectively degrades peroxides and also shows antioxidant activity. The conjugation of ebselen and TPP only slightly enhanced the mitochondrial uptake of ebselen, because the ebselen moiety covalently binds to proteins. MitoPeroxidase enters the mitochondria, and mitochondrial GSH or thioredoxin converts it to effective antioxidants that can remove peroxides and prevent oxidative stress [68]. Recently, Stoyanovsky et al. developed MitoPeroxidase-2 [69], a modified version of MitoPeroxidase (Fig. 32.4). Whereas MitoPeroxidase showed cytotoxicity in mouse embryonic cells at concentrations of 10–20 μM, MitoPeroxidase-2 showed comparatively lower cytotoxicity at a concentration of ~40 μM. In a test of the therapeutic potential of MitoPeroxidase-2 against ionizing radiation-induced cell death, MitoPeroxidase-2 showed radiomitigative properties in radiation-exposed mice [69]. The application of the MitoPeroxidases has so far been limited, so more clinical investigations are required to test their therapeutic potential in CVDs.

SkQ

SkQ is an organic, cationic plastoquinone derivative containing positively charged phosphonium or rhodamine moieties (Fig. 32.5) [70]. SkQ was developed by Antonenko et al. to overcome the unexpected negative effect of MitoQ, which

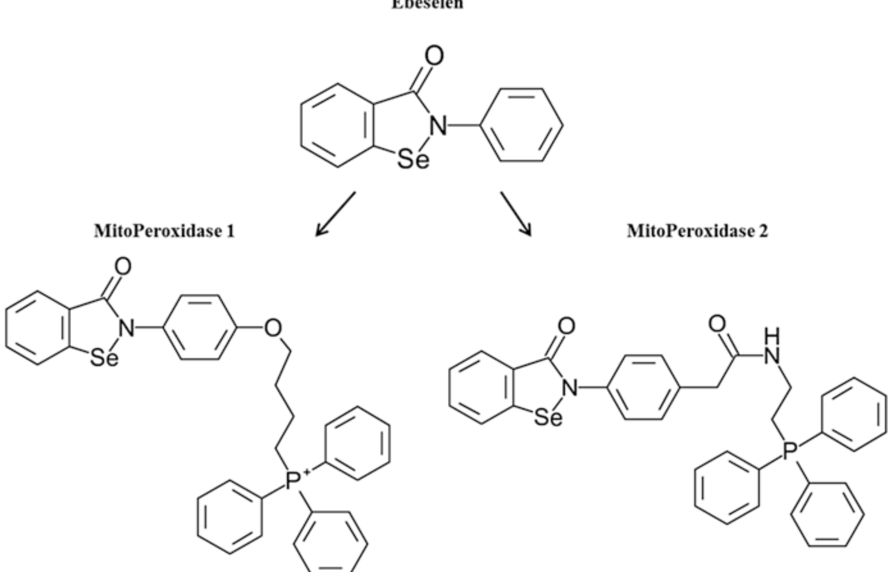

Fig. 32.4 Structure of ebselen, MitoPeroxidase 1 and 2

Fig. 32.5 Structure of SkQ

sometimes acts as a pro-oxidant, depending on the treatment dose [37]. For that purpose, the ubiquinone in MitoQ was replaced with plastoquinone, which has better antioxidant capacity. SkQ strongly targets the mitochondria and has remarkably enhanced antioxidant potential, which inhibited H_2O_2-induced apoptosis at very low (picomolar) concentrations in cell culture [70]. A nanomolar oral dose of SkQ1 (0.02 nmol/day for 3 weeks) abolished ROS-induced or Ischemia/Reperfusion (IR)-induced cardiac arrhythmia in ex vivo rat hearts. Higher doses of SkQ1 (125–250 nmol/day for 3 weeks) decreased MI in an in vivo IR model, lowering plasma levels of cardiac damage markers including lactate dehydrogenase and creatin kinase [71]. SkQ1 treatment also effectively attenuated age-associated myocardial diseases, cardiac hypertrophy, and myocardial fibrosis in a mouse model of aging [72].

SkQ has been used to treat various age-related pathologies [73]. A successful Phase II clinical trial (NCT02121301) showed that SkQ1 is a safe and effective treatment for dry-eye syndrome [74]. Preclinical and clinical studies of SkQ to treat CVDs have been limited. Because SkQ showed low toxicity and high antioxidant potential at low doses, it would likely be worthwhile to test the therapeutic potential of SkQ in cardiovascular disorders.

MitoGSH and MitoNAC

MitoGSH and MitoNAC are mitochondria-targeted forms of gluthione choline ester and *N*-acetyl-l-cysteine choline ester, respectively (Fig. 32.6). Both compounds were developed by the Shey-Shing Sheu group [33]. The hydrophilic forms of GSH and NAC were designed to easily enter and accumulate in the mitochondria. MitoGSH protected mitochondria from H_2O_2-induced oxidative stress in neonatal rat ventricular myocytes. The treatment of those cells with 50 μM MitoGSH preserved the mitochondrial membrane potential against H_2O_2-induced depolarization [33]. There have since been no follow-up studies of MitoGSH and MitoNAC.

Fig. 32.6 Structure of MitoGSH and MitoNAC [33]

MitoGSH

MitoNAC

SS Peptides

SS (Szeto-Schiller) peptides (SS-01, 02, 31, and 20) are short-sequence, antioxidant tetrapeptides that were designed by Szeto and Schiller to target the inner mitochondrial membrane (IMM) [75]. Because mitochondrial ETCs are located in the IMM, targeting the IMM could be a brilliant strategy to reduce mitochondrial ROS levels. The SS peptides possess alternating basic and aromatic residues, providing them with ROS scavenging ability (see [75, 76] for detailed sequences). The cationic SS peptides interact with the negative charge of the IMM potential to enter the mitochondria, reaching concentrations in the IMM that are up to 1,000-fold higher than those in the cytoplasm.

SS-02 and SS-31 prevented tBHP-induced ROS overproduction and sequential membrane-potential depolarization and MPTP opening in neuronal cells [77]. SS-31 pretreatment in a mouse model of islet transplantation prevented mitochondrial depolarization, reduced apoptosis, increased cell yield, and improved post-transplantation function in the islet cells, suggesting that SS-31 might have clinical value in organ transplantation [78]. In other animal models, SS-31 treatment protected kidneys from IR injury [79, 80], unilateral ureteral obstruction [81], and diabetic nephropathy [82] by reducing mitochondrial damage and preserving mitochondrial energy metabolism. SS-31 protected neurons from damage in models of cognitive deficits [83, 84] and protected or recovered mitochondrial function in skeletal muscle damaged by aging [85] and burn trauma [86, 87].

Both SS-02 and SS-31 showed significant cardioprotective effects in ex vivo animal models of IR [75, 88]. Treatment with SS-02 (100 μM) or SS-31 (1 nM) during the reperfusion period significantly improved the contractility of IR-injured hearts. Cho et al. tested the cardioprotective effects of both peptides in an in vivo rat model of left anterior descending artery ligation and found that they significantly reduced IR-induced arrhythmia, lipid peroxidation, and infarct size [89]. In a study of hyper-

tensive cardiomyopathy, 4 weeks of SS-31 treatment attenuated angiotensin II (Ang)-induced cardiomyopathy without changing blood pressure [90]; however, treatment with SS-20, a non-antioxidant peptide, did not protect the heart at all. Those results suggest that the cardioprotective effects of SS-02 and SS-31 are facilitated by the mitochondria-targeted antioxidative properties of the peptides.

Bendavia (or Elamipretide), a drug form of SS-31, was developed by Stealth BioTherapeutics and applied in clinical trials to treat heart failure. The PREVIEW study – a Phase I trial (NCT02388464) to study the safety, tolerability, and pharmacokinetics of escalating doses of Bendavia in patients with heart failure – was completed in September 2015. According to Stealth BioTherapeutics, the Bendavia treatments demonstrated acceptable safety and tolerability and improved heart function in the patients (report not yet published).

The Phase II EMBRACE trial (NCT01572909) [91] enrolled 300 patients with ST-segment elevation myocardial infarction (STEMI). The patients received intravenous infusion of either Bendavia (0.05 mg/kg/h) or a placebo (60 mL/h). The primary endpoint was infarct size. Secondary endpoints included physiological and clinical outcomes [91]. The EMBRACE study was completed in November 2015, and its results are yet to be released.

In addition to the studies of CVDs, the Phase II MMPOWER trial (NCT02367014) was conducted to evaluate the safety and efficacy of Bendavia in 36 patients with mitochondrial myopathy associated with genetically confirmed mitochondrial disease. The patients received Bendavia infusions over a period of 5 days. The study was completed in May 2016. According to Dr. Amel Karaa, the trial investigator, Bendavia passed its safety and tolerability endpoints and enhanced the exercise capacity of the patients (data unpublished).

Edaravone

Edaravone (also known as MCI-186 or Radicut) is a scavenger of free radicals that has been used to treat acute ischemic stroke (Fig. 32.7). There is substantial clinical and experimental evidence demonstrating the neurovascular-protective effects of edaravone (for detail information see the review article [92]). The cardioprotective effects of edaravone have been studied in various conditions including diabetic cardiomyopathy [93], cardiac stem-cell therapy [94], and isoproterenol-induced cardiac toxicity [95]. It is unclear whether edaravone specifically targets the mitochondria or accumulates in any particular mitochondrial compartment. Edaravone did, however, demonstrate remarkable mitochondria-protective potential in a model of myocardial infraction [96].

In a Phase IV clinical trial (NCT00265239) examining the effects of edaravone in patients with acute myocardial infarction, edaravone prevented myocardial injury following IR [97]. Intravenous infusion of edaravone (30 mg) before myocardial reperfusion reduced myocardial infarction and enhanced ejection fraction at early periods of reperfusion [97].

Edravone

NecroX

Idebenone

Fig. 32.7 Structure of edravone, NecroX and idebenone

Idebenone

Idebenone (also known as CV-2619) is a CoQ10 analog developed in the 1980s by Takeda Pharmaceuticals (Fig. 32.7) [98]. Idebenone has properties similar to those of CoQ10, a mitochondrial coenzyme that acts as an antioxidant and electron carrier [99, 100]. Idebenone differs structurally from CoQ10 in that it has a shorter lipophilic tail, which reduces its lipophilicity. The extreme lipophilicity of CoQ10 limits its localization and action to membranes, whereas idebenone localizes and functions in membranes, the cytoplasm, and the mitochondrial matrix [98]. In the mitochondria, idebenone plays multiple roles including those of an electron carrier in the ETC, a complex I inhibitor, a complex II-III activator, and an antioxidant (for the detailed mechanism, see the review [98]).

Because of its unique properties related to mitochondrial regulation, pharmaceutical companies developed idebenone to treat mitochondrial dysfunction in diseases including Alzheimer disease [101], FRDA (Phase II, NCT00229632; Phase III, NCT01303406) [102, 103], Duchenne muscular dystrophy (Phase III, NCT01027884) [104, 105], mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS; Phase II, NCT00887562) and Leber's hereditary

optic neuropathy (LHON; Phase II, NCT00747487) [106]. Based on the results of clinical trials, the European Medicines Agency approved idebenone (Raxone) for the treatment of LHON, and the drug has been available on the European market since 2015 [106].

The therapeutic potential of idebenone in cardiac hypertrophy or cardiomyopathy was investigated in patients with FRDA [102, 103, 107–110]. The results suggested that idebenone has positive effects on cardiac hypertrophy and cardiomyopathy; however, the efficacy of idebenone is still debated with regards to the conditions of the studies [103]. The cardioprotective effect of idebenone needs to be investigated further in the broad range of heart diseases.

NecroX (LC28-0126)

NecroX (C25H31N3O3S · 2CH4O3S) is a novel mitochondria-targeted antioxidant developed by LG life science (Fig. 32.7) [111]. NecroX shows strong antioxidant effects in in vitro models of oxidative stresses induced by tBHP, doxorubicin, CCl4, or hypoxic injury. NecroX prevented oxidative stress-induced mitochondrial dysfunction [112, 113] and showed therapeutic potential in animal models of diseases including hepatic IR injury [114], myocardial fibrosis [115], myocardial IR injury [113], drug-induced liver injury [116], allergic inflammation [117], renal IR injury [118], and atherosclerosis [119, 120].

A Phase I clinical trial of NecroX (named LC28-0126 at clinicaltrials.gov, NCT01737424) to investigate the drug's safety, tolerability, and pharmacokinetics in healthy males was finished successfully. A Phase II trial (NCT02070471) to evaluate the efficacy, safety and pharmacokinetics of NecroX prior to percutaneous coronary intervention in patients with STEMI was completed in September 2015. An additional Phase II trial (NCT02770664) to further study NecroX under those conditions has been registered and is recruiting patients.

Astaxanthin

Astaxanthin is a membrane-permeable dietary carotenoid found in various marine organisms (shrimp, lobster, crab, and salmon), algae, fungi, and plants (Fig. 32.8) [35, 121]. The antioxidant potential of astaxanthin is 100–1,000 times greater than those of other organic antioxidants including vitamins C and E, CoQ10, and polyphenols [35]. The mitochondrial localization of astaxanthin was tested in blastocysts [122]. The antioxidant and mitochondria-protective effects of astaxanthin were demonstrated in human mesangial cells in a model of diabetic nephropathy [123], in neuronal cells [124, 125], in blastocysts [122], in vitamin E-deficient rats [126], in a model of Parkinson's disease [127, 128], in a model of liver IR injury

Astaxanthin

Fig. 32.8 Structure of astaxanthin

[129], and in models of CVDs including various animal models of IR injury and hypertensive rats [121, 130–134].

From 2000 to 2010, various clinical trials investigated the therapeutic potential of astaxanthin in both healthy and disease conditions. In healthy individuals, astaxanthin showed dose safety, antioxidant effects, reduced oxidation of lipids, and anti-inflammation effects, which might help to reduce the risk of CVDs (for detailed information about the clinical trials, see the review of Fassett and Coombes [121]).

In recent human trials, the effects of pure or antioxidant-containing medical-food formulations of astaxanthin on the cardiovascular system were tested. In a Phase IV clinical trial (NCT01490229) entitled "Ezetimibe Versus Nutraceuticals in Statin-intolerant Patients (ECLIPSE)", an astaxanthin-containing nutraceutical improved the lipid profiles of statin-intolerant patients with coronary heart disease without producing any adverse side effects [135]. Similarly, 8 weeks of treatment with an astaxanthin-containing medical food (PDL-0101) significantly reduced triacylglycerol levels in patients with hyperlipidemia (Pilot study, NCT02582424) [136]. In contrast, 12 months of treatment with astaxanthin alone showed no beneficial effects in recipients of renal transplants (ACTRN12608000159358) [137].

Albeit there are not yet any direct studies of the effects of astaxanthin in cardiovascular patients, the demonstrated safety, antioxidant potential, and beneficial metabolic effects of astaxanthin suggest that it could have therapeutic potential in CVDs.

Next-Generation Mitochondria-Targeted Antioxidants

Recently, several novel mitochondria-targeted antioxidants were developed that can be considered next-generation drug candidates [138–141].

Mitochonic Acid 5 (MA-5) is a new indole derivative synthesized by Suzuki et al. [138]. MA-5 enhanced ATP production and reduced mitochondrial ROS levels without affecting the activity of the mitochondrial OXPHOS complex [139]. MA-5 can bind to mitofilin, an IMM protein. In mice with dysfunctional mitochondria and

IR injury, MA-5 improved the function of cardiac and renal mitochondria [139]. The mitochondria-targeted clinical potential of MA-5 was tested in fibroblasts of patients with different mitochondrial diseases including Leigh syndrome, MELAS, LHON, and Kearns-Sayre syndrome. MA-5 prolonged cell survival and increased the ATP production in all the fibroblasts with failed mitochondrial OXPHOS [138]. Those results suggest that MA-5 could have therapeutic value for patients with genetic mitochondrial diseases and mitochondria-associated cardiac and renal diseases because of its ATP compensation and antioxidant effects.

Mitochondrial CPP 1 (mtCPP-1), a novel mitochondria-targeting peptide, is a modified version of SS-31 with enhanced mitochondrial targeting and antioxidant capacity [140]. One of a series of developed peptides (mtCPP-1~9), mtCPP-1 showed greater mitochondria-targeting efficiency (1.8-fold) and antioxidant potential (twofold) than an equivalent concentration of SS-31. Remarkably, mtCCP-1 did not affect the mitochondrial membrane potential and ATP level, which were reduced by SS-31 treatment [140]. Those advantages make mtCCP-1 more suitable for clinical application and overcome some of the shortcomings of previous mitochondria-targeting peptides.

Kyotorphin-nitroxide hybrid molecules are novel synthetic lipophilic molecules that conjugate nitronyl nitroxide to Kyotrophin (KTP; l-Tyr-l-Arg) [141]. Among the ten such compounds synthesized, compounds 9i and 9j showed strong antioxidant effects and protected mitochondria against IR injury in vitro [141]. Investigation of the exact working mechanism and follow-up studies are needed to further develop these compounds as therapeutic candidates.

Conclusion

The integrity of mitochondrial function is essential for cardiac homeostasis. Excess mitochondrial ROS in cardiomyocytes leads inevitably to oxidative stress, damaging the mitochondria, causing heart function to deteriorate. Antioxidant therapy was suggested as a promising therapeutic strategy for a variety of cardiovascular disorders, but a number of critical obstacles blocked the successful connection between scientific benches and clinical beds. Although only a few clinical studies showed benefits of mitochondria-targeted antioxidants in the treatment of cardiovascular disorders, properly timed treatments with optimal mitochondria-targeting antioxidants are likely to achieve breakthrough therapeutic results in the near future. In addition to the development of optimal mitochondria-targeted antioxidants, techniques to selectively deliver the drugs to cardiac tissues (e.g. ventricle, atrium, and coronary arteries or nodes) and to damaged mitochondria are also necessary for successful clinical treatment.

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