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Yong-Xiao Wang *Editor*

Pulmonary Vasculature Redox Signaling in Health and Disease

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Preface

This book is composed of 26 chapters contributed by numerous outstanding basic, translational, and physician scientists in the fields of pulmonary vasculature redox signaling in health and disease; thus, it offers a widespread and comprehensive overview for academic and industrial scientists, postdoctoral fellows, and graduate students who are interested in redox signaling in health and disease and/or normal and pathological functions of the pulmonary vasculature. The book may also be very valuable for clinicians, medical students, and allied health professionals.

Redox signaling is a major molecular process involved in almost every physiologic cellular response in the pulmonary vasculature including energy metabolism, host defense, gene expression, contraction, proliferation, and migration. Aberrancy in this important signaling pathway leads to a critical role in the development of nearly all pulmonary diseases, such as pulmonary hypertension, cor pulmonale, pulmonary edema, and vasculitis, among others. These well-recognized concepts with recent advances have been comprehensively described by Prof. Jeremy Ward in his chapter "From Physiological Redox Signalling to Oxidant Stress."

Key members of redox signaling are reactive oxygen species (ROS), e.g., superoxide and hydrogen peroxide. These well-known molecules usually participate in specific oxidation or reduction modifications of one or more targeted molecules in order to mediate pulmonary vasculature cellular responses. ROS can be produced by mitochondria, NADPH oxidase (Nox), or other sources. In the chapter "Crosstalk Between Mitochondrial Reactive Oxygen Species and Sarcoplasmic Reticulum Calcium in Pulmonary Arterial Smooth Muscle Cells," Dr. Tengyao Song et al. have delivered detailed information with respect to the contribution and crosstalk of mitochondria and Nox in hypoxia-induced ROS production in pulmonary artery smooth muscle cells (PASMCs). These authors have further reported new data demonstrating that reciprocal interplays between mitochondrial ROS and sarcoplasmic reticulum Ca^{2+} signaling are important to hypoxic cellular responses in PASMCs and hypoxic pulmonary hypertension. In support, Drs. Qiujun Yu and Stephen Chan have highlighted the importance of mitochondrial ROS in pulmonary vascular endothelial cells to mediate the development of pulmonary hypertension in their chapter titled "Mitochondrial and Metabolic Drivers of Pulmonary Vascular Endothelial Dysfunction in Pulmonary Hypertension." In the chapter "Adventitial Fibroblast Nox4 Expression and ROS Signaling in Pulmonary Arterial Hypertension," Profs. Scott Barman

and David Fulton have further provided emerging evidence that Nox4-mediated ROS signaling in adventitial fibroblasts plays an important role in pulmonary hypertension. Similarly, Drs. Megha Sharma and Adeleye Afolayan have meticulously presented convincing evidence for the critical roles of mitochondrial and Nox-mediated redox in the development of pulmonary hypertension of newborns in their chapter "Redox Signaling and Persistent Pulmonary Hypertension of the Newborn."

Typically, ROS are metabolized and degraded by superoxide dismutase (SOD), glutathione peroxidase, catalase, or other antioxidant enzymes. This fact, together with the view that increased ROS production may cause cardiovascular disease development and progression, has led to preclinical and clinical trials of antioxidant interventions for cardiovascular disorders including pulmonary hypertension. Prof. Nozik-Grayck and her associates have provided a thorough overview of the roles of different SOD isoforms in the physiological and pathological cellular responses in the pulmonary circulation in their chapter titled "Redox regulation of the superoxide dismutases SOD3 and SOD2 in the pulmonary circulation." Consistent with the therapeutic roles of endogenous antioxidant enzymes, Dr. Gerald Maarman has reported new and ample data to draw notable attention to the potential effectiveness of melatonin as a natural antioxidant pulmonary hypertension therapy in his chapter "Natural Antioxidants as Potential Therapy, and a Promising Role for Melatonin Against Pulmonary Hypertension."

The potential roles of sophisticated redox signaling in the pulmonary vasculature are further reinforced by the recent experimental indications that reactive nitrogen species (e.g., NO) and reactive sulfur species (e.g., H_2S) are likely to be important for a number of physiological and pathological pulmonary vascular cellular responses. In the chapter "A Brief Overview of Nitric Oxide and Reactive Oxygen Species Signaling in Hypoxia-Induced Pulmonary Hypertension," Profs. Ariel Jaitovich and David Jourd'heuil have provided a systemic summary of the interactive roles of NO and ROS in the development of pulmonary hypertension. Largely based on their own research, Prof. You-Yang Zhao and his colleague have articulated new molecular mechanisms for the roles of NO and relevant nitrative stress in pulmonary hypertension in their chapter "Molecular Basis of Nitrative Stress in the Pathogenesis of Pulmonary Hypertension." In complement, the chapter "Redox Mechanisms Influencing cGMP Signaling in Pulmonary Vascular Physiology and Pathophysiology" from Prof. Michael Wolin's group has systematically elaborated the functional importance of redox-mediated, NO-dependent cGMP signaling in physiological and pathological cellular responses in the pulmonary vasculature. Equally interestingly, Drs. Jesus Prieto-Lloret and Philip I Aaronson have contributed an excellent chapter, "Hydrogen Sulfide as an O_2 Sensor: A Critical Analysis," that provides a comprehensive indication that H_2S may function as an O_2 sensor and play an important role in hypoxic responses in pulmonary vascular cells.

Redox signaling may mediate cellular responses in a temporally and spatially dynamic manner. Redox molecular processes can also occur in a specific fashion, dependent on cell type. The pulmonary vasculature is composed of different types of cells including smooth muscle cells, endothelial cells,

adventitial cells, fibroblasts, neutrophils, macrophages, lymphocytes, and stem/progenitor cells. Thus, each of these distinctive types of cells may produce its own specific redox signaling in response to distinctive stimuli. The functional crosstalks within and among individual cells are likely to further make redox signaling more effective and specific in the pulmonary vasculature. In addition to the aforementioned chapters, Dr. Karthik Suresh and Larissa Shimoda elegantly review the reciprocal roles of ROS and Ca^{2+} signaling in endothelial cells as key players in mediating pulmonary hypertension in their chapter "Endothelial Cell Reactive Oxygen Species and Ca^{2+} Signaling in Pulmonary Hypertension." Another well-written chapter, "Metabolic Reprogramming and Redox Signaling in Pulmonary Hypertension" by Dr. Lydie Plecitá-Hlavatá et al., has provided a systemic overview of redox-dependent metabolic reprogramming in almost all types of cells in the pulmonary vasculature and their contributions in the development of pulmonary hypertension.

Redox molecules have their own intrinsic physicochemical properties (e.g., redox potential, life time, and diffusive ability), diverse physiological functions, unique spatial and temporal profiles, and also distinctive metabolic products. The contribution of specific redox molecules and relevant cellular processes may vary with the development and progression of different pulmonary vascular diseases. The complexity and diversity of redox systems indicate that general antioxidants may not have sufficient accessibility to target molecules to produce specific actions. These may well explain the relatively low efficiency of generalized antioxidants in clinical trials. In the chapter titled "Subcellular Redox Signaling," Prof. Qinghua Hu's team has provided broad and detailed discussions on the subcellular ROS signaling in PASMCs, with particular focus on the mechanisms of subcellular ROS production and potential use of exogenous mitochondria in the treatment of pulmonary hypertension. Compatibly, Drs. Ryota Hashimoto and Sachin Gupte have further dedicatedly depicted the significance of interactive roles of redox molecules between the cytosol and mitochondria in pulmonary hypertension in their chapter "Pentose Shunt, Glucose-6-Phosphate Dehydrogenase and NADPH Redox, and Stem cells in Pulmonary Hypertension."

Indubitably, innovative state-of-the-art methods and techniques are very helpful in the elucidation of redox functions and processes in the cell. For instance, the use of the powerful electron paramagnetic resonance (EPR) spectrometry makes in vitro and in vivo studies of previously indescribable redox molecules possible. Recently identified redox biosensors may specifically and clearly outline changes of redox molecule levels in cells and even in separate cellular compartments, collecting important data that would clarify previously conflicting results. Similarly, novel and highly selective labeling agents are now available to investigators to help detect uncommon redox modifications within and outside the cell. Furthermore, innovative proteomic, gene mapping and other methods have been introduced to monitor posttranslational modifications of redox proteins and enzymes as well as complex redox responses. These modern approaches and techniques have opened the door to entirely new areas of redox studies, and more importantly, will pave the way for life-saving interventions. Very appreciatorily, Prof. Steven Qian

and his associate have updated the current techniques and methods for measurements of intracellular ROS in their chapter "Techniques for Detecting Reactive Oxygen Species in Pulmonary Vasculature Redox Signaling."

The precise functional roles, signaling processes, and molecular mechanisms of redox molecules, particularly in the formation and progression, are very complex and still remain mostly elusive. Further basic and translational research on redox signaling in the pulmonary vasculature will significantly promote discoveries of new and more effective antioxidants and redox-regulatory drugs for treatment of pulmonary vascular diseases. Dr. Annarita Di Mise along with Prof. Yun-Min Zheng has detailed the current knowledge on the possible essential roles of transcription factors for the initiation and progression of pulmonary hypertension in the chapter "Role of Transcription Factors in Pulmonary Artery Smooth Muscle Cells: Focus on Pathogenesis of Hypoxia Pulmonary Artery Hypertension." The chapter that Prof. Laura Bosc and his colleagues have written, "Altered Redox Balance in the Development of Chronic Hypoxia-Induced Pulmonary Hypertension," expounds the significant contribution of redox-dependent activation of the nuclear factor activated T-cells in pulmonary hypertension. Moreover, my own group has composed a chapter titled "Emerging Role of MicroRNAs and Long Noncoding RNAs in Health and Disease Pulmonary Vasculature" to further elucidate the functional impacts of the transcriptional and nontranscriptional regulation of various key molecules by microRNAs and long noncoding RNAs in physiological and pathological responses in pulmonary vascular cells.

Recent studies reveal that redox signaling is necessary for the normal function and development of the pulmonary vasculature. Prof. Christina Pabelick and her associates fully explicate the importance of redox-mediated hyperoxic signaling in postnatal vascular and alveolar development in their chapter titled "Effects of Hyperoxia on the Developing Airway and Pulmonary Vasculature." In complement, the chapter by Dr. Michael Thompson et al. with a title "Hypoxia and Local Inflammation in Pulmonary Artery Structure and Function" exquisitely delineates whether and how redox signaling mediates the effects of hypoxia and inflammation on pulmonary structure and function.

In addition to the well-documented pulmonary hypertension as described above, a series of recent studies demonstrate that redox signaling plays a key role in several other acute and chronic pulmonary diseases; the role of redox singling in each disease is mediated by one or more unique molecular mechanisms. Prof. Stephen Black's team has provided a full review highlighting how the Nox4-relied ROS signaling in endothelial cells and fibroblasts contributes to acute lung injury and respiratory distress syndrome, two of the most common and severe pulmonary illnesses, in the chapter titled "ROS Signaling in the Pathogenesis of Acute Lung injury (ALI) and Acute Respiratory Distress Syndrome." The chapter "Lung Ischemia/Reperfusion Injury: The Role of Reactive Oxygen Species," written by Prof. Norbert Weissmann and his colleagues, includes an extensive review about the actions of ROS and reactive nitrogen species with Nox, xanthine oxidases, nitric oxide synthases, and mitochondria to involve in ischemia/reperfusion-mediated

lung injury. The two excellent laboratories led by Prof. Yunchao Su and Li Zuo, respectively, contribute the chapter "Redox-Dependent Calpain Signaling in Airway and Pulmonary Vascular and Remodeling in COPD" and "Reactive Oxygen Species in COPD-Related Vascular Remodeling." The former chapter focuses on the role of reactive oxygen and nitrogen species-reliant calpain signaling in the development of airway and pulmonary vascular remodeling in COPD, while the latter chapter emphasizes the potential utilization of anti-inflammatory and antioxidative agents against ROS-mediated impairments of pulmonary functions in treatments of COPD.

A major cause of death in pulmonary diseases, particularly COPD, is right ventricular failure. It is not surprising that Prof. Yuichiro Suzuki's laboratory has conducted a series of excellent investigations with respect to the functional significance of right ventricular redox signaling for many years. As such, he and his associate have written an elegant chapter "Redox Signaling in the Right Ventricle," in which they thoughtfully deliberate the current compelling molecular and biochemical understandings of redox signaling-mediated right ventricular functional abnormalities and failure in pulmonary hypertension.

Finally, I sincerely express my wholehearted gratitude to all of the authors for their dedicated and diligent contributions. Many of the authors of this book have played a unique role as well as the reviewers; as such, their additional efforts are further highly appreciated. I also wish to thank Ms. Dana Bigelow, Associate Editor at Springer Nature in New York, and Mr. Silembarasan Panneerselvam, Project Coordinator (Books) for Springer Nature, for their kind patience and assistance.

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Dr. Yong-Xiao Wang is a Full Professor in the Department of Molecular and Cellular Physiology at Albany Medical College, Albany, New York, USA. He received his M.D. at the Wannan Medical College (China), Ph.D. at the Fourth Military Medical University (China), and postdoctoral training at the Technical University of Munich (Germany) as well as the University of Pennsylvania (USA). Dr. Wang's primary research interests have focused on basic, translational, and drug discovery research concerning cardiac arrhythmias and hypertrophy, pulmonary hypertension, asthma, and diabetes for over 30 years. In particular, he has had extensive research experience in the studies of the molecular geneses, regulatory mechanisms, network pathways, physiological functions, and the critical roles of calcium, redox, inflammatory, and other signaling in the aforementioned illnesses. Dr. Wang has been the corresponding author, first author, and key contributor in numerous publications in highly peer-reviewed journals including ***Antioxid Redox Signal***, ***Proc Natl Acad Sci USA***, ***Free Radic Biol Med***, ***Cell Calcium***, ***J Gen Physiol***, ***J Biol Chem***, ***FASEB J***, ***Nature***, ***Circ Res***, and more. He has been the editor of several academic books in the field including a recent one entitled ***Calcium Signaling in Airway Smooth Muscle Cells*** that was published by Springer in 2014. Dr. Wang has been the principal investigator on multiple research grants and awards from the National Institutes of Health, American Heart Association, American Diabetes Association, and other agencies. He has collaborated with numerous well-known scientists, served on various grant review panels, been the Editor-In-Chief and Editorial Board Member for several scientific journals, and trained a number of scholars, some of whom have become well-known independent investigators in the field.

Adventitial Fibroblast Nox4 Expression and ROS Signaling in Pulmonary Arterial Hypertension

Scott A. Barman and David Fulton

1 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a progressive disease of the lung vasculature, which is characterized by sustained pulmonary arterial pressure, resulting in increased pulmonary vascular resistance, with eventual right heart failure [1]. Vascular remodeling caused by the medial hyperplasia of pulmonary artery (PA) smooth muscle cells is a hallmark feature of PAH [2], which causes occlusion of the vessels [2]. In most forms of PAH, muscularization of small distal PA occurs [3], and is further characterized by excessive vascular cell proliferation, inward remodeling, rarefaction, and a loss of compliance of the pulmonary blood vessels [3–5]. Increased resistance to blood flow and more rigid blood vessels (loss of vascular compliance) leads to failure of the right ventricle and eventual death. PAH is more frequent in women than men, and left untreated has a survival time of 5–7 years post diagnosis [6]. From a therapeutic standpoint, there are a number of vasodilator drugs that are indicated for the treatment of PAH, but none of the current therapeutics offers long-term success for survival due to limited effectiveness and unwanted side effects [7], and more importantly, do not address the underlying causes of the disease [1].

2 Reactive Oxygen Species, NADPH Oxidase and PAH

The generation of reactive oxygen species (ROS) is well recognized as a pathophysiological mechanism underlying the vascular remodeling and proliferation that occurs in PAH. The major ROS that are produced in the pulmonary vasculature are superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and hydroperoxyl radical ($HO^{\cdot}{}_2$) [8]. Of these ROS, both O_2^{-} and H_2O_2 activate multiple signaling pathways that cause cell proliferation and apoptosis, elevated vascular tone, fibrosis, and inflammation, which are all hallmark signs of PAH [8]. However, the cellular origin and functional significance of ROS in PAH remain poorly delineated. Elevated levels of ROS in PAH occur because there is increased production and decreased enzymatic degradation of the ROS moieties, of which, evidence exists for both phenomena in the underlying etiology of elevated

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pulmonary arterial pressure and pulmonary arterial resistance [9–13]. The major intracellular sources of ROS include the mitochondrial electron transport chain, abnormal oxygenase activity, and the NADPH oxidase family (Noxes) [14, 15]. The human genome encodes five Nox isoforms, and four of these isoforms, Nox1, Nox2, Nox4, and Nox5 are expressed in vascular cells. In relation to other sources of ROS, Nox enzymes are regarded as professional ROS generators and are capable of generating high levels of ROS in a spatial and temporal manner. Nox1, Nox2, Nox3, and Nox4 are bound to p22phox, and Nox1 and Nox2 are activated by binding numerous cytosolic subunits, including p47phox, p67phox or NOXO1 and NOXA1. In contrast, Nox4 is regarded as a constitutively active enzyme with ROS levels primarily controlled by changes in gene expression [16, 17]. In mice, genetic deletion of Nox2 has been shown to reverse hypoxia-initiated PAH [10], and Nox1 has been shown to be an important signaling mediator for both systemic and pulmonary arterial hypertension [18, 19].

Specific to the scope of this review, increased expression of Nox4 has been reported in human PAH [20]. However, despite these observations, the functional significance of Nox4 in the development of PAH is poorly understood. Nox4 is expressed in all three layers of the vascular wall [21, 22], is constitutively active [23], and whose expression is increased by a plethora of diverse stimuli including angiotensin II, TGF-β, TNF-α, γ and hypoxia [24, 25]. In addition to human PAH, Nox4 expression is upregulated in the pulmonary vasculature of hypoxia-exposed mice, and in rat models of PAH [20, 22]. Evidence suggests that Nox2 may be involved in the induction of Nox4 to cause ROS production and subsequent pulmonary arterial smooth muscle cell (PASMC) proliferation that is characteristic of PAH [20]. Specifically, it is thought that initial activation of Nox2 induces the production of Nox4 in pulmonary endothelium to initiate events that cause pulmonary arterial remodeling [20].

Evidence also suggests that Nox4-derived ROS mediates both rodent and human PASMC proliferation under hypoxic conditions [25, 26]. In particular, during hypoxia, Nox4 is induced by TGF-β, which promotes smooth muscle cell proliferation in pulmonary arteries, a major cause of pulmonary arterial remodeling [27], and specific growth factors such as insulin-like growth factor binding protein (IGFBP-3) increases Nox4 gene expression, resulting in PASMC proliferation and subsequent medial thickening [27]. Further, Nox4 has been shown to be important in hypoxia-inducible factor 2α (HIF-2α) expression and transcription, which suggests an important relationship exists between Nox4 and HIF-2α, as well as TGF-β in pulmonary vascular remodeling in PAH [8].

3 Nox4 Expression in PAH

As stated above, reactive oxygen species (ROS) are important regulators of pulmonary vascular remodeling, and abundant evidence supports a prominent role for Nox4 in the pathogenesis of PAH [20, 28]. Nox4 is the major NADPH oxidase homolog expressed in human PASMCs [29], and its expression both at the mRNA and protein level is significantly increased in lungs from patients with idiopathic pulmonary arterial hypertension (IPAH) compared to healthy lungs [20], which suggests a correlation between Nox4 and the onset of PAH. In experimental rodent models of PAH, Nox4 expression is increased. Specifically, Nox4 is upregulated in chronic hypoxia-induced PAH in mice [20, 28, 30], and monocrotaline (MCT)-treated rats [22, 31]. Nox4 mediates the hypoxia-induced growth of human PASMCs [27], and silencing Nox4 expression by RNA interference decreases human PASMC and fibroblast proliferation [30, 32, 33]. Severe forms of PAH are associated with plexiform lesions, which are comprised of proliferating endothelial cells and elevated levels of angiogenic factors such as VEGF [34, 35]. Pneumonectomy increases the severity of PAH in animals treated with MCT, and has been shown to stimulate the formation of lesions that are morphologically similar to plexiform lesions [36]. Pneumonectomy also further increases the expression of Nox4 in MCT-treated animals [31] but it is not currently known whether Nox4 expression contributes to the formation of these lesions. Collectively, these findings support the premise for Nox4 expression being inherently involved in pulmonary vascular

remodeling by promoting arterial medial smooth muscle proliferation and adventitial fibroblast activation in PAH. Reports on the location of Nox4 expression in pulmonary arteries varies as Nox4 has been observed in the media of both normotensive and hypertensive pulmonary arteries [20], as well as in endothelial cells and fibroblasts [21, 22, 37, 38].

4 Enzymatic Properties of Nox4

Nox4 is unique in that it is the only Nox enzyme which is constitutively active. Nox4 colocalizes with and directly binds the integral membrane protein p22phox, which is essential for the Nox4 activity [39] to stabilize p22phox expression [40]. The binding and activation of Nox4 by p22phox does not depend on the proline rich region of p22phox, which is important in the regulation of Nox1, Nox2, and Nox3 [41]. A further distinction is that Nox4 does not require the binding of cytosolic proteins for ROS production [40], and instead produces ROS constitutively. This is due to unique characteristics of the C-terminus of Nox4 that facilitates the constitutive transfer of electrons from NADPH to FAD [16]. Another distinguishing feature of Nox4 compared to the other Nox enzymes is that robust production of H_2O_2 can be detected which contrasts from a mixture of superoxide and H_2O_2 from Nox1, Nox2, Nox3, and Nox5 [40, 42, 43]. The mechanism underlying the preferential production of H_2O_2 versus superoxide is related to the presence of a highly conserved histidine residue in the E-loop of Nox4 that promotes the rapid dismutation of superoxide before it leaves the enzyme [44].

5 Role of the Adventitial Fibroblast and Nox4 in PAH

Remodeled pulmonary arteries in PAH are characterized by increased stiffness [45, 46], secondary to collagen and elastin deposition, a process which is regulated by the adventitial fibroblast. The fibroblast, a primary cell type of the adventitia, contributes to the perpetual reorganizing of the extracellular matrix, secretion of growth factors and chemokines, as well as inflammatory cytokines. Studies show that TGF-β1 induces Nox4 expression in pulmonary fibroblasts and adventitial fibroblasts surrounding pulmonary vessels, suggesting that Nox4 is a component of the TGF-β1 signaling pathway [29, 47, 48]. TGF-β1 via Nox4 may contribute toward disease pathology through transcriptional activation of extracellular matrix components such as collagen and through a number of mediators known to exacerbate the extent of fibrosis and vascular remodeling, including PAI-1 and HIF-1α [49]. TGF-β1 is known to induce PAI-1 expression in fibroblasts through Nox4-dependent ROS production and increased activation of p38 MAPK and JNK [50]. In addition, NOX4 promotes myofibroblast secretion of extracellular matrix (ECM) proteins and production of fibronectin via TGF-β1 signaling, which promotes cellular fibrogenesis [47]. In vascular diseases such as PAH, an increase in collagen and ECM matrix proteins would promote increased tissue fibrosis as well as vascular stiffness through a decrease in tissue and vessel compliance [45, 46]. Recent studies show that the pulmonary arterial remodeling that occurs in hypoxia-induced PAH is characterized by the emergence of adventitial fibroblasts, which recruit inflammatory cells and adhesion proteins, promoting a pro-inflammatory and proliferative environment, leading to vascular remodeling [51]. Fibroblasts also influence and promote the inflammatory response by manipulating leukocyte recruitment, survival, and behavior [52], a phenomenon that appears to be regulated by Nox [53]. In addition, a subset of circulating bone marrow derived cells termed fibrocytes that possess genetic markers and behaviors consistent with both fibroblasts and macrophages can also be found in the adventitia [54, 55], and fibrocytes have the capability to differentiate into collagen-producing fibroblasts and myofibroblasts [56–58]. It has been shown that Nox enzymes and elevated ROS stimulate fibroblast proliferation [30, 47, 59]; however, the contribution of specific Nox isoforms to adventitial fibroblast proliferation and the development of PAH is still poorly understood.

Cultured pulmonary adventitial fibroblasts express Nox4 [30], and Nox4 expression is upregulated by many different stimuli including

hypoxia, inflammatory mediators, and fibrotic moieties such as TGF-β, and TNF-α, which contribute to pulmonary fibroblast activation and proliferation [8]. Further, Nox4-induced ROS production by TGF-β in human fibroblasts occurs extracellularly, which is in contrast to that observed in human PASMC, where Nox4 activation by TGF-β leads to intracellular ROS production [29]. The cellular effects and mechanisms of extracellular versus intracellular generation of Nox4 are poorly understood in PAH. In many cell types, Nox4 generates low-level, predominantly intracellular ROS constitutively and in response to a variety of stimuli [60]. For example, in vascular smooth muscle cells (VSMC), ROS generation is predominantly intracellular [61], and in vascular endothelial cells (VEC), many NAD(P)H oxidase subunits are located in the nucleus, with ROS production occurring in a "nucleus-rich" fraction [62]. This prominent labeling of Nox4 in the nucleus provides a source of ROS that can potentially activate many downstream targets, which include transcription factors such as AP-1 proteins c-Fos and c-Jun, implicated in growth and differentiation processes, and NF-κB, which is involved in inflammatory reactions and apoptosis [63]. Extracellularly, TGF-β1 increases ROS release (presumably through Nox4) by human fetal lung fibroblasts in a transcriptionally mediated manner, and the ROS produced by this NADPH oxidase activity is H_2O_2 [15, 64]. In addition, ROS production in human pulmonary arterial smooth muscle cells (HPASMC) via TGF-β1 is mediated by transcriptional induction of Nox4 expression [29]. However, in contrast to lung fibroblasts [15, 64], TGF-β1 stimulation in HPASMC induces intracellular generation of ROS derived from Nox4 localized to the endoplasmic reticulum, which does not result in extracellular release of H_2O_2 [29].

Studies done with siRNA to knockdown Nox4 has lead to the conclusions that Nox4-mediated ROS production stimulates cellular proliferation and inhibits apoptosis in pulmonary fibroblasts [30]. Interestingly, Nox4 upregulation by TGF-β is inhibited by N-acetylcysteine and DPI, which suggests that Nox4-induced ROS production regulates Nox4 gene expression [48]. Further, specific inhibition of Nox4 prevents pulmonary fibroblasts from mediating TGF-β-induced myofibroblast differentiation as well as producing collagen [48].

In a recent study, Barman et al. [22] did immunofluorescence staining of lung sections from MCT-treated rats and in lungs from normal (control) and human PAH (Fig. 1). In control rat pulmonary arteries (PA), Nox4 was primarily detected in the adventitial cells with secondary labeling on the intima (endothelial cells) (Fig. 1; top panel). In 4-week MCT-treated rats, using two different antibodies that are selective for Nox4 (Epitomics and Abcam), there was a dramatic increase in Nox4-positive cells in the adventitia (Fig. 1; MCT, left panels). In PA from MCT-treated rats, Nox4-positive cells were also detected in the remodeled medial layer agreeing with previous studies [20]. Nox4 also exhibited a staining pattern in sections of human lung from normal individuals and PAH (Fig. 1, lower panels) that was consistent with Nox4 expression in the MCT-treated rat lungs. In lung sections from animals treated with the specific Nox4 inhibitor (VCC202273; C) [22], MCT-stimulated pulmonary arterial remodeling and Nox4 adventitial expression were significantly attenuated (Fig. 1; MCT + C).

Barman and colleagues [22] also determined the location of ROS production in hypertensive PA using immunofluorescence imaging for 8-hydroxydeoxyguanosine (8-OHdG), a DNA nucleoside that is generated by ROS. As shown in Fig. 2, the highest signal for ROS was observed in the adventitia, which overlapped significantly with the fibroblast marker, fibroblast activating protein (FAP) (Fig. 2a; MCT). ROS levels in the adventitial layer was decreased in lung sections treated with the Nox4 inhibitor VCC202273 (C) (Fig. 2a; MCT + C), suggesting that the elevated adventitial ROS production in hypertensive PA derives from increased Nox4 expression. In addition, there was significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellular fibronectin, CD90) as well as the monocytic cell marker CD11B (Fig. 2b).

Nox4 also modifies fibroblast function in human lung. Using an adenovirus that expresses Nox4, Barman et al. [22] showed that Nox4-transduced

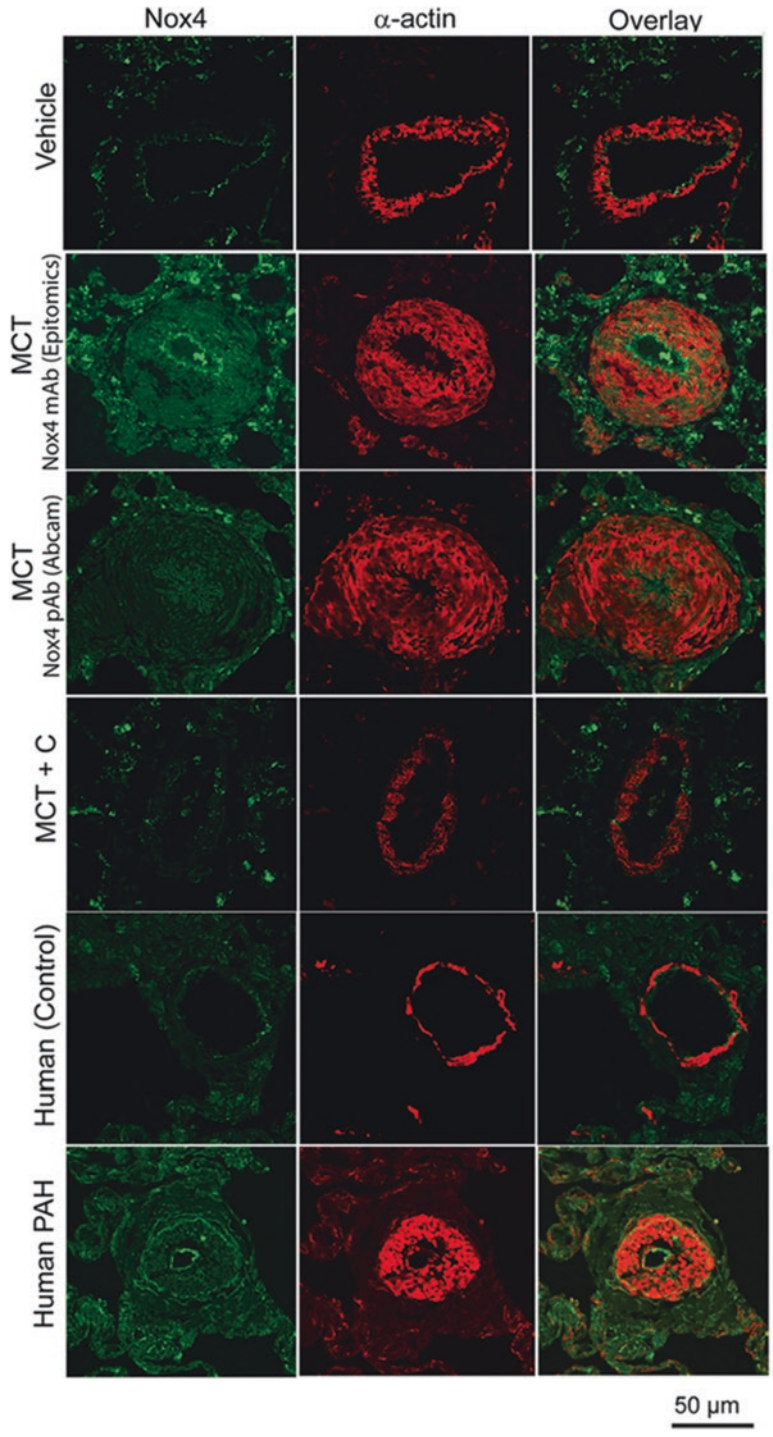

Fig. 1 Nox4 expression is upregulated in the adventitia in rat and human PAH. Confocal images of lung sections from control, experimental PAH (4-week MCT), and human PAH (IPAH undergoing lung transplant). Sections were stained with Nox4 and α-actin antibodies. Nox4 is highly expressed in cells of the adventitia (and intima) in 4-week MCT-treated rats and human PAH. There is also an abundance of Nox4-expressed cells present in the remodeled PA medial layer but devoid of α-actin expression in the MCT and human PAH. In the presence of Nox4 VCC202273 (C), (**MCT + C**), Nox4 expression is similar to vehicle-treated PA in the MCT-treated group. (Reproduced from Barman et al. (2014) Arterioscler Thromb Vasc Biol 34:1704–1715)

fibroblasts exhibited a robust increase in cellular proliferation as demonstrated by real-time changes in electrical impedance using electric cell-substrate impedance Sensing arrays (ECIS) (Fig. 3a). In addition, fibroblasts transduced with the Nox4-adenovirus displayed increased cell proliferation (total cell number, Fig. 3b), and the number of viable cells as measured via the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyletrazolium bromide (MTT) assay (Fig. 3c).

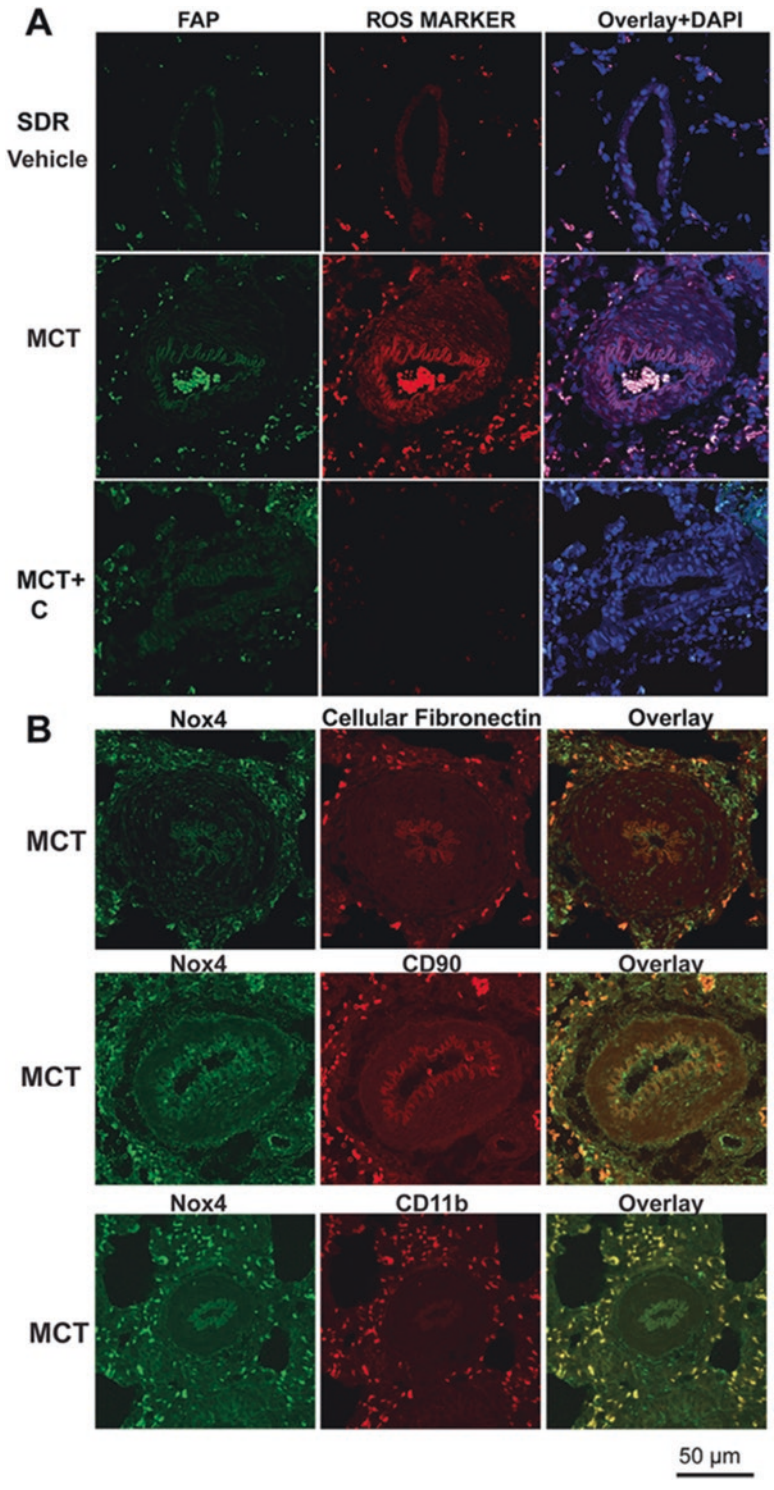

Fig. 2 Nox4 expression and reactive oxygen species (ROS) production is localized in the adventitia. (**a**) Sections of control and 4-week MCT-treated rat lungs co-stained for fibroblast activation protein (FAP), 8 Hydroxy-2'dexoyguanosine (ROS marker) and DAPI. (**b**) Co-staining for Nox4 and cellular fibronectin, CD90 and CD11b ROS production is elevated in PA adventitia from 4-week MCT-treated rats, which overlapped significantly with the fibroblast marker fibroblast activating protein (FAP) (**a; MCT**). ROS are decreased to control (vehicle) levels by the Nox4 inhibitor VCC202273 (**c**) (**A; MCT + C**). In MCT-treated PA, there is significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellular fibronectin, CD90) as well as the monocytic cell marker CD11B (**b**). (Reprinted from Barman et al. (2014) Arterioscler Thromb Vasc Biol 34:1704–1715)

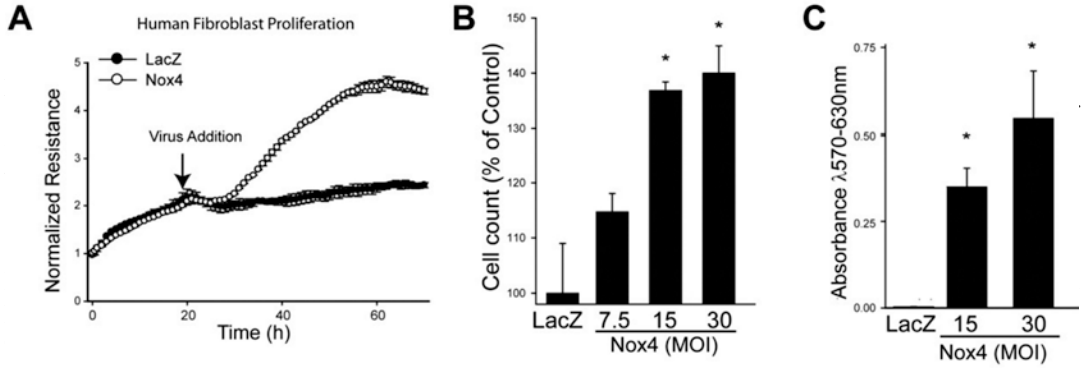

Fig. 3 Nox4 stimulates fibroblast proliferation and migration (**a**) Electrical impedance (ECIS) of human lung fibroblasts in the presence/absence of Nox4 adenovirus. Nox4-transduced fibroblasts exhibit a robust increase in cellular proliferation in real time using ECIS. (**b**) Nox4 increases fibroblast cell number, and (**c**) the number of viable cells using MTT assay. * Significantly different from Lac Z, $p < 0.05$ ($n = 3$–6 per group). (Reprinted from Barman et al. (2014) Arterioscler Thromb Vasc Biol 34:1704–1715)

robust than those observed in fibroblasts.

The functional relevance of Nox4 in adventitial cells is not well described especially in the realm of PAH. The *tunica externa* or adventitia is a loosely defined collection of cells including fibroblasts and immune cells, collagen, and elastic fibers that encircle the tunica media and intima layers of the blood vessel [66]. The adventitia orchestrates inflammation and vascular proliferation in response to injury, atherosclerosis and both pulmonary and systemic hypertension [67]. The fibroblast is a primary cell type of the adventitia, responsible for the continual reorganization of the extracellular matrix via matrix deposition and secretion of growth factors, chemokines and inflammatory cytokines [68]. Aberrant vascular remodeling in PAH occurs through increased inflammation, proliferation, and fibrosis, processes that collectively yield more muscular and less compliant pulmonary blood vessels [69]. Fibroblasts promote PAH by actively secreting matrix proteins, growth factors as well as promoting the inflammatory response by manipulating leukocyte recruitment and behavior [52, 70]. Supportive of this phenomenon, Barman et al. [22] observed that increased expression of Nox4, in the absence of other stimuli, was sufficient to increase fibroblast migration and proliferation. Similarly, it has been shown that silencing Nox4 in fibroblasts decreases the ability of stimuli such as TGF-β to increase matrix and induce contractile gene expression, which is consistent the number and behavior of adventitial fibroblasts that are inherently involved in PAH.

TGF-β1, a proliferative autocrine growth factor implicated in the pathophysiological vascular remodeling in PAH [73] robustly increases both Nox4 mRNA and protein levels in human lung fibroblasts. When comparing the ability of TGF-β1 to drive Nox4 expression in intimal cells (endothelial), medial cells (smooth muscle), or adventitial cells (fibroblasts), the greatest expression of Nox4 occurs in fibroblasts [22]. This observation by Barman and colleagues [22] is in agreement with previous studies [29, 74], and others have shown that TGF-β1 can upregulate Nox4 expression in other cell types including human cardiac fibroblasts, airway smooth muscle and vascular smooth muscle [29, 65, 75]. A role for fibroblasts in pathologic remodeling in PAH is further supported by studies in transgenic mice with fibroblast specific activation by TGF-β1 signaling, which develop mild PAH with medial hypertrophy, inflammation and fibrosis [76]. While studies strongly support a role for fibroblast TGF-β1 signaling in aberrant pulmonary vascular remodeling, PAH can be further exacerbated with additional stress on the endothelium and reflects the important contributions of multiple cell types in the development of PAH in addition to adventitial signaling.

In summary, Nox4 has gained considerable attention as a primary source of ROS, and cellular proliferation in the pathogenesis of both idiopathic pulmonary fibrosis and PAH, and connects Nox4

as a common variable in fibroblasts (and other perivascular cells) that contributes to the proliferation and remodeling of hypertensive pulmonary arterioles. The remodeling of pulmonary blood vessels requires the "joint effort" of all three vascular layers and while numerous studies have proposed a central role for endothelial cells ("inside out" remodeling), it is also readily apparent that vascular remodeling can be driven by changes in the adventitia (i.e., "outside in" remodeling). In both humans and animal models of PAH, prominent inflammation, activation and restructuring of the adventitia is observed [58]. The adventitial location of Nox4 is therefore highly suited to orchestrate the changes in vascular inflammation, matrix deposition, and subsequent vascular remodeling that occur in PAH. From a therapeutic standpoint, current treatments for PAH are ineffective in the long term, and merely prolong the disease, with a focus on ameliorating increased pulmonary vascular tone and improving quality of life. The development of more efficacious Nox4 inhibitors may be a viable direction to pursue in the quest to halt the morphological progression of PAH. It remains to be determined whether modalities that target adventitial Nox4 and the production of ROS in combination with current therapeutic approaches will have superior efficacy, as well as newly found success in the continuing battle against PAH and other pulmonary vascular diseases.

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Role of Transcription Factors in Pulmonary Artery Smooth Muscle Cells: An Important Link to Hypoxic Pulmonary Hypertension

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Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
AP-1	Activator protein-1
BK_{Ca}	Ca^{2+}-activated potassium channel
C/EBP	CCAAT/enhancer binding protein
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CBP	CREB binding protein
CCL11	C-C motif chemokine 11
COPD	Chronic obstructive pulmonary disease
CREB	cAMP response element-binding protein
CS	Cigarette smoke
CXCL	Chemokine ligand
ET-1	Endothelin-1
ETC	Electron-transport chain
FOXO	Forkhead box protein O
GATA	Erythroid transcription factor
HDAC2	Histone deacetylase 2
HIF-1	Hypoxia inducible factor-1
IFN	Interferon
IgE	Immunoglobulin E
IKK	IκB kinase
IL	Interleukin
IP_3R	Inositol triphosphate receptor
IκB	Inhibitor of NF-κB
JNK	jun-N-terminal kinase
MEF	Myocyte enhancer factor
NFAT	Nuclear factor of activated T lymphocytes
NF-κB	Nuclear factor-κB
Nox	NADPH oxidase
NRF2	Nuclear erythroid 2-related factor 2
PAEC	Pulmonary artery endothelial cell
PASMC	Pulmonary artery smooth muscle cell
PDGFR	Platelet-derived growth factor receptor
PH	Pulmonary hypertension
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SOCE	Store-operated Ca^{2+} entry
SOCS	Suppressor of cytokine signaling
SR	Sarcoplasmic reticulum
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
TAK	Transforming growth factor activating kinase
TBP	TATA binding protein
TF	Transcription factor
Th2	T-helper type-2
TNF	Tumor necrosis factor

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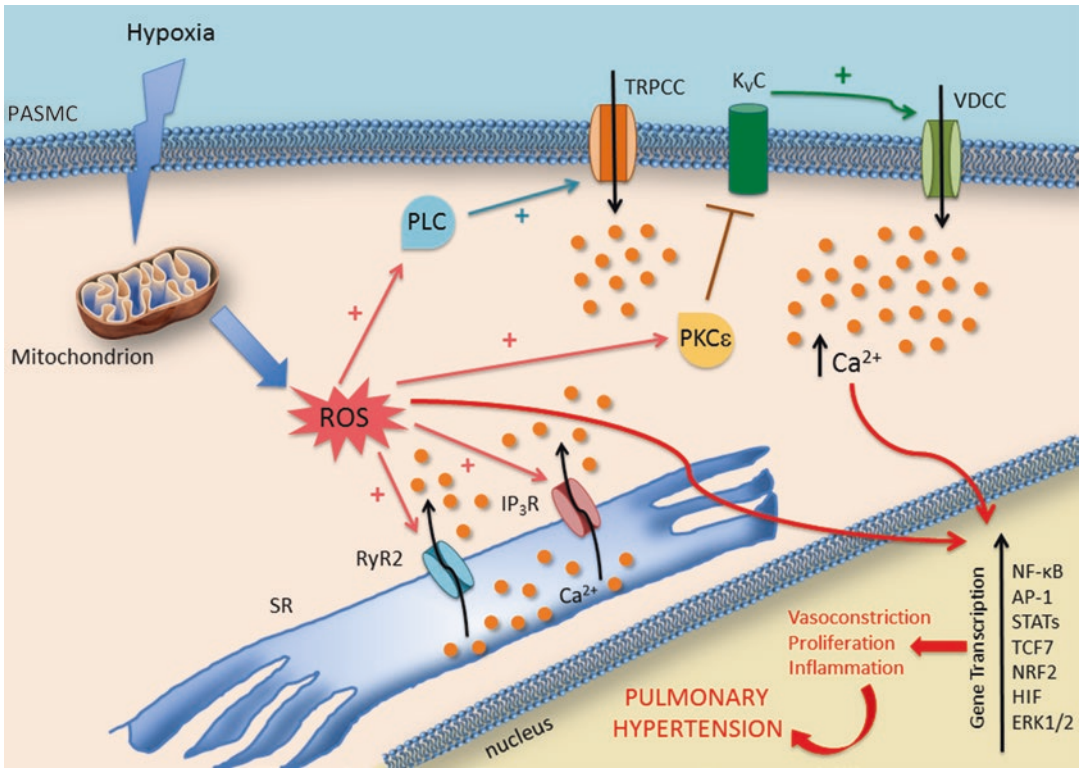
Treg	Regulatory T cells
VDCC	Voltage-dependent Ca^{2+} channel
VEGF	Vascular endothelial growth factor

1 Introduction

Hypoxia-induced or related pulmonary hypertension (PH), is a mysterious killer that is characterized by tremendous pathologic complexity. It is a considerable cause of morbidity and mortality in many respiratory diseases. The limited understanding of the basic cellular and molecular mechanisms governing PH is the major reason for the lack of effective therapeutic interventions and the subsequent unchanged morbidity and mortality of this disease [1].

With regard to underlying mechanisms, recent evidence suggests that inflammation plays an important role in the pathogenesis of PH [2–6]. In particular, acute and chronic alveolar and/or bronchial inflammation are thought to be central to the pathogenesis of many lung disorders such as asthma, chronic obstructive pulmonary disease (COPD), adult respiratory distress syndrome, and idiopathic pulmonary fibrosis. These disorders may cause acute hypoxia that induces pulmonary artery vasoconstriction, whereas chronic hypoxia promotes pulmonary arterial wall remodeling via the induction of cell proliferation in all three layers, particularly in the pulmonary arterial smooth muscle cells (PASMCs) of the tunica media [7, 8]. These observations suggest that the pulmonary vasculature in PH patients is phenotypically different from that in normal subjects because of inheritable or acquired mutations (or polymorphisms) of certain genes that are specifically involved in regulating proliferation, apoptosis, and differentiation in PASMCs and pulmonary arterial endothelial cells (Fig. 1).

It is now recognized that transcription factors (TFs) are instrumental in immune and inflammatory responses during lung diseases and PH. Understanding the function and regulation of TFs is fundamental to the investigation of lung diseases and may provide novel therapeutic strategies. In this book chapter we focus on recent progress in our understanding of the vital roles of the TFs in the functional and structural modulations of PASMCs, and in the development of PH.



2 Roles of Transcription Factors in Lung Diseases

2.1 NF-κB

A key element of immune and inflammatory responses is a family of pleiotropic transcription factors termed nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). The mammalian NF-κB family consists of five proteins, RelA (also known as transcription factor p65), RelB, cRel, NF-κB1 (also known as p105/p50) and NF-κB2 (also known as p100/p52). All of these proteins share the N-terminus of a related DNA-binding, dimerization and nuclear localization domain, the Rel homology domain. This domain enables the association with either one of the members of the IκB (inhibitor of NF-κB) protein family, IκBα, IκBβ, or IκBε. The C-terminus of RelA, RelB and cRel contains transcriptional activation domains, which mediate interactions with basal transcription factors and cofactors, such as TATA binding protein (TBP), the transcription factor II B (TFIIB), adenovirus early region 1A (E1A)-binding protein 300kD (EP300, also known as p300) and CREB binding protein (CBP) [9, 10]. The other two members, NF-κB1 and NF-κB2, encode longer precursor proteins that can be processed, either during translation or through phosphorylation-induced partial proteolysis, in order to the activate respective DNA-binding forms p50 and p52 [11].

NF-κB activity can be induced by numerous and various stimuli such as tumor necrosis factor (TNF), interleukin-1 (IL-1), lipopolysaccharide, and DNA damage or hypoxia. NF-κB activation triggers signaling pathways that ultimately initiate a complex transcriptional program, allowing the cell to respond to environmental stress [12].

In unstimulated cells, NF-κB dimers are retained in their inactive cytoplasmic form by binding to a member of the IκB family, ensuring low basal transcriptional activity. Following inflammatory stimuli, the IκB kinase (IKK) complex phosphorylates IκBα at serines 32 and 36, leading to its ubiquitination and degradation through the 26S proteasome pathway. This exposes the nuclear localization sequence of NF-κB, allowing its entry into the nucleus, where it binds to specific 9–10 base pair DNA sequences (κB sites) and upregulates the transcription of genes downstream of the κB motif [12, 13].

It was discovered that transforming growth factor activating kinase 1 (TAK1), a member of the mitogen-activated protein kinase family, was required for IKK activation [14]. Upon exposure to hypoxia, Ca^{2+} is released from cellular compartments such as the sarcoplasmic reticulum (SR). This increase in $[Ca^{2+}]_i$ activates calcium/calmodulin-dependent kinase II (CaMKII) that leads to the activation of TAK1. Subsequently, TAK1 forms a complex with its binding protein and then is brought to the IKK complex by the K63-ubiquitin chains (promoted by Ubc13-XIAP complex), leading to IKK activation and hence phosphorylation of IκBα [14, 15].

In lung pathologies, NF-κB has the important role of regulating the expression of inflammatory mediators such as cytokines, chemokines and cell adhesion molecules. Regulation of their expression influences the type and quantity of inflammatory cells that infiltrate airway tissue in chronic obstructive pulmonary diseases. NF-κB activation in asthma and COPD occurs mostly in response to IL1β and TNF-α or elicited by the

Fig. 1. A schematic diagram illustrating the signaling mechanism for ROS-dependent activation of various transcription factors in pulmonary artery smooth muscle cells to mediate the development of pulmonary hypertension. Hypoxia induces a rise in $[ROS]_i$, opens RyR2 (ryanodine receptor-2/Ca^{2+} release channel) and IP_3R (inositol triphosphate receptor/Ca^{2+} release channel) on the SR (sarcoplasmic reticulum), activates TRPCC (transient receptor potential canonical channel) and VDCC (voltage-dependent Ca^{2+} channel) on the plasmalemmal membrane, inhibits K_VC (voltage-dependent K^+ channel), and increases $[Ca^{2+}]_i$. The increased $[ROS]_i$ and $[Ca^{2+}]_i$ synergistically cause activation of various TFs (transcription factors) including NF-κB (nuclear factor-κB), AP-1 (activator protein-1), CREB (cAMP response element-binding protein), HIF-1α (hypoxia inducible factor-1α), and other transcription factors. The activated TFs result in the increased transcription of the contractile, proliferative, and pro-inflammatory genes to mediate the development of pulmonary hypertension

activation of toll like receptors during bacterial or viral exacerbation [16, 17]. Cell-based studies of normal PASMCs demonstrate that NF-κB mediates cytokine-induced release of endothelin-1 [18]. Concerning NF-κB implications in PH development, interesting studies have demonstrated that NF-κB inhibition attenuates PH in the monocrotaline model in rats [19, 20].

Furthermore, asthmatic patients were shown to display enhanced p65 nuclear expression in bronchial epithelial cell, increased p65 DNA binding in lung tissue and elevated levels of NF-κB-regulated inflammatory mediators [21]. The ability of NF-κB to enhance expression of inflammatory mediators is regulated by post-translational modifications of p65 [22]. However, the degree of NF-κB phosphorylation and acetylation in asthma is unknown yet.

Hypoxia can directly activate NF-κB through a pathway involving prolyl hydroxylase (PHD)-mediated hydroxylation and activation of IKKβ, leading to IκBα degradation and NF-κB nuclear localization [23, 24]. Within its activation loop, IKKβ contains an evolutionarily conserved consensus motif (LXXLAP, where X is any amino acid) for hydroxylation by PHDs. Under normoxic conditions, PHD1 and PHD2 hydroxylate the LXXLAP motif that suppresses IKKβ activity [23].

NF-kB is activated in COPD lungs, particularly in alveolar macrophages and airway epithelial cells, and further activates multiple inflammatory genes that contribute to persistent inflammation and oxidative stress [25, 26], even after the discontinuation of smoking.

Moreover, it has been shown that NF-κB signaling is redox sensitive being influenced by the changes in the oxidant–antioxidant balance that occurs in the airways diseases [27].

2.2 AP-1

During hypoxia, an increase in $[Ca^{2+}]_i$ has a pivotal role in PASMCs contraction and consequent PH. Moreover, an increase in calcium has been demonstrated to enhance expression of various TFs including activator protein-1 (AP-1) that regulates expression of genes involved in cell proliferation/differentiation, transformation, apoptosis, pulmonary defense, inflammation and immune responses [28]. This family of proteins includes sequence-specific DNA-binding transcription factors and consists of homodimers or heterodimers that are formed by Jun (c-Jun, JunB, and JunD), and Fos (c-Fos, FosB, Fra-1, and Fra-2), and binds the promoters of target genes [29]. These proteins are also referred to as "immediate-early genes" and "early response proto-oncogenes," due to their capability to be activated transiently and rapidly in response to various external mitogenic and toxic stimuli [30]. In general, the mRNA levels of c-Jun, JunB, JunD, c-Fos, and FosB reach a peak within 15–30 min of stimulation and returns to basal level within 1–2 h [31]. The expression of Fra-1 and Fra-2 mRNA mainly occurs between 30 and 60 min, peaking at 90–180 min. However, the mRNA expression remains elevated above basal level for 2–24 h, depending upon the stimuli [31].

Regulation of AP-1 activity is critical for the determination of cell fate, and occurs at various levels, including dimer composition, transcriptional and posttranslational, and interaction with accessory proteins [32]. It has been reported that hypoxia-induced AP-1 can activate the transcription of the vascular endothelial growth factor (VEGF) [33, 34]. It was also found that the elevation of $[Ca^{2+}]_i$ caused an increase in expression and phosphorylation of c-Jun protein. Notably, the appearance of functional AP-1 was proved by the stimulation of AP-1-dependent transcription (AP-1-Luc). Using the K252a protein kinase inhibitor, it was demonstrated that increased $[Ca^{2+}]_i$ induced AP-1 transcriptional activity and that the expression of the two hypoxic genes was coupled. The protein kinase inhibitor K252a, abolished c-Jun induction and AP-1-dependent reporter expression caused by $[Ca^{2+}]_i$ or hypoxia [35].

AP-1 may be activated via protein kinase C (PKC) and by various cytokines, including TNF-α and IL-1β, via several types of protein tyrosine kinase and mitogen-activated protein kinases, which themselves activate a cascade of intracellular kinases [29]. Certain cytokine signals rapidly increase the transcription of the fos-gene, resulting in increased synthesis of Fos

protein. Other signals lead to activation of kinases that phosphorylate c-Jun, which in turn results in its increased activation. Specific Jun and Fos kinases are now recognized and suspected to play a key role in the regulation of cellular responsiveness to cytokine signals.

c-Fos and c-Jun were first identified as oncogenic genes activated by the FBJ murine osteosarcoma virus [36] and avian sarcoma virus [37], respectively. Thus, they were shown to have a high relevance in cancer development and progression [38]. While c-Fos and c-Jun are the most studied TFs in the cancer field, their role and contribution to PH are still not fully addressed. In a recent study, it was shown that they are involved in vascular remodeling underlying PH [39]. Various growth factors involved in vascular remodeling such as interleukin-6 (IL-6) [40] or platelet derived growth factor-BB (PDGF-BB) [41] have been reported to lead to c-Jun expression increase. It was also observed that c-Jun and c-Fos were highly expressed in the smooth muscle layer of the vessel wall in lungs in both animal models and human PH samples [39]. Furthermore, exposure of mice to hypoxia enhanced the lung/pulmonary artery expression of c-Fos after only 3 h, but not in the primary human PASMCs [39]. This implies that the changes in c-Fos expression are not a direct effect of hypoxia, but rather a secondary response. Therefore, presumably hypoxia activates c-Fos expression in human PASMCs by intermediate factors since hypoxia has been reported to increase levels of factors such as PDGF-BB, TGF-β, connective tissue growth factor (CTGF), and endothelin-1 (ET-1) both in vitro and in vivo [42]. Accordingly, it was found that very short exposures (3 h) of mice to hypoxia led to upregulation of ET-1 mRNA in mouse lung homogenates that was not evident in chronic conditions. Moreover, increased expression of c-Fos in bronchial epithelial cells had been reported in asthmatic airways [43] and in patients with severe, treatment-insensitive asthma [44, 45]. In contrast, expression of c-Jun was neither altered in pulmonary arteries from hypoxia-exposed mice, nor in human PASMCs exposed to hypoxia or ET-1 [39]. However, similar to c-Fos, the increased c-Jun expression was observed in the lungs of patients with idiopathic pulmonary artery hypertension. This supports the notion that multiple signaling molecules are involved in the development of PH [46]. In the MCT rat model, which is also an inflammatory and lung injury model [42], c-Jun was upregulated, further supporting the need of more initiating stimuli to develop vascular remodeling.

Recent studies showed that sirtuin 1 (SIRT1) decreased c-Fos and c-Jun acetylation induced by p300 and inhibited the transcriptional activity of AP-1 and subsequent cyclo-oxygenase-2 expression and PGE2 generation [47]. Thus, AP-1 may play a critical role in mediating expression of various inflammatory proteins. There is evidence that many of the stimuli relevant to asthma that activate NF-κB will also activate AP-1 [48].

Moreover, inhibitors of c-Jun N-terminal protein kinases (JNKs) are being developed [49] that show effectiveness against bronchial hyperresponsiveness, bronchoalveolar lavage (BAL) inflammatory cells, and airway remodeling in animal models of asthma [50]. Interestingly, JNKs are also involved in T-cell class switching, and their inhibitors may also have a profound immunomodulatory role [49].

2.3 STAT

Signal transducers and activators of transcription (STAT) is a family of cytoplasmic transcription factors. Today, seven STAT members have been identified: STATs 1, 2, 3, 4, 5a, 5b, and 6. STAT1, STAT3, STAT4, STAT5a, and STAT5b all form homodimers, whereas STAT1 and STAT2, and STAT1 and STAT3 may also form heterodimers, depending on the concentration and nature of the activating ligand. The STATs are latent cytoplasmic proteins that are promptly activated by tyrosine phosphorylation by the cytokine receptor associated JAK (Janus) kinases after cytokine exposure. This event leads to the recruitment of inactive STAT monomers through interaction with their Src homology 2 (SH2) domains, and subsequent dimerization. The resulting functional STAT dimer is then capable of migrating directly

to the nucleus where it can bind DNA and directly activate cytokine responsive gene transcription.

Among STATs proteins, STAT6 has been identified as a potential therapeutic target for allergic asthma [51, 52]. It undergoes tyrosine phosphorylation by JAKs in response to cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13) exposure. IL-4 and IL-13 each bind to two receptor complexes and shares receptor subunit. IL-4 binds to its cognate receptor complex consisting of the IL-4 receptor α chain (IL-4Rα) and the common gamma chain (γc) to form the type I receptor [53]. IL-4 first binds to IL-4Rα subunit with high affinity followed by dimerization with γc receptor subunit and subsequent JAK-STAT6 activation [54]. Both IL-4 and IL-13 bind to the shared type II receptor complex made up of IL-4Rα and IL-13Rα1, resulting in the activation of the STAT6-mediated signaling pathway. This pathway is critical to the development of Th-2 type inflammation characteristic of asthma and antiparasitic responses [55, 56]. Once phosphorylated, STAT6 is transported to the nucleus where it regulates gene expression in various cell types, a critical event for the balance between host immune defense and allergic inflammatory responses [57]. The principle lung cells that are profoundly altered by STAT6 signaling during inflammatory responses include T and B lymphocytes, macrophages, and structural cells including airway epithelial and PASMs. In lung epithelium, STAT6 mediates the effect of IL-13 to induce airway hyperreactivity and mucus production [52]. The expression and activation of STAT6 have been observed in primary human bronchial epithelium indicating that STAT6 contributes to the function in these cells [58].

The generation of mice lacking STAT6 ($STAT6^{-/-}$) has led to an intensive investigation into the role of STAT6 in numerous lung disease models. STAT6 knockout mice have no response to IL-4, do not develop Th2 cells in response to IL-4, and fail to produce IgE, bronchial hyperresponsiveness, or bronchoalveolar lavage eosinophilia and Treg induction after allergen sensitization, indicating the critical role of STAT6 in allergic responses [59, 60]. Moreover, mice expressing a constitutively active STAT6 are predisposed toward allergic disease [61].

Another consequence of STAT6-dependent activation of the IL-14/IL13 receptor complex is Pendrin-induced expression of MUC5AC (Mucin 5A), a major mucin in asthma, in bronchial epithelial cells [62, 63]. The expression of MUC5A and the calcium-dependent chloride channel 1 (hCLCA1) or mouse homolog Gob 5 (mCLCA3), is increased in airway mucus-producing cells in patients with asthma [64]. The induction of MUC5A and Gob 5 in airway epithelial cells was shown to be completely abrogated in STAT6 knockout mice [65].

The suppressor of cytokine signaling (SOCS) family of proteins downregulate specific cytokine signals [66]. SOCS2 inhibits Th2 development, and its absence enhances STAT5 and STAT6 activation favoring Th2 polarization [67]. In animal models of allergen-induced airway inflammation, the expression of the chemokines CCL11, CCL17, and CCL22 involves regulation by STAT6 [68]. STAT6 has been reported to be overexpressed in bronchial biopsies from asthmatic patients in some [58], but not all [69] studies. In addition, STAT6 gene polymorphisms are associated with asthma-related symptoms, but not with the risk of developing asthma [70, 71]. Genistein, a flavonoid in legumes and some herbal medicines, decreases airway inflammation in animal models of allergic asthma and this is associated with a downregulation of erythroid transcription factor (GATA)-3 and STAT6 and the upregulation of T-bet [72].

In order to induce an efficient gene expression, the multiple actions of several enhancer binding proteins are required, some activated by distinct signaling pathways. This paradigm is also true for STAT6-dependent transcription [51]. As mentioned above, STAT6 plays an important role in the expression of several chemokines, included the chemoattractant eotaxin or CCL11. Eotaxin is induced by NF-κB, which is activated by TNF-α, and IL-4 activated STAT6 in an airway epithelial cell line [73]. STAT6 and members of the NF-κB family of proteins act in conjunction with each other to regulate transcription of multiple IL-4-induced genes.

Furthermore, the signal peptide of eosinophil cationic protein functions as a regulator for enhancing macrophage migration through the upregulation of the transcriptional factors STAT1 and STAT2 [74]. In vitro CCL5 expression in airway epithelial cells is STAT1 dependent [75] and IL-4 and IL-13 activate STAT1 in bronchial SMCs, lung epithelium, endothelium, and fibroblasts [76, 77]. Thymic stromal lymphopoietin (TSLP) is a cytokine which may be involved in the pathogenesis of bronchial asthma. The TSLP receptor (TSLPR) mediates gene regulation not only through STAT5 and STAT3 but also via STAT1 [78]. Also STAT1 is a critical intracellular signaling molecule for the production of type I interferons (IFNs) (α/β) and IFN-γ and resistance to viral respiratory infections [79, 80]. SOCS1 gene polymorphisms associated with an increased susceptibility to adult asthma enhance the transcriptional level of SOCS1 in human airway epithelial cells, and induce higher levels of protein expression of SOCS1 and lower phosphorylation of STAT1 stimulated with IFN-β [81]. STAT1 expression is elevated in animal models of asthma [82] and in the lower airways of stable asthmatics, but not of COPD patients [83, 84].

An interesting study indicated that STAT3 is a substrate for calpain, a critical mediator of cell death that is triggered by calcium signals. The ability of calpain to cleave a growing number of substrates also suggests a potentially important role for this enzyme in the regulation of cell death. Both in vivo and in vitro experiments have demonstrated that calpain-mediated cleavage is a common feature of STAT3 and STAT5 [85]. Given that calpain has been implicated in many cellular processes, including cell proliferation, apoptosis, and differentiation [86], cleavage of STAT5 by activated calpain may also inhibit STAT5-mediated signaling in additional situations. For example, strong T cell receptor (TCR) signaling, which is likely to increase intracellular free calcium and thereby induce more calpain activation, is well known to drive Th1 polarization [87]. As mentioned before, since STAT5 has been suggested to play a critical role in Th2 cell differentiation [88], it is possible that TCR-mediated calpain activation induces STAT5 cleavage, thereby impairing Th2 cell differentiation [89].

2.4 TCF7

Transcription factor 7 (TCF7) is involved in the pathogenesis of lung diseases. The expression of TCF7 gene is regulated by a number of factors through multiple signaling pathways. TCF7 is enhanced by β-catenin and TCF7L2 (also known as TCF4) as a downstream target gene of Wnt pathway [90]. At least 16 different protein isoforms have been found that present distinct functional properties due to the presence of two different promoters. Thus, the mRNAs transcribed can be translated into two groups of proteins with different functions. The first promoter generates mRNA encoding a full-length activating form (FL-TCF7) (42–60 kDa), while the second intronic promoter produces a truncated, dominant-negative isoform of TCF7 (dnTCF7) (25–40 kDa) [91, 92]. FL-TCF7 acts as a transcription activator, while dnTCF7 works as a transcription repressor. Both isoforms of TCF7 could bind with Groucho corepressors and function as a transcription repressor without β-catenin signaling. When β-catenin accumulates in the nucleus, FL-TCF7 could interact with it and induce the transcriptional activation. The dnTCF7 isoform lacks the N-terminal β-catenin binding domain and was proposed to play a negative role in transcription regulation [93]. The FL-TCF7 was considered to have dual functions in regulating gene transcription through the interaction with different proteins. In addition to Groucho family proteins, the β-catenin/TCF7-mediated transcription process could also be interfered directly by a multidomain protein, Bcr (breakpoint cluster region), to dissociate β-catenin/TCF7 complex and downregulate the level of β-catenin/TCF7 target genes such c-Myc [94, 95].

TCF7 resides on human chromosome 5q31.1, where was proposed as a candidate locus associated with asthma and allergy through genome-wide screens [96]. A recent study has demonstrated that TCF7 is required for the

development of ILC2 cells (Group 2 innate lymphoid cells), a type of innate lymphocyte that produces Th2 cell-associated cytokines and innate type 2 immunity [97]. Notably, both Th2 cells and ILC2 cells were found to be involved in asthma. In an experimental model of ovalbumin-induced asthma, it was demonstrated that TCF7 was required in the production of the Th2 cytokine, IL-4. These TCF7-deficient mice produced less IL-4 and showed less airway inflammation, while the normal mice showed more inflammatory cell infiltration in perivascular and peribronchial areas after asthma induction. A diminished GATA3-1b expression was also detected in TCF7-deficient mice, suggesting that TCF7 induce IL-4 through GATA-3 pathway and contribute the development of airway inflammation [98]. ILC2 induced by TCF7 also contributes to Th2 response, and is closely related to asthma. A recent study found that during experimental asthma, ILC2 proliferates and produces IL-13, which contributes to allergy and the worsening of asthma [99]. The lack of TCF7 will lead to the deficiency of ILC2, and contributes relieving type 2 inflammations. Thus, it is reasonable to assume that inhibition of TCF7 in the airway may play a protective role in allergic asthma, and might be considered as a promising target for the future treatment of asthma [90].

TCF was also found correlated to COPD and Wnt/β-catenin signaling was found to be related with lung development and repair after lung injury. Based on these findings, it was proposed that the Wnt/β-catenin signaling pathway is associated with emphysema, a main feature of COPD with alveolar airspace enlargement, parenchymal tissue destruction, and impaired pulmonary regeneration [100]. The end results of the Wnt/β--catenin signaling pathway and the expression of its target genes, such as TCF/LEF genes family, were decreased in lung tissues of patients/animals with COPD where TCF7 even was not expressed [101, 102]. Increased activation of Wnt/β-catenin showed therapeutic effects in experimental emphysema by increasing pulmonary repair and decreasing airspace enlargement, and restored the structure and function of alveolar epithelial cells [101]. These studies suggest that the upregulation of the Wnt signaling pathway might be a therapeutic strategy for emphysema.

2.5 NRF2

The transcription factor NRF2 controls the expression of the antioxidant response element (ARE)-regulated antioxidant and cytoprotective genes. Thus, it has an essential protective role in the lungs against oxidative airway diseases [103]. Studies in animal models as well as human studies of cigarette smoke (CS)-induced COPD, confirmed the antioxidant and anti-inflammatory role of NRF2. Some of these studies have shown the presence of decreased activity and/or expression of NRF2 in the airway and lung cells of patients with stable COPD [104, 105] or pulmonary emphysema [106]. For this reason, NRF2 promises to be an attractive therapeutic target for intervention and prevention in COPD.

NRF2 is a Cap'n'collar basic region leucine zipper (CNC-bZIP) TF [107]. Its expression is abundant in tissues where detoxification reactions commonly occur such as the intestine, lung and kidney [108]. In the lung, NRF2 is mainly expressed in alveolar macrophages and epithelial cells. Under normal conditions, NRF2 is sequestrated in the cytosol where its expression is maintained at low basal levels through the specific binding of the evolutionarily conserved N-terminal Neh2 regulatory domain of NRF2 to its cytosolic inhibitor Kelchlike ECH associated protein 1 (KEAP1) [109]. This event leads to the ubiquitination of NRF2 through the cullin-3 (CUL3) E3 ubiquitin ligase complex and to its proteasomal degradation [103, 108]. By contrast, the protein DJ-1 stabilizes NRF2 by preventing its association with KEAP1 [110].

Upon exposure to oxidative stress, NRF2 is released from KEAP1 and translocates into the nucleus where it forms heterodimers with other bZIP transcription factors including small Maf proteins, c-Jun, activating transcription factor-4,

c-Fos, and Fra-1. Subsequently, it binds to a cis-element ARE in target gene promoters, upregulating their expression [108, 111]. Coactivator proteins CREB-binding protein/p300 (CBP/p300) and ARE-binding protein-1 are supposed to bind NRF2 heterodimers leading to the regulation of ARE-dependent genes expression such as glutathione S-transferases (GSTs), NADPH, quinone oxidoreductase 1 (NQO1), HO1, and UDP-glucuronyl transferase 1a6, which are responsible for the antioxidant and detoxification response [103].

Chronic CS exposure for 6 months increases NRF2 mRNA and protein expression in the terminal bronchiolar epithelium in mouse lungs. This correlates with the induction of antioxidant and detoxification genes, including cytochrome P450 1b1, glutathione reductase, thioredoxin reductase, and members of the GST family [112]. NRF2 knockout mice exhibit a higher susceptibility to CS-induced pulmonary emphysema [113, 114]. Compared with wild-type littermates, $NRF2^{-/-}$ mice show more pronounced inflammation, increased numbers of apoptotic endothelial and alveolar type II epithelial cells and decreased antioxidant and antiprotease gene expression in alveolar macrophages [103, 115]. The available data underline the protective effects of NRF2, which activates the transcription of antiproteases as well as antioxidants in alveolar macrophages, thereby maintaining a balance between proteases and antiproteases, as well as oxidants and antioxidants.

Interestingly, several studies have shown the presence of decreased activity and/or expression of NRF2 in the airway and lung cells of patients with stable COPD [104, 105] or pulmonary emphysema [106]. In alveolar macrophages from patients with COPD, S-nitrosylation of Histone deacetylase 2 (HDAC2) is increased and this abolishes its GR-transrepression activity promoting glucocorticoid resistance [116]. Treatment with sulforaphane, a small-molecule activator of NRF2 is able to denitrosylate HDAC2, restoring glucocorticoid sensitivity in alveolar macrophages obtained from patients with stable COPD [116], suggesting the potential role of NRF2 activators for the treatment of COPD [117, 118]. However, large genetic studies have been unable to find a significant association between polymorphisms in the NRF2 pathway and the rate of decline of lung function [119]. Moreover, the antibody used in the previous studies using COPD tissues, does not appear to be specific for NRF2 [120]. This highlights the necessity of further human studies in this area using validated specific antibodies.

2.6 HIF-1

Hypoxia inducible factor (HIF) senses and coordinates cellular responses to hypoxia. HIF is a heterodimer consisting of one of three α subunits and a β subunit. HIF-β is constitutively expressed, whereas HIF-αis induced by hypoxia. HIF-1α is the most well-established member of the HIF family.

HIF-1α is a transcriptional activator that regulates the expression of genes involved in the response to hypoxia in most mammalian cells [121]. Under normoxia, HIF-1α is prolyl hydroxylated at P402 and P564 in its oxygen-dependent degradation domain (ODD). This leads to its degradation through the binding of the HIF-α unit to the E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) at L574 [122, 123]. Hypoxic conditions stabilize the α-subunit and lead to its nuclear translocation, formation of a dimer with HIF-1β, and recruitment of transcriptional coactivators [124]. This complex binds to an enhancer domain of the hypoxia responsive element (HRE) located either at the 5′ or 3′ region of target genes, including heme oxygenase-1, VEGF, glucose transporter (GLUT)-1, and GLUT-4 [125].

HIF-1β is expressed constitutively, whereas HIF-1α expression in the lung is regulated by the inspired O_2 concentration [126]. Homozygous-null knockout mice that completely lack HIF-1α expression die at mid-gestation owing to the failure of embryonic vascularization [127–129]. Mice heterozygous for the null allele $HIF\text{-}1\alpha^{+/-}$, and thus partly deficient for HIF-1α expression, develop normally and are indistinguishable from

their wild-type littermates under normoxic conditions. Wild-type mice exposed to 10% O_2 develops PH in response to chronic hypoxia. Medial wall thickening in pulmonary arterioles results in increased pulmonary arterial pressure and right ventricular hypertrophy. The remodeling is progressive and eventually leads to cor pulmonale. In HIF-1α$^{+/-}$ mice, the hypoxia-induced muscularization of pulmonary arterioles is significantly impaired, resulting in significantly less medial wall thickening, PH, and right ventricular hypertrophy after 3 weeks at 10% O_2 [130]. Although the pathophysiology of PH is complex [131], a major component is the actions of peptides, such as endothelin-1 (ET-1) and angiotensin II that induces SMC contraction and hypertrophy. ET-1 expression is induced within the pulmonary vasculature of hypoxic rats [132], and ETA receptor antagonists prevent/reverse chronic hypoxic PH [133]. A HIF-1-binding site in the ET-1 gene promoter is required for hypoxia-induced transcription [134], suggesting that ET-1 mRNA expression by hypoxic PAECs is mediated by HIF-1. ET-1 increases $[Ca^{2+}]_i$ in PASMCs [135] and induces generation of ROS and activation of extracellular signal-regulated kinase (ERK)1/2 in a variety of vascular smooth muscle preparations [136, 137]. It was verified that ET-1 increased ROS levels and activated ERK1/2 in PASMCs and that increased $[Ca^{2+}]_i$ was necessary for both responses. The activation of ERK1/2 depended on increased ROS levels, since antioxidants blocked ET-1-dependent ERK1/2 activation. ET-1 also increased $[Ca^{2+}]_i$ and ROS levels in aortic smooth muscle cells, consistent with previous reports [138], but did not activate ERK1/2 or induce HIF-1α protein accumulation. It has been demonstrated that ET-1 is essential for induction of HIF-1α in PASMCs during moderate hypoxia via a mechanism requiring Ca^{2+} influx through VGCCs, increased ROS levels, and activation of ERK1/2, even if the mechanism by which ROS lead to ERK1/2 activation in PASMCs is still unclear [139]. Expression of angiotensin-converting enzyme, which converts angiotensin I to angiotensin II, is also induced within the pulmonary vasculature of hypoxic rats [140]. The administration of captopril, an inhibitor, orlosartan, a type 1 angiotensin receptor antagonist, also attenuates the development of hypoxic PH [141]. Angiotensin II, which induces vascular SMC hypertrophy, has recently been shown to induce HIF-1α expression [142]. These results suggest that HIF-1 might be required for the angiotensin-induced hypertrophy of vascular SMCs in the hypoxic lung.

Forkhead box M1 (FoxM1) is a transcription factor that regulates cell cycle progression, and it has been implicated in cancer cell lines [143–145]. In response to proliferative signaling, it translocates to the nucleus and promotes G1/S and G2/M transitions, leading to the progression of mitosis via its downstream targets [146]. Recent evidence suggests that the FoxM1 promoter contains HIF response elements, and that hypoxia induces FoxM1 in some cancer cell lines [143–145, 147]. In the lung, FoxM1 was reported to be required for normal pulmonary vascular development [148, 149]. The loss of FoxM1 also attenuates epithelial and endothelial repair after lung injury [148–151]. The lungs of Rosa26-FoxM1B mice exhibit earlier nuclear localization of FoxM1 with its downstream targets, and increased cell proliferation, in response to butylated hydroxytoluene-induced lung injury [152]. Recently, forkhead transcription factor (FOXO) isoforms have been identified as key regulators of cellular proliferation [153, 154], and a decreased FOXO1 expression has been demonstrated in pulmonary vessels and PASMCs of human and experimental PH lungs [155].

3 Redox-Sensitive Signaling in SMC Regulation: Role of TFs

The cellular and molecular mechanisms underlying the unique hypoxic pulmonary vasoconstriction and associated PH, remains largely elusive. However, many investigators recently provided extensive evidence showing that hypoxia elevates intracellular ROS in PASMCs [156–167]. This is consistent with the contribution of ROS to the initiation or maintenance or both of numerous physiologic and pathologic cellular responses in virtually all types of cells. Intracellular ROS can

be generated by multiple resources including the mitochondrial electron-transport chain (ETC), NADPH oxidase (Nox), xanthine oxidase, cyclooxygenase, and cytochrome P450. Among these resources, the mitochondrial ETC and Nox [160–164, 166, 168–172] have been shown to be essential for the hypoxic increase or decrease in intracellular ROS in PASMCs. A number of publications suggest that the hypoxic increase or decrease in intracellular ROS can directly affect the activity of ion channels, leading to a large increase in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in PASMCs. For instance, hypoxia may inhibit voltage-dependent $K^+(K_V)$ channels by affecting intracellular ROS [168, 170, 173]. Presumably, hypoxic inhibition of K_V channels would result in membrane depolarization, activation of voltage-dependent Ca^{2+} (Ca_V) channels, and extracellular Ca^{2+} influx, resulting in an increase in $[Ca^{2+}]_i$. ROS also may trigger ryanodine receptors/Ca^{2+} release channels (RyRs) to induce Ca^{2+} release from the SR, contributing to the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs [174, 175]. Increased $[Ca^{2+}]_i$ is a most important factor for cell contraction, and recent studies have demonstrated that the hypoxia-induced increase in $[Ca^{2+}]_i$ and cell contraction are intimately related in PASMCs [175–177]. Pharmacologic and genetic interventions that inhibit or eliminate the hypoxic increase in $[Ca^{2+}]_i$ can correspondingly inhibit or eliminate the hypoxic contraction [162, 163, 178, 179]. However, it has been found that hypoxia normally causes neither an increase in $[Ca^{2+}]_i$ nor a contraction in systemic (e.g., cerebral and mesenteric) artery SMCs [162, 180, 181]. In addition to the direct effect, ROS also may activate intermediate signaling molecules, such as PKCε, to regulate specific ion channels in concert with ROS, contributing to the hypoxic increase in $[Ca^{2+}]_i$ and associated contraction in PASMCs [162]. Recent work demonstrates that mitochondrial ROS-dependent activation of PKCε can significantly augment Nox activity and lead to a further increase in intracellular ROS generation. This provides a positive-feedback mechanism that augments intracellular ROS generation further, and thus contribute to the hypoxic increase in intracellular ROS and $[Ca^{2+}]_i$ [163]. It has also been reported that serotonin [182] and ET-1 [136] cause the production of ROS via Nox in PASMCs. GATA4 plays an important role in the regulation of growth of PASMCs, and antioxidants inhibit serotonin-induced GATA4 phosphorylation and activation [183]. The serotonin signal for the nuclear translocation of ERK and subsequent GATA4 phosphorylation are dependent on the activation of RhoA and Rho kinase [184]. In response to serotonin, ERK has also been shown to activate GATA4 via monoamine oxidase-A-dependent production of hydrogen peroxide (H_2O_2), which promotes the translocation of phosphorylated ERK to the nucleus [185]. All of these above studies indicate that $[Ca^{2+}]_i$ is an important second messenger in SMCs.

Therefore, vascular SMCs (VSMCs) exercise tight control of the $[Ca^{2+}]_i$ by expressing a wide repertoire of Ca^{2+} channels and transporters. In these cells, $[Ca^{2+}]_i$ is tightly associated with gene transcription in a stimulus-specific manner, so that different Ca^{2+} signaling patterns activate distinct TFs and yield specific functional outcomes. Transcription regulation in VSMCs modulates the expression of patterns of genes, including genes coding for contractile and cytoskeleton proteins, and those promoting proliferation and cell growth. Depending on their gene expression, VSMCs can exist in different functional states or phenotypes. The majority of healthy VSMCs show a contractile phenotype, characterized by high contractile ability and a low proliferative rate. However, VSMCs can undergo phenotypic modulation with different physiological and pathological stimuli, whereby they start to proliferate, migrate, and synthesize excessive extracellular matrix. CREB is a TF that regulates gene expression by binding to cAMP response elements (CREs) in the promoter regions of target genes in a variety of tissues, including VSMCs [186–190]. In VSMCs, CREB activation is initiated by a rise in $[Ca^{2+}]_i$ and the subsequent recruitment of CaMKII or CaMKIV [187]. Interestingly, both CaMKII and CaMKIV phosphorylate CREB at Ser133 and thereby induce CREB activation, but CaMKII can also phosphorylate CREB at the second site, Ser142, and thereby induce negative regulation [191].

Moreover, Pulver et al. [192] showed that Ca^{2+} depletion of the SR by thapsigargin and subsequent store-operated Ca^{2+} entry (SOCE) via SOC channels (SOCCs) increased phospho-CREB nuclear content and the transcription of CREB-dependent genes in both cultured and intact VSMCs. In line with these findings, small interfering RNA downregulation of stromal interaction molecule-1 decreases thapsigargin-induced CREB phosphorylation in human coronary artery SMCs [193]. IP_3R-dependent Ca^{2+} release from the SR has also been shown to activate CREB [194]. In contrast, RyR-dependent Ca^{2+} release suppresses CREB phosphorylation by a negative feedback mechanism involving voltage-dependent Ca^{2+} channels (VDCCs) [187]. RyR Ca^{2+} release causes activation of BK_{Ca} channels and thereby membrane hyperpolarization and subsequent closure of VDCCs [195].

NFAT is a hyperphosphorylated cytosolic protein, dephosphorylation of which by the Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin permits nuclear translocation of active NFAT.

In the nucleus, NFAT associates with transcription coactivators, which include activator protein 1 (AP1), myocyte enhancer factor 2 (MEF2), and members of the GATA family, and promotes gene expression [196]. Regulation of NFAT activity in VSMCs is highly dependent on temporal and spatial aspects of Ca^{2+} signaling. Sustained depolarization and subsequent Ca^{2+} influx through VDCCs alone fail to produce NFAT nuclear accumulation, both in ileal and vascular SMCs [197, 198]. The inositol triphosphate-dependent Ca^{2+} release from the SR has been shown to induce NFATc3 activation [197]. It has also been shown that Ca^{2+} sparks originating from RyRs have an inhibitory effect on UTP-induced NFATc3 activation, which is independent on BK_{Ca} channel-mediated membrane hyperpolarization. Thus, the mechanism for the inhibitory action of Ca^{2+} sparks differs between CREB and NFAT signaling. Strong evidence has been provided that SOCE activates NFAT in PASMCs, where a disrupted SOCE pathway reduces hypoxia-induced NFAT nuclear translocation [199, 200]. There is solid evidence that NFAT activation is strongly involved in the vascular remodeling accompanying the development of HPH, largely because of its positive effect on VSMC proliferation, and also possibly hypertrophy [199–201]. Moreover, this role of NFAT as the proliferative TF contrasts with other data showing that the VSMC contractile phenotype marker genes, encoding components of the Ca^{2+} signalosome as well as Ca^{2+}-dependent and KV channels, are also regulated by NFAT [202–206]. The reason for these contrasting findings is unclear, but this indicates that NFAT transcription signaling could be further fine-tuned to mediate specific changes in the vascular wall.

SRF is a protein that binds to CArG elements in the DNA and regulates the expression of genes containing CArG boxes in their promoter regions. SRF plays an important role in VSMC transcription control, because the majority of genes encoding contractile proteins, such as smooth muscle myosin heavy chain, smooth muscle α-actin, h-caldesmon, and desmin, contain one or more CArG boxes in their promoters [207, 208]. Myocardin is a potent SRF transcription coactivator, and highly specialized in induction of expression of all CArG-dependent VSMC contractile phenotype marker genes, whereas it does not activate an early response c-fos gene that is also CArG element/SRF-dependent [209, 210]. SRF and its cofactor myocardin are thus the key players in VSMC differentiation and the onset of a VSCMC contractile phenotype. Unlike CREB and NFAT Ca^{2+}-dependent TFs, SRF is not directly activated by Ca^{2+}-dependent kinase/phosphatase, but it is indirectly regulated by Ca^{2+} influx. Ca^{2+} entry through VDCCs and subsequent activation of the RhoA–ROCK kinase pathway promotes binding of SRF to the CArG promoter regions of VSMC contractile protein genes but not c-fos genes. The SRF transcription pathway can therefore lead to expression of genes that maintain the contractile phenotype or those that mediate cell growth.

Other than those described above, the PPARs (PPAR-α, −β, −γ, and -δ), which belong to the nuclear receptor superfamily, are established vasoprotective TFs in systemic atherosclerotic diseases [211]; however, its role in pulmonary

vascular disease has not been explored incomparable depth. Upon ligand activation, PPARγ heterodimerizes with the retinoid X receptor and regulates multiple target genes, such as those encoding adiponectin, interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), ET-1, and the endogenous endothelial nitric oxide (NO) synthase (eNOS) inhibitor asymmetric dimethylarginine (ADMA), many of which are strongly implicated in the pathobiology of PH [212]. PPARγ activation ultimately inhibits the G1 → S phase transition that is mandatory for cell cycle progression and VSMC proliferation, e.g., by stabilizing the cyclin-dependent kinase inhibitor $p27^{KIP1}$ [213] or inhibiting telomerase [214]. By blocking important survival pathways downstream of activated PDGFR-β such as those regulated by phosphatidylinositol 3-kinase (PI3K), PPARγ agonists also induce apoptosis of proliferating SMCs [211].

4 Conclusion and Future Perspective

Oxidative stress and increased Ca^{2+} signaling are critical to the hypoxia-induced cellular responses in PASMCs. Together, they make significant contributions to the pathological changes in the function and structure of the lung vasculature. The subsequent pathologic pulmonary vascular changes are mediated by the upregulation or downregulation of redox and Ca^{2+}-sensitive TFs. Indeed, experimental evidence indicates that the regulated TFs and gene expression play a vital role in pro-inflammatory, immunological, and other multiple cellular responses, leading to PH. Additional studies are required to further elucidate the specific interactions, signaling pathways, and molecular processes of oxidative stress- and Ca^{2+}-dependent TFs and associated genes. These studies may provide important information regarding the molecular pathogenesis of PH and other chronic pulmonary diseases. Furthermore, verification of the roles of known TFs and genes and identification of new candidates may create novel and effective therapeutic treatments for lung diseases and PH.

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Molecular Basis of Nitrative Stress in the Pathogenesis of Pulmonary Hypertension

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1 Introduction

Pulmonary hypertension (PH) is a progressive condition that was responsible for 5.5 deaths per 100,000 people in the USA in 2001, a number that increased to 6.5 deaths per 100,000 people in 2010 (National Vital Statistics System, Centers for Disease Control and Prevention, USA) [1]. The pathogenesis of PH is characterized by progressive increases in pulmonary vascular resistance and pulmonary artery pressure (>25 mmHg at rest). Features of PH include pulmonary vascular remodeling, endothelial dysfunction, impaired vasoconstriction, and intravascular thrombosis [2–4]. Causes of PH have been classified in an expert consensus as class I to V (Table 1) [2]. In severe cases of PH (e.g., idiopathic pulmonary arterial hypertension, IPAH), treatment options are limited, and lack of treatment can lead to right heart failure and premature lethality. Current pharmacological therapies targeting abnormalities in the prostacyclin, nitric oxide, and endothelin pathways can improve IPAH symptoms and lead to modest survival benefits, but do not reverse the disease pathogenesis [5, 6]. Lung transplantation remains the best option for this devastating disease.

In healthy individuals, the prevention of pulmonary vascular remodeling and the preservation of a normal pulmonary tension appear to be controlled by cyclic guanosine monophosphate (cGMP)-dependent activation of protein kinase G (PKG); this occurs downstream of nitric oxide (NO) production and subsequent activation of soluble guanylate cyclase (sGC) (Fig. 1). Genetic deletion of PKG-1α induces PH in mice [7], demonstrating the causal role of PKG dysfunction in the pathogenesis of PH. Therapeutic agents targeting this pathway by either inhibiting phosphodiesterase type 5 (PDE5)-dependent cGMP degradation or activating sGC-derived cGMP production have been shown to be effective in improving the symptoms of PH in humans. Early PH typically involves dysregulation of vasoactive pathways including reduced vasodilator pathway signaling (e.g., through impaired bioavailability

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of NO and downregulation of prostaglandin signaling), and enhanced vasoconstrictor pathway signaling (e.g., through increased production of endothelin-1 and reactive oxygen species, ROS) [8]. Nevertheless, the precise molecular mechanisms that are responsible for aberrant pulmonary vascular remodeling and vasoconstriction in PH patients have not been fully defined.

Reactive nitrogen species (RNS) are usually unstable nitrogen-centered free radicals containing unpaired electrons (Table 2). RNS regulate many physiological processes including differentiation, metabolism, migration, and proliferation. These messenger molecules are also heavily involved in the nitrative modification of proteins that regulate PH pathogenesis. NO, for example, is formed by the NO synthase (NOS) enzymes and is stable under anoxic conditions. In the presence of excessive superoxide anion ($O_2^{\bullet-}$) (i.e., oxidative stress), however, NO is converted to peroxynitrite ($ONOO^-$) (i.e., nitrative stress) (Fig. 2a). In general, when superoxide formation occurs at a threefold greater rate than NO synthesis, NO is being quantitatively converted to peroxynitrite, which leads to decreased NO bioavailability, and induces posttranslational modifications (e.g., nitrates tyrosine residues) of proteins and resultant dysregulation of protein functions [9]. Nitrative stress-induced dysregulation of molecular signaling pathways have been implicated in the pathogenesis of PH in both animal models and patients [10–12].

Given that the lung tissue of patients with severe PH demonstrate prominent levels of nitrative as well as oxidative stress [13, 14], delineation of the mechanistic role of oxidative/nitrative stress in the pathogenesis of PH and the signaling pathways regulating oxidative/nitrative stress in the pulmonary vasculature has become an actively pursued area of research. The aim of this chapter is to review mechanisms that mediate nitrative stress-induced PH, delineate molecular sources of ROS and RNS in the context of PH, and describe evidence of nitrative stress in PH patients.

Table 1 Clinical categories of pulmonary hypertension

Class	Name
I	Pulmonary arterial hypertension
II	Pulmonary hypertension owing to left heart disease
III	Pulmonary hypertension associated with lung disease and/or hypoxemia
IV	Pulmonary hypertension due to chronic thrombotic and/or embolic disease
V	Pulmonary hypertension with unclear multifactorial mechanisms

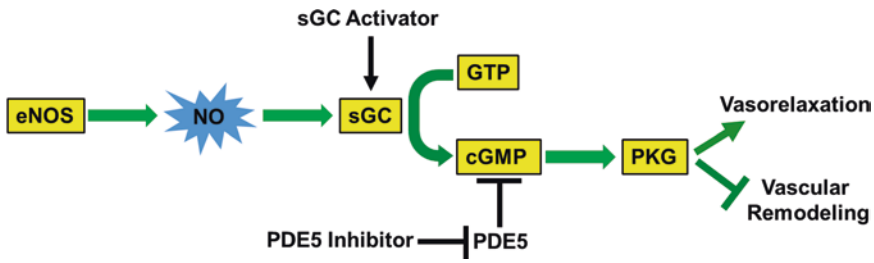

Fig. 1 Nitric oxide signaling maintains vascular homeostasis and inhibits the development of pulmonary hypertension. eNOS generates basal levels of NO, which activate sGC; this in turn leads to cGMP production and subsequent activation of cGMP-dependent PKG. Activated PKG causes vasorelaxation and inhibits pulmonary vascular smooth muscle cell proliferation which underlies the mechanisms of pulmonary vascular remodeling, and thereby preserves normal pulmonary tension. Decreased NO bioavailability leading to impaired PKG activity is a common feature of PH. FDA-approved drugs by activating sGC (Riociguat) or inhibiting PDE5-mediated cGMP degradation (Sildenafil, Tadalafil) have been shown effectiveness in improving the symptom of PH and promoting modest survival, demonstrating the fundamental role of this signaling pathway in maintaining pulmonary vascular homeostasis. *cGMP* cyclic guanosine monophosphate, *eNOS* endothelial nitric oxide synthase, *GTP* guanosine triphosphate, *NO* nitric oxide, *PKG* protein kinase G, *sGC* soluble guanylate cyclase

Table 2 Reactive nitrogen species

Name	Formula
Dinitrogen trioxide	N_2O_3
Nitric oxide	·no
Nitrite	NO_2^-
Nitrogen dioxide	$^{\bullet}NO_2$
Nitronium ion	NO_2^+
Nitrosothiols	RSNOs
Nitrosyl cation	No^+
Nitrosyl chloride	NO_2Cl
Nitrous acid	HNO_2
Nitrous oxide	N_2O
Nitroxyl anion	No^-
Peroxynitrite	$ONOO^-$

2 Molecular Mechanisms of Nitrative Stress in the Pathogenesis of Pulmonary Hypertension

Selectivity for tyrosine nitration by peroxynitrite. As mentioned above, excessive increases in NO and ROS levels give rise to peroxynitrite, a potent, diffusible, and damaging oxidant [15–17]. It has been shown that high levels of peroxynitrite are cytotoxic and induce death of vascular endothelial cells and smooth muscle cells, which may contribute to the pathogenesis of PH [18, 19]. Even at sublethal concentration,

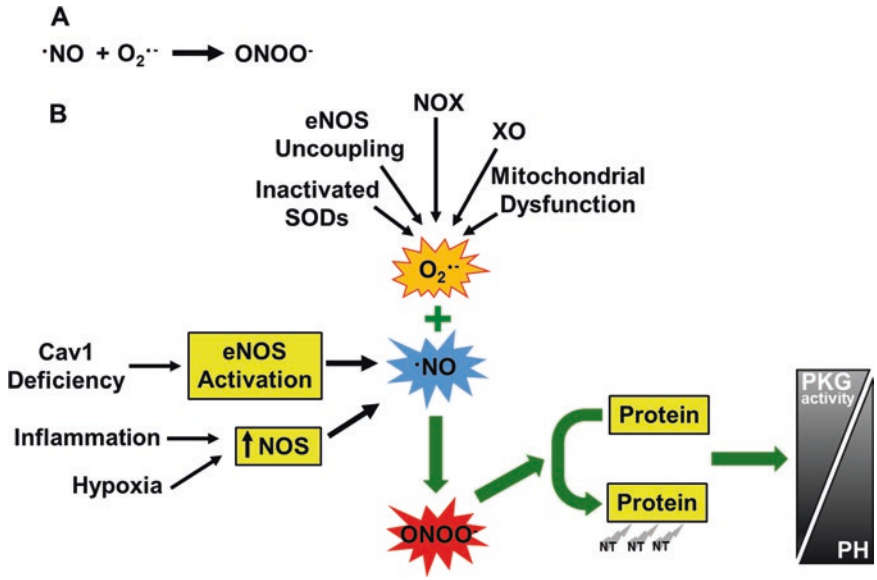

Fig. 2 Molecular basis of nitrative stress in the pathogenesis of PH. (**a**) Generation of peroxynitrite. In the presence of excessive superoxide anion (i.e., oxidative stress), nitric oxide and the reactive oxygen species, superoxide anion, react to form the potent, diffusible and damaging oxidant peroxynitrite. $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite. (**b**) Peroxynitrite induces tyrosine nitration of proteins leading to endothelial dysfunction, vasoconstriction and vascular remodeling and contributes to the pathogenesis of PH. Under various conditions, such as tissue inflammation, hypoxia, and Cav1 deficiency, excessive NO is generated because of increased expression of NOS or activation of eNOS (secondary to Cav1 deficiency). Under these conditions, excessive superoxide is also generated, which is attributable to increased activities of NOX (mainly NOX2 and NOX4) and xanthine oxidase (XO), and decreased antioxidant enzyme (SODs) activities, as well as eNOS uncoupling and mitochondrial dysfunction. Excessive NO and supeorxide anion leads to generation of peroxynitrite which induces tyrosine nitration of proteins and resultant dysfunction. For example, PKG nitration leads to impaired PKG activity which in turn induces vasoconstriction and vascular remodeling and thereby PH. The roles of nitration of other proteins in the pathogenesis of PH are listed in Table 3. *NT* tyrosine nitration

Table 3 Proteins modified by peroxynitrite and involved with pulmonary hypertension

Name	Action of nitration	Pathological function of nitration	Refs
PKG	Inhibition	Pulmonary vasoconstriction and remodeling	[10, 11]
Prostacyclin synthase	Inhibition	Decreased production of vasodilator prostacyclin and increased production of vasoconstrictors	[25, 26]
eNOS	Inhibition	Induction of eNOS uncoupling generating superoxide and decreasing NO production	[11, 55, 59]
Mitochondrial SOD	Inhibition	Increased oxidative and nitrative stress	[30, 31]
ERK	Activation	Vascular cell proliferation: Vascular remodeling	[33, 39]
PKC	Activation	Vascular cell proliferation: Vascular remodeling	[36, 39]
p85PI3K	Inhibition (of PI3K)	Endothelial cell apoptosis and endothelial dysfunction	[40]
Src kinase	Activation	Vascular cell proliferation and migration: Vascular remodeling	[41]

eNOS endothelial nitric oxide synthase, *ERK* extracellular signal-related kinase, *PI3K* phosphoinositide 3 kinase, *PK* protein kinase, *SOD* superoxide dismutase

peroxynitrite reacts with amino acids leading to protein modifications such as tyrosine nitration. Nitration of tyrosine residues involves the addition of a nitro group ($-NO_2$) to the hydroxyl group on the tyrosine residue. Peroxynitrite-induced tyrosine nitration is selective for certain tyrosine residues, which is not directed by specific tyrosine-containing signatures within the primary sequences, by the abundance of the proteins, or by the total amount of tyrosine. Instead, it is attributable to the local environment in which the tyrosine residue resides and the proximity of the protein to the nitrating agents [20]. The presence of a proximal negatively charged Glu or Asp residue promotes the selective nitration of tyrosine, provided there is no Cys or Met near the tyrosine residue, as Cys or Met would otherwise preferentially react with nitrating agents [21]. Additionally, the presence of enzymatic metal cofactors near the tyrosine residue is likely to confer specificity to nitration due to the metal-catalyzed formation of $^{\cdot}NO_2$ from $ONOO^-$. Tyrosine nitration can be enhanced by the presence of heme-containing proteins (e.g., prostacyclin synthase) or in the presence of hydrogen peroxide through the generation of the nitrogen dioxide free radical by heme-peroxidases (e.g., myeloperoxidase) [22, 23]. Specific molecules that can be modified by peroxynitrite and could be involved in the pathogenesis of PH are given in Table 3.

Nitration of protein kinase G impairs its activity and induces PH. In the lungs of Caveolin 1 (Cav1)-deficient mice that develop PH, there are enhanced levels of nitrotyrosine (a surrogate marker for peroxynitrite) [11]. Tyrosine nitration of PKG is also enhanced in the lung of these mice versus wild types, while PKG activity is impaired at baseline or with its activator, cGMP [11]. In the same study, authors demonstrated that alterations in PKG activity that are induced by Cav1 deficiency are eNOS-dependent and occur at least partly through nitration of PKG-1α tyrosine residues 345 and 549, which results in decreased kinase activity (Fig. 2b). Genetic deletion of eNOS in Cav1 null mice results in normalization of the hypertensive pulmonary phenotype. Finally, this study also showed that PH phenotypes observed in Cav1 null mice could be reversed by treatment of these mice with either a superoxide dismutase mimetic (MnTMPyP, which scavenges superoxide) or the NOS inhibitor, L-NAME [11]. Additionally, in Cav1 null mice, restoration of PKG activity through increased expression of PKG attenuates PH.

Another genetic study also demonstrated the causal role of impaired PKG activity in the pathogenesis of PH by showing that *Prkg1* (encoding PKG-1) knockout mice develop PH [7]. Decreased PKG activity induces vasoconstriction and vascular remodeling partly through activation of Rho A/Rho kinase signaling [7]. Together, these studies provide unequivocal evidence for the role of nitrative stress-induced tyrosine nitration of PKG and the resultant inhibition of its activity in the pathogenesis of PH.

In another study, it has been shown that nitration of tyrosine 247 in PKG-1α results in decreased cGMP binding and thereby decreased PKG activity in pulmonary artery smooth muscle cells [12]. Tyrosine nitration of PKG has also been shown to occur in ovine fetal intrapulmonary veins in a hypoxia-dependent manner that is endothelial NOS (eNOS)-independent but through increased levels of nitrite and nitrate [10]. As little as 30 min exposure to hypoxia induces PKG tyrosine nitration and inhibition of its activity, which is attributable to hypoxia-induced impairment of pulmonary vessel vasorelaxation [10].

Nitration and inactivation of prostacyclin synthase induces vasoconstriction. Prostacyclin, generated primarily by the vascular endothelium, is a potent vasodilator through activation of adenylate cyclase in vascular smooth muscle cells, which increases synthesis of cyclic adenosine monophosphate (cAMP). It has been shown that prostacyclin synthase can be nitrated at residue 430 and that this results in impairment of its activity [24]. Prostacyclin synthase nitration was also observed to be increased in pulmonary arterial endothelial cells from newborn lambs with persistent PH [25]. Inactivation of prostacyclin synthase through tyrosine nitration impairs production of the potent vasodilator prostacyclin, but also promotes the generation of the vasoconstrictors prostaglandin H2 (PGH2) and thromboxane A2, and thereby contributes to vasoconstriction and development of PH [26, 27].

Nitration of endothelial nitric oxide synthase leads to endothelial nitric oxide synthase uncoupling and endothelial dysfunction. Studies show that eNOS can be modified by peroxynitrite through tyrosine nitration, which results in impairment of eNOS activity and diminished synthesis of the vasodilator, NO [27]. Furthermore, peroxynitrite-mediated damage to eNOS induces eNOS uncoupling, which occurs when eNOS is not coupled with its cofactors or substrate, and the synthase activity is redirected away from generation of NO to superoxide [28, 29]. In eNOS uncoupling, superoxide is generated by dissociation of the ferrous-dioxygen complex from the oxygenase domain [28, 29]. eNOS uncoupling has been shown to be involved in eNOS-dependent tyrosine nitration of prostacyclin synthase. Thus, nitration of eNOS and its resultant uncoupling leads to decreased bioavailability of the vasodilators, NO and prostacyclin, and augmented oxidative/nitrative stress.

Nitration of the antioxidant enzyme manganese superoxide dismutase contributes to oxidative stress and PH. Given that the reaction between NO and superoxide anion leads to generation of the potent oxidant peroxynitrite, dysregulation of antioxidant enzymes that scavenge and reduce levels of superoxide will enhance oxidative and nitrative stress and thereby contribute to the pathogenesis of PH. It has been shown that the mitochondrial manganese superoxide dismutase (MnSOD) is nitrated at residue 34, which results in inactivation of its activity [30]. Tyrosine nitration of MnSOD has been demonstrated in vivo in fetal lambs with persistent PH [31]. This study shows that decreased MnSOD activity contributes to oxidative stress and resultant endothelial dysfunction and thereby facilitates the development of persistent PH.

Nitration of key signaling molecules involved in pulmonary vascular remodeling. Extracellular signal-related kinase (ERK), p38 mitogen-activated protein (MAP) kinase, and protein kinase C (PKC) are important mediators of pulmonary vascular cell proliferation underlying pulmonary vascular remodeling. It has been shown that peroxynitrite can activate these key signaling molecules [32–36]. Peroxynitrite also

activates nuclear factor kappa B (NF-kB), which in turn induces transcription of inducible NOS (iNOS, see Sect. 3). NF-kB is activated in the pulmonary vessels of end-stage IPAH patients [37], while NF-kB inhibition reduces experimental PH in mice [38]. Importantly, another study demonstrates that peroxynitrite activates pulmonary artery smooth muscle cell and endothelial cell proliferation through activation of ERK and PKC [39]. Additionally, it has been shown that peroxynitrite induces nitration of p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [40], and Src kinase [41]. Nitration of p85 inhibits its binding to the catalytic domain of PI3K and thereby attenuates PI3K activity, whereas nitration of Src kinase leads to Src activation. These signaling molecules play an important role in pulmonary vascular cell survival, migration and proliferation. However, tyrosine nitration of these molecules in pulmonary vascular cells or in lung tissue from animal models of PH or from patients with PH has not yet been reported. Future study is warranted to assess the role of tyrosine nitration of these molecules in the regulation of pulmonary vascular remodeling.

3 Molecular Sources of Reactive Nitrogen Species in Pulmonary Hypertension

Induced expression of endothelial nitric oxide synthase. While controversy exists regarding the expression levels of eNOS in the lungs of PH patients compared with controls, eNOS is robustly expressed in the plexiform lesions of IPAH lungs [42], which may contribute to nitrative stress in these lesions. Animal models of PH have also consistently demonstrated increases in the levels of eNOS mRNA, protein, and activity [43–46]. Despite these inconsistent findings, which may be a product of the different stages of disease progression being assessed, it is widely believed that NO signaling is impaired in PH patients. It has been suggested that impaired NO bioavailability and activity during PH is a result of diminished NOS cofactor availability [47] or eNOS uncoupling [48], as opposed to reductions in NOS levels per se. Reducing intracellular tetrahydrobiopterin levels, for example, reduces NO synthesis and enhances superoxide generation in endothelial cells and isolated blood vessels [49], while administration of tetrahydrobiopterin inhibits superoxide production in a dose-dependent manner [50].

Activation of endothelial nitric oxide synthase secondary to Caveolin 1 deficiency. eNOS-derived NO is in general considered to be beneficial and plays an important role in maintaining vascular homeostasis through the activation of PKG (Fig. 1). Activity of eNOS is regulated by its interaction with effector molecules including Cav1. It has been shown that eNOS activity is negatively regulated by Cav1 binding to eNOS [51]. Administration of the Cav1 scaffolding domain inhibits eNOS activity [52]. In Cav1-deficient mice compared with wild type controls, eNOS is activated in blood vessels [53], NO levels in plasma and lung are increased [11, 54]. Cav1 deficiency induces PH as shown by increased pulmonary vascular resistance, medial thickness, and muscularization, along with enhanced right ventricle: left ventricle plus septum weight ratio [11, 54]. PH did not occur, however, when both Cav1 and eNOS were genetically deleted, or when NOS was inhibited pharmacologically by administration of L-NAME to Cav1 knockout mice [11, 55]. These experimental studies together demonstrate the essential requirement for eNOS activation secondary to Cav1 deficiency in the pathogenesis of PH. Cav1 deficiency has been shown in several animal models of PH [56–58] and in the lungs of IPAH patients, which is associated with eNOS activation and tyrosine nitration of other proteins including PKG [11, 57, 59, 60].

Induced expression of inducible nitric oxide synthase and neuronal nitric oxide synthase. Studies of hypoxia-induced PH have shown increased levels of iNOS mRNA and protein in the lungs of rats exposed to hypoxia compared with normoxia [61–64]. Inhibition of iNOS attenuates hypoxia-induced PH in rats [64], indicating the pathogenic role of iNOS-derived NO in hypoxia-induced PH. Another study has shown

similar increases in the levels of neuronal NOS (nNOS) gene and protein expression in the lungs of rats following exposure to hypoxia [47]. Furthermore, nNOS-derived NO is the source of peroxynitrite that causes neuron damage in ischemic stroke [65].

Dietary nitrite. Along with the L-arginine-NO signaling pathway, the diet is a major source of nitrite in humans [8]. The enterosalivary circulation of nitrate originates from intake of leafy greens and vegetables, along with food additives and preservatives. Salivary glands also concentrate nitrates from the plasma and secrete them into the mouth. Symbiotic bacteria in the mouth then reduces nitrate to nitrite, which enters the gastrointestinal tract. From here, nitrite can be absorbed into the circulation or reduced to form NO in the stomach. The enzymatic and nonenzymatic formation of NO from nitrite has been reviewed elsewhere [8]. Nonenzymatic production of NO, for example, is enhanced by copper, ascorbate, and polyphenols, while enzymatic NO generation has been shown in metal-containing proteins such as carbonic anhydrase, hemoglobin, and myoglobin [8]. Little is known, however, about the role of dietary nitrite-derived NO in nitrative stress-induced pathogenesis of PH. In fact, several studies show that inhalation of a low dose of nebulized sodium nitrite is protective from PH in animal models [66, 67].

4 Molecular Sources of Reactive Oxygen Species in Pulmonary Hypertension

Nicotinamide adenine dinucleotide phosphate oxidases. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) exist in numerous isoforms, namely NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase 1, and dual oxidase 2. NOX enzymes reduce oxygen to superoxide anion ($O_2^{\bullet-}$). NOX5 is not present in rats or mice, and only NOX2 and NOX4 are expressed in the lungs [68]. NOX enyzmes are the predominant source of ROS in endothelial cells [69], fibroblasts [70], and vascular smooth muscle cells [71]. In pulmonary artery smooth muscle cells, for example, NOX4 expression and $O_2^{\bullet-}$ levels are increased by treatment with transforming growth factor β [72]. In chronic hypoxia models of rodent PH, pulmonary vascular remodeling and PH are caused by NOX2- and NOX4-dependent generation of ROS [73, 74], while NOX2 and NOX4 deletion reverses PH through decreases in ROS levels [75]. In a rat model of monocrotaline-induced PH, however, induction of PH was dependent on NOX1 but not NOX4 [76]. Although the type of hypertensive insult may determine which NOX isoform is activated, their dysregulation is well established in the pathogenesis of PH [77].

Xanthine oxidase. Xanthine oxidase converts purine to uric acid under normoxia, but under hypoxic conditions that commonly occur in PH patients, hypoxanthine is formed from adenosine triphosphate, and oxygen is reduced to hydrogen peroxide and superoxide anion [78]. Xanthine oxidase level is enhanced in the circulation of PAH patients, along with increased xanthine oxidase activity in the pulmonary arteries [79]. Furthermore, xanthine oxidase activity is enhanced in a rodent model of PH and blood pressures can be restored by treatment of these animals with specific xanthine oxidase inhibitors [80, 81].

Endothelial nitric oxide synthase uncoupling. Paradoxical decreases in NO signaling that occur while eNOS expression is unaltered or even raised during PH may be a result of eNOS uncoupling. In this process, electrons being transferred to the NOS oxygenase domain from the reductase domain to form L-arginine are instead diverted to molecular oxygen, which results in the generation of superoxide anion instead of NO (for detailed review, see [48]). eNOS uncoupling is associated with changes in the quaternary structure of the enzyme, i.e., decreased assembly of the homodimer but increased assembly of the monomer. Uncoupling of eNOS is stimulated by depletion of cofactors L-arginine and tetrahydrobiopterin (BH4), or increased dihydrobiopterin (BH2) [82]. Mice that

have low levels of BH4 or decreased BH4:BH2 ratios exhibit PH [83]. Administration of BH4 or a BH4 analogue attenuates PH in rat models challenged with monocrotaline or hypoxia [82, 84]. These studies provide strong evidence for a pathogenic role of eNOS uncoupling-derived ROS in PH.

Mitochondrial electron transport chain dysfunction. Abnormal function of the mitochondrial electron transport chain leads to increased ROS generation in PH [85]. Elevated levels of pyruvate dehydrogenase kinase lead to reduced levels of acetyl-CoA and a shift to aerobic glycolysis, which promotes pulmonary hyperproliferation [86]. It has also been suggested that removal of mitochondrial hydrogen peroxide leads to hypoxic pulmonary vasoconstriction, and nevertheless, increased levels of mitochondrial ROS contribute to hypoxic pulmonary vasoconstriction and hypoxic PH [87, 88].

Antioxidant enzyme dysfunction. Given that the reaction between NO and superoxide anion leads to generation of the potent oxidant peroxynitrite, enzymes that scavenge and reduce levels of superoxide will reduce peroxynitrite-induced injury. While NO reacts with oxyhemoglobin and is rapidly converted to nitrate in erythrocytes, superoxide is removed by SOD enzymes [89]. Mitochondrial SOD is rendered inactive, however, under conditions of nitrative stress when increases in peroxynitrite lead to nitration of tyrosine residue 34 in vitro [30]. Tyrosine nitration of SOD has also been demonstrated in vivo in newborn lambs with persistent PH [31]. Although mitochondrial SOD nitration is seen in human renal allografts [30], it is unknown whether mitochondrial SOD is nitrated and inactivated in lung tissues of patients with severe PH such as IPAH. However, one study has shown decreased activities of SOD isoforms (including CuZnSOD, ECSOD, and MnSOD) in airway epithelial cells and in bronchial tissues of IPAH patients [90], suggesting that levels of superoxide could be increased in IPAH lung tissue.

5 Nitrative Stress in Pulmonary Hypertension Patients

Human studies that provide evidence for a role of nitrative stress in the pathogenesis of PH can be observational studies of nitrative stress factors in PH patients or studies showing an impact of therapies that target nitrative stress signaling pathways on clinical outcome. For example, levels of peroxynitrite are elevated in the lungs of patients with severe PH, and these levels are thought to be raised in part by conditions of tissue hypoxia and inflammation [13, 80]. Prominent tyrosine nitration, a hallmark of nitrative stress, is evident in lung tissues of severe PH patients including IPAH patients [13]. Increased tyrosine nitration of PKG, for instance, is observed in lung tissues of IPAH patients [12]. The activity of eNOS is markedly increased in the lungs of IPAH patients compared with healthy controls, without significant change in its protein levels [11]. There is also evidence of high levels of eNOS in the plexiform lesions of lungs from IPAH patients [42], although eNOS levels have been reported to be upregulated [43], unaltered [91], or downregulated [92] in the lungs of PH patients versus control subjects. 8-hydroxyguanosine, the product of the reaction between superoxide and guanine, is markedly increased in the endothelium of plexiform and concentric lesions from IPAH patients but not in control subjects [13]. In the lungs of the same IPAH patients, the amount and activity of MnSOD was lower compared with controls. Another study also shows marked decreases in the expression and activity of all three of the SOD isoforms in lungs of IPAH patients [90]. In contrast, expression and activity of some of the ROS-generating enzymes are markedly increased in lung tissues of IPAH patients compared with controls; NOX4 expression was markedly increased in the pulmonary vasculature of IPAH patients (i.e., predominantly in the thickened medial layer of pulmonary arteries) [93]. Xanthine oxidase level is also enhanced in the circulation of IPAH patients, along with increased xanthine oxidase activity in the pulmonary arteries [79]. Together these studies demonstrate

increased oxidative and nitrative stress in PH patients, especially in IPAH patients.

The presence of nitrative and oxidative stress in PH patients suggests that antioxidant therapy could prove beneficial in the clinic. Despite examples of antioxidant therapy suppressing PH in animal models, such treatments in human PH have proven to be predominantly ineffective [94]. The antioxidant, coenzyme Q, for instance, gave rise to a modest improvement in right ventricle function, without lengthening 6-min walking time [95]. Large-scale randomized clinical studies of therapies that reduce nitrative stress will continue to improve our understanding of the importance of the nitrative stress pathways in the pathogenesis of PH in humans.

6 Conclusions

Increased oxidative/nitrative stress is a hallmark of severe PH in patients including those with IPAH. Peroxynitrite, nitrotyrosine, and nitration of specific proteins are prominent in lung tissues of IPAH patients. Recent studies have demonstrated that increased nitrative stress induces cytotoxicity in pulmonary vascular cells, post-translational modification (tyrosine nitration) of proteins and resultant dysregulation of their functions (Table 3), and decreased bioavailability of vasodilators NO and prostacyclin. Nitrative stress-induced tyrosine nitration of PKG results in impairment of its function (via decreased kinase activity or cGMP binding), which causes vasoconstriction and vascular remodeling leading to PH. Nitration of prostacyclin synthase inhibits its ability to synthesize the vasodilator, prostacyclin, while eNOS nitration induces eNOS uncoupling leading to decreased NO production and increased superoxide generation. Nitration-mediated inhibition of antioxidant enzymes such as MnSOD enhances superoxide levels and oxidative/nitrative stress. Nitrative stress also leads to activation of key signaling molecules such as ERK and PKC, which promotes pulmonary vascular cell proliferation that underlies pulmonary vascular remodeling. Thus, nitrative stress plays an important role in the pathogenesis of PH by inducing vasoconstriction and pulmonary vascular remodeling (Fig. 2b, and Table 3). Although current approaches that restore aberrant nitrative signaling and alleviate nitrative stress can reduce PH in animal models, the potential benefit of such treatments on patient survival is yet to be conclusively proven. Development of novel pathologically relevant animal models of PH [91] and of therapies that inhibit nitrative stress (thereby normalizing the functions of key proteins such as PKG) could ultimately lead to improved clinical outcome in patients with PH.

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Pentose Shunt, Glucose-6-Phosphate Dehydrogenase, NADPH Redox, and Stem Cells in Pulmonary Hypertension

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1 Pulmonary Hypertension

Pulmonary hypertension is classified into five groups: pulmonary arterial hypertension (PAH, Group 1), pulmonary hypertension due to left heart disease (Group 2), pulmonary hypertension due to chronic lung disease and/or hypoxia (Group 3), chronic thromboembolic pulmonary hypertension (Group 4), and pulmonary hypertension due to unclear multifactorial mechanisms (Group 5) [1].

Pulmonary hypertension is a progressive disease characterized by pulmonary vasoconstriction and vascular remodeling, which cause increased pulmonary vascular resistance and ultimately right ventricular failure [2–4]. Both "unusual endothelial cells" and "unusual smooth muscle cells" with apoptosis-resistant proliferative properties appear in pulmonary hypertension and cause pulmonary vasoconstriction and vascular remodeling that includes: intimal thickening, medial hypertrophy, and adventitial muscularity.

It appears that one of the first hits to occur in pulmonary hypertension is disruption of the endothelial layer, which triggers the release of growth factors and cytokines to promote proliferation and survival of remained endothelial cells (Fig. 1). However, these factors also change the cells to "unusual endothelial cells" and "unusual smooth muscle cells." In this context, we use the term "unusual cells" as no-usual cells in healthy state, although "unusual cells" might be essential for repair and may be normal in pathological state. For example, expression of platelet-derived growth factor (PDGF) and its receptor is increased in lung tissue with PAH [5]. In addition, isolated smooth muscle cells (SMCs) from patients with PAH show a higher rate of growth in response to PDGF stimulation [6]. Endothelin-1 (ET-1) signaling is mediated through two receptor subtypes, ET type A receptor (ET_AR) and ET type B receptor (ET_BR). ET-1 induces contraction and proliferation of SMCs through ET_AR, which is highly expressed in SMCs but not in endothelial cells (ECs). On the other hand, ET-1 accelerates the production of vasodilators such as nitric oxide (NO) and prostaglandin I_2 (PGI_2) in ECs via ET_BR, which is mostly expressed in ECs [7, 8]. In pulmonary hypertension, expression of ET-1 increases in lung vascular endothelial cells [9, 10], ET_A increases [11] and ET_BR decreases [12] in lung. These reports suggest that increased ET-1 in pulmonary hypertension enhances contraction of pulmonary artery SMCs (PASMCs) through increased ET_AR and indirectly through decreased ET_BR in ECs. Increased ET-1 and

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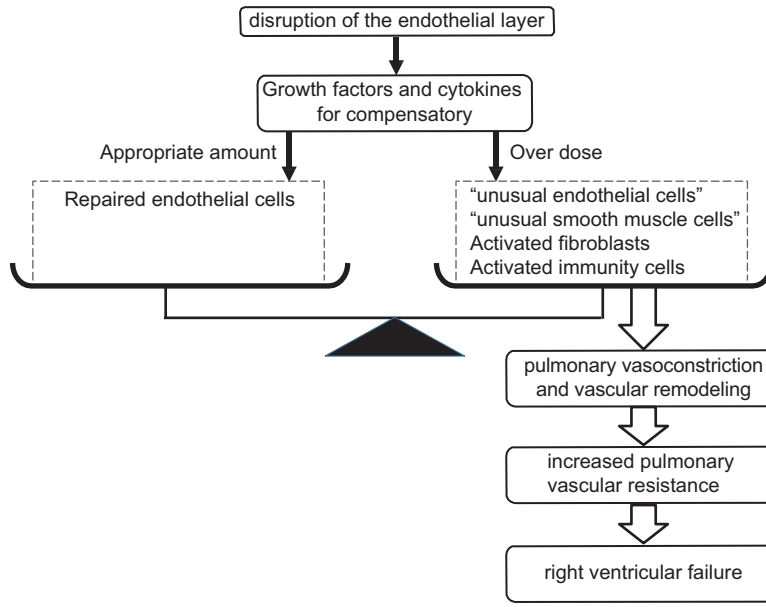

Fig. 1 A schematic illustration demonstrating the endothelial dysfunction lead to pulmonary artery remodeling and constriction elicits pulmonary hypertension and heart failure

PDGF also enhances proliferation of PASMCs through increased ET_AR and PDGF receptor.

Increased inflammation is another significant contributor to the pathogenesis of pulmonary hypertension. Inflammatory cells (such as T and B lymphocytes, mast cells, and macrophages) and inflammatory cytokines (such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)) are increased in pulmonary hypertension [13, 14]. For example, TNF-α contributes to change PASMCs to a proliferative and apoptosis-resistant phenotype [15]. IL-6 promotes polarization of alveolar macrophages to M2 phenotype through T cell activation. M2 macrophages enhance the proliferation of PASMCs by releasing soluble factors such as CXCL12 [16]. To this regard, pro-inflammatory perivascular fibroblast-derived IL-6 activate M2 macrophages and PASMCs leading to outside-in remodeling of pulmonary arteries [17]. Because pulmonary hypertension is a multifaceted disease and many other factors, cells, and signaling are involved in the pathogenesis of pulmonary hypertension, we recommend referring to other review articles [2, 4, 13, 14] for more information about pulmonary hypertension.

2 Warburg Effect and Pulmonary Hypertension

One hallmark of cancer is the metabolic switch, which is known as the Warburg effect and leads to greater glucose uptake and aerobic glycolysis [18, 19]. Furthermore, glucose flux is diverted into the pentose phosphate pathway (PPP) due to inhibition of phosphofructose kinase-1 and increased expression of the pyruvate kinase M2 in cancer cells and in hypoxic cells [20]. Aerobic glycolysis is observed in human pulmonary endothelial cells isolated from PAH [21]. Some studies have shown a greater lung glucose uptake (measured by glucose analogue, fluorine-18-labeled 2-fluoro-2-deoxyglucose (^{18}FDG)) in PAH patients when compared with the control subjects [22, 23]. In addition, glucose uptake is also observed in rats with pulmonary hypertension and glucose transporter-1 (Glut1) upregulation is observed in both pulmonary endothelial cells and smooth muscle cells [24]. Aerobic glycolysis is upregulated in macrophages of hypoxic calves and iPAH patients [25]. These reports

suggest that the Warburg effect exists in pulmonary hypertension. Based on these and other observations, it has been proposed that a reprogramming of metabolism plays a critical role in the pathogenesis of pulmonary hypertension and is a druggable target.

3 Glucose Metabolism, Pentose Shunt, and Glucose-6-Phosphate Dehydrogenase in Pulmonary Hypertension

3.1 Glucose Metabolism is Altered in Pulmonary Hypertension

Glycolysis, glucose flux through the pentose phosphate pathway (PPP), and the activity of NADPH producing isocitrate dehydrogenase-1 and -2 are increased in endothelial cells with BMPR2 mutation and in PAs of idiopathic- and heritable-PAH patients [26–28]. Expression of enzymes in the glycolytic pathway (e.g., hexokinase-1, 6-phosphofructokinase and pyruvate kinase M2) and the PPP (e.g., 6-phosphogluconolactonase, transaldolase-1 and transketolase) is increased in PASMCs of PAH patients with *BMPR2* exon1–8 deletion [27]. Studies suggest that the expression and activity of glucose-6-phosphate dehydrogenase and PPP are increased in: (1) endothelin-1 treated PASMCs from PAH patients with *BMPR2* exon1–8 deletion [27]; (2) lung fibroblasts from idiopathic PAH patients and calves with HPH [25]; (3) hypoxic rat PASMCs [29]; (4) PASMCs derived from 4-week-old lambs exposed to increased pulmonary blood flow [30]; and (5) lungs of HPH and monocrotaline-induced PH rat models [29, 31]. Interestingly, PPP activity is increased before PH develops [29, 32]. This suggests that an increase of glucose-6-phosphate dehydrogenase (G6PD) activity has a temporal relationship with PH. G6PD, the rate-limiting enzyme in the PPP, is a major supplier of cellular NADPH (60% by G6PD + 40% by isocitrate dehydrogenase and other sources) for: anabolic reactions and NADPH oxidases-derived superoxide production [33]. Excess NADPH generation contributes to pathogenic "reductive stress" in the cardiovascular system [34].

3.2 Glucose-6-Phosphate Dehydrogenase Inhibition Relaxes Pulmonary Arteries, Reduces Inflammatory Cytokines and Cell Proliferation, and Induces Cell Apoptosis

G6PD is the rate-limiting enzyme in the PPP, which is a metabolic pathway parallel to glycolysis. G6PD catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconolactone and the formation of NADPH from $NADP^+$. Thus, NADPH is mainly produced by G6PD and 6-phosphoglucoronate dehydrogenase in the oxidative branch of the PPP. The NADPH in turn maintains the supply of reduced glutathione [35, 36].

We originally found that G6PD-derived NADPH redox plays a critical role in regulating function of voltage-gated Ca^{2+} and K^+ channels through redox reaction and modulates intracellular Ca^{2+} in vascular smooth muscle cells and that inhibition of G6PD with dehydroepiandrosterone and epiandroterone (noncompetitive inhibitors) and 6-aminonicotinamide (a competitive inhibitor) relaxes precontracted aorta and pulmonary arteries [37]. We recently found that: (1) hypoxia-induced contraction of isolated PAs from G6PD-deficient, as compared to wild-type, mice is blunted [38]; and (2) 6-aminonicotinamide, a competitive inhibitor of G6PD, prevents hypoxia-induced downregulation of contractile protein expression in isolated bovine PAs [39] and reduces expression of pro-inflammatory TNFα in hypoxic bovine PAs and rat PASMCs [40].

The PPP produces ribose sugar that is essential for de novo synthesis of RNA and DNA. G6PD-derived NADPH catalyzes fatty acid metabolism and lipid synthesis. Lipids are required for the formation of membranes in proliferating cells. Activation of the PPP supports K ras-induced, anchorage-independent cellular growth [41]. Conversely, Inhibition of G6PD increases the rate of apoptosis of *Xenopus laevis* oocytes, HEK293 cells, esophageal squamous cell carcinoma, and melanoma cells [42–45]. The NADPH redox also controls the activities of cell cycle enzymes and caspases that trigger apoptotic

cell death. Therefore, G6PD-derived NADPH plays a key role in stimulating proliferation and inhibiting apoptosis of cells [46]. Ectopic expression of G6PD increases rat PASMC proliferation [29] and contributes to the HIF1α-induced endothelial cell growth [47].

3.3 Glucose-6-Phosphate Dehydrogenase Inhibitor Treatment Reduces Pulmonary Hypertension

We and others have shown that dehydroepiandrosterone, which potently inhibits G6PD activity among other actions in the cell [48–50], reduces pulmonary vascular resistance in HPH and PAH rats [29, 51–54], and arterial elastance and heart failure in PAH rats [52, 55]. Interestingly, low levels of dehydroepiandrosterone sulfate are associated with severity of PAH in humans [56], and an ongoing Phase III clinical trial (NCT00581087) reports that dehydroepiandrosterone improves pulmonary hemodynamics in patients with COPD-associated PH [57].

4 G6PD and CD133 (+) Cells in Pulmonary Hypertension

4.1 CD133 (+) Cells

CD133 is a transmembrane pentaspan protein and is also known as Prominin-1 or AC133. As CD133 was first identified in neuroepithelial stem cells in mice [58] and in the same year in human hematopoietic stem cells (HSCs; [59]), CD133 is one of the stem cell (and/or progenitor) markers (Fig. 2). Accordingly as the CD133 studies proceed, relationships between CD133 positive cells (CD133 (+) cells) and some diseases have been revealed gradually. The hottest field of CD133 in medical research may be cancer stem cells. Singh et al. [60, 61], have identified CD133 (+) cell subpopulation from human brain tumors as brain tumor stem cells. Injection of these CD133 (+) cells produced a tumor in transplanted mouse brain and was a phenocopy of the patient's original tumor. CD133 (+) cancer stem cells have also been found in several other tumor types including gastric, colon, liver, pancreatic, prostate, ovarian, breast, and lung cancer [62, 63]. For more information about structural properties of CD133 and cancer stem cells, we recommend referring to the article by Shmelkov et al. [64].

4.2 Glucose Metabolism and G6PD in CD133 (+) Cells

Metabolism plays a significant role in progenitor cell phenotypic fate. It is well known that the metabolic status of HSCs is distinct from that of their differentiated progeny [65]. Like cancer cells, HSCs derive their energy from aerobic glycolysis instead of mitochondrial oxidative phosphorylation and Meis1 regulates HSC metabolism through transcriptional activation of HIF1α [66]. G6PD activity is increased in hypoxic CD133 (+) cells and increased G6PD activity upregulates expression of HIF1α, cyclin A, and phosphohistone H3 [67]. This maintains the CD133 (+) cells in highly proliferative state. Increased G6PD plays a critical role in CD133 (+) cell proliferation and growth, and co-localizes with CD133 (+) cells in the perivascular region of lungs from rats with hypoxia-induced pulmonary hypertension. Inhibition of G6PD by dehydroepiandrosterone in pulmonary arterial hypertensive rats nearly abolished CD133 (+) cell accumulation around pulmonary arteries and the formation of occlusive lesions [67].

4.3 CD133 (+) Cells Increase in Pulmonary Hypertension

The evidence of relationship between pulmonary hypertension and CD133 (+) progenitor cells has accumulated for the past decade. CD133 (+) cells accumulate around the pulmonary artery in hypoxia-induced pulmonary hypertension (Fig. 3). Papa et al. reported that systemic sclerosis patients, about 30% of whom show pulmonary hypertension, have more circulating CD133 (+) progenitor cells in peripheral blood [68, 69].

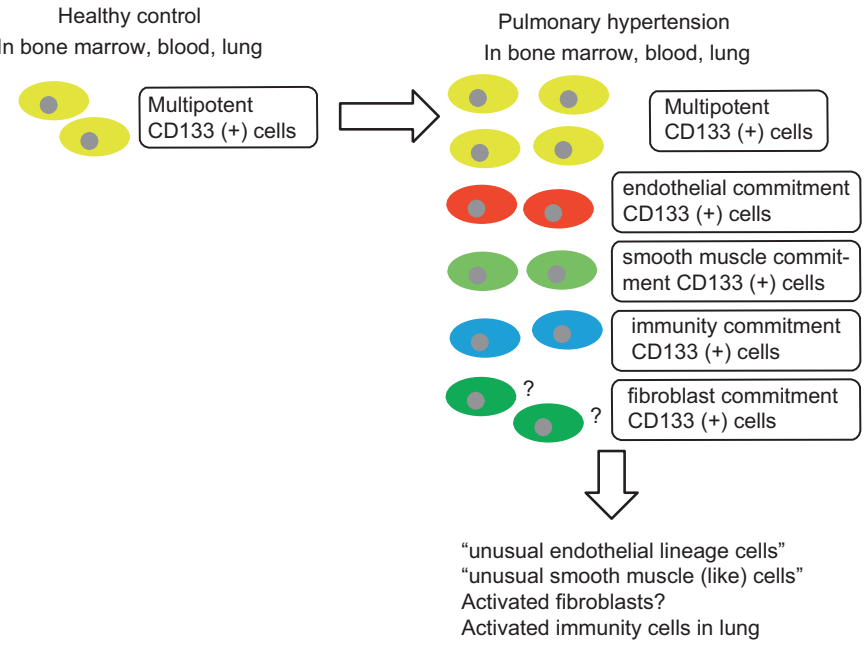

Fig. 2 Multipotent bone marrow-derived CD133 (+) stem cells give rise to endothelial, smooth muscle, immune, and fibroblast cells and play a critical role in the pathogenesis of pulmonary artery remodeling and hypertension

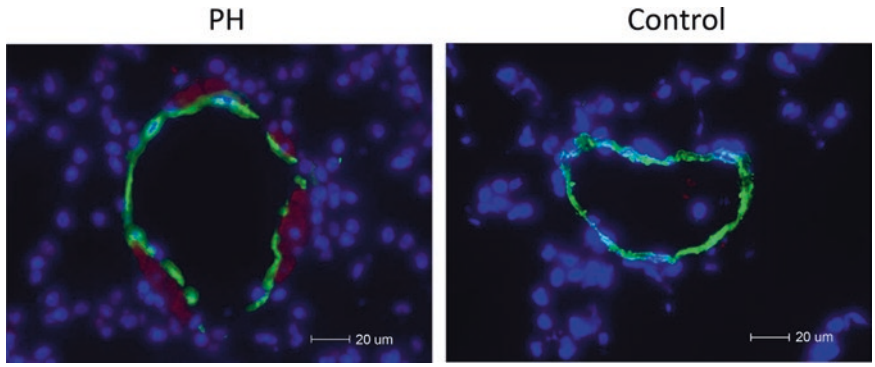

Fig. 3 CD133 (+) cells appear in perivascular region and particularly around the vessel wall before pulmonary artery remodels and pressures increase in mice exposed to hypoxia (10% O2) as compared to normoxic control

Others also have reported that CD133 (+) cell numbers are increased in blood from patients with other forms of pulmonary hypertensions as compared to healthy controls [70–73]. Not just in humans, CD133 (+) cells increase in blood of beagle dogs after treatment of monocrotaline, which is used to induce pulmonary hypertension [74]. We also observe that hypoxia increases the number of CD133 (+) cells in mice blood (manuscript in preparation). CD133 (+) cells are also increased in lung vessels of pulmonary hypertension patients [75]. CD133 (+) cells in lung also express CD34 [73], CD31 [76], or CD34 and VEGFR-2 [77]. As familial PAH is associated with mutations in bone morphogenetic protein type II receptor (BMPR2), mice with BMPR2 mutation result in increased right ventricular systolic pressures accompanied with pulmonary

vascular remodeling. In these mutant mice, there are substantial numbers of CD133 (+) cells in lung blood vessels [78]. These reports suggest that CD133 (+) cells increases in pulmonary hypertension. Asosingh et al. [79] reported that transplantation of bone marrow CD133 (+) cells from patients with pulmonary hypertension into mice resulted in angioproliferative pulmonary vascular remodeling, right ventricular failure, and death.

4.4 CD133 (+) Cells Exacerbate Pulmonary Arterial Hypertension

What is the role of CD133 (+) cells in pulmonary hypertension? The numbers of CD133 (+) cells in blood of PAH patients have correlative relationship with pulmonary artery pressure [70, 80–83] and cardiac dysfunction [84]. Xenograft of bone marrow CD133 (+) cells of PAH patients led to lung endothelial injury and widespread in situ thrombosis in immunodeficient NOD-SCID mice. Engraftment of PAH-CD133 (+) cells also results in hypertrophic right ventricle [79]. These reports suggest that CD133 (+) cells exacerbate pulmonary hypertension. The next question is how does CD133 (+) cells exacerbate pulmonary hypertension? As described in Sect. 1, both "unusual endothelial cells" and "unusual smooth muscle cells" with apoptosis-resistant and proliferative properties appear in pulmonary hypertension. These cells cause pulmonary vasoconstriction and vascular remodeling. CD133 (+) progenitor cells have multipotent differentiation potential [79]. In that regards, CD133 (+) cells expressing endothelial cell marker (CD31) are also detected in the endothelium of plexiform lesions in PAH patients [76]. In addition, numbers of colony-forming units of CD133 (+) endothelial-like cells (CFU-ECs) are elevated in blood [70]. CFU-ECs from PAH patients have greater affinity for angiogenic tubes, and spontaneously form disorganized cell clusters that increase in size in the presence of transforming growth factor β (TGF-β) or bone morphogenetic protein-2 (BMP-2) [70]. It is also noteworthy that endothelial progenitor cells (EPCs) isolated from BMPR2-associated PAH show high proliferation activity and impaired tube-formation activity [76]. Although this CFU-ECs and EPCs were not purified as CD133 (+) cells, these reports suggest that CD133 (+) endothelial lineage cells are "unusual cells" with high proliferative and impaired normal tube-formation activity, which cause vascular remodeling. CD133 (+) cells expressing smooth muscle cell marker, smooth muscle actin (SMA), are also detected in occlusive lesions and perivascular areas in lung vessels of pulmonary hypertension mice [78] and PAH patients [75]. In addition, human CD133 (+) cells under coculture with PASMCs differentiate into smooth muscle-like cells in vitro and this transition is blocked by G6PD inhibition [67]. Differences of differentiation potential of CD133 (+) progenitor cells have been reported in pulmonary hypertension. For example, bone marrow CD133 (+) cells give rise to all hematopoietic lineages (erythroid, myeloid, monocyte/macrophage, and megakaryocytes), and the CFU-granulocyte and monocyte (CFU-GM) colony formation is increased in PAH patients [73]. Higher number of multilineage colonies of CFU-granulocyte, erythrocyte, monocyte, megakaryocyte and CFU-GM formed from CD133 (+) cells has been reported in PAH patients [79]. These studies indicate that there are more bone marrow-derived multipotent CD133 (+) progenitor cells with a greater myeloid commitment that increase immune cells in pulmonary hypertension. Thus increased CD133 (+) progenitor cells differentiate into "unusual endothelial lineage cells," "unusual smooth muscle-like cells," and immunity cells, which contributes to remodeling in pulmonary hypertension.

In conclusion, we have discussed the latest developments in the metabolic status and the role of G6PD/PPP-dependent redox signaling in the regulation of phenotypic fate of various cell types, including CD133(+) cells, that contribute remodeling of pulmonary arteries in hypertensive lungs and G6PD as a potential druggable target to abrogate PH.

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Redox Regulation of the Superoxide Dismutases SOD3 and SOD2 in the Pulmonary Circulation

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1 Introduction

When evaluating the role of redox-regulating signaling in pulmonary vascular diseases, it is intriguing to consider the modulation of key antioxidant enzymes like superoxide dismutase (SOD) because SOD isoforms are regulated by redox reactions, and, in turn, modulate downstream redox sensitive processes. The emerging field of redox biology is built upon understanding the regulation and consequences of tightly controlled and specific reduction–oxidation reactions that are critical for diverse cellular processes including cell signaling. Of relevance, both the site of production of specific reactive oxygen and nitrogen species and the site of the antioxidant defenses are highly compartmentalized within the cell. For example, superoxide is generated during oxidative phosphorylation in the mitochondria as well as by a number of enzymatic sources within the cytosol and at the cell membrane. In the pulmonary circulation, these sources include the mitochondrial electron transport chain, NADPH oxidases (NOX1–4, Duox1,2), nitric oxide synthases, and xanthine oxidase; this important topic has been thoroughly reviewed recently [1]. In parallel with these different cellular sites of superoxide production, the three SOD isoforms are also specifically localized to the cytosol (SOD1), mitochondria (SOD2) or extracellular compartment (SOD3). This chapter focuses on the role of redox mechanisms regulating SOD2 and SOD3, with an emphasis on these processes in the setting of pulmonary hypertension.

The superoxide dismutases are an important family of antioxidant enzymes that catalyze the rapid dismutation of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (~6.7×10^{-9} M^{-1} s^{-1}). Three human isoforms, cytosolic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular EC-SOD (SOD3), have been identified and characterized. The first SOD isoform, SOD1 was discovered by Fridovich and McCord in 1969 [2]. However, while SOD1 is essential for cellular homeostasis and there is a body of literature investigating the use of recombinant human SOD1 in the treatment of pulmonary hypertension, the level and activity of SOD1 has not been found significantly perturbed in human pulmonary hypertension [3–5].

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This chapter focuses on the regulation of SOD3 and SOD2 activity since accumulating evidence suggests that impaired activity of these enzymes contribute to pulmonary hypertension.

2 The Extracellular SOD3 Enzyme

2.1 Characterization of SOD3

The eukaryotic superoxide dismutase enzyme SOD3 was first detected in human serum and subsequently isolated from human lung [6–8]. Human SOD3 exists as a tetrameric protein comprised of four identical monomers, producing a mature enzyme of approximately 132 kDa. One key feature of SOD3 is its extracellular matrix (ECM) binding region in the C-terminal part of each subunit that allows it to bind to the ECM and accounts for its high expression in the vasculature. Although the exact molecular interactions have not yet been described, Antonyuk et al. have reported detailed high-resolution structural analysis of SOD3 including two grooves that are likely to represent binding sites for heparan sulfate and collagen [9]. Unlike many other organs, SOD3 is the most abundant SOD isoform in the vasculature, accounting for 60–70% of total SOD activity in human systemic and pulmonary arteries. The ECM binding region is susceptible to cleavage by proteolytic enzymes including furin, which changes the distribution of SOD3, releasing cleaved SOD3 into the extracellular fluids [10–13]. The formation of tetramers containing different combinations of cleaved and uncleaved SOD3 monomers leads to three different classes of SOD3 defined by their heparin-binding affinity [7, 14, 15]. A known polymorphism in human *SOD3* within the ECM binding region, R213G, decreases ECM binding affinity and results in a redistribution of SOD3 from the tissue into extracellular fluids [16]. SOD3 expression and activity is decreased in multiple animal models of PH and in the lungs of individuals with end-stage IPAH [4, 5, 12, 17].

2.2 Redox Regulation of SOD3

The mature secreted SOD3 monomer contains 222 amino acid residues and combines to generate a homotetramer of approximately 132 kDa [7, 18]. Posttranslational modifications include an N-linked glycosylation and the formation of intra- and inter-subunit disulfide bonds. Important regions of SOD3 include an N-terminal region responsible for the formation of the tetramer, an enzymatic domain coordinating zinc and copper ions and a positively charged ECM binding region in the C-terminal. These features of SOD3 are essential to its structure, function, and localization. Specific aspects of SOD3 biology, including disulfide bond formation, proteolytic cleavage of the ECM binding region, regulation of enzyme activity, and regulation of gene expression are influenced by the redox state.

2.2.1 Intrasubunit Disulfide Bonds

Five of the six native cysteine residues in SOD3 (Cys45, Cys107, Cys189, Cys190, and Cys195) participate in intra-subunit disulfide bond formation and protein folding that results in active (aEC-SOD) and inactive (iEC-SOD) forms whereas Cys219 is involved in the formation of an inter-subunit disulfide bond [19]. The active isoenzyme is stabilized by two disulfide bonds within the subunit between Cys45-Cys190 and Cys107-Cys189, whereas the inactive counterpart forms two disulfide bridges between Cys107-Cys195 and Cys189-Cys190 [20]. The active SOD3 enzyme disulfide pattern is homologous to that of SOD1 [21], critical for active-site conformation in this SOD isoform as well. Dimers formed by these two different SOD3 states in vivo lead to three different combinations of SOD3 dimers (homodimers of aa and ii, heterodimers of ai) with discreet enzyme activity [22]. These dimers assemble into tetramers of various SOD activity synthesized intracellularly before secretion [23]. While Cys195 participates in the disulfide bond formation and protein folding of human iEC-SOD monomer, the absence of this cysteine residue in the primary sequences of SOD3 in mouse [6], rat [24], and rabbit [20, 25] accounts for the exclu-

sive formation of the active SOD3 form in these species. The critical role of Cys195 in determining active vs. inactive states of SOD3 was confirmed by an elegant study in which an expression vector encoding human SOD3 was transfected into mouse cells that do not normally synthesize the inactive enzyme, showing that these cells were now able to synthesize both active and inactive SOD3. Moreover, when mouse SOD3 that lacks Cys195 was expressed in a human cell line that do not generate human SOD3, the resulting product derived from the mouse SOD3 was exclusively aEC-SOD [26]. Collectively, these studies suggest that the interplay between the reduction and oxidation of disulfide bonds in a given subunit may provide a redox-sensitive switch to determine SOD3 activity.

2.2.2 Intersubunit Disulfide Bond

The C-terminus of SOD3 contains a cluster of six basic amino acid residues [18] essential for binding of SOD3 to heparan sulfate [27, 28], collagen type I [29], and other cell surface-associated sulfated glucosaminoglycans [30] within the ECM. The conserved cysteine, Cys219 located within the C-terminus, forms an inter-subunit linkage between two full-length SOD3 monomers, bringing the two ECM binding regions in close proximity. The interaction of the positively charged ECM binding region in SOD3 with the negatively charged matrix components is responsible for the abundant localization of SOD3 to the lung and pulmonary vasculature [31, 32]. Recent data has also provided new insight into the redox-sensitive intracellular equilibrium between monomeric and dimeric SOD3, and confirmed that Cys219 impacts the susceptibility of the C-terminus to proteolytic degradation. Briefly, Cys219 can undergo disulfide bond formation to form dimers or react via cysteinylation or another yet unidentified protein interaction to maintain SOD3 as a monomer. Once secreted, the SOD3 dimers are resistant to proteolysis, while the monomeric form of SOD3 is susceptible to proteolysis in the setting of oxidative stress [33]. These data collectively demonstrate the importance of the cysteine residues and their redox reactions on the structure and localization of SOD3.

2.2.3 Proteolytic Cleavage of SOD3

Redox reactions not only can impact SOD3 directly by influencing cysteine disulfide bond formation, as discussed above, but also can modulate other key processes that in turn influence SOD3 structure or gene expression. Proteolysis is a major regulator of SOD3 biodistribution and contributes to the loss of bound SOD3 in various lung diseases [11–13]. Though the proteolytic processing of SOD3 has not been directly studied in PH, a number of proteolytic enzymes are increased in human PAH and animal models of PH. For example, among the complex vascular abnormalities involved in establishing PAH, there is an imbalance of matrix metalloproteases, adamalysins, serine elastases, and their inhibitors are involved in the excessive ECM synthesis and abnormal degradation during the remodeling process. Thus, there is a potential link between redox-regulated signaling and loss of SOD3 in the pulmonary vasculature in addition to the lung parenchyma [34].

Early studies reported that tetrameric SOD3 existed in multiple states that are characterized by different binding affinity to heparin. Three different classes of SOD3 are determined by the ratio of monomers containing the C-terminal ECM binding region (intact; Trp1-Ala222) vs. those lacking this ECM binding region (cleaved; Trp1-Glu209). To identify if the heterogeneity in heparin affinity is important for tissue biodistribution, the first studies showed that SOD3 could undergo limited proteolysis by endoprotease LysC, trypsin, and plasmin in vitro with significant loss of heparin binding affinity [7, 14, 15, 27, 35]. Subsequently, it was demonstrated that proteolytic processing of SOD3 occurs intracellularly in a two-step mechanism, which first involves the cleavage of Arg215-Glu216 bond by the endoprotease furin after passage through the Golgi compartment followed by the trimming of remaining basic amino acid residues back to Glu209 by a carboxypeptidase [10, 36, 37]. Cys219, discussed above, is not within the target site for furin, but instead the bond formation of Cys219 determines the susceptibility of SOD3 to furin and other carboxypeptidases to cleave the ECM binding region and release SOD3 from its

bound state within the matrix. Interestingly, a common human single nucleotide polymorphism in SOD3 results in a substitution of the Arg213 for glycine (R213G). This form of SOD3 can still be cleaved by furin but is resistant to complete processing by the carboxypeptidases, generating a mature C-terminus at Gly213 [37]. Recently, Morales et al. reported that neutrophil -derived elastase and cathepsin G cleave the N-terminal part of SOD3, which dissociates SOD3 and results in the formation of monomers that can diffuse into tissue [38]. Though the presence of cleaved vs. uncleaved forms of SOD3 has not been specifically tested in pulmonary vascular diseases, these data cumulatively suggest that the upregulation of proteases in the pulmonary circulation may regulate SOD3 distribution.

2.3 Regulation of SOD3 Activity

Multiple studies have demonstrated that SOD1 and SOD2 enzyme activity can be modulated by posttranslational modifications including nitration, phosphorylation, oxidation, glutathionylation, or glycation [39]. In contrast, there is very limited data on the posttranslational modifications that regulate SOD3 [39]. For example, in SOD1, a series of histidine residues involved in the coordination of catalytic metal ions, Cu and Zn, is oxidized by H_2O_2 [40, 41]. Since the amino acid sequence in the enzymatic region of SOD1 and SOD3 are highly homologous [18], and share a similar structural fold [9, 21], it is plausible that the same residues that are targets in SOD1 will also be subject to oxidative modifications in SOD3. Though a comprehensive evaluation of specific amino acid residues susceptible to protein modifications have not been performed for SOD3, it has been shown to be modified by hypochlorous acid (HOCl) and H_2O_2 oxidation that alters its biodistribution and enzymatic activity, respectively [38, 42–44]. In one study, neutrophil-derived HOCl at the inflammatory site of lung injury has been shown to target SOD3 for protein folding destabilization by oxidative modification of the N-terminal region and the formation of intermolecular crosslinking, which the authors speculate increase its susceptibility for proteolysis by neutrophil-secreted proteases. The resultant proteolyzed SOD3 is thought to lead to its change in its biodistribution from the ECM to BALF [38]. In an another study, H_2O_2 has been shown to inhibit/disrupt SOD3 enzyme activity in a peroxidase reaction involving the oxidation of histidine residues in the copper-containing catalytic core [11] similar to that described for SOD1 [45]. The inhibition of SOD3 enzymatic activity has also been demonstrated in a lamb model of persistent pulmonary hypertension of the newborn (PPHN) by H_2O_2 and this activity was restored by intratracheal administration of PEG-catalase [44]. We also previously found that the loss of SOD3 activity immunoprecipitated from lungs of hyperoxia exposed neonatal rats showed a higher nitrotyrosine content [46]. Another level of regulation of SOD3 is via the redox-sensitive membrane bound copper transport protein, Menkes ATPase, ATP7A. This copper ATPase is critical for providing the copper to the active site of SOD3 and its function is impaired by oxidation of cysteine residues [47]. These data suggest that SOD3 activity, similar to the other SODs, may be regulated by these specific redox-dependent protein modifications.

2.4 Regulation of SOD3 Gene Expression

One unexplored area of redox-dependent regulation is the transcriptional regulation of *SOD3* through the modulation of transcription factors that bind to the promoter region, or epigenetic mechanisms including DNA methylation, histone acetylation, and miRNA. The initial physiologic response to oxidative stress is an adaptive increase in antioxidant enzyme activity. However, unlike *SOD1* and *SOD2*, the *SOD3* gene does not appear to be regulated directly by the transcription factors, Nrf2 (nuclear factor E2-related factor), a classic redox-regulated transcription factor, nor hypoxia inducible factor HIF-1α. However, *SOD3* transcription is induced by the Sp1/Sp3 transcription factors as well as a number of cytokines including TNF-α, that are induced by other redox sensitive transcription factors in the setting of inflammation and oxidative stress [48]. On the other hand, the cell specific expres-

sion of *SOD3* in the pulmonary circulation and lung, and the loss of *SOD3* in cancers and pulmonary artery smooth muscle cells from individuals with IPAH can be regulated by DNA methylation or histone acetylation [49]. We recently reported that in IPAH human pulmonary artery smooth muscle cells, *SOD3* expression can be increased by molecular silencing or pharmacologic inhibition of Class I HDAC3 [5, 50–52]. There is also indirect evidence that miR21 could regulate *SOD3* expression in PAH, as this specific miRNA is increased in PAH and found to inhibit *SOD3* expression in lung epithelial cells [53–55]. The specific mechanisms responsible for these processes, both redox-regulated transcription factor activation and epigenetic regulation, require further investigation.

2.5 Redox Regulation by SOD3 in Pulmonary Hypertension

The biologic impact of SOD3 has been attributed to its ability to limit reactions of extracellular $O_2^{\bullet-}$ and preserve nitric oxide bioactivity in blood vessels [4, 56]. Thus, alterations in vascular SOD3 directly affect the redox state of the pulmonary circulation and modulate redox sensitive signaling pathways. This was demonstrated in chronically hypoxic mice in which overexpression of lung SOD3 prevented the hypoxia-induced decrease in lung GSH/GSSG [17]. Our lab and others have found that augmenting lung SOD3 either in genetically engineered mice with lung specific overexpression of SOD3 or using adenoviral SOD3 gene delivery protects in rodent models of chronic hypoxia, monocrotaline or bleomycin induced pulmonary hypertension [17, 57, 58]. Several studies also demonstrate that lack of total body SOD3 or vascular SOD3 augments chronic hypoxia or monocrotaline induced PH [59, 60]. In these models, several redox sensitive targets are modulated in the setting of increased or decreased SOD3. In the mouse model of chronic hypoxic PH, overexpression of lung SOD3 prevented the hypoxic induction of the transcription factor early growth response-1 (Egr-1), and its downstream target tissue factor [17]. We observed a similar attenuation in the induction of Egr-1 as well as TGF-β gene expression in the mice overexpressing lung SOD3 in the bleomycin-induced model of lung fibrosis and PH [58]. Conversely, in pulmonary artery smooth muscle cells, knock down of SOD3 increased Egr-1 and enhanced cell proliferation [61]. Furthermore, in mice with decreased vascular SOD3 due to an inducible knock out of SOD3 in smooth muscle cells, we observed a decrease in eNOS and GTP cyclohydrolase-1, the rate-limiting enzyme in the synthesis of tetrahydrobiopterin [59]. Collectively, redox-regulated processes are key to both the regulation of SOD3 and effects of low vascular SOD3 in pulmonary hypertension and substantial work is needed to more fully understand this process and harness this information to improve therapies.

3 The Mitochondrial SOD2 Enzyme

3.1 Characterization of SOD2

After the isolation of SOD2 from prokaryotic cells [62] and later from eukaryotic cells [2, 63], Barra and coworkers described the primary structure of human SOD2 [64]. The native enzyme was suggested to be composed of two identical polypeptide subunits containing 196 amino acid residues each. One feature that distinguished SOD2 of eukaryotic sources was the presence of cysteine residues which are absent in prokaryotic SOD2. When comparing different species, there was less sequence homology between SOD2s that observed for SOD1 between species. Also, no sequence homology was found between human SOD2 and SOD1. However, the predicted secondary structure and the optical characteristics of human SOD2 are similar to SOD2 isolated from *S. cerevisiae* as well as from prokaryotes including *E. coli* and *B. stearothermophilus* [65]. The overall structure of SOD2 is composed of a mixed α-helix and β-sheet in agreement with the described for the crystalized form of SOD2 from *T. thermophilus* [66].

The SOD2 protein has been purified and crystalized from human liver to characterize its subunit association and sulfhydryl reactivity. Matsuda and coworkers [67] described the human SOD2 as a tetrameric enzyme consisting of four identical subunits, and identified four reactive thiols per tetramer under non-denaturating conditions, and eight reactive thiols/tetramer when denaturating conditions were employed. They concluded that one out of the two cysteine residues in the SOD2 monomer was exposed and accessible for reaction with Ellmans' reagent and sensitive to alkylation by iodoacetamide. The remaining cysteine residue (Cys140) was buried and is only accessible for reaction when the protein is completely denaturated. They did not find changes in the enzymatic activity when wild type SOD2 enzyme was treated with thiols.

3.2 Redox Regulation of SOD2

The tetrameric interface of the SOD2 molecule has been analyzed using the wild type and two polymorphic variants at residues Ile58 and Leu60. The Ile58 to Thr58 variant has been found in the SOD2 gene in two out of six human cDNAs libraries [68, 69]. In addition, the coding mutation Leu60 to Phe60, was described in the T-cell leukemia-derived cell line [70]. Both Thr58 and Phe60 SOD2 variants produce a defective protein with modified assembly and activity. Both are located in the four-helix bundle interface. The hydrophobic residue Ile58, is the largest contributor of the buried surface area interacting with six other residues [71]. Replacing Ile58 with the hydrophilic Thr58 destabilizes the four-helix bundle, leading to decreased enzyme activity by a reduction in tetramer thermostability. Heat treatment of wild-type SOD2 at 41.7 °C for 3 hours does not alter its activity, but completely inactivates the Thr58 variant. In addition, the half-life at 37 °C was calculated to be 3.1 years for the wild-type human SOD2, and only 3.2 hours for the variant [72]. The Thr58 and Phe60 variants with weakened dimer–dimer interaction expose the buried Cys140 residue. Both showed thermal instability at 55 °C, and lose activity at 37 °C in the presence of 5 mM *N*-ethylmaleimide, whereas wild type SOD2 does not. When serine was substituted for Cys140 by site directed mutagenesis, both double mutants Ile58Thr/Cys140Ser and Leu60Phe/Cys140Ser lose their increased thiol sensitivity and show no activity loss in the presence of *N*-ethylmaleimide [73]. The authors suggest that cells expressing these SOD2 variants may be compromised at a physiologic body temperature, and may compensate by inducing SOD2 expression.

3.3 Regulation of SOD2 Activity

SOD2 inhibits peroxynitrite formation in mitochondria, and it may be itself inactivated by excess peroxynitrite [74]. In vitro, peroxynitrite-mediated nitration of Tyr34 of human SOD2 results in enzyme inactivation [75–78]. This process has been implicated in renal failure in which, though the quantity of SOD2 protein is increased in rejected human renal allografts, the activity level is impaired. MacMillan-Crow et al. demonstrated that peroxynitrite inactivates SOD2 enzymatic activity by tyrosine nitration and the presence of a nitrated and dysfunctional enzyme in rejected human renal allografts strongly supports the relevance of this process in vivo [79]. Furthermore, decrease in SOD2 activity has been associated with mitochondria dysfunction, over production and leakage of ROS and the consequent redox changes in the cytoplasm. Significant reduction of SOD2 specific activity has been documented in human (IPAH) and rat (FHR) pulmonary smooth muscle cells [80] and in total lungs from PH patients [81]. However, the mechanisms of SOD2 activity inhibition in PH remain to be elucidated.

3.4 Regulation of SOD2 Gene Transcription

The molecular structure and the organization of the human *SOD2* gene was first described by Wan et al. [82]. Both human and mouse SOD2 have two regulatory regions, promoter and enhancer. The transcription initiation site is local-

ized after a G + C rich (78%) promoter region containing a cluster of seven Sp1 and three AP-2 consensus sequences, with no TATA box or CAAT box. In addition, the 3′ flanking region of the *SOD2* gene contains one NF-κβ consensus sequence. The authors suggested that the presence of such sequences may play a role in the regulation of the expression of the human *SOD2* gene. The Sp1 binding sites in the 5′ region of the *SOD2* gene are necessary for the synergistic induction of SOD2 expression by interacting with the distal enhancer through a DNA loop [83]. There is a sequence containing 11 consecutive guanine nucleotides responsible for the D-loop structure which allow the binding of the RNA-binding protein [84], and thus the complex interaction of Sp1, NF-κβ, and p53 factors to regulate the constitutive and inductive expression of the *SOD2* gene [85]. Previously, it was shown the functional mechanism of basal SOD2 expression regulated by the Sp1/Sp3 ratio, where Sp3 binding have a lower effect in the induction of the *SOD2* gene [86]. Binding of AP-2 to the SOD2 promoter has been proposed as a negative regulator of the expression of the *SOD2* gene through a mechanism involving the Sp1–AP-2 ratio [87]. Also, a FOXO3 binding site located in the 5′ flanking region has been proposed to exert a regulatory role in the transcription of the *SOD2* gene [88, 89]. The promoter region is metabolically controlled, and polymorphisms in three loci of the *SOD2* gene have been associated with the levels of expression of the gene in cancer cell lines [90]. An enhancer element in intron 2 of the gene has been described as well [90, 91]. This region confers inducibility by inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interferon-γ [92, 93]. This enhancer element is responsive to inflammatory and perhaps hormonal regulators. Also, it is one polymorphism located near the enhancer region of the *SOD2* gene (G1677 T) between two potential AP-1-binding consensus sequences. The activity of the AP-1 binding protein is redox regulated. We identified an association of T allele with a decreased risk of lung cancer [94]. There are several studies regarding the redox regulation of the human *SOD2* gene.

Early studies of SOD2 biosynthesis regulation were done in *E. coli* by Gardner and Fridovich [95]. They show inhibition of SOD2 synthesis and suppression of transcription associated with changes in the redox state of the cells by using thiols such as glutathione, dithiothreitol and *β*-mercaptoethanol. They suggest that thiols directly may reduce a regulatory protein which suppresses transcription in its reduced state, but not in its oxidized one. One protein was isolated from S-30 extracts, which can interact with the palindromic region located near the transcription start site. Another possible option was that thiols can prevent the formation of one "compound" which causes increased transcription of the *SOD2* gene. The candidate was an aminoacyl *t*-RNA synthetase, because it is sensitive to oxidative inactivation, and because it can be protected by thiols. Later it was shown that depletion of glutathione by using thiol-modulating agents, such as *N*-ethylmaleimide, induces an increase of SOD2 activity in human lung fibroblasts [96].

Reactive thiols have been associated with the activation of NF-κβ and the consequent SOD2 expression by cytokines in human lung adenocarcinoma cells [97, 98]. Das and collaborators described how cytokines such as TNF-α or IL-1 enhance the activity of NF-κβ, which results in the activation of SOD2 expression, and how thiols (*N*-acetyl cysteine, dithiothreitol, and *β*-mercaptoethanol) modulate the regulation of SOD2 expression through the cytokine-NF-κβ and/or AP-1 activation. TNF-α and H_2O_2 appear to inhibit the induction of human *SOD2* gene through different pathways [99]. NF-κβ-DNA binding and expression of *SOD2* gen induced by TNF-α is inhibited by pretreatment with *N*-acetyl cysteine. Also, H_2O_2 pretreatment increased expression of SOD2 mRNA, while pretreatment with *N*-acetyl cysteine blocked that induction. Also, addition of diamide, which oxidizes sulfhydryl groups, increases the expression of *SOD2* gene [99]. However, no consistent redox regulation or changes in AP-1-DNA binding by modulated thiols on human *SOD2* gene expression were found [98, 99]. The nuclear factor NF-κβ exists in the cytoplasm in an inactive disulfide form, and its reduction may be required to allow

its DNA binding activity. The p50 subunit of NF-κβ is an disulfide-linked dimer, and mutations in the Cys62 residue alters the DNA binding and thiol sensitivity of NF-κβ [97]. Dhar et al. showed in vivo reduction of SOD2 induction mediated by p50 repression after 12-*O*-tetradecanoylphorbol-13-acetate treatment [100].

Superoxide and peroxynitrite have been suggested to regulate the *SOD2* gene expression. Earlier studies in human tracheal epithelial cells showed a 2–3 fold increase in SOD2 message levels when exposed to superoxide produced by the xanthine/xanthine oxidase system [101]. However, no direct mechanism of the gene regulation by superoxide has been described. In contrast, peroxynitrite appears to regulate SOD2 expression in the rat SOD2 promoter in vitro. Experiments using a promoter-luciferase reporter assay driven by the rat SOD2 promoter showed an increase in SOD2 message levels in lung epithelial cells when exposed to peroxynitrite [102]. The authors suggest that such induction of expression may involve a direct activation of intracellular signaling pathways, or indirect activation of an oxidant sensitive nuclear factor.

The SOD2 promoter contains CpG islands, which could be target for methylation and therefore epigenetically regulate SOD expression. Cytosine methylation in the SOD2 promoter has been associated with epigenetic silencing of the *SOD2* gene expression in cancer [103–105]. Likewise, high levels of histone acetylation of the proximal promoter of the *SOD2* gene were associated with decrease of the SOD2 expression in cancer cell lines [106, 107]. The inhibition of methyl transferases in pancreatic carcinoma cell lines reduced the methylation levels in the SOD2 promoter and reestablished the SOD2 transcription levels [108].

3.5 Redox Regulation by SOD2 in Pulmonary Hypertension

Oxidative stress is a product of either the increased production of reactive oxygen species (ROS) and/or the diminishing of antioxidants. The mitochondria are the main sources of $O_2^{\bullet-}$ production, and their damage and consequent changes on the cellular redox balance contribute to the development and worsening of pulmonary hypertension.

Reduction of catalase and SOD2 has been shown in the development of pulmonary hypertension (PH) in a lamb model of increased postnatal pulmonary blood flow [109]. Increased lipid peroxidation and reduced plasma glutathione peroxidase is correlated in patients with cardiac insufficiency and pulmonary hypertension. The use of antioxidants improves the prognosis in patients with PPH [110]. Early studies showed than SOD2 protects pulmonary epithelial cells from oxygen injury [111]. Because of the chronic inflammatory environment created under those circumstances, a vascular endothelial dysfunction is expected to be associated with late complications. It is possible the existence of a unifying mechanism linking mitochondrial dysfunction, reactive oxygen species unbalance, endothelial dysfunction, and unregulated proliferation observed in chronic pathologies such as obesity, type 2 diabetes, cardiovascular disease, and pulmonary arterial hypertension. There are evidences of a redox signaling (O_2-sensitive) pathway involved in the intersection of pulmonary hypertension and cancer. Some new mitochondrial abnormalities, such as activation of a pyruvate dehydrogenase kinase, SOD2 deficiency, and fragmentation and hyperpolarization of the mitochondrial reticulum have been found in PH [112]. The use of anti-inflammatory drugs has shown to decrease superoxide radicals improving the response of pulmonary arteries to vasodilators in a lamb persistent pulmonary hypertension model [113].

Decreased SOD enzymes have been associated with PH. SOD2 deficiency has been identified in the pulmonary arteries and plexiform lesions of PAH [80, 81]. In a fawn-hooded rat model (FHR), it was observed downregulation of SOD2 preceding the development of PAH [80]. Archer's group discovered a selective hypermethylation of the CpG islands on the intron 2 of the *SOD2* gene in pulmonary smooth muscle cells (PASMC), but not in the aortic smooth muscle cells of the fawn hooded rat. Such hypermethylation reduces its expression ~50% compared to

PASMCs from genetically matched consomic rats, which contributes to the proliferative, anti-apoptotic phenotype of the PASMC of the FHR [114]. This study demonstrated that SOD2 methylation is important in the development of PAH and contributes to HIF-1α activation and the development of an apoptotic resistant state with marked proliferation. Previously, it was showed that SOD2 regulate and stabilize HIF-1α via $O_2^{\bullet-}$ availability [115]. Overall, we conclude that both SOD3 and SOD2 are regulated by oxidative stress and redox-dependent signaling mechanisms and impaired activity of these enzymes alters downstream redox-sensitive targets and contributes to the pathogenesis of pulmonary hypertension.

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A Brief Overview of Nitric Oxide and Reactive Oxygen Species Signaling in Hypoxia-Induced Pulmonary Hypertension

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1 Introduction

In general, chronic hypoxia leads to pulmonary artery remodeling driven by smooth muscle proliferation and increase in wall thickness, which causes increase in flow resistance. This phenomenon leads to pressure overload of the heart right ventricle (RV) potentially causing its failure, the main driver of mortality in patients with chronic obstructive disease [1]. The remodeling process is initiated by oxygen sensors present in vascular cells that detect a decrease in partial pressure of oxygen in the blood (pO_2), and then activate a signaling system that leads to acute constriction of pulmonary arteries [2]. Eventually, this acute phase is "consolidated" by architectural remodeling of the vascular wall that perpetuates lumen narrowing [2]. Interestingly, although RV dysfunction seems to be caused by pressure overload secondary to increase in pulmonary vascular resistance, new evidence suggest that these two phenomena could develop independently in such a way that RV dysfunction occurs even if PH is prevented [3].

Among many signaling molecules, the contribution of reactive oxygen species (ROS) and NO to the pathophysiology of PH is complex and partially elucidated in the context of hypoxia-mediated pulmonary hypertension (World Health Organization Class 3). Some of the cellular signals found to participate in the hypoxia-induced PH seem to be also relevant in other models of pulmonary vascular remodeling that occur under normoxia [4]. In this short review, we outline the salient results that provide a foundation to the delineation of the mechanisms by which aberrant production of NO and ROS may contribute to the pathogenesis of PH.

2 Nitric Oxide and Pulmonary Hypertension

2.1 Relevant Nitric Oxide Biochemistry

Nitric oxide (NO) is a paracrine and autocrine messenger molecule that is derived from the five electron oxidation of L-arginine [5]. This reaction is catalyzed by nitric oxide synthase (NOS; Fig. 1), of which three isoforms have been described in mammals, neuronal NOS

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(nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3) [6]. Tissues and cells conserve NO through nitrosylation and nitrosation of biomolecules and NO itself can be released upon reductive decomposition of functional groups such as S-nitrosothiols (Fig. 1; [7]). The reaction of NO with metals to form nitrosyl or nitroso species is also an important step determining many of the functional effects of NO such as the activation of soluble guanylate cyclase (sGC) or inhibition of cytochrome c oxidase [8]. In addition, the nitrosation of thiols by reactive species derived from NO serves as a posttranslational modification that modulates protein function such as certain caspases [9].

An alternative source of NO is derived from the reduction of nitrite (NO_2^-; Fig. 1) that proceeds at low pH and under hypoxia [10]. The significance of this reaction is as an alternative source of NO at sites where NOS might be inhibited due to the lack of molecular oxygen but where hypoxic and acidic reduction of diet or pharmacologically derived NO_2^- is possible. Deoxyhemoglobin is an important site upon which NO_2^- is reduced to NO, although the chemical pathway and mechanism by which NO may escape erythrocytes still need clarification [11]. Whether non-erythrocytic cells mediate hypoxic NO_2^- reduction in the lung is also under investigation with multiple intracellular activities identified consisting of additional globins (Myoglobin [12], Neuroglobin [13], and Cytoglobin [14]) and molybdenum-containing proteins (xanthine dehydrogenase [15], sulfite oxidase [16], aldehyde oxidase and mitochondria amidoxine reducing component [17]), potentially

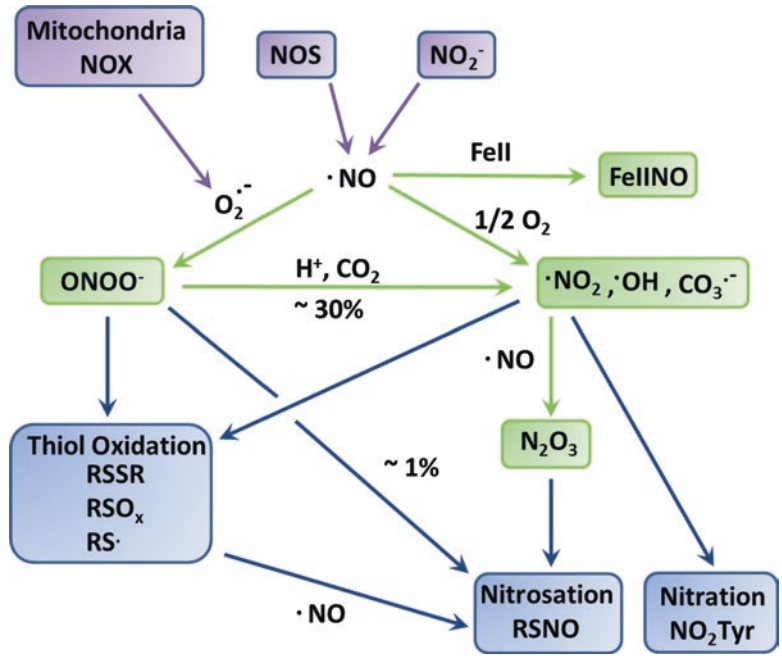

Fig. 1 Biological chemistry of nitric oxide relevant to pulmonary hypertension. Important sources of nitric oxide (NO) include nitric oxide synthase (NOS) activity and nitrite (NO_2^-)-reduction under hypoxia and in acidic environments. NO reacts with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$). Superoxide may be derived from multiple sources including mitochondrial and NADPH oxidase (NOX) activities. Peroxynitrite—upon protonation or combination with carbon dioxide (CO_2)—yields a number of free radicals, including nitrogen dioxide (·NO_2), and the hydroxyl (·OH) and carbonate ($CO_3^{\cdot-}$) radicals. NO reacts with metals such as iron (FeII) to form a metal-nitrosyls (FeIINO) such as the one found in soluble guanylate cyclase. Nitric oxide also reacts with molecular oxygen to form nitrogen dioxide and dinitrogen trioxide (N_2O_3). All together these species may be involved in oxidative (such as thiol oxidation), nitrosation (thiol nitrosation, RSNO), and nitration (tyrosine nitration, NO_2Tyr) reactions with biological targets. See Text for details

contributing to this activity in the pulmonary vasculature.

One of the most significant reactions of nitric oxide (NO) is its combination with superoxide ($O_2^{\bullet-}$) at a diffusion-limited rate (Fig. 1; [18]). The product of this reaction, peroxynitrite ($ONOO^-$/ONOOH), is a one and two electron oxidant, which modifies DNA, proteins, lipids, and sugars by way of oxidation, nitration, and nitrosation [18–20]. The biochemical reactivity of peroxynitrite in physiologically relevant settings may be dominated by its reaction with thiols and transition metals but also with excess carbon dioxide to yield a nitrosoperoxocarboxylate anion ($ONOOCO_2^-$) that partially decomposes to nitrogen dioxide and the carbonate radical [21]. Under most conditions, peroxynitrite might not coexist with NO or $O_2^{\bullet-}$ because superoxide dismutase and oxyhemoglobin insure limited availability of these molecules in excess of peroxynitrite. However, during conditions characterized by high NO synthase activity and multiple cellular sources of $O_2^{\bullet-}$, peroxynitrite-mediated reactions combined with those of excess NO or $O_2^{\bullet-}$ may become important. The formation of peroxynitrite in vivo, inferred from the formation of stable footprints such as 3-nitrotyrosine, represents an important mediator of tissue injury and dysfunction that limits NO bioavailability [22].

2.2 Nitric Oxide Signaling in Pulmonary Hypertension

The bioavailability and signaling of NO is decreased in experimental models and in patients with PH [23–26]. For example, Giaid and Saleh provided some evidence that the expression of eNOS was decreased in the vascular endothelium of pulmonary arteries in a cohort of patients with pulmonary hypertension with different grades of arteriopathy [27]. The dysfunction is usually considered to be decreased vasoprotection including depleted vasodilatory, antimigratory, and antiproliferative functions. However, loss of eNOS is also associated with a decrease in muscularization of small pulmonary vessels during chronic hypoxia in the mouse due to a decrease in proliferative capacity [25]. The underlying mechanism has been relatively well studied in animal models and is usually considered to be multifactorial through changes in eNOS expression and uncoupling [28], alteration in L-arginine metabolism [29, 30], and increased NO consumption through $O_2^{\bullet-}$. Although conflicting results exist regarding the levels of eNOS expression during PH, it is possible that—if increased—eNOS in the context of PH is uncoupled, meaning that a fraction of its activity is diverted towards the production of other reactive species such as $O_2^{\bullet-}$. Increased ROS production is associated with endothelial dysfunction and NADPH oxidase (NOX)-derived $O_2^{\bullet-}$ limits NO production and downstream signaling through eNOS uncoupling [31]. Overall, this provides conditions conducive to decrease NO bioavailability and increase oxidative and nitrative stress through the formation of peroxynitrite or metal-catalyzed nitration. Insufficient stimulation of sGC by NO reduces cGMP production and downstream effector activation such as cGMP-dependent protein kinase (PKG) [28]. In addition, downstream nitration or oxidation of target molecules such as PKG may lead to amplification of the inhibitory effect associated with NO inactivation [28].

2.3 Therapeutics Based on Direct Targeting of cGMP and NO Surrogates' Delivery

With a key role for the dysregulation of NO signaling in PH (including WHO Group 3 PH), therapeutic strategies aimed at restoring the NO/cGMP pathway have received increasing attention. A novel class of drugs that directly stimulates sGC independently of NO is now aggressively pursued for the treatment of PH. One such molecule, riociguat (Fig. 2), has been approved for the treatment of pulmonary artery hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH) [32, 33]. Riociguat has also shown significant therapeutic effects in patients with other types of PH including interstitial lung disease PH and PH associated

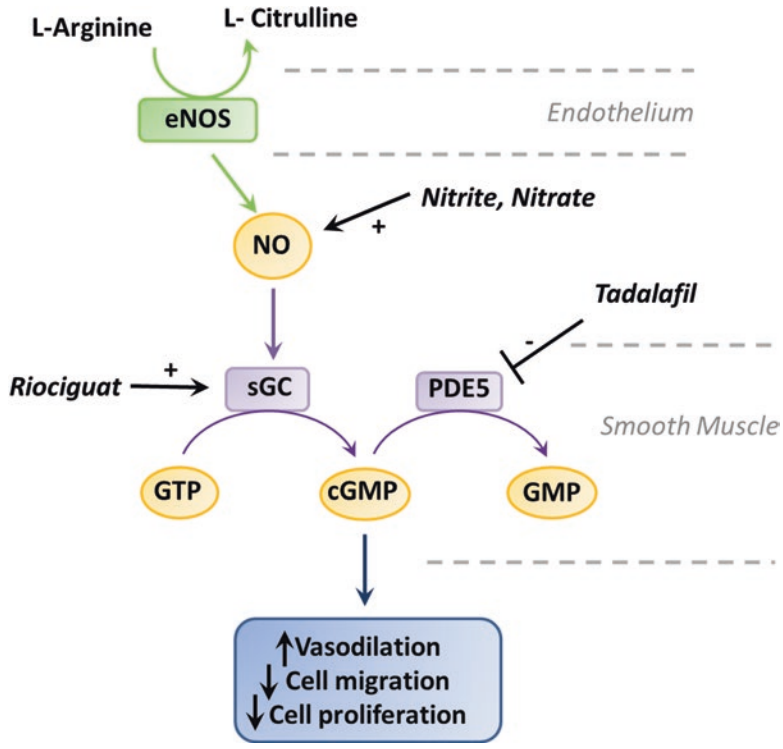

Fig. 2 Mechanism of action of NO in the vasculature and therapeutic targets. Nitric oxide (NO) is generated from the oxidation of L-arginine to L-citrulline by endothelium nitric oxide synthase (eNOS). NO diffuses into target cells such as smooth muscle cells to bind and activate soluble guanylate cyclase (sGC), which in turn generates cyclic GMP (cGMP) from GTP to promote vasodilation, and inhibit cell migration and proliferation. The signal is turned off upon cGMP hydrolysis to GMP by phosphodiesterase 5 (PDE5). Inhibition of this pathway is thought to contribute to the pathogenesis of pulmonary hypertension (PH). Increase NO biovailability through nitrite or nitrate delivery, stimulating sGC with Riociguat, or inhibiting PDE5 with Tadalafil all provides therapeutic means for the treatment of certain type of PH. See Text for details

with chronic obstructive pulmonary disease (COPD) [34]. The NO/cGMP pathway may also be targeted by inhibitors of phosphodiesterase type 5 (PDE-5) causing inhibition of the breakdown of cGMP by PDE-5. One such molecule, Tadalafil (Fig. 2), has been shown to reduce clinical worsening and improve hemodynamic outcomes in patients with PAH and is use in the clinic in this specific setting [35].

A number of preclinical studies have also indicated the beneficial effect of providing an alternate source of NO in the form of pharmacological delivery of NO_2^- or nitrate (NO_3^-) to alleviate PH [36–38]. In the context of hypoxia-induced PH in the mouse, inhaled nebulized NO_2^- inhibits and reverse preestablish PH and high right ventricular pressure. In this case, the effect of NO_2^- has been shown to be inhibited by a xanthine oxidase inhibitor or through diet-mediated inhibition of molybdenum-containing enzymes [38]. Dietary NO_3^- (which can be reduced to NO_2^-through the entero-salivary cycle) also reduced pulmonary vascular remodeling in mouse exposed to hypoxia for 3 weeks [36]. Interestingly, this effect required eNOS in addition to xanthine oxidase, suggesting a role for eNOS as a nitrite reductase. In a recent early phase II pilot study, Simon and coworkers have shown that inhaled NO_2^- provides some hemodynamics improvement in a small group ($n = 6$) of patients with PH due to lung disease or hypoxia, although these effects were less than those observed in patients with WHO Group 2 PH [39].

3 Reactive Oxygen Species and Pulmonary Hypertension

3.1 Significant Sources of ROS

While signaling pathways centered on NO bioavailability are key therapeutic targets for the treatment of PH, the production of ROS is also an essential contributor to hypoxia-induced PH. In this case, cellular respiration is an important source of ROS [40]. Accordingly, ROS role in the development of PH is strongly suggested by studies showing profound alteration in mitochondrial structure and function in that context. Using human and rodent models, Ryan and colleagues found evidence of mitochondrial fragmentation [41], which is associated with a decrease in the expression of mitofusin-2 (MFN2), a molecular regulator that promotes the fusion of mitochondria into long tubular structures. Also, PGC1α, a transcriptional activator of mitochondrial biogenesis was found to be downregulated in that context. Adenoviral overexpression of MFN2 increased mitochondrial fusion, decreased proliferation, lessened the severity of PH, and improved exercise capacity in the rodent model [41]. These results suggest that decreases in MFN2 and PGC1α contribute to pulmonary vascular remodeling and provide indirect evidence of a potential role of mitochondrial-derived ROS in the pathophysiology of PH.

Although not completely elucidated in one single model, compelling evidence from different laboratories indicates that low oxygen stimulation leads to mitochondrial production of ROS, which serves as an activator of prolyl-4-hydroxylases which in turn induce activation of Hypoxia-inducible factor 1 (HIF-1), a necessary event that triggers vascular remodeling and narrowing of the pulmonary arteries (Fig. 3). We present, in the following paragraphs, a concise review of the current evidence supporting this mechanism.

During hypoxia, mitochondria from vascular cells release superoxide ($O_2^{\bullet-}$) from complex III to the intermembrane space, where it is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase [40, 42]. The H_2O_2 then enters the cytosol, where it activates multiple responses contributing to smooth muscle contraction and remodeling. The mechanism of hypoxia-driven ROS generation was first suspected using pulmonary artery cell homogenates, which suggested that superoxide generation increased during hypoxia in an effect that was inhibited by diphenylene iodonium, a flavoptrotein inhibitor of NADPH oxidase but not by the mitochondrial inhibitor myxothiazol, which blocks electron entry into complex III [43]. Chandel et al. first demonstrated that mitochondrial ROS signals control gene transcription in hypoxia [44].

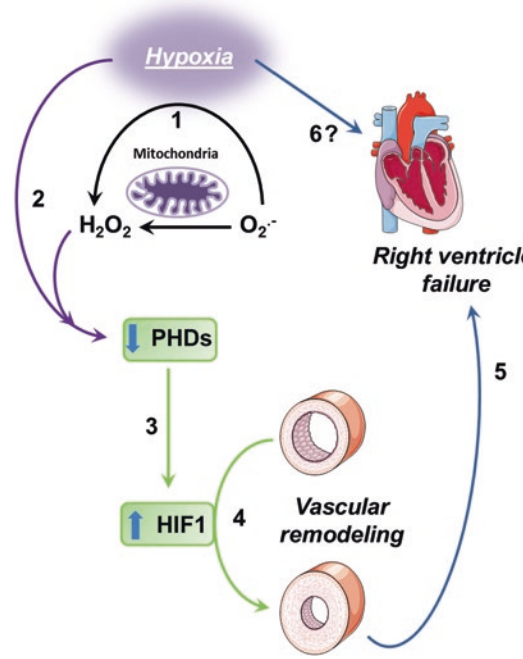

Fig. 3 Potential mechanisms of hypoxia-induced pulmonary hypertension: (1) hypoxia causes cells to release superoxide ($O_2^{\bullet-}$) which is converted to hydrogen peroxide (H_2O_2). (2) Both low oxygen and ROS production cause a reduction in hydroxylase activity of PHDs. (3) Lower PHDs activity causes a reduction of HIF-1α proline-402 and proline-564 hydroxylation, which leads to dissociation from VHL and stabilization of the transcriptional activity of HIF-1α. (4) Increase in the transcriptional activity of HIF-1α causes pulmonary vascular remodeling. (5) Increased pulmonary vascular resistance leads to right ventricle failure. (6) Hypoxia could also contribute to right ventricle remodeling and failure independent of HIF-1α [3]

Specifically, using mitochondrial inhibitors, ρ0 cells lacking a functional electron transport chain (ETC), and ROS-sensitive fluorescent chemical probes, they showed that under hypoxia ETC is required for ROS-dependent stabilization of the HIF-1α transcription factor subunit [44]. Also, the same group found that even though anoxia is the most extreme form of hypoxia, these two stimulations operate differently on downstream signaling: the activation of HIF-1 under hypoxia requires mitochondrial ETC, whereas, under anoxia, HIF-1 is activated without involvement of ETC [45].

The specific mechanisms linking hypoxia and ROS generation are not completely understood, but evidence indicates that they involve oxidation of complex III, which requires cytochrome c and leads to the formation of $O_2^{\bullet-}$ that is later ejected to the intermembrane space due to electrical gradient. Indeed, when cytochrome c is absent, complex III remains fully reduced, which prevents ROS generation under hypoxia and are unable to stabilize HIF-1α [46]. These findings implicate electron flux through complex III as a critical event in the detection of hypoxia in cells [46]. Waypa et al. demonstrated that hypoxic pulmonary vasoconstriction required electron flux through complex III, and that increases in ROS generation were responsible for eliciting the hypoxic response [47, 48]. Specifically, these authors demonstrated that acute production of ROS during hypoxia pulmonary artery smooth muscle cells depends on the Rieske iron–sulfur protein subunit of complex III, as reflected by PH attenuation in animals with deletion of this gene using a Cre/loxP system [48].

3.2 Signaling Pathways Associated with Increased ROS in Pulmonary Hypertension

3.2.1 ROS Cause HIF-1 Activation

HIF-1 is a highly conserved transcription factor present in almost all cell types [2]. It is tightly regulated by O_2 availability, and modulates the expression of hundreds of genes. HIF-1 exists as a heterodimer, consisting of HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed, whereas HIF-1α is found at very low levels under normoxic conditions [49]. In this context, HIF-1α protein is ubiquitinated and degraded by the proteasomal pathway; however, acute exposure of pulmonary arterial smooth muscle cells (PASMCs) or endothelial cells (ECs) to hypoxia (1% O_2) causes increased HIF-1α protein levels and HIF-1 DNA-binding activity. Thus, HIF-1α confers sensitivity and specificity for hypoxic induction of HIF-1 transcriptional activity [2, 49].

Under normoxia, HIF-1 is associated to von Hippel–Lindau protein (VHL), which recruits an E3-ubiquitin protein ligase Elongin 2 and 3, Cullin 2, and RBX1 [50, 51]. Binding of VHL depends on hydroxylation of HIF-1 proline-402 and 564 in well-oxygenated cells [52]. Three prolyl-4-hydroxylase domain proteins (PHDs) that hydroxylate proline-402 and 564 in an O_2-dependent manner are identified in mammalian cells [53, 54]. These proteins, known as PHD1, PHD2, and PHD3 are members of a superfamily of dioxygenases that contain Fe(II) in their catalytic center and utilize O_2 and α-ketoglutarate as substrates. Reduction of Fe(III) to Fe(II) in the catalytic center by ascorbate is required for a subsequent catalytic cycle. The observed reduction in hydroxylase activity under hypoxic has been proposed to be due to substrate (O_2) limitation [53, 55] and/or by an increase in mitochondrial production of ROS that may oxidize Fe(II) and inactivate the PHDs [46, 56, 57]. Thus, hypoxia can lead to PDHs deactivation and HIF-1 stabilization via a direct effect of either low oxygen, or also ROS on PDHs (Fig. 3). Importantly, at least in the acute phase, the generation of ROS appears to be a necessary step in the process of PH under hypoxia [48].

3.2.2 HIF-1 Mediates Hypoxia-driven Pulmonary Hypertension

Seminal work by Shimoda and coworkers [58] established the effects of chronic hypoxia (CH) on heterozygous mice lacking one copy of the HIF-1 gene (homozygous animals could not be used due to intrauterine lethality). Compared

with wild-type control animals, heterozygous HIF-1a mice demonstrated impaired lung vascular remodeling in chronic hypoxia and attenuated RV hypertrophic responses [58–61]. This was associated with lower level of vascular smooth muscle hypertrophy, attenuated upregulation of transient potential receptor proteins and Na^+/H^+ exchanger-isoform 1, and failure to suppress the expression of plasma membrane K1 channels during CH [58]. Recently, using a Cre/loxP system smooth muscle-specific conditional deletion of HIF-1, Ball et al. demonstrated that HIF-1 is critical as a mediator of pulmonary arterial remodeling under hypoxia. Interestingly, they also found that loss of HIF-1 function in smooth muscle did not affect hypoxic cardiac remodeling [3]; suggesting that the cardiac hypertrophy response is not directly coupled to the increase in pulmonary artery pressure (Fig. 3). This last finding challenges the "hemodynamic dogma" that states that the right ventricular hypertrophy and eventual failure depend purely on pressure overload due to increase of pulmonary vascular resistance and suggests that ventricular and vascular remodeling are distinct and somewhat independent processes. Similar challenges are emerging regarding the left ventricular remodeling in connection to systemic hypertension [62].

3.2.3 Mechanisms of HIF1-driven Vascular Wall Remodeling

Chronic hypoxia induces functional and structural changes in the endothelial and smooth muscle cells, and fibroblasts that make up the intima, media, and adventitia of pulmonary arterial wall thus contributing to pulmonary hypertension [63]. The effect of hypoxia on vascular remodeling has been mostly studied in PASMC. Acute hypoxia leads to an increase in intracellular calcium $[Ca^{2+}]_i$ that is reversible upon reoxygenation. In contrast, chronic hypoxia causes a sustained increase on $[Ca^{2+}]_i$ which remains elevated even after return to normoxia [64]. This effect is mediated by store-operated Ca^{2+} channels, which are activated by depletion of intracellular Ca^{2+} stores during chronic hypoxia [65]. These channels are composed of transient receptor potential (TRP) proteins, which are under the control of HIF-1 [65]. Indeed, infection with an adenovirus encoding a constitutively active form of HIF-1α increases TRPC1 and TRPC6 expression under nonhypoxic conditions.

Hypoxia inhibits opening of voltage-gated K^+ channels Kv1.5 and Kv2.1, which contributes to PASMC depolarization [66]. The expression of these channels is also decreased in PASMCs subjected to chronic hypoxia in vivo or ex vivo [67, 68], and these changes in gene expression are also HIF-1α dependent [4]. In addition to increased $[Ca^{2+}]_i$, chronic hypoxia also results in increased intracellular pH (pHi) in PASMCs, an effect that is due to HIF-1-dependent expression of the sodium–hydrogen exchanger NHE1 [60]. Increased $[Ca^{2+}]_i$ and pH_i contribute to the activation of signal transduction pathways that promote PASMC hypertrophy and hyperplasia, which leads to the medial thickening of pulmonary arterioles, which is the pathological hallmark of hypoxia-induced PAH. Indeed, exposure of WT but not *Hif-1*$^{-/-}$ mice to chronic hypoxia induces PASMC alkalinization and hypertrophy [58, 60].

The demonstration that mice partially deficient in HIF-1α expression are protected against the development of PAH suggests that pharmacological inhibition of HIF-1 may be of therapeutic benefit in this clinical context. This hypothesis was tested by the daily administration of digoxin, a cardiac glycoside that has been used to treat congestive heart failure and cardiac arrhythmias for decades, which inhibits the synthesis of HIF-1α protein [69]. Treatment with digoxin attenuated the development of right ventricular hypertrophy and prevented the changes in pulmonary vascular $[Ca^{2+}]_i$ and pH_i, remodeling, and pressure that occur in mice exposed to chronic hypoxia [70].

The role of HIF-1 in the pathogenesis of PH is not restricted to hypoxia-induced PH. The spontaneous development of PAH in fawn-hooded rats is associated with increased HIF-1α expression, HIF-dependent reductions in K^+ currents and Kv1.5 expression, increased PDK1 expression, and a switch from oxidative to glycolytic metabolism in pulmonary artery smooth muscle cells [4]. These metabolic changes appear to play a critical role in the pathogenesis of PAH because treatment

of animals with dichloroacetate, an inhibitor of PDK1, leads to regression of PAH [71].

4 Conclusion

Dysregulation in NO and ROS production have been established in the context of PH including hypoxia/disease-induced PH and there is now sufficient evidence to indicate that decreased NO bioavailability and increased mitochondrial-derived $O_2^{\bullet-}/H_2O_2$ production are central to the pathogenesis of PH. Whether these two arms of redox biology converge on common molecular pathways and pathologies is still debatable and will require additional investigation. What is clear is that the elucidation of the signaling pathways downstream of NO and ROS and their pathophysiological alterations will continue to provide a foundation for the rational design and clinical implementation of new therapies targeting PH.

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Altered Redox Balance in the Development of Chronic Hypoxia-induced Pulmonary Hypertension

Nikki L. Jernigan, Thomas C. Resta, and Laura V. Gonzalez Bosc

1 Phenotypic Changes in Pulmonary Arterial Smooth Muscle Cells

Phenotypic alterations in pulmonary arterial smooth muscle cells (PASMC) are thought to play prominent roles in the development of both enhanced vasoconstrictor reactivity and arterial remodeling components of hypoxia-associated PH. Chronic hypoxia (CH) causes morphological changes of pulmonary arteries including the migration and distal extension of "smooth muscle"-like cells into previously non-muscularized, pre-capillary vessels. In addition, CH results in thickening of the medial and adventitial vessel layers. Medial thickening is attributable to hypertrophy and proliferation of smooth muscle cells as well as increased deposition of extracellular matrix protein, like collagen and elastin [1]. The adventitial thickening is caused by accumulation of fibroblasts, myofibroblasts, and extracellular matrix proteins [1]. Circulating vasoactive factors, hypoxia, and mechanical stress act to stimulate a cascade of intracellular signaling mechanisms that collectively act to control SMC contractility, growth, and differentiation. These mechanisms include changes to PASMC Ca^{2+} homeostasis, Ca^{2+} sensitivity, and activation of transcription factors.

1.1 Changes in PASMC Ca^{2+} Homeostasis

Resting PASMC intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is significantly elevated 1.5 to 2 fold in patients with idiopathic pulmonary arterial hypertension [2]; as well as in various animal models of PH [3–7]. The elevation in resting $[Ca^{2+}]_i$ can be attributed to persistent vascular smooth muscle (VSM) depolarization, mechanical stimuli, alveolar hypoxia, and enhanced Ca^{2+} influx in response to receptor stimulation. PASMC $[Ca^{2+}]_i$ homeostasis requires the coordination of several fundamental systems including release of Ca^{2+} from sarcoplasmic reticulum (SR), Ca^{2+} entry through plasma membrane channels, Ca^{2+} sequestration into cytoplasmic organelles, and Ca^{2+} extrusion from the cell. The persistent elevation in $[Ca^{2+}]_i$ is accompanied by anomalies in intracellular signaling cascades and changes in the expression, function, and regulation of ion channels and transporters involved in PASMC Ca^{2+} homeostasis.

Chelation and removal of extracellular Ca^{2+} reduces PASMC $[Ca^{2+}]_i$ similar to control levels,

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indicating that a constant Ca^{2+} influx is required for maintenance of elevated resting PASMC $[Ca^{2+}]_i$ in PH [3–5]. Ca^{2+} influx occurs through both voltage-dependent L- and T-type Ca^{2+} channels, and voltage-independent receptor-operated channels (ROC) and store-operated channels (SOC) (see Fig. 1). In PASMC, store-operated Ca^{2+} entry (SOCE) is greatly augmented and associated with the pathological progression of PH. Patch clamp studies reveal two different types of ionic current evoked by store-depletion: (1) a current mediated by a Ca^{2+} release-activated channel (CRAC) displaying high selectivity for Ca^{2+} [8] and (2) a non-selective cation channel current [9, 10]. Many agree that stromal interaction molecule 1 (STIM1) is the endoplasmic/sarcoplasmic reticulum Ca^{2+} sensor relaying the signal to plasma membrane Ca^{2+}-permeable ion channels [11, 12]. In PASMCs, STIM1 has been shown to interact with both the CRAC-related channel, Orai1, and the classical/canonical transient receptor potential 1 channel (TRPC1) [13, 14] In addition, our laboratory has recently shown a novel role for the Ca^{2+}-permeable cation channel, acid sensing ion channel 1 (ASIC1) to mediate enhanced SOCE following CH [3, 15]. Additionally, we show that ASIC1 null mice are protected from development of hypoxic PH and right ventricular hypertrophy [16]. It may therefore be likely that SOCE is not mediated by a single molecule/channel, but rather involves a dynamic and highly regulated process mediated by several proteins and ion channels.

1.2 Calcium Sensitization and Actin Polymerization

In addition to elevated PASMC $[Ca^{2+}]_i$ levels, signaling mechanisms that enhance the sensitivity of the contractile apparatus to Ca^{2+} contribute to pulmonary arterial constriction and associated PH resulting from exposure to CH. PASMC contraction is determined primarily by the phosphorylation of Ser^{19} of the 20 kDa regulatory myosin light chain (MLC_{20}) through the balance of activities of Ca^{2+}/calmodulin-activated myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). It is well established that MLCP activity is highly regulated to mediate changes in the sensitivity of the contractile apparatus to Ca^{2+} and thus VSM tone [17–25]. For example, stimulation of many G-protein-coupled receptors leads to inactivation of MLCP, allowing accumulation

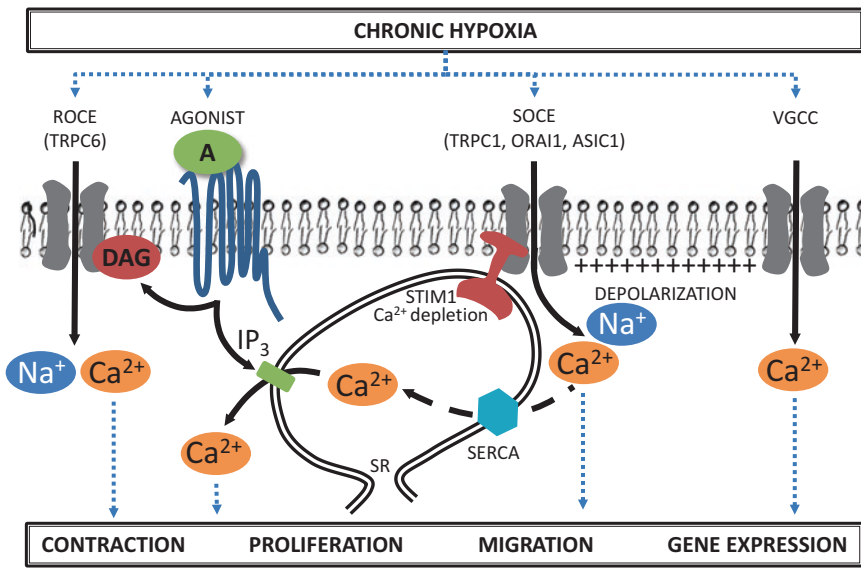

Fig. 1 Chronic hypoxia-induced mechanisms leading to enhanced intracellular Ca^{2+} in pulmonary arterial smooth muscle cells

of phosphorylated MLC and greater contraction for a given $[Ca^{2+}]_i$. This phenomenon of MLC phosphorylation occurring independently of changes in VSM $[Ca^{2+}]_i$ is referred to as myofilament Ca^{2+} sensitization. Activation of the RhoA/Rho kinase (ROK) pathway and consequent inhibition of MLCP are central to receptor-mediated Ca^{2+} sensitization of VSM [17, 18, 22–26], although roles for protein kinase C (PKC)-dependent inhibition of MLCP [19, 23, 24, 27–29] and MLCP-independent mechanisms have also been described [25, 30, 31].

In addition to Ca^{2+} sensitization, dynamic cytoskeletal reorganization is integral to force generation in smooth muscle [32–38]. Actin polymerization is thought to promote contraction independent of changes in $[Ca^{2+}]_i$ or MLC phosphorylation by enhancing transmission of force to the extracellular matrix [33, 357] and by rapidly increasing the assembly of contractile units ([32, 39, 40]; R. [41]). Both ROK and PKC contribute to actin polymerization in PASMC, thus providing an additional mechanism by which these mediators augment myocyte contractility independent of changes in $[Ca^{2+}]_i$ [36, 42–45].

Stimulation of RhoA/ROK signaling in PASMC plays an important role in mediating the vasoconstriction and associated PH resulting from CH [46–53], as well as in a rat model of severe pulmonary arterial hypertension (PAH) [54]. ROK inhibition also improves pulmonary hemodynamics in PAH patients [55], and pulmonary arteries (PA) from patients with idiopathic PAH display elevated ROK activity and enhanced ROK-dependent contraction to serotonin [56]. In agreement with these studies, we and others have identified a major contribution of RhoA/ROK-mediated Ca^{2+} sensitization to the development of pressure-induced (myogenic) pulmonary arterial constriction, as well as enhanced vasoconstriction to receptor mediated agonists and depolarizing stimuli in small pulmonary arteries from animals exposed to CH [46–53, 57]. Although the mechanisms by which CH activates ROK signaling in PASMC are not fully understood, recent studies support a role for oxidant stress as an initiating event. These mechanisms are described below in Sect. 3.

1.3 Activation of Transcription Factors: Role of NFAT

Calcium (Ca^{2+}) is the most common signal used by living organisms to convey information to many different cellular processes. Several Ca^{2+}-dependent transcription factors have been implicated in the development of both PH and PAH, such as cAMP response element binding protein (CREB) [58–60]; Garat [61–63], nuclear factor kappa-light-chain-enhancer of activated B cells (NFκβ) [64–68] and nuclear factor of activated T-cells (NFAT) [69–76]. In addition to Ca^{2+}-dependent transcription factors, hypoxia-inducible factor-1α (HIF-1α) has been implicated in CH-induced PH [77–79]. However, this section focuses on the role of NFAT in hypoxic PH.

The NFAT family consists of four members (NFAT1/c2, NFAT2/c1, NFAT3/c4, and NFAT4/c3) that share the property of Ca^{2+}-dependent nuclear translocation, and a fifth member—NFAT5 or tonicity-responsive enhancer binding protein—which is Ca^{2+}-independent and shares limited homology with the other family members [reviewed in 80–82]. NFAT family members share an N-terminal NFAT homology region (NHR) that contains the primary transactivation domain and elements necessary for regulated nuclear translocation, and a C-terminal DNA binding domain that shows moderate sequence homology to the DNA binding domains of Rel-family proteins including NFκB [reviewed in [80–82]. NFAT activation is regulated primarily through control of its subcellular localization [82]. Calcineurin, upon activation by Ca^{2+} and calmodulin binding, dephosphorylates NFAT, consequently exposing nuclear localization signals allowing for NFAT nuclear translocation. NFAT nuclear accumulation is also controlled by a series of Ser/Thr kinases such as glycogen synthase kinase 3 β (GSK3β), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKinase II) and c-jun N-terminal kinases 1 and 2 (JNK1/2) [reviewed in [80, 82]]. Under non-stimulated conditions, these kinases keep NFAT in a highly phosphorylated state in the cytosol. After stimulation and once the Ca^{2+} signal returns to baseline, they rephosphorylate NFAT inducing its

nuclear export by the CRM1 exportin [reviewed in [80, 82]]. JNK1 also targets NFAT by phosphorylating sites required for NFAT interaction with calcineurin, resulting in a disruption of calcineurin binding and inhibition of NFAT nuclear import [83]. In the presence of nitric oxide (NO), NFAT nuclear import is enhanced and export is decreased via protein kinase G (PKG)-mediated inhibition of JNK1/2 [84–86]).

NFAT binds DNA with very low affinity in the absence of a cofactor so that formation of an NFAT-cofactor-DNA ternary complex is required for significant NFAT-mediated transcriptional activity. NFAT activation is generally associated with an increase in gene expression [81, 82]. However, NFAT can also function as a repressor of gene transcription [87–90]. NFAT regulates the expression of genes in a diverse array of both immune and non-immune cells [82, 91]. NFAT appears to regulate the expression of smooth muscle-specific myosin heavy chain (MHC) and α-actin genes [85, 86, 92], and to maintain the differentiated smooth muscle phenotype through regulation of α1 integrin and caldesmon expression [93] and to regulate VSM contractility through the down-regulation of voltage-dependent potassium channel 2.1 (K_V 2.1) and β-subunit of large conductance potassium channel (BK) [87, 90] and up-regulation of TRPC6 [94]. A number of other transcription factors implicated in the regulation of smooth muscle-specific gene expression act as NFAT cofactors, including Egr-1[95, 96], AP-1 [97, 98], YY1 [99], the MADS family member, MEF2 [100–102], and members of the GATA family [92, 103] [reviewed in [81]]. NFAT activity is closely linked to developmental processes that involve modulation of cellular phenotypes such as cardiac heart valve formation [104] and embryonic vasculogenesis [105, 106]. NFAT also contributes to pathological cardiac hypertrophy [103, 107, 108].

In the pulmonary circulation, NFATc2, NFATc3 and NFATc4 expression have been reported [69, 72, 109]. Our group was the first to demonstrate that exposure to CH activates NFATc3 in mouse pulmonary arterial smooth muscle as early as 2 days post-exposure [72]. We discovered that NFATc3 is actually the only NFAT isoform activated in mouse pulmonary arteries in response to CH. In addition, we have shown that NFATc3 is required for CH-induced right ventricular (RV) hypertrophy, up-regulation of smooth muscle-α-actin and vascular remodeling [72]. Almost simultaneously, Bonnet S et al. reported that >60% and ~20% of PASMC from PAH patients have NFATc2 and NFATc3 in the nucleus, respectively [69]. They implicated NFATc2 in decreased $K_V1.5$ expression and current, and increased $[Ca^{2+}]_i$ in PASMC from PAH patients and from monocrotaline-PH rats suggesting NFATc2 plays a role in the development of PAH. A few years later, we demonstrated that NFATc3 is required for CH-induced PH and that is implicated in PASMC early proliferative response [110] followed by redifferentiation through upregulation of guanylyl cyclase α1 [73]. The mechanisms implicated in NFAT activation in hypoxic PH are described in Sect. 4.

2 ROS in Hypoxic Pulmonary Hypertension

Reactive oxygen species (ROS) encompass a variety of diverse chemical species including superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). They are generated as a by-product of cellular respiration and metabolism or by specialized enzymes involved in redox signaling include the mitochondrial electron transport chain, xanthine oxidase, cytochrome *P*-450, lipoxygenases, nitric oxide synthase (NOS), peroxisomes, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [111–113]. Under normal conditions, ROS are essential signaling molecules that are tightly regulated to maintain physiological homeostasis, regulate cellular proliferation, and sustain host defense. Whereas augmented ROS levels can stimulate oxidative stress signaling pathways and damage cellular proteins, lipids, and DNA, lower than normal ROS levels can disrupt oxidative regulation of cell proliferation and host defense [111]. An imbalance in ROS homeostasis has been implicated in the progression of

various disease states, including PH. However, whether ROS are increased or decreased is still largely debated [112, 114, 115]. This controversy may be a result of various influences on the integrated cellular signaling of ROS by site of production (i.e., cell type), precise species, local concentration, and antioxidant availability.

2.1 Regulation of $^{\bullet}O_2^-$ and H_2O_2 Levels in Hypoxia

Both endogenous $^{\bullet}O_2^-$ and H_2O_2 are physiologically important second messengers that regulate a variety of downstream signaling pathways. Numerous studies show that increased ROS and subsequent oxidative stress contribute to the development of PH [112]. Several lines of evidence, including studies from our laboratory, suggest that CH increases $^{\bullet}O_2^-$ generation. A number of sources have been implicated including xanthine oxidase [116–118], NADPH oxidase (NOX) [51, 119–126], uncoupled nitric oxide (NO) synthase [124, 127], the mitochondrial respiratory chain [122, 128], and altered antioxidant capacity [114, 120, 129–134] (see Fig. 2). Irrespective of the source, our laboratory has demonstrated an important contribution of $^{\bullet}O_2^-$ to induce RhoA activation, myofilament Ca^{2+} sensitization, and enhanced vasoconstriction in hypertensive pulmonary arteries [47, 49, 51, 57]. This is discussed in more detail in Sect. 3.

Although evidence suggests $^{\bullet}O_2^-$ is augmented due to increased generation of $^{\bullet}O_2^-$, it is also evident that degradation of $^{\bullet}O_2^-$ is limited following CH. In contrast to $^{\bullet}O_2^-$ levels, our laboratory and others have shown CH decreases H_2O_2 levels in several different species [120, 129, 133, 134]. Superoxide is highly reactive and rapidly dismutates to H_2O_2; therefore, an additional means of regulating $^{\bullet}O_2^-$and H_2O_2 levels is through superoxide dismutase (SOD). Various models of PH are associated with dysregulation of SOD, and possibly other antioxidant pathways, that could explain the lower H_2O_2 levels [114, 120, 129, 130, 134].

Fawn-hooded rats, which develop spontaneous PH, have an epigenetic silencing of SOD2 expression/activity, resulting in decreased H_2O_2 production [114, 129]. Although in our recent studies we did not detect differences in SOD2 expression or activity following exposure to CH, we did find that CH decreased SOD1 and SOD3 expression and activity [133], which is consistent with studies from 3- and 10-day-old CH piglets [120, 130] and a lamb model of persistent PH of the newborn [135]. CH also decreases lung SOD3 protein expression and activity in mice [136], whereas overexpression of SOD3 attenuates both hypoxic- and monocrotaline-induced PH [136, 137]. Furthermore, our laboratory has recently shown that $SOD1^{-/-}$ mice develop spontaneous PH, and this response is associated with increases in pulmonary arterial $^{\bullet}O_2^-$ and decreases in H_2O_2 levels similar to exposing wildtype mice to CH [134], suggesting CH exposure is as effective as SOD1 knockout in decreasing H_2O_2 production in pulmonary arteries. These animal studies further correlate with studies showing significantly reduced SOD mRNA and SOD activity in patients with idiopathic pulmonary arterial hypertension (IPAH) compared to healthy individuals [131, 132].

Although SOD can be regulated by a variety of transcription factors, HIF-1α and HIF-2α directly bind the hypoxia-responsive element of

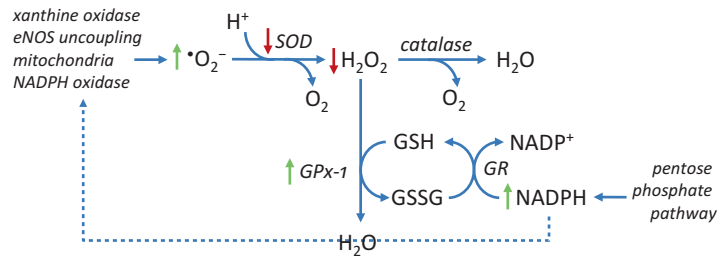

Fig. 2 Regulation of reactive oxygen species and redox following chronic hypoxia

SOD2 and SOD1 promoter, resulting in negative regulation of gene expression [138–140]. Likewise, a loss of SOD1, SOD2, and SOD3 leads to the hypoxic activation of HIF-1α, suggesting that stabilization of HIF-1α is redox sensitive and closely linked to SOD function and the oxidant–antioxidant equilibrium [141–144]. In addition to transcriptional regulation, the enzymatic activity of SOD may be altered in PH. We found that ET-1, which is implicated in the pathogenesis of hypoxic PH [71], increases $^{\bullet}O_2^-$ while decreasing H_2O_2 production through the decrease in SOD1 activity without affecting SOD1 protein levels [145]. Together, these data suggest a dysregulation of SOD in PH that disrupts the ROS homeostasis leading to increased PASMC $^{\bullet}O_2^-$ levels and a paradoxical decrease in H_2O_2 levels.

In addition to SOD, other antioxidant enzymes responsible for the degradation of H_2O_2, namely catalase and glutathione peroxidase (GPx-1), may contribute to a reduction in H_2O_2 in PH (Fig. 2). Our laboratory and others have shown that catalase expression and activity were similar between control and CH exposed rats [133], piglets [120] or humans with IPAH [131]. However, consistent with earlier studies [146–148], we recently found that expression and activity of glutathione peroxidase (GPx-1) was augmented in pulmonary arteries from CH rats [133]. This increase in GPx-1 expression and activity could contribute to the overall lower levels of H_2O_2 in PH. Additionally, CH enhances the activity of the pentose phosphate pathway; thereby increasing glucose-6-phosphate dehydrogenase (G6PD) activity and NADPH levels [149]. The production of $^{\bullet}O_2^-$ from NOX is dependent on NADPH and this increased activity of this pathway likely contributes to greater $^{\bullet}O_2^-$ production in PH (Fig. 2). Indeed, inhibition of G6PD with dehydroepiandrosterone suppressed hypoxia-induced increase in mean pulmonary arterial pressure, RV hypertrophy, elevated NADPH levels and HIF-1α expression [150]. Because CH alters the state of various redox couples, it is difficult to determine the physiological impact on the overall cellular redox environment. This is further complicated by the fact that redox responses can differ markedly among subcellular compartments [151]. Nonetheless, changes to the redox potential represent an important mechanism in the pathology of PH.

3 NADPH Oxidase-derived $^{\bullet}O_2^-$ Promotes Activation of RhoA/ROK

CH increases reactivity to vasoconstrictor stimuli through ROS-dependent signaling in VSM [49, 57, 152]. Such mechanisms may involve impaired NO-signaling in pulmonary VSM due to oxidation of soluble guanylyl cyclase, as suggested by effects of the pharmacologic activator of oxidized (and thus NO-insensitive) soluble guanylyl cyclase, HMR1766, to reduce PH in CH mice [153]. However, considering that vasoconstrictor mechanisms involving both ROK and ROS are important contributing factors to the development of PH [117, 121, 125], and evidence that $^{\bullet}O_2^-$ mediates RhoA/ROK-dependent VSM contraction in ductus arteriosus [154], aorta [155, 156], mouse tail artery [157] and pulmonary arteries [158], several groups have investigated a potential link between ROS and ROK-dependent VSM Ca^{2+} sensitization in mediating CH-induced increases in pulmonary vasoconstrictor reactivity. Consistent with this possibility, Chi and colleagues used a novel cytosolic redox sensor (RoGFP) in primary cultures of rat PASMC to demonstrate an effect of prolonged hypoxia (24 hr) to shift the redox status of the cytosol to a more oxidized state [159]. This oxidant response to hypoxia was associated with an increase in RhoA activity, an effect that was mimicked by exogenous H_2O_2. However, scavenging of ROS did not prevent hypoxic induction of RhoA activity, suggesting that ROS are sufficient but not required for RhoA activation in this setting. Studies by our group have additionally revealed a signaling relationship between ET-1 receptor stimulation, $^{\bullet}O_2^-$ generation and RhoA-induced myofilament Ca^{2+} sensitization in mediating effects of CH to augment vasoconstrictor sensitivity to ET-1 in small pulmonary arteries [49]. We later identified $^{\bullet}O_2^-$ as an intracellular second messenger linking pulmonary VSM membrane

depolarization to RhoA activation through a unique Ca^{2+} -independent mechanism in small pulmonary arteries from CH rats, resulting in enhanced ROK-mediated vasoconstriction [57]. Interestingly, $^{\bullet}O_2^-$ mediates elevated basal VSM RhoA activity in CH pulmonary arteries [49], suggesting an additional role for $^{\bullet}O_2^-$ -induced RhoA activation in the ROK-dependent myogenic vasoconstrictor response observed in these vessels [47].

More recent studies have begun to investigate enzymatic sources of $^{\bullet}O_2^-$ that mediate augmented vasoconstrictor reactivity following CH. Consistent with evidence that depolarization stimulates NOX isoforms to produce $^{\bullet}O_2^-$ in macula densa [160] and endothelial cells [161–163], we found that enhanced vasoconstriction and $^{\bullet}O_2^-$ generation in response to depolarizing concentrations of KCl in arteries from CH rats were abolished by pretreatment with general inhibition of NOX isoforms, as well as by selective inhibition of either NOX2 or the NOX2 accessory protein, Rac1 [51]. Together, these findings may provide a mechanistic basis for previous studies that have established a crucial role for NOX2 and ROS [116, 125, 164] in the development of hypoxic PH.

3.1 Role of Epidermal Growth Factor Receptor in the Activation of RhoA and Oxidant Signaling

EGFR is a receptor tyrosine kinase comprised of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain that mediates the tyrosine kinase activity of the receptor [165]. Ligand stimulation of EGFR by epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and amphiregulin promotes receptor dimerization and autophosphorylation of key tyrosine residues in the intracellular domain, thereby coupling EGFR to distal signaling pathways involved in growth, proliferation, and cell contraction. EGFR is transactivated by a variety of stimuli, including G protein-coupled receptor (GPCR) stimulation [166–170], depolarization [171–173], and mechanical deformation of cell membranes [174–178]. EGFR therefore serves to integrate information from distinct upstream pathways to downstream cellular responses. Interestingly, a Src kinase (Src)-EGFR signaling pathway is activated by such stimuli in other cell types [168, 178–180], and represents a potential signaling mediator that links membrane stretch, depolarization and receptor stimulation to NOX2 activation, vasoconstriction and arterial remodeling in the hypertensive pulmonary circulation (Fig. 3).

It has been suggested that EGFR is not a promising target for treatment of PH based on: (1) the variable efficacy of EGFR antagonists to prevent monocrotaline-induced PH in rats, (2) the lack of effect of EGFR inhibitors to attenuate PH in chronically hypoxic mice, and (3) evidence that lung EGFR levels are not altered in either human PAH or rodent models of PH [181]. Nevertheless, it is possible that EGFR signaling in PH is enhanced independent of changes in expression, and that the therapeutic potential of EGFR inhibitors varies depending on the species or model of PH. Consistent with this possibility, we have identified a unique effect of CH exposure in rats to couple depolarization-induced EGFR activation to NOX2-mediated pulmonary arterial constriction without a change in EGFR protein levels, a response not present in control vessels [51] (see Fig. 3). EGFR additionally contributes to PH in mice that overexpress the EGFR ligand TGF-α [182], and chronic EGFR inhibition improves survival, lowers right ventricular systolic pressure, and attenuates both arterial remodeling and right ventricular hypertrophy in monocrotaline-treated rats [181, 183, 184]. EGFR activation associated with monocrotaline exposure is thought to result from oxidative dimerization of EGFR through a Src- and ligand-independent mechanism [184]. However, whether a similar mechanism of ligand-independent EGFR stimulation accounts for enhanced EGFR-dependent vasoconstrictor reactivity in CH-induced PH remains to be determined.

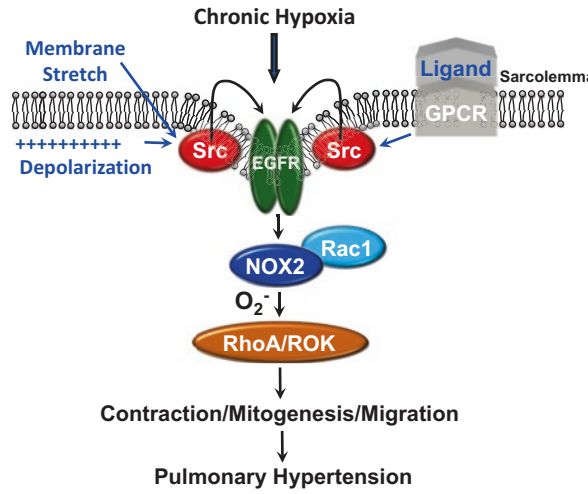

Fig. 3 Proposed signaling mechanism in pulmonary vascular smooth muscle that confers mechanical, electrical and chemical transduction to NOX2/RhoA-mediated vasoconstriction, arterial remodeling and pulmonary hypertension following chronic hypoxia. See text for details

3.2 Effects of RhoA on NFAT Activation and Cytoskeleton

The RhoA/ROK pathway is essential for NFAT activation in B cells [185, 186]. We and others have also shown that RhoA/ROK signaling regulates NFAT activity in PASMC [109]. In rat PASMC, the ROK inhibitor, Y-27632, significantly attenuates phenylephrine-induced NFATc4 nuclear localization [109]. In human PASMC, we demonstrated that the ROK inhibitor, fasudil, significantly decreases ET-1-induced NFATc3-GFP nuclear import. In vivo, we found that CH increases lung RhoA activity which is dependent on endothelin A receptor (ET_AR) activation [71]. It is well established that the expression of ET-1is increased in both humans and animals with PH [187–189]. Also, CH increases the vasoconstrictor sensitivity to ET-1 through Ca^{2+}-dependent and -independent mechanisms. ET-1 through activation of Gq/11-coupled receptors increases $[Ca^{2+}]_i$ by causing release of Ca^{2+} from intracellular stores and by influx through L-type Ca^{2+} channels . In addition, it activates RhoA/ROK independently of increases in $[Ca^{2+}]_i$ [49, 53]. ET-1 through RhoA/ROK causes reorganization of the cytoskeleton (stress-fiber formation) [190–192]. The cytoskeleton of smooth muscle cells is a filamentous network consisting largely of filamentous actin (F-actin), and to a lesser extent, monomeric actin (G-actin). The increase in the proportion of F-actin that occurs in response to the stimulation of smooth muscle cells and the essential role of stimulus-induced actin polymerization and cytoskeletal dynamics in the generation of mechanical tension has been convincingly documented in VSM [reviewed in [33, 35, 192]]. In addition, actin cytoskeleton polymerization has been implicated in the transport of transcription factors to the nucleus [193]. We reported that ET-1 significantly increases the F/G actin ratio in human PASMC. This increase is prevented by fasudil and cytochalasin B [71]. Cytochalasin B blocks addition of actin monomer (G-actin) to filamentous actin (F-actin). Furthermore, cytochalasin B and jasplakinolide, an actin stabilizing agent [194], inhibited ET-1-induced NFATc3 nuclear import [71]. Similar findings were obtained when these treatments were applied to isolated mouse intrapulmonary arteries [71].

Actin cytoskeleton dynamics are regulated by many signaling pathways. In general, receptor activation and/or integrin ligation activates protein kinases and/or small GTPases, which in turn regulate the functional status of the actin regulatory proteins and eventually cause actin filament assembly or structural reorganization [reviewed in [35]]. In particular, it has been reported that nuclear import of nuclear factor κB (NF-κB; which, along with NFAT, is a member of the Rel family of transcription factors), requires activation of RhoA/ROK [193]. Stimulation of RhoA/

ROK enhances LIM kinase activity, leading to phosphorylation and inactivation of cofilin (actin regulatory protein) [193, 195]. When cofilin is phosphorylated, it no longer binds to actin, thus facilitating actin polymerization [195] and NF-κB transport to the nucleus [193]. ET-1, in a ROK-dependent manner, increases phospho-cofilin/cofilin ratio in pulmonary artery rings and cultured PASMC [196]. Hypoxia has also been shown to increase phospho-cofilin levels in PASMC [197] in a RhoA-dependent manner [43] but whether it is downstream of ET-1 receptors activation is unknown. In summary, NFATc3 nuclear transport depends on a dynamic reorganization of the actin cytoskeleton and is similar to the mechanism of NF-κB nuclear transport [193].

ROS play an important role in the regulation of both the RhoA/ROK pathway [49, 57] and downstream actin cytoskeleton polymerization [198]. We have demonstrated that an increase in $^{\bullet}O_2^-$ levels is required for NFAT activation in PASMC by ET-1 [145], CH and in superoxide dismutase 1 $(SOD1)^{-/-}$ mice [134]. Furthermore, we reported that $^{\bullet}O_2^-$ is required for ET-1-induced increases in cytoskeletal actin polymerization but not for ET-1-induced increases in $[Ca^{2+}]_i$ in human PASMC [145]. Therefore, $^{\bullet}O_2^-$ appears to regulate NFAT nuclear import by increasing actin cytoskeleton polymerization through RhoA/ROK activation. Other groups have also shown that ROS mediate NFAT activation. In T cells, doxorubicin stimulates Ca^{2+}/calcineurin-dependent activation of NFAT through mitochondria-derived ROS however which oxidant is involved it was not determined [199]. In mouse epidermal and human lung bronchoepithelial cells, exposure to silica activates NFAT in these cells by increasing $^{\bullet}O_2^-$ and decreasing H_2O_2 levels through a calcineurin-dependent mechanism [200]. On the contrary, in primary bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC), $^{\bullet}O_2^-$ reversibly inhibits calcineurin activity and calcineurin-dependent NFAT activation suggesting the involvement of reversible oxidative modifications of calcineurin [201]. This study and a previous study conducted in vitro suggest that calcineurin-dependent gene expression in endothelial cells is sensitive to redox regulation [201, 202].

4 H_2O_2 in the Pulmonary Circulation

Although $^{\bullet}O_2^-$ is generally associated with contraction of pulmonary arteries, both contraction and relaxation have been observed in response to H_2O_2 [reviewed in [203]]. This varied response to H_2O_2 in the pulmonary circulation appears to be dependent on the level of vascular tone present and the concentration of H_2O_2 applied. In addition, H_2O_2 production in the lung has been shown to be oxygen dependent with hyperoxia increasing [204] and hypoxia decreasing H_2O_2 levels [205, 206]. At physiological concentrations, H_2O_2 is a vasodilatory and anti-proliferative signaling molecule [206, 207]. In response to acute hypoxia, basal production of H_2O_2 in the rat pulmonary circulation is diminished [205, 206]. This prompted the general notion that, under normoxic conditions, H_2O_2 inhibits smooth muscle contraction, whereas under hypoxia H_2O_2 levels fall, resulting in hypoxic pulmonary vasoconstriction. A decrease in H_2O_2 levels has also been reported following long-term exposure to CH [120, 129, 133, 134, 208], as well as in other experimental models of spontaneous PH and in humans with IPAH [114, 129, 134]. This decrease in H_2O_2 is thought to contribute to pro-proliferative and anti-apoptotic effects that are, in part, mediated by HIF-1α and involve a decrease in $K_v1.5$ channel expression and elevated $[Ca^{2+}]_i$ levels [129].

4.1 Effects of H_2O_2 on Ion Channel Function and Calcium

The mechanism of intracellular Ca^{2+} regulation by ROS in PASMC is controversial. There are two opposing paradigms currently discussed in the literature which have the same effector; inactivation of $K_v1.5$ channels leading to membrane depolarization-mediated Ca^{2+} entry. One para-

digm proposes that channel inhibition is mediated by reduction [129, 205, 209–212] and the other by an increase in H_2O_2 [126, 213–222]. There could be many reasons why it has been difficult to achieve consensus. The use of different cell passages or tissue preparations, the targeted vascular cell studied, species, methods used to detect H_2O_2, and the stimulus used to affect ROS levels, such as hypoxia or exogenous vs. endogenous H_2O_2.

In studies in which H_2O_2 increases $[Ca^{2+}]_i$ and constricts pulmonary arteries; the responses were unaffected by removal of extracellular Ca^{2+} or by non-selective channel inhibitors, arguing against involvement of store- and receptor-operated Ca^{2+} channels [215, 223]. Although the effect of H_2O_2 on SOCE can vary from tissue to tissue, in VSM and endothelial cells H_2O_2 seems to cause a profound inhibition of SOCE [133, 224–227]. Our laboratory has recently shown that H_2O_2 inhibits ASIC1-dependent SOCE in rat PASMC [133]. Furthermore, removing H_2O_2 through the addition of catalase or the glutathione peroxidase mimetic, ebselen, increased SOCE in rat PASMC. We also found that in pulmonary arteries from $SOD1^{-/-}$ mice, with reduced levels of H_2O_2 production, arterial wall Ca^{2+} is increased through an ASIC1-dependent SOCE mechanism [133]. The enhanced SOCE in PASMC from CH animals could be inhibited by H_2O_2, but catalase and/or ebselen did not further augment SOCE from vehicle conditions. These data suggest that the lower endogenous levels of H_2O_2 in PASMC from CH animals facilitate the activation of SOCE [133]. However, whether this is due to a direct effect of decreased H_2O_2 levels on ASIC1 or an indirect effect mediated by a shift in the cytosolic redox environment is currently unknown. ASIC1 is a known redox sensitive ion channel [228–231], as many ion channels and receptors are, and a better understanding of the regulation of various ion channels by the cellular redox state will expand our knowledge regarding the phenotypic changes in PASMC under disease states.

4.2 H_2O_2 as an Anti-proliferative and Pro-apoptotic Factor: Role of NFAT

Increased $^{\bullet}O_2^-$ together with reduced H_2O_2 are implicated in NFAT activation in a mouse epidermal cell line [200] but the mechanism is unknown. It is possible that changes in $[Ca^{2+}]_i$ are involved since Ca^{2+} influx and Ca^{2+} release from intracellular stores are required for NFAT activation [80–86]. In rat cardiac fibroblasts [232], angiotensin II (AngII)-induced NFAT activation is both Ca^{2+}- and H_2O_2-dependent but H_2O_2 does not affect $[Ca^{2+}]_i$ or NFAT nuclear import in these cells suggesting ROS mediates AngII-induced NFAT activation without an increase in $[Ca^{2+}]_i$. This study also revealed that stimulation of AngII receptor type 1 (AT1R) activates $G\alpha_{12/13}$ which increases ROS production through Rho-mediated Rac activation of NOX. NOX-mediated production of H_2O_2 then activates JNK increasing AP-1 levels in the nucleus synergistically contributing to NFAT activation with Ca^{2+}/calcineurin-dependent NFAT nuclear import. On the contrary, in human and mouse PASMC, we demonstrated that a reduction in H_2O_2 induced either by ET-1 or knockout of SOD1, leads to NFATc3 nuclear import through a Ca^{2+}-dependent mechanism [134, 145]. Since, we have also found that ET-1- and CH-induced NFATc3 nuclear import requires Ca^{2+} influx through ASIC1 channels [233], it is reasonable to conclude that stimuli that cause a reduction in H_2O_2 intracellular levels would lead to activation of ASIC1 causing an increase in $[Ca^{2+}]_i$, calcineurin-dependent NFATc3 nuclear import and activation in PASMC. On the other hand, it has been shown that H_2O_2 inhibits calcineurin/NFAT activity in immune cells [234]. Therefore, these findings suggest that the effect of H_2O_2 on the calcineurin/NFAT pathway is cell type-dependent and may also be stimulus-dependent.

We have demonstrated that NFATc3 is required for CH-induced PASMC proliferation in mice [110]. This proliferative response was detected after 2 days of CH and it was no longer

present after 7 days [110]. After 7 days of CH, both SM α-actin and guanylyl cyclase α1 were upregulated in an NFATc3-dependent manner suggesting that NFATc3 plays a role in hypertrophy and/or redifferentiation of PASMC [72, 73]. Since CH decreases H_2O_2 [133, 134, 145] and increases ASIC1-dependent $[Ca^{2+}]_i$ [16, 133] leading to NFATc3 activation in PASMC [233], and NFATc3 is required for CH-induced pulmonary arterial remodeling in PASMC, it supports a role for H_2O_2 as an anti-proliferative signal in PASMC. As discussed above, H_2O_2 regulates VSM tone and $[Ca^{2+}]_i$ but has also been shown to initiate vascular myocyte proliferation, cell adhesion, and migration [235]. The hyperplastic effects of H_2O_2 have been mainly reported in the context of atherosclerosis and in the systemic circulation [236, 237]. In PASMC, exogenous H_2O_2 induces PASMC proliferation and migration presumably through depletion of transcription factor cAMP response element binding protein (CREB) [60] or extracellular signal-regulated protein kinases (ERK1 and ERK2) [238]. However, interpretation of the physiological role of H_2O_2 in regulation of PASMC function is often complicated by the use of exogenous H_2O_2 or the SOD mimetic, tempol, the latter of which may inadvertently raise H_2O_2 levels. Consistent with a role for $^{\bullet}O_2^-$ as a mitogenic signal, Li et al. demonstrated that $^{\bullet}O_2^-$ but not H_2O_2 was mitogenic to VSMC when ROS was increased using xanthine oxidase/xanthine or H_2O_2-Fe(II) [239]. This group and others actually found that H_2O_2 causes both systemic and pulmonary VSMC apoptosis [239, 240]. The pro-apoptotic effect of H_2O_2 is consistent with reports showing that SOD-mimetic therapy regresses PAH in vivo. Along this lines, PAH is characterized by a reduction in the expression and activity of mitochondrial SOD2, leading to a decrease in H_2O_2 production in PASMC together with a hyperproliferative phenotype and resistance to apoptosis [114]. NFATc2 has been implicated in both PASMC proliferation and resistance to apoptosis in PAH [69]. Similarly, we have shown that CH-induced PASMC proliferation and hypertrophy and PH require NFATc3 activation. The mechanism underlying these cellular responses involves a decrease in SOD1 [133, 134] in pulmonary arteries leading to elevated $^{\bullet}O_2^-$ and decreased H_2O_2 levels. $^{\bullet}O_2^-$ enhances the sensitivity of the contractile apparatus to Ca^{2+} via RhoA/ROK and increased actin polymerization. This same mechanism is involved in NFATc3 nuclear import. The low intracellular H_2O_2 concentration augments ASIC1-dependent SOCE and $[Ca^{2+}]_i$ activating calcineurin which in turns dephosphorylates NFATc3 promoting nuclear import. We have also demonstrated that the PDZ-binding protein known as protein interacting with C kinase (PICK1) binds calcineurin B and ASIC1 in PASMC [233, 241]. This interaction was previously reported in neurons [242]. The scaffolding properties of PICK1 enable macromolecular complexes to form, assembling molecules into signaling domains. We observed a complex mechanism of NFATc3 activation in mouse PASMC that involves Ca^{2+} influx through ASIC1 and downstream regulation by PICK1 [233]. Furthermore, we have shown that calcineurin limits ASIC1-mediated SOCE through dephosphorylation of the channel in rat PASMC in a PICK1-dependent mechanism [241]. Interestingly, PICK1 is redox-sensitive and can interact with cytoskeletal proteins providing a potential link between the increased $^{\bullet}O_2^-/H_2O_2$ ratio, ASIC1 activation, calcineurin, and the cytoskeleton in the NFATc3 activation pathway. A diagram depicting this pathway is included in Fig. 4.

5 Conclusions

This chapter summarizes recent advances in our understanding of altered redox balance in the development of chronic hypoxia-induced pulmonary hypertension. Such redox changes are characterized by a paradoxical increase in the ratio of vascular $^{\bullet}O_2^-$ to H_2O_2 levels associated with decreased SOD activity as well as greater $^{\bullet}O_2^-$ generation from a variety of enzymatic sources. This redox imbalance is thought to provide a major contribution to both vasoconstrictor and arterial remodeling components of pulmonary hypertension by increasing PASMC

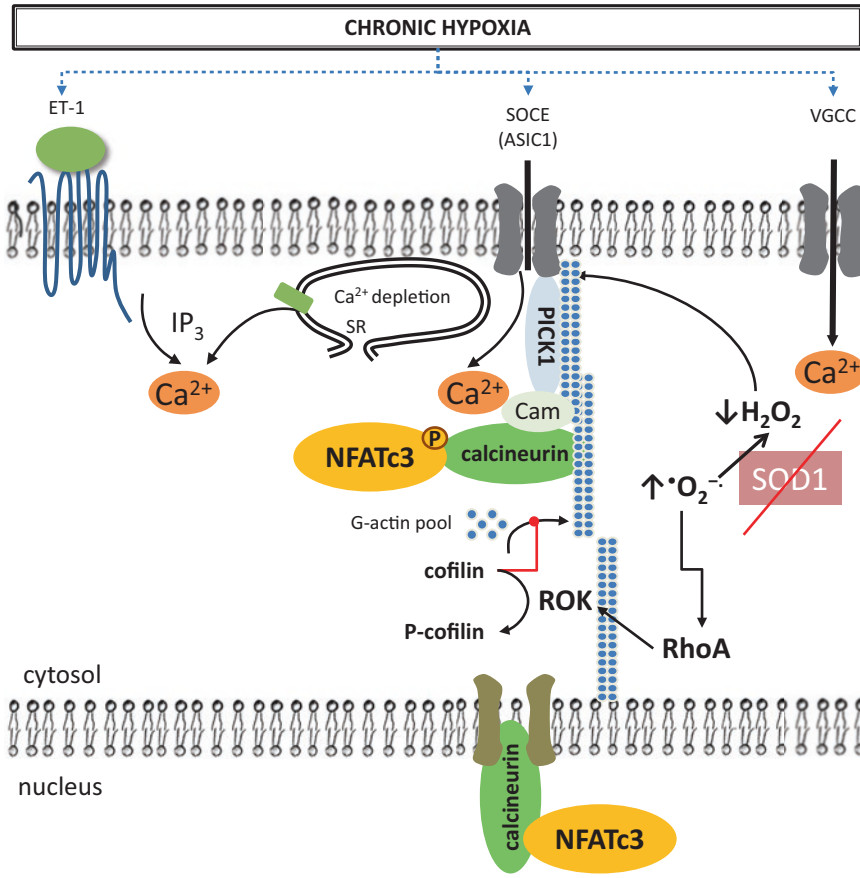

Fig. 4 CH increases $^{\bullet}O_2^-/H_2O_2$ ratio. Reduced H_2O_2 increases Ca^{2+} thereby activating calcineurin, which dephosphorylates NFATc3. Decreased H_2O_2 activates ASIC1 forming a complex with calcineurin B facilitated by PICK-1. This macromolecular complex interacts with the cytoskeleton allowing the calcineurin/NFATc3 complex to be imported into the nucleus. Increased $^{\bullet}O_2^-$ activates RhoA/ROK, leading to cofilin phosphorylation, actin polymerization, and trafficking of the calcineurin/NFATc3 complex to the nucleus. Then, NFATc3 regulates transcription of genes involved in pulmonary arterial remodeling and contractility therefore contributing to the development of pulmonary hypertension

Ca^{2+} influx and myofilament Ca^{2+} sensitivity, and by mediating pro-proliferative, pro-migratory, and anti-apoptotic effects through activation of NFAT and other transcription factors. While we have made critical advances in characterizing the cellular signaling pathways involved in each of these responses, a greater appreciation of the functional relationships between these pathways and the stimuli by which they are induced is needed to better understand the etiology of PH and to develop more effective treatments and preventative measures for this condition.

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ROS Signaling in the Pathogenesis of Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS)

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1 Pathophysiology

Acute respiratory distress syndrome (ARDS) is a critical noncardiogenic syndrome caused by heterogeneous pathologic factors, and is characterized by acute development of respiratory failure, bilateral diffuse lung infiltrations, and severe hypoxemia. The severity of ARDS is associated with poor prognosis and higher mortality. The first description of ARDS was published in 1967 by Ashbaugh et al. [1] where it was defined as acute lung injury developed after various traumas, drug ingestion, aspiration, bacterial or viral pneumonia, sepsis, etc. Thus, ARDS is a syndrome characterized by tachypnea, hypoxemia and loss of lung compliance and diffuse alveolar infiltrations that does not respond to ordinary methods of respiratory therapy, closely resembling infantile respiratory distress syndrome [1]. The new ARDS definition, formulated in 2012 (the Berlin definition), divided ARDS into three categories using hypoxemia as one of main diagnostic parameters. According to the Berlin definition, hypoxemia is defined as decreased arterial blood oxygen tension (PaO_2) to fraction of inspired oxygen (FiO_2) ratio with 201–300 mm Hg for mild ARDS, 101–200 mm Hg for moderate ARDS, and ≤100 mm Hg for severe ARDS [2]. Acute lung injury (ALI), which is similar to mild ARDS, has been excluded from the new definition. Under normal conditions, the dynamic equilibrium between fluid formation and clearance across lung epithelium is strictly regulated [3, 4] and the pulmonary edema associated with ARDS is the result of a loss of the barrier functions of the lung capillary endothelium and alveolar epithelium resulting in vascular leakage and extravascular water accumulation in the lung. Thus, impaired alveolar liquid clearance is characteristic for the majority of patients with lung injury [5]. Similarly, injury to the lung endothelium also leads to fluid hyperpermeability, increased production of pro-inflammatory factors and increased expression of the adhesion molecules needed for leukocyte recruitment and neutrophil migration across the endothelium into the lung. These activated neutrophils induce tissue damage by secreting cytotoxic agents such as granular enzymes, pro-inflammatory cytokines, ROS, and bioactive lipids. The variety of pathologic stimuli leading to ARDS is indicative of the existence of numerous independent risk factors associated with the syndrome. These factors may have either a direct effect on ARDS severity or may potentiate complications by activating inflammatory processes or impairing lung function. However, sepsis is the main risk factor,

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although not all severe sepsis patients suffer from ARDS. Although it should be noted that ARDS developed in sepsis patients is associated with fourfold higher risk of mortality (14 vs. 60%) [6]. Comparative analysis of clinical data shows a good correlation between the Berlin definition of ARDS severity and mortality rates (27%, 35%, and 45%, for mild, moderate, and severe ARDS, respectively), increased lung weight by CT scan, and duration of mechanical ventilation in survivors (6, 12, and 19 days) [2]. Studies have also revealed a correlation between ARDS severity categories and the volume of extravascular liquid accumulated in the lungs (16.1, 17.2, and 19.1 ml/kg) [7]. Early detection is the best way to attenuate the development of ALI/ARDS. However, standard chest X-ray diagnostics may be insensitive for the early phase of ARDS when pulmonary edema is hard to detect and other imaging systems such as lung ultrasound, CT scan, and positron emission tomography are being evaluated [8–12]. Other approaches are investigating the possibility of using biomarkers to identify early signs of ARDS. Several diagnostic methods based on a clearance of isotope-labeled low-molecular weight compounds or pulmonary vessel leakage assessment [13], evaluation of the levels of VEGF, interleukin-2 (IL-2), interleukin-8 (IL-8), and other pro-inflammatory markers in BALF and plasma samples [14–16] have been proposed. Further, a thorough comparative study of biomarkers of inflammation, fibroblast activation, proteolytic injury, lung endothelial and epithelial injury in severe sepsis patients with or without ARDS has identified at least five biomarkers characteristic for ARDS that appear to be suitable for further diagnostics in plasma or bronchoalveolar lavage fluid (BALF) samples: surfactant protein-D (SP-D), receptor for advanced glycation end-products (RAGE), IL-8, club cell secretory protein (CC-16), and interleukin-6 (IL-6) [16]. Indeed, a critical care randomized trial demonstrated that early alveolar damage can be identified by the presence of SP-D in blood. Significantly increased levels of SP-D is a strong independent predictor that the patient suffers from ARDS who will not recover [17]. Due to heterogeneity of ALI/ARDS etiology and complexity of the syndrome, there is no efficient therapy available. Mechanical lung ventilation as a supportive clinical approach for oxygenation of healing lungs is a standard therapeutic method for ARDS patients. However, mechanical ventilation itself can lead to biotrauma increasing lung inflammation and worsening clinical condition due to ventilation-induced lung injury (VILI) [18–20]. Recent studies have demonstrated that injured lungs can be optimally supported by low-tidal volume ventilation and that this requires personalization of the settings [20–23]. Lung ventilation can also be supplemented by anti-inflammatory medicine. First applied for ARDS treatment by Ashbaugh and colleagues [1], corticosteroids are still considered valuable for the treatment [24], as a major anti-inflammatory agent.

Excessive ROS generated by the injured endothelium/epithelium as well as recruited leukocytes plays a major role in ARDS progression and lung damage. Oxidative stress can be a cause of the endothelial and epithelial barrier dysfunctions resulting in massive neutrophil penetration across the barriers followed by secretion of cytotoxic agents (Fig. 1). ROS upregulate the expression of pro-inflammatory cytokines and adhesion molecules amplifying the tissue damage and pulmonary edema. Thus, a proper oxidant–antioxidant balance is critical for vasculature homeostasis. Therefore, the systems responsible for excessive ROS production can be therapeutic targets in ARDS treatment. The following sections summarize our current knowledge regarding ROS generation and their effects on ARDS development and discuss possible approaches to prevent or minimize ROS-induced pulmonary damage.

2 Biological Origins of ROS in Vasculature

2.1 Cellular Free Radicals

Free radical intermediates generated during the reduction–oxidation (redox) reaction involving the conversion of molecular O_2 to water are called

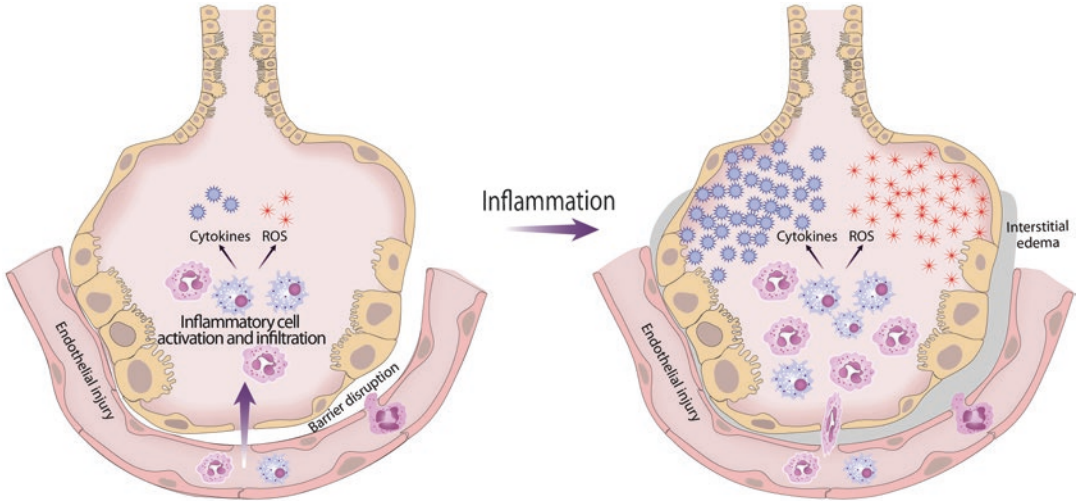

Fig. 1 Dysfunction of microvascular endothelium and alveolar epithelium in ARDS. Polymorphonuclear leukocytes (PMNs) and macrophages infiltrate the inflamed region through the microvascular blood vessels releasing cytotoxic factors such as pro-inflammatory cytokines and ROS. Theese cytokines and ROS contribute to the endothelial and epithelial dysfunction resulting in leakage of fluids from circulation into the interstitial space and alveoli. This results in pulmonary edema and impaired gas exchange. Sources of inflammation range from bacterial infections to mechanical ventilation

ROS. Molecular oxygen (O_2) as such is a free radical having two unpaired electrons with the same spin quantum number or parallel spin. When O_2 tries to oxidize a non-radical by accepting a pair of electrons with antiparallel spin, these electrons do not match the spin number in O_2. Therefore, the reaction of O_2 with non-radicals is thermodynamically unfavorable. However, O_2 can readily accept single electron transfers from other free radicals [25]. For example, during aerobic respiration, cytochrome oxidase of mitochondria catalyzes four such single electron transfers to molecular O_2 from two reduced heme (Fe^{2+}) and two copper (Cu^{+}) ions coupled with proton translocation resulting into molecular water, energy, and oxidized cytochrome [26, 27]. The cytochrome oxidase enzyme operates under severe constraints to prevent release of partially reduced, toxic, and high energy oxygen free radicals. Cells can efficiently reduce almost 95% of molecular oxygen that we consume to water by aerobic respiration. When a single electron is transferred to molecular O_2, the resulting product is a superoxide ($O_2^{\bullet-}$) free radical. Another electron transfer to $O_2^{\bullet-}$ results into peroxide (O_2^{2-}) ion. This reaction may be spontaneous dismutation or catalyzed by superoxide dismutase (SOD) [28]. Hydrogen peroxide when completely reduced is converted to molecular water and O_2. Partially reduced hydrogen peroxide in the presence of transition metals yield the most potent hydroxyl ($OH^{\cdot}$) free radical [29]. Together, these oxygen free radicals are called ROS. The other important free radical in the vasculature is nitric oxide ($NO^{\cdot}$). Under physiological conditions, $O_2^{\bullet-}$ reacts with $NO^{\cdot}$ to form a highly oxidative, reactive nitrogen species (RNS), peroxynitrite ($ONOO^{-}$). These oxidizing and nitrating free radicals can severely damage cellular macromolecules such as lipids, proteins and DNA even under normal physiological conditions [30]. Therefore, antioxidant mechanisms that neutralize the highly oxidative free radicals are critical for cell survival.

2.2 ROS in Vascular Tissue

The generation of ROS is an unavoidable consequence of living in an oxygen rich environment and organisms have evolved with elaborate

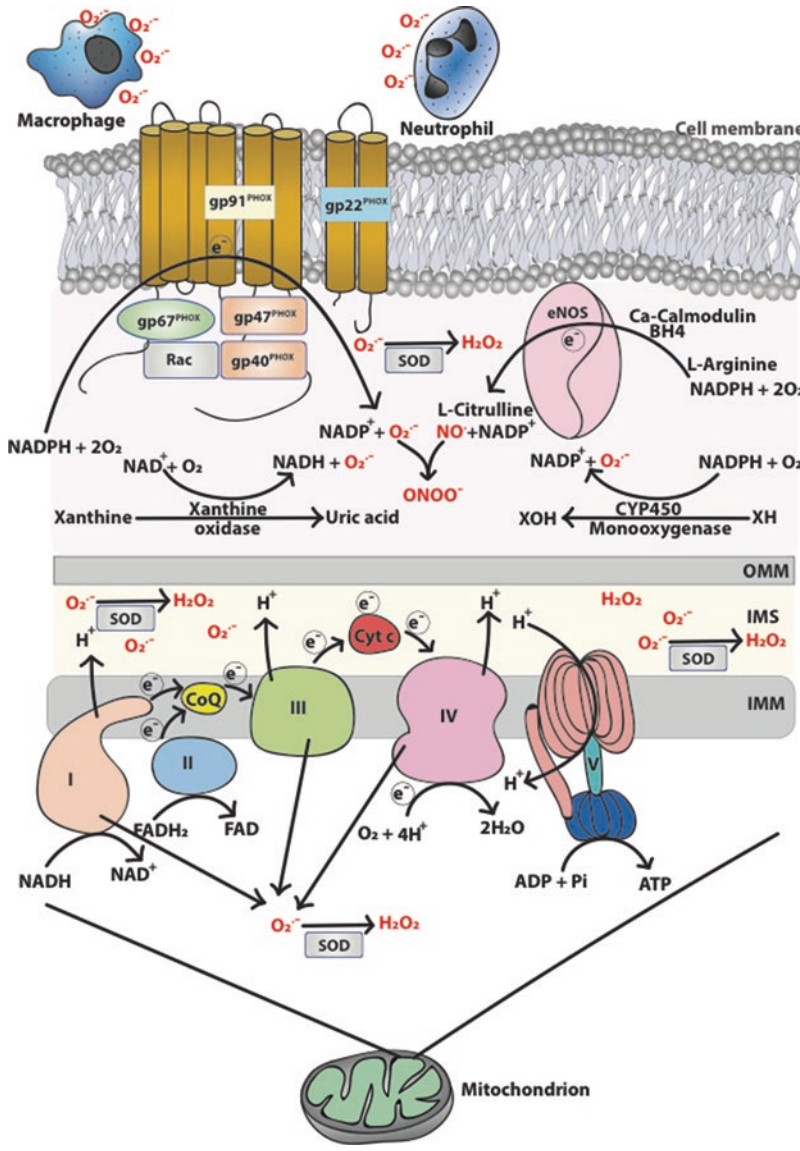

Fig. 2 Sources of reactive oxygen species. Mitochondria, NADPH oxidase, xanthine oxidase, and eNOS are the major contributors of ROS in cells of vasculature during active metabolism. NADPH oxidase in phagocytic cells such as macrophages and neutrophils that are resident in blood vessels contribute to a significant amount of superoxide ($O_2^{\cdot-}$). Endothelial NOS (eNOS) generates NO free radicals that interact with $O_2^{\cdot-}$ to generate peroxynitrite. Peroxynitrite induces nitrasative stress on cells by nitrating proteins and altering signaling pathways. When eNOS is uncoupled, it can generate superoxide. Oxidative phosphorylations in mitochondria are a source of $O_2^{\cdot-}$. Especially complexes I, III, and IV generate $O_2^{\cdot-}$ when there is a leak of electrons at subsequent transfer stages. $O_2^{\cdot-}$ generated in mitochondria is often immediately dismutated to H_2O_2 by SOD which can cross mitochondrial membrane as well as cell membranes. Other lesser sources of ROS are cytochrome P450 enzymes which often generate $O_2^{\cdot-}$ during detoxification of xenobiotics and they are predominantly expressed in hepatic tissue. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, BH_4 tetrahydrobiopterin, *Ca-Calmodulin* calcium and calmodulin, *CoQ* coenzyme Q, *Cyt c* cytochrome c, *eNOS* endothelial nitric oxide synthase, $FADH_2$ flavin adenine dinucleotide, H_2O_2 hydrogen peroxide, *IMM* inner mitochondrial membrane, *IMS* inter-mitochondrial membrane space, *NADH* nicotinamide adenine dinucleotide, *NADPH* nicotinamide adenine dinucleotide phosphate, NO nitric oxide, *OMM* outer mitochondrial membrane, *Pi* inorganic phosphate, O_2^- superoxide free radical, $ONOO^-$ peroxynitrite free radical, *SOD* superoxide dismutase, complex I—NADH oxidoreductase (I), complex II—succinate dehydrogenase (II), complex III—cytochrome *c* reductase (III), complex IV—cytochrome *c* oxidase (IV), complex V—ATP synthase (V), *XH* xenobiotic, *XOH* alcohol/aldehyde form of xenobiotic

mechanisms to detoxify these ROS. As shown in Fig. 2 both enzymatic (NAPDH oxidase [NOX], xanthine oxidase, and uncoupled nitric oxide sythase [NOS]) and nonenzymatic (mitochondrial) sources can be major ROS producers in endothelium. The endothelium is more than just a single layer of cells lining the lumen of blood vessels. It is intimately involved in maintaining the homeostasis of vascular tissue. ROS can influence many functions of the endothelium. The endothelium acts as a barrier to prevent leakage of the contents of circulation. It also generates NO which mediates vasorelaxation. Endothelial cells are susceptible to increased ROS generation under various pathological conditions. For example, ROS dependent expression of adhesion molecules such as ICAM-1 and VCAM-1 by endothelial cells can recruit immune cells that themselves express enzymes that generate high levels of ROS. Neutrophils and macrophages recruited to the site of inflammation generate free radicals that contribute to the pool of ROS causing oxidative stress (Figs. 1 and 2). It has been noted that neutrophils can kill endothelial cells by generating ROS [31]. In the lungs multiple cell types including, endothelial cells, neutrophils, eosinophils, alveolar macrophages, and alveolar epithelial cells are major ROS generators. Fibroblasts, perivascular adipocytes and vascular smooth muscle cells are also significant sources of ROS in the vasculature. Within these cells, several enzymes are involved in generating ROS. These include NOX, uncoupled NOS, dysfunctional mitochondria, and xanthine oxidase. There is evidence that all these systems may be involved in the oxidative stress associated with ALI/ARDS and antioxidants have been shown to reduce the severity of ALI/ARDS in multiple mouse models including lipopolysaccharide (LPS) [32–34], influenza A [35], hyperoxia [36], toxic gas [37], ischemia–reperfusion (I/R) [38], sepsis [39, 40], acid aspiration [41], burn and smoke inhalation [42] as well as high tidal mechanical ventilation [43–45]. Figure 2 illustrates the biological origins of ROS in a cell. The following sections will deal with the major ROS generating systems in the pulmonary vasculature and how they are involved in the pathogenesis of ARDS.

2.3 Xanthine Oxidase

Xanthine oxidoreductase (XOR) belongs to the molybdoenzyme family with two interconvertible forms, O_2 dependent type O xanthine oxidase (XO) and NAD-dependent type D xanthine dehydrogenase (XDH). It catalyzes the oxidation of hypoxanthine to xanthine and uric acid in purine metabolism [46, 47]. XOR is a homodimer, and each monomer consists of three domains each harboring cofactors molybdopterin (Mo–Co), two iron–sulfur centers [2Fe-2S], and flavin adenine dinucleotide (FAD) arranged linearly in the order of their redox potentials [48]. In the process of purine metabolism, XO generates ROS, $O_2^{\cdot-}$ and hydrogen peroxide. Early studies using isolated rabbit lungs perfused with XO increased the permeability of pulmonary microvascular endothelial cells implicating the role of XO in lung injury [49]. Reperfusion of rabbit lungs were with XO inhibitor allopurinol or superoxide scavenger, SOD decreased the lung injury [50]. In a VILI animal model, application of high tidal volume mechanical ventilation (HTMV) activated XOR and increased the pulmonary capillary permeability [51]. Treatment of endothelial cells directly with ROS or with XO decreases the transendothelial electrical resistance (TEER) and increases the permeability of macromolecules [52]. Oxidative stress is known to induce apoptosis of epithelial cells during VILI [53]. VILI also induces p38 MAPK mediated inflammatory lung injury [54] and activation of p38 increases XOR enzymatic activity. Pharmacological inhibition of p38-XOR attenuates VILI induced lung injury [55]. These studies indicate a significant role of XOR in ROS mediated lung injury.

2.4 Uncoupled Endothelial Nitric Oxide Synthase

Under normal physiological conditions, endothelial nitric oxide synthase (eNOS) functions as a homodimer to produce the vasodilator signaling molecule, NO. NO is a free radical capable of reacting with ROS to generate RNS [56]. eNOS requires molecular O_2 and L-arginine as

substrates along with cofactors NADPH, 6(R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD, and FMN [57] to produce NO and L-citrulline as a by-products. To understand the role of eNOS in ROS generation, we need to first understand the structure and mechanism of action of eNOS. The C-terminus reductase domain of one monomer in the eNOS homodimer is linked to N-terminus oxidase domain of the other monomer. The homodimer is stabilized by a zinc thiolate cluster which include phylogenetically conserved cysteine residues that bind zinc ion in a tetrahedral conformation [58]. The reductase domain binds to NADPH, FMN, and FAD cofactors, and oxygenase domain binds to cofactor BH_4, and substrates L-arginine and O_2 [59]. The oxygenase domain also carries the prosthetic heme cofactor. Calcium–calmodulin binding sequence is located in the center between the reductase and oxygenase domains. Binding of calcium–calmodulin aligns the two domains for an efficient transfer of electrons from reductase domain to the heme on the oxidase domain, therefore making the eNOS homodimer catalytically active [60]. Two electrons donated by NADPH are transported through flavins, FAD and FMN and subsequently to the heme of the oxidase domain to activate O_2. Reduced oxygen is incorporated into the guanidine group of L-arginine in two steps; step one includes hydroxylation of L-arginine to N^{ω}-hydroxy-L-arginine intermediate and in the second step N^{ω}-hydroxy-L-arginine oxidized to L-citrulline and NO [61]. Since NO generation is a tightly regulated process, pathophysiological conditions causing deficiency of any of these cofactors can lead to generation of superoxide $O_2^{\bullet-}$ which is called uncoupling. Under uncoupled conditions, eNOS can synthesize $O_2^{\bullet-}$ at the heme although this requires binding of calcium–calmodulin. $O_2^{\bullet-}$ generation by eNOS appears to be primarily dependent on the availability of BH_4 rather than L-arginine [62]. It is thought that BH_4 couples L-arginine oxidation to NADPH reduction by preventing the disassociation of the ferrous-dioxygen complex of heme [63], suggesting that BH_4 can be used as pharmacological agent to treat vascular diseases. BH_4 has also been implicated in the maintenance of mitochondrial redox balance [64]. The mechanism by which eNOS becomes uncoupled appears to be through increases in the endogenous NOS uncoupler, asymmetric dimethylarginine (ADMA) and the generation of peroxynitrite [65, 66]. Peroxynitrite can add a nitro group ($-NO_2$) to one ortho carbon of tyrosine's phenolic ring to form 3-nitrotyrosine (3-NT), a process called protein nitration. Protein tyrosine nitration dramatically alters the pKa of the tyrosine hydroxyl group producing structural and function changes within affected proteins [67]. Peroxynitrite can also cause uncoupling of eNOS via the oxidation of zinc thiolate clusters and the formation of disulfide bonds between the monomers [68]. The phosphorylation of eNOS at T495, mediated by protein kinase C (PKC), impairs NO production in the endothelial cells [69] and enhances eNOS uncoupling. pT495 eNOS can be translocated to mitochondria [70, 71] where it increases mitochondrial derived ROS. Proteomic studies have begun to identify nitrated proteins that may be important in ALI/ARDS. The proteins identified so far include sphingosine-1-phosphate lyase 1 (SIP lyase 1) [72], Rho-GTPase-activating protein 5 (RHOGAP5) [72] and RhoA itself [73]. However, RhoA is the only nitrated protein validated being involved ALI/ARDS [72, 74]. Uncoupled eNOS has aslo been shown to be involved in the lung injury associated with G^+ bacterial infections [70], smoke inhalation [75], and high tidal mechanical ventilation [76].

2.5 Mitochondrial Respiratory Chain

Mitochondria are the respiratory centers of the cell where ATP is produced by reducing O_2 to water. A series of single electron transfers are performed across four electron transport complexes (ETC) [77]. The four ETC are arranged in the order of increasing redox potential, between -32 V (NADH of complex I) and +39 V (cytochrome *a3* of complex IV). Complex I (NADH dehydrogenase),

complex III (cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) pump protons H^+ into the mitochondrial intermembrane space which contribute to the mitochondrial membrane potential that will ultimately drive the ATP synthase motor to generate high energy ATP from ADP and inorganic phosphate. The transfer of electrons across these electron transport carriers is usually highly efficient. However, there is 1–2% leak of electrons that react with O_2 to generate $O_2^{\bullet-}$. Complex I and III are the major contributors of $O_2^{\bullet-}$ in mitochondrion [78]. Electrons donated by NADH to complex I are transported through flavin complex, series of Fe–S clusters to ubiquinone Q. Superoxide can be generated at each of these electron transport steps by complex I [79–81]. Furthermore, any blockade in the electron transport downstream of complex I can result in significant generation of $O_2^{\bullet-}$ by complex I. The presence of a higher NADH/NAD^+ ratio can drive more $O_2^{\bullet-}$ generation into the mitochondrial matrix by complex I [81]. Complex I is major source of $O_2^{\bullet-}$ in skeletal and neural cells whereas complex III is the major source of $O_2^{\bullet-}$ in endothelial cells. Therefore, cell type as well as the metabolic state of the cell determines the source of $O_2^{\bullet-}$ in mitochondria. Complex II (succinate dehydrogenase) is not a significant source of $O_2^{\cdot-}$ in mitochondria. Complex III on the other hand generates $O_2^{\cdot-}$ during the Q cycle which involves transfer of electrons from complex I and II to ubiquinone resulting in reduction of ubiquinone to ubiquinol [82, 83] resulting in the release of $O_2^{\bullet-}$ to both sides of the inner mitochondrial membrane [84]. Oxidation of ubiquinol involves donation of two electrons to cytochrome *c* as single electron transfers through reiske iron–sulfur protein and cytochrome *c*1, resulting in unstable intermediate ubisemiquinone. Ubisemiquinone radical donates the single electron to O_2 to generate $O_2^{\cdot-}$ [85]. $O_2^{\cdot-}$ generated by mitochondria is converted to H_2O_2 by manganese SOD (MnSOD) that can cross the mitochondrial membrane into cytoplasmic compartment. $O_2^{\bullet-}$ and H_2O_2 form the pool of mitochondrial ROS (mtROS). Genetic mutations in nuclear and mitochondrial genes encoding the proteins of mitochondrial respiratory complexes can lead dysfunction of specific electron transport complexes. Defects in complexes I and III can lead to significant increase in mtROS and subsequent pathological conditions [86]. Increase in mtROS can alter the signaling of redox sensitive transcription factors such as HIF-1α which can further alter the metabolic state of the cell [87]. Other important aspects of mtROS is regulation of inflammasome [88], activation of caspases [89] and regulation cell death by apoptosis [90]. Therefore, ROS generated by mitochondria have very broad implications on cellular homeostasis. Excessive demand for ATP or damage to any of the ETC components can result in increased ROS leakage by complexes I, III and IV and consume the antioxidant defenses. This can lead to rupture and release of mitochondrial components including mitochondrial DNA (mtDNA) [91]. Release of mtDNA can induce an inflammatory response [92] through activation of TLR9/NLRP3 inflammasome [93]. Excessive ROS can activate pro-apoptotic Bcl-2 family proteins by increasing mitochondrial permeability to drive the MMP, release cytochrome c, mtDNA [94], and pro-apoptotic caspase-3 and -9. This leads to the activation of intrinsic or mitochondrial driven cell death by apoptosis [95]. Mitophagy removes excessive ROS generating mitochondria to avoid cell death by self-destructive inflammatory response [96]. This is evident by the activation of NLRP3 inflammasome when mitophagy is inhibited [97]. Under normal breathing, lung epithelial cells are adapted to cyclic stretch. However, under mechanical ventilated conditions, increased levels of mitochondrial ROS can be generated by epithelial cells when subjected to cyclic stretch as a result of direct distention of mitochondria. This ROS production is dependent upon the time and magnitude of stretch [98]. Excessive ROS generation can lead to loss of mitochondrial function and apoptosis of lung epithelial cells [99]. Viral infections can also alter the mitochondrial dynamics leading to excessive mtROS generation, mitochondrial biogenesis, and altered mitochondrial β-oxidation [100, 101]. Given the central role of mitochondria in cell physiology, antioxidants to combat the deleterious ROS generated by the mitochondrion could be a potential target for developing therapeutic strategies for ALI/ARDS.

2.6 Cytochrome P450

Cytochrome P450 (CYP) belongs to the family of membrane bound heme-thiolate enzymes involved in oxidative metabolism of a variety of hydrophobic endogenous macromolecules and exogenous compounds such drugs, carcinogens, and xenobiotics by monooxygenation reaction [102]. Most CYP enzymes are predominantly expressed in the liver. Some isoforms of CYP such as 2B, 2C8, 2C9, 2C10, 2J2 are expressed in endothelium and vascular smooth muscle cells where they play important roles in arachidonic acid metabolism and in the maintenance of vascular homeostasis and tone. CYP at resting state have a hexa-coordinated low spin heme (LS) having water molecule weakly bound as the sixth axial ligand. The water molecule is then displaced by the substrate resulting in a penta-coordinated high spin (HS) heme. This LS to HS transition of heme increases its redox potential. HS ferric heme is reduced to ferrous heme by accepting electrons donated by a redox partner; in this case, NADPH-dependent cytochrome P450 reductase, a diflavoprotein that contains FAD and FMN. Oxygen binds to ferrous heme followed by a series of oxyferrous intermediates leading to activation of O_2. Second electron transfer from redox partner to oxyferrous intermediate results in a series of intermediates leading to heterolytic cleavage of O–O bond and generation of highly reactive oxyferryl intermediate which is responsible for monooxygenation of substrates [103]. As an unwanted consequence, activated oxygen at the heme of CYP can lead to generation of ROS, leading to uncoupling of CYP. Indeed, the ROS generated from CYP2C in coronary artery endothelial cells has been shown to impair NO mediated vasorelaxation [104]. Also, the activation of endothelial CYP by hemodynamic stimulus such as cyclic stretch also leads to increased production of $O_2^{\cdot-}$ [105], suggesting that CYP activation could be and important factor in the oxidative stress associated HTMV. The increased ROS production in lung epithelial cells exposed to sulfur mustard [106] or environmental pollutants has also been shown to be dependent on increased CYP1A1 enzyme activity [107]. Other CYPs have also been implicated in the oxidative lung injury associated with hyperoxia (CYP1A1 and CYP1A2) [108, 109] and alcohol abuse (CYP2E1) [110]. Therefore, CYP enzymes likely also contribute to the pool of ROS that can mediate lung injury.

2.7 NADPH Oxidase (NOX)

An increase in leukocyte respiration was observed when these cells exposed to bacteria as early as 1933 by Baldridge and Gerard. During this respiratory burst, the leukocytes generate ROS, superoxide, hydrogen peroxide and hydroxyl free radicals to kill the phagocytosed pathogens [111, 112]. NOX is the enzyme that catalyzes reduction of oxygen to generate superoxide using NADPH [113]. Therefore, NADPH oxidase is often referred as the "professional ROS producer." The phagocytic NOX (NOX2) is a multicomponent enzyme with two membrane bound subunits ($gp91^{PHOX}$, $p22^{PHOX}$) and three cytosolic subunits ($p67^{PHOX}$, $p47^{PHOX}$, and $p40^{PHOX}$). In addition to these subunits, small GTPase Rac1 or Rac2 may be associated with NAPDH oxidase [114]. Membrane bound subunits $gp91^{PHOX}$, $p22^{PHOX}$ form heterodimeric flavoprotein called cytochrome b_{558}. When the cytosolic components migrate to the membrane, the NOX complex can now accept electrons to transfer to O_2 and generate $O_2^{\cdot-}$ [115]. Initially NOX was thought to be expressed only in phagocytic immune cells (hence the name PHOX), but later other homologues of NOX were discovered in non-phagocytic cells types and were designated as NOX family of NADPH oxidases [116]. Seven different NOX isoforms have been identified: NOX1, NOX2, NOX3, NOX4, NOX5, Duox1, and Duox2. Only NOX1, NOX2 and NOX4 are expressed in vasculature and are all implicated with ROS mediated vascular diseases [117]. When exposed to TNFα, human aorta smooth muscle cells rapidly induced ROS generation which mediated by NF-κB induced upregulation of NOX1 and NOX4 [118]. Aldosterone induced expression of NOX1 and superoxide generation which was mediated through PKC delta in

vascular smooth muscle cells [119]. When rat vascular smooth muscle cells are exposed to cigarette smoke extract, NOX1 derived superoxide causes cellular toxicity [120]. Neutrophils, the majority of circulating white blood cells play an important role host defense mechanism against invading pathogen and NOX2 is responsible for the respiratory burst and superoxide generation. Chronic granulomatous is a disease caused by genetic mutations in NOX2 subunits, especially in $gp91^{PHOX}$ and observed predominantly in males because of the presence of gene on the X chromosome [121]. Phagocytic cells with defective $gp91^{PHOX}$ are unable to produce superoxide and patients are susceptible to severe infections [122]. On the other hand, increased expression of $p22^{PHOX}$ by activation of p38-Erk1/2-MAPKinase pathway resulted in ROS mediated endothelial dysfunction in type 2 diabetes mice [123]. NOX2 and NOX4 are also known to mediate ROS dependent proliferative response in microvascular endothelial cells [124]. Rac1 pharmacological inhibition improved function of endothelial cells obtained from vein grafts of patients who underwent bypass surgery due to severe vascular disease. Rac1 inhibition not only reduced NOX dependent ROS but also increased eNOS function by suppressing ROCK1 which is a negative regulator of eNOS [125]. NOX4 is a constitutive producer of ROS at basal levels unlike NOX1 and NOX2 which are signal activated. However, NOX4 increases ROS generation on demand when cells are exposed to inflammatory stimulus. TGFβ induced expression of NOX4 in vascular smooth muscle cells along with inflammatory phenotype commonly seen in atherosclerosis and aging [126]. NOX4 is mainly involved in maintenance of basal ROS mediated signaling of vasculature. NOX4 knockout mice developed cardiac dilation, contractile dysfunction and cardiac failure due to chronic overload suggesting the importance of NOX4 mediated ROS signaling in cardiac function [127]. Together, NOX isoforms contribute widely being beneficial in innate immunity and basal ROS mediated signaling and deleterious in the development several vascular pathological conditions.

Analysis of lung sections and BALF from patients with ARDS show massive accumulation of PMNs especially neutrophils [128]. These cells produce very high levels of ROS that exacerbates the inflammatory responses in lungs. ROS generated by LPS exposure has been shown to be NOX1 dependent in macrophages [129] and NOX2 dependent in the LPS-challenged lung [130]. LPS activation of TLR4 receptor induced NOX mediated ROS production which subsequently lead to activation of pro-inflammatory NF-κB [131] and TNFα signaling [132, 133]. ROS generated by NADPH oxidase of PMNs during hemorrhagic shock which is a known cause of ARDS, activates NOX of endothelial cells through HMGB1, TLR4, and Rac1 signaling pathway [134]. A significant decrease in hyperoxia mediated ROS production was observed in lung epithelial and capillary endothelial cells of NOX1 knockout mice compared to wild type mice, thereby preserving the alveolocapillary barrier [135]. Epithelial cells under cyclic stretch produce ROS and it is NOX dependent [98]. Knockout of NOX1 prevented lung injury in mice exposed to hyperoxia [136]. Inhibition of NOX4 highly expressed in epithelial cells and fibroblasts can prevent epithelial cell death and prevent ROS mediated epithelial cell deatch, inflammation and lung fibrosis [137]. In a gastric acid aspiration mice model, it was shown that NOX was able to limit lung injury by Nrf2 mediated decrease of PMN airway accumulation [138]. NADPH oxidase can also limit lung injury by activation of redox sensitive anti-inflammatory transcription factor NRF2 [139]. Largely described, above studies significantly implicate the role of NOX in ARDS/ALI. Therefore, inhibitors of NOX may utility in the treatment of ARDS.

3 Cellular Defenses Against Oxidative Stress

Since oxidants have the capacity to react in an indiscriminate manner leading to damage of almost any cellular component, an extensive

range of antioxidant defenses have evolved to protect the cell from the oxidant-induced damage. As shown in Fig. 3, there are several enzyme systems that catalyze reactions to neutralize free radicals and ROS. These form the body's endogenous defense mechanisms to help protect against oxidant induced cell damage. The main cellular antioxidant enzymes and their involvement in protection against ARDS will be discussed below.

3.1 Superoxide Dismutase

The enzyme superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide (H_2O_2) [140]. The H_2O_2 must then be removed by catalase or glutathione peroxidase. There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution. Copper zinc superoxide dismutase (CuZnSOD) is found in the cytoplasm and organelles of virtually all mammalian cells. It has two protein subunits, each containing a catalytically active copper and zinc atom. Manganese superoxide dismutase (MnSOD) is found in the mitochondria of almost all cells. It consists of four protein subunits, each containing a single manganese atom. The amino acid sequence of MnSOD is entirely dissimilar to that of CuZnSOD and it is not inhibited by cyanide, allowing MnSOD activity to be distinguished from that of CuZnSOD in mixtures of

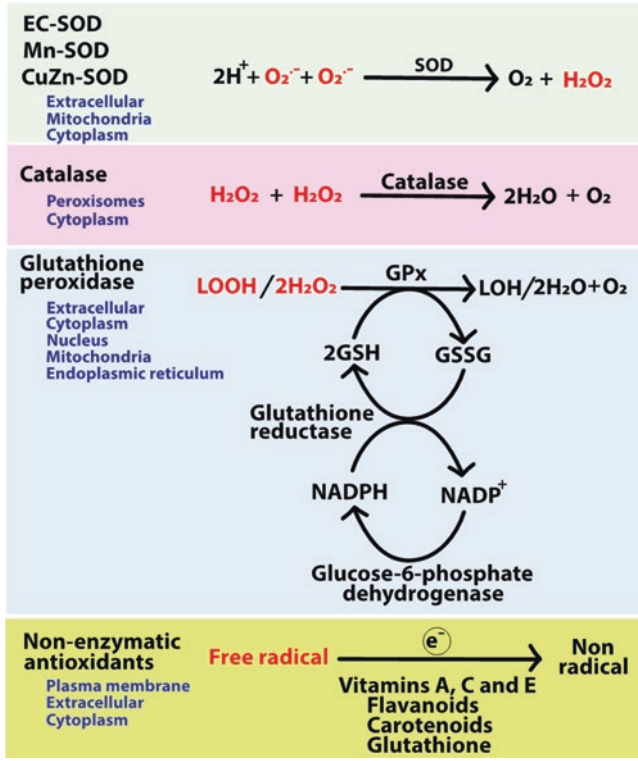

Fig. 3. The antioxidant system in cells. Enzymatic and nonenzymatic antioxidants catalyze reactions to neutralize free radicals by donating electrons. Enzymatic antioxidants catalyze reactions to neutralize specific free radicals such that superoxide dismutase (SOD) dismutates superoxide to hydrogen peroxide (H_2O_2), and catalase and glutathione peroxidase (GPx) convert hydrogen peroxide to water. GPx also converts lipid hyroperoxides (LOOH) to lipid alcohols or aldehydes (LOH). Glutathione reductase replenishes reduced glutathione (GSH) pools from oxidized glutathione (GSSG) using NADPH as reducing equivalents. Nonenzymatic antioxidants such as vitamins, flavonoids and glutathione can also reduce free radicals by donating electrons

the two enzymes. Extracellular superoxide dismutase (EC-SOD) is a secretory copper containing SOD distinct from the CuZnSOD. EC-SOD is synthesized by only a few cell types, including fibroblasts and endothelial cells. A number of studies have shown that exogenously administered antioxidant enzymes, particularly when encapsulated in lipid vesicles (liposomes) or conjugated to polyethylene glycol to prolong biological half-life and aid delivery to cells, can protect against oxidant damage and mitigate the severity of acute pulmonary injury. A synthetic Mn-containing superoxide dismutase mimetic (SODm), M40403, inhibits endotoxin-induced production of TNF-α and IL-6 in alveolar macrophages [141]. MnTMPYP, a superoxide dismutase mimetic, restored the inflammatory responses to LPS challenge including reduced lung myeloperoxidase activity and vascular permeability in mice [142]. In addition to SODs, several other antioxidant agents have been studied in therapeutic applications for lung injury. This includes EUK-8, a synthetic low molecular weight compound with powerful SOD, catalase, and oxyradical scavenging properties. Treatment with EUK-8 ameliorated pulmonary dysfunction in a porcine model of LPS-induced adult respiratory distress syndrome [143]. EUK-8 significantly attenuated many of the features of LPS-induced acute lung injury such as arterial hypoxemia, pulmonary hypertension, decreased dynamic pulmonary compliance and pulmonary edema. The authors concluded that EUK-8 prevents many of the manifestations of LPS-induced adult respiratory distress syndrome by detoxifying reactive oxygen metabolites without affecting the release of other important proinflammatory mediators. In another study, endothelium targeted EUK-134 accumulated in lungs after intravascular injection, providing >60% protection against pulmonary edema in endotoxin-challenged mice [34]. The superoxide scavenger Manganese (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP) played a protective role in alleviating acute inflammatory response and lung injury [144].

3.2 Catalase

Catalase was the first antioxidant enzyme to be characterized; it catalyzes the two-stage conversion of H_2O_2 to water and oxygen. Catalase consists of four protein subunits, each containing a heme group and a molecule of NADPH [145]. The rate constant for the reactions described above is extremely high, implying that it is virtually impossible to saturate the enzyme in vivo. Catalase is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating H_2O_2. It has been shown that in sheep pretreatment of intraperitoneal injections of catalase attenuated changes in pulmonary arterial pressure, lung lymph flow, and arterial leukocyte counts and oxygen tension after endotoxin infusions [146]. Another study showed that catalase prevents increased lung vascular permeability during air emboli in unanesthetized sheep [147]. In a study targeting catalase to the pulmonary endothelium showed alleviated oxidative stress and reduced acute lung transplantation injury [148]. These studies indicate that H_2O_2 plays a role in the pathogenesis of the acute lung injury and catalase is an important player in development of ALI/ARDS.

3.3 Glutathione and Related Enzymes

Reduced glutathione (GSH) is a major source of thiol groups in the cell [149]. GSH can function directly as an antioxidant, scavenging a variety of radical species, as well as participating in the reactions of glutathione peroxidase. Glutathione peroxidases (GPx) catalyze the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxide (LOOH) [150]. Other peroxides, including LOOH, also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. GPx require selenium at the active site. Their predominant subcellular distribution is in the cytosol and mitochondria, suggesting that GPx is the main scavenger of H_2O_2 in

subcellular compartments. The activity of the enzyme is dependent on the constant availability of GSH. The ratio of reduced to oxidized glutathione (GSSG) is usually kept very high as a result of the activity of the enzyme glutathione reductase [151]. The NADPH required by this enzyme to replenish the supply of GSH is provided by the pentose phosphate pathway. Glutathione reductase is a flavine nucleotide dependent enzyme and has a similar tissue distribution to GPx. GSH supplementation has been shown to attenuate lipopolysaccharide (LPS)-induced mitochondrial dysfunction in a mouse model of acute lung injury [32]. Selenium, a GPx cofactor, activates GPx in vivo and attenuates lipid peroxidation and lung injury early after paraquat intoxication in rats, but did not affect the survival [152]. Ebselen, an organoselenium compound, mimics GPx activity and showed protective action in animal model of pleurisy [153]. BXT-51072 and BXT-51077, selenium-containing GPx mimics, prevented TNF- and neutrophil-induced endothelial alterations through the downregulation of endothelial proinflammatory responses [154].

3.4 Nonenzymatic Antioxidants

Whenever a free radical interacts with another molecule, secondary radicals may be generated that will further react with the available targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralized by an antioxidant. Antioxidants are molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable by-products. The most important lipid phase antioxidant is probably vitamin E (tocopherol). It quickly reacts with a peroxyl radical to form a relatively stable tocopheroxyl radical, with the excess charge associated with the extra electron being dispersed across the chromanol ring. The dietary supplement γ-tocopherol (γT), a natural form of vitamin E, inhibited LPS-induced increase in BAL fluid total cells, neutrophils, protein, and secreted mucins, along with tissue neutrophil influx [155]. Pretreatment with vitamin E has also been shown to ameliorate acute lung injury induced by burn and smoke inhalation in sheep [156]. The carotenoids are a group of lipid-soluble antioxidants based around an isoprenoid carbon skeleton. The most important of these is beta-carotene, although at least 20 others may be present in membranes and lipoproteins. They are particularly efficient scavengers of singlet oxygen, but also trap peroxyl radicals at low oxygen pressure with an efficiency at least as great as that of alpha-tocopherol. The other important role of certain carotenoids is as precursors of an antioxidant, vitamin A. Flavonoids are a large group of polyphenolic antioxidants found in many fruits, vegetables and beverages such as tea and wine. Over 4000 flavonoids have been identified and they are divided into several groups according to their chemical structure, including flavonols (quercetin and kaempherol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein). There is evidence that augmenting the intake of flavonoids might improve biochemical indices of oxidative damage and epidemiological studies suggest an inverse relation between flavonoid intake and incidence of chronic diseases [157]. Many flavonoids such as epigallocatechin-3-gallate, xanthohumol, casticin, astilbin, naringenin, apigenin, and baicalin have been shown to protect against ALI [158–164]. Resveratrol, a polyphenolic compound, reduced acute lung injury which was accompanied by activation of Sirtuin1 (Sirt1) and downregulation of NF-κB [165, 166]. α-Lipoic acid (ALA), a cofactor, is essential for energy production and the regulation of carbohydrate and protein metabolism. ALA is synthesized in vivo, however, when ALA is supplemented in the diet, it is readily absorbed and acts as a redox modulator and antioxidant [167]. ALA reduces oxidative stress and prevents against oleic acid-induced ALI [168]. ALA protects against LPS induced acute lung injury (ALI) through activation of heme oxygenase 1 (HO-1) and suppression of NF-κB-mediated inflammatory responses [169]. Qualitatively the most important aqueous phase

antioxidant is vitamin C (ascorbate). Ascorbate has been shown to scavenge superoxide, H_2O_2, hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals and singlet oxygen. Ascorbate undergoes a two electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate during its antioxidant action. Ascorbic acid administered intraperitoneally following lipopolysaccharide infusion attenuated proinflammatory and procoagulant states that induce lung vascular injury in mice model of sepsis [170]. Ascorbic acid (AA) also prevented the toxic effects of zinc oxide nanoparticles (ZnO NPs) inhalation induced acute pulmonary dysfunction including oxidative stress, inflammation, and injury [171].

4 ROS Damaging Effects on the Endothelial Barrier

The pulmonary endothelium, as a semipermeable interface, coordinates the influx and efflux of fluids, solutes, macromolecules and cells from the blood vessel lumen, over the interstitium to the alveolar lumen [172]. Consisting of a thin monolayer of endothelial cells which are connected by a vast amount of junctional proteins, the endothelium forms a tight barrier and lines the entire circulatory system. The pulmonary blood vessel system by itself disposed about a blood surface of 130 m^2. Moreover, by an alveolar-capillary barrier thickness of only slightly more than 0.5 µm, the capillary network forms a dense meshwork close to the alveolar lumen that guarantees an efficient gas exchange [173, 174]. For the exchange of substrates between the blood and alveolar lumen two pathways controlling endothelial barrier have been identified: The transcellular pathway that transports substrates through the body of the cells and the paracellular pathway that transfers material through the intercellular spaces between the cells.

Under normal physiological conditions, fluids and solutes with a molecular radius under 3 nm, utilize the paracellular system to enter the endothelial barrier by a hydrostatic pressure gradient between the intra- and perivascular space. Within the transcellular pathway, aquaporins are the main agents for the transmission of fluids. Macromolecules having molecular radius over 3 nm are actively transcellular transported over caveolae-mediated vesicular carriers [175, 176]. This system ensures that only a highly restricted volume of plasma protein and blood cells can enter into the pulmonary interstitium and thereby avoid alveolar flooding [177]. However, in pathological conditions such as ALI and ARDS, lung endothelial cells are activated or even damaged, which leads to a phenotypic shift with massive functional impairments of the vascular endothelium. Increased vascular permeability combined by an enhanced expression of adhesion molecules creates an excellent environment for trafficking of inflammatory cells and chemotactic substances through the blood barrier [178]. The following section describes the importance and role of ROS as key signaling molecules in the progression of ALI and ARDS by elevated vascular permeability and PMN migration.

4.1 Increased Vascular Permeability Triggered by ROS

To facilitate structure maintenance by adhesion and transmission of mutual information, endothelial cells continuously interact with the extracellular matrix, the basement membrane, and other surrounding cells via specialized protein complexes. The vascular endothelium does not have a rigid and inflexible architecture. Rather it possesses a dynamic structure defined by moment-to-moment changes in the cytoskeleton, cell–cell, cell–basal membrane, and cell–extracellular matrix interactions, by which the transcellular and paracellular transport of fluids and substrates between blood and alveolar lumen is ensured [175] (Fig. 4).

The transcellular transport mechanism is mediated by water channels and vesicle-dependent uptakes of substrates via endocytosis at the apical endothelial, transport across the interior cell body via transcytosis, and substrate release via exocytosis at the basolateral mem-

brane. Hereby, the endocytosis and exocytosis is carried out with individual vesicles shuttles, or interconnected vesiculo-vacuolar organelles (VVOs) that form channel-like structures [179, 180]. Both, caveolae-mediated vesicle transport options, open up the possibility to transit substantial fluid and substrate volumes of around 15–20% of the total cell volume within a very short time [181, 182]. During situations of high ROS levels such as ALI and ARDS, there are changes in the caveolae-based transport. It has been shown that the primary structural protein caveolin-1 required for caveolae formation is affected in a different manner by various ROS. While O_2- and H_2O_2 downregulate caveolin-1 expression, $OH^{\cdot}$ increases it [183]. Moreover, several studies have been demonstrated that the combination of different ROS generated by increased endogenous ROS production correlates with increased cellular transcytosis. Therefore, Angiotensin-II mediated ROS enhancement increases transcellular permeability. The dithiothreitol (DTT)-mediated attenuation of ROS levels leads to a decrease in transcytosis [184]. LPS caused expression of ROS through multiple mechanisms [74, 185, 186], induces mRNA and protein expression and thereby increase the vascular transcellular permeability [187, 188]. Increased caveolin-1 phosphorylation after LPS treatment has also been associated with increased transcellular permeability [188, 189]. Lungs from caveolin-$1^{-/-}$ mice showed a remarkable attenuation of transcellular permeability after LPS treatment [190]. Even thrombin stimulates the transport of fluorescently labeled albumin transcytosis across a confluent

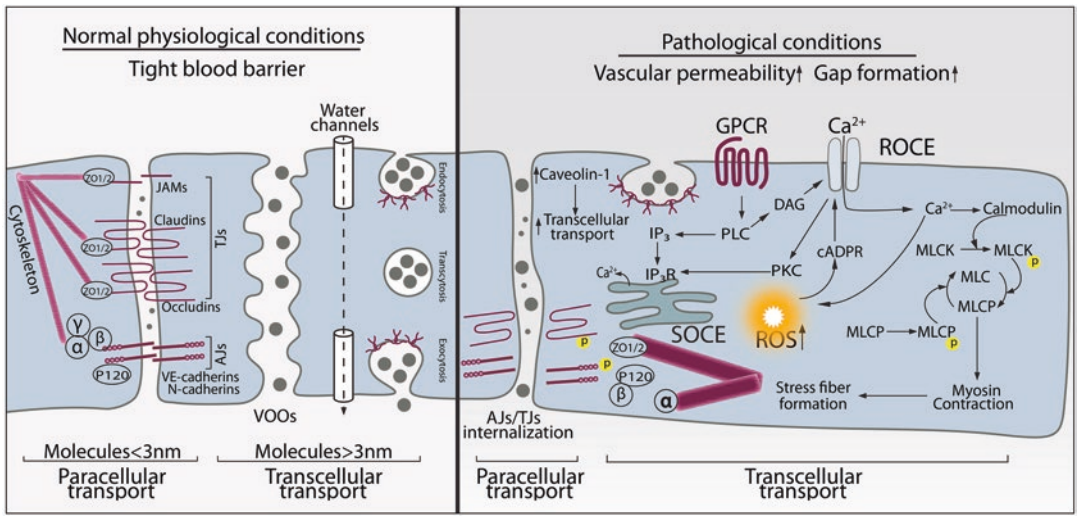

Fig. 4. Comparison of the transcellular and paracellular transport in physiological and pathological conditions. The transport of fluids, solutes, and macromolecules occur over transcellular and paracellular pathways. Under physiological conditions both transcellular and the paracellular transport are highly restricted, whereas under pathological conditions increased vascular permeability can be observed. ROS have several distinct impacts on the endothelial barrier. Initially, within the transcellular pathway caveolin-1 is affected by ROS leading to increased vascular permeability. ROS mainly influence the paracellular pathway through decreased expression and oligomerization of the junctional proteins as well as increases in the phosphorylation of junctional proteins on both serine and tyrosine residues. Both ROCE and SOCE are affected by ROS leading to increased endothelial Ca^{2+} influx. This increases calcium/calmodulin-dependent phosphorylation of myosin light chains leading to myosin contraction. Both ROCE and also SOCE are affected by ROS. *AJs* adherens junctions, *cADPR* cyclic adenosine triphosphate ribose, *DAG* diacylglycerol, *ER* endoplasmatic reticulum, *GPCR* G protein coupled receptor, *IP_3* inositol triphosphate, *JAMs* junctional adhesion molecules, *MLC* myosin light chain, *MLCK* myosin light chain kinase, *MLCP* myosin light chain phosphatase, *PLC* phospholipase C, *PKC* protein kinase C, *ROCE* receptor-operated calcium entry, *ROS* reactive oxygen species, *SOCE* store-operated calcium entry, *TJ* tight junctions, *TRPC/M* transient receptor potential canonical/melastatin, *VOOs* vesiculo-vacuolar organelles, *ZO* zonula occludens

human lung microvascular endothelial cells (HLMVECs) monolayer via enhanced caveolin-1 de novo synthesis [191]. However, caveolin-1 mediated increase of vascular permeability based on increase ROS production is a highly concentration- and time-dependent process [187, 188, 192].

Even through enhanced ROS levels affect the transcellular pathway, under pathophysiological conditions the paracellular transport mechanism is responsible for the predominant amount of blood fluid and proteins passage across the microvascular endothelium. In general, to seal endothelial cells to a tight monolayer together, two different types of intercellular junctions have been characterized as cell-cell adhesive barrier structures: adherens junctions (AJs) and tight junctions (TJs) [175]. Endothelial AJs are mainly composed of two different kinds of transmembrane proteins of the cadherin family, the vascular endothelial cadherin (VE-cadherin) and the neuronal cadherin (N-cadherin). The intercellular protein domains of the transmembrane proteins are connected to the cytoskeleton by α-, β-, γ-, and p120 catenins [193, 194]. The extracellular protein domains of the transmembrane proteins are attached to other extracellular protein domains of adjacent endothelial cells by Ca^{2+}-dependent homotypic interactions. The binding of Ca^{2+} to the negative charged amino acid residues localized on the extracellular cadherin domain causes a change in the protein conformation, which ensures the formation of a tight endothelial monolayer [195]. The structure of endothelial TJs is similar to AJs. However, compared to AJs, TJs consist of transmembrane proteins such as claudins, occludins, and junctional adhesion molecules (JAMs) [175], [196–198]. Claudins and occludins possess four transmembrane domains and two extracellular loops. The intercellular domains of the tight junctions are connected to the cytoskeleton by cytoplasmic adaptor proteins such as zonula occludens (ZO)1, -2 and -3, AF6, or cingullin combined with downstream situated α-catenins. As in AJs, the extracellular loop domains of occludin and claudins form homotypic bindings to extracellular domains of adjacent endothelial cells. JAMs belong to the immunoglobin (Ig) superfamily of proteins. Compared to claudins and occludins, JAMs are composed of only single-pass transmembrane proteins. Currently, three different isoforms of JAMs are known, JAM-A, JAM-B, and JAM-C. The expression pattern of the JAMs varies significantly in different kinds of tissue. JAM-A and JAM-C are predominantly expressed in endothelial cells [199]. The main differences of AJs and TJs exist in pore size or rather potential passing molecule size and appearance. TJs only represent 20% of all existing endothelial junctions and possess a mean pore size of approximately 1 nm. AJs are the most ubiquitous endothelial junctions and have a mean pore size of about 3 nm [175].

In the last decade, an increasing amount of studies described that an enhanced ROS production plays a critical role in initiating the junctional disassembly within ALI and ARDS development [200, 201]. The damage of AJs is mediated by a phosphorylation of serine and tyrosine residues localized in VE-cadherin, β-, and p120-catenin. Only VE-cadherin has five tyrosine residues (Y645, Y658, Y685, Y731, and Y733) and one serine residue (S665) that all can be phosphorylated [202–204]. Vascular endothelial growth factor (VEGF) treatment demonstrated an increase in vascular permeability in both, arteriolar, venular and capillary vessels [205]. Moreover, the treatment of HLMVECs with VEGF leads to an enhanced vascular permeability by VE-cadherin and β-catenin tyrosine phosphorylation. Using N-acetylcysteine as free radical scavenger identified increased ROS production as an essential factor of vascular barrier impairment [206]. Increased endothelial junctional disassembly by enhanced tyrosine phosphorylation of VE-cadherin and the associated proteins β-catenin, γ-catenin, and p120-catenin was also measured after the treatment with thrombin [207]. LPS-induced ROS production enhances endothelial barrier dysfunction via elevated protein tyrosine phosphatase oxidation and an associated decrease in their activity [208]. LPS can then promote the internalization of VE-cadherin from the plasma membrane to intracellular compartments [209]. An increase in TJs

disassembly due to enhanced ROS production has also been demonstrated in several studies. Oxidative stress decreases the expression of TJs significantly [210–213]. In addition to this, oxidative stress reduces the oligomerization of remaining TJs, which leads to an increased vascular permeabilization [214]. One indicator for oxidative stress is the ratio of GSH to GSSG. The oligomerization of occludin itself is regulated by this ratio. Under physiological conditions, GSH is about 30 to 100-fold higher compared to GSSG and similar amounts of occludin monomers and oligomers can be detected [215, 216]. During pathological conditions caused by oxidative stress a dramatically shift to higher GSSG levels and reduced occludin oligomer values occurs [210, 217]. TJs can also undergo serine and tyrosine residue phosphorylation leading to barrier dysfunction [218].

Thus, enhanced ROS production leads to increased vascular permeability by reinforced junctional protein disassembly. In addition to this, the stimulation of endothelial cells induces actin cytoskeleton shortening. By the motion of myosin along the actin filaments, pronounced thicker actin cytoskeleton with a huge amount of contractile actin bundles, called the stress fibers, are developed. Thus, an increasing number of inter-endothelial gaps are formed and expanded, leading to the pathologically altered vascular permeability [172, 219]. The dynamic interaction between AJs or TJs and the actin cytoskeleton is disrupted by myosin light chain (MLC) phosphorylation with accompanying cytoskeleton endothelial cell contraction [220]. The MLC kinase (MLCK) phosphorylates MLC in a Ca^{2+}/calmodulin mediated reaction. Small Rho-GTPases such as Rho, Rac, and Cdc42 are key regulators of the actin cytoskeleton by activation of MLCK and other cytoskeletal-remodeling agents [221]. While RhoA mainly regulates stress fiber formation [222], Rac and Cdc42 promote the formation of lamellipodia and filopodia [223, 224]. Together with the Rho kinase (ROCK), small Rho-GTPases can reinforce the effect of MLC by inhibiting of the MLC phosphatase (MLCP) [225]. Rho-GTPase activity is inhibited by guanine nucleotide exchange factors (GEFs), which replace guanine triphosphate (GTP) with guanine diphosphate (GDP). Through the exchange of GDP for GTP, GTPase-activating proteins (GAPs) induce activation [226]. The deletion or inhibition of MLCK210, the endothelial cell-specific MLCK isoform, protects mice from experimental pulmonary edema, inflammation, and death [227]. However, a basal level of MLCK is essential due to physiological vascular permeability. A pathological increase of MLCK leads to an abnormal enhancement of vascular permeability followed by blood barrier disruption. Based on enhanced ROS production, various inflammatory agents such as LPS [228], thrombin [229, 230], or VEGF [231] have been shown to enhance Ca^{2+} influx in the endothelium in two distinct pathways: store-operated calcium entry (SOCE) and receptor-operated calcium entry (ROCE). The initial increase via SOCE is due to a Ca^{2+} release from stores within the endoplasmatic reticulum (ER) by an inositol triphosphate (IP_3)-dependent reaction [232]. At the activated G protein coupled receptors (GPCR) bound phospholipase C (PLC) cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 binds to an IP_3 receptor on the ER, which releases Ca^{2+} from the ER in the cytosol. DAG, in turn, connects SOCE to ROCE. Within this second mechanism, DAG activates transient receptor potential canonical/melastatin channels (TRPC/TRPM), whereby Ca^{2+} enters into the endothelium and activates calmodulin. Finally, calmodulin triggers the RhoA-dependent myosin contraction via MLC phosphorylation [233]. Furthermore, PKC localized on TRPC/TRPM is activated by phosphorylation and promotes SOCE [234]. Increased cyclic adenosine triphosphate ribose (cADPR) expression caused by enhanced ROS production contributes to ROCE via the activation of TRPC/TRPM [235, 236]. The actin cytoskeleton is not the only cellular structure that contributes to vascular permeability. Microtubules, the filamentous biopolymers formed by the polymerization of α, β-tubulin dimers, also modulate vascular permeability [237–239]. The ROS-dependent disassembly of the microtubule network after LPS treatment has also been reported. The stabilization

of microtubules with epothilone B or the inhibition of the guanine nucleotide exchange factor (GEF)-H1 suppressed LPS-induced barrier disruptive effects in vitro and significantly improve vascular permeability in vivo [240]. Inhibition of microtubule destabilization with taxol leads to barrier improvement during LPS treatment in vivo [241]. Microtubule dynamics also depends on ROS levels, as enhanced dynamics are observed in a ROS-free environment, whereas increased dynamic instability occurs in the presence of ROS [242].

Thus, the overall consensus in the field is that ROS can reduce the tightness of the endothelial barrier by causing the disruption of endothelial cell junctions in combination with enhanced cytoskeleton contraction and microtubule destabilization.

4.2 Transendothelial Leukocyte Migration Affected by Increased ROS Formations

Endothelial cell adhesion followed by transendothelial migration of inflammatory cells play an important role in the pathology of ALI and ARDS. Mainly occurring in postcapillary venules, the process is mediated by six sequential steps: (1) tethering and rolling, (2) activation, (3) adhesion, (4) crawling, (5) transendothelial migration, and finally (6) diapedesis (Fig. 5). The migration of inflammatory cells across the endothelium happens through paracellular or more rarely through transcellular transport [243, 244] and is mediated by adhesive interactions between cell adhesion molecules (CAMs) expressed in both activated endothelial cells and migrating PMNs. Selectins, integrin, and members of the immunoglobulin (Ig) superfamily are the three families of adhesion molecules that are crucial for PMNs transmigration.

Selectins, responsible for the initial tethering and rolling of PMNs on endothelial cells, are membrane glycoproteins which are classified in three different subtypes: P-selectins, L-selectins and E-selectins. P-selectins, also known as granule membrane proteins-140, are expressed in platelets or endothelial cells and stored in Weibel-Palade bodies, and can be rapidly recruited to the cell surface during inflammation. L-selectins formed in activated PMNs, contribute to PMN rolling and are quickly shed from the cell surface, via a protease-dependent mechanism, upon activation. E-selectins are not expressed under baseline conditions. Only cytokine stimulation leads to increased expression of E-selectins in endothelial cells [245–248]. Integrins, are heterodimeric transmembrane receptors consisting of two noncovalently linked transmembrane glycoproteins, one α and one β subunit at a time. Within the mammalian genome, 18 α subunit and 8 β subunit genes have been identified that can assemble into 24 different integrin combinations characterized by distinct binding properties and tissue distributions [249, 250]. The most common integrins in PMNs are β_2-integrins. Integrins are activated by binding of endothelial secreted chemokines like CXCL1 and CXCL8 to PMNs existing chemokine receptors CXCR1 and CXCR2. As a result, integrins change their conformation from bent low-affinity to fully extended high-affinity state, bind to an Ig superfamily member and arrest the PMNs on the endothelial cell surface [251, 252]. Compared to the adhesion by selectins to integrins, the integrins-mediated adhesion is a strong and solid binding to the vascular endothelium and represents an essential factor for PMNs transendothelial migration [253]. All Ig superfamily members like intercellular cell adhesion ICAM-1 and -2, vascular cell adhesion molecule 1 (VCAM-1), JAMs (JAM-A, -B, and -C), platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31), CD99 antigen-like 2 (CD99L2), and endothelial cell-selective adhesion molecule (ESAM) possess a structural Ig domain and are expressed in endothelial cells, platelets, and PMNs [254–256].

In the last decade, mounting evidence has supported the idea that extravasation of PMNs in response to inflammatory stimuli is regulated by increased oxidative stress. Initially, increased ROS levels produced in the injured tissue can function as chemoattractant for immune cells [248, 257, 258]. Moreover, ROS transmit signals from activated cell surface receptors and act

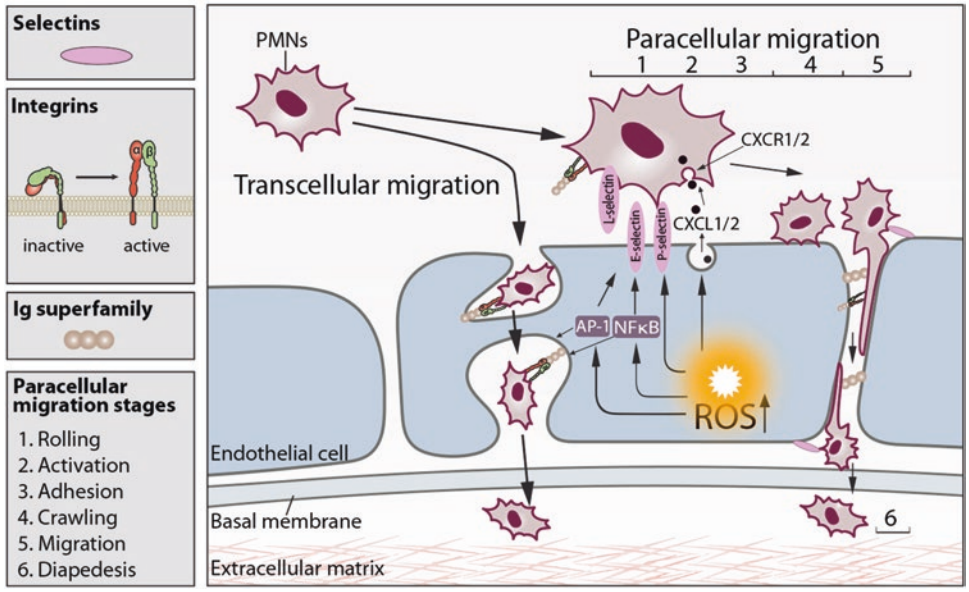

Fig. 5. ROS-mediated endothelial polymorphonuclear leukocyte migration. Over both transcellular and paracellular pathways, polymorphonuclear leukocytes (PMNs) pass through the endothelium to migrate from the blood lumen into the alveolar lumen. In both pathways selectin, integrins, and immunoglobulins (Ig) help facilitate this cellular migration. The transcellular migration of PMNs through the cell body is a rare event. More commonly paracellular migration occurs. This requires a number of migration steps: (1) tethering and rolling; (2) activation; (3) adhesion; (4) crawling; (5) transendothelial migration, and (6) diapedesis. Within each migration steps, varying cell adhesion molecules (CAMs) are needed. Selectins modulate the initially tethering and rolling of PMNs on the inner surface of the blood vessel, whereby PMNs start to slow down. Based on the rolling, endothelial cells are activated to release chemokines (CXCL1 or CXCL8) that transmit and bind to chemokine receptors (CXCLR1 and CXCLR8) localized on the surface of the PMNs. This causes PMN localized integrins to change from a low-affinity state (inactive bent conformation) to a high-affinity state (active fully extended conformation) forming a firm adhesion to CAMs of the Ig superfamily. The ultimate entry of the PMNs into the blood barrier occurs over these established CAMs formations. Increased ROS leads to enhanced invasion of immune cells into the injured tissue. ROS also regulate the expression CAMs both directly and through transcription factors (NF-κB, AP-1) that exert major influences on PMN migration. *NF-κB* nuclear factor-kappa-B, *PMNs* polymorphonuclear leukocytes, *ROS* reactive oxygen species

intrinsically within migrating cells and the surrounding tissue to promote migration. Thereby, increased ROS production can regulate the expression of endothelial CAMs by a direct transcription-independent activation or by a transcription-dependent mechanism via redox-sensitive transcription factors like nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [259]. For instance, the exposure of endothelial cells to inflammatory stimuli like H_2O_2 [260], LPS or cytokines [254, 261] leads to a reinforced PMNs migration by increased endothelial CAMs expression. After 1 h treatment with H_2O_2 or t-butylhydroperoxide, in HUVECs there is an increase in transcription-independent surface expression of P-selectin followed by PMNs adhesion. Prior treatment of the cells with anti-P-selectin antibody or an antioxidant decreases the PMNs adhesion significantly [262]. Moreover, the exposure of human umbilical vein endothelial cells (HUVECs) to 60 min anoxia followed by 10 h reoxygenation causes a biphasic early transcription-independent and late transcription-dependent neutrophil adhesion response correlated to endothelial ROS production [263]. In contrast to the ROS-mediated expression of P-selectin, the expression of ICAM-1, VCAM-1, and E-selectin is only regulated on transcriptional level [264]. NF-κB as one of the transcription factors is involved in the expression of many inflammatory and immune response CAMs like E-selectin [265, 266], ICAM-1 [266], and VCAM-1 [267]. The

binding of inducible redox sensitive transcription factors in the promoter regions of the CAMs leads to their increased expression and activation [268, 269]. H_2O_2 increases the ICAM-1 expression while the pretreatment with an antioxidant or the application of an ICAM-1 antibody abolished the H_2O_2-induced PMNs adhesion [260]. VCAM-1 expression can also be induced by NOX associated ROS generation that promotes lymphocyte migration across the endothelial cell layer. The inhibition of NOX with diphenyleneiodonium or apocynin blocked the migration [270]. Rats exposed to hypoxia have increased ROS production and enhanced NF-κB and CAMs expression and the transvascular leakage is abrogated by nifedipine treatment due to reductions in oxidative stress and NF-κB [271].

5 Potential Therapies

5.1 N-Acetylcysteine (NAC)

NAC is a precursor for GSH, an antioxidant present in high levels in the normal lung. Lavage from patients with ALI/ARDS is deficient in GSH, and GSH levels are also below average in some pulmonary fibrotic disorders. Increased intracellular levels of GSH reduce production of pro-inflammatory cytokines like TNFα and IL-1. In addition to promoting GSH production, NAC also has direct antioxidant properties because of its thiol group, and it can scavenge reactive oxidants including hydrogen peroxide, superoxide anion, and hypochlorus acid. Animal studies indicate that NAC has significant protective effects against acute pulmonary injury from hyperoxia, endotoxin, or GSH synthesis inhibition [272–274]. The antioxidant effects of liposomally entrapped NAC is more effective as evaluated in rodents challenged with LPS [273]. In one study the benefits of NAC treatment in the management of ARDS were assessed by measuring patient's intracellular glutathione and plasma antioxidant biomarkers and outcome [275]. Treatment by NAC apart from increasing intracellular glutathione also increased extracellular total antioxidant power, total thiol status, and the outcome of the patients. The authors suggested that patients with ARDS could potentially benefit from NAC supplementation. In a randomized, double-blind, placebo-controlled, prospective clinical trial the levels of glutathione and cysteine in patients with ARDS were determined and the effect of NAC treatment examined [276]. The study concluded that repletion of glutathione may safely be accomplished with NAC in patients with ALI/ARDS which may shorten the duration of lung injury. It has also been shown that NAC protects against H9N2 virus-induced acute lung injury [277]. Repetitive post-treatment of NAC in LPS-exposed attenuates the extent of ALI through the inhibition of NF-κB activation [278]. NAC also improves respiratory function, but not survival, in adults with ALI/ARDS. A double-blind, placebo-controlled study in 48 patients at five centers found that treatment with NAC increased cardiac index and decreased the number of days of ALI without improving mortality [276]. No adverse side effects have been reported from the use of NAC in patients with ALI/ARDS. Given the potential benefits of NAC supplementation studies are needed to investigate the utility of NAC in combination therapies for ALI/ARDS.

5.2 Vasodilator Gases

NO is an important endogenous gaseous mediator in several physiological processes in vivo. One of its most important action is potent vasodilation, which results from decreased calcium in vascular smooth muscle cell cytoplasm following an NO-dependent increase in cyclic-GMP [279]. Inhaled NO affects gas exchange by increasing blood flow in ventilated areas. Administration of NO by inhalation has been shown to acutely improve hypoxemia associated with pulmonary hypertension in humans and animals [280–282]. Inhaled NO results in a transient improvement in oxygenation without any effect on mortality in both adults and children with ARDS [283]. Hydrogen sulfide (H_2S), another signaling gas, is produced by the catalytic conversion of L-cysteine by two enzymes: CBS (cystathionine β-synthase)

and CSE (cystathionine γ-lyase). H_2S reduces inflammation and protects tissues from injury especially in the gastrointestinal tract [284, 285]. Hydrogen sulfide pretreatment aslo exerts protective effects on both hyperoxia and LPS induced ALI [286, 287]. Recent findings suggest that carbon monoxide (CO) can also act as an endogenous defensive gaseous molecule to reduce inflammation and organ injury [288–290]. The endogenous production of CO occurs through the activity of bot the constitutive heme oxygenase 2 (HO-2) and inducible heme oxygenase 1 (HO-1). The therapeutic potential of CO has been shown in models of acid-induced lung injury, HTMV, endotoxin challenge, and cecal ligation and puncture induced-sepsis [291]. The efficacy of NO, H_2S, and CO in humans with ALI/ARDS remains unclear and awaits further controlled clinical studies.

5.3 NADPH Oxidase (NOX) Inhibitors

NOX is the primary generator of $O_2^{-\cdot}$ and is responsible for the initiation of the ROS generation cascade. NOX is a unique target and inhibiting NOX would reduce $O_2^{-\cdot}$ production which would result in less available $O_2^{-\cdot}$ for the generation of H_2O_2 and $ONOO^{-\cdot}$, subsequently reducing OH generation and increasing NO bioavailability as a result. NOX is an important contributor of oxidant production and is an upstream actor in oxidative stress-induced acute lung injury involving JNK and ERK pathways [135, 292]. There are several NOX inhibitors currently being studied, however the most common is apocynin, which is a NOX inhibitor that preferentially blocks NOX-2 at low doses. Apocynin acts by preventing the assembly of the NOX enzyme subunits. The administration of apocynin, a NOX inhibitor, reduces lipid peroxidation, suppresses the NF-κB pathway, attenuates lung injury, and improves survival in rat hemorrhagic shock and LPS models [293, 294]. Similarly, the inhibition of NOX-2 activity ameliorates influenza A virus-induced lung inflammation, indicating that pharmacologically targeting NOX-2 may have therapeutic potential in ALI [295]. The NOX inhibitor diphenyleneiodonium chloride, attenuates oleic acid-induced lung injury [296].

5.4 Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) Activators

Nrf2 presents a potential target for reducing oxidative stress in ALI/ARDS. Nrf2 is found in the cytoplasm of many mammalian cells and is responsible for the regulation of various antioxidants and cytoprotective genes, acting as a "master switch" for these genes. In response to oxidative stress, Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) of target genes, along with other binding factors and cofactors, resulting in the induction of stress response genes. Nrf-2 activators such as andrographolide sulfonate [297, 298] and CDDO-Me [299, 300] have been shown to attenuate acute lung in lung in animal models of ALI.

5.5 Activated Protein C

Protein C is a vitamin K-dependent plasma protein zymogen. Activated protein C (APC) inactivates factors (F) Va and VIIIa and downregulates thrombin generation. The cytoprotective effects of APC involve gene expression profile alterations, anti-inflammatory and anti-apoptotic activity, and endothelial barrier stabilization [301]. However, its utility in ALI/ARDS is still controversial. The infusion of recombinant human activated protein C (rh-APC) in patients with ARDS showed attenuation of pulmonary coagulopathy and injury without any side effects [302]. However, in another randomized, saline-controlled, single-blinded clinical trial with rh-APC did not find any improvement in increased alveolocapillary permeability or the clinical course of ARDS patients [303]. Thus, further studies are required to confirm the role of rh-APC as therapeutic candidate for ALI/ARDS.

6 Conclusion

Studies over the last decade have clearly demonstrated that ROS can increase the permeability of the pulmonary endothelial bed and that this oxidative stress plays a major role in the pathogenesis of ALI and ARDS. The source of the ROS is complex and a number of pathways are involved. However, it is hoped that tackling the overproduction of ROS, and the decreased defense capacity, during ALI/ARDS may open up a new field of therapeutic approaches for a disease that has not seen significant advances, despite 50 years of investigations.

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Redox-Dependent Calpain Signaling in Airway and Pulmonary Vascular Remodeling in COPD

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1 Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive and life-threatening lung disease affecting more than 20 million individuals in the USA and predicted to become the third leading cause of death worldwide by 2030. COPD is characterized by irreversible airway obstruction mainly attributable to the pulmonary vascular and airway remodeling which is the most critical process in the pathogenesis of COPD. The primary cause of COPD is inhaling air pollutants including cigarette smoke (CS) and secondhand smoke. Large amount of reactive oxygen and nitrogen species (RONS) from CS or released from lung cells can contribute to the development of airway and pulmonary vascular remodeling. Calpains are a conserved family of calcium-regulated, non-lysosomal, neutral cysteine endopeptidases which are involved in several physiological and pathological processes. Growing body of evidence indicates that CS and its constituent are able to regulate directly or indirectly the protein levels and activities of calpain indicating its implication in the pathological processes in COPD. This chapter focuses on the RONS production systems in the airway and pulmonary vasculature and the potential effect of RONS on calpain signaling in the pathogenesis of COPD.

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2 Calpain Signaling

Calpain is a family of neutral, calcium-dependent, non-lysosomal cysteine proteases which exist in a wide range of organisms such as mammals, invertebrates, plants, fungi, yeasts, and bacteria [1]. They act as regulatory enzymes and perform precise and limited cleavage of substrate proteins irreversibly changing their functions [2]. The human genome contains 15 genes that encode the catalytic subunits of calpain, and two genes encoding the regulatory subunits [3]. Some of the

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calpains show ubiquitous expression (calpain-1, 2, 5, 7, 10, 13, 14, 15, and 16), while others are restricted to specific tissues (calpain 3, 6, 8, 9, 11, and 12) [4]. Classical or typical calpains contain penta-EF-hand motif in the calcium-binding domain (IV) in addition to the N-terminal domain (I), protease domain (II), and C2-like domain (III) in the catalytic subunit. Nonclassical or atypical calpains include the domain I and II, but not all comprise the penta-EF-hand domain or C2-like domain and some of them have other types of domains [5]. Calpain-1 and calpain-2 are the two major typical and well-characterized members of the calpain family which differ in their sensitivity to calcium in vitro. Calpain-1 (μ-calpain) requires micromolar level of Ca^{2+}, while calpain-2 (m-calpain) requires millimolar Ca^{2+} concentration for half-maximal activation in vitro [6]. These ubiquitously expressed isoforms are heterodimers composed of a different larger catalytic subunit (80 kDa) including the N-terminal anchoring domain (DI), the catalytic domain (DII), the Ca^{2+}- and phospholipid binding domain (DIII), and the penta–EF-hand domain (DIV) as well as the smaller regulatory subunit (30 kDa) comprising of the N-terminal hydrophobic domain (DV) and the penta-EF-hand domain (DVI) [7]. Calpain is abundant in the cytoplasm therefore tight regulation of the protease is essential for normal cell function. The activity of calpain is modified by several mechanisms: (a) limited autolysis of the N-terminus of both subunits [8]; (b) binding to specific membrane phospholipids [9]; (c) posttranslational modifications such as phosphorylation of the calpain-2 at Ser50 by extracellular signal-regulated kinases 1/2 (ERK 1/2) which increases calcium sensitivity and the catalytic activity [10] or phosphorylation of the Ser369/Thr370 residues of calpain-2 by protein kinase A (PKA) inhibiting the calpain activity [11]; (d) interaction with the endogenous inhibitor calpastatin [12]; and (e) binding to activator proteins [13]. Calpain activity has been shown to be critical for wide range of basic cellular processes including cell motility [14], cell proliferation [15], cell cycle [16], signal transduction [17] and apoptosis [18]. Several pathological conditions are attributed to the overactivation of calpain including Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke, traumatic brain injury, cataract formation, muscular dystrophies, myocardial infarcts, ischemia, rheumatoid arthritis, cancer, and pulmonary hypertension. Genetic alterations in the calpain genes have been also shown to be associated with human diseases. For example, defects in calpain-3 inactivation cause limb girdle muscular dystrophy type 2A; downregulation of calpain-9 is linked to human gastric cancer; and mutations of the calpain-10 gene contribute to the development of type II or non-insulin-dependent diabetes mellitus [19–21].

3 RONS in the Airway and Pulmonary Vasculature

RONS can be produced by many types of cells including inflammatory, endothelial, adventitial, and smooth muscle cells in the airway and lung vasculature. Under normal conditions, generation of these species is well controlled by enzymatic and nonenzymatic reactions. RONS at physiological level contribute to a number of physiological processes such as cell proliferation, differentiation, migration, apoptosis, cytoskeletal movement, and vascular contraction and relaxation [22, 23]. However, overproduction of RONS due to disturbed balance between oxidants and antioxidants leads to oxidative and nitrosative stress causing pathological responses [24–28].

3.1 Production of Reactive Oxygen Species (ROS)

ROS are relatively unstable small molecules formed as by-products in reactions involving oxygen. They consist of free oxygen radicals and non-radicals. Free radicals contain one or more unpaired electrons in atomic or molecular orbitals such as superoxide ($O_2^{\cdot -}$), hydroxyl (OH·), peroxyl (RO_2·), lipid peroxyl (LOO·), and alkoxyl (RO·). Non-radicals are created from free radi-

cals by sharing their unpaired electrons such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and lipid peroxide (LOOH) [29]. At low to moderate concentrations, ROS have beneficial effects and play important role in normal airway and vascular physiology as well as maintain vascular integrity. However, at high concentrations they can damage lipids, proteins, carbohydrates, and nucleic acids contributing to the pathogenesis of lung diseases [30–32]. ROS can also act as a second messenger in the signal transduction pathways [33, 34]. There are three major ROS including superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) in the airway and pulmonary vasculature. The initial reaction in the ROS production is usually one electron transfer to molecular oxygen to create superoxide anion ($O_2^{\cdot-}$). Superoxide is water soluble, is highly unstable, and has a short half-life. Superoxide can be dismutated into more stable and lipid soluble hydrogen peroxide (H_2O_2) via a spontaneous reaction or catalyzed by superoxide dismutase (SOD). Superoxide can react with nitric oxide to form extremely reactive peroxynitrite ($ONOO^-$) Superoxide can also react with hydrogen peroxide in iron ion catalyzed process via Haber–Weiss reaction to generate hydroxyl radicals (OH·) [35]. Biological sources of ROS include the mitochondrial electron transport chain and several enzymatic system such as NADPH oxidases, xanthine oxidoreductase (XOR), and nitric oxide synthase (NOS).

3.2 ROS Production from Mitochondria

Mitochondria represent a major source for ROS production in the cardiovascular system. During oxidative phosphorylation, the mitochondrial respiratory chain governs the electron transfer to the molecular oxygen which is reduced to water at the terminal cytochrome oxidase (Complex IV). Superoxide can also be generated as a by-product at the proximal sites. Under physiological conditions, approximately 1–3% of the electrons can leak from the electron transport chain (ETC) yielding ROS. The production of superoxide occurs at complex I, II, and III. The complexes I and III (NADH:ubiquinone oxidoreductase and coenzyme Q: cytochrome c oxidoreductase, respectively) are considered as the major sites of ROS production in the mitochondria [36–38]. Superoxide is converted into more stable hydrogen peroxide by manganese superoxide dismutase (Mn-SOD) and then further converted to water by catalase or thiol peroxidases (e.g., glutathione and thioredoxin peroxidases) in the mitochondrial matrix. Superoxide can also be dismutated by copper/zinc superoxide dismutase (Cu,Zn-SOD) or scavenged by cytochrome c in the mitochondrial intermembrane space, or released from the mitochondria into cytosol through voltage-dependent anion channels (VDAC) [39, 40]. Mitochondria are also a source of reactive nitrogen species such as nitric oxide (NO·) which is produced by nitric oxide synthases (NOS) during the conversion of L-arginine to citrulline. Nitric oxide can block the respiration by binding to the heme groups of cytochromes contributing to elevated superoxide formation. Superoxide and nitric oxide can form peroxynitrite. These RONS cause oxidative and nitrosative damage of numerous mitochondrial structures such as proteins, DNA, and lipids, leading to the development of pathological processes [41–43]. In patients with COPD excessive mitochondrial RONS levels are also associated with mitochondrial dysfunction, contributing to the pathophysiology of COPD [44]. Mitochondria also produce hydroxyl radicals (OH·) which are one of the strongest oxidants and can easily interact with unsaturated lipids causing lipid peroxidation. Lipid peroxidation generates hydroperoxides (LOOH) as primary products and reactive carbonyl species, e.g., malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) as end products of the reaction. They induce irreversible modification of cellular components, causing lipoxidative damage to mitochondrial protein, phospholipids and DNA which is associated with a variety of pathophysiological states [45–47].

3.3 ROS from NADPH Oxidases (NOX)

The NADPH oxidases are membrane-associated proteins that catalyze the superoxide production via electron transfer from NADPH to molecular oxygen [48]. The NADPH oxidase family has seven members: five mammalian NOX isoforms such as Nox1, Nox2 (gp91phox), Nox3, Nox4, and Nox5 and two related enzymes dual oxidase 1 and 2 (DUOX1 and 2). The family members have a common core structure consisting of six transmembrane domains at the N-terminal part including two hemes, and a relatively long C-terminal cytosolic part containing FAD and NADPH-binding domains [49, 50]. Nox5 and Duox 1/2 have additional domains at their N-terminal region. For example, Nox5 contains four Ca^{2+}-binding EF-hand domains; and DUOX enzymes have two Ca^{2+}-binding EF-hand domains, an additional membrane-spanning domain, and a peroxidase-like domain. Due to the EF-hand domains these oxidases are regulated by Ca^{2+} signaling [51]. The major NADPH oxidase isoforms are DUOX1 and 2 in the lower and upper airways and Nox1, Nox2, Nox4, and Nox5 in human vascular cells. The tissue expression, subcellular localization, regulatory mechanisms, activation and physiological function of these enzymes are fairly different [52–55]. Nox2, also known as the phagocyte NADPH oxidase, is the prototype Nox. The enzyme complex consists of the plasma membrane spanning flavocytochrome b558 heterodimer that is composed of the gp91phox and p22phox proteins, and cytosolic protein subunits such as p67phox, p47phox, p40phox, and the small GTP-binding protein Rac. During stimulation, p47phox becomes phosphorylated and translocates to the membrane-bound cytochrome b558 with the activator subunit p67phox and p40phox. Rac also translocates to the membrane independently of p47phox and p67phox. Association of the cytosolic subunits with the transmembrane heterodimer leads to the activation of the enzyme. The assembled and activated NADPH oxidase catalyzes the electron transfer from NADPH via FAD and two hemes to molecular oxygen resulting in the formation of $O_2{\cdot}^-$ [56–58]. Nox1 requires two cytosolic subunits such as the p47phox homolog, NADPH oxidase organizer subunit 1 (NOXO1) and the p67phox homolog, NADPH oxidase activator subunit 1 (NOXA1) for its enzyme activity [59, 60]. In contrast, Nox4 is different from Nox1 and Nox2, and seems to be constitutively active enzyme, and its activation does not depend on the cytosolic subunits p47phox and p67phox or their homologs. ROS production of Nox4 requires association with membrane subunit p22phox and generation of H_2O_2 rather than superoxide [61–63]. Nox5 is directly activated by calcium binding to the EF-hand motifs at the N-terminus and by subsequent conformational changes. The enzymatic activity of Nox5 does not require p22phox or the cytosolic subunits. Binding of calcium-bound calmodulin or phosphorylation increases sensitivity to calcium [64–66]. DOUX enzymes contain two EF-hand motifs between the NADPH oxidase and peroxidase homology domains indicating the role of calcium in the regulation of the enzyme activities. Interestingly, the NADPH oxidase domain generated superoxide is quickly converted to H_2O_2 by the peroxidase-like domain, therefore the H_2O_2 production theoretically occurs without formation of detectable amounts of superoxide [67–69]. The NADPH oxidases play a critical role in the physiological responses of the airway and vascular cells such as host defense, mucin expression, maintenance of barrier function as well as cell growth, proliferation, migration, differentiation and apoptosis. They are also implicated in several airway and vascular pathologies including pulmonary diseases such as COPD, pulmonary hypertension, asthma as well as atherosclerosis, inflammation, hypertension, restenosis, diabetes, and metabolic syndrome [48, 54, 55, 70].

3.4 ROS from Xanthine Oxidoreductase (XOR)

Xanthine oxidoreductase is a molybdenum-containing flavoprotein and a potential source of ROS in the airway and pulmonary vasculature. XOR is a homodimer with a molecular mass of approximately 300 kDa, and each subunit of the enzyme comprises of four redox centers: two nonidentical [2Fe–2S] centers at the N-terminus, a central flavin adenine dinucleotide (FAD) cofactor, and one molybdopterin cofactor (Mo-pt) at the C-terminus [71]. XOR has two isoforms: xanthine oxidase (XO) and xanthine dehydrogenase (XDH) which are encoded by the same gene. The enzyme primarily exists as a dehydrogenase which can be converted into an oxidase by reversible oxidation of cysteine residues or by irreversible proteolysis. XOR governs the final two steps in purine degradation. It also catalyzes the oxidation of hypoxanthine and xanthine to uric acid and transfers electrons from xanthine to NAD+ and molecular oxygen to produce NADH, hydrogen peroxide, and superoxide. XDH mostly contributes to the generation of NADH, whereas XO is more likely responsible for the production of ROS. For this reason, the absolute amount of XO and the ratio of XO to XDH is very important to determine the ROS generation in the vasculature [71–76]. XOR can also act as a nitrate/nitrite reductase by catalyzing the reduction of nitrate to nitrite and nitrite to nitric oxide (NO·) via electron transfer supplied by either xanthine or NADH under hypoxic and inflammatory conditions [77–81]. Superoxide can rapidly interact with nitric oxide to form the powerful and cytotoxic non-radical oxidant species peroxynitrite ($ONOO^-$) [82, 83]. XOR is mainly expressed in the endothelium, and its expression and activity are significantly increased in various pathological conditions such as ischemia–reperfusion injury, endothelial dysfunction, metabolic syndrome, airway inflammation, and vascular remodeling in PAH and COPD [84–91].

3.5 Production of Reactive Nitrogen Species (RNS)

RNS are nitrogen-centered molecules including free radical nitric oxide (NO·) and nitric dioxide (NO_2·), as well as several non-radicals, such as peroxynitrite ($ONOO^-$), its protonated form peroxynitrous acid (ONOOH) and alkylperoxynitrite (ROONO). Nitric oxide is a small, uncharged molecule which has a short half-life and contains one unpaired electron. Production of nitric oxide is catalyzed by the nitric oxide synthases (NOSs) which convert the L-arginine to L-citrulline and nitric oxide [92, 93]. It serves as a second messenger in various physiological processes including vasodilation, neurotransmission, apoptosis and immune response [94]. Nitric oxide is relatively unreactive, but it has the ability to react with other radical species to generate more reactive derivatives. For example, diffusion-limited reaction between nitric oxide and superoxide radical produces the toxic peroxynitrite [95]. Peroxynitrite is a strong oxidant which can react with a wide range of biological substrates causing oxidation of DNA, lipids, and proteins and nitration of tyrosine residues of the proteins [96]. It can also react with CO_2 leading to the formation of a reactive intermediate nitrosoperoxocarbonate ($ONOOCO_2^-$), the decomposition of which provides additional two radicals, nitrogen dioxide and carbonate radical anion (CO_3·). The protonation of peroxynitrite generates peroxynitrous acid (ONOOH) that homolyzes into nitrogen dioxide and hydroxyl radical (OH·). It can also undergo nitration on free or protein bound tyrosine to form 3-nitrotyrosine which is a detectable in vivo marker for the RNS formation. Nitric oxide can rapidly react with O_2 to form the very reactive oxidant nitric dioxide (NO_2·). Peroxidases can also produce nitric dioxide as an intermediate product by catalyzing the nitrite oxidation which is also able to induce nitration on protein tyrosine residues [96–99]. Overproduction of RNS, especially high levels of peroxynitrite and its derivatives contribute to nitrosative stress which is

associated with several pathological conditions including cancer, neurodegenerative disorders, chronic inflammatory diseases, circulatory shock, diabetes, vascular diseases and chronic diseases of the airways such as asthma and COPD [100–103].

3.6 Nitric Oxide Synthase (NOS)

Nitric oxide synthases are a family of enzymes which catalyze the formation of nitric oxide (NO·) and L-citrulline from L-arginine via five electron oxidative reactions. Cofactors such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), iron protoporphyrin IX (heme) and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) are essential for the proper enzyme function. There are three different isoforms of the family: neuronal NO synthase (nNOS or NOS1), inducible NO synthase (iNOS or NOS2) and endothelial NO synthase (eNOS or NOS3). All NOS isoforms are expressed in the airways and vasculature. nNOS and eNOS are calcium-dependent and are constitutively expressed in neurons and endothelial cells, respectively. iNOS is independent of calcium concentration and is inducible by endotoxin or inflammatory cytokines in various cell types [104–106]. Active NOS is a homodimeric enzyme complex in which each monomer consist of an oxygenase domain in the N-terminus and a reductase domain in the C-terminus that are connected via a regulatory calmodulin-binding domain. The homodimer is structurally stabilized by the zinc tetrathiolate (ZnS_4) cluster which is formed by a zinc ion and two cysteines from each monomer. The oxygenase domain contains binding sites for heme, cofactor BH_4 and substrate L-arginine as well as forms the active site of the enzyme. The reductase domain binds the two flavinic cofactors FAD, FMN and cosubstrate NADPH [92, 107, 108]. During NO synthesis, electron transfer occurs from NADPH via FAD and FMN to the heme which catalyses the substrate reaction. This electron flow is triggered by calcium-induced calmodulin binding in case of nNOS and eNOS. In contrast, iNOS binds the calmodulin at low calcium level, therefore it does not depend on calcium [109]. At low concentrations, NO diffuses into adjacent cells and stimulates the soluble guanylate cyclase (sGC) which converts guanosine triphosphate (GTP) to second messenger cyclic guanosine monophosphate (cGMP). The increased level of cGMP can modify the activity of numerous target proteins such as protein kinases and ion channels. NO produced by eNOS play role in the regulation of vascular tone and platelet aggregation. NO generated by nNOS is a very important neurotransmitter in the central nervous system and contribute to the regulation of synaptic transmission. Higher concentrations of NO is produced by iNOS in response to endotoxin or inflammatory stimuli has cytotoxic effect and it is involved in the host defense [110–112]. Under some circumstances, the absence or limited availability of the substrate L-arginine or the critical cofactor BH_4, the enzymes become in a dysfunctional state termed NOS uncoupling. In these conditions, electrons transferring from the NADPH to the molecular oxygen produce superoxide rather than L-arginine to generate nitric oxide, leading to pathogenic processes [95, 113–115].

3.7 Antioxidant Defenses

Antioxidant can be defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [116]. Under physiological conditions, RONS are generated as a normal product of aerobic metabolism and play important role in wide range of cellular processes. The levels of RONS are tightly controlled by the regulation of the RONS-generating enzymes and by antioxidant defenses. In pathophysiological circumstances, excessive production and accumulation of RONS and/or decreased ability of antioxidant defenses create an imbalance in the RONS homeostasis. Elevated RONS bioavailability causes irreversible changes of biological molecules including proteins, lipids and DNA, inhibiting their normal function as well as leading to oxidative and nitro-

sative stress [117–119]. These stress conditions contribute to the development of a number of diseases, including cancer, ischemia–perfusion, diabetes, neurological disorders, atherosclerosis, hypertension, asthma, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, COPD, as well as ageing [31]. Therefore, the main purpose of the antioxidants is to counterbalance the production of RONS. They can be classified into two groups: enzymatic and nonenzymatic. The major enzymatic antioxidants in pulmonary vasculature include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). There are three forms of SOD: Cu/ZnSOD (SOD1) in the cytoplasm, MnSOD (SOD2) in the mitochondria, and the extracellular SOD in the extracellular space (EC-SOD or SOD3). SODs catalyze the conversion of superoxide into hydrogen peroxide and oxygen. Catalase decomposes hydrogen peroxide to water and oxygen. Glutathione peroxidases catalyze the reduction of lipid hydroperoxides to alcohols and the decomposition of hydrogen peroxide to water and oxygen. In addition, heme oxygenases and reductases including thioredoxins (TRXs), peroxiredoxins (PRXs), and glutaredoxins (GRXs) are also involved in the enzymatic antioxidant defense. Nonenzymatic antioxidants are small molecular-weight substances including ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione (GSH), uric acid, carotenoids (beta-carotene), flavonoids, and trace metals such as selenium which serve as direct scavengers of ROS [120–122].

4 Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory disease of the respiratory system which is a major cause of morbidity and mortality throughout the world and represents the third leading cause of death in the USA [123]. It is characterized by airflow limitation which is associated with an abnormal inflammatory response of the lungs to noxious particles or gases [124]. Spirometry is used for diagnosis and evaluation of COPD. Post-bronchodilator forced expiratory volume in 1 s (FEV_1) is less than 80% of predicted as well as the ratio of FEV_1 to forced vital capacity (FVC) (FEV_1/FVC ratio) less than 0.7 confirm the presence of airflow obstruction [125]. In contrast to asthma, the airflow limitation in COPD is largely irreversible [126]. Pathological changes of COPD include chronic bronchitis, emphysema, as well as inflammation and remodeling of small/large airways and vasculature. Several processes are implicated in the development of COPD including oxidative stress (disturbed balance between oxidants and antioxidants), inflammation, degradation of extracellular matrix (imbalance between proteases and antiproteases), defects in tissue repair mechanisms and apoptosis of lung cells [127, 128]. The major risk factor for the development of COPD is the exposure to cigarette smoke (CS) which contains more than 4700 chemical components including about 10^{15} reactive species per puff in the gas phase. These are short-lived oxidants such as superoxide, nitric oxide and peroxynitrite. The tar phase of the CS contains several relatively stable free radicals such as quinone/hydroquinone (Q/QH2) complex, hydrogen peroxide and hydroxyl ions. The tar has more than 10^{18} free radicals per gram [129, 130]. In addition to CS, increased burden of oxidants originated from exogenous sources such as environmental air pollutants and from endogenous sources such as RONS generated by inflammatory cells also contribute to the pathogenesis of COPD. Numerous inflammatory cells including neutrophils, macrophages, T-lymphocytes, and eosinophils are involved in the chronic inflammatory processes of airways which are important features of COPD. Increased number of inflammatory cells in COPD produce and release several inflammatory mediators and tissue-degrading enzymes which play critical role in the lung tissue destruction, bronchoconstriction, airway remodeling, and excess mucus production [126, 131]. Other risk factors related to COPD are environmental tobacco exposure, age, body mass index, education level, history of tuberculosis, hospitalization for respiratory problems before the age of 10 years, family history of COPD and

years worked in dusty jobs [132]. Treatments for COPD such as short-acting/long-acting bronchodilators, long-acting anticholinergic agents and corticosteroids are slightly able to improve symptoms, quality of life, and exacerbation rates, but not the mortality rate [133]. However, long-term oxygen therapy and pulmonary rehabilitation may improve mortality and morbidity [131].

4.1 Remodeling in COPD

One of the main pathological feature of COPD is the remodeling and thickening of airway walls which can contribute to the airway lumen narrowing and airflow obstruction. Structural changes of the airways comprise increased airway smooth muscle (ASM) mass, disproportionate deposition of extracellular matrix (ECM) proteins, neovascularization, mucous gland and goblet cell hyperplasia and epithelial disruption [134, 135]. Significant increase in the thickness of the ASM layer is a key event in the airway remodeling which is more dominant in the small airways compared to the large airways. Variety of stimuli including inflammatory mediators and growth factors induced signaling cause hyperplasia and hypertrophia of ASM cells as well as evoke the accumulation and deposition of ECM components such as collagens, elastin, fibronectin, laminin, and proteoglycans around the smooth muscle cells leading to the thickening of small airway walls and reduction of lumen calibre. Moreover, ECM can influence the phenotypic switch of ASM cells from contractile to proliferative/synthetic phenotype contributing to the proliferation and migration of the ASM cells and the secretion of chemokines, cytokines and ECM proteins [136, 137]. Inflammation is also involved in the airflow limitation in the small airways. Infiltration of the airway wall by inflammatory cells such as neutrophils, macrophages, CD8 cells and B cells and accumulation of inflammatory mucous exudates in the lumen are associated with lumenal narrowing and progression of COPD [138]. In addition, the number of the goblet cells is elevated in the epithelium of peripheral airways which are mostly represented in the central airways [139]. Structural changes of the lung parenchyma is also presented in COPD. Destruction of alveolar walls and parenchymal tissue due to the excessive proteolysis and/or inappropriate repair mechanisms are associated with chronic infiltration of inflammatory cells and inflammation which are responsible for the airflow obstruction [140]. Remodeling of the large airways also occurs in patients with COPD including thickening of epithelial, subepithelial and ASM layers as well as reticular basement membrane (RBM). Hyperplasia of goblet cells and mucus-secreting submucosal glands, metaplasia of squamous cell and loss of ciliated epithelial cells are important pathological features of the large airways in COPD [141]. COPD is also associated with abnormal deposition of ECM components such as collagen I and versican in the subepithelial layer as well as increased depositions of collagens (e.g., collagen I, III, and IV), fibronectin and laminin can be detected in the surface epithelial basement membrane (SEBM) of the bronchial walls and vasculature [142, 143]. In addition, RBM thickness is increased due to the ECM deposition apposing the lamina reticularis as well as inflammation by eosinophils [144, 145]. Moreover, the ASM mass is significantly greater in the central airways attributable to the growth and proliferation of AMC cells [142].

Pulmonary vascular remodeling is another critical feature of COPD which is characterized by thickening of the vessel wall leading to narrowing of the vascular lumen, elevation of vascular resistance and development of pulmonary hypertension [146]. All vessel wall layers are affected in COPD, though the enlargements of the intima and media are the major contributors to the remodeling. Degree of intimal and medial thickening is correlated with the deterioration in lung function and the severity of the disease [147]. Augmentation of the intima thickness is associated with intense deposition of collagen and intimal hyperplasia including increased proliferation of a subpopulation of less differentiated vascular smooth muscle cells that express smooth

muscle alpha-actin and vimentin, but not desmin (VSMC) [148, 149]. Endothelial dysfunction also occurs in pulmonary arteries of COPD patients [150]. Hypertrophy and hyperplasia of VSMC as well as medial accumulation of fibroblasts and inflammatory cells contribute to the muscularization and thickening of the media layer. Furthermore, over-deposition of ECM proteins such as collagen III and laminin in vascular media and adventitia occurs [147]. Infiltration of increased number of inflammatory cells mostly activated T lymphocytes can be observed in the adventitia of pulmonary arteries in COPD patients which play an important role in the chronic inflammatory response and pathogenesis of the disease [151].

Angiogenesis is an important event in the development of COPD which governs the formation of new blood vessels from a preexisting vasculature. Wide range of stimuli including inflammation, shear stress, numerous angiogenic factors such as growth factors (e.g., vascular endothelial growth factor [VEGF], fibroblast growth factors [FGFs], epidermal growth factor [EGF], platelet-derived growth factor [PDGF], angiopoietin-1 [Ang-1] as well as chemokines and cytokines promote the angiogenesis. Variety of cell types such as macrophages, lymphocytes, epithelial cells, fibroblasts, and SMC can produce and release angiogenic factors regulating the critical steps in angiogenesis including survival, proliferation, differentiation, and migration of the endothelial cells and tubes formation [152–154]. Nevertheless, the regulation of angiogenesis in COPD is not fully understood yet. VEGF is one of the crucial player in the angiogenesis. It was reported that expression of VEGF and its receptors are significantly increased in smokers and patients with moderate COPD compared to the nonsmokers [155, 156]. In contrast, patients with severe emphysema showed reduced VEGF and VEGFR-2 receptor expression which was associated with increased endothelial apoptosis [157, 158]. These observations suggest that expression levels of VEGF highly depend on the severity of COPD and may be implicated in the pathogenesis of the disease [159, 160].

4.2 RONS on Calpain in COPD

Oxidative/nitrosative stress, due to the oxidant–antioxidant imbalance, have a critical role in the pathogenesis of COPD. RONS are exogenously derived from cigarette smoke (CS) or air pollutants, or endogenously released from activated inflammatory cells such as neutrophils, alveolar macrophages and eosinophils or other respiratory cells such as epithelial, endothelial, and smooth muscle cells. They are implicated in various pathological processes including chronic inflammation; oxidation and nitration of cellular substances such as lipids, proteins, carbohydrates, and nucleic acids causing damage to the lung cells and apoptosis; activation of redox-sensitive transcriptions factors and chromatin regulation contributing to increased gene expression of inflammatory mediators and cytokines; inactivation of antiproteases and activation of metalloproteinases leading to imbalance of the protease–antiprotease system and ECM degradation; defects in tissue repair mechanisms, as well as abnormal proliferation of ASM cells [161, 162]. The main enzyme systems which are responsible for the production of RONS such as mitochondria, NADPH oxidase, xanthan-oxidase, and iNOS are upregulated in COPD patients [88, 163, 164]. Several intracellular signaling pathways are implicated in the RONS-mediated processes involving extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, and PI3K/Akt, in addition, activation of these pathways can contribute to the pathological responses in COPD [165].

CS and its components are major risk factors in COPD and are implicated in the development of airway and pulmonary vascular remodeling. One of the main effector protein which mediates these processes is the calcium-activated enzyme namely calpain. Calpain is a family of calcium-dependent, cytoplasmic neutral cysteine endopeptidases that act through limited proteolysis of substrate proteins in mammalian cells. Heijink and colleagues revealed that cigarette smoke extract (CSE) causes disruption of cell–cell contacts leading to epithelial barrier dysfunction in

bronchial epithelial cells via degradation of tight junction (TJ) proteins such as zonula occludens-1 (ZO-1) and occludin by EGFR-dependent calpain activation. Inhibition of EGFR tyrosine kinase and/or inhibition of calpain prevent the CSE-induced permeability increase as well as the cleavage of TJ components, respectively, emphasizing their participation in the reduced epithelial integrity evoked by CSE [166]. One report demonstrated that CSE induces calpain-1 expression via activation of ERK signaling pathway in bronchial epithelial cells contributing to distributional abnormalities and increased cleavage of adherens junction proteins such as E-cadherin and β-catenin consequently leading to transepithelial electrical resistance reduction and barrier disruption [167]. Calpain-1 specific inhibitor ALLM and ERK inhibitor U0126 diminish the CSE-induced epithelial barrier disruption. Moreover, active form of vitamin D, 1α,25-Dihydroxyvitamin D3, reduces these pathological changes via inhibiting ERK phosphorylation and activation and calpain-1 expression suggesting its protective role against CSE damage in the airway epithelium by down-regulating the ERK signaling pathway [167]. It was recently published by Wang and colleagues that exposure to particulate matter (PM) air pollution causes tight junction protein ZO-1 degradation and increases in vascular permeability via calpain activation in human lung microvascular endothelial cells (HLMVECs). They found that PM induces ROS production, ROS-dependent endothelial permeability increase and degradation of ZO-1 as well as provokes calcium leakage via ROS-activated transient receptor potential cation channel member M2 (TRPM2) leading to elevated calpain activation. Using either the ROS scavenger, N-acetylcysteine (NAC) or calpain inhibitors such as calpeptin and ALLN or calcium chelator BAPTA-AM, the PM-induced decrease in transendothelial electrical resistance and ZO-1 degradation are attenuated. These data suggest that ROS-mediated calpain activation has a crucial role in PM-induced endothelial barrier disruption [168]. Chun and Prince reported that calpain can also alter the epithelial junctions to promote the polymorphonuclear leukocytes (PMNs) transmigration via activation of toll-like receptors (TLR) and interleukin-8 (IL-8) production in response to inhaled pathogens. Patients with COPD are highly susceptible for infections which may have significant role in acute exacerbations. Inflammation and infiltration of inflammatory cells to the airways may contribute to the airflow obstruction in COPD. They found that activation of TLR2 signaling due to the inhaled pathogens causes calpain activation, which in turn, cleaves the transmembrane components such as occludin and E-cadherin leading to elevated PMN transmigration. The selective calpain inhibitor calpeptin almost completely blocks the PMN mobilization into the airway lumen indicating the relevance of calpain in the PMN transmigration and pulmonary inflammation [169]. The role of calpain in lung inflammation was further strengthened by Liu and colleagues. They demonstrated that mechanical ventilation causes rapid elevation in calpain activation as well as enhances the neutrophil infiltration into the lung causing pulmonary vascular barrier disruption and edema formation. Calpain inhibition by either calpain inhibitor I or specific siRNAs against calpain-1 and 2 prevented the inflammatory responses evoked by hyperinflation of the lung. In addition to this, inhibition of calpain also abolishes the eNOS-mediated NO production and subsequent phosphorylation of the transmembrane protein ICAM-1 after mechanical ventilation. These findings indicates that activation of calpain regulates early lung inflammation through eNOS/NO-dependent ICAM-1 phosphorylation and recruitment of neutrophils in response to lung hyperinflation [170]. Activated calpain can also cleave other critical cytoskeletal proteins such as myristoylated alanine rich C-kinase substrate (MARCKS) which plays a role in regulation of airway mucin secretion. Hypersecretion of mucin has a significant role in the pathophysiology of COPD. It has been shown that phorbol-12-myristate-13-acetate (PMA) induces the activation of protein kinase C (PKC) pathway, contributing to elevated calpain activity and mucin secretion in human bronchial epithelial cells. Calpain inhibitors Z-LLY-FMK (FMK) and Z-LLY-CHO (CHO) significantly diminish the

calpain activity and mucin secretion induced by PMA. PMA also increased the cleavage of MARCKS. They proposed that calpain-evoked MARCKS cleavage may have an important role in regulation of airway mucin secretion [171].

CSE can inhibit endothelial monolayer wound repair and angiogenesis. We previously reported that CSE results in dose-dependent inhibition of tube formation, proliferation and migration of pulmonary artery endothelial cells (PAEC) as well as decreases in calpain activity. Calpain specific inhibitor, calpain inhibitor-1 potentiates inhibitory effect of CSE. Downregulation of calpastatin which is an endogenous calpain inhibitor prevents CSE-induced reduction in calpain activity and angiogenesis. Taken together, these data suggest that CSE diminishes angiogenesis through calpain inhibition which may contribute to the remodeling in COPD [172]. Edirisinghe and colleagues revealed that inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) enhances cigarette smoke-induced oxidative stress and inflammatory responses causing endothelial dysfunction. They found that CS and VEGFR-2 inhibitor NVP-AAD777 increases oxidative stress, macrophage infiltration and the levels of proinflammatory mediators as well as decreases the levels of VEGF and VEGFR-2, phosphorylation of VEGFR-2, and levels of phosphorylated and total eNOS protein in mouse lung. In addition, they showed that CS exposure or VEGFR-2 inhibition results in increases in ROS production as well as reduction in VEGFR-2 expression, eNOS levels, and VEGF-induced VEGFR-2 phosphorylation in human lung microvascular endothelial cells (HMVEC-Ls) contributing to diminished endothelial cell migration and angiogenesis induced by VEGF [173]. More importantly, we recently reported that VEGF causes dose- and time-dependent increases in calpain activity and protein levels of calpain-2 in human pulmonary microvascular endothelial cells (PMEC). Downregulation of the calpain activity with either calpain-2 specific siRNA or by overexpressing the endogenous calpain inhibitor calpastatin prevented the increases in angiogenesis induced by VEGF, which further support the idea that calpain may have role in the CSE-induced inhibition of endothelial angiogenesis [174]. Calpain–calpastatin system also play a role in the cigarette smoke-induced inhibition of endothelial NOS in pulmonary artery endothelial cells (PAECs). Decreases in the NO production and impaired endothelium-dependent vasodilation evoked by cigarette smoke may lead to the progression of pulmonary vascular diseases in smokers. Our group previously published that CSE causes an irreversible, time- and dose-dependent inhibition of eNOS activity as well as causes time-dependent decreases in mRNA and protein levels of eNOS [175]. We also revealed the implication of calpain in the regulation of eNOS activity in hypoxic PAEC. Exposure to hypoxia reduces the eNOS activity, but not the protein levels, which are prevented by calpain specific inhibitors, calpain inhibitor I and calpeptin. Hypoxia also decreases the protein content of heat shock protein 90 (HSP90) which is attenuated by both calpain inhibitors. Moreover, HSP90 specific inhibitor geldanamycin mimicked the effect of hypoxia via diminishing the eNOS activity. These findings show that hypoxia-induced decrease in eNOS activity is attributable to the calpain-mediated reduction of HSP90 protein level [176]. Our additional study discovered the involvement of calpain–calpastatin system in the inhibition of eNOS induced by CSE in PAECs. We found that CSE exposure results in decreases in the transcription of eNOS gene and calpain activity as well as increases the protein levels of calpastatin. We also found that overexpression of calpastatin can mimic the inhibitory effects of CSE. Diminished activities of calpain and eNOS as well as lower mRNA and protein levels of eNOS were observed in calpastatin-overexpressing PAECs. In addition, downregulation of the calpastatin protein level by antisense oligodeoxyribonucleotides prevented the CSE-induced reduction in calpain activity as well as it prevented the decreases in eNOS gene transcription rate, eNOS protein levels, eNOS activity, and NO release induced by CSE. Together, these data indicate that CSE-induced inhibition of calpain activity and consequently reduced eNOS expression and activity are associated with an

increase in the protein levels of calpastatin [177]. More recently, we reported that nitric oxide can affect the angiogenesis of lung microvascular endothelial cell in a dose-dependent manner via regulation of calpain activity. At lower concentrations, NO enhances angiogenesis, however, higher levels of NO inhibit angiogenesis and calpain activity as well as increase the calpain-1 nitrosylation. Decreased calpain activity is attributable to the nitrosylation of calpain which may have role in the anti-angiogenic effect of NO [178].

A recent report shows that acrolein which is a highly reactive, unsaturated aldehyde in cigarette smoke can induce the ROS production in different cell types and it is associated with COPD [179, 180]. Tanel and colleagues found that calpain and other proteases have role in the ER-mediated apoptosis of lung cells induced by acrolein. They showed that acrolein increases the activities of calpain and calpase-7 leading to the activation of ER initiator caspase-4 and apoptosis. The elevated calpain activity was prevented by calcium chelator BAPTA-AM or calpain inhibitor. In addition, acrolein reduced the protein levels of calpain inhibitor calpastatin during calpain activation. Moreover, acrolein-induced apoptosis was partially inhibited by calpain inhibitors and completely blocked by BAPTA-AM. These findings revealed the participation of calpain as well as caspase-7 and -4 in acrolein-induced apoptosis in human lung cells [181]. Ryter and colleagues investigated the pathogenic implication of autophagy in cigarette smoke-induced COPD. They found that autophagy is increased in lung tissue derived from COPD patients and revealed that activation of autophagic proteins is associated with epithelial cell apoptosis in response to CS [182]. Other group reported the implication of calpain in the autophagy/apoptosis switches. Yousefi and colleagues demonstrated that apoptosis is related to calpain-mediated cleavage of autophagy protein 5 (Atg5) which is responsible for the autophagosomes formation and increased the susceptibility towards apoptosis. They found that calpain cleaved fragment of Atg5 facilitates apoptotic cell death by translocating from the cytosol to mitochondria and then binding to and consequently inhibiting the anti-apoptotic Bcl-xL molecule leading to cytochrome c release and caspase activation [183].

Increased ROS production and activation of proteolytic system such as calpain pathway, ubiquitin–proteasome (UP), the caspase and autophagy-lysosomal system (AL) are associated with skeletal muscle atrophy in COPD [184]. Chronic hypoxemia is one of the contributors to skeletal muscle dysfunction [185]. Chaudhary and colleagues revealed the involvement of calpains and UP pathway in the chronic hypobaric hypoxia-induced skeletal muscle atrophy. They found that exposure to hypoxia simultaneously elevates the activities of chymotrypsin-like enzyme of the UP pathway and calpains as well as enhances the expressions of ubiquitinated proteins and calpain-1, leading to increased myofibrillar protein degradation. They proposed that chronic hypoxia-induced oxidative stress may play an important role in the skeletal muscle loss via ub-proteasome and calpains pathways [185]. It was also demonstrated that hypoxia increases both activity and mRNA levels of calpain in pulmonary artery endothelial cells (PAECs). Inhibition of the transcription by actinomycin D prevented the increases in calpain gene expression and activity suggesting that acute hypoxia enhances calpain activity via transcriptional process in PAECs [186]. The implication of calpain in the development of skeletal muscle atrophy in response to ROS was further evidenced by other groups. McClung and colleagues demonstrated that calpain-1 is essential for myotube atrophy induced by hydrogen peroxide. They showed that exposure to H_2O_2 induces C2C12 myotube oxidative damage and atrophy. Downregulation of calpain-1 by siRNA, but not calpain-2 or caspase 3 proteases, attenuated the H_2O_2-induced myotube atrophy, suggesting the requirement of calpain-1 in this process [187]. In addition, it was reported by Dargelos and colleagues that H_2O_2 administration causes up-regulation of both calpain-1 and calpain-2 expression and increases their proteolytic activities in LHCN-M2 human myoblasts. Interestingly, they also found that natural antioxidant extracted from pine bark

(Oligopin®) prevents the H_2O_2-induced effects and up-regulation of calpain. These findings indicate that ROS-induced oxidative stress is involved in the skeletal muscle dysfunction via calpain-dependent pathways [188]. Recent study also revealed that oxidative stress is required for the activation of calpain and caspase-3 in the diaphragm during prolonged mechanical ventilation (MV), resulting in myofiber atrophy and contractile dysfunction. They also showed that the prevention of MV-induced oxidative stress via the antioxidant Trolox abolishes calpain and caspase-3 activation, as well as prevents diaphragmatic atrophy and contractile dysfunction evoked by MV [189]. Smuder and colleagues also confirmed the role of calpain and/or caspase-3 in the oxidative stress-induced myofibrillar protein breakdown. They demonstrated that oxidative stress causes augmented oxidative modification of myofibrillar proteins, leading to their accelerated proteolytic degradation by calpain-1, calpain-2, and caspase-3. They conclude that oxidation of myofibrillar proteins increases their susceptibility to degradation through calpain and caspase-3 in response to oxidative stress [190]. Taking together, these reports show that elevation in ROS production in the skeletal muscle is associated with the activation of calpain.

Increased ROS formation and calpain activation are also associated with pulmonary hypertension (PH), a common complication of COPD. The critical mechanisms leading to PH in COPD are hypoxic vasoconstriction and remodeling of pulmonary arteries which is characterized by increased smooth muscle cell proliferation [191]. Elevated expressions of genes encoding smooth muscle mitogens such as PDGF have been found in COPD. Xing and colleagues demonstrated that CS exposure significantly increased the protein and mRNA levels of PDGF isoform B (PDGF-B) and PDGF receptor beta (PDGFRβ) in pulmonary artery of rats. In addition, they also showed that CSE induces the rat PASMC proliferation which was inhibited by PDGFR inhibitor imatinib. This finding indicates that PDGF signaling is one of the major contributor to the CSE-induced PAH [192]. More importantly, our publication provides evidence that calpain mediates the proliferation and collagen synthesis of PASMCs induced by EGF and PDGF in pulmonary vascular remodeling. We found that EGF and PDGF cause significantly elevation in the calpain activity, cell proliferation and collagen synthesis in PASMCs which were attenuated by specific calpain inhibitor MDL28170 or using siRNAs targeted against calpain-1 and calpain-2. Moreover, we revealed that inhibition of the calpain activity by conditional knockout of calpain 4 or by calpain inhibitor MDL28170 prevents the hypoxia and monocrotaline (MCT)-induced progression of pulmonary vascular remodeling demonstrating the critical role of calpain in this process [193]. More recently, we further confirmed that mediators of PAH including PDGF, serotonin, H_2O_2, endothelin-1, and interleukin-6 markedly enhance the calpain activity, cell proliferation and protein levels of collagen-I of PASMCs. These processes were abolished by downregulation of calpain-2 by specific siRNA or using extracellular signal-regulated kinases (ERK) inhibitor PD98059, indicating the involvement of ERK1/2 in the PAH mediators induced activation of calpain-2 in pulmonary vascular remodeling. Furthermore, we demonstrated that smooth muscle-specific knockout of calpain 4 diminishes and knockout of calpastatin potentiates the chronic hypoxia-induced pulmonary vascular remodeling suggesting that calpain activation is an important signaling pathway in the pathogenesis of PH [10]. The implication of the calpain/calpastatin system in the progression of PH was further confirmed by Wan and colleagues. They found elevated lung expression of calpastatin, calpain-1, and calpain-2 proteins and activities occurring both intracellularly and extracellularly in patients with iPAH and in mouse models of PH induced by either hypoxia or overexpression of the serotonin transporter (5HTT) in SMCs. Overexpression of calpastatin or calpain inhibition by calpain inhibitor PD150606 decreased the protein levels of calpain-1 and 2 as well as reduced the intracellular and extracellular calpain activities in mouse models which were associated with attenuated PH severity. In addition, in PASMCs derived from WT and calpastatin overexpressing transgenic mice,

exogenous calpastatin or calpain inhibitor PD150606 diminished the cell proliferation and migration, suggesting the inhibitory role of extracellular calpastatin in the proliferation and migration of PASMC induced by various stimuli. Thus, calpastatin acts as an endocrine factor which markedly decreases the severity of PH in mice mainly via the inhibition of extracellular calpain activity [194].

5 Summary

In conclusion, there is convincing evidence that RONS and the members of the calpain family, primarily the two major isoforms calpain-1 and calpain-2, have a mediating role in the pathogenesis of COPD. Targeting the redox-dependent calpain signaling pathways using multidisciplinary approaches could provide therapeutic intervention in the pathogenesis and the progression of airway and pulmonary vascular remodeling in COPD.

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Natural Antioxidants as Potential Therapy, and a Promising Role for Melatonin Against Pulmonary Hypertension

Gerald J. Maarman

Abbreviations

MCT	Monocrotaline
MPAP	Mean pulmonary arterial pressure
PA	Pulmonary artery
PAAT	Pulmonary artery acceleration time
PAP	Pulmonary arterial pressure
PASMCs	Pulmonary arterial smooth muscle cells
PH	Pulmonary hypertension
PVR	Pulmonary vascular resistance
RV	Right ventricle/right ventricular

1 Introduction: Antioxidant Therapy in Animal Models of Pulmonary Hypertension

Pulmonary hypertension (PH) is a disease with multifactorial etiology, and a range of factors are involved in its pathophysiology [1–6]. These factors include inflammatory cytokines, growth factors, elastases, and vasoconstrictors such as thromboxane-A2, endothelin-1, and serotonin [1–6], activated endothelial cell colony-forming cells, reduced circulation of vasodilators (prostacyclin, nitric oxide, and vascular endothelial peptide) and mutations of bone morphogenetic protein receptor-2 (BMPR-2) [6–13]. The general understanding is that these factors may trigger the pathologic development of PH, but how exactly the development ensues is not well understood.

Aside from the aforementioned factors, a prominent role has been identified for reactive oxygen species (ROS) [2, 14–17] and elevated oxidative stress [14, 18–20]. As depicted in Fig. 1, trigger factors disrupt normal mitochondrial function which increases ROS beyond intracellular antioxidant capacity. Trigger factors may also activate other ROS sources (NADPH oxidases, cyclooxygenases, and lipoxygenases) that contribute to the excessive production of ROS in the pulmonary vasculature (Fig. 1). These ROS cause pulmonary vascular endothelial damage [18, 21–24] that leads to increased proliferation apoptosis of endothelial cells, resistance to apoptosis and proliferation of pulmonary arterial smooth muscle cells (PASMCs) [6, 8, 9, 23, 25]. This culminates in pathologic remodeling of small pulmonary arterioles with a histological derangement that reflects hypertrophy of the vascular muscle layers (intima, media, and adventitia), intimal fibrosis, in situ thrombosis, and plexiform lesions in severe PH

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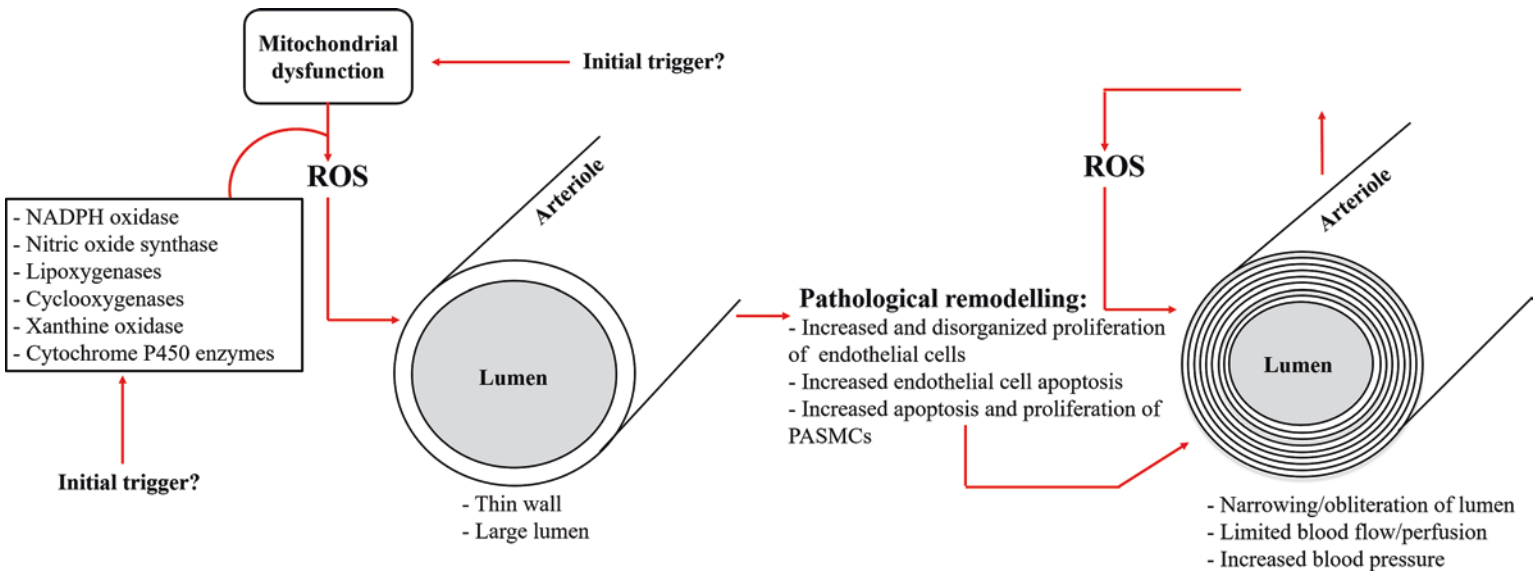

Fig. 1 This is a representation of the process during which trigger factors (known and unknown) disrupt normal mitochondrial function to increase ROS. These trigger factors also activate other ROS sources (NADPH oxidases, cyclooxygenases, and lipoxygenases) that contribute to the excessive production of ROS in the pulmonary vasculature. ROS cause pulmonary vascular endothelial damage that leads to increased proliferation and apoptosis of endothelial cells, resistance to apoptosis and proliferation of pulmonary arterial smooth muscle cells. This culminates in hypertrophy of the vascular muscle layers (intima, media, and adventitia), intimal fibrosis, in situ thrombosis, and plexiform lesions. The derangement and pathologic remodeling of the pulmonary arteries may also increase mitochondrial dysfunction that further elevates the production of ROS

[26, 27]. The characteristic histological derangement narrows and obliterates the lumens of small pulmonary arterioles, increases pulmonary arterial pressure and ultimately causes full-blown PH (Fig. 1). The derangement and pathologic remodeling of the pulmonary arteries may also increase mitochondrial dysfunction that further elevates the production of ROS and the cycle continues (Fig. 1).

Antioxidant therapy in experimental studies reduces or stunts the pulmonary vascular remodeling process [28–33], and, therefore, PH pathophysiology is considered to be strongly dependent on ROS [34–36]. The efficacy of antioxidants against PH suggest that antioxidant therapy may be a possible therapeutic strategy in PH [18, 22]. A number of studies have investigated the impact of antioxidants in animal models of PH [28–33], and a range of experimental antioxidants show health benefit [37–48]. These include antioxidant medicinal plant extracts (*Moringa oleifera, Mimosa pigra, Kelussia odoratissima,* and *Rhodiola sachalinensis*) and biological compounds resveratrol, *N*-acetyl cysteine, and melatonin (Table 1).

Table 1 Natural antioxidants that display promise as possible therapy against the adverse effects of pulmonary hypertension (PH)

Type of antioxidant agent	Reported benefits in PH
Antioxidant extracts from medicinal plants	
Moringa oleifera [38]	Decreases PAP and PA remodeling, and increases PA relaxation
Mimosa pigra [39]	Decreases PAP and PA remodeling, increases PA relaxation, and reduces RV remodeling
Kelussia odoratissima [40]	Inhibits the proliferation of PASMCs, causes vasodilatation, and reduces PA remodeling
Rhodiola sachalinensis [76]	Reduces MPAP, RV hypertrophy, and PA endothelial growth factor expression
Biological compounds as antioxidants	
Resveratrol [184]	Reduces PA remodeling, inhibits oxidative stress and inflammation, and improves PAAT
N-Acetylcysteine [37]	Decreases lung inflammation, PA remodeling, and PVR
Melatonin [124, 129, 131]	Reduces PASMC proliferation, lung inflammation, PAP, and PVR. Increases PA vasodilatation. Improves RV function, plasma oxidative stress, antioxidant capacity, and RV fibrosis

2 Antioxidant Medicinal Plant Extracts

Medicinal plants have high antioxidant and anti-inflammatory characteristics, and provide health benefits against many pathologies [49–55]. Underlying mechanisms for their beneficial effects include the downregulation of vasoconstriction, improvement of beta cell responsiveness, inhibition of pro-inflammatory cytokines, and stunting of apoptosis and fibrosis [56–60]. Chen et al. [38] prepared an antioxidant extract from the plant *Moringa oleifera*, and administered it to rats with monocrotaline (MCT)-induced PH. The extract was given either 14 or 21 days after MCT injection (60 mg/kg) and decreased pulmonary arterial pressure, increased pulmonary artery relaxation, reduced pulmonary artery medial thickening and increased lung SOD activity (Table 1). What makes this study so important is the fact that *Moringa oleifera* treatment commenced 14 or 21 days after MCT injection, when PH was fully progressed. Usually without any intervention, MCT rats die around day 21. Therefore, these findings support the notion that antioxidant treatment is still effective, even in far progressed PH and RV failure. This has an important clinical implication, as PH patients are often diagnosed late, when the disease is already far progressed and severe [61–64]. In theory, administration of antioxidants may very well confer health benefit in fully developed and severe PH. Future studies could investigate the effectiveness of antioxidant extracts for patients with established and severe PH.

Mimosa pigra is a plant used in traditional medicine in Indonesia and South America for minor ailments and cardiovascular diseases [65–67]. In rats with chronic hypoxia-induced PH, *Mimosa pigra* extract induced endothelium-dependent, nitric oxide-mediated relaxation of

the pulmonary artery, reduced pulmonary artery pressure, and decreased cardiac and pulmonary remodeling [39] (Table 1). Other extracts include *Kelussia odoratissima Mozzaf*, a type of wild celery [68–70], and the root of *Rhodiola sachalinensis*, a plant popular in traditional medicine of Siberia and Asia, used for the prevention of high altitude sickness [71–75] (Table 1). *Rhodiola sachalinensis* reduced mean pulmonary artery pressure and right ventricular (RV) hypertrophy, in rats with high altitude-induced PH [76].

The true efficacy of medicinal plant extracts against human disease has been extensively debated over the years [77–81]. This debate mainly originates from the fact that minor changes in the chemical structure of these extracts may profoundly impact its activity [77–81]. These changes may either enhance its beneficial effects or cause unwanted side effects [77–81]. Unfortunately, not many studies investigate the benefits of these antioxidant plant extracts in preclinical models of PH. Therefore, the current body data does not permit sufficient evidence for a proper, critical evaluation of the use of these plant extracts for PH in the clinical setting. Future studies should investigate the safety and chemical stability of these antioxidant plant extracts in animal models of PH. Nonetheless, their potent antioxidant properties and disease-ameliorating effects make them good candidates for *adjunctive* antioxidant therapy against PH (Fig. 2).

3 Biological Compounds as Antioxidants

Resveratrol is a non-flavonoid polyphenol compound with strong antioxidant, anti-inflammatory, and endothelial protective properties [82–84]. A number of studies have demonstrated that resveratrol treatment in rats with MCT-induced PH, abolished pulmonary artery endothelial dysfunction [82], improved pulmonary artery acceleration time [85], and reduced medial thickening of intrapulmonary arteries [86] (Table 1). Additional benefits of included reduced expression of inflammatory cytokines (tumor necrosis factor-alpha,

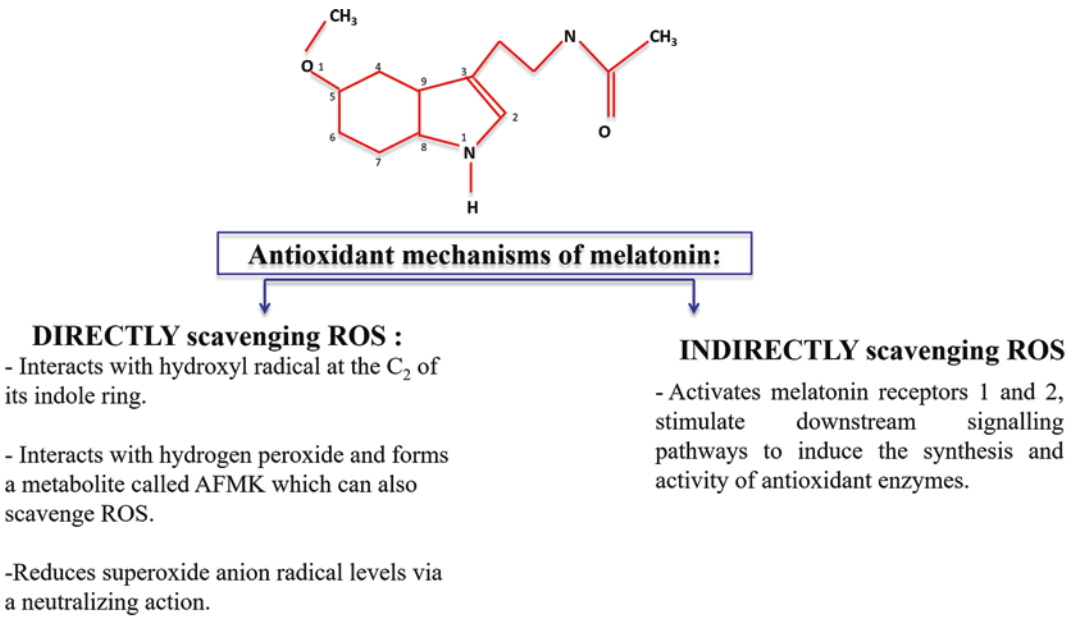

Fig. 2 Melatonin and its antioxidant and antihypertensive properties. A schematic showing the chemical structure of melatonin and its direct and indirect antioxidant mechanisms. *CH_3* methyl group, *O* oxygen, *N* nitrogen, *AFMK* *N*-acetyl-*N*-formyl-5-methoxykenuramine, *ROS* reactive oxygen species, *C_2* Carbon-2

interleukin 1 beta, interleukin 6, and platelet-derived growth factor-alpha/beta) and reduced leukocyte infiltration in the lung [82]. These findings demonstrate that resveratrol is an effective antioxidant therapy against experimental PH.

In spite of its effectiveness, concern has been raised regarding to safety of resveratrol treatment for humans [87]. This stems from supraphysiological doses administered in preclinical studies, and the lack of studies investigating its potential toxicity [87]. However, this concern is challenged by all the aforementioned studies of resveratrol treatment in PH. These studies administered low doses for extended periods, with no reported side effects. Moreover, a recent meta-analysis demonstrated that resveratrol doses greater than 150 mg/day reduce hypertension in humans [88]. Therefore, although further human trials with resveratrol and PH are required, current evidence suggests resveratrol as promising therapeutic strategy in PH.

Another biological compound with antioxidant properties is *N*-acetylcysteine which is derived from the amino acid L-cysteine [89–93]. *N*-acetylcysteine has been proposed as a treatment for idiopathic pulmonary fibrosis, bronchitis, and lung ischemia–reperfusion injury [89–93]. It is an analogue and precursor of glutathione synthesis that restores cellular redox status and inhibits pro-inflammatory cytokines (tumor necrosis factor-alpha and interleukin-1-beta) [94–96]. *N*-acetylcysteine also decreases the activation of nuclear factor kappa-B, to decrease inflammatory cell accumulation in the lungs of rats with mechanically induced lung injury [97–100]. Chaumais et al. [37] injected rats with MCT (60 mg/kg) and kept them untreated for 14 days, after which *N*-acetylcysteine (500 mg/kg/day) was administered for an additional 14 days. *N*-acetylcysteine improved PH, decreased pulmonary vascular remodeling, reduced lung inflammatory cell deposition, and improved RV function, RV hypertrophy, and fibrosis (Table 1). Taken together, these findings suggest that *N*-acetylcysteine may be an effective antioxidant therapy against PH, especially considering its good tolerability, safety, and clinical efficacy [101–105]. However, further research is required.

4 Melatonin: A Promising Therapy Against Oxidative Stress-Related Pathology

Melatonin is an indoleamine synthesized by the pineal gland, is lipophilic, and is able to cross cell membranes [106–109]. It is also secreted by entero-endocrine cells in the upper gastrointestinal tract [110] and produced by plants, fruits, generated during the process of milk production and the fermentation of wine [111]. Melatonin achieves its antioxidant effects by either directly scavenging ROS or by indirectly stimulating the synthesis/activity of antioxidant enzymes [112]. It has also been shown to be a hundred times more effective in scavenging ROS compared to vitamin E [113, 114].

Small-scale clinical and preclinical studies investigating the benefit of melatonin against human disease show promising results [83, 84, 87, 115–118], while no such study has yet been done on PH patients. In one particular study, healthy volunteers were randomly divided into two groups: one group received oral administration of melatonin (6 mg) for 2 weeks, and the other group no melatonin [119]. Blood samples were collected before melatonin administration, on the 7th and 14th day of melatonin treatment, and on the 10th day after the last dose of melatonin. Melatonin increased plasma antioxidant ferric reducing ability and decreased lipid/deoxyribonucleic acid peroxidation. Furthermore, these beneficial effects of melatonin were maintained for 10 days after discontinuation of melatonin treatment [119]. These data demonstrate that melatonin can increase the antioxidant ability of the plasma in the absence of pathology, and could therefore be applied as preventive therapy in a clinical setting.

In a randomized, double-blinded, placebo-controlled, crossover trial involving 16 men with untreated hypertension, repeated oral doses of melatonin (2.5 mg/kg for 3 weeks) reduced systolic and diastolic blood pressure [116]. In another study where normotensive and hypertensive women were treated with melatonin (3 mg/kg) for 3 weeks, investigators observed a significant reduction in the nocturnal systolic diastolic and mean arterial pressure [120]. It is known that

systolic and diastolic blood pressures fluctuate in humans with hypertension, being at its highest during the day and lowest at night [106–108]. Patients who do not display a lower blood pressure at night have a higher risk for cardiovascular mortality [121]. These two studies are very crucial, as they show melatonin's ability to reduce nocturnal blood pressure in hypertensive patients, which in theory should reduce their risk of cardiovascular mortality. Moreover, in a randomized, double-blinded, placebo-controlled study on 36 patients with clinically stable moderate to severe chronic obstructive pulmonary disease, melatonin treatment (3 mg capsule, once a day before bedtime for 3 months) reduced oxidative stress and improved dyspnea [122]. These findings are clinical relevant to PH, because chronic obstructive pulmonary disease is a well-known risk factor for PH [122]. Therefore, the findings from clinical trials using melatonin in concert demonstrate that melatonin holds promise as therapeutic strategy against PH.

4.1 Melatonin Antioxidant Therapy in Models of Pulmonary Hypertension

Das and colleagues examined the effect of chronic hypoxia, on the influence of melatonin-induced vasoreactivity of the pulmonary artery [123]. Melatonin (10^{-4} M) inhibited phenylephrine-induced constriction and enhanced relaxation in response to acetylcholine. These melatonin-induced changes were blocked by the melatonin-receptor antagonist luzindole, confirming the presence of melatonin receptors (1 and 2) on the pulmonary artery wall. This strongly supports melatonin as a modulator of pulmonary vascular tone and therefore its relevance to PH.

In a study by Jin et al. [124] rats received melatonin treatment (15 mg/kg/day, via intraperitoneal injection) every morning prior to hypoxia exposure (10% fraction of inspired oxygen), for 1 week before and 4 weeks during hypoxia. Melatonin attenuated RV systolic pressure, improved RV hypertrophy, and mitigated pulmonary vascular remodeling (Table 1). In the same study, PASMCs were exposed to hypoxia (1% oxygen compared to normal 21% oxygen) for 48 h. Hereafter, the cells were treated with melatonin (1 nM, 100 nM, 10 μM, and 1 mM) for an additional 4 h. Melatonin suppressed the hypoxia-induced PASMC proliferation and expression of proliferating cell nuclear antigen, hypoxia-inducible factor-1 alpha, and nuclear factor-kappa-B [124] (Table 1). These data show that melatonin is effective in protecting against the adverse effects of hypoxia-induced PH on the pulmonary vasculature and heart. Chronic hypoxia-induced PH usually occurs in people with high altitude sickness or chronic obstructive pulmonary disease [125, 126]. Therefore, the efficacy of melatonin to protect against the adverse effects of this type of PH is encouraging and suggests that it may be a possible antioxidant therapy against hypoxia-induced PH setting.

PH of the newborn has severe cardiovascular and neurological consequences [127, 128], and oxidative stress is considered a main cause, but the underlying mechanisms remain to be elucidated [127, 128]. In a study by Torres et al. [129], 12 newborn sheep were gestated, born, and raised at 3600 m above sea level. After 3 days of exposure to hypoxia, they received daily melatonin treatment (1 mg/kg/day) for 8 days. Melatonin decreased pulmonary pressure and resistance and improved the vasodilation of the small pulmonary arteries [129]. These improvements were associated with enhanced nitric oxide-dependent and nitric oxide independent vasodilator components and with increased nitric oxide bioavailability in lung tissue. Lastly, melatonin also increased the lumen diameter and caused a mild decrease in the wall of the pulmonary arteries [129] (Table 1). These findings have recently been corroborated by Thakor et al. [130], who showed that melatonin improved fetal cardiometabolic responses to acute hypoxia, by increasing nitric oxide bioavailability. Taken together, these data suggest that melatonin can improve the pulmonary vascular responses to stressors such as hypoxia and hyperbaric pressures, in newborn animals with fairly underdeveloped pulmonary structures.

In our laboratory, we recently administered oral melatonin treatment (75 ng/L, or 6 mg/kg) to rats that had been injected with MCT (80 mg/kg) [131]. In a clinically relevant design, MCT rats

were allowed to develop PH and RV remodeling for 14 days. Melatonin treatment was started on day 14, and administered daily for an additional 14 days. In this study, melatonin improved lung edema, stunted RV hypertrophy, and improved RV function. It also reduced interstitial cardiac and lung fibrosis, enhanced plasma antioxidant capacity, and reduced oxidative stress [131]. Findings from our laboratory strongly support the use of melatonin as an adjuvant antioxidant therapy to prevent PH or reduce PH that has fully progressed or is severe.

4.2 Mechanisms of Melatonin's Beneficial Effects Against Pulmonary Hypertension

Melatonin can directly neutralize the highly toxic hydroxyl radical [132], by reacting with hydroxyl radical at the C-2 position of melatonin's indole ring [133]. Similarly, melatonin can also interact with hydrogen peroxide, and form the by-products water and *N*-acetyl-*N*-formyl-5-methoxy-kynuramine [134]. Therefore, melatonin's antioxidant effects are receptor independent, and usually occur at low physiological concentrations of melatonin [109, 135].

Melatonin can also be metabolized in the liver to its daughter molecules, *N*-acetyl-*N*-formyl-5-methoxy-kynuramine and 3-hydroxymelatonin, which also have antioxidant abilities [112, 136]. Tan et al. [112, 134] showed that *N*-acetyl-*N*-formyl-5-methoxy-kynuramine display antioxidant properties similar to melatonin itself, by reducing oxidative damage in cells exposed to hydrogen peroxide, glutamate, or amyloid-beta. Therefore, the potent antioxidant effects of melatonin are significantly amplified in vivo, due to its own antioxidant properties and that of its downstream daughter molecules.

In the study by Jin et al. [124], melatonin reduced pulmonary arterial muscularization and proliferation of PASMCs. In PH, PASMC proliferation is caused by impairment of the transforming growth factor-beta (TGF-β)–BMPR-2–Smad signaling axis [12, 13, 137–141]. The BMPR-2 protein forms part of the TGF superfamily of proteins, where TGF-β signaling is initiated by signals transmitted from small mothers against decapentaplegia (Smad) molecules. These Smad molecules facilitate the binding of TGF-β ligands to the TGF-β-receptor [137–140]. Subsequently, Smad 2 and 3, or Smad 1, 5, and 8 are phosphorylated and co-localized with Smad 4 that translocates to the nucleus, where it modulate transcription of target genes. The general understanding is that signaling via TGF-β/Smad 2 and 3 induces PASMC proliferation and resistance to apoptosis, whereas BMPR-2/Smad 1, 5, and 8 induce the opposite effects [137–140]. This means that neither BMPR-2 nor TGF-β acts independently, but forms part of the TGF-β–BMP–Smad signaling axis, which controls apoptosis and PASMC proliferation in PH [137–140]. Melatonin is known to modulate the TGF-β–BMP–Smad signaling axis [142–144], and, therefore, this is a possible mechanism how melatonin can reduce PASMC proliferation in PH.

Another mechanism underlining melatonin's ability to improve vasodilation in PH comprises elevation of plasma levels of L-arginine, a substrate used by endothelial nitric oxide synthase to produce nitric oxide [145–147]. Additionally, melatonin also causes vasodilation by reducing circulating levels of asymmetric dimethyl arginine, the natural inhibitor of nitric oxide [123]. Moreover, melatonin can bind to its receptors, activating intracellular pathways that reduce intracellular calcium levels and elevate cyclic-GMP to cause vasodilation [123]. In addition to this, it is well known that excessive ROS can decrease the bioavailability of nitric oxide and thereby reduce vasodilation [148, 149]. In concert, it is therefore likely that, as a main mechanism, melatonin improves PH symptoms by reducing plasma ROS, and increasing nitric oxide-induced vasodilation [129, 131].

4.3 Strategies to Further Improve the Efficacy of Melatonin

Orally administered antioxidants, such as melatonin, are usually easily destroyed by acids/enzymes in the gastrointestinal tract, as a result only a small portion is absorbed [150]. The low

clinical success with some antioxidant therapies has been ascribed to low intestinal absorption rates [151]. There is thus a need to develop methods that improves the delivery of antioxidants to the pathologic site of interest [150, 151]. Various approaches have been implemented to increase the bioavailability of antioxidants such as melatonin. Although melatonin is a highly effective antioxidant, it undergoes extensive first-pass metabolism, during which the majority of it is lost and excreted in the urine [152–155]. A number of studies have investigated the bioavailability of melatonin after oral administration in humans [156–158].

In their experiments, DeMuro et al. [156] observed that oral melatonin at doses 2–4 mg has a bioavailability of approximately 15%. Due to this relatively low bioavailability, Bartoli et al. [159] developed an oral melatonin emulsion spray, administered it sublingually to healthy individuals, and compared the bioavailability to that of a melatonin capsule. This spray is absorbed via the oral mucosa and avoids the first-pass effect in the liver [159]. In this study, the amount of melatonin that reached systemic circulation was twice as high as with the pill form, even though the dose of melatonin was the same for both the spray and the pills (5 mg) [159]. Similar work was done by Mao et al. [160] who prepared starch microspheres (particle size of 30–60 μm) for intranasal administration of melatonin, by an emulsification cross-linking technique. In this study, the entrapment ratio of melatonin in the microspheres was 11.0%, and melatonin was released from the microspheres in a sustained manner. The absorption rate was rapid (7.8 min) and the absolute bioavailability was increased to 84%. However, without undermining the importance of these studies, one needs to critically evaluate their findings.

In the study by DeMuro et al. [156] after oral melatonin administration (pills of 2–4 mg), 85% of melatonin was lost due to first-pass metabolism. However, despite the low bioavailability of only 15%, the mean concentration of melatonin in the serum of these individuals reached peak values of approximately 4000–5000 pg/mL after melatonin administration. These serum concentrations are much higher than physiological peak levels and could therefore be the reason why, in most studies, the melatonin is still highly effective despite low bioavailability. These studies, managed to increase the bioavailability of melatonin in order to increase its efficacy in long-term treatment [161], and future studies could investigate the efficacy of these approaches against PH.

Another approach to improve the efficacy of antioxidants, is the use of small lipid nanoparticles [162]. These small nanoparticles are between 1 and 100 nm in size and include nano-encapsulation and nano-dendrimers, which act as drug delivery vehicles to circumvent the destructive milieu of the gastrointestinal tract [150, 151, 163–165]. These nanoparticles can serve as drug delivery vehicles, and have consequently been tested for cardiovascular and lung diseases [47, 166–171]. What makes them favorable for improved drug delivery is their composition of oil, water, co-surfactant, and surfactant, and they have an interfacial tension near zero, thus giving them long-term stability [162]. Therefore, lipid nanoparticles makes them suitable for delivery of drugs such as melatonin [162]. In a study by Priano et al. [172] melatonin was incorporated into small lipid nanoparticles, and administered to healthy subjects. In this study, compared to oral melatonin, melatonin-loaded nanoparticles had a better half-life and elimination rate and improved plasma melatonin levels post-administration [172]. These data strongly suggest that antioxidant nanoparticle drug delivery can improve the efficacy of melatonin, and perhaps lend greater ability against PH [45], but further research is required.

4.4 Caution to Be Taken with Excessive Antioxidant Therapy

Although ROS are detrimental to pulmonary vasculature and the heart in PH, their biological functions and relevance in health and disease are complex [34, 35]. These ROS play two major

roles, which include (1) the induction of injury to cellular components, and (2) their involvement in cell signaling pathways and antioxidant defense [34, 35]. The general understanding is that low, non-cytotoxic concentrations of ROS triggers mitochondrial hormesis [173–177]. During hormesis, low concentrations of mitochondrial ROS activate mitochondrial biogenesis and antioxidant mechanisms, in order to counteract oxidative stress and to reestablish cellular homeostasis [173–177].

Piantadosi et al. [178] demonstrated that ROS play a crucial role in mitochondrial biogenesis. In their experiments, ROS activated Akt/PKB, which caused translocation of nuclear factor erythroid 2-related factor-2 to the nucleus. Nuclear factor erythroid 2-related factor-2 occupies multiple antioxidant response element motifs in the promoter region of nuclear respiratory factor-1, and this induces mitochondrial biogenesis via peroxisome proliferator-activated receptor-gamma coactivator-1-alpha [178]. This suggests that ROS enable cells to sense ROS-induced damage, employ inherent antioxidant mechanisms, and counteract damage as an adaptive response [178].

Moreover, ROS have been shown to trigger the activation of rat sarcoma protein (Ras) that induces the recruitment of phosphatidylinositol-3′-kinase to Ras [179]. This ROS-triggered activation of Ras is required for the activation of cytoprotective, prosurvival, downstream signals such as Akt and mitogen-activated kinases [179]. It is believed that although antioxidant therapy is beneficial in experimental PH, it should be administered with caution, as ROS play an important role in health and disease [34, 35]. This physiological balance of ROS should be kept in mind when antioxidant treatments are administered, as complete depletion of ROS may be detrimental. The pathogenesis of PH is complex and not well understood, and although oxidative stress is instrumental, is it not the only mechanism [15, 180–182]. Therefore, most antioxidant therapies should currently only be considered as part of an adjunctive therapeutic strategy for PH, and not a single-line therapy [29, 35, 129, 183].

5 Conclusion

Oxidative stress plays an instrumental role in the pathogenesis of PH, which is reflected by the protection afforded by antioxidant therapy. Antioxidant therapy is therefore a promising therapy against PH. This chapter comprehensively reviewed a range of natural antioxidants that have shown impact against PH, with melatonin as the most superior. Melatonin's efficacy to protect against disease is supported by decades of research, and recent studies have also confirmed these findings in animal models of PH. Considering the convincing preclinical evidence, antioxidant therapy remains a promising therapeutic strategy against PH. Clinical studies should be performed to further assess the efficacy of antioxidants such as melatonin as a therapy against PH.

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Effects of Hyperoxia on the Developing Airway and Pulmonary Vasculature

Christina M. Pabelick, Michael A. Thompson, and Rodney D. Britt Jr

1 Introduction

Prematurity remains a significant health care issue in the USA. Over 500,000 premature babies are born every year with approximately 3.6% of these births occurring at <34 weeks gestation [1–4]. Although neonatal care, including administration of antenatal corticosteroids, surfactant, lower supplemental O_2 levels, and better ventilation strategies, has improved the outcome of preterm infants, a significant number of former preterm infants remain at risk of developing chronic lung disease [5–9]. By necessity, supplemental oxygen (hyperoxia), with or without intubation and mechanical ventilation, is administered to preterm infants. In addition to other perinatal factors, hyperoxia is a key contributor to neonatal and pediatric lung diseases, including airway disease (wheezing and asthma) and bronchopulmonary dysplasia (BPD) [10–12].

In the neonatal intensive care unit (NICU), preterm infants are often exposed to hyperoxia for prolonged periods, increasing generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [13]. Hyperoxia shifts the redox balance towards a pro-oxidant environment contributing to manifestation of disease in the airway, pulmonary vasculature, and/or alveoli. Preterm infants exposed to supplemental oxygen develop inflammation, fibrosis, and impaired postnatal lung development, resulting in abnormal lung function that can chronically persist into adolescence or adulthood [14]. Accordingly, there has been great emphasis on developing novel therapies, particularly antioxidant strategies that could alleviate the oxidative burden and prevent lung disease in preterm infants. However, successful application of such therapies has been limited thus far.

In addition to contributions to disease pathogenesis, redox signaling is also important for lung development and homeostasis [15]. As second messengers, ROS and RNS regulate pathways that are key for postnatal lung development [16]. Whether in lung development or chronic lung disease, it remains critically

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important to understand redox signaling in the neonatal lung.

This chapter discusses the role of redox-mediated signaling and how hyperoxia affects neonatal lung development and disease with an emphasis on the airway, pulmonary microvasculature, and alveoli.

2 Hyperoxia and Redox Signaling in Developing Airway

A consistent observation has been that preterm infants, with or without BPD, continue to be at high risk for airway hyperreactivity throughout infancy and childhood [1, 14, 17–19]. Unlike early preterm infants (<32 weeks gestational age), late preterm infants (32–36 weeks gestational age) usually receive less supplemental oxygen and are at lower risk of developing BPD but remain at risk of recurrent wheezing and asthma. It is important to note that infants with BPD can also develop airway disease. Yet structural and functional analyses indicate airway dysfunction, but no deficits in alveolar structures in late preterm infants [20]. It is not clear whether it is due to a higher supplemental oxygen concentration or less development that leads to a higher risk of developing BPD in early preterm infants. Nevertheless, these differences between early and late preterm infants raise new questions about how hyperoxia can impact the developing airway and whether there is a certain threshold regarding the detrimental effects of hyperoxia. This has led to recent practice changes in the NICU involving use of moderate levels of supplemental oxygen (40–60% O_2) when possible. Recent in vitro and in vivo studies have begun to explore how varying levels of oxygen affect airway structure and function to promote airway disease in the developing lung [21–24]. While the airway epithelium is an important component of the airway, airway smooth muscle (ASM) is key to determining airway tone and reactivity. This section discusses recent studies examining the effects of hyperoxia on airway contractility and remodeling, with a focus on developing ASM.

Intracellular calcium ($[Ca^{2+}]_i$) regulates numerous cellular processes including contractility and proliferation [25]. Understanding of signaling mechanisms that regulate ASM $[Ca^{2+}]_i$ and contractility are largely from studies in adult ASM [26–29]. $[Ca^{2+}]_i$ regulation in ASM involve sarcoplasmic reticulum (SR) Ca^{2+} release via inositol triphosphate receptor (IP_3R) and ryanodine receptor (RyR) channels, Ca^{2+} influx via Ca^{2+} channels, and store-operated Ca^{2+} entry (SOCE) through recently identified STIM and Orai proteins [30, 31]. However, the mechanisms by which $[Ca^{2+}]_i$ is modulated in the developing ASM is still largely unknown, especially in the context of hyperoxia exposure. Previous studies using newborn rat [32, 33], mouse [34–39] and sheep [40, 41] models have proposed potential mechanisms that lead to airway hyperreactivity. For example, in rat pups hyperoxia exposure (95% for 7 days) disrupted the NO–cGMP axis and lowered cGMP levels, which are known to reduce Ca^{2+} influx, leading to reduced ASM contractility [37, 42]. Another study in rat pups showed that hyperoxia altered Ca^{2+} sensitivity in lung parenchymal strips by inhibiting MLC phosphatase suggesting that hyperoxia effects on contractility may occur through RhoA activation [23]. However, these studies provide only limited information on the underlying mechanisms leading to hyperoxia-induced airway hyperreactivity.

Some studies have examined the effect of hyperoxia [43] and inflammation [44] on human ASM cells or tissue in adults. But these do not serve as adequate models for the developing lung given the likely phenotypic differences and metabolic needs of fetal lung cells during development. Recent experiments have utilized human fetal ASM cells to model ASM during the neonatal period. These cells were isolated from the fetal lung between 18 and 22 weeks gestational age (canalicular stage) and express $[Ca^{2+}]_i$ signaling and contractile proteins [45]. These human fetal ASM cells also express a number of agonist receptors, such the histamine (H1R) and muscarinic receptors (mAChR), as well as contractile proteins actin and myosin [45]. Functionally, human fetal ASM show substantial Ca^{2+} influx in response to bronchoconstrictors, histamine and

acetylcholine [45]. SOCE via TRPC3, STIM1, and Orai1 may be more prominent than SR channels, IP_3R, RyR, and sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) [45]. Interestingly, $[Ca^{2+}]_i$ responses to histamine and acetylcholine were robust but generally smaller and slower than adult ASM which may be indicative of a more proliferative (compared to contractile) phenotype. However, developmental differences regarding capacity of synthetic (proliferative and remodeling) versus pro-contractile phenotypes between fetal and adult ASM have yet to be explored [46].

Another set of studies examined the effect of moderate hyperoxia (50% O_2) on agonist-induced $[Ca^{2+}]_i$ responses in human fetal ASM [47, 48]. Here, fetal ASM exposed to hyperoxia display a greater $[Ca^{2+}]_i$ peak response to histamine than those exposed to normoxia alone. There is also evidence to suggest that soluble guanylate cyclase (sGC) activity may be affected by hyperoxia which can modulate SR Ca^{2+} loading [49], Ca^{2+} oscillations [50] and relaxation mechanisms [51, 52]. Additionally, hyperoxia increases secretion of neurotrophins, growth factors that promote nerve growth and differentiation but also found abundantly in the fetal airway [53–56]. Moderate hyperoxia stimulates secretion of BDNF, a neurotrophin, which acts in an autocrine/paracrine fashion by binding to its receptor, Trk-B, to promote activity of Ca^{2+} regulatory proteins including IP_3R and RYR, and Orai1 [47, 57]. These effects were found to be mediated by increased cAMP levels, which stimulate secretory pathways via Epac proteins [47]. Effects of hyperoxia on release of pro-contractile factors, such as BDNF, through cAMP-mediated mechanisms create an interesting dichotomy in ASM hypercontractility vs. relaxation, since cAMP is also involved in bronchodilation.

In addition to $[Ca^{2+}]_i$ and contractility, airway remodeling is another factor that contributes to airway narrowing and stiffening. The effect of hyperoxia on ASM proliferation and remodeling, especially in human models, is largely unexplored. Recent studies have examined the proliferative properties of developing human fetal ASM and their responsiveness to increasing levels of oxygen. These experiments indicate that fetal ASM proliferate in response to serum at a higher rate than adult ASM [45, 58], a result not unexpected given their importance in airway development. In fetal ASM, Hartman et al. assessed the effects different oxygen exposures (10–90% O_2) on fetal ASM proliferation. These studies observed increased fetal ASM proliferation following exposure to 30–50% O_2 [45]. These observations were confirmed by measuring protein expression of proliferation markers such as cyclin E and proliferative cellular nuclear antigen (PCNA) which steadily increased up to 40% O_2. Correspondingly, expression of an anti-apoptotic maker, BCL2, was also significantly elevated. However, once O_2 levels approached 60% and beyond there was a phenotypic shift towards apoptosis as indicated by increased expression of pro-apoptotic markers such as caspase 9, cytochrome C, and p27Kip1 [45]. One of the classic indications of cellular apoptosis is increased mitochondria fragmentation which is regulated by mitochondrial fusion/fission proteins, Mfn2 (fusion) and Drp1 (fission). Under normoxic conditions or moderate levels of hyperoxia the mitochondria have a more elongated, fusiform appearance and correspondingly express higher levels of Mfn2 and lower expression of Drp1. As O_2 levels increased beyond 40% the Mfn2:Drp1 ratio reversed [45]. As a result, mitochondrial fragmentation increased upon exposure to >60% O_2 where ASM apoptosis was observed. Together, these data indicate that in addition to promoting hypercontractility, hyperoxia has differential effects on the developing lung, depending on O_2 concentration. Whether at moderate (30–50% O_2) or high levels (>60% O_2), hyperoxia promotes conditions that alter ASM structure that can ultimately lead to airway dysfunction, via ASM remodeling or apoptosis.

In the normal developing lung, the organization of collagens play an important role in providing structure for the extracellular matrix (ECM) deposition. In airway remodeling, ECM deposition is increased causing the airway to stiffen. Aberrant ECM regulation in preterm infants contributes to diminished lung function, recurrent wheeze and asthma [28, 59]. Following

hyperoxia exposure, newborn mouse pups display thickened airways that are attributed to an increased ASM layer and collagen deposition [38, 39]. Analysis of the lungs via histological examination and mRNA expression confirmed increased production of collagen I and III, although, interestingly elastin was not altered [38].

Transforming growth factor-β (TGFβ) and connective tissue growth factor (CTGF) are among the factors that elevate ECM deposition in the lung. Both TGFβ and CTGF are increased in the airway of neonatal pups exposed to hyperoxia [38]. TGFβ increases proliferation, collagen deposition, and CTGF secretion in asthmatic adult ASM [60, 61]. Additionally, TGFβ promotes remodeling in fetal ASM [62]. Regulation of ECM composition requires an intricate balance between ECM protein production and degradation which is regulated by matrix metalloproteases (MMP) and tissue inhibitors of matrix metalloproteases (TIMP). Recent studies in fetal ASM show increases in collagen deposition, as well as MMP-9 activity following exposure to 50% O_2 [63]. Effects of 50% O_2 were attributed to reduced caveolin-1 expression, which has been shown to negatively regulate pro-proliferative and fibrotic signaling pathways [63]. Together, these data indicate that moderate hyperoxia significantly contributes to fetal ASM remodeling by enhancing proliferation, ECM deposition and remodeling. However, strategies, such as enhancing antioxidant pathways, to inhibit the effects of moderate hyperoxia on ASM remodeling have yet to be evaluated.

The airway epithelium serves as a protective barrier for the airway and plays key roles in modulating airway inflammation, remodeling, and contractility. The epithelium is vulnerable to hyperoxia exposure resulting in disruption of the barrier and infiltration of inflammatory mediators and fluid accumulation [64]. In vitro studies show hyperoxia stimulates the release of pro-inflammatory cytokines from airway epithelial cells which may influence airway remodeling [65, 66]. Animal studies using elevated levels of O_2 have been somewhat contradictory in terms of effects on the airway epithelium with respect to remodeling. Studies in mice show hyperoxia can increase epithelial layer thickness [32]. However moderate hyperoxia in neonatal rats found that remodeling is primarily in the airway smooth muscle and not the epithelial layer [67]. These differences could involve differential effects of oxygen concentration on the airway.

An important modulator of bronchoconstriction/dilation is nitric oxide (NO) which is generated by the airway epithelium and non-adrenergic non-cholinergic (NANC) nerves [68–71]. Clinically, inhaled NO has not been shown to be significantly effective in preventing BPD [40, 71–74], but there are indications that endogenously produced NO may promote bronchodilation [73]. Recent in vivo experiments in rat pups have shown that hyperoxia decreases NO production through reduction in nitric oxide synthase (NOS) activity [37, 75, 76] though underlying mechanisms still need to be determined. The contribution of arginases to disease pathophysiology, including lung disease, is being increasingly recognized [77–80]. Epithelial arginases have played a role in adult asthma pathogenesis [81–85], including diversion of L-arginine from NOS/NO pathway leading to impaired bronchodilation. Decreases in NO may also occur through reduced availability of L-arginine. There has been interest in L-arginine supplementation for treatment of adult asthma [82, 83, 85–88]. However, there is little information on arginase function in developing airway epithelium. Therefore, the overall role of the L-arginine-Arginase-eNOS pathway in modulating the neonatal airway is still to be determined.

3 Hyperoxia and Redox Signaling in the Pulmonary Vasculature and Alveoli

Proper formation and organization of venous and arterial microcapillary networks within alveoli are essential for effective gas exchange and nutrient distribution. The developing lung is sensitive to drastic shifts in redox homeostasis, thus maintaining an adequate redox balance is critical for normal pulmonary vascular and alveolar development [89]. Generation of vasculature, via

angiogenesis or vasculogenesis, is regulated by signaling mechanisms that involve redox-mediated signaling [90]. Postnatally, development of the pulmonary vasculature within saccular structures is critical for formation of alveoli, the site of oxygen absorption into pulmonary capillaries and carbon dioxide release [89].

Hyperoxia alters the redox balance, increasing oxidative stress in the lung and impairing vascular and alveolar development, which is the hallmark feature of BPD [91]. In BPD, hyperoxia hinders vascular development during alveologenesis resulting in reduced density of the alveolar capillary bed [92] and diminished alveolar surface area [13]. Alveologenesis is a process that begins after birth and extends well into adolescence, thus preterm infants with BPD also have impaired lung function that can persist into adulthood [93]. Given the structural and functional deficits of infants with BPD, it is important to understand how hyperoxia disrupts postnatal vascular and alveolar development. This section will review emerging concepts and mechanisms in the understanding of how redox signaling pathways and hyperoxia affects the microcapillary networks during alveolar development.

Hypoxia inducible factor (HIF) is a transcription factor that senses hypoxia within the cell and increases expression of important pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [94]. Early studies demonstrated that loss of HIF isoforms, HIF1α, HIF2α, or HIF3α, results in reduced alveolar growth [95]. Hyperoxia reduces expression of HIF2α and VEGF during postnatal days 9–12, a period of rapid alveolar growth in newborn rats [96]. More recently, studies using conditional HIF overexpression in the lung illustrate a more complex role of HIF in postnatal vascular and alveolar development. Overexpression of HIF1α in alveolar type II cells increased VEGF expression but did not improve hyperoxia-induced lung injury in newborn mice [97]. This is in contrast to studies showing overexpression of HIF1α during hyperoxia, via administration of adenovirus, increases expression of pro-angiogenic factors, e.g., VEGF, eNOS, and Tie2, to stimulate alveolar development and capillary formation [98]. These data suggest that HIF-mediated mechanisms play an important role in alveolarization under normoxic conditions. However, during hyperoxia, specific enhancement of HIF-VEGF signaling in the vasculature, but not the alveolar epithelium, may be required to overcome the adverse effects of hyperoxia on formation alveoli.

VEGF is a key factor for angiogenesis [99, 100], is secreted by alveolar type II cells and plays a central role in the development of alveolar capillary beds [101]. Inhibition of VEGF-VEGFR signaling in newborn mice impairs alveolar and vascular formation in the postnatal lung [95, 102, 103]. Similar to HIF, VEGF expression is reduced in newborn mice exposed to hyperoxia [96]. The downstream effects of VEGF include increasing NOS expression and surfactant production [104, 105]. Another mediator downstream of VEGF signaling, hepatocyte growth factor (HGF), was shown to enhance angiogenesis and alveolarization during hyperoxia [106]. However, the role of VEGF in the developing lung is complicated by studies in alveolar type II cells where VEGF overexpression causes lung injury [104, 107]. These data indicate that VEGF is important in postnatal development, but therapeutic administration of VEGF in infants with BPD would need to be performed cautiously to ensure increasing alveolarization without detrimental effects (e.g., promoting lung injury).

While HIF has a clear role in alveolar development, nuclear factor E2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that is important for adaptation to heightened oxidative stress [108]. Upon activation, Nrf2 translocates to the nucleus to upregulate expression and activity of antioxidant systems, including glutathione, thioredoxin, catalase, and superoxide dismutase [108]. Loss of Nrf2 augments newborn hyperoxic lung injury, resulting in less alveolarization and greater inflammation [109, 110]. Therapeutically targeting Nrf2, via thioredoxin reductase inhibition, was recently shown to activate Nrf2 [111, 112]. In newborn mice, inhibition of thioredoxin reductase improves hyperoxia-induced deficits in alveolarization [113]. For the neonatal lung, Nrf2 is an important regulator for adaptation to

hyperoxia, thus targeting Nrf2 may serve as a useful strategy to enhance endogenous antioxidant responses and boost alveolarization.

The nitric oxide (NO)-soluble guanylate cyclase (sGC)-cyclic GMP (cGMP) signaling cascade regulates multiple cellular processes in the lung including bronchodilation, vasodilation, angiogenesis, and inflammation [114]. Endothelial release of NO stimulates sGC to produce cGMP, which induces vasodilation [115]. In the developing lung, NO promotes angiogenesis [116], while lack of sGC disrupts alveolar development [117]. In the pulmonary vasculature, hyperoxia increases phosphodiesterase 5 (PDE5; degrades cGMP) activity and induces sGC degradation, via oxidation, in pulmonary artery smooth muscle cells [118]. Inhibition of PDE5 activity, leading to enhanced cGMP levels during hyperoxia improves alveolarization in newborn mice [119].

For the alveolar epithelium, hyperoxia disrupts NO–sGC signaling by inhibiting NO uptake by alveolar type II cells [120] and reducing NO-induced surfactant production by alveolar type II cells [121]. Administration of inhaled NO improves alveolarization in preterm lamb [40] and baboon [122] models of BPD. The benefits of inhaled NO are attributed to reducing hyperoxia-induced inflammation [123, 124] and enhancing VEGF expression [125]. Although beneficial effects of inhaled NO have been shown in preclinical models of BPD, use of inhaled NO therapy for preterm infants at risk of BPD has been relatively unsuccessful [52, 126]. Together these mechanisms emphasize the importance of NO–sGC signaling in both alveolar and vascular compartments.

In recent years, pharmacologically targeting sGC to promote postnatal lung development has emerged as an alternative to inhaled NO therapy and may be an attractive therapeutic option for infants with chronic lung disease [114]. sGC modulators are a class of drugs that stimulate sGC activity and increase cGMP levels independent of NO [127]. sGC modulators improve vascular function in neonatal lambs with pulmonary hypertension [118, 128]. Effects of sGC modulators on alveolar growth are highlighted by a recent study demonstrating improved alveolarization in cigarette smoke-exposed adult mice [129]. These data suggest that use of sGC modulators to boost cGMP levels could improve vascular function and alveolar development in preterm infants. However these ideas have yet to be explored.

In addition to NO, hydrogen sulfide (H_2S) and carbon monoxide (CO) are additional gasotransmitters recently shown to affect alveolarization. H_2S is endogenously produced by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). In the lung, both enzymes are expressed by multiple cell types, including pulmonary artery smooth muscle, endothelial, and airway smooth muscle cells [130]. Newborn mice with no CBS or CSE expression have reduced alveolarization and thicker blood vessels with greater α-smooth muscle actin staining compared to wild type mice [131]. Although the mechanisms that disrupt vascular development are unclear, loss of CBS impairs endothelial cell tube formation, in vitro. During newborn hyperoxia exposure, systemic H_2S administration improves alveolarization and vascularization, while reducing inflammation [132, 133]. Additionally, H_2S improved vascular function by preventing hyperoxia-induced pulmonary hypertension [133]. The protective effects of H_2S have been attributed to promoting alveolar type II cell proliferation [132] and increasing the antioxidant capacity of pulmonary artery endothelial cells [133]. However, these studies were performed in vitro and need to be confirmed in vivo.

Heme oxygenase-1 (HO-1) is an antioxidant enzyme that degrades heme into iron, bilirubin, and CO [134]. HO-1 is known to be protective in vascular and epithelial cells [135] and inhibit inflammation in models of lung disease, including chronic obstructive pulmonary disease and acute lung injury [136]. Expression of HO-1 peaks early in life and helps the neonatal lung adapt to the transition to a normoxic environment [137]. HO-$1^{-/-}$ newborn mice have larger alveolar spaces and reduced expression of surfactant protein [138]. Effects of newborn hyperoxia exposure are exacerbated further in HO-$1^{-/-}$ mice who have more apoptosis in alveolar epithelial cells [139]. HO-1 overexpression in alveolar type

II cells during hyperoxia protected newborn mice from vascular remodeling, but did not improve alveolarization [140]. Administration of CO during hyperoxia reduces lung inflammation and improves alveolarization [141]. These data show that HO-1 is important for postnatal lung development, while administration of CO could protect the lung during hyperoxia. Although there are potential protective effects of H_2S and CO for neonates at risk of BPD, further investigation into their physiological effects in human neonatal lung will be important to determine their therapeutic potential.

4 Summary and Conclusions

In this chapter, we discuss the complex role of hyperoxia-induced redox signaling in the neonatal lung. Recent studies reveal the importance of several redox signaling mechanisms, e.g., HIF and NO–sGC. By altering the redox balance, hyperoxia exposure disrupts this critical process in preterm infants, causing significant deficits in alveolar formation. Using transgenic animal models and in vitro models, studies have identified multiple pathways that could be potential targets for enhancing alveolarization in preterm infants. HIF-mediated pathways are critical for development of alveolar capillary beds and alveoli. Animal models suggest that this approach, for example VEGF administration, could be beneficial; however, these efforts have not been fully tested clinically. Additional therapeutic avenues such as enhancing endogenous Nrf2-induced antioxidant systems [142] or soluble guanylate cyclase [114] are other intriguing options. The above-described effects of hyperoxia on alveologenesis are summarized in Fig. 1.

Less is known about the role of hyperoxia and redox signaling mechanisms in airway development, which primarily occurs in utero (16–26 weeks gestational age). In the postnatal lung, redox mechanisms likely do play an important role given the redox sensitivity of contractile regulatory proteins [143] and ASM proliferation [144, 145]. Studies in developing ASM and newborn mice show that moderate hyperoxia (40–50% O_2) adversely affects developing ASM and airway epithelium to promote airway hypercontractility and remodeling. The underlying mechanisms by which moderate hyperoxia promotes airway dysfunction remains poorly understood. Studies in human fetal ASM suggest a role for

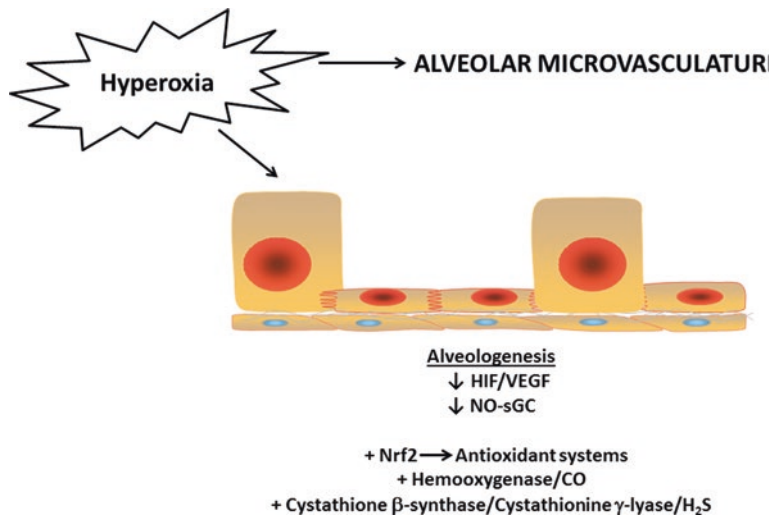

Fig. 1 Effect of hyperoxia on alveologenesis. The alveolar microvasculature is critical for alveologenesis. Hyperoxia diminishes alveologenesis by reducing HIF-VEGF and NO-sGC signaling. The transcription factor Nrf2, via antioxidant pathways, protects against the detrimental effects of hyperoxia and may additionally promote alveologenesis. CO and H_2S also promote alveologenesis. (+) indicates promotion of alveologenesis

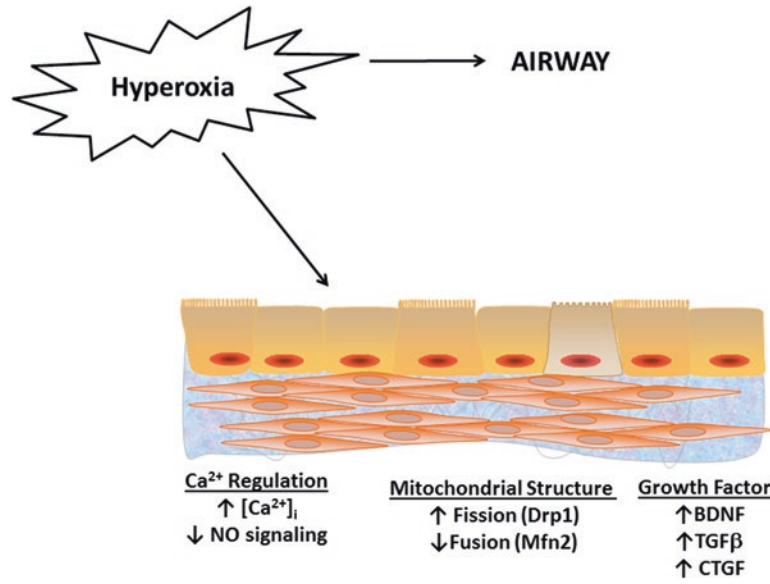

Fig. 2 Effect of hyperoxia on airways. Hyperoxia affects Ca^{2+} regulation by increasing $[Ca^{2+}]_i$, and reducing NO signaling. Hyperoxia shifts the equilibrium of mitochondrial structure by increasing fission and reducing fusion. Lastly, hyperoxia increases growth factors like BDNF, TGFβ, and CTGF. All these changes increase contractility, proliferation, and extracellular matrix deposition, which are all features in airway disease

$[Ca^{2+}]_i$ regulation [47, 48], caveolae [63], and mitochondria [45]. Investigations should aim to assess strategies to enhance bronchodilation and inhibit hyperoxia-induced remodeling. Here, stimulation the NO–sGC signaling axis has potential [48, 114]. The effects of hyperoxia on the airways are summarized in Fig. 2.

Redox signaling mechanisms have proven to be highly relevant to lung development and pathogenesis of neonatal lung disease. Maintenance of a homeostatic redox balance is critical, but unfortunately hyperoxia shifts this balance and creates an environment that favors lung disease in preterm infants. Novel therapeutic strategies for targeting neonatal lung diseases are needed for at risk preterm infants. In the developing lung, the roles of redox signaling are complex. They are important for normal postnatal lung development, contribute to neonatal lung disease, and serve as potential therapeutic avenues for treating alveolar deficits and airway disease.

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Lung Ischaemia–Reperfusion Injury: The Role of Reactive Oxygen Species

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1 Introduction

Ischaemia–reperfusion injury is a severe multifaceted pathological condition that becomes manifest in blood flow deprived and therefore oxygen (O_2)-starved organs/tissues after restoration of blood perfusion and reoxygenation [73]. This paradoxical increase of tissue damage in response to reoxygenation has steadily gained attention as an important component of numerous diseases [98]. A great variety of pathologies, including myocardial infarction [277, 278], ischaemic stroke [186], trauma [242, 251], surgical procedures such as organ transplantation and circulatory arrest [87] can be complicated by ischaemia–reperfusion injury [73, 122]. The initial disruption of arterial blood supply in these diseases can be triggered by an embolus, thrombus, rupture of an atherosclerotic plaque, surgical intervention, etc. and may lead to tissue/organ ischaemia, resulting in an undersupply of oxygen and nutrients that ultimately results in severe metabolic and molecular alterations in the affected tissues as well as in accumulation of toxic metabolites. The susceptibility to ischaemia differs between tissues/organs [122]; for example, the brain can survive for only a few minutes during ischaemia [145], while the lung is able to tolerate ischaemia for several hours [41]. The ischaemic phase primes the tissue for reperfusion injury by induction of vascular and endothelial dysfunctions [96]. Restoration of blood supply during the reperfusion phase, which is the only effective treatment to prevent ischaemia-induced irreversible injury, surprisingly, exacerbates the tissue injury [73] resulting in profound metabolic and cellular derangement, subsequently leading to cell death and end organ damage [84, 96]. It was suggested that the sudden reoxygenation of the previously ischaemic tissue/organs triggers a unique reperfusion injury via reoxygenation-induced cellular pathways [98]. In addition, reperfusion causes local as well as systemic inflammatory responses that impair local tissue/organ function and trigger a multi-organ dysfunction syndrome.

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Previously, the lung was considered to be relatively resistant to ischaemic triggers due to the fact that O_2 in the lung is supplied by a dual circulation through pulmonary arteries (pulmonary circulation) and bronchial arteries (bronchial circulation) as well as by the alveolar ventilation that exposes lungs to O_2-rich alveolar air [267]. However, several studies indicate a relevance of lung ischaemia–reperfusion injury (LIRI) in clinical practice including organ transplantation [61, 62, 267]. Reperfusion of the ischaemic lung evokes an increase of microvascular permeability and a vigorous immune response, and the subsequent development of pulmonary oedema can result in impaired oxygenation (hypoxaemia) [62].

Despite extensive research, the complete mechanisms and the essential signal transduction pathways underlying LIRI have not yet been fully identified and a specific treatment is not yet available. Additionally, as the transplantation of various organs including the lung has become a routine surgical procedure, strategies how to effectively prevent LIRI are absolutely necessary. Alterations in reactive oxygen species (ROS) concentration have been suggested as a key cellular signalling mechanism underlying LIRI, and inhibition of excessive ROS generation has been suggested as a therapeutic approach to prevent and/or treat LIRI. This concept arose from the fact that reperfusion injury is triggered by the reintroduction of molecular O_2 to the ischaemic lung, and ROS production is enhanced by increased O_2 availability [80, 98].

Against this background, we summarise here the major current evidence for the contribution of altered cellular ROS production to the development of LIRI and give an overview of approaches to target the cellular ROS alterations to limit or prevent LIRI.

2 The Causes of Lung Ischaemia–Reperfusion Injury

Reperfusion injury has been extensively studied in various organs including the brain [197], heart [278], kidney [136], skeletal muscle [187], liver [51], ovary [112], testicle [193], intestine [99] and lung [71, 192, 265, 267]. Despite some similarities including increased cellular apoptosis and necrosis, as well as impaired vascular function and permeability, there are some tissue specific differences in the manifestation of ischaemia–reperfusion injury reflecting their unique properties [98]. In this regard, the lung is a relatively ischaemia resistant organ which can survive in severe ischaemia for several hours [265].

The lung is the central organ of the respiratory system that is responsible for gas exchange across the air and blood interface. During the breathing process, O_2, which is a crucial substrate in cellular metabolism and bioenergetics [163], diffuses from the alveolar space into blood, while carbon dioxide (CO_2), which is produced mainly by cellular respiration, is released into the air (Fig. 1). Due to this primary function, the lung has a dual circulatory system [169, 170, 206, 208, 220, 260, 280]: (1) the pulmonary circulation which transports deoxygenated blood (approximately 99% of the total pulmonary blood flow) from the right ventricle of the heart through the pulmonary arteries to the pulmonary capillaries for gas exchange (also called “vasa publica”, as they serve for supply of the whole body) [137] and (2) the bronchial circulation which delivers the oxygenated blood (approximately only 1% of the total pulmonary blood flow [86]) for supply of the pulmonary structures including the trachea, bronchi, bronchial branches, pulmonary arteries, regional lymph nodes, visceral pleura etc. through bronchial arteries (also called “vasa privata” as they serve only for supply of the lung), which mainly arise directly from the descending aorta [137, 169, 190, 260] (Fig. 1). However the alveoli, including the respiratory epithelium, are exclusively supplied by the pulmonary circulation. Thus, in the past, the lung has been considered to be relatively resistant to ischaemia–reperfusion injury as the lung has a high availability of O_2 through both ventilation and dual blood circulation. However, increasing evidence indicates that lungs, like other organs, suffer from LIRI in situations that impair pulmonary blood flow through pulmonary arteries [61, 267]. The common

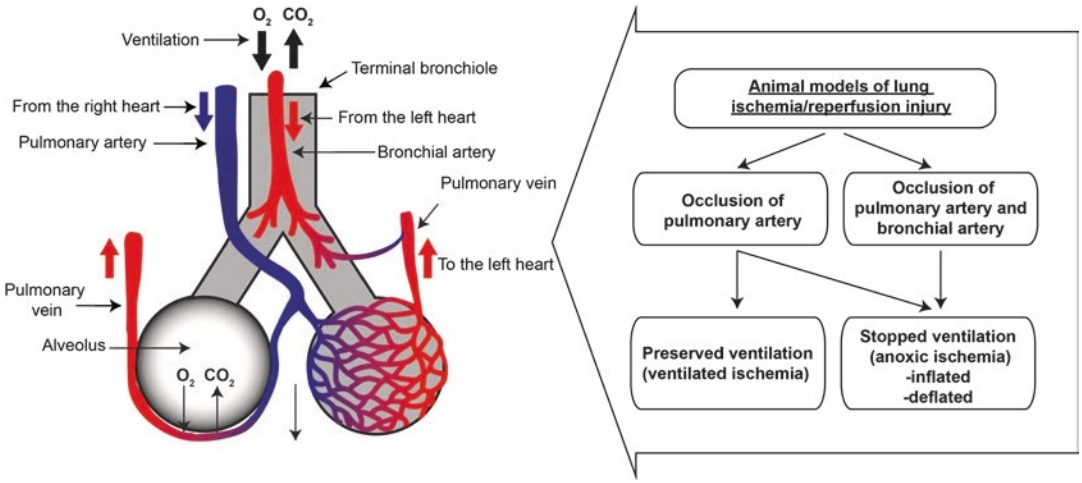

Fig. 1 The characteristics of lung perfusion and animal models of lung ischaemia–reperfusion injury. The lung has a dual circulation composed of (1) the pulmonary circulation which carries deoxygenated blood from the right heart to the alveoli through the pulmonary arteries for gas exchange and supply of the body and (2) the bronchial circulation which delivers oxygenated blood from the aorta to the anatomical structures of the lung including bronchi and regional lymph nodes through bronchial arteries. Deoxygenated blood from bronchial circulation drains into the pulmonary veins. Therefore, pulmonary veins which carry oxygenated blood to the left ventricle also carry a small amount of deoxygenated blood. There are two experimental setups of in vivo animal models of lung ischaemia–reperfusion injury in which ischaemia can be created either (1) by occlusion of the main pulmonary artery or its branches with preserved bronchial circulation or (2) by the clamping the hilum, which interrupts both pulmonary and bronchial circulation as well as ventilation. Regarding ventilation, there are also two experimental setups: (1) ventilated ischaemia, when ischaemia is accompanied by preserved ventilation and (2) anoxic ischaemia, when ischaemia is accompanied by stopped ventilation either in an inflated or deflated state

causes of occlusion are emboli, thrombi, the acute chest syndrome in sickle cell patients and surgical procedures [8, 11, 89, 115, 130, 146, 244]. The restoration of blood flow after occlusion of pulmonary arteries causes LIRI [267]. For example LIRI occurs in the early postoperative period in patients that underwent thromboendarterectomy for pulmonary emboli [115]. These patients develop noncardiac pulmonary oedema in areas of the lung subjected to thromboendarterectomy.

Unlike pulmonary occlusion by a thromboembolic event or surgical clamping, in case of lung transplantation LIRI in donor lungs is triggered not only by a complete interruption of blood flow in the lung through both pulmonary and bronchial arteries, but also by a complete cessation of ventilation. Therefore, LIRI during lung transplantation is also known as anoxic ischaemia [267]. Since 1962, when Hardy performed the first human lung transplantation, it has become the routine and only possible therapeutic option for extremely sick patients including those with severe pulmonary arterial hypertension and advanced interstitial lung diseases [181]. Every year the number of patients undergoing lung transplantation as well as the age of the recipient for the transplantation has steadily increased [181]. In this respect, 4,111 lung transplantations were carried out worldwide in 2013 [283] according to the International Society for Heart and Lung Transplantation (ISHLT). Two-hundred and ninety-six lung transplantations were performed in 14 German transplantation centres in 2015 according to the German Foundation for Organ Transplantation (DSO—Deutsche Stiftung Organtransplantation). Despite improving the lung transplantation procedure over years, posttransplantational complications including primary graft dysfunction and chronic lung allograft dysfunction are still a major concern [33–35, 241]. LIRI plays an important role in both of these conditions [81, 192]. Accordingly, LIRI is the main cause of pri-

mary graft failure [41] after lung transplantation which has a reported incidence ranging between 11 and 57% in single-institution studies and has a major impact on early mortality after lung transplantation [241].

LIRI as a result of sole occlusion of the bronchial arteries has not yet been investigated; however, some investigations show the relevance of the bronchial blood supply for the lung [101, 185, 249]. The interruption of the bronchial circulation in children with congenital heart diseases who experienced surgical stripping of their bronchial arteries for creation of a new pulmonary trunk leads to ischaemic necrosis of the airway mucosa developing bronchial stenosis, dehiscence, or loss of small airways [101]. Additionally, the airway clearance of inert insoluble particles was significantly impaired in experiments where the bronchial circulation was stopped [259]. Moreover, necrosis of airway mucosa and alterations of airway clearance may contribute to the chronic lung allograft dysfunction after lung transplantation without re-anastomosis of the bronchial arteries [249]. Therefore, it was suggested that restoration of the bronchial circulation could be beneficial in lung transplantation [249]. Indeed, bronchial artery revascularisation during a bilateral sequential lung transplantation reduces airway ischaemia, preventing the development of necrosis of the airway mucosa [101, 185, 249] and has clinical advantages including an increase of long-term survival as well as a decrease of the frequency of graft dysfunction [185, 249]. Despite the obvious beneficial effect of bronchial revascularisation following lung transplantation, ischaemic–reperfusion injury of tissues supplied via bronchial arteries could occur. However, the effect of reperfusion injury in this situation has not been investigated, yet.

Additionally, the lung, as other organs, can suffer from systemic effects of the ischaemia–reperfusion injury in various tissues located remotely from lung [212].

3 Animal Models of LIRI

Different in vivo [153], or in vitro models, such as the isolated lung [71] and lung slices [15], next to cellular models [50] of various animals including pigs [102], dogs [157], rabbits [71], rats [155] and mice [265], as well as investigations in humans [65], are widely used to study the molecular mechanisms underlying LIRI in the search for possible treatment options [62, 167]. In vivo, different experimental set-ups for LIRI have been suggested to reflect the various diseases [167]. Due to the dual circulation in the lung, there are two experimental set-ups of in vivo animal models of LIRI where ischaemia can be induced either (1) by clamping or by balloon occlusion [153] of the main pulmonary artery or its branches with preserved bronchial circulation or (2) by clamping the hilum to interrupt both pulmonary and bronchial circulation as well as to stop ventilation [265] (Fig. 1). Regarding ventilation, there are also two experimental set-ups: (1) ventilated ischaemia, when ischaemia is accompanied by preserved ventilation [71] or (2) anoxic ischaemia, when ischaemia comes along with the interruption of ventilation either in the inflated or deflated state [167, 265] (Fig. 1). Additionally, ventilated ischaemia can be divided according to the composition of the ventilated gas mixture: hypoxic (less than 21% O_2) or anoxic ventilation (0% O_2) [83, 290]. All these variables as well as the duration of the ischaemic and reperfusion phases have influence on the severity of LIRI. It has been shown that lung inflation is one of the most important factors affecting the severity of LIRI [167]. Reperfusion after 4 h of hilar occlusion with lung deflation results in pulmonary oedema and death, while inflation of the lung prevents LIRI development [211]. Not surprisingly, the duration of the ischaemic phase has an influence on the severity of LIRI and the longer it lasts the more severe is LIRI [167]. Additionally, there are species specific differences in the severity of LIRI, for instance, rabbits are more sensitive to LIRI in comparison to dogs [167].

4 Molecular Mechanisms of Ischaemia–Reperfusion-Induced Changes in the Lung

4.1 Cell Signalling Pathways Underlying LIRI

The severity of the ischaemic injury depends on the tissue specific susceptibility to ischaemia and the duration of the ischaemic period [84, 98]. Reperfusion of the ischaemic lung induces lung injury beyond that of the initial ischaemic damage and results in formation of noncardiogenic pulmonary oedema as well as acute lung injury (ALI) which is a severe clinical syndrome with substantial morbidity and mortality [118, 152, 167]. The cellular mechanisms underlying LIRI are multifactorial [80, 267] (Fig. 2). Ischaemia initiates a decrease in ATP concentration [82], a switch from aerobic to anaerobic cellular metabolism [82, 88] and an increase of intracellular calcium influx [265]. The fast decrease of ATP concentration leads to the accumulation of degradation products of ATP including hypoxanthine [98], which later is used to produce ROS by the xanthine oxidase (XO) during reperfusion [246]. Diminished ATP also limits the function of various ATP-dependent cellular ion pumps [80], which results in intracellular accumulation of various ions including sodium and calcium [122]. The accumulation of sodium can disturb the balance of water flow across the cellular membrane [61], while the increased concentration of intracellular calcium activates several calcium-sensitive enzymes, and thereby leads to vasoconstriction and degradation of membrane phospholipids [192]. Additionally, an ischaemia-induced increase in calcium concentration can occur via pathways other than through ATP-depletion, e.g., via NOS2-mediated ROS release in mice [265]. Ion imbalance in mitochondria can lead to their swelling and activation of apoptosis [80] (Fig. 2). The switch to anaerobic metabolism results in an accumulation of lactate and decrease of cellular pH [82, 88]. Besides its direct cytotoxic effects [273], the accumulation of hydrogen ions contributes to an increase of intracellular sodium via activation of the Na^+/H^+ exchanger [122]. Although reperfusion restores the delivery of O_2 and nutrition as well as washes out the toxic metabolites and normalises pH, it leads to severe reperfusion damage. The mechanism of the reperfusion injury is complex and mostly involves increased generation of ROS (which is discussed later) and a vigorous inflammatory response [123]. For example, upon reintroduction of blood to the ischaemic tissue, blood delivers also leukocytes which could exacerbate tissue injury releasing pro-inflammatory cytokines and ROS after their activation by damaged tissue [123]. Moreover, reperfusion initiates the metabolism of the accumulated waste products such as hypoxanthine [246] and succinate that both can increase ROS generation [48]. In conclusion, despite the diversity of cellular events underlying LIRI, alterations in ROS levels could play a key role in LIRI, especially during the reperfusion phase [80]. Therefore, this review focuses on the role of increased ROS concentration for molecular mechanisms initiating LIRI.

4.2 LIRI-Induced Oedema Formation

Increased endothelial permeability is a main cause of LIRI-induced oedema formation. LIRI induces an increase of endothelial permeability via: (1) cellular calcium overload, (2) activation of the immune response, (3) enhancement of platelet aggregation, (4) gap formation in the endothelial layer and (5) increase of pulmonary vascular resistance (PVR). Additionally, both the rise of epithelial permeability and the decreased ability of the alveolar epithelium to actively clear alveolar fluid contribute to the increase of extravascular water accumulation [261].

The LIRI-induced injury of various lung cells initiates increased expression and release of signalling factors (e.g. ROS [265]) and disruption of the ionic cellular homeostasis [243] that can lead to the intracellular calcium overload. The increased intracellular calcium concentration could contribute to an increase of microvascular permeability in various experimental models of LIRI via: (1) an increase of vasoconstriction

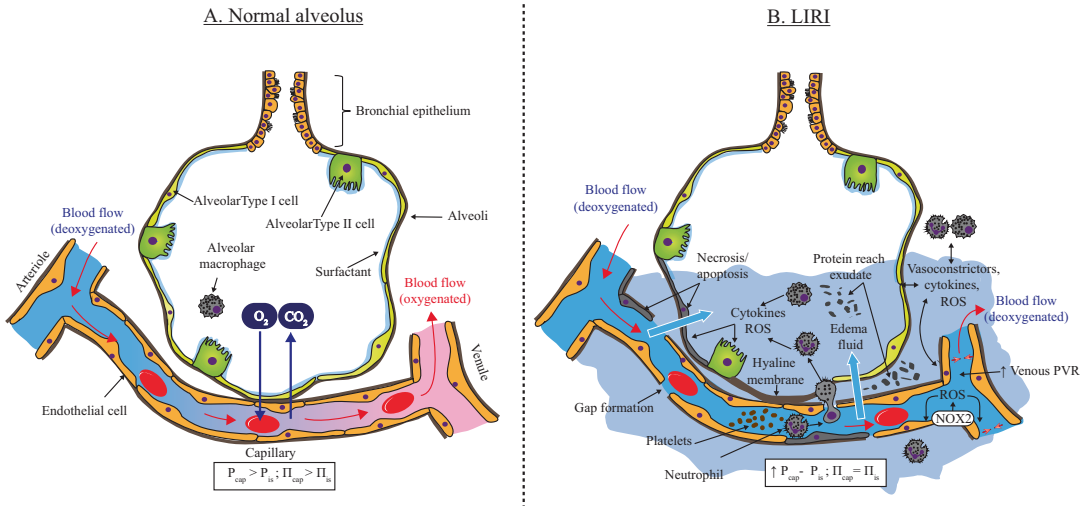

Fig. 2 The mechanism of lung ischaemia–reperfusion injury. (**a**) Theory of alveolar–capillary region. During the breathing process, oxygen (O_2) diffuses through the alveolar–capillary membrane from the alveolar space into the blood, while carbon dioxide (CO_2) is released into air. The alveolar–capillary membrane is composed of an endothelial cell monolayer at the capillary site of pulmonary circulation, an alveolar epithelial cell monolayer from the alveolar site with their basement membranes fused into a single layer. (**b**) Lung ischaemia–reperfusion injury (LIRI). Ischaemia–reperfusion of the lung triggers a cascade of various cellular events initiating increased microvascular permeability leading to interstitial and alveolar oedema. LIRI induces augmented microvascular permeability via increased: (1) immune response, which is characterised by the activation of alveolar macrophages (not shown) and recruitment of neutrophils, (2) platelet aggregation and (3) level of apoptosis/necrosis. Activated macrophages, recruited neutrophils, aggregated platelets and dysfunctional/damaged lung cells release ROS and inflammatory mediators that increase microvascular permeability. For instance, the increased production of nicotinamide adenine dinucleotide phosphate-oxidase (NOX2)-derived ROS in endothelial cells is suggested to be an important cellular signal that orchestrates endothelial dysfunction and the rise of endothelial permeability via increased intracellular calcium concentration. Increased calcium concentration could initiate gap formation in the endothelial layer. The increased microvascular permeability causes extravasation of a protein rich fluid into the interstitial space that eradicates the transvascular osmotic (or oncotic) pressure difference that further increases water leakage into the extravascular space ($\Pi_{cap} = \Pi_{is}$). The imbalance between vasodilators and vasoconstrictors as well as the accumulation of inflammatory mediators lead to a rise of the venous (postcapillary) pulmonary resistance (PVR) that elevates the transcapillary hydrostatic pressure ($\uparrow P_{cap} - P_{is}$) contributing to pulmonary oedema formation. Together these cellular events can result in gas exchange disturbance leading to severe systemic hypoxemia. Morphologically, LIRI-induced oedema is characterised by neutrophil infiltration, hyaline membrane deposition and a protein-rich exudate in both interstitial and alveolar spaces. *Abbreviations*: H_2O_2 hydrogen peroxide, CO_2 carbon dioxide, O_2 oxygen, *NOX2* nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase 2, *PVR* pulmonary vascular resistance, *ROS* reactive oxygen species, P_{cap} hydrostatic pressure within capillary lumen, P_{is} hydrostatic pressure within interstitial space, Π_{cap} protein osmotic (or oncotic) pressure in capillary lumen, Π_{is} protein osmotic (or oncotic) pressure in interstitial space

[218], (2) changes in the cellular shape [265], (3) widening of the interendothelial junctions and gap formation [92] and (4) triggering of apoptosis [114]. For example, the accumulation of mitochondrial calcium can induce the release of pro-apoptotic factors via both a rupture of the mitochondrial membrane during swelling of mitochondria and opening of mitochondrial permeability transition pores [123]. Accordingly, the amount of apoptotic pneumocytes was negatively correlated to the quotient of arterial O_2 partial pressure and fractional inspired O_2 (PaO_2/FiO_2) in arterial blood, while it was positively correlated to the severity of oedema formation during LIRI in an experimental canine model of pulmonary thromboembolism [63]. Therefore, application of the calcium blockers nifedipine, diltiazem [127] or verapamil [202] prevented endothelial

damage after reperfusion [202] and oedema formation [127].

Activation of apoptosis and necrosis can also occur via other mechanisms such as increased ROS [80] and immune response during LIRI [234, 291]. LIRI activates sterile innate [139, 204] and adaptive immune responses [274] which are mainly driven by enhanced generation of chemokines and cytokines (e.g. tumour necrosis factor alpha, TNF-α and interleukins, IL1β, 2 and 8) by lung cells such as EC, alveolar type II cells, vascular smooth muscle cells and resident macrophages [141, 227]. The increased response of the innate immune system, which is the first line of defence against pathogen invasion during LIRI, is characterised by neutrophilic infiltration [139] of extravascular and alveolar spaces and activation of resident macrophages [204] (Fig. 2). Therefore, depletion of alveolar macrophages [204] or treatment with a neutrophil-specific depleting antibody [139] significantly attenuates LIRI. This increased innate immune response in LIRI can be mediated by the Toll-like receptor-4 (an innate immune receptor), as mice that carry a point mutation in TLR4 [204] as well as TLR4 knockout [230] are protected against LIRI. LIRI-induced transmigration of activated leukocytes into the extravascular space promotes the increase of microvascular permeability through release of toxic ROS, proteases and elastases, as well as through the increase of gap formation in the endothelial layer [72]. For example neutrophil adhesion to the endothelial lining triggers leukocytic β2 integrin-mediated release of the protein azurocidin 1 which induces calcium-dependent cytoskeletal rearrangement and intercellular gap formation in endothelial-cell (EC) monolayers [92]. Moreover, LIRI-induced inflammation mediates increased necrosis and apoptosis of pulmonary cells [234, 291] via increased levels of pro-inflammatory cytokines, such as TNF-α and IL1β, 2 and 8 that contribute to pulmonary dysfunction [183] (Fig. 2). In line with these observations, application of a TLR4 inhibitor reduced the LIRI-induced rearrangement of the cytoskeletal actin and gap formation [285].

In addition, the LIRI-induced tissue injury exposes many cellular proteins and signalling factors (e.g. cytokines) to the blood stream, where they initiate platelet aggregation and coagulation [80, 192, 267]. Activated platelets adhere to the pulmonary endothelium initiating microvascular constriction, microthrombus formation, leukocyte adhesion to the vascular wall and release several vasoactive mediators such as serotonin, thromboxane A_2, platelet activating factor and ROS, thus, contributing to oedema formation [66, 80, 192, 267] (Fig. 2).

There is no doubt that reperfusion during LIRI induces the increase of pulmonary vascular resistance (PVR), but the specific contribution of the arterial or venous vessels to this increase is under debate. Although different groups have reported an increased PVR during LIRI, Löckinger et al. did not find an increase of pulmonary capillary pressure in isolated rabbit lungs [151], suggesting that LIRI induced the increase of the arterial pressure, while, in contrast, Luh et al. described an increased pulmonary capillary wedge pressure in an in vivo LIRI model in pigs [156]. Moreover, the pulmonary capillary pressure was increased in pigs which underwent a lung autotransplantation [233]. In this study, reimplantation of the caudal lobe and its reperfusion for 30 min resulted in significantly increased pulmonary capillary pressure, lipid peroxidation metabolites, release of proinflammatory cytokines and chemokines (TNF-α, IL-1β and monocyte chemoattractant protein-1 (MCP-1)) as well as neutrophil activation [233]. Moreover, it has been well documented that in ALI which is one of the major complications of LIRI, the imbalance between vasodilators and vasoconstrictors including the accumulation of inflammatory mediators increases the PVR, mostly of the venous (postcapillary) site [90]. Accordingly, various inflammatory mediators such as leukotrienes and histamine have been demonstrated to selectively increase the venous PVR [90], while inhaled nitric oxide has decreased the pulmonary capillary pressure by lowering the venous PVR [19]. Both, increased pulmonary arterial and venous pressure can lead to oedema formation. Increased pulmonary venous pressure raises the transcapillary hydrostatic pressure, which is the difference between the hydrostatic pressure within the

capillary lumen and the hydrostatic pressure in interstitial space ($P_{cap} - P_{is}$, respectively), resulting in oedema formation [90, 178] (Fig. 2). The increased arterial pressure could be accompanied by unevenly distributed blood flow within the lung resulting in hypercirculation in a portion of the capillaries and thus exposure of them to very high hydrostatic pressures and promoting water leakage [178].

In addition, the increase of microvascular permeability can culminate in extravasation of a protein rich fluid into the interstitial and alveolar space, which then eliminates the transcapillary protein osmotic (oncotic) pressure difference (the interstitial protein osmotic pressure (Π_{is}) becomes identical to intravascular protein osmotic pressure (Π_{cap})) [178]. The decrease of the transcapillary protein osmotic pressure ($\Pi_{cap} - \Pi_{is}$), which retains water in the blood vessels, further increases water leakage into the extravascular space (Fig. 2).

The interaction between the capillary pressure, the protein osmotic pressure and the microvascular permeability, which define pulmonary oedema formation, is described by Ernest Starling's equation:

$$\text{Rate of filtration of fluid} = K_f \left(\left[P_{cap} - P_{is} \right] - \sigma \left[\Pi_{cap} - \Pi_{is} \right] \right)$$

where K_f is the coefficient which characterises the hydraulic conductivity and filtration surface area, and σ is the endothelial permeability for proteins [90, 178]. LIRI induces the shift of the Ernest Starling's equation in favour of oedema formation, as described above.

Finally, the LIRI-induced oedema results in gas exchange abnormalities and causes ventilation/perfusion mismatches with an increased shunt fraction that leads to severe systemic hypoxemia [151, 267]. The "no-reflow phenomenon" [180] and formation of hyaline membranes [189] also contributes to systemic hypoxemia. The "no-reflow phenomenon" can occur after prolonged ischaemia and is characterised by significant microvascular damage that leads to heterogeneity of blood flow distribution with persistent obstruction of vessels and subsequently results in ischaemia despite reperfusion [61, 80]. Besides systemic hypoxemia, LIRI induces various systemic effects affecting different organs such as the heart and liver by activation of neutrophil sequestration and increased release of significant amounts of ROS into the circulation [77]. Furthermore, Palazzo et al. have demonstrated that LIRI of one lung can lead to similar, but less severe injury in the contralateral nonischaemic lung in a canine model, proving systemic effects of LIRI [196].

5 The Role of ROS for the Molecular Mechanisms Underlying LIRI

The term ROS comprises highly reactive free oxygen radicals such as a superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($HO^{\bullet}$), and non-radicals like hydrogen peroxide (H_2O_2) [18]. Previously, ROS were considered only as "toxic" injuring agents related to the development and/or progression of numerous diseases. However, nowadays, more evidence indicates that ROS also act as essential cellular signalling molecules in various important physiological pathways [56, 177]. In low physiological concentrations, ROS regulate diverse cellular events, such as differentiation [144], proliferation [195], migration [223] and secretion [97]. In contrast, excessive pathological ROS production or release and/or insufficient ROS removal by intracellular and extracellular antioxidant systems results in different pathologies and diseases [221], including LIRI [80, 192, 265].

Several cellular and extracellular systems including the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) [173], xanthine oxidases (XO) [104], cyclooxygenases, cytochrome P450 enzymes [284], lipoxygenases [46], NO synthases (NOS) [219] and mitochondria [236] are capable of producing ROS. They are localised in various cellular compartments, including the plasma membrane [20, 23, 24, 68, 134, 250, 286], nucleus [47, 95] and endoplasmatic reticulum [142, 286]. Due to the short half-life of ROS, especially $O_2^{\bullet -}$, their signalling

functions are likely to be controlled by their local subcellular release as well as by the local subcellular environment [164]. Additionally, extracellular generation and release of more stable ROS into the circulation may have direct effects on neighbouring cells [74] or on various remote organs [77]. All of these cellular ROS sources have been intensively studied with regard to their contribution to the molecular mechanisms underlying LIRI [80, 192].

Mitochondria and NOXs are the main sources of $O_2^{\bullet-}$ [57] which converts spontaneously or is processed by superoxide dismutases (SOD1–3) to H_2O_2. In contrast to $O_2^{\bullet-}$, H_2O_2 is a stable signalling molecule which easily passes through biological membranes and plays a key role in various physiological as well as in pathological signalling pathways via oxidation of cysteine residues [13, 124]. Furthermore, H_2O_2 converts into $HO^{\bullet}$ by the Fenton reaction. $HO^{\bullet}$ induces oxidative damage of proteins by their carbonylation [268]. In addition, $O_2^{\bullet-}$ reacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), which belongs to the reactive nitrogen species (RNS) [271]. $ONOO^-$ easily penetrates through cellular membranes, and it can modify numerous lipids, DNA [9, 43] and proteins via the nitration of tyrosine and tryptophan residues as well as S-nitrosation of cysteine residues [106, 119, 258]. $O_2^{\bullet-}$ also has direct effects on haem-containing enzymes by interaction with their iron sulphur clusters [91]. The ROS-defence system comprises several enzymes specialised in the detoxification of ROS and non-enzymatic antioxidants [177]. Most importantly, as written above, SOD converts $O_2^{\bullet-}$ into H_2O_2 that is further deactivated by catalase to water and O_2 [12, 177]. Furthermore, H_2O_2 can be deactivated by several glutathione peroxidases (GPx1–8) to reduced glutathione and water and by the peroxiredoxins (PRDX1–6) to water [12].

As described above, the cellular mechanisms underlying LIRI are multifactorial and the complete understanding of these pathways is still lacking [80, 192]. ROS have long been suggested to be signalling molecules in LIRI-induced cellular mechanisms, especially during reperfusion, when tissue is exposed to high levels of O_2 [80]. However, the cellular source of this increased ROS is still under debate. Numerous evidence exist that ROS are increased in the reperfusion phase of LIRI [71, 80, 192] including reports which detected increased ROS levels during LIRI [214, 290] or studies that demonstrated the effectiveness of ROS scavengers for treatment or prevention of LIRI [1, 85, 93, 111, 150, 207, 209]. For instance, 1 h reperfusion entails a significant increase of both $O_2^{\bullet-}$ and $ONOO^-$ levels measured by electron spin resonance and by observation of tyrosine nitrosation in lung tissue, respectively [94]. While seeming paradoxical at first, several investigators also observed that oxidative stress during ischaemia commences before reperfusion [131, 265, 293]. In this regard, a considerable molecular O_2 concentration was still present in the heart after reduction of O_2 delivery by about 90% [17]. This residual molecular O_2, which is also present in the early phase of anoxic ischaemia or during hypoxic ischaemia, is a critical element for ROS generation as the ischaemia-mediated impairment of the mitochondrial electron transport chain can facilitate the conversion of residual O_2 into $O_2^{\bullet-}$ due to the fact that reduced components of the respiratory chain are prone to transfer electrons to O_2 under specific conditions [17, 292].

Among various potential sources of ROS or RNS within cells, NOXs [265, 275, 282], XO [3, 4, 100, 129, 160, 290], NOS [71, 85, 117, 270] and mitochondria [149, 226, 238] play the most important role in LIRI. Therefore, the role of these cellular ROS sources for the development of LIRI is given below in detail. Ischaemia–reperfusion of the lungs increases ROS production mostly in EC, alveolar type II cells, vascular smooth muscle cells and macrophages [61, 192]. The LIRI-induced increase in ROS triggers alteration of the activity of various enzymes and ion channels [265], increase of lipid membrane peroxidation [113], DNA damage [123] and activation of transcriptional factors [117], resulting in direct damage of cells [123] or in activation of various cellular signalling pathways underlying LIRI [80, 265] including apoptosis [183], calcium overload [265] and increase of innate immune response [222]. For example, the

peroxidation of lipids in the cellular membrane directly leads to its damage and therefore to increased cellular permeability [38], while NOX2-mediated ROS increase endothelial permeability not directly but via increased calcium influx into EC (see details below) [265]. Additionally, the ROS-mediated promotion of innate immunity during LIRI contributes to the rise of microvascular permeability and therefore oedema formation as discussed above. For instance, H_2O_2 could promote the activation of innate immunity [230] by increasing the surface level of TLR4 in macrophages [203] or by increasing the synthesis of platelet-activating factor by the endothelium which initiates adhesion of platelets and neutrophils [147]. Moreover, ROS mediate the activation of alveolar macrophages triggering a release of pro-inflammatory cytokines including interleukins (IL-8, IL-12 and IL-18) and tumour necrosis factor alpha (TNF-α), which impairs lung function and triggers recruitment of neutrophils [222]. Besides releasing pro-inflammatory cytokines, these recruited neutrophils [269] and activated alveolar macrophages [232] generate additional ROS. Thus, the LIRI-induced increase of ROS creates a self-perpetuating mechanism where ROS can activate the immune system which in turn further increases ROS release. ROS also contribute to the increase of PVR via a release of vascular vasoconstrictive and decrease of vasodilative factors. In this regard, increased ROS during LIRI induces mobilisation of arachidonic acid which is metabolised to produce the vasoconstrictive substance thromboxane [80], whereas it decreases the concentration of the vasodilative molecule NO via increased NO consumption during $ONOO^-$ formation [80]. Furthermore, ROS could activate apoptosis of lung cells in LIRI via both intrinsic and extrinsic pathways [80, 183] that contribute to the pulmonary dysfunction and lung oedema formation [288].

Taken together, these ROS-mediated cellular events induced oedema formation during LIRI [192], while the effective removal of excessive ROS could protect against LIRI in animal models [224]. Therefore, antioxidative therapy could be one approach to treat LIRI [1, 5, 111, 150, 207, 209]. Furthermore, alterations in the ROS level can play a key role during ischaemic preconditioning [253, 254] which is a therapeutic approach based on the observation that a brief exposure to ischaemia prior ischaemia–reperfusion attenuates LIRI [60].

5.1 The Role of Mitochondria in LIRI

Since 1966, when the first report demonstrated that mitochondria can produce ROS [116], these organelles have been considered to be the most important source of ROS within a cell during various physiological as well as pathological conditions [39, 236]. Most of the O_2 consumed by mammalians (approximately 90–95%) is utilised to supply cellular energy through oxidative phosphorylation [10, 210], while only ~0.2% to 1–2% of the O_2, which is consumed by the mitochondrial electron transport system, is used to produce ROS [14]. Thus, a small number of electrons can "leak" to O_2 forming $O_2^{\bullet-}$ during ATP production by mitochondria. The specific sites of electron leakage are not precisely known, but most probably complexes I and III of the respiratory chain are predominantly responsible for $O_2^{\bullet-}$ release during oxidative phosphorylation [12]. Complex I produces and releases $O_2^{\bullet-}$ only into the matrix of mitochondria, while complex III can release $O_2^{\bullet-}$ to both sides of the inner mitochondrial membrane [176]. Within the mitochondrial matrix concentrations of $O_2^{\bullet-}$ in the range from 10 to 200 pM in different experiments were detected [177]. This wide variance in mitochondrial $O_2^{\bullet-}$ concentration can be explained at least by: (1) the extremely short half-life of ROS, (2) significant variation in ROS release between mitochondria originating from different species, tissues and subcellular compartments [237] and (3) the discrepancy in methodological approaches used to measure ROS such as differences in materials and location (measurement of ROS in vivo, in isolated cells, in isolated mitochondria), duration of measurements and probes used to measure ROS (HPLC, fluorescence- or luminescence-based approaches etc.) [164]. In

summary, mitochondria, in addition to their primary function to produce ATP, participate in various cellular signalling pathways including the initiation of apoptotic cell death, regulation of cellular calcium homeostasis and in generation and detoxification of cellular ROS which is the focus of this review.

For a long time mitochondria have been considered a primary ROS source in the cellular processes underlying ischaemia–reperfusion injury in various organs and tissues [98] including the lung [238] (Fig. 4). Not surprisingly, the ischaemic phase of LIRI reduces mitochondrial activity and mitochondrial viability [226] due to O_2 deprivation in rat lungs. It has been demonstrated that ischaemia decreased NADH/FAD (reduced nicotinamide adenine dinucleotide to flavin adenine dinucleotide) autofluorescence [226], and impaired the function of complexes I, II and III [107, 238] as well as mitochondrial biogenesis [276]. But surprisingly, the decrease of mitochondrial function was accompanied by mitochondrial membrane hyperpolarisation (increase of mitochondrial membrane potential ($\Delta\psi_m$)) measured by a fluorescent dye called JC1 during ischaemia in rats [238]. However, the increase of $\Delta\psi_m$ has been described in experiments in which mitochondria were isolated from the ischaemic rat lung, and were exposed to normoxia during measurement [238]. Consequently, it is possible that increase of $\Delta\psi_m$ in this study was due to re-exposure of mitochondria to O_2 but not induced by ischaemia itself. In contrast, ischaemia–reperfusion of the rabbit lung resulted in a decrease of $\Delta\psi_m$ in isolated mitochondria measured by rhodamine 123 [207]. In addition to the different methodological approaches regarding the fluorescent dye used to measure $\Delta\psi_m$, this discrepancy should be further addressed. Nevertheless, $\Delta\psi_m$ hyperpolarisation obviously plays a crucial role in brain ischaemia–reperfusion injury [108, 121, 215]. Hüttemann et al. have suggested an elegant theory which can explain the cellular mechanisms of ischaemia–reperfusion-induced $\Delta\psi_m$ hyperpolarisation in the brain [108, 121, 215]. Briefly, the ischaemia-mediated increase of the inner mitochondrial calcium concentration activates mitochondrial phosphatases that dephosphorylate key enzymes of the respiratory chain, which results in an increase of their activity during reperfusion. Increased mitochondrial chain activity leads to $\Delta\psi_m$ hyperpolarisation which promotes the increase of mitochondrial ROS generation. Although this mechanism has not been shown during LIRI, given the general direct relationship of $\Delta\psi_m$ and mitochondrial ROS production, mitochondrial hyperpolarisation during LIRI also might enhance ROS production [135, 140, 225].

Recently, a new hypothesis has been proposed to explain the increase of mitochondrial ROS release during myocardial ischaemia–reperfusion injury in mice [48, 49]. In this study, the fumarate overflow from purine nucleotide breakdown and partial reversal of the malate–aspartate shuttle in the ischaemic phase led to a reversed function of the succinate dehydrogenase which induces a selective accumulation of the citric acid cycle intermediate succinate [48]. After reperfusion, the accumulated succinate is rapidly re-oxidised by the succinate dehydrogenase that extensively increases ROS generation by reverse electron transport at mitochondrial complex I [48]. The pharmacological inhibition of the ischaemic succinate accumulation by dimethyl malonate ameliorated the in vivo ischaemia–reperfusion injury in the heart [48]. Moreover, inhibition of complex I by rotenone or by S-nitrosation also protected against cardiac ischaemia–reperfusion injury, proving that complex I can be the primary source of increased ROS in this pathology [200]. If this mechanism also occurs in LIRI, it should be further investigated.

Moreover, complex III of the respiratory chain is the most prominent source of increased ROS during hypoxia in pulmonary artery smooth muscle cells isolated from mice [262, 263]. Deletion of Rieske iron–sulphur protein (RISP) subunit in complex III in mice abrogated the hypoxia-induced increase of ROS and cytosolic calcium concentration in isolated pulmonary artery smooth muscle cells, as well as attenuated the acute hypoxia-induced increase in right ventricular systolic pressure in vivo [262]. It may be that hypoxia and ischaemia share similar mechanisms which could alter mitochondrial function, leading to increased mitochondrial ROS generation by complex III.

In addition, the mitochondria could be a target of increased oxidative stress in LIRI. In this regard, mass spectrometry of heart tissues after ischaemia–reperfusion in mice has revealed that mitochondrial proteins such as the 24 and the 30 kDa subunits of complex I, the Rieske iron–sulphur protein of complex III, the alpha subunit of the ATP synthase and the voltage-dependent anion channel are the major targets of $ONOO^-$-mediated nitration [149]. Nitration of mitochondrial proteins can contribute to the suppression of mitochondrial respiration [149]. Moreover, preconditioning with the antioxidant glutathione improved mitochondrial viability and normalises $\Delta\psi_m$ hyperpolarisation in LIRI [238]. All of these data suggest that a reciprocal interaction is possible, where an increase of intracellular or mitochondrial ROS concentration can directly or indirectly modify mitochondrial function and in turn regulate mitochondrial ROS production. However, more studies dedicated to this subject, also in view of a possible self-perpetuating interaction of mitochondria and other cellular ROS sources, are needed.

In terms of therapeutic relevance of mitochondrial ROS in LIRI, recently mitochondria-targeted antioxidants such as mito-tempo [184], MitoQ [235] and XJB-5-131 [76] have become available. Unfortunately, these antioxidants have not yet been tested in LIRI. However, the administration of MitoQ to the donor heart protected against ischaemia–reperfusion injury in mice [59], and XJB-5-131 treatment improved post-ischaemic–reperfusion recovery of aged rat hearts [76].

Besides changes in ROS release, mitochondria could participate in cellular mechanisms underlying LIRI via activation of apoptosis/necrosis [132] and modulation of cytosolic calcium concentration [30].

5.2 The Role of NADPH Oxidases in LIRI

NOXs are the only known enzymes that generate ROS as their primary function [18, 23, 26, 257]. Therefore NOXs are called "professional" producers of ROS [7]. NOXs are membrane-bound multi-subunit complexes enzymes, which catalyse the transfer of electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to molecular O_2 and thereby produce ROS [18, 182]. Until now, seven NOX isoforms have been identified in mammals: NOX1–5 and Dual oxidases 1/2 (DUOX) [6, 25, 68, 182]. The expression of NOXs has been found in different cellular structures, such as the endoplasmic reticulum [142], nuclei [47, 166, 171], perinuclear endosomes [42, 148, 172], focal adhesion regions [159, 231] and possibly mitochondria [37] which could explain the multiplicity of the NOXs functions within the cell [164]. Moreover, NOXs are widely expressed in pulmonary cells including vascular (ECs, smooth muscle cells and fibroblasts) [164], immune (neutrophils and macrophages) [198] and alveolar type II cells [256]. NOX1, 2, 3, 5 produce $O_2^{\bullet-}$ [18, 174, 182], while NOX4, and DUOX 1 and 2 are H_2O_2-producing enzymes [255].

The important role of NOXs-mediated ROS in LIRI has been proven in numerous studies [265, 275, 282]. The increased extracellular release of H_2O_2 from EC previously exposed to hypoxia for 24 h has been markedly reduced by applications of the flavoproteins and the unspecific NOXs inhibitor diphenyliodonium in a dose-dependent manner [294]. Apocynin, which can inhibit NOXs in myeloperoxidase (MPO)-dependent manner, prevented LIRI in sheep [67] and mouse lungs [265]. Regarding the isoform of NOXs which is responsible for LIRI, our group has demonstrated that NOX2-derived ROS plays a crucial role in LIRI via interaction with the canonical transient receptor potential channels 6 (TRPC6) [265] (Fig. 3). In this study, the global deletion of the catalytic subunit of NOX2 (previously known as $gp91^{phox}$) as well as the global TRPC6 knockout prevented LIRI in isolated mouse lung experiments as well as in in vivo mouse models of LIRI, while global TRPC1, TRPC4, NOX1 and NOX4 knockout mice exhibited no effect on LIRI formation. To test whether NOX2 and TRPC6 in leukocytes or in EC were responsible for the protective effect, chimeric mice were created by transplanting bone marrow from NOX2 knockout mice into lethally irradiated wild type mice (NOX2 KO to

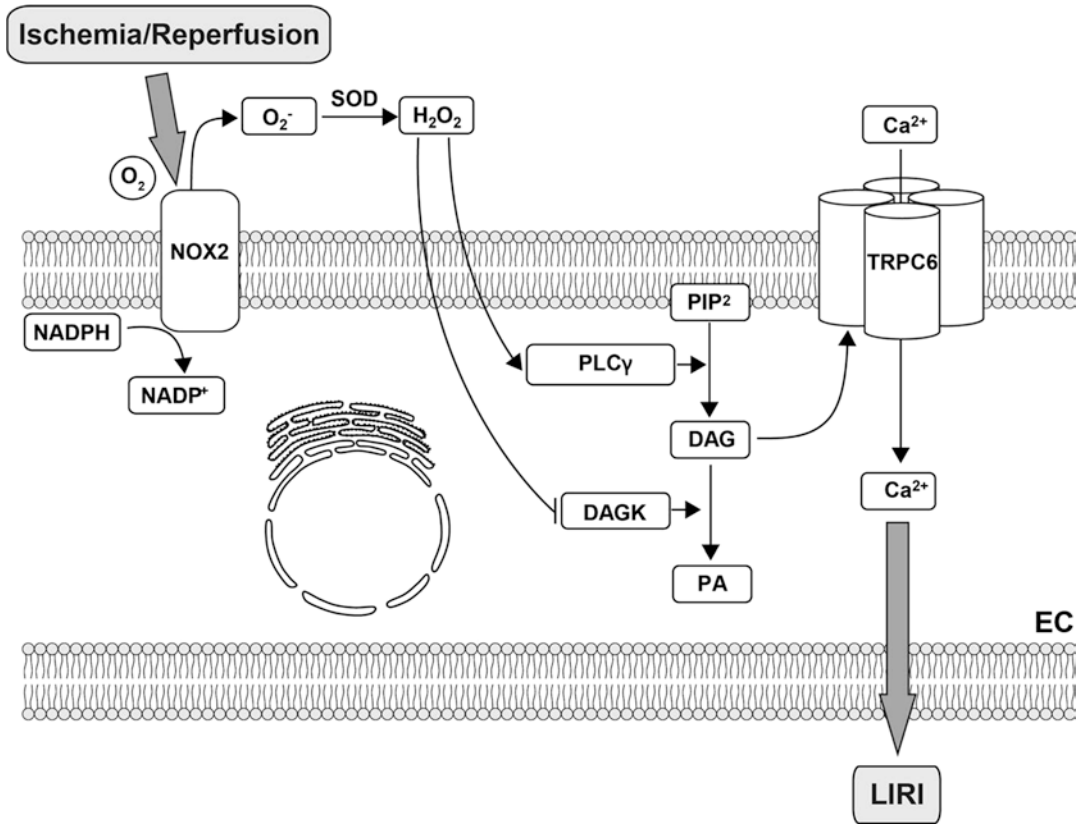

Fig. 3 The possible role of NOX2 and TRPC channels in lung ischaemia–reperfusion injury of mice. Ischaemia–reperfusion induces the increase of superoxide ($O_2^{\bullet-}$) production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX2) in endothelial cells. Afterwards, dismutation of $O_2^{\bullet-}$ to H_2O_2 initiates the influx of extracellular calcium through transient receptor potential cation, subfamily C, member 6 (TRPC6) channels. NOX2-mediated H_2O_2 activates TRPC6 by the accumulation of DAG (diacylglycerol) via both a stimulation of phospholipase C activity or/and inhibition of DAG kinases. The subsequent influx of calcium leads to an increase of endothelial permeability and thus contributes to lung ischaemia–reperfusion injury. *Abbreviations*: *DAG* diacylglycerol, *DAGK* DAG kinase, *Ca^{2+}* calcium, *EC* endothelial cell, *LIRI* lung ischaemia–reperfusion injury, *NOX2* nicotinamide adenine dinucleotide phosphate (NADPH) oxidases 2, *PA* phosphatidic acid, *PLCγ* phospholipase C, *PIP_2* phosphatidylinositol-4,5-bisposphate

WT chimera) and vice versa (WT to NOX2 KO chimera). Isolated lung experiments demonstrated that NOX2 KO to WT chimera showed the same severity of LIRI as WT mice, while WT to NOX2 KO chimera were protected against LIRI, suggesting that NOX2 in EC were responsible for the protective phenotype. The same experiments with TRPC6 KO mice demonstrated that WT to TRPC6 chimera were also protected against LIRI. Moreover, cell specific deletion of the catalytic subunit of NOX2 in EC led to the same result as was found in chimeric mice, preventing LIRI formation. It was revealed that LIRI triggers an increase of NOX2-mediated release of $O_2^{\bullet-}$ into the extracellular environment, with subsequent conversion of $O_2^{\bullet-}$ to H_2O_2, which enters into the EC and activates TRPC6 by inhibition of DAG kinase η1/2 activity and activation of PLCγ, both resulting in DAG accumulation [265] (Fig. 3). Accumulation of DAG resulted in activation of TRPC6 and an increase of the intracellular calcium concentration. Increased calcium concentration in the EC can lead to changes in EC morphology as well as to increased endothelial permeability [247, 248] (Fig. 3). Interestingly, in our study, ROS and the intracellular calcium

concentration were increased in pulmonary EC during the early ischaemic state of LIRI [265] suggesting that NOX2-mediated ROS can prime EC to reperfusion injury.

Depolarisation of the cellular membrane during ischaemia has been suggested as the driving force which can induce the activation of NOX2-mediated ROS release by EC in LIRI [40, 287]. In these studies, lung ischaemia initiated membrane depolarisation as well as the increase of ROS release in EC [40, 287]. NOX2 deletion prevented the ROS increase in EC, but not the membrane depolarisation, while deletion of $K_{ir}6.2$, which is a major subunit of the ATP-sensitive potassium (K_{ATP}) channel, inhibited both ROS increase and membrane depolarisation in EC during ischaemia, suggesting that K_{ATP} channel-dependent membrane depolarisation activated the NOX2-mediated ROS release [287]. Cell membrane depolarisation could act via PI3K (phosphoinositide 3-kinase)/Akt1 (RAC-alpha serine/threonine-protein kinase) pathways as both the PI3K inhibitor wortmannin and knockout of Akt1 greatly diminished the activation of NOX2, whereas they did not affect membrane depolarisation of EC during ischaemia [40].

Yang et al. found that global deletion of cytosolic subunit $p47^{phox}$ reduced LIRI and decreased neutrophil infiltration and lipid peroxidation [275]. However, in contrast to our study, they found that $p47^{phox}$ deletion in bone marrow-derived cells was responsible for the protective phenotype as WT mice, in which $p47^{phox}$ KO bone marrow cells were transplanted after irradiation, did not develop LIRI, while $p47^{phox}$ KO mice after bone marrow transplantation of WT mice resulted in LIRI development. However, $p47^{phox}$ also regulates NOX1 activity replacing the NOXO1 function [282]. Therefore, it is possible that decreasing the function of both NOX1 and NOX2, in bone marrow-derived cells was protective against LIRI as demonstrated in this study, while in our experiments only NOX2 KO was protective in ECs. Recently, Sharma et al. have published a follow-up study demonstrating that NOX2 or $p47^{phox}$ deletion in invariant natural killer T (iNKT) significantly attenuated LIRI and increased of interleukin 17 secretion [228]. They also found that activation of NOX2 occurred via adenosine receptors $A_{2A}R$, as an $A_{2A}R$ agonist prevented the effect of NOX2 KO in LIRI. The discrepancy in these studies should be addressed in further investigations. However, in these studies, mice were exposed to a much shorter ischaemic period; therefore, LIRI was much milder than in our experiments. Additionally, endothelial NOX2 could trigger the initial event of LIRI, while the bone-marrow derived NADPH oxidases could participate in the later stages of LIRI, when secondary neutrophil invasion can occur after endothelial injury.

5.3 The Role of Xanthine Oxidoreductases in LIRI

The Xanthine oxidoreductase (XOR) is a dimeric molybdenum-containing flavoprotein that consists of two identical subunits of approximately 145 kDa each [16, 32, 194]. XOR is widely expressed in many organs including the lung [133], where it is present in its constitutive active dehydrogenase form, which is termed xanthine dehydrogenase (XDH) [16]. XDH can be converted in its oxidase form, named xanthine oxidase (XO), by the oxidation of sulphhydryl residues or by proteolysis under several pathological conditions only in mammals [16, 32, 194]. Both XDH and XO primarily catalyse the oxidation of hypoxanthine to xanthine and xanthine to uric acid [16, 32, 168, 194]. However, only XO can generate ROS via direct transfer of electrons to molecular O_2 instead of NAD^+ producing mostly H_2O_2 and less $O_2^{\bullet-}$ [168, 205]. Besides their primary function to degrade hypoxanthine, XOR participates in a variety of biochemical reactions including the oxidation of different endogenous metabolites such as purines, pterins and aromatic heterocycles, as well as the detoxification/activation of both endogenous compounds and xenobiotics [194, 205]. The elevated level of XOR-mediated ROS has been found in various pathological situations, particularly in those which are related to ischaemic–reperfusion injury [16]. A few theories have been proposed to explain the XOR-mediated increase

of ROS during ischaemic–reperfusion injury. According to these theories, ischaemia induces the accumulation of hypoxanthine and xanthine from the catabolism of ATP [98]. For instance, the concentrations of hypoxanthine and xanthine in physiological conditions are 1–3 μM, while in hypoxia their cumulative concentration can reach 50–100 μM [31]. During reperfusion the accumulated pool of both hypoxanthine and xanthine can lead to a burst of $O_2^{\bullet-}$ and H_2O_2 production through the following possible pathways: (1) increased accumulation of XO, which is converted from XHD during ischaemia [168], (2) the pathological ability of XHD, which does not produce ROS in physiological conditions, to generate the ROS in acidic conditions (pH ~ 6.5) or under reduced conditions when higher proportion of the NAD(H) pool is in the reduced state [162, 213] or (3) ischaemia- [246] and/or cytokine (such as IL-1, IL-6 and TNF-α) [69, 201] -mediated increased expression and/or activity of XO. The relevance of these different pathways in real life situations should be further clarified. However, it is possible that ischaemia–reperfusion induces the increase in XOR-mediated ROS production in a species-, tissue- and cell-type specific manner. For instance, complete conversion of XHD into XO has been reported in rat intestine [98], while the pathological ability of XHD to produce ROS has been detected in human umbilical vein EC [289] during ischaemia–reperfusion. Additionally, XOR can contribute to the systemic effects of ischaemia–reperfusion injury [245, 279]. Experiments in rats revealed that the ischaemia–reperfusion injury of the intestine [245] or liver [279] triggered a release of XO into the systemic circulation. In case of the ischaemia–reperfusion injury of the intestine, increased XO level in the circulation induced neutrophil retention, transvascular leak and decreased ATP levels in the lung [245].

The role of XOR in LIRI is still under investigation and the data regarding beneficial effects of the XO inhibitor allopurinol during LIRI are controversial [267]. It has been demonstrated that the activity of XOR was increased only during anoxic (anoxic ventilation) but not during oxygenated (95% O_2 ventilation) ischaemia–reperfusion in the isolated rat lung [290]. In this study both anoxic as well as oxygenated ischaemia–reperfusion induced an increase of ROS measured by 2′,7′-dichlordihydrofluorescein and the increase of lipid peroxidation measured by thiobarbituric acid reactive substances. However, the inhibitor of XO allopurinol repressed both the increased ROS and lipid peroxidation levels only during anoxic ischaemia–reperfusion, but not in LIRI after oxygenated ischaemia–reperfusion [290] suggesting that XO was activated only in LIRI induced by anoxic ischaemia–reperfusion. These data correlated with the finding that the ATP level was decreased (which indicates increased hypoxanthine and xanthine levels) only during anoxic but not hypoxic (5% O_2) ventilation in LIRI [83]. On the cellular level, XO activation in alveolar type II cells resulted in DNA strand breakage, diminished mitochondrial integrity, and enhanced lipid peroxidation [50]. The effect of XO was reversed by application of glutathione peroxidase, suggesting that XO acted via H_2O_2 generation [50]. The treatment with allopurinol and its metabolite oxypurinol has been effective to prevent ischaemia–reperfusion injury in different tissues/organs of various animal models [98]. Therefore, the effect of allopurinol on LIRI was intensively investigated in vitro [3, 4, 129, 160] and in vivo [120]. Allopurinol inhibited LIRI in experiments which were conducted in either blood free- [129, 160] or blood-perfused [3, 4] isolated ventilated lungs of different animals including rats [160], rabbits [3, 129] and dogs [4] (Fig. 4). Inhibition of XOR by a tungsten also known as wolfram enriched diet [27] also decreased the endothelial injury [100] and LIRI development [3, 100] in isolated lung experiments. In contrast, in vivo experiments in rabbits, where LIRI was induced by ligation of the right or left hilum to stop both ventilation and perfusion, did not reveal any difference between allopurinol-treated and control groups [120]. The reason for this discrepancy has not yet been elucidated.

5.4 The Role of NO Synthases in LIRI

Since the discovery of NO as an essential endothelium-derived volatile vasodilator molecule, which regulates cardiovascular homeostasis, its role under physiological and pathological conditions has been extensively studied. For discovery of the signalling function of NO, R.F. Furchgott, F. Murad and L. Ignarro jointly were awarded with the Nobel Prize in Physiology or Medicine in 1998. NO is generated by NO synthases (NOS) that catalyse the conversion of L-arginine and O_2 into L-citrulline in a complex reaction using several cofactors and coenzymes such as NADPH, tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), flavine mononucleotide (FMN) and protoporphyrin IX [22, 36]. In mammals, three isoforms of NOS are known: the neuronal NOS (nNOS or NOS1), the inducible NOS (iNOS or NOS2) and the endothelial NOS (eNOS or NOS3) [52]. Both nNOS and eNOS are constitutively expressed in different cellular compartments such as caveolae [79], the sarcoplasmic reticulum [272] and mitochondria [29] of different tissues including the lungs [229]. In contrast, the expression of iNOS is induced by a wide range of stimuli, most prominently by endotoxin and endogenous pro-inflammatory mediators, which stimulate the rapid production of large fluxes of NO [2, 143]. Within cells, NO activates the soluble guanylate cyclase (sGC) that converts guanosine-5′-triphosphate (GTP) into the second messenger cyclic GMP (cGMP) [78]. cGMP mediates the activity of cGMP-dependent protein kinases (PKGs) and cGMP-gated cation channels [36]. The most well-known effect of activation of the NO–sGC–cGMP pathway is vasorelaxation [109]. Besides vasorelaxation, the NO–sGC–cGMP pathway controls many other physiological processes, including the immune response [22], cell growth [28] and proliferation [175], and neuronal transmission [266]. Moreover, besides sGC, NO reacts with other iron-containing proteins including haemoglobin, myoglobin, cytochrome p450, aconitase and catalase, modulating their functions [106]. However, not all cellular effects of NO are mediated by the NO–sGC–cGMP pathway. NO may also exert its function through RNS-mediated oxidative modifications of many proteins, as NO is a common progenitor of RNS: NO_2, N_2O_2, NO_2^-, NO_3^- etc. [165, 199], especially, in situations when a high and sustained flux of NO occurs [192]. Additionally, NO can react with $O_2^{\bullet-}$ to produce another member of RNS, which is $ONOO^-$ [271]. $ONOO^-$ itself is not an oxidant, but it produces powerful oxidants such as NO_2, $HO^\bullet$ and CO_3^- [106]. NO and its RNS derivates mediate the irreversible nitration of tyrosine and tryptophan residues of various proteins [128], fatty acids [53] and the nucleotide guanosine (inducing effects on DNA synthesis) [252] as well as S-nitrosation of cysteine residues [216], leading to "nitrative" and "nitrosative" stress, respectively. Moreover, all three forms of NOS can produce the $O_2^{\bullet-}$ anion instead of NO [158]. This phenomenon is named NOS uncoupling, as $O_2^{\bullet-}$ generation mainly occurs when NOS are not coupled with their cofactors or substrates.

NOS-mediated NO and RNS play an important role in LIRI [75, 113, 179]. Surprisingly, both therapeutic strategies either inhibiting NO [70, 154, 224] or activating NO related pathways [125, 239] appear to be protective in several experimental models of LIRI. These experiments demonstrated that NO, and probably RNS, are a double-edged sword in LIRI. This paradoxical dichotomy of NO related effects can be explained at least by the following unsolved questions: (1) which NO source (eNOS, nNOs or iNOS) is active in LIRI [239] and (2) in which phase (ischaemia or reperfusion) of LIRI the alteration in NO concentration occurs [75, 240]. Inhaled NO delivered at the beginning of the reperfusion phase worsened LIRI, and this increased injury could be prevented by treating the animals with SOD before reperfusion [75]. These data suggest that an increase of $ONOO^-$ release is important for activation of early cellular events in the reperfusion phase of LIRI. In contrast, Sugimoto et al. revealed that addition of nitrite, which is an endogenous source of NO, to the preservation solution before lung transplantation (during the ischaemic phase) in rats resulted in higher levels

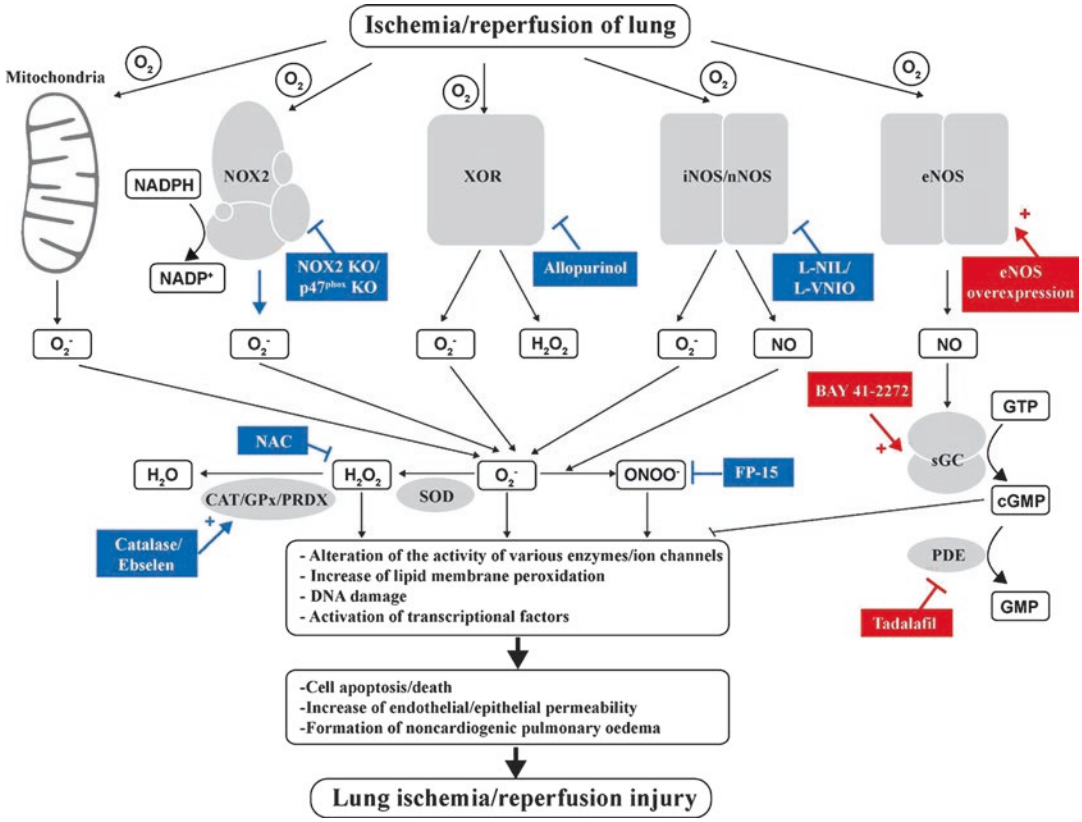

Fig. 4 The role of ROS in lung ischaemia–reperfusion injury (LIRI). Ischaemia–reperfusion of the lung could trigger an increase of superoxide ($O_2^{\bullet-}$) production by: (1) mitochondria, (2) NADPH oxidases (particularly NOX2), (3) xanthine oxidoreductases (XOR), (4) the inducible or neuronal nitric oxide synthase (iNOS/nNOS) via uncoupling. XOR also could directly produce hydrogen peroxide (H_2O_2) in LIRI. LIRI-induced $O_2^{\bullet-}$ is dismutated by superoxide dismutases (SOD) into H_2O_2. Additionally, $O_2^{\bullet-}$ reacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$) which belongs to the family of reactive nitrogen species (RNS). All of these three molecules, $O_2^{\bullet-}$, H_2O_2 and $ONOO^-$ can directly damage various cellular components such as lipid membrane and DNA or affect different cellular signalling pathways including the activity of various enzymes or ion channels and transcriptional factors within lung cells, initiating their dysfunction, apoptosis/necrosis and increase of endothelial/epithelial permeability, thus leading to noncardiogenic pulmonary oedema formation. In contrast, endothelial NOS (eNOS)-derived nitric oxide (NO) protects against LIRI via the NO–sGC–cGMP pathway. The inhibition of ROS generation in various animal models by: (1) the unspecific ROS scavenger *N*-acetylcysteine (NAC), (2) knockout of NOX2 (NOX2 and cytosolic subunit of NOX2 $p47^{phox}$ KO), (3) the XOR specific inhibitor allopurinol and (4) iNOS/nNOS specific inhibitors L-NIL and L-VNIO, respectively, attenuate LIRI formation. The ROS detoxification by application of: (1) catalase, (2) the glutathione peroxidase mimetic ebselen or (3) the $ONOO^-$ decomposition catalyst FP-15, also attenuates LIRI formation. In addition, activation of the NO–sGC–cGMP pathway by: (1) overexpression of eNOS, (2) application of the sGC activator BAY 41-2272 or (3) treatment with the phosphodiesterase inhibitor tadalafil, protects against LIRI. In conclusion, enhancement of ROS and RNS plays an important role in cellular mechanisms underlying LIRI, while eNOS-derived NO protects against its development. *Abbreviations*: *BAY 41-2272* 3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine, *cGMP* cyclic guanosine monophosphate, *GMP* guanosine-5′-monophosphate, *GTP* guanosine-5′-triphosphate, *GPx* glutathione peroxidases, *H_2O* water, *L-NIL* L-N^6-(1-iminoethyl)lysine, *L-VNIO* N5-(1-imino-3-butenyl)-L-ornithine, *NADPH* reduced form of nicotinamide adenine dinucleotide phosphate, *$NADP^+$* nicotinamide adenine dinucleotide phosphate, *O_2* oxygen, *sGC* soluble guanylyl cyclase, *PDE* phosphodiesterase, *PRDX* peroxiredoxins

of tissue oxygenation, lower levels of cytokines and neutrophil infiltration as well as a lower level of oxidative damage than in untreated lungs [240]. Moreover, inhalation of NO started 10 min after the beginning of reperfusion inhibited the increase of vascular permeability as well as neutrophil infiltration in LIRI [75]. Thus, NO has a divergent effect in LIRI and the application of NO is beneficial during the ischaemic [240] or late reperfusion phase [75], while NO seems to be harmful during the early reperfusion phase of LIRI [75].

The relation between the activity of specific NOS isoform and the level of NO in LIRI has been extensively studied [75, 85, 113, 117, 179, 224]. The survival of eNOS knock out mice was reduced due to severe pulmonary oedema following LIRI [125] and the adenovirus-mediated gene transfer of eNOS improved oxygenation and decreased neutrophil sequestration in transplanted lung isografts [239]. In contrast, the application of the nNOS specific inhibitor vinyl-L-NIO [70] as well as the application of the specific inhibitors of the iNOS, N6-(1-iminoethyl)-L-lysine (L-NIL) [270] and *N*-[3-(aminomethyl)benzyl]acetamidine (1400 W), prevented LIRI-induced RNS generation, increase of PVR and oedema formation [224] (Fig. 4). Additionally, 1400 W attenuated platelet adhesion in LIRI [191]. We are not aware of any experiments regarding nNOS or iNOS specific knockout in LIRI; however, ischaemia–reperfusion-induced cardiac injury was significantly lower in nNOS knockout mice than in wild type mice [154]. These data suggest that eNOS-derived NO plays a positive role and protects against LIRI, whereas the local release of large amounts of iNOS- or nNOS-derived NO activates damaging mechanisms promoting LIRI formation via increased $ONOO^-$ (Fig. 4). It has been shown that the expression of iNOS was increased in rats [85, 117] and in rabbits [191] during LIRI. Moreover, the levels of the stable degradation products of NO, nitrite and nitrate, as well as the levels of nitrotyrosine were significantly higher during LIRI in rat lungs [113], indicating the possibility of an increased activity of NOS in LIRI. The increased activity of NOS could lead to an excess of NO production which later can be converted to $ONOO^-$ and initiates lipid peroxidation as demonstrated [71, 113]. In these experiments the unspecific NOS inhibitor N^G-nitro-L-arginine (L-NAME) decreased the increased $ONOO^-$ level to normal values proving that LIRI can trigger an increase of the $ONOO^-$ levels via NOS activation [113]. The application of L-NAME not only decreased the levels of lipid peroxidation, but also significantly attenuated LIRI in isolated rabbit lungs [71]. Moreover, pretreatment with the water-soluble iron containing metalloporphyrin FP-15 that acts as a catalyst for degradation of $ONOO^-$ reduced the pulmonary vascular permeability by 61% compared to control animals [179] (Fig. 4). In this study the protective effects of enhanced $ONOO^-$ decomposition correlated with a 72% reduction in tissue myeloperoxidase content, marked reductions in brochoalveolar lavage leukocyte accumulation and diminished expression of pro-inflammatory chemokines [179]. Thus, $ONOO^-$ could be a key player in the cellular mechanisms of LIRI. It is also possible that LIRI induces NOS uncoupling as demonstrated by Egemnazarov et al. [71] (Fig. 4). According to these data, LIRI could initiate NOS uncoupling, leading to a paradoxical NOS-mediated $O_2^{\bullet-}$ production instead of NO that results in increased $ONOO^-$ levels and decreased availability of NO to activate the sGC–cGMP pathway, respectively. Therefore, application of both the inhibitor of NOS, L-NAME, and stimulator of sGC, BAY 41–2272 (3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H–pyrazolo[3,4-b]pyridine), prevented the development of LIRI [71] (Fig. 4). These data have been confirmed in experiments with a long-acting oral phosphodiesterase inhibitor, tadalafil, which increases cGMP levels [264] (Fig. 4). The application of tadalafil significantly improved lung performance after LIRI. In summary, iNOS or nNOS could initiate LIRI via direct production of $O_2^{\bullet-}$ or excess production of NO which increases $ONOO^-$ levels.

Finally, NO and $ONOO^-$ play an important role in preconditioning. The pre-treatment with NO-releasing sodium nitroprusside prior to LIRI prevented LIRI-induced oedema formation, and

L-NAME abolished the beneficial effect of precondition by sodium nitroprusside [126]. The beneficial effects of NO in preconditioning are probably mediated through its effects on mitochondrial function including the attenuation of reperfusion-induced ROS generation by complex I, inhibition of mitochondria-mediated apoptosis, regulation of mitochondrial K_{ATP} channels etc. [60]. Furthermore, the pre-treatment with $ONOO^-$ prior to LIRI also prevented LIRI-induced oedema formation [253, 254]. Two possibilities which can explain these findings are: (1) physiological concentrations of $ONOO^-$ can activate beneficial cellular pathways prior LIRI or (2) $ONOO^-$ activates cellular defence mechanisms which later protect the lung against LIRI. However, further studies are necessary to reveal the beneficial role of $ONOO^-$ in preconditioning.

6 Antioxidant Therapy of Lung Ischaemia–Reperfusion Injury

Despite tremendous research, to date there is no specific treatment to prevent and/or treat LIRI available [80]. The current therapeutic approach for LIRI is to use a symptomatic as well as supportive therapy and treat life-threatening complications of LIRI such as ALI [267]. In the case of lung transplantation, the best strategy to prevent development of LIRI is to use hypothermic preservation and an optimised lung preservation solution [45]. Possibly normothermic ex vivo lung perfusion (EVLP) may also improve the state of organs for transplantation [54]. Although lung transplantation has significantly improved in recent years, primary graft dysfunction due to LIRI still remains a serious complication of lung transplantation [161]. Therefore, a better understanding of the molecular mechanisms underlying LIRI, particularly the role of ROS in these pathways, is warranted. The effectiveness of ROS scavengers/antioxidants for prevention or inhibition of cellular pathways that are involved in the ischaemia–reperfusion injury and thus for prevention or treatment of LIRI has been confirmed in different experiments [85, 93, 111, 150, 207]. The application of nonspecific antioxidants [85, 93, 111], specific inhibitors of ROS-producing enzymes [265, 275], the genetic manipulations of cellular ROS sources (e.g. knockout of NOX2) [265, 275] or increased ROS detoxification including the application of catalase and glutathione peroxidase [21, 138, 188] prevented the development of LIRI.

For a long time, a variety of antioxidants has been tested during LIRI [1, 85, 93, 111, 150, 207, 209]. For example, *N*-acetylcysteine (NAC), which is a powerful antioxidant acting via direct scavenging of H_2O_2 and $O_2^{\bullet -}$ as well as via an increased biosynthesis of the antioxidant glutathione [217], significantly decreases inflammation and tissue damage in LIRI [85, 93] as well as attenuates the capillary "no-reflow phenomenon" after normothermic ischaemia of the lung [180] (Fig. 4). Moreover, treatment of the donor and recipient with NAC effectively protects lungs from primary graft dysfunction after single left-lung transplantation in pigs [110]. NAC inhibits accumulation of macrophages and lymphocytes, decreases IL-1 release and expression of cleaved caspase 3, NF-κB, IκB-α and TNF-α during LIRI [85, 93]. Application of NAC 5 min after the beginning of reperfusion significantly inhibits lipid peroxidation and attenuates LIRI, suggesting that NAC can not only protect from but also reverse LIRI development [85]. Besides LIRI, NAC has proved its effectiveness in treatment of ischaemia–reperfusion injury of the human liver in a prospective randomised Phase II clinical trial [55]. In this study, the application of NAC prior to liver transplantation significantly improved graft survival after 1 year and decreased the risk of primary dysfunction. Application of other nonspecific antioxidants also attenuated LIRI in animal models [1, 5, 111, 150, 207, 209]. For example the application of melatonin resulted in reduced lipid peroxidation and better oxygenation after single-lung transplantation in the rat [111].

To verify the cellular ROS source which is responsible for the cellular mechanisms underlying LIRI, several specific inhibitors or knockout mice were tested during LIRI [3, 4, 129, 160,

265, 275] as discussed above. For example, two independent studies have demonstrated that NOX2 played a crucial role in LIRI [265, 275]. In these studies, both NOX2 as well as $p47^{phox}$ knockout mice were protected against LIRI [265, 275] (Fig. 4). Despite the discrepancy about which cell type (endothelial [265] or bone marrow-derived cells [228, 275]) induces the increase of NOX2-mediated ROS release in LIRI, both of these studies found that the non-specific NOXs inhibitor apocynin attenuates LIRI in the mouse model [265, 275].

Increase of ROS detoxification by overexpression of or treatment with cellular components of the antioxidative defence system such as SOD and catalase has been proposed as an alternative therapeutic approach for treatment of ischaemia–reperfusion injury [44, 64, 281]. Overexpression of ROS catabolising enzymes including SOD [64, 281], catalase [44] and glutathione peroxidase [281] prevented the development of ischaemia–reperfusion injury in various tissues/organs. There is little knowledge about the beneficial effects of respective therapeutic approaches during LIRI. Danel et al. have studied the effect of intratracheal injection of an adenoviral vector encoding the human SOD1, catalase, or a mixture of both on LIRI development [58]. In this study overexpression of SOD worsened LIRI and concomitant overexpression of catalase prevented this adverse effect, but did not protect against LIRI [58]. In contrast, a single intravenous infusion of the mixture of both SOD and catalase a before the ischaemic phase of LIRI resulted in a less severe injury in the contralateral lung [21], while infusing SOD concomitantly with catalase two times (before and during the reperfusion phase) attenuated LIRI [105]. Moreover, immunotargeting catalase to the pulmonary endothelium alleviated oxidative stress and reduced acute lung transplantation injury [138] as well as attenuated in vivo LIRI development in rats [188] (Fig. 4). The detoxification of H_2O_2 with the glutathione peroxidase mimetic Ebselen resulted in improvement of the transplanted rat lung function 24 h after transplantation [103] (Fig. 4). Taken together, these data suggest that increased cellular H_2O_2 levels could trigger the LIRI-induced injury; therefore, the detoxification of H_2O_2 could possibly be used as a new therapeutic approach to treat and/or prevent LIRI (Fig. 4).

7 Conclusion

LIRI is a severe pathological condition that manifests during reperfusion of the previously ischaemic lung. During ischaemia, the interruption of the blood supply to the lungs leads to an imbalance between the metabolic supply and demand that finally results in hypoxia or anoxia, substrate depletion and accumulation of (toxic) metabolites, which activate different cellular pathways priming the lung to reperfusion injury. Reperfusion initiates a cascade of various cellular events that result in worsening of the ischaemic tissue injury ultimately leading to life-threatening lung oedema. Currently, there is no specific treatment for LIRI. Compelling findings from animal and human studies indicate an important role of ROS for cellular mechanisms underlying LIRI including the activation of an increased immune response (innate and adaptive), promotion of endothelial permeability, and increase of PVR. Several ROS sources such as mitochondria, NOXs, XOR and NOS may contribute to ROS production during LIRI. Particularly, increased production of H_2O_2 by NOX2 can be an important cellular signal that triggers the rise of endothelial permeability. Furthermore, iNOS and nNOS-mediated increase of $ONOO^-$ levels could be an important player in LIRI-induced oedema formation. In summary, a better understanding of ROS-mediated signalling pathways underlying LIRI is crucial to prevent or treat LIRI.

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Redox Mechanisms Influencing cGMP Signaling in Pulmonary Vascular Physiology and Pathophysiology

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Abbreviations

ALA	δ-Aminolevulinic acid
Ang II	Angiotensin II
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
DHEA	Dehydroepiandrosterone
EDNO	Endothelium-derived nitric oxide
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
Fe^{2+}	Ferrous
FECH	Ferrochelatase
H_2O_2	Hydrogen peroxide
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOX	NADPH oxidase
PA	Pulmonary arteries
PH	Pulmonary hypertension
PKG	Protein kinase G
PKG1α	Protein kinase G 1α
PpIX	Protoporphyrin IX
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
Zn-PpIX	Zinc-protoporphyrin IX

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1 Introduction

The earliest studies detected evidence suggesting the cyclic GMP generating activity of the cytosolic or soluble form of guanylate cyclase (sGC) from lung and/or other partially purified tissue preparations was modulated by redox processes influenced by autooxidation, hydrogen peroxide, lipid peroxides, thiols, superoxide dismutase, ascorbate, and drugs potentially releasing nitric oxide (NO) [1]. Subsequently, the formation of nitrosothiols (RSNO) and the availability of ferrous (Fe^{2+}) heme were proposed for explaining sGC sites mediating activation by NO [2, 3]. Furchgott, Ignarro, and Murad received the Nobel Prize in Physiology or Medicine in 1998 for identifying nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF), which appeared to function as a physiological regulator of sGC. The initial work of Louis Ignarro evolved from studies conducted in bovine pulmonary arteries (PA) [4] and the similarities

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between superoxide inhibition of EDRF and NO was a key factor used by Ignarro LJ et al. [5] in identifying NO. A major interest of our lab has been elucidating aspects of multiple additional mechanisms through which redox can control sGC and cGMP signaling in PA [6–8]. Some of these mechanisms seem to participate in pulmonary artery hypoxic pulmonary vasoconstriction (HPV) [6] and changes that occur in pulmonary hypertension (PH) [9, 10]. There is now substantial evidence for a loss of endothelium-derived nitric oxide (EDNO) [11] and perhaps its ability to stimulate sGC [12, 13] in various forms of PH. NO and drugs including the phosphodiesterase-5 (PDE-5) inhibitor Sildenafil and the sGC stimulator Riociguat are now used to treat PH. The properties of cyclic guanosine monophosphate (cGMP) signaling suggest that it may normally function to attenuate vascular pathophysiological actions of stimuli promoting pulmonary hypertension development.

2 Organization of cGMP Signaling in Pulmonary Arteries

Different redox systems can regulate sGC and/or cGMP-associated signaling mechanisms, which in turn leads to relaxation of vascular smooth muscle (VSM) in pulmonary arteries. In smooth muscle tissue, cGMP is well established as an activator of type 1 and 2 forms of Protein Kinase G (PKG) present in vascular smooth muscle. More recently, a thiol oxidation resulting in a disulfide bond between the two subunits of PKG1α has been identified as a cGMP-independent activator of this system [14]. Activation of PKG is known to promote the opening of calcium-activated potassium channels which leads to cell hyperpolarization and relaxation. PKG activates sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump on sarcoplasmic reticulum (SR) which pumps calcium back to sarcoplasmic reticulum (SR). As this store of calcium fills, extracellular calcium influx is also likely to be decreased. Thus, PKG signaling decreases intracellular calcium through multiple mechanisms, and this leads to smooth muscle relaxation. PKG inhibits Rho Kinase (a kinase which inhibits Myosin light chain (MLC) Phosphatase) and leads to relaxation of smooth muscle [15]. While there may be differences in the systems activated by cGMP versus thiol oxidation activation of PKG due to different docking properties of these active forms of PKG [16], both of these activation mechanisms show many similarities in the way PKG regulates vascular smooth muscle relaxation and remodeling processes [17, 18].

Some of the cyclic nucleotide-metabolizing phosphodiesterases are cGMP selective, and the type 5 isoform of this enzyme (PDE5) appears to be a major cGMP-selective phosphodiesterase in vascular smooth muscle. Thus, PDE5 may normally function in the pulmonary vasculature to remove cGMP generated in response to prevailing NO levels, by converting it to GMP. Under these conditions, inhibition of PDE5 causes smooth muscle relaxation by increasing cGMP, which decreases the levels and actions of calcium through PKG. NO may also activate K^+ channels independent of cGMP, which would also lead to hyperpolarization and relaxation. Therefore, inhibitors of cGMP-dependent phosphodiesterase, by increasing intracellular cGMP, enhance smooth muscle relaxation associated with lowering pulmonary arterial pressure under conditions promoting pulmonary hypertension.

NO/cGMP signaling pathway also has important pro-apoptotic and antimitogenic effects on vascular smooth muscle (VSM) and endothelial cells which play an essential role in pulmonary vascular remodeling. It has been documented that activation of the NO/cGMP pathway inhibits the proliferation of bronchial smooth muscle and vascular smooth muscle cells from the systemic [19–21] and pulmonary circulations [22–24]. Actions such as PKG-mediated inhibition of Rho kinase activation could be a factor in processes such as inhibition of myosin light chain phosphatase activity associated with decreasing the sensitivity of the contractile apparatus to calcium and in attenuating smooth muscle growth-associated remodeling processes promoted by Rho kinase. Figure 1 shows some relationships associated

with actions of cGMP signaling mechanisms that potentially contribute to pulmonary vascular function and disease processes.

3 Redox Modulation of cGMP Signaling in Pulmonary Arteries

There appear to be many redox regulatory interactions influencing cGMP signaling that have been detected in pulmonary arteries. The discussion of these pathways are organized around considering how different individual cellular redox processes are designed to function, and potentially regulate aspects of cGMP signaling, in relation to other systems they could influence in physiological and pathophysiological regulation of the pulmonary vasculature. Redox regulation of the sGC heme and thiols can occur directly by systems such as NO, and reactive oxygen and NO-derived species. The function of redox systems such as NADPH/NADP and NADH/NAD, and related thiol redox control mechanisms in subcellular regions are key factors indirectly controlling the function and depletion of sGC through interactions illustrated in Fig. 2 and processes described in Table 1. While the activity of PKG enzymes are controlled by redox systems influencing changes in the levels of cGMP, the activity of PKG1α is also directly controlled by systems regulating the disulfide between its subunits, which activates this form of PKG in a cGMP-independent manner. While the expression of key enzymes participating in cGMP signaling such as PDE5 [25] may also be regulated by redox, it is currently difficult to discuss the mechanisms involved due to limitations in what is known.

3.1 Redox Regulation of sGC Through Its Heme Group

The ferrous or Fe^{2+} heme form of sGC is known to bind and be required for activation by low nanomolar concentrations of NO. This activation appears to occur through NO binding disrupting a histidine bond of sGC to Fe^{2+} of its heme [26, 27]. When the heme of sGC is oxidized to its ferric Fe^{3+} form, it does not readily bind or become activated by the low levels of NO present in the vasculature. Oxidant stress conditions could potentially oxidize the heme of sGC, and, the product of NO reacting with superoxide, peroxynitrite is a well documented oxidant of the heme of sGC [28, 29]. Oxidation of the sGC heme to its ferric Fe^{3+} form may

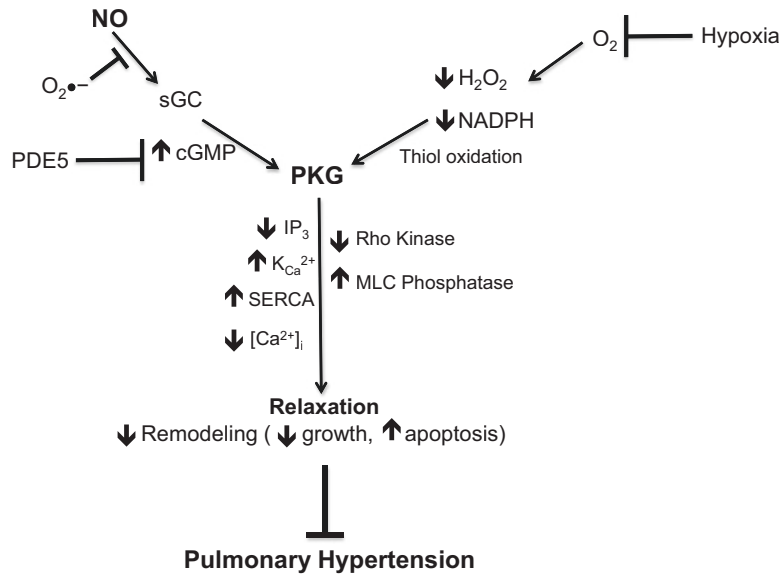

Fig. 1 cGMP-related signaling mechanisms regulating pulmonary vascular relaxation and remodeling

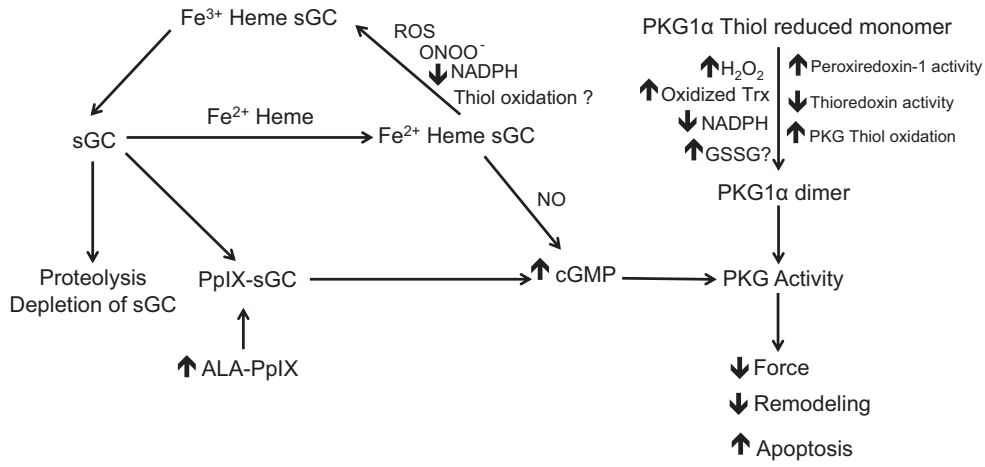

Fig. 2 Nitric oxide, reactive oxygen species, thiol and heme redox, and heme biosynthesis control mechanisms that regulate the production of cGMP by sGC and/or a cGMP-independent mechanism regulating protein kinase G

result in the release of heme and formation of heme-free form of sGC. Heme-free sGC seems to preferentially bind some sGC activator drugs at the site normally occupied by heme. While sGC appears to undergo proteolytic depletion once its heme is oxidized, this process appears to be prevented when agents such as sGC activators bind the sGC heme site [29–31]. These sGC activator drugs may actually be mimicking a potentially endogenous mechanism of activating heme-free sGC through binding protoporphyrin IX (PpIX) at the heme site [30]. PpIX is normally used as the substrate for insertion of Fe^{2+} by ferrochelatase, during biosynthesis of heme in mitochondria. When there is a deficiency in Fe^{2+} needed for heme biosynthesis, ferrochelatase (FECH) can insert Zn into PpIX generating zinc-protoporphyrin IX (Zn-PpIX). Zn-PpIX could potentially bind the heme site of sGC in a manner that might prevent both its stimulation by NO and its degradation by proteolysis. While heat shock protein-90 appears to participate in the binding of heme to sGC [32], the role of this protein under conditions promoting sGC heme oxidation and depletion remain to be better defined. While the oxidation of heme of sGC under pathophysiological conditions and its association with enhanced sGC stimulation by sGC activators under these conditions are rather well documented processes, most of the hypothesized relationships between the function of ferrochelatase in heme biosynthesis and sGC regulation remain to be investigated.

3.2 Redox Regulation of sGC Through Modulation of Its Thiol Groups

There appear to be multiple ways through which thiol groups can influence the activity of sGC, and sGC has an unusually large number of thiols [33]. Thiols potentially have a major influence of the generation of NO from potential NO-donors through processes such as forming S-nitrosothiols (RSNO) which release NO. Cysteine is one of the least abundant residues in proteins, but it is also one of the most conserved. Cysteine can undergo a variety of thiol oxidations, including disulfide (S-S), sulfenic acid (S–OH), nitrosylation (RSNO), and sulfhydration reactions. The ability of NO to stimulate sGC by its Fe^{2+} heme-dependent activation mechanism appears to require certain specific thiols, and oxidation or modification of these thiols may prevent activation by NO.

Various forms of sGC thiol oxidation can play a role in the development of cardiovascu-

Table 1 Redox processes modulating vascular cGMP signaling

Redox species and systems	Mechanisms regulating cGMP signaling
Nitric oxide (NO)	• NO binds the Fe^{2+} heme of soluble guanylate cyclase (sGC) promoting cGMP generation and cGMP-mediated protein kinase G (PKG) activation
Superoxide	• Superoxide reacts with NO forming peroxynitrite and RNS
Hydrogen peroxide (H_2O_2)	• Peroxide metabolism by peroxiredoxin-1 promotes cGMP-independent PKG1α activation by disulfide formation between its subunits, a process inhibited by cGMP • Peroxide metabolism by catalase promotes cGMP generation by sGC, a process inhibited by NO
Reactive nitrogen species (RNS)	• Peroxynitrite oxidizes the heme of Fe^{2+}sGC, preventing its activation by NO • Peroxynitrite and RNS formation may have additional effects oxidizing thiols and other functional groups in ways that may generate molecules that subsequently release NO in a delayed time-dependent manner
Heme	• Oxidation of the heme of Fe^{2+}sGC prevents its activation by NO and promotes proteolytic depletion of sGC • Physiological porphyrins such as protoporphyrin IX (PpIX) and Zn-PpIX potentially prevent the depletion of heme oxidized sGC
Thiols	• Reduced thiols can be associated with enabling NO stimulation of sGC and minimizing cGMP-independent PKG1α activation by disulfide formation
NADPH	• Cytosolic NADPH may control the maintenance of the sGC heme in its Fe^{2+} form and reduced thiols needed for stimulation by NO • NADPH has a major role in influencing ROS regulation of sGC and PKG by its being a key source of electrons for superoxide and peroxide generation by Nox oxidases, and by its role in maintaining the reduced thiol status of key systems such as glutathione and thioredoxin which are used to consume peroxides • Cytosolic NADPH generated by glucose-6-phosphate dehydrogenase and the pentose phosphate pathway of glucose metabolism has a major role in maintaining the reduced thiol status of PKG1α, and oxidation of NADPH promotes cGMP-independent PKG1α activation by disulfide formation

lar diseases by decreasing the NO-dependent production of cGMP and thus the vascular reactivity. This thiol-based resistance to NO (increased peripheral resistance) appears to be detected in hypertension [33]. Studies have shown that the disulfide inducer diamide and the attenuation of nicotinamide adenine dinucleotide phosphate generation (NADPH) via the pentose phosphate pathway inhibitor 6-aminonicotinamide decrease the relaxation of pulmonary arteries and NO-stimulated sGC activity [34]. Dithiotheritol (DTT), the thiol reductant, can reverse these inhibitory effects. Perturbations to the redox status of cells in the pulmonary vasculature due to activation of reactive oxygen species (ROS)—generating enzymes, such as xanthine oxidase, NADPH oxidase (NOX), or via disrupted electron transport chain (ETC) function in mitochondria promote pulmonary vasculopathy that is characterized by intimal thickening, impaired $NO^{\bullet}$-dependent vasodilation and perivascular fibrosis [35]. In general, the types of sGC thiol modifications under these types of pathophysiological conditions remain to be defined, and it is likely they go beyond NADPH redox controlled reversible disulfide regulation of its sensitivity to stimulation by NO, to more difficult to reverse modifications of sGC thiols that could impair the function of this system.

3.3 Redox Regulation of PKG1α Through Modulation of Its Thiol Groups

It was reported by Eaton and colleagues that exposure of arteries to hydrogen peroxide could promote relaxation by activating protein kinase G 1α (PKG1α) through formation of a disulfide bond between its two subunits, in a manner independent of cGMP [14]. Inhibition of thioredoxin reductase was also observed to enhance this relaxation. Our lab studied these processes in pulmonary arteries and identified peroxiredoxin-1 as a peroxide-metabolizing enzyme which potentially promotes PKG1α disulfide formation and activation. In addition, we provided evidence that the control of cytosolic NADPH oxidation resulting from decreased glucose-6-phosphate dehydrogenase (G6PD) activity and the pentose phosphate pathway (PPP) could function to promote thiol oxidation-mediated PKG1α activation [7]. This potentially occurs through decreasing NADPH availability needed to support the activity of thioredoxin reductase for reversing the actions of oxidant processes promoting PKG1α activation by its disulfide form.

3.4 Coordination of the Regulation of cGMP Signaling by Reactive Oxygen and NO-Derived Species

Multiple interactions of reactive oxygen and NO-derived species are described in Table 1. When the levels of ROS are low, the sGC system is likely to be most sensitive to stimulation by NO as a result of thiols and heme being maintained in their reduced forms. As superoxide levels increase in any extracellular or subcellular region, it is likely to scavenge and attenuate the actions of NO by reacting with it to form peroxynitrite. This is because NO is a dissolved gas which readily diffuses across extracellular and intracellular membranes. Increased superoxide formation drives the generation of peroxide, which could stimulate both sGC generation of cGMP and/or promote cGMP-independent peroxide activation of PKG1α generally as a result of the actions of peroxide-metabolizing enzymes such as catalase and peroxiredoxin-1, respectively, causing redox changes that influence sGC and/or PKG1α. Under these conditions, sGC stimulation by NO might also be attenuated by peroxide promoting thiol oxidation. While peroxynitrite is also known to oxidize the sGC heme and thiols, the importance of this interaction in pathophysiological dysfunction needs to be better defined. As the levels of reactive oxygen and/or NO-derived species increase, many other signaling mechanisms beyond those associated with promoting cGMP signaling become activated in various subcellular regions, and the actions of these other regulatory systems are likely to dominate the biological effects that are seen. There are many different processes controlling cellular systems generating ROS, and the stimuli activating these oxidases often define the roles of various potential signaling mechanisms in the responses observed.

3.5 Coordination of the Regulation of cGMP Signaling by Subcellular Thiol Redox Systems

Cellular thiol redox systems such as glutathione and thioredoxin are oxidized through the functions of hydrogen peroxide (H_2O_2)-metabolizing enzymes such as glutathione peroxidases and peroxiredoxins and enzymes including glutathione and thioredoxin reductases maintain these systems in their reduced forms based on the availability of cofactors such as NADPH. Thus, the redox status of thiol influencing sGC and PKG signaling in the subcellular regions these enzymes are located should dominate regulation of these systems by processes described previously in Sects. 3.2 and 3.3. Again, as described for ROS, there are many different processes controlling cellular thiol redox systems, and subcellular metabolic perturbations often define the roles of various potential signaling mechanisms in the responses observed.

3.6 Coordination of the Regulation of cGMP Signaling by Subcellular NADH and NADPH Redox Systems

The redox status of NADH and NADPH in subcellular regions are usually influenced by multiple regulatory processes and metabolism-associated factors. They have a potential influence on cGMP signaling through their roles in supporting the generation of ROS by enzymes such as NOX oxidases and the mitochondrial electron transport chain, and as cofactors for influencing thiol and heme redox regulation. When biological factors such as hypoxia or stimulation of oxidases regulate NADH or NADPH redox systems they have the potential to regulate cGMP signaling through the pathways described above.

4 Redox Regulation of Pulmonary Vascular Function Through cGMP Signaling

The properties of redox regulation of cGMP signaling in pulmonary arteries isolated from normal healthy animals and limited information available from studies on properties of vascular reactivity the pulmonary circulation in vitro or in vivo indicate that sGC normally exists in its NO responsive form, suggesting the sGC heme is in its NO-binding ferrous form, with sGC thiols influencing sensitivity to NO being reduced as well. Studies primarily from our laboratory have also documented what appears to be a basal partial activation of both sGC and PKG by endogenous H_2O_2 in pulmonary arteries under aerobic conditions, which are both attenuated by hypoxia associated with a hypoxic pulmonary vasoconstriction response [36]. The unusual property of pulmonary arteries compared to systemic arteries driving this aerobic generation of vasodilator levels of peroxide seems to be increased activity of glucose-6-phosphate dehydrogenase for supporting higher rates of basal peroxide generation by a Nox oxidase, which may be Nox4 [37]. There is often evidence for baseline levels of endothelium-derived NO somewhat depressing responses to pulmonary vasoconstrictors including hypoxia and for endogenous NO having a major influence on blood flow shunting responses by the pulmonary circulation at birth [38].

Many conditions or agents participating in the control of pulmonary vascular function, such as the shear forces of flow, the actions of increased pressure, vasoactive mediators and hypoxia have often been observed to regulate cGMP signaling aspects of pulmonary vascular function through modulating endothelium-derived NO. Many of these conditions or agents are also likely to modulate redox systems in pulmonary arterial smooth muscle which could influence cGMP-related signaling by additional mechanisms. For example, there is a large literature of the effects of hypoxia being mediated by redox mechanisms regulating a variety of systems, including our focus [6, 36] on redox regulation of sGC/cGMP and cGMP-independent PKG mechanisms. Under these types of conditions, many redox changes that are occurring could potentially have prominent influences on processes such as the activation of contractile and remodeling mechanisms, in addition to influencing cGMP-related signaling. Only a few studies have examined relationships between the influence of physiological conditions on oxidant-redox systems and their simultaneous influence on multiple other redox-regulated processes, including assessing what is happening with cGMP-related signaling. This is probably due to how difficult it currently is to document how each redox system functions in subcellular regions and how these redox changes influence aspects of cGMP signaling versus other processes influenced by redox. In addition, as discussed in Sect. 2, PKG regulates many processes controlling vascular function, and many of these processes have been suggested to be redox regulated without considering if PKG is involved. Thus, the properties of these systems suggest hypothesizing that there may be many conditions where the redox regulation of cGMP signaling (beyond the role of endothelium-derived NO) has regulatory roles in physiological processes influencing pulmonary vascular function.

5 Pathophysiological Regulation of Pulmonary Vascular Function Through Altered cGMP Signaling

The endothelium is a known semipermeable layer between the vascular and extravascular fluid compartments. It is involved in the regulation of vascular tone, differentiation and growth. In pulmonary hypertension (PH), vascular responses to injury caused by increased pressure, flow (shear stress), hypoxia, drugs (dexfenfluramine), etc. are mediated partly through the endothelial cell dysfunction. The endothelium releases several vasoactive substances, such as endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2 as well as the growth factors such as transforming growth factor-β, fibroblast growth factor-2, vascular endothelial growth factor, and platelet-derived growth factor. These factors influence the growth of the underlying smooth muscle layer and thus result in vascular remodeling and the progression of PH [39–42]. There are several intracellular posttranslational modifications such as phosphorylation (e.g., Ser1177, Ser65, and Thr495), S-nitrosylation (e.g., Cys94 and Cys99), and palmitoylation, which can regulate endothelial Nitric Oxide Synthase (eNOS) activation [43]. The extracellular signaling pathways such as G-protein-coupled receptor signal transduction, Akt/PKB (protein kinase B) signaling via sphingosine 1-phosphate and vascular endothelial growth factor via phosphatase calcineurin are involved in eNOS activation [44–46]. Studies have shown that there is decreased pulmonary vascular eNOS activity in various animal models of pulmonary hypertension as well as in human patients with PH [47, 48]. Loss of NO bioavailability is associated with increased pulmonary vascular smooth muscle cell mitogenesis and impaired endothelium-dependent and -independent vasodilation. Hypoxia plays an important role in development of PH. Hypoxia can induce posttranslational modifications of eNOS and/or caveolin-1 that in turn lead to a decrease in calcium sensing by eNOS. This causes dissociation of eNOS from its regulatory proteins (heat shock protein 90 and calmodulin) and thus hypoxia decreases eNOS activity [46]. Many of these processes lead to conditions that are associated with a loss of the favorable effects of EDNO and its regulation of sGC, and a progression of PH development.

In humans, increased levels of ROS generation in pulmonary vasculature may occur as a pathological response to various conditions such as chronic hypoxia, increased pulmonary vascular blood flow (secondary to intracardiac shunt) or due to impaired antioxidant enzyme function (glutathione peroxidase deficiency in sickle cell anemia-associated PH) [49]. ROS by oxidizing the enzyme cofactors such as tetrahydrobiopterin (BH4) may inhibit eNOS activity. Additionally, superoxide by reacting with NO generates peroxynitrite ($ONOO^{-\bullet}$) and inactivates NO. Furthermore, superoxide also reacts with nitrite (either derived from diet or as a stable NO by-product) and generates peroxynitrate (O_2NOO), which is a potential NOS-independent source of NO [50, 51]. This decrease in NO synthesis leads to a decreased activation of sGC and thus decreased cGMP levels.

Endothelin-1 (ET-1) regulates pulmonary vascular tone by its interaction with the vasoconstrictor endothelin-type A (ETA) and endothelin-type B (ETB) receptors in pulmonary vascular smooth muscle cells and vasodilatory ETB receptors in pulmonary vascular endothelium. Stimuli associated with pulmonary vascular injury, such as cytokines that mediate vascular inflammation [52] as well as stimuli such as increased ROS generation in the pulmonary vasculature [53], hypoxia [54] decreased levels of bioavailable NO [55] can significantly upregulate ET-1 gene expression levels in right ventricular cardiac myocytes, pulmonary arterial endothelial and vascular smooth muscle cells. ET-1 immunohistochemical analysis demonstrates significantly increased immunoreactivity in pulmonary arterial endothelial and smooth muscle cells of plexiform lesions compared to blood vessels harvested from normal controls [56]. Thus, ET-1 is an important factor generated under conditions leading to PH that contributes to the progression of this disease process. Our studies have found that

ET-1 promotes in pulmonary arteries a loss of mitochondrial superoxide dismutase (SOD) expression associated with increased mitochondrial superoxide, and activation of other signaling associated with pulmonary arterial smooth muscle remodeling that appear to be prevented by using δ-aminolevulinic acid (ALA) to generate protoporphyrin ix (PpIX), an activator of sGC [10]. Treatment of mice with ALA prevented the development of hypoxia-induced PH in mice associated with actions similar to its effects on the actions of ET-1 observed in isolated pulmonary arteries. These observations suggest cGMP-activated PKG signaling by an sGC activator has actions that go beyond vasodilation, which can prevent fundamental processes contributing to the progression of PH. A sGC activator has been observed to increase vasodilator actions in a neonatal model of pulmonary hypertension, suggesting sGC heme oxidation is potentially an additional factor in this disease process [12, 13].

The actions of Angiotensin II (Ang II) on its endothelial type-2 receptors in the pulmonary circulation releasing NO has been suggested to be a dominant factor in protecting the pulmonary circulation from Ang II generated by systemic hypertension acting on its type 1 receptor in pulmonary arteries [57, 58]. In the regulation of systemic vascular reactivity, Ang II activation of its type 1 receptor and NO/cGMP normally function as a vasoconstrictors or vasodilators, respectively. The countervailing influences of Ang II and NO/cGMP on vascular smooth muscle cell (VSMC) growth have been well documented. Ang II stimulates, whereas NO/cGMP inhibits, VSMC growth, through different mechanisms. Studies have shown that infusion of Ang II into rats significantly decreased the expression of both sGC subunits in blood vessels, associated with decreased PKG-mediated phosphorylation of vasodilator-stimulated phospho protein (VASP) [59]. The inhibitory effects of Ang II on sGC are likely mediated by increased production of superoxide in response to Ang II. These results suggest that a decrease in PKG activity occurred in response to Ang II treatment. Ang II may exhibit inhibitory effects on cGMP accumulation through an additional mechanism in systemic arteries potentially mediated by increased Ca^{2+} promoting activation of Ca^{2+}/calmodulin-stimulated phosphodiesterase-1A1 [60]. Our studies in isolated coronary arteries [61] suggest that Ang II may function to deplete sGC by increasing mitochondrial and extramitochondrial superoxide. Increased mitochondrial superoxide is associated with a depletion of mitochondrial SOD and ferrochelatase (which has an iron–sulfur center that could be disrupted by superoxide) and a depletion of heme. Whereas, increased cytosolic superoxide may promote sGC heme oxidation and depletion. Promoting PKG activation by the accumulation of PpIX or by 8-bromo-cGMP attenuated all of these actions of Ang II. Our ongoing studies in pulmonary arteries [62] suggest that the direct effects of Ang II on endothelium-rubbed pulmonary arteries are consistent with it having actions similar to those described for coronary arteries, except that ferrochelatase and sGC are not depleted. Since Ang II (and ET-1) increased the detection of fluorescence associated with PpIX accumulation in pulmonary arteries [10, 63], there may be disruption of mitochondrial iron availability needed for heme biosynthesis, and sGC might be protected by PpIX and/or Zn-PpIX binding to its heme site.

6 Therapeutic Targeting of PA cGMP Signaling

Pulmonary hypertension is a syndrome in which vasoconstriction, inflammation, and remodeling of small pulmonary arteries increase pulmonary vascular resistance, leading to right ventricular hypertrophy and failure. While vasodilator-related therapies have shown beneficial effects in the treatment of this rather poorly understood disease, the high rate of mortality remains a major concern. Sildenafil, a selective inhibitor for cGMP phosphodiesterases, is beneficial in the treatment of pulmonary hypertension [11]. Tadalafil, a phosphodiesterase type 5 inhibitor, gained FDA approval in 2003 and is indicated for patients with pulmonary arterial hypertension to improve exercise capacity. Drugs stimulating sGC either through heme-dependent actions

potentially enhancing the actions of endogenous NO (stimulators) or by directly activating sGC in a heme-independent manner (activators) have also showed potential for treatment of pulmonary hypertension in humans [64]. These studies lead to the sGC stimulator Riociguat recently being approved for treating some forms of PH. While the direct activators of sGC, which bind its heme site where PpIX binds, evolved into clinical trials for Cinaciguat, these were stopped during phase IIb studies due to adverse effects associated with it promoting systemic hypotension [65]. Endothelin-1 receptor antagonists bosentan, ambrisentan, and macitentan are also used for treatment of pulmonary hypertension. Prostacyclin analogues such as Epoprostenol, Treprostinil and Iloprost are used as a therapy for pulmonary hypertension. The mechanism of action of prostacyclin analogues includes direct vasodilation of pulmonary and systemic arterial vascular beds, inhibition of platelet aggregation and antiproliferative effects [66]. Thus activation of cGMP signaling plays an important role for treatment of pulmonary hypertension. Clinical trials with dehydroepiandrosterone (DHEA), an activator of PKG through dimerization [67], treatment has reported significant improvement in 6-min walk test, pulmonary hemodynamics and diffusing capacity of the lung of patients with chronic obstructive pulmonary disease-associated PH, without worsening gas exchange, as do other pharmacological treatments of PH [68]. Another potential therapy for the treatment of pulmonary hypertension can be δ-aminolevulinic acid (ALA). ALA, the biosynthetic precursor to heme, by increasing PpIX, shows promising beneficial effects for the treatment of this disease in the mouse model of hypoxia-induced hypertension [9, 10]. The heme-independent activators of sGC act in a manner similar to PpIX by binding the heme site of sGC after its endogenous heme has been oxidized by vascular disease-associated oxidant stress [29]. The use of ALA as a therapeutic approach uses mechanisms (outlined in Fig. 2), which go beyond the drug activators of sGC in that it may take advantage of additional disease-associated cellular mechanisms such as using a potentially disrupted heme biosynthetic pathway to accumulate the PpIX activator of sGC. Perhaps once increased cGMP reversed processes such as mitochondrial oxidant stress, which contribute to disease progression, improved availability of heme might also be a contributing beneficial factor of this new therapeutic approach. Figure 3 shows various mechanisms by which therapeutic targeting of cGMP signaling is useful for the treatment of pulmonary vascular disease.

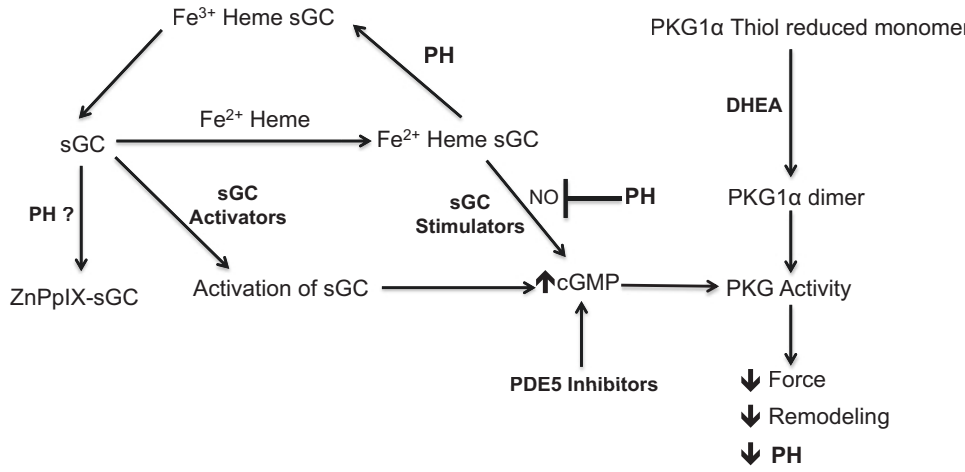

Fig. 3 Therapeutic targeting of cGMP signaling for the treatment of pulmonary vascular disease

7 Future Perspectives

The multiple different redox processes controlling sGC and PKG signaling suggest that these systems are sensors for many aspects of different physiological and pathophysiological processes that remain to be defined. They are among the most sensitive sensors for signaling promoted by NO and peroxide, and inhibition of NO by superoxide. Routes to regulation by NADPH, NADH, thiol and heme redox, and heme biosynthesis provide routes through which aspects of metabolism can influence cGMP signaling. In addition to regulating smooth muscle contractile function, evidence is emerging for cGMP signaling having important roles in controlling aspects of signaling mechanisms modulating ROS generation, and inflammatory, fibrotic cell death signaling processes that participate in often beneficial actions against pathophysiological vascular remodeling. The significance and most aspects of these processes remain to be elucidated.

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Metabolic Reprogramming and Redox Signaling in Pulmonary Hypertension

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1 Introduction

Pulmonary hypertension (PH) is a serious and often fatal pulmonary vascular condition that is becoming increasingly prevalent worldwide. PH occurs in a variety of clinical conditions and is associated with a broad spectrum of pathologic abnormalities in the pulmonary arteries of affected patients. All patients with PH exhibit a common set of clinical signs, such as exertional dyspnea, marked exercise limitation, and in severe cases, right heart failure and death. Because of the diverse causes and mechanisms contributing, PH has been classified into five categories related to common clinical parameters, potential etiologic mechanisms and pathological, pathophysiological, and therapeutic characteristics [1, 2]. PH in all categories is characterized by a complex panvasculopathy involving excessive proliferation and apoptosis resistance of multiple cell types, as well as inflammation and fibrosis throughout the vasculature. The characteristics of this remodeling have led many to hypothesize that the cellular and molecular features of PH resemble hallmark characteristics of cancer cell behavior [3–6]. In cancer, it is widely recognized that changes in metabolism in the cancer cell, as well as cells in surrounding stromal cells, including fibroblasts and macrophages, are essential for cancer cells to proliferate, migrate, and exhibit pro-inflammatory characteristics [7–9]. Because of this there have been intense efforts in oncology to define the molecular mechanisms that underlie the coordinated cross talk between cancer cells and cells in their immediate microenvironment. Indeed, while it is acknowledged that there is evidence to suggest that metabolic rewiring is orchestrated by the concerted action of oncogenes and tumor suppressor genes, it is now recognized that in some circumstances altered metabolism can play a primary role in oncogenesis. Indeed, aberrant metabolism, rather than simply an epigenetic phenomenon of

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oncogenic reprogramming, is now thought to play a key role in oncogenesis with the power to control both genetic and epigenetic events in cells [8, 10]. Certainly, questions arise as to whether this may be the case in PH as well.

Metabolic adaptations akin to aerobic glycolysis ("Warburg-like"), historically assigned to cancer cells, have also recently been reported in PH where hypoxia has significant regulatory role [3, 5, 11–13]. These changes have been described to occur in smooth muscle cells (SMCs), endothelial cells (ECs), and fibroblasts (Fibs) [11, 13–16]. Additional data in support of the importance of this metabolic adaptation are supported by 18-fluorodeoxyglucose (18FDG) PET imaging, which has demonstrated increased glucose uptake and metabolism in PH patients as well as in the monocrotaline rat model of PH [13, 17]. Furthermore, 18FDG uptake and gene-expression studies in pulmonary arterial fibroblasts, isolated from iPAH patients, tend support to the concept that a proliferative and inflammatory pulmonary vascular pathology contributes to the lung 18FDG PET signal [13]. This study also showed that 18FDG uptake occurs in perivascular mononuclear cells, which accumulate in the adventitial perivascular regions. In vivo studies in the monocrotaline model demonstrated a close correlation between lung18FDG uptake and pulmonary vascular remodeling. Importantly, enhancement of oxidative metabolism with dichloroacetate-mediated inhibition of the enzyme pyruvate dehydrogenase kinase attenuated PH and vascular remodeling in this model and also reduced expression of the glucose transporter (Glut1) typically upregulated in cells exhibiting high glycolysis. These findings correlated with reduced 18FDG PET signals, which were associated with decreased peripheral vascular muscularization and inflammatory cell accumulation. Collectively, these in vivo and ex vivo observations support a "metabolic hypothesis" for the pathogenesis of PH, whereby a rearrangement of the mitochondrial and cytosolic metabolism, known as the "Warburg effect," might explain, at least partially, the molecular and functional abnormalities seen in PH cells, including excessive proliferation, apoptosis resistance, and inflammatory activation [12].

2 Metabolic Reprogramming in Cells of Pulmonary Artery Wall in PH Pathology

Excessive proliferation and apoptotic resistance of ECs, SMCs, and Fibs from the PH vessel wall phenocopies the Warburg-like metabolic reprogramming observed in highly proliferating cancer cells. Incomplete oxidation of glucose through aerobic glycolysis depresses catabolism through mitochondria and fuels anabolic reactions of amino acids, lipids and nucleosides to sustain proliferation. This is often induced by dysregulation of oncogenes, hypoxic signaling and aberrant responses in cancer cells [18]. The metabolic switch needs to cover the energy demands of highly proliferating cells, such as DNA replication and gene expression, macromolecule biosynthesis, ion gradients' homeostasis and maintenance of cellular structure. Transcriptional upregulation and increased expression of glycolytic enzymes is observed in these cells and hypoxic signaling can play a pivotal role in these responses (see Fig. 1). Generalized or microenvironmental hypoxia can participate in the development of pulmonary hypertension (PH) through regulation of the oxygen sensitive subunit of hypoxia inducible factor (HIF) [19]. Consistent with a hallmark of metabolic reprogramming in proliferating cancer cells, cells from the hypertensive pulmonary vasculature express specific isoforms of glycolytic enzymes that promote faster fluxes through glycolysis, such as glucose transporter 1 (GLUT1), phosphofructokinase 3 (PFKB3) and pyruvate dehydrogenase kinase 1, and lactate dehydrogenase A (LDHA) [3, 12, 20]. Increased uptake of glucose not only provides a rate-limiting substrate to promote glycolysis, but also results in the upregulation of the pentose phosphate pathway. Hexokinase responsible for glucose phosphorylation, the initial step of glycolysis, was found to be able to suppress apoptosis by reversible binding to VDAC in mitochondrial membrane, thus preventing cytochrome c release [21]. Similar to highly proliferating cancer cells, activation of the oxidative branch of the pentose phosphate pathway in PH cells provides reducing

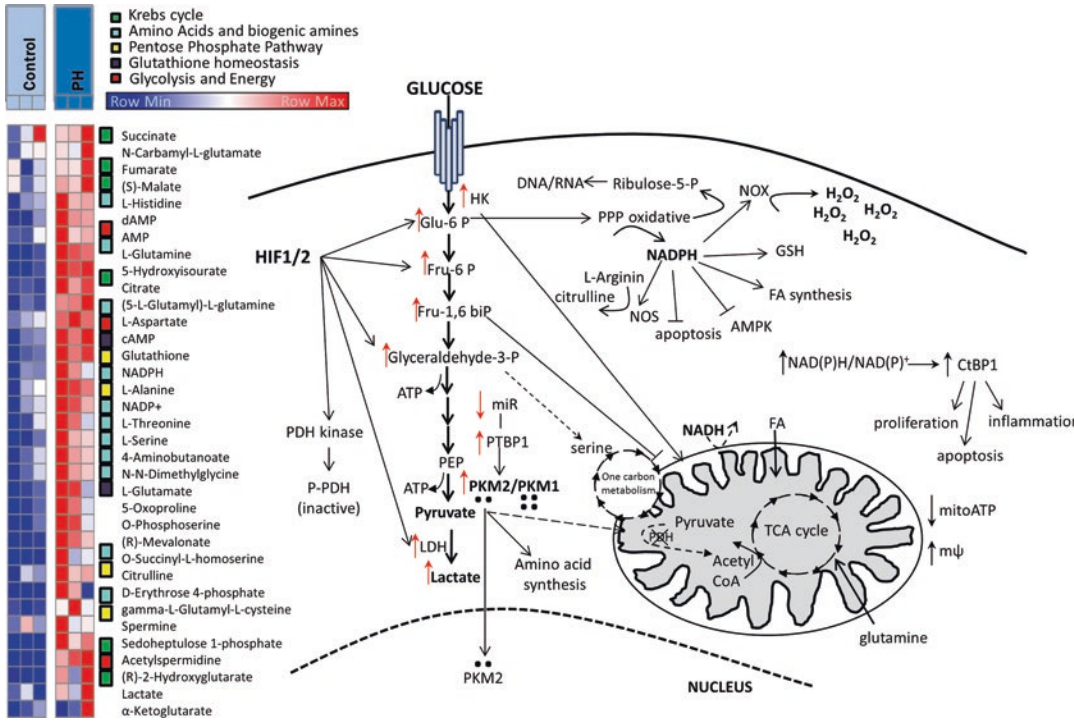

Fig. 1 Anabolic metabolism of hypertensive cells from pulmonary artery wall. Increased glycolytic pathway, where numerous enzymes is upregulated by HIF1/2. Enhanced pentose phosphate pathway, leading to NADPH production. Altered mitochondrial metabolism with suppressed oxidative phosphorylation. Elevated one carbon metabolism. Metabolic changes in glycolysis, Krebs cycle, redox homeostasis, and amino acid metabolism (color coded) are documented by UHPLC-mass spectrometry metabolomics data (*left*) of calf control and pulmonary hypertensive fibroblasts from pulmonary arteries. Relative metabolite quantities were graphed through heat maps, upon Z score normalization of values determined across samples (*blue*, lowest values; *red*, highest values). Each sample is represented by three independent experiments depicted in columns. All metabolic changes are leading to increased NAD(P)H, which then participates in several signaling reactions

equivalents (NADPH) to sustain NADPH-dependent anabolic reactions (e.g., de novo synthesis of fatty acids) and, at the same time, it generates ribose phosphate moieties for the de novo synthesis and salvage of nucleosides—the building blocks of RNA and DNA. It can inhibit full AMPK activation, which increases fatty acid and cholesterol synthesis while inhibiting acetyl-CoA carboxylase 1 and 2, otherwise participating in fatty acid oxidation (see Fig. 2). In addition to serving as a key cofactor for anabolic reactions, NADPH represents a key-reducing equivalent to preserve the redox poise. Indeed, NADPH is used as an antioxidant substrate to reduce oxidized glutathione or to promote pro-oxidant reactions through the activity of NADPH oxidase (NOX). Moreover, NADPH derived from the pentose phosphate pathway seems to be important during the initial phase of PH development for vasoconstriction of pulmonary arteries, as it forms an essential cofactor for nitric oxide (NO) production by NO synthase (NOS) [22]. In agreement with this, a deficiency of glucose-6-phosphate dehydrogenase in the African, Middle East, and Asian population was associated with enhanced endothelial oxidative stress, decreased NO bioavailability and increased risk for vascular disease such as PH [23]. Interestingly, glucose-6-phosphate dehydrogenase was also found to be involved under certain conditions in apoptosis [24]. Excess NADPH can then induce calcium/calmodulin-dependent kinase II to phosphorylate, thus inhibit pro-apoptotic caspase2 activation.

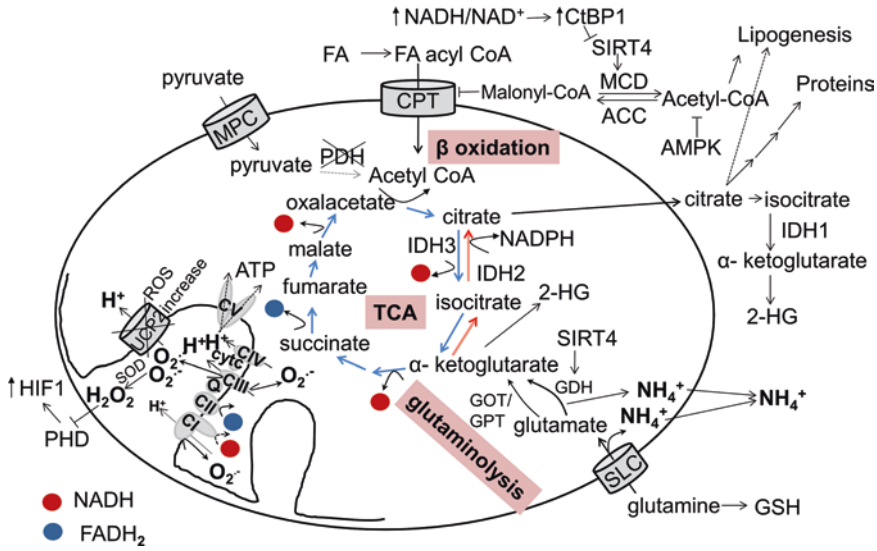

Fig. 2 Mitochondrial metabolism of hypertensive cells from pulmonary artery wall. Pyruvate oxidation is inhibited. β-oxidation is induced, since there is low level of inhibitory metabolite malonyl-CoA. Glutaminolysis is induced leading among others to production of α ketoglutarate, which incorporated into TCA cycle can serve for catabolic reactions to fuel further respiratory chain through succinate production or can serve for anabolic reactions in reversed flow of TCA cycle called reductive carboxylation. It will then provide citrate for lipogenesis. Electron transport chain is retarded, mainly through complex I, leading to increased production of superoxide, which after dismutation to H_2O_2 participates in stabilization of HIF1 and further signaling reactions

Increased glucose oxidation also promotes the accumulation of reducing equivalents other than NADPH, such as NADH. Increased NADH/NAD^+ ratios are promoted by increased fluxes through glycolysis, consistent with the law of mass action indicating that excess lactic fermentation under hypoxic conditions unbalances the NAD-cofactor poise towards the accumulation of reduced equivalents. Hypoxia also compromises physiological electron transfer through the electron transport chain, resulting in the incapacity to regenerate reducing equivalents (NADH, $FADH_2$) in the mitochondria owing to the lack of oxygen as the final acceptor of electrons in the electron transport chain. Disruption of the normal electron flow in the electron transport chain does not impair mitochondrial Krebs cycle reactions upstream to succinate dehydrogenase (SDH) (as part of complex II of the electron transport chain), though it promotes metabolic bottlenecks in complex I, which is involved in the reactions catalyzing the conversion of malate to oxaloacetate [25, 26]. Isocitrate dehydrogenase (IDH) activity either in cytosol (IDH1) or in mitochondria (IDH2,3), which can generate NADH or NADPH, depending on the isoform of the enzyme catalyzing the reaction and redox equilibrium, has been reported to be increased in PH patients [14]. Further, increased NAD(P)H can significantly impact redox status and also the regulation of proteins using NAD(P)H as a cofactor. The transcriptional repressor C-terminal-binding protein1 (CtBP1) is such an example. Oligomerization of CtBP1 into active dimers is dependent on the presence of increased free NADH and NADH/NAD^+ ratios [27]. CtBP1 interacts with gene specific transcriptional factors besides recruiting several epigenetic modifying enzymes on the target genes [27]. Recently, the coauthors of this chapter found its increased expression in fibroblasts generated from the pulmonary arteries of chronically hypoxic calves or patients with iPAH (termed PH-Fibs) that were characterized by an aerobic glycolytic state and exhibited increased free NADH, even under normoxic conditions and with enhanced proliferation and inflammation. Genetic or chemical suppression of CtBP1 led to downregulation of proliferation (and upregulation of

the cyclin-dependent kinase inhibitor genes, P21 and P15), apoptosis (e.g., upregulation of NOXA and PERP) and upregulation of anti-inflammatory (HMOX1) markers [28]. Other studies showed that CtBP1 also participates in the maintenance of metabolic homeostasis through the inhibition of apoptosis. In this respect, it was found that the transcriptional repressor CtBP1 directly inhibits pro-apoptotic Bax or SIRT4, the repressor of glutaminolysis in mitochondria. The activation of glutaminolysis promotes the generation of alternative substrate for mitochondrial catabolism and also release of the acidification pressure of highly proliferative lactate-producing cells [29, 30]. These studies highlight the importance of metabolic regulation on gene expression during PH development and identify feed forward mechanisms at the crossroads of transcriptional regulation and metabolic reprogramming.

Another glycolytic intermediate, fructose-1,6-bisphosphate, was shown to inhibit mitochondrial respiratory rate in highly glycolytic cells, an observation that may contribute to explain the so-called Crabtree effect—i.e., the reliance on glycolysis rather than the tricarboxylic acid cycle (TCA) to generate high energy compounds (ATP) [31].

Analogous to cancer, metabolic reprogramming in PH is accompanied by post-transcriptional reprogramming triggering the expression of specific isoforms of key metabolic enzymes. For example, an isoform switch in pyruvate kinase M plays an important role in the glycolytic state of highly proliferative cells. PKM1 and 2 isoforms are alternative splice isoforms of pyruvate kinase M. While PKM1 is expressed in terminally differentiated tissues, PKM2 isoform is expressed mostly in proliferating cells and tissues with anabolic functions (cancer, embryonic cells) and cells with high intrinsic self-renewal properties. While PKM1 has high catalytic activity, PKM2 is allosterically regulated between a high and a low activity state. PKM2 exists in the catalytically distinct tetrameric and dimeric states [32] and is present usually in its less active dimer form in highly proliferative cells. This leads to inhibition of the last step of glycolysis and to accumulation of glycolytic intermediates including lactate for biosynthetic processes. The dissociation of PKM2 tetramer into dimers is a reversible process and determines the balance between anabolic and catabolic phases of cell metabolism. Besides direct activation of PKM2 transcription by HIF1, PKM2 also directly interacts with HIF1 and thus promotes transactivation of genes with HIF response elements in their promoters [33]. This regulatory mechanism contributes to the escalation of the efficiency of glycolytic fluxes. The PKM2 isoform is also subject to many regulatory post-translational modifications such as phosphorylation, which participates in the conversion of the tetrameric form into the dimeric one [34]. On the other hand, PKM2 is positively allosterically regulated by fructose-1,6 bisphosphate and serine, which is produced from glycolytic intermediate 3-phosphoglycerate [35]. Moreover, oxidation of cysteine 358 in PKM2 impairs enzymatic function and decreases its activity, leading to the accumulation of glucose-6-phosphate and increased glucose flux through pentose phosphate pathway [36], thus contributing to redox homeostasis. Such complex regulation of this protein isoform is crucial for highly proliferating cells as a variety of molecules further interact with PKM2. Specifically, dimeric PKM2 was shown to translocate into the nucleus, where it can act as an active protein kinase to phosphorylate specific nuclear proteins such as STAT3 and β-catenin [29, 37, 38]. PKM2 can interact directly with the HIF-1α subunit in the nucleus to promote transactivation of HIF-1α target genes by enhancing HIF-1 binding and recruitment of p300 [39]. PKM2 gene transcription is also activated by HIF-1, which creates a positive feedback loop that promotes HIF-1 transactivation and alters glucose metabolism [39]. The coauthors of this chapter recently found that PMK2/PKM1 ratio is increased in PH Fibs from calves and iPAH humans [40]. Moreover, this alternative splicing resulting in the increased PKM2/PKM1 ratio was found to be under direct control of polypyrimidine-tract binding protein 1 (PTBP1) and miR124 acting upstream. Genetic manipulations of miR124 (miR-124 mimic) or PTBP1 (siPTBP1) normalized the PKM splicing ratio. This induced mitochondrial activity and

returned metabolism and proliferation toward that of normal control fibroblasts. Collectively this evidence supports the idea that PKM2 is a crucial enzyme controlling overall metabolic state of cells during the development of PH.

PKM catalyzes the final step of glycolysis, which results in the production of pyruvate. The destiny of pyruvate is another crucial point in highly proliferating cells. Under physiological conditions in most cells, pyruvate enters mitochondria where is further processed by pyruvate dehydrogenase (PDH) to acetyl-CoA fueling the TCA cycle and mitochondrial respiratory chain. However, under hypoxic conditions, the activity of mitochondrial respiratory chain is disrupted, as anticipated above, and cells require alternative pathways to regenerate reducing equivalents (NADH to NAD^+) to keep glycolysis going. Reduction of pyruvate to lactate through lactic fermentation serves this purpose under hypoxic conditions. This process can be enzymatically regulated under hypoxic conditions in PH. Indeed, inactivating pyruvate oxidation in mitochondria by PDH phosphorylation in hypoxia is directly regulated by HIF1α. This transcription factor induces PDH kinase in proliferating cells, an adaptation that can become independent from oxygen availability in cancers undergoing constitutive HIF activation or PDH kinase mutations [41]. Recently, we have shown that this metabolic adaptation also occurs in PH Fibs from chronic hypoxia exposed calves and iPAH humans [16]. Suppressed glucose oxidation was reflected by decreased mitochondrial bioenergetics (see below).

Termination of the glycolytic pathway by production of lactate is important for the later stage of hypoxia in cancer cells. It was found that accumulated lactate can stabilize NDRG3 protein which binds to c-Raf and mediates hypoxic cell growth through c-Raf-ERK pathway in various types of cancer cells [42].

Recent interest in one-carbon metabolism has been generated by experiments showing its importance in rapidly proliferating cells. One-carbon metabolism contributes key substrates for de novo synthesis of purine bases, participates in redox homeostasis by regulating the synthesis of amino acid substrates for glutathione synthesis and directly contributes to the redox poise through NADPH-generating reactions [43–49]. Notably, glycolytic isoform switch in cancer modulates the activity of rate limiting enzymes of one-carbon metabolism, as documented by evidence indicating a role for serine as an allosteric modulator for PKM2 activity [49]. Further studies will be necessary to investigate the role of this pathway in PH development.

3 Metabolic Remodeling in Macrophage Activation

Pulmonary arterial wall remodeling during PH development develops in concert with the innate immune system where macrophages play a pivotal role. Macrophages contribute to maintenance of tissue homeostasis [50, 51] through paracrine communication with tissue parenchymal cells [52], but also by sensing and responding to environmental cues and signals [53]. They are innate immune cells known to be important in the initiation and propagation of sterile inflammatory processes, like chronic inflammatory pathologies such as rheumatoid arthritis and remodeling pathologies such as liver fibrosis and vascular remodeling as it occurs in association with pulmonary hypertension [51, 54]. Indeed, macrophages have been recognized to promote tumor growth [55]. They are equipped with a sophisticated system to detect microenvironmental differences across various organs and conditions, and exhibit a high degree of functional versatility/plasticity in order to adequately contribute to homeostatic tissue maintenance, infectious and inflammatory processes and to tissue repair processes [51]. Continuous adjustments in the metabolic programming of macrophages have been suggested to be a key component in the regulation of their gene transcriptional profile and thus their functional phenotype within these processes [56]. There is now consensus that metabolic reprogramming consistent with a Warburg-like state is a prerequisite for inflammatory macrophages (here referred to as macrophages stimulated with lipopolysaccharide (LPS) or with LPS in combination with interferon gamma (IFNg) to

generate pro-inflammatory mediators [57, 58]. Metabolic reprogramming results at least in part from LPS mediated increases in HIF1 and expression of HIF1 target genes, encoding for enzymes of the glycolysis pathway [57–59]. LPS also promotes PKM2 expression, and IDH and SDH suppression [58]. Accumulated succinate derived from SDH suppression can generate ROS by reverse electron transfer on Complex I of respiratory chain, which has been suggested to be critical for activation of genes encoding for pro-inflammatory mediators [60]. LPS, especially in combination with IFNg, also upregulates expression of citrulline-arginine-succinate cycle enzymes, such as iNOS (inducible nitric oxide synthase), ASS (argininosuccinate synthetase), ASL (arginine succinate lyase), and Arginase1 [61]. As a result, inflammatory macrophages consume large amounts of glucose to generate lactate, accumulate citrate, succinate, and produce nitric oxide (NO).

Lactate and succinate have also been suggested to induce macrophage activation, thus providing a potential autocrine and paracrine feed forward loop [55]. Citrate, as a critical substrate for fatty acid synthesis and precursor for leukotrienes and prostaglandins, has been shown to be also important macrophage derived pro-inflammatory mediator production [62]. Succinate can positively regulate pro-inflammatory IL1b through HIF1α stabilization [57] as well as iNOS [63] and Arginase1 [54, 55]. Citrulline derived from iNOS metabolism of arginine can react with aspartate to regenerate arginine [58], which is a critical event under arginine limiting conditions [61]. In addition, this reaction generates fumarate, which is a critical step in replenishing the reprogrammed TCA cycle when SDH is reduced [58].

While these concepts help explain how inflammatory macrophages that are activated by LPS and IFNg use metabolic reprogramming to mount generation of pro-inflammatory mediators, much less is known about if and how metabolic programming underlies macrophage phenotypes in chronic inflammatory conditions that are not characterized by LPS, but instead involve a more complex microenvironmental makeup, e.g., cancer or rheumatoid arthritis, or vascular remodeling. In these conditions a metabolic synergy or symbiosis between macrophages and cancer cells and/or macrophages and fibroblasts might be a key mechanistic foundation for chronic inflammatory activation towards a pro-cancer or pro-remodeling phenotype. In such a metabolic symbiosis both cells, the macrophage and the tissue cell (for instance the fibroblast) undergo overlapping but also distinct metabolic reprogramming, such that both anabolic and catabolic pathways are engaged. As a result catabolic metabolites and anabolic substrates can be exchanged and shared between the two cells in order to sustain the respective metabolic reprogramming and thus functional phenotype. For example, cancer cell derived lactate has been shown to induce activation of HIF1 and expression of Arg1 in cancer associated macrophages, which in turn promotes generation of polyamines (anabolic pathway) through utilization of Arginine by Arginase1 [55]. In that study, polyamines were proposed to sustain cancer growth, since macrophages genetically deficient in Arginase1 were less capable of promoting cancer growth in vivo. Recent studies investigating the mechanism of vascular remodeling in PH have identified perivascular fibroblasts with a distinct pro-inflammatory phenotype characterized by generation of cytokines and chemokines [16, 54, 64]. Intriguingly, the activated fibroblast is capable of activating macrophage expression of Arginase1 at least in part through paracrine IL6 [54, 64]. As pointed out previously, these fibroblasts display metabolic reprogramming similar to that observed in inflammatory macrophages, including generation of lactate. Inhibition of glycolysis prevents inflammatory activation of these fibroblasts. Therefore, a metabolic symbiosis could potentially underlie the macrophage fibroblast crosstalk in vascular remodeling associated with PH in which fibroblast derived lactate (catabolism of glucose) and fibroblast derived IL6 (anabolic cytokine generation as a result of rewired TCA cycle and mitochondrial ROS production [16]) activate macrophage expression of HIF1 and Arginase1 [54]. Macrophage derived polyamines downstream of Arginase1 expression and macrophage derived IL1b (as a catabolic event mediated

by HIF induced Il1 generation) could in turn activate fibroblasts and promote their proliferation.

4 Mitochondrial Alterations and Superoxide Production in Pulmonary Arterial Hypertension

Observations of incomplete glucose oxidation terminating in lactate production in PH cells lead to the discovery of altered mitochondrial bioenergetics, similar to cancer cells (see Fig. 2). These changes have been described for cells from all layers of the hypertensive pulmonary artery wall [15, 16]. The role of mitochondria in apoptosis resistance, redox and calcium homeostasis assign them a central position within the cell which can determine cell fate. Changes in fluxes in the TCA cycle provide intermediates for biosynthetic pathways in highly proliferative cells. Thus, mitochondrial respiration in PH cells is still operating and needs to be preserved [16]. To overcome suppressed pyruvate oxidation, mitochondria adjust the fluxes from carbohydrates (pyruvate) to other significant substrate sources, such as fatty acid oxidation or glutaminolysis (Fig. 2). Glycolysis followed by pyruvate oxidation is assumed to contribute to 50–70% of total ATP production in cancer cells with the remainder contributed by mitochondrial oxidation of glutamine and fatty acids [65–67].

The feedback mechanism in which phosphorylation of PDH, leading to its downregulation, is critical for the mitochondrial substrate switch to fatty acid oxidation was described originally by Randle [68]. Upregulation of the expression of genes involved in fatty acid oxidation as well as an increase in metabolic intermediates was found in lung tissue from PH patients [69]. The importance of fatty acid oxidation in PH development is evident from the experiment where mice lacking malonyl-coA decarboxylase (MDC), which otherwise induces fatty acid oxidation at the expense of its synthesis, do not develop PH upon chronic hypoxic exposure [70]. Interestingly, MDC can be deacetylated by SIRT4 and thus inactivated [71]. This is consistent with our finding of increased CtBP1 levels in hypoxic PH-Fibs in that inhibition of SIRT4 by increased CtBP1 activity allows MDC to be active and drive fatty acid oxidation in mitochondria [28].

Another potential fuel for the TCA cycle is glutamine, the most abundant amino acid in blood. It is processed through glutaminolysis to α-ketoglutarate which can enter the TCA cycle. In highly proliferative cells, glutaminolysis plays a major anaplerotic role, since α-ketoglutarate production either fuels the TCA cycle or provides a carbon backbone for the biosynthesis of nonessential amino acids. We have observed elevation of glutamine and α-ketoglutarate in PH Fibs (see Fig. 1). Glutamine catabolism can thus provide a carbon source that supports further proliferation. In highly proliferative cells, it can also serve as the source of nitrogen for transamination reactions, which is necessary for GSH synthesis (serine and thus cysteine/glycine anabolism depends on glutamine for rate-limiting transamination reactions branching from glycolysis) and protect the cell from excessive acidification, by converting pyruvate into alanine, thus preventing accumulation of excess lactate in highly glycolytic cells. Under hypoxic conditions glutamine can also contribute to de novo lipogenesis by fueling reductive carboxylation reactions (reverse fluxes through the TCA cycle) [72] (See Fig. 2). Among others, α-ketoglutarate can give rise to 2-hydroxyglutarate, a metabolite that accumulates significantly in response to hypoxia in cells and plasma [73]. On the other hand, certain types of cancer cells (e.g., glioma) are characterized by IDH 1/2 mutations or a switch in the expression pattern of IDH isoforms in favor of NADPH-generating IDH1 [74]. Some of these mutations, together with excess glutaminolysis contribute to increased α-ketoglutarate–citrate ratio [75], which in turn promotes reductive carboxylation fluxes to sustain acetyl-CoA generation and anabolic reactions (e.g., de novo synthesis of fatty acids). We have found that the α-ketoglutarate–citrate ratio was significantly increased in PH Fibs [64]. On the other hand, 2-hydroxyglutarate has been described as oncometabolite and was found to elicit significant epigenetic changes that can contribute to cancer phenotype [76]. Citrate

can be also processed through oxaloacetate or malate for amino acid synthesis required for building proteins.

Mitochondrial bioenergetics terminates with the production of ATP where the respiratory chain is the key part of this machinery. Mitochondrial respiratory capacity is defined by availability of substrate, i.e., reducing equivalents coming either from the cytosol (NADH) or mitochondrial TCA cycle (NADH and FADH) and integrity of electron transport supercomplexes. The optimal flow of electrons through respiratory supercomplexes then defines the maximal production of ATP by complex V. However, in rapidly proliferating cells pyruvate stays in the cytosol instead of fueling TCA cycle and possibly the respiratory chain (Fig. 1). In that case, glutaminolysis or fatty acid oxidation can significantly substitute for the fuel in the TCA cycle. This might be the case of PH-Fibs, which were found to have enhanced succinate levels in mitochondria [16]. Succinate can then, in addition to other TCA cycle intermediates like α-ketoglutarate or fumarate, regulate histone and DNA methylation [77]. Besides shaping epigenetic regulation of DNA expression, upregulation of succinate and fumarate can also stimulate HIF1 stabilization though inhibition of HIF-prolyl hydroxylase, thus enhancing the Warburg effect.

Mitochondria of PH Fibs are hyperpolarized, reflecting less efficient oxidative phosphorylation [16]. This can be due to several factors, primarily involving mitochondrial respiratory chain complexes, some of which (Complex I, III and IV) are pumping protons across the mitochondrial membrane, which at insufficient protons backflow via ATP synthase creates increased membrane potential. However, the involvement of fatty acid stimulated uncoupling proteins in attenuation of mitochondrial membrane potential is also well recognized. Resulting mild uncoupling has antioxidant effect. Studies using mitochondrial uncoupling protein 2 deficient (UCP2−/−) mice showed that vascular remodeling with development of pulmonary hypertension occurred primarily via increased ROS production, not by regulation of membrane potential itself [78]. Another study explained spontaneous PH development in UCP2−/− mice by decreased mitochondrial calcium levels [79]. Many mitochondrial enzymes are calcium dependent so loss of UCP2 could lead to decreased mitochondrial bioenergetics. Another reason for hyperpolarized mitochondria in cells from the hypertensive vessel wall was attributed to translocation of hexokinase II into mitochondria via a GCK3-β dependent mechanism. This was shown to inhibit the voltage dependent anion channel (VDAC) and thus sustain mitochondrial hyperpolarization [80].

Mitochondrial membrane potential together with substrate availability, local oxygen concentration and electron flow through respiratory chain determines the mitochondrial redox status. Moreover, mitochondria contain numerous redox active metals, i.e., Fe–S cluster, cytochromes and thus are a primary site for single-electron reactions in the cell. The primary reactive oxygen species (ROS) produced in mitochondria is superoxide, which is formed mainly within complexes I and III of the respiratory chain [81–84]. The respiratory complexes with multiple redox centers form electron transport chain, which normally facilitates transfer of electrons to their final acceptor, molecular oxygen. It is reduced by four electrons to water at complex IV. The premature single electron reduction of molecular oxygen gives rise to superoxide formation. This can happen when electron flow is either retarded (i.e., ATP synthase is inhibited, cytochrome c or coenzyme Q cycling is retarded) or there is substrate pressure (high load of NADH, $FADH_2$) on respiratory complexes. Notably Complex I elevates superoxide formation at $NADH \gg NAD^+$ and at reverse electron transport after succinate accumulation [84]. Also alterations in the assembly of electron transport chain complexes can lead to stoichiometric mismatches, which can result in delay of electron flow on sites of the complexes mediating production of superoxide. It is important to realize that antioxidant redox couples (i.e., GSH/GSSG) are closely linked to the metabolic redox couples (NADH, $FADH_2$) which serves also as the substrate of respiratory chain [82]. There are two sites within the complex I, where superoxide can be formed [85]. The first site is the flavin in the NADH-oxidizing site, producing superoxide at $NADH \gg NAD^+$, i.e., high substrate

pressure and the ubiquinone-reducing site, pumping superoxide at high protonmotive force or retarded proton pumping [86] or under condition of reverse electron pumping. Both sites produce superoxide into mitochondrial matrix. In complex III, superoxide is thought to arise from the quinol oxidizing site, producing superoxide about equally into mitochondrial intermembrane space and matrix. It was proposed that Complex I driven increased superoxide production is predominantly deleterious as they can react in high concentration with mitochondrial DNA or other matrix components vulnerable to oxidative damage, whereas Complex III driven superoxide may rather serve as a second messenger in cellular signaling. Their various but different targets were experimentally determined [87]. However, other sites of mitochondrial superoxide production were also suggested in context of various cell types. This involves complex II [88], glycerol phosphate dehydrogenase [89], pyruvate dehydrogenase [90], or α-ketoglutarate dehydrogenase [91]. How the individual ROS source contributes to increased ROS production during PH development remains to be elucidated.

Additional regulation of superoxide production might come directly from alterations in Complex I and III. For example, mutations in 9 out of 13 assembly factors of Complex I were proven as disease causing due to impairments in complex biogenesis [92]. There is evidence regarding direct downregulation of subunits of Complex I (NDUFA4L2) in murine embryonic fibroblasts [93] regulating further superoxide production. We have recently shown altered activity of Complex I in PH-Fibs from chronically hypoxic calves due to downregulation of the assembly subunit NDUFS4. This can cause complex I instability which leads to an increased disconnection of electron influx of the NADH dehydrogenase module from the complex I holocomplex. Subsequent mitochondrial hyperpolarization and increased superoxide production occurs [16]. Deficiency of NDUFS4 was shown previously to cause aberrant mitochondrial morphology and elevated ROS production in primary fibroblasts of NDUFS4 knockout mice, a model of Leigh syndrome [94]. Specific inhibition of Complex I, in this case NDUFV1 gene knockdown, reduces NAD^+/NADH ratio, however, does not drastically inhibit oxidative phosphorylation. This was shown to significantly enhance metastatic activity and cell proliferation [95]. On the other hand, enhancement of Complex I was shown to increase tumor cell autophagy and to inhibit proliferation in vivo. Based on these data from cancer research, Complex I seem to be involved in efficiency of proliferation when glycolysis takes place.

Mitochondrial superoxide production was shown to be increased by hypoxia in pulmonary artery SMCs [96, 97]. However, another group suggested it was decreased [98]. This discrepancy might be explained by differences in the techniques used for ROS detection, the experimental PH model used, the level of PH development, and handling of the cells in vitro. We have confirmed accumulation of mitochondrial superoxide in PH-Fibs from chronically hypoxic calves grown and studied under normoxic conditions [16]. Mitochondrial superoxide production from Complex III has unique role in hypoxia as produced superoxide was shown to be crucial for stabilization of HIF1α [99]. Superoxide burst inhibits HIF-prolyl hydroxylases, which under normoxic conditions hydroxylate oxygen sensitive HIF1α thus priming it for proteolytic degradation. That this superoxide production is crucial for response of pulmonary artery SMCs in hypoxia and further PH development was confirmed by Sabharwal et al., who targeted peroxiredoxin5 into mitochondrial intermembrane space of these cells [100]. ROS decay by peroxiredoxin5 then suppressed ROS production from mitochondria and the acute activation of cytosolic calcium, which regulates vasoconstriction, the initial step in PH development.

Mitochondrial ROS production can be regulated by SIRT3 protein, the predominant mitochondrial deacetylase. This enzyme requires NAD^+ for its activity. Thus changes in nutrient status can be directed through this protein to mitochondrial redox metabolism. The observed increased ratio of NADH/NAD^+ in PH-Fibs [28] together with increased glycolysis will repress SIRT3 activity. This was confirmed in human and

rat PH cells. Moreover, the human iPAH was associated with loss of function polymorphism of SIRT3 protein [101]. The targets of SIRT3, such as IDH2, SDH A, Lon protease, or NDUFA9 subunit of Complex I remain acetylated and inactivated [102]. Inactivated SDH A can then lead to accumulation of succinate. In case of the complexes of electron transport chain, the acetylation can negatively regulate assembly or subunits interaction. This might negatively impact superoxide production. SIRT3 was also shown to regulate PDHA1, one of the components of PDH complex [103]. The decreased activity of SIRT3 in PH cells might keep PDHA1 acetylated, which will attenuate its activity and induce its phosphorylation, thus directing pyruvate into lactate production. Moreover, SIRT3 knockout mice were shown to develop spontaneous PH [101]. SMCs from pulmonary arteries derived from SIRT3 knockout mice showed enhanced stabilization of HIF1 and STAT3 phosphorylation, increased phosphorylation of PDH and thus suppressed glucose oxidation in favor of glycolysis. Thus, the metabolic regulations observed in SIRT3 knockout mice contribute to PH development.

Efficiency of mitochondrial ATP synthesis was previously associated with mitochondrial morphology. Low respiratory mitochondrial bioenergetics was associated with condensed state of cristae structure (i.e., cristae expansion and matrix condensation) in situ and mitochondrial fission. Such mitochondrial ultrastructure was found to accompany mitochondria linked diseases such as diabetes and selected neurological diseases [104]. On the other hand, orthodox cristae state (i.e., matrix expansion and thin cristae) in situ and fused mitochondrial morphology corresponds with higher respiratory capacity. We have recently shown that under chronic hypoxia (72 h), metabolism of hepatocellular cancer cells (HepG2) establishes Warburg effect and downregulated mitochondrial bioenergetics is accompanied by condensed mitochondrial cristae structure [16]. A metabolic link is obvious as proteins participating in mitochondrial fusion and fission are often GTPases (Opa1, Mfn1 and 2, and Drp1) posttranslationally modified and moreover, often sensitive to mitochondrial membrane potential (Opa1). Also redox homeostasis will be important regulator [105]. Indeed, there are several reports showing that mitochondria of SMCs or Fibs from humans or experimental models with PH are fragmented with corresponding downregulation of pro-fusion proteins (Mfn2 and Opa1) and upregulated pro-fission proteins (Drp1 and Fis1) [16, 106]. Moreover, Drp1 was shown to be phosphorylated thus activated by cyclin B CDK1 in PH mitochondria of PH cells, while Mfn2 was suggested to be downregulated through transcriptional coactivator PGC1 or platelet-derived growth factor and endothelin-1 [106]. How mitochondrial dynamics in concert with mitochondrial bioenergetics participate in PH development remains to be elucidated. However, mitochondrial fusion/fission can modify rates of cell proliferation and apoptotic resistance. Thus, increased fission of mitochondria in PH cells might augment proliferation and inhibit apoptosis.

5 Activation of NADPH Oxidases in Pulmonary Arterial Hypertension

The activity of mitochondrial produced superoxide is often restricted to close proximity of its origin. Although, it can be dismutated into more diffusible hydrogen peroxide (H_2O_2), it is suggested to have signaling properties rather than pro-oxidative features outside the mitochondria. In contrast both pro-oxidative status and signaling function in cells is provided by NADPH oxidases (NOXs) (Figs. 1 and 2). Vascular NOXs are multi-subunit complexes bound to plasma membranes. Based on real-time PCR, NOX1, NOX2, and NOX4 isoforms were found to be expressed in ECs (predominantly NOX4), SMCs (predominantly NOX1,4) and Fibs (predominantly NOX4) in pulmonary arteries [107]. Expression of NOX5 was found to be present only in pulmonary artery ECs and SMCs [108]. It is important to mention, that mRNA expression profiles do not correspond to protein expression and enzyme activity. Yet, because currently

available antibodies are highly nonspecific, it is a widely used method for localization of quantification. NOXs associate in membranes with signaling domains, such as calveolae (NOX1, NOX2, and NOX4) or lipid rafts (NOX2 and NOX4). They catalyze the transfer of electrons from NADPH to molecular oxygen and thus generating ROS. Vascular NOXs produce superoxide, except for NOX4 isoform, which primarily produces H_2O_2. The stability of ROS produced by NOXs makes them likely to act as signaling molecules. Activation of NOX1,2 requires interaction with additional proteins. NOX5 is activated directly through calcium binding. The NOX2 catalytic subunit needs to be associated with p22phox membrane regulatory subunit and p47phox, p40phos, Rac2, and p67phox cytosolic regulatory subunits. Activation of this enzymatic isoform thus requires both assembly and phosphorylation steps. Similarly, NOX1 forms a complex with p22phox. NOX5 is lacking p22phox regulatory subunit for its function. NOX4 activity is different from other NOXs, as it is the only constitutively expressed NOX enzyme. It does not require p22phox regulatory subunit, but the polymerase delta-interacting protein (Poldip2) and the p47phox-related adaptor protein Tks5 have been reported to modestly enhance its activity [109, 110]. Thus its activity is regulated mainly by its expression and availability of reducing equivalents. Moreover, this isoform is upregulated under hypoxic conditions, as the promoter of this protein isoform has a HIF binding site [111]. Together with enhanced glycolysis and pentose phosphate pathway and suppressed mitochondrial respiration in PH arterial cells (being NADH and NADPH producers), NOX4 activity is significantly elevated [16, 112]. As such, it can regulate cellular function through growth factors (TGF-β and PPAR-γ), cytokines, HIF, vasoactive agents (endothelin-1) or G protein-coupled receptor agonists allowing it to regulate enzymes and ion channels with proliferative, inflammatory, and apoptotic properties. Indeed, increased NOX4 activity was sufficient to enha,nce fibroblast migration and proliferation [112, 113]. Similarly, it has been shown to be critical for HIF2 transcriptional activity in renal carcinoma [114]. Another target was described to be Ca^{2+} permeable cation channel TRPC, through which 60% of Ca^{2+} enters the pulmonary artery SMC. It can thus initiate vasoconstriction, later remodeling of pulmonary artery and also endothelial barrier dysfunction [115]. Its importance highlight the fact that it was found to be the only isoform present in all layers of pulmonary artery which is increased in various models of PH [112]. Thus, NOX4 was suggested to play a key role in chronic hypoxia-induced pulmonary vascular remodeling, which can be suppressed using new NOX1/4 inhibitor (GKT137831) [116]. Unfortunately, many previous studies using NOXs inhibitors such as diphenyliodonium (DPI) or apocynin cannot be interpreted as being solely due to NOX activity involvement because, for example, DPI was found to be a general flavoprotein inhibitor and apocynin was described to behave like an antioxidant in the cell. However, new generation inhibitors are also not specific for individual protein isoforms. Therefore, the results of these studies often reflect manipulation of redox status within whole cell, i.e., mitochondrial and other sources.

The subcellular localization of NOX4 in vascular cells is more complex than other NOX isoforms. In lung ECs, expression of native NOX4 is predominantly localized to the nucleus as compared to endoplasmic reticulum, mitochondria or plasma membrane [117]. NOX4 has a cis-acting ARE sequence, which is regulated by Nrf2 under stress conditions (i.e., hyperoxia), and, thus, the catalyzed H_2O_2 signal is part of a cellular adaptation response to oxidative stress. For that, nuclear localization of NOX4 is important and its expression can be regulated. The Km of NOX4 for oxygen has been described to be unusually high. This together with non-inducible production of H_2O_2 could mean that it responds directly and acutely to oxygen tension with output of the signal molecule (H_2O_2), rather than responding to external signals via intermediate signaling mechanisms such as phosphorylation of regulatory subunits or changes in cellular calcium levels. Thus NOX4 signaling may participate in both acute mechanisms and slower ones involving induction of NOX4 expression [118]. As mentioned previously,

enhanced glycolytic metabolism of PH arterial cells is similar in certain ways to cancer metabolism. Cancer cells with compromised mitochondrial function due to p53 loss showed increased NOX4 activity and enhanced sensitivity to NOX4 inhibition. Activation of NOX4 in that case was suggested to provide additional NAD^+ to support the highly active glycolysis [119] in addition to regulation of several signaling pathways (JAK-STAT, protein kinase C, MAPK, AKT, and inflammatory mediators via the NF-κB pathways etc.). TGF-β was described to be involved in pathophysiological vascular remodeling in PH and interestingly, to increase NOX4 protein levels [120]. Protein kinases C, angiotensin II, and cyclic adenosine monophosphate are other stimuli that modulate NOX4 expression.

Another isoform found in PH cells, NOX2, was originally found to be highly expressed in phagocytes. In mice, NOX2 deletion reverses hypoxia induced PH [121]. However, how NOX2 contributes to PH is not fully understood. It might regulate inflammation as a part of vessel remodeling.

Thus, NOXs are important sources of ROS production in PH pathology; however, the activity of individual isoforms present, their regulation and signaling properties need to be better determined in relation to PH development [16].

6 Interplay Between Mitochondria and NADPH Oxidases

The nonspecific nature of in situ ROS detection methods combined with a disregard of ROS compartmentalization within a cell has led to still unresolved controversies regarding the contribution of individual ROS sources to cell signaling. However, it is suggested that substantial interplay between different sources exists. For example, mitochondrial ROS can be amplified by cytoplasmic NOX and vice versa, leading to feed-forward process that augments the pro-oxidative status required for pathological signaling in PH. There is evidence regarding mitochondrial redox involvement in NOX activity [122, 123]. One report by Rathore et al. [122] showed that inhibition of mitochondrial ROS production either with rotenone (Complex I) and myxothiazol (Complex III) or by mitochondrial Gpx1 overexpression prevented activation of NOX in pulmonary artery SMCs under hypoxic conditions. This points out a requirement of mitochondrial ROS for HIF stabilization, which can then further activate NOXs in pulmonary arteries through a mitochondrial ROS-PKCε-NOX4 axis. Wedgwood et al. described another example where attenuation of stretch-induced NOX4 expression was found to be a consequence of mitochondrial complex III inhibition in fetal pulmonary artery SMCs [123]. Moreover, Complex III lies upstream of NF-κB and NOX4 and is sensitive to ROS. Enhanced H_2O_2 production by NOX4 activity then leads to activation of genes necessary for cell cycle progression and thus induction of vascular remodeling.

On the other hand, changes in NOX activity also have an impact on mitochondrial redox status. For example, PKC dependent activation of NOXs by angiotensin is an early response seen in endothelial cells. This induces mitochondrial superoxide production, which becomes deleterious for mitochondrial function. It opens the mitochondrial K_{ATP} channel, causes matrix alkalization, swelling, mild mitochondrial uncoupling and further ROS production [124]. Another report describes inhibition of mitochondrial Complex I (NDUFA9 subunit) by increased NOX4 activity in HUVEC cells [125]. NOX was suggested to affect the synthesis and/or stability of Complex I subunits encoded in the mitochondria.

Based on the evidence above it is obvious that mitochondrial ROS interact with NOXs and collectively induce a pro-oxidative redox status in cells which further participates in signaling during PH development. Adesina et al. performed elegant experiments to establish this relationship [126]. They overexpressed mitochondrial superoxide dismutase (SOD2) in mice, which converts superoxide to H_2O_2 in mitochondria. This lead to enhanced mtH_2O_2 production, which induced NOX activity (NOX2 and NOX4 isoforms), leading to pulmonary vascular cell proliferation. On the other hand, mice with increased expression of mitochondria-targeted catalase, which

decompose mitochondrial H_2O_2, suppressed NOX expression and attenuated ECs proliferation (i.e., downregulated cyclinD1 and PCNA) in hypoxia induced PH [126]. This suggests that mitochondrial redox balance might be suitable target for pharmacological treatment of PH.

7 Other ROS Sources and Antioxidant Balance

Most intracellular ROS are derived from the superoxide radical. Mitochondrial superoxide production is not the only cellular source. It was shown that the exposure of pulmonary artery endothelial cells to hypoxic conditions results in increased activity of xanthine oxidase, which then generates a surplus of superoxide [127]. Enhanced xanthine oxidase activity was also reported in the arteries of PH patients [128]. ROS produced by xanthine oxidase upregulates Erg1 via ERK1/2 and increases its phosphorylation thus enhancing proliferation and reducing apoptosis [129]. However, superoxide can be converted to non-radical H_2O_2 by superoxide dismutases (SOD), present in mitochondria as well as the cytosol. H_2O_2 has a capacity to cross the membranes and its stability is much higher. This allows H_2O_2 to encounter susceptible residues on target molecules and display selectivity. Such signaling includes reversible oxidation of cysteines. This can cause allosteric changes of proteins to modify their activity or can affect binding with partners and thus alter signaling cascades. Interestingly, pulmonary hypertensive SMCs show decreased mitochondrial manganese superoxide dismutase (MnSOD), due to hypermethylation of the SOD2 gene [130]. Similarly, the disruption of extracellular SOD (EC-SOD) either globally or in specific cells of pulmonary arteries, results in worsening of experimental PH [131–133]. Decreased activity of EC-SOD in chronically hypoxic calves was shown to lead to upregulation of Erk1 via ERK1/2 [129]. The decreased enzymatic antioxidant protection in PH thus enables excessive production of ROS which can proceed to oxidative stress, the phenotype accompanying PH pathology. Several studies confirmed enhanced lipid peroxidation in human PH samples, i.e., increased F2 isoprostane and plasma malondialdehyde [134, 135]. On the other hand, nonenzymatic antioxidants, such as thioredoxin, were found to increase catalytic activity in many cancer cells. Moreover, the level of its activity was associated with aggressive tumor growth and poorer prognosis. Enhanced thioredoxin activity was shown to significantly increase HIF1α under both normoxic and hypoxic conditions [136]. This was confirmed to be valid in SMCs of chronic hypoxia-induced PH in mice, which is interesting with respect to downregulated enzymatic antioxidant protection. Increased activity of thioredoxin1 was reported to modulate HIF1 activation and subsequent phosphatidylinositol 3 kinase (PI3K)–seronine/threonine kinase (Akt) activation inducing proliferation [137]. Collectively, these data show that redox maintenance in PH is complex and compartmentalized. Thus, we need to consider not only the levels and type of ROS produced, but also state of specific antioxidant defense. ROS has naturally compartmentalized signaling properties; however, while exceeding antioxidant capacity they can establish rather pathological oxidative stress. Thus, cellular redox balance is important feature of cell proliferation in PH development.

In the pulmonary circulation the production of nitric oxide (NO) by ECs is crucial for normal function, as it allows vessel vasodilation. It also regulates vascular SMC proliferation and migration [138]. There is general consensus that bioavailability of NO is decreased and NO signaling is impaired during PH development. However, conflicting results exist concerning endothelial nitric oxide synthase (eNOS) expression and activity. Diminished NO signaling can be explained by eNOS uncoupling, which is a dysfunctional state of eNOS enzyme forming superoxide rather than NO [139, 140]. NO works almost exclusively as a signaling molecule, because it becomes injurious only after reacting with superoxide to form peroxynitrite anion. This can happen in PH when superoxide levels are increased. Peroxynitrite can then induce loss of function of protein kinase G and lead to the development of PH [141]. Peroxynitrite can also

activate ERK [142], p38 MAP kinase [113], and protein kinase C [143].

Thus it is increasingly evident that redox balance is diverted toward pro-oxidative status during the development of PH. However, we are still missing detailed knowledge regarding the interplay and importance of individual ROS/NO sources in PH.

8 Summary

Pulmonary vascular remodeling in PH results from a synergy of changes in metabolism and redox balance in resident and recruited cells within the microenvironment of the artery wall. It involves endothelial cells, smooth muscle cells, fibroblasts, cells of immune system such as macrophages and the sympathetic nervous system. A prominent characteristic of the pulmonary artery remodeling is enhanced proliferation and apoptosis resistance of each of the resident cells comprising the vessel wall. Rapid or uncontrolled proliferation requires metabolic changes in cells in order to provide sufficient quantities of building blocks for protein synthesis and cell proliferation. Thus, it is not surprising that cells of hypertensive pulmonary artery wall shift toward glycolysis while suppressing catabolic reactions of mitochondrial metabolism. This provides cells with anabolic intermediates required for synthesis of amino acids, lipids, and nucleotides. This altered metabolism resembles the Warburg-like effect described for rapidly proliferating cancer cells. The cells increase glucose uptake, increase expression of enzymes of glycolytic pathways, and enhance the pentose phosphate pathway and one carbon metabolism, and thus the glycolytic pathway becomes a crucial source of energy production and metabolic intermediates. Mitochondrial bioenergetics is largely suppressed; fluxes of TCA cycle intermediates become altered to maintain mitochondrial membrane potential, to change redox signaling and to support production of metabolites for growth. Adjustment of mitochondrial energy metabolism gives rise to generation of ROS, being the initial provider of redox signaling, which is further amplified in cytosol by upregulation of NOX activity. We believe that the increased oxidative status supports the proliferation and together with activated cells of immune system participates in remodeling of pulmonary artery wall in PH. Chronic inflammation accompanying development of PH was described to require macrophage activation, whose first step includes metabolic changes. Thus, such metabolic and redox synergy might be key factors in pro-remodeling phenotype of cells that ultimately leads to severe PH and right ventricular failure.

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Hydrogen Sulfide as an O_2 Sensor: A Critical Analysis

Jesus Prieto-Lloret and Philip I. Aaronson

1 Introduction

It has been 20 years since Hosoki et al. [1] demonstrated that rat aortic homogenates produce the gas hydrogen sulfide (hereafter referred to as "sulfide" to include the species H_2S and HS^- which exist in a relative proportion of about 20% and 80%, respectively, at physiological pH), and that application of exogenous sulfide caused vasodilation of this artery. It has subsequently been shown that sulfide can exert antihypertensive, anti-inflammatory, antioxidant, and pro-angiogenic effects in animals, and there is increasing interest in the possibility that sulfide-releasing drugs could be used to treat conditions such as hypertension and heart failure. Chronic treatment with sulfide via intraperitoneal (i.p.) injection also reduces pulmonary artery pressure and ameliorates pulmonary vascular remodelling in animal models of pulmonary hypertension [2].

Notwithstanding its blood pressure-lowering effects in the systemic vasculature, and also in the pulmonary vasculature when used as a chronic treatment in experimental models of pulmonary hypertension, the *acute* response to application of sulfide to isolated pulmonary arteries (PA) or perfused lungs in vitro tends to be an immediate contraction and a rise in pulmonary arterial blood pressure, respectively [3]. Intriguingly, this contraction shares a number of properties with hypoxic pulmonary vasoconstriction (HPV), a physiological mechanism which couples alveolar hypoxia to an increase in the resistance of local pulmonary arteries, thereby diverting the flow of blood from poorly to well oxygenated regions of the lung and in this way maintaining the ventilation–perfusion ratio and thereby maintaining O_2 saturation [4].

Based in part on the apparent similarity between the acute effects of hypoxia and sulfide application on PA constriction, Kenneth Olson and colleagues proposed that sulfide acts as an O_2 sensor; in particular they described a model in which HPV is due to a hypoxia-induced rise in the intracellular sulfide concentration in PA smooth muscle cells [3]. This group later proposed that an analogous sulfide-based mechanism mediates O_2 sensing in trout gills, which are homologous to the mammalian carotid body [5].

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In this chapter we provide a description and critical analysis of the proposal that sulfide is an O_2 sensor, both in PA and in the carotid body. We begin by presenting a synopsis of what is known about the synthesis and metabolism of sulfide, and also describe briefly its interaction with cysteine residues on proteins—proposed to be the main mechanism by which it is thought to regulate various cellular processes.

2 Sulfide as a Physiological Signaling Molecule

Figure 1 summarizes basic aspects of the mechanisms by which sulfide is synthesized, is metabolized, and exerts its cellular effects. There are four enzymatic pathways by which sulfide is thought to be synthesized in the body. The enzyme cystathionine-γ-lyase (CSE), which is viewed as being the most important source of sulfide in the cardiovascular system [6], synthesizes sulfide mainly from L-cysteine and utilizes pyridoxal 5′-phosphate (PLP, vitamin B_6) as a cofactor. Another PLP-requiring enzyme, cystathione β-synthase (CBS), predominates in the central nervous system, and forms sulfide mainly by condensing L-cysteine and homocysteine. The third pathway synthesizes sulfide in two steps. In the first, L-cysteine aminotransferase (CAT) produces 3-mercaptopyruvate (3-MP) from L-cysteine and α-ketoglutarate. 3-MP is then used by the enzyme 3-mercaptopyruvate sulfurtransferase (3-MST) to form 3-MST persulfide, from which sulfide can then be released by cellular reducing agents which may include thioredoxin and dihydrolipoic acid [7]. It is thought that whereas sulfide synthesis by CSE and CBS occurs in the cytoplasm, its formation by the CAT-3-MST pathway occurs mainly in the mitochondria. 3-MP can also be formed from D-cysteine by D-amino acid oxidase, a process thought to occur in the brain and kidney [8].

Sulfide is metabolized via oxidation, first to thiosulfate ($S_2O_3^{2-}$) and ultimately to sulfate (SO_4^{2-}). The initial step of this process involves a mitochondrial "sulfide oxidation unit" comprising three enzymes, sulfide quinone oxoreductase (SQR), dioxygenase, and sulfur transferase. Interestingly, this process causes the sequential transfer of electrons from sulfide to SQR, and then into the electron transport chain (ETC) at ubiquinone, resulting a stimulation of mitochondrial respiration at very low (nM) sulfide concentrations. At higher concentrations (μM), however, sulfide blocks cytochrome C oxidase, causing a block of the ETC which accounts for its poisonous nature [9, 10].

Current knowledge regarding intracellular sulfide levels, and how these are regulated and compartmentalized, is rudimentary. However, several mechanisms regulating sulfide synthesis have been identified, e.g., CSE has been shown to be stimulated by Ca^{2+}–calmodulin [11], although this has been disputed [12], and is inhibited by protein kinase G-mediated phosphorylation [13]. It is also thought that sulfide is stored in cells as sulfane sulfur bound to cysteine residues, such that the release of these stores might play the predominant role in controlling its intracellular concentration.

An additional level of complexity arises because sulfide coexists with and gives rise to other related chemical species, each of which can act as a signaling molecule in its own right. For example, sulfide can form sulfur chains, termed polysulfides (H_2S_n), and there is evidence that these can also be produced directly from 3-MP by 3-MST [14]. Sulfide and polysulfides are thought to exert their effects on cells by interacting with cysteine residues to modulate the formation of disulfide bonds within target proteins, thereby affecting their conformation and function. Sulfide itself can reduce and therefore break disulfide bonds, whereas cysteine residues oxidized under oxidative conditions or by reaction with NO can be sulfhydrated by polysulfides, which can then result in disulfide bond formation [15]. Sulfide can also react with nitric oxide (NO) to form polysulfides and nitrosopersulfide ($SSNO^-$) [16] as well as nitroxyl (HNO) [17]. This is but one example of the extensive network of interactions which have been demonstrated to exist between the sulfide and NO pathways and are thought to result in their mutual synergism [2].

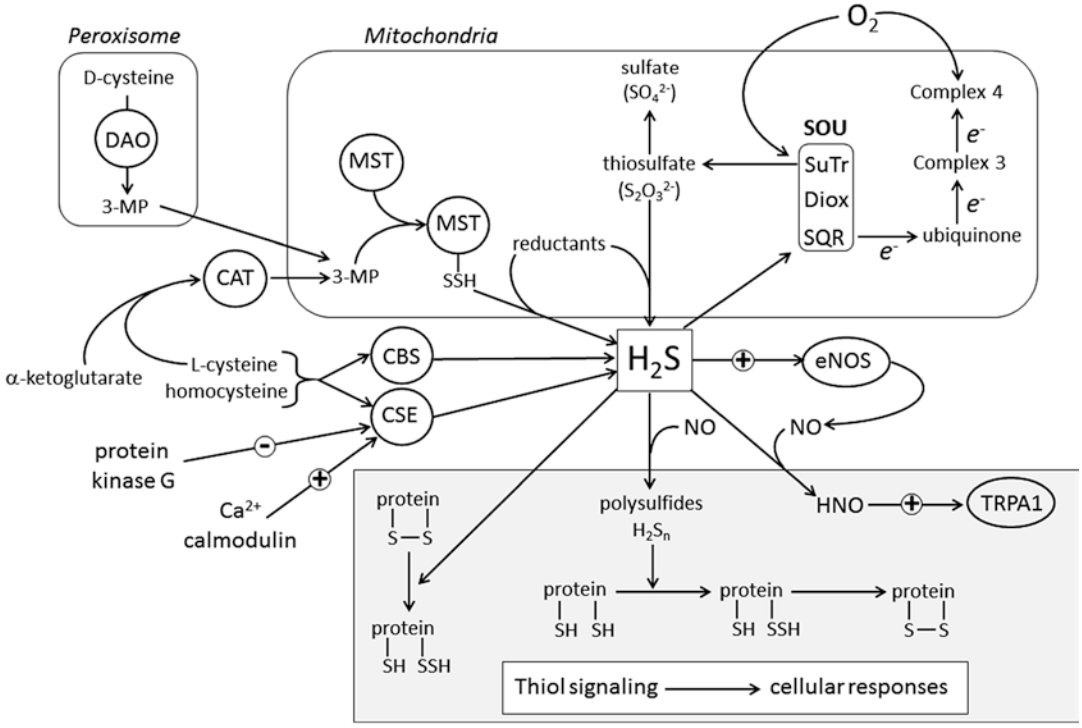

Fig. 1 Basic aspects of the synthesis, metabolism, and cellular effects of sulfide. Sulfide is synthesized by four enzymatic pathways: (1) by cystathionine-γ-lyase (CSE) and (2) cystathione β-synthase (CBS) from L-cysteine and homocysteine, (3) by cysteine aminotransferase (CAT) and then 3-mercaptopyruvate sulfurtransferase (3-MST) from L-cysteine and α-ketoglutarate, and (4) by D-amino acid oxidase (DAO) and then 3-MST from D-cysteine. Sulfide can also be generated by the reduction of thiosulfate. Sulfide is thought to be metabolized mainly by the mitochondrial sulfide oxidation unit (SOU) which consists of three enzymes operating sequentially: sulfide quinone oxoreductase (SQR), dioxygenase (Diox), and a sulfur transferase (SuTr). The oxidation of sulfide by SQR results in electrons being passed into the electron transport chain via ubiquinone. Sulfide is thought to exert its effects on cells mainly by causing thiol signaling and consequent changes in protein configuration. Sulfide itself can reduce disulfide bonds. It can also be converted to polysulfides, e.g., by reacting with nitric oxide (NO), which can oxidize cysteine residues, leading to the formation of persulfides (−SSH) and disulfide bonds. Sulfide has multiple interactions with the NO pathway; for example it promotes the dimerization and activation of endothelial nitric oxide synthase (eNOS). It can also react with NO to form nitroxyl (HNO), which can act as a vasodilator and also activates TRPA1. This figure is based on information drawn mainly from Yang et al. [11], Olson et al. [7], Szabo et al. [10], Eberhardt et al. [17], Yuan et al. [13], Cortese-Krott et al. [16], and Kimura [15]

Despite the burgeoning literature on the effects of sulfide on various body systems, many basic aspects of its physiology remain unclear and controversial. Relatively little is known about how its synthesis is regulated, and no proven methods exist for measuring either its concentration or its compartmentalization within cells. Much of what has been observed with regard to its possible physiological functions has emerged from studies which have examined the functional effects, on animals, tissues or cells, of imposing changes in the ambient sulfide concentration, although this has seldom been measured or well controlled in these experiments. Most often, its concentrations have been raised by applying exogenous sulfide, typically in concentrations which are almost certainly supra-physiological. Another widespread approach has been to examine the effects of preventing the synthesis of endogenous sulfide using either antagonists of sulfide-synthesizing enzymes or mice in which one of these enzymes (most often CSE) has been knocked out. However, the validity of these approaches is potentially limited by the

non-selectivity of the antagonists, which are often applied in very high concentrations, and by the fact that CSE knockout mice demonstrate markedly elevated levels of homocysteine, which has well-documented deleterious effects, particularly on vascular function [18].

Studies of the effects of sulfide in the cardiovascular system have focused mainly on unravelling its interactions with NO, examining the mechanisms underlying its vasodilating and pro-angiogenic properties, and exploring its therapeutic potential in animal models of hypertension, ischemia and heart failure (see review by Brampton and Aaronson [2]). On the other hand, the proposal that sulfide is involved in O_2 sensing in PA [3] and more recent work showing that it plays a similar role in the carotid body [5, 19] has also generated an increasing amount of interest. We examine these models and the evidence supporting them in Sects. 3 and 4, respectively.

3 Hydrogen Sulfide as a Putative O_2 Sensor in Pulmonary Arteries

Studies by Olson's laboratory revealed that exogenously applied sulfide and hypoxia caused similar contractile responses in a wide range of blood vessels from a diverse group of vertebrate species, even though the responses themselves varied widely between the different preparations. For example, both sulfide and hypoxia caused relaxation of rat aorta, whereas both contracted bovine PA or lamprey dorsal aorta [3, 20, 21]. In isolated rat PA, a preparation widely used to study HPV, hypoxia elicits a characteristic biphasic contraction when applied to arteries which have been slightly pre-constricted by an agonist (e.g., $PGF_{2\alpha}$ or U46619 which act on prostaglandin TP receptors or the α-adrenoceptor ligand phenylephrine; [4]); this "pre-tone" is often used in studies of HPV to amplify the response. Olson et al. [3] also observed a biphasic contraction with an intervening relaxation in rat PA upon applying 1000 μM NaHS in the presence of U46619. Figure 2 illustrates this complex response in these arteries, in this case evoked by 500 μM Na_2S in the presence of $PGF_{2\alpha}$. Interestingly, lower concentrations of Na_2S (10 and 30 μM Na_2S) evoke only a monophasic response (Prieto-Lloret & Aaronson, unpublished).

Olson et al. [3] proposed that the remarkable similarity between the effects of hypoxia and sulfide that they consistently observed in many types of blood vessels arises because the contractile effects of hypoxia are mediated by a rise in the intracellular sulfide concentration, this being due to an attenuation of oxidative sulfide metabolism consequent on the reduced O_2 concentration. The cellular sulfide concentration would thus act as an O_2 sensor. The apparent ubiquity of this mechanism, as reflected by the similar effects of sulfide and hypoxia in numerous blood vessels from many species, some ancient, implied that it had arisen early in evolution and had been conserved.

In particular, the observation that sulfide mimicked the effect of hypoxia in PA from rats and cows presented by Olson et al. [3] indicated that this mechanism might be responsible for HPV. Evidence for this model was subsequently expanded upon in a series of papers from this laboratory [7, 22–25], and in addition to the similarity between the contractile responses to sulfide and hypoxia in across a range of species and types of blood vessels described above, includes a number of key observations:

1. Lung tissues and PA express enzymes which synthesize sulfide.
2. Lung homogenates and pulmonary vascular preparations produce sulfide in an oxygen-dependent manner.
3. The sulfide precursor cysteine enhances hypoxia-induced contractile effects.
4. Hypoxia and sulfide antagonize each other's contractile effects.
5. Antagonists of sulfide-synthesizing enzymes inhibit hypoxia-induced contractile effects.

Below, we provide a critical analysis of these findings and the opposing evidence in an effort to evaluate the validity of the concept that sulfide acts as an O_2 sensor in HPV, and also speculate

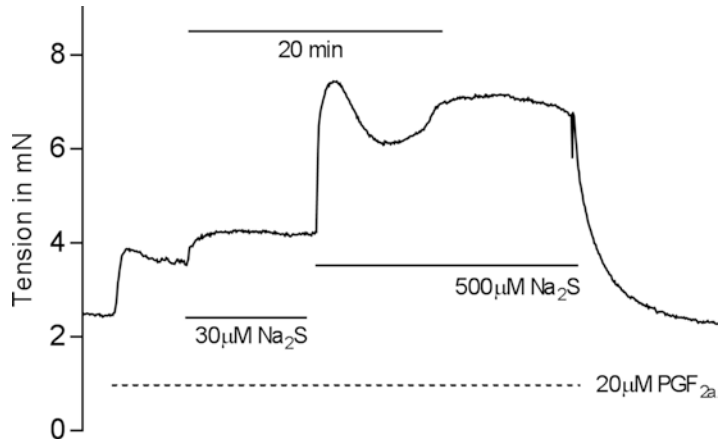

Fig. 2 Contractile effects of 30 and 500 μM Na_2S sulfide in rat pulmonary artery. A small PA suspended in a myograph and gassed with 20% O_2, 5% CO_2, and 75% N_2 was pre-contracted with 20 μM PGF_{2a} and then was exposed to 30 μM and 500 μM Na_2S sequentially. The artery was then washed in control physiological saline, causing a full relaxation. Note that 30 μM Na_2S caused a sustained monophasic contraction, whereas 500 μM Na_2S evoked a complex response consisting of a rapid contraction, a partial relaxation, and then a second more slowly developing contraction

about alternative possibilities as to why the contractile effects of sulfide and hypoxia may resemble each other. We note that for reasons of space we have limited ourselves mainly to examining studies carried out in PA, although much of the evidence favoring a role for sulfide in O_2 sensing in the vasculature has come from studies examining the involvement of sulfide in the hypoxia-induced vascular relaxation, which is more commonly observed in a range of systemic arteries (see for example [23]).

3.1 Lung Tissues and Pulmonary Arteries Express Enzymes Which Synthesize Sulfide

A number of studies have demonstrated that lung homogenate and/or PA smooth muscle from rats expresses mRNA for CSE [26–28]; in contrast, neither protein nor mRNA for CBS were detected [22, 27]. CSE and 3-MST proteins are present in sea lion PA and PA smooth muscle cells (PASMC) from bovine PA, whereas CBS is present in the endothelium from the latter arteries [24]. In addition, all three enzymes were shown by immunohistochemistry to be present in alveoli, small airways and PA endothelium, in lung slices from both species. CSE and 3-MST protein was also detected in rat lung homogenates [22].

3.2 Lung Homogenates and Pulmonary Vascular Preparations Produce Sulfide in an Oxygen-Dependent Manner

Whereas previous studies had demonstrated that lung homogenates supplemented with exogenous cysteine and PLP generated sulfide under anoxic conditions [26, 28], Olson et al. [24] were the first to examine the effect on sulfide levels of introducing variable concentrations of O_2 into the homogenates, in this case from cow and sea lion lung. L-cysteine and the CSE/CBS cofactor PLP were added to promote sulfide synthesis. Sulfide levels rose under severely hypoxic conditions, but then fell rapidly when an amount of air sufficient to raise the pO_2 to ~7 mmHg was added to the reaction chamber. Further experiments in which the rate of sulfide consumption was recorded showed that this was half maximal at pO_2 values of 3.2, 6, and 0.8 mmHg in bovine lung homogenate, suspensions of cultured bovine PASMC, and cow heart mitochondria,

respectively. Measurements of HPV in intact cow PA demonstrated that contraction developed as the O_2 concentration was decreased over a similar range, consistent with the possibility that the increase in tension development was associated with a fall in sulfide metabolism and a consequent rise in its cellular concentration.

Madden et al. [22] measured sulfide accumulation by rat lung homogenate under severely hypoxic conditions. Addition of 1 mM L-cysteine in the presence of PLP had no effect on sulfide levels, whereas sulfide increased after 1 mM α-ketoglutarate was added, implying that the CAT-3-MST pathway rather than CSE was primarily responsible for sulfide synthesis in rat lung. This rise in sulfide was reversed by introduction of air into the reaction chamber. Similar effects were observed when sulfide release from intact rat PA was recorded, although introduction of air into the chamber caused only a small fall in the sulfide concentration.

In a recent study, Krause et al. [29] used AzMC, a fluorescent sulfide sensor, to record sulfide production over 6 h by porcine tracheal epithelium under hypoxic and hyperoxic conditions. AzMC fluorescence showed little increase over this time period in hyperoxic conditions, whereas a slow but marked increase occurred with hypoxia. Interestingly, parallel measurements of polysulfide levels using another fluorescent probe, PP4, showed that these were reduced by hypoxia, leading the authors to suggest that hypoxia was causing the conversion of the polysulfides to sulfide. However, an alternative explanation for these results is that since both probes were present in the bathing solution throughout the period during which the measurements were made, it is possible that the apparent suppression of sulfide production by hyperoxia reflected the extracellular conversion of sulfide released by the cells into polysulfides, which would be predicted to occur in the presence of oxygen but not in its absence.

Although these results are, in general, in accord with the idea that hypoxia increases cellular sulfide concentrations, the evidence for this remains both sparse and indirect. As far we are aware, experiments in vascular preparations have only examined the relationship between pO_2 and the rate of sulfide *consumption*, although Peng et al. [19] recorded the predicted inverse relationship between sulfide *production* and pO_2 in carotid body homogenates. The proposed hypoxia-induced increase in O_2 and sulfide concentrations within cells has never been observed, and will probably remain unverified until better methods for measuring intracellular sulfide are available.

3.3 The Sulfide Precursor Cysteine Enhances Hypoxia-Induced Contractile Effects

There is general agreement that adding exogenous sulfide precursors such as L-cysteine to the physiological saline solution bathing isolated PA or lungs amplifies the vasoconstricting effects of hypoxia. This was first shown by Olson et al. [3], who reported that 1 mM L-cysteine doubled the hypoxia-induced contraction in both lamprey dorsal aorta and bovine PA. This effect seemed to be specific for the response to hypoxia, as the contraction evoked by high K^+ solution was unaffected by cysteine. Madden et al. [22] reported that both L-cysteine and glutathione, which is converted to L-cysteine in cells, strongly enhanced HPV when added to the physiological saline solution perfusing isolated rat lungs, whereas neither substance had any effect on the response to angiotensin II. Moreover, applying α-ketoglutarate, which would putatively stimulate the CAT-3-MST pathway for sulfide synthesis, also potentiated HPV but not the effect of angiotensin II.

Olson et al. [24] similarly found that both cysteine and glutathione strongly enhanced HPV in bovine PA, but made the unexpected observation that this did not occur if PA were pre-constricted with the thromboxane A_2 mimetic U46619 before hypoxia was imposed. Pre-constriction itself enhanced HPV, and the authors argued that this was somehow masking the effect of the sulfide. It remains unclear, however, why this should occur, since the potentiating effect of the sulfide precursors on HPV was substantial, and likely to be due

to a set of mechanisms differing substantially from those causing U46619-induced amplification of HPV.

Prieto-Lloret et al. [27] observed that 1 mM L-cysteine, on its own or in combination with 1 mM α-ketoglutarate, similarly enhanced HPV in isolated small PA from the rat. However, cysteine exerted a quantitatively similar potentiation of the contraction elicited by $PGF_{2\alpha}$, implying that HPV was not unique in its sensitivity to an increased cellular synthesis of sulfide. In contrast to what was reported by Madden et al. [22], α-ketoglutarate applied on its own did not cause any potentiation of HPV. Prieto-Lloret and Aaronson [30] also demonstrated that treating isolated rat PA with either 3-MP or D-cysteine, which would be predicted to increase cellular synthesis of sulfide via 3-MST, increased both HPV and the contraction to $PGF_{2\alpha}$.

Taken as a whole, these results show that interventions designed to increase the cellular synthesis of sulfide potentiate HPV, as would be predicted if this response is due to a hypoxia-induced rise in the intracellular sulfide concentration. However, it is apparent that sulfide precursors have a similar effect on at least one type of vasoconstrictor-mediated contraction. Moreover, this effect is of similar magnitude even though $PGF_{2\alpha}$ is unlikely to mimic hypoxia in raising the sulfide concentration, a finding which on the face of it appears to be inconsistent with the concept that hypoxia raises the cellular sulfide concentration by suppressing its metabolism.

3.4 Hypoxia and Sulfide Antagonize Each Other's Contractile Effects

Olson et al. [3] observed that whereas both hypoxia and 300 μM sulfide contracted bovine PA when added separately, application of sulfide caused relaxation if arteries were already contracted by hypoxia, and vice versa. This interaction, which they described as being competitive, did not occur when either stimulus was applied to arteries pre-constricted with a vasoconstrictor, e.g., U46619. Based on this observation, they argued that the contractile responses to hypoxia and sulfide must be mediated by the same pathway, such that once this had been activated by one of these stimuli the other was unable to have its usual effect. Although the authors did not discuss the nature of this putative competitive effect further in this paper, implicit in their argument is the idea that both the level of hypoxia and the concentration of sulfide in these experiments must have been maximally stimulating the common pathway, since an additive or synergistic effect on contraction would be predicted if the first stimulus applied was submaximal. Notably, 100% N_2 was used to induce HPV in these experiments, suggesting that the hypoxic stimulus was indeed maximal. Similarly, the response to a range of concentrations of sulfide was inhibited by severe hypoxia in the vasculature of trout gills [25]. On the other hand, Olson et al. [31] reported that hypoxia induced with 100% N_2 strongly potentiated the contraction evoked by 1–100 μM H_2S in hagfish dorsal aorta, and had no effect on the response to higher concentrations of H_2S. It would therefore appear that the same severe level of hypoxia can either inhibit or potentiate the response to sulfide, possibly depending on the preparation.

3.5 Antagonists of Sulfide-Synthesizing Enzymes Inhibit Hypoxia-Induced Contractile Effects

To date, the most direct evidence supporting a role of H_2S as an O_2 sensor in HPV comes from two reports demonstrating that antagonists of sulfide-synthesizing enzymes blocked this response [3, 22]. The antagonists used included propargylglycine (PAG or PPG), β-cyanoalanine (BCA), hydroxylamine (HA), aminooxyacetic acid (AOA or AOAA), and arginine. PPG and BCA are relatively selective blockers of CSE, whereas HA and AOA block CSE and CBS over a similar concentration range [32] and arginine blocks CAT.

Olson et al. [3] reported that AOA attenuated and HA abolished HPV in isolated bovine PA, whereas PPG had no effect. Given the lack of effect of PPG, these results imply that CBS was the likely source of sulfide during HPV; however, this conclusion seems inconsistent with a subsequent study [24] which demonstrated that CBS protein could not be detected in PASMC from these arteries, although weak expression was detected in endothelial cells. Madden et al. [22] observed that PPG essentially abolished HPV in isolated rat perfused lung. In addition, application of α-ketoglutarate on its own strongly enhanced HPV, and this effect of α-ketoglutarate was absent in the presence of the aspartate, which antagonizes sulfide production by CAT. Importantly, none of these drugs affected the constricting response to angiotensin II in the rat lung. These results showed that the CAT-3-MST pathway in rat lung could potentially mediate sufficient sulfide synthesis to potentiate HPV, although since the effect of aspartate on HPV was not examined in the absence of exogenously applied α-ketoglutarate the physiological role of this pathway in HPV remained undefined.

In addition to the sulfide produced on an ongoing basis by CSE or CAT-3-MST, cells may also contain a large amount of sulfide which is stored as sulfane sulfur bound to cysteine residues (e.g., present in mitochondrial 3-MST). Olson et al. [7] have proposed that the sulfide metabolite thiosulfate, which is generated within mitochondria, may also act as an additional reservoir of sulfide. Both types of store would be expected to give rise to sulfide under reducing conditions. In light of evidence that hypoxia is associated with a reduction of reactive oxygen species (ROS) levels within the intramitochondrial compartment in PASMCs [33], it was suggested that sulfide might be regenerated from thiosulfate during HPV, thereby causing a rise in its cellular concentration which would be potentiated by the simultaneous inhibition of oxidative sulfide metabolism [7]. This process would allow hypoxia to cause a rise in cellular sulfide which might be relatively insensitive to inhibitors of sulfide-synthesizing enzymes. In support of this concept, they demonstrated that the reducing agents dithiothreitol (DTT) and dihydrolipoic acid increased the release of sulfide from tissues under hypoxic but not normoxic conditions, and also that both reductants enhanced HPV.

In contrast to these findings, we have recently presented evidence that neither enzymatic production of sulfide nor cellular stores which can be mobilized by reducing agents appear to play a role in HPV in isolated small PA from the rat [27]. Our experiments showed that application of 1 mM L-cysteine increased the amplitude of both HPV and the contraction to $PGF_{2\alpha}$ by ~50%. The L-cysteine-induced potentiation of both types of contraction was abolished by the CSE blocker PPG, consistent with the idea that L-cysteine was increasing tension development by increasing sulfide synthesis via CSE, and that PPG was effectively blocking this enzyme. However, PPG had no effect on HPV in the absence of cysteine, or in the presence of a concentration of L-cysteine similar to that which exists in plasma (10 μM, [34]). HPV was also enhanced by the combination of L-cysteine and α-ketoglutarate, and this response was largely blocked by the CAT inhibitor aspartate, implying that sulfide synthesis by CAT-3-MST could also potentiate HPV. However, as with PPG, aspartate had no effect on HPV under control conditions. In further experiments, we were also unable to confirm the finding of Olson et al. [7] that the reductant DTT potentiated HPV, showing instead that DTT virtually abolished this response.

This study also showed that HA and BCA, in concentrations lower than those used by Olson et al. [3], had marked effects of their own on the contraction evoked by $PGF_{2\alpha}$ or the ROS donor LY83583, with BCA enhancing and HA suppressing both responses. AOA also strongly inhibited the LY82583 contraction. These drugs therefore exert effects on responses which are unlikely to involve sulfide synthesis, rendering their use as blockers of potentially sulfide-requiring responses problematic.

3.6 Possible Mechanisms of Sulfide-Induced Contraction

The mechanisms by which a rise in the cellular sulfide concentration could cause PA constriction remain obscure, as does an explanation of why sulfide causes opposite effects on tension development in different arteries. One clue to both findings may have been provided by Skovgaard and Olson [25] in a report which concluded that sulfide raises the levels of ROS in the vasculature of the trout gill, a preparation resembling PA in that it constricts to hypoxia. In support of this concept, they found that constriction to both hypoxia and sulfide was strongly and similarly inhibited by block of the mitochondrial ETC, an important cellular source of ROS, at complexes I, III, and IV, and also by DDC, an inhibitor of the conversion of superoxide to hydrogen peroxide by superoxide dismutase. These results are particularly interesting in light of evidence that hydrogen peroxide constricts PA [35], whereas it generally dilates systemic arteries (e.g., [36]).

We have similarly presented preliminary evidence that the sulfide-induced contraction is largely dependent on mitochondrial ROS production in rat PA [37]. Since it has been proposed that HPV is also triggered by mitochondrial ROS production (see review by Sylvester et al. [4]), we speculate that the similarity between the hypoxia- and sulfide-induced contractions in PA could be due to their parallel dependence on this mechanism rather than the existence of a sulfide-mediated O_2 sensing pathway. This would not explain why sulfide apparently antagonizes HPV (Fig. 1 and [3]), although it may be that at concentrations of sulfide high enough to block the ETC, mitochondrial depolarization may attenuate ROS production [38]. On the other hand, modest increases in sulfide, which are insufficient to block the ETC and cause mitochondrial hyperpolarization, could occur in cells during hypoxia. This could promote ROS production by stimulating electron transport ([9], see Sect. 2), thereby causing HPV. In this case, it is possible that low concentrations of exogenously applied sulfide or supplementation of solutions with a sulfide donor such as cysteine might mimic HPV; this, however, has apparently not been reported.

3.7 Summary: Does Sulfide Play a Role in HPV?

The concept that sulfide is an O_2 sensor in PA and other blood vessels is a compelling one, as it provides a straightforward explanation for how graded levels of hypoxia in the physiologically relevant range could lead to proportionate changes in vascular resistance. The reported similarity of the effects of hypoxia and exogenously applied sulfide in blood vessels from more than a dozen diverse species [23] is consistent with the possibility that sulfide arose as a ubiquitous O_2 sensor relatively early in the evolution of vertebrates.

However, despite the elegance of the model from a theoretical standpoint, as described above the experimental evidence regarding a role for sulfide in HPV is incomplete and often contradictory. Perhaps the most important missing piece of the puzzle is direct evidence establishing the existence of the putative inverse relationship between the concentrations of O_2 and sulfide in cells which lies at the heart of the hypothesis. Instead, this relationship has been inferred from observations that injecting O_2 into anoxic tissue homogenates causes a concentration-dependent disappearance of sulfide. The ability of hypoxia to raise the sulfide concentration, and of O_2 to consume sulfide, has been demonstrated in tissue homogenates [19, 22, 24]. However, Yuan et al. [13] have recently demonstrated that changes in PO_2 do not affect sulfide production in homogenates of HEK-293 cells overexpressing CSE unless HO-2 is also expressed, implying that oxidative sulfide metabolism per se may not be sufficient to regulate cellular sulfide levels. In any case, it is difficult to extrapolate from homogenates to intact cells, especially because the former are supplemented with an effectively inexhaustible supply of exogenous L-cysteine and often pyridoxal 5′-phosphate. Moreover, even if it is the case that hypoxia does raise sulfide levels in cells, it is not evident that these

levels would be sufficient to have any contractile effect, since the concentrations of exogenous sulfide which have been shown to mimic HPV are very high (≥300 μM).

In addition, our experiments using PPG and the CAT antagonist aspartate, neither of which have a significant non-selective effect on vascular contraction, indicate that blockade of sulfide synthesis by CSE and CAT-3-MST has no effect whatsoever on hypoxia-induced contractions in rat PA. [27]. It is also the case that the resemblance between HPV and the contractile response to sulfide in isolated rat PA, which is perhaps the most widely used preparation for studying HPV in vitro, is not particularly close, although both stimuli do cause a biphasic contraction separated by a relaxation. This is illustrated in Fig. 3, which shows the effects on tension of hypoxia (pO_2 6–9 mmHg) and 300 μM NaHS, both applied in the presence of 10 μM $PGF_{2\alpha}$ pre-constriction. It is particularly noteworthy that the first phase of HPV in this experiment (and in numerous studies we have published previously (e.g., [39])) is much larger and more sustained than the first phase of the sulfide response, even though this is the point at which the sulfide concentration is likely to be maximal (since it is rapidly lost from solutions open to the air). Figure 3 also illustrates that applying this concentration of sulfide during HPV causes a transient but profound inhibition of contraction, suggesting that sulfide actually antagonizes the effects of hypoxia.

Determination of the putative contribution of a given signal to a biological response is often approached by examining three criteria. Firstly, does the exogenous application of the signal mimic the response? Secondly, does blockade of the endogenous generation of the signal prevent the response? Thirdly, are the pathways activated by exogenous application of the signal the same as those which occur during the response? With regard to a role for sulfide in HPV, existing evidence provides the strongest support for the first criterion, although there remain important

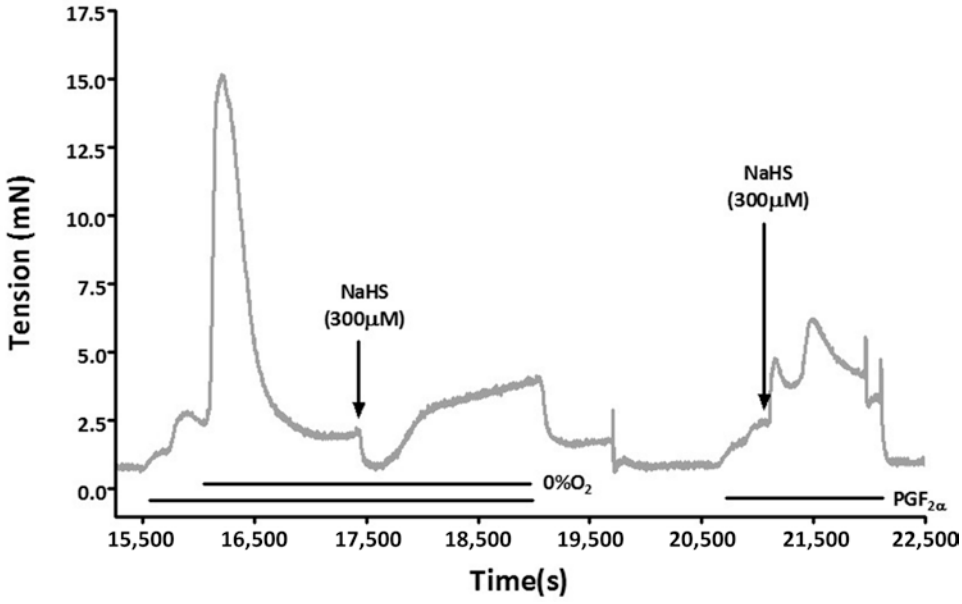

Fig. 3 Hypoxia- and sulfide induced contractions in isolated rat pulmonary artery. A small PA was pre-contracted with 10 μM PGF_{2a} and then exposed to severe hypoxia (5% CO_2, balance N_2). 300 μM NaHS was applied to the solution during the sustained phase of the hypoxic contraction, causing a transient abolition of the response. The artery was washed in control physiological saline and re-exposed to $PGF_{2\alpha}$, and 300 μM NaHS was added to the solution again. The myograph chamber was open to the solution, meaning that the sulfide concentration in the bath would have fallen rapidly as H_2S outgassed; this probably accounts for the transience of the sulfide responses

questions about whether the concentrations of sulfide which are required to cause PA to constrict are physiologically relevant. The evidence supporting the second criterion is very tenuous; the drugs used to antagonise sulfide synthesis are far from ideal and the apparent role of CBS in HPV suggested by the blocking effects of HA and AOA but not PPG [3] is difficult to understand given the lack of CBS expression in PASMC [24]. Finally, since virtually nothing is known about the mechanisms by which sulfide causes PA to constrict (or, for that matter, causes mammalian systemic arteries to relax), the question of whether third criterion applies cannot be addressed. It is therefore apparent that a role for sulfide as an important O_2 sensor in HPV remains a provocative but unproven hypothesis.

4 Hydrogen Sulfide as a Putative O_2 Sensor in the Carotid Body

The carotid body (CB), located at the bifurcation of the carotid artery, was first studied by Fernando de Castro in the 1920s, and was later defined as a sensory organ for O_2 levels by Heymans, a discovery that led to him receiving the Nobel Prize in 1938. Research over the years has determined that within the CB, the chemoreceptor (type 1 or glomus) cells are responsible for O_2 detection via the following steps: (1) depolarization by hypoxia; (2) neurotransmitter release; (3) activation of afferent nerve fibers; and (4) signal integration in the brainstem to induce adaptive responses to hypoxia. Over the years the "blood detector" function of the CB has been extended beyond hypoxia, with its role in the transduction of acidosis/hypercapnia [40], glucose [41] and insulin levels [42] having also been described. The transduction cascades for these stimuli, which may share some steps, are not fully understood. For the purpose of our review we will specifically focus on evidence relating to sulfide-dependent O_2 sensing in the CB. This is only one of several separate mechanisms which have been proposed to explain how glomus cells sense hypoxia, although it has been suggested that each of these makes a contribution to this process depending on the extent of the hypoxic stimulus [43].

Evidence for a role of sulfide in chemoreceptors was first demonstrated in trout gills, the first pair of which serve as O_2 sensors in this species which are homologous to the mammalian CB [5]. In 2010, Peng et al. reported that CSE is expressed in murine glomus cells, and that their response to hypoxia was markedly impaired in CSE knockout mice and in mice injected with PPG. Hypoxia caused a marked rise in sulfide production by CB homogenates. Ventilation was also slowed under hypoxic conditions in the $CSE^{-/-}$ mice, although since the same effect was recorded in normoxia it is unclear as to whether the ventilatory response to hypoxia per se was specifically CSE-dependent. The same year, Li et al. [44] reported that pharmacological inhibition of CBS, but not CSE, attenuated the suppression of the BK_{Ca} current and activation of CB afferents evoked by hypoxia in mice. The apparent discrepancy between these results and those of Peng et al. [19] as to which sulfide-synthesizing enzyme is important is puzzling, although it is noteworthy that the drugs used by Li et al. [44] as selective blockers of CBS (AOA and HA) were subsequently shown to antagonize both CSE and CBS over similar concentration ranges [32].

These and subsequent observations [13, 45] gave rise to a model for glomus cell O_2 sensing which draws on earlier evidence that hypoxia-induced activation of glomus cells is due to a fall in the carbon monoxide (CO) concentration which occurs because its production by hemoxygenase requires oxygen [46]. The new evidence indicated that the high ambient CO levels in normoxia cause a stimulation of protein kinase G (PKG)-dependent phosphorylation of Ser377 of CSE which results in a suppression sulfide synthesis [13]. Hypoxia results in a reduction in the activity of hemoxygenase 2 (HO-2), leading to a decreased CO production. As a consequence, CSE inhibition is relieved, allowing an increase in H_2S levels and subsequent activation of the carotid sinus nerve which is mediated by BK_{Ca} channel activation [44, 47], depolarization and an increase in type 1 cell $[Ca^{2+}]_i$. Although most of

the work characterizing these mechanisms was carried out in mouse, Jiao et al. [48] have shown that application of exogenous sulfide also stimulates the activity of the carotid sinus nerve in ex vivo CB preparations from rat, rabbit, and cat, although a higher concentration was required to do so in the latter species.

Additional evidence for a role of sulfide in glomus cell O_2 sensing came from a study which examined the role of sulfide in causing CB-mediated breathing instability (e.g., increased episodes of apnea) which occurs in a rat model of heart failure [49]. The irreversible inhibitor CSE blocker PPG strongly reduced the incidence of apnea, while also reversing the increased CB afferent and chemoreflex responses. The effect of PPG on chemoreceptor O_2 sensing was also studied in spontaneously hypertensive rats, in which the CB demonstrates an increased sensitivity to hypoxia [50] that is thought to contribute to the elevation of blood pressure. Peng et al. [51] showed that sulfide production by CB homogenates was higher in SHR compared to normotensive Sprague-Dawley controls, possibly due to a decreased generation of CO by HO-2. PPG virtually abolished sulfide production by CB homogenates in both strains, indicating its importance in chemoreceptor cell sulfide synthesis in this species. Daily i.p. injection of PPG also caused an attenuation in the rise in BP over a 5-week period in young SHR, and this was equivalent to that recorded in age-matched rats subjected to CB ablation. The treatment with PPG did not reduce blood pressure further in CB-ablated animals, implying that the elevated CB sulfide levels were responsible for the hypertension development, through an overactivity of the CB [51]. These studies indicate that disturbances in the close interaction between CO and sulfide levels, by altering the output of the CB in response to hypoxia, can potentially lead to pathological conditions such as hypertension, pulmonary edema, and poor ventilatory adaptation to hypoxia [52].

The evidence supporting a role for sulfide as an O_2 sensing in the carotid body is much stronger than that favoring its involvement in HPV, mainly because, as described above, all the necessary cellular components of a sulfide-dependent pathway leading hypoxia to cell depolarization have been identified. Moreover, the contribution of sulfide to O_2 sensing in glomus cells is supported by work using the $CSE^{-/-}$ mouse, which on the other hand has not been used to study HPV. However, the involvement of sulfide in O_2 sensing in the CB has been challenged by a study which showed that exogenous sulfide caused an increase in $[Ca2^+]_i$ in glomus cells from rat which resulted from an inhibition of a voltage insensitive K^+ current which had the properties of a TASK channel [53]. However, sulfide inhibited mitochondrial function over the same concentration (EC_{50} near 10 μM) as it increased $[Ca2^+]_i$. Additionally, another mitochondrial blocker, cyanide, caused a similar inhibition of TASK and rise in $[Ca2^+]_i$ and the effects of sulfide and cyanide on the K^+ current were not additive—implying that the block by sulfide of the TASK channel was secondary to its effect on the mitochondria. Although this concept does not appear to directly rule out a role for sulfide in O_2 sensing, the author calculated that given the high membrane permeability of sulfide, the CB cells would need to synthesize sulfide at an unfeasibly high rate in order to maintain an intracellular concentration of sulfide high enough to block the mitochondria and thereby inhibit the TASK current. Along the same lines, the suppression of BK_{Ca} channel activity which is proposed to contribute to sulfide-dependent activation in glomus cells has been shown to occur only at very high concentrations (≥100 μM) [54].

It is also worth pointing out that whereas Peng et al. [19] provided extensive evidence that sulfide potentiates CB signaling and that CSE knockout attenuated ventilation in vivo, the latter effect was of similar magnitude under normoxic and hypoxic conditions. This implies that sulfide may facilitate CB function in a tonic manner rather than contribute specifically to O_2 sensing.

5 Discussion

Figure 4 summarizes the pathways proposed to underlie sulfide-mediated O_2 sensing in PASMC and glomus cells. The concept that the cellular

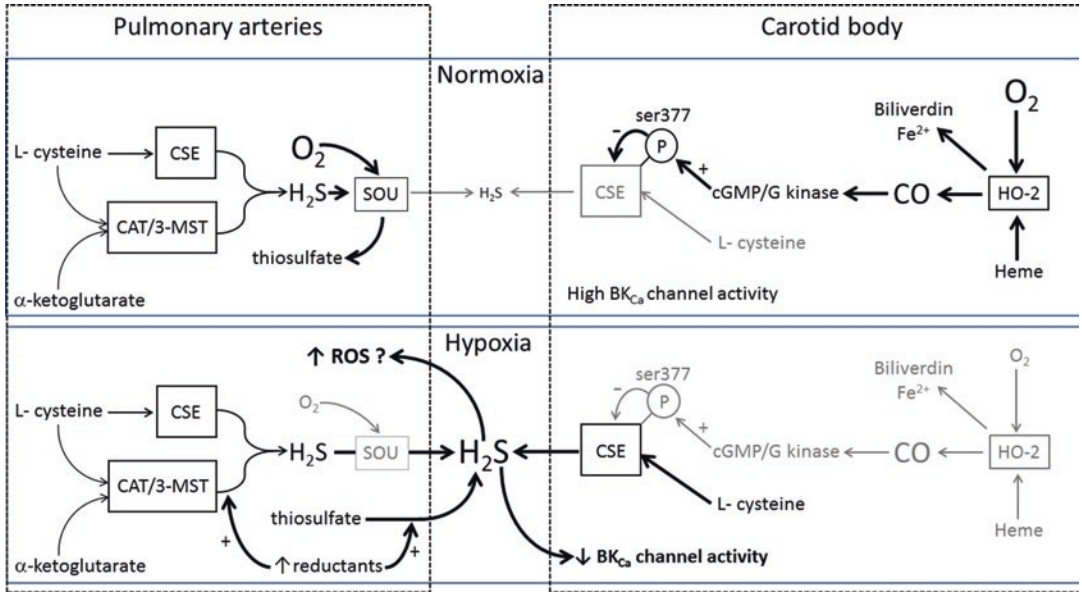

Fig. 4 Proposed involvement of sulfide in O_2 sensing in pulmonary artery smooth muscle and glomus cells. Under normoxic conditions (*upper box*), intracellular sulfide concentrations are low in both types of cells due to rapid oxidative sulfide metabolism by the SOU in PASMC and suppression of CSE activity due to its phosphorylation by protein kinase G, which is promoted by high CO levels, in glomus cells. As a result, sulfide levels are insufficient to cause contraction of PASMC or inhibition of BK_{Ca} channel activity in glomus cells. Hypoxia depresses SOU-mediated oxidation of sulfide in PASMCs, and leads to a reduction of the mitochondrial redox state, causing the regeneration of sulfide from its metabolite thiosulfate and from sulfane sulfur, in which form sulfide is bound to 3-MST as it is produced by this enzyme. These effects raise the intracellular [sulfide], which may lead to an increase in mitochondrial ROS production and, as result, PASMC contraction. In glomus cells, hypoxia diminishes the synthesis of CO by HO-2, resulting in an inhibition of protein kinase G and a reduced phosphorylation of CSE, thus relieving the inhibition of the enzyme so sulfide synthesis increases. Higher sulfide levels suppress the opening of BK_{Ca} and TASK channels, causing membrane depolarization and release of neurotransmitters. Processes which are more or less highly activated in normoxia or hypoxia are indicated by *bold black font/thicker lines* and *gray font/thinner lines*, respectively. The figure is based on information drawn mainly from Olson et al. [3], Skovgaard and Olson [25], Olson et al. [7], Peng et al. [19], and Yuan et al. [13]

H_2S concentration acts as an O_2 sensor in the vasculature and the carotid body is an elegant one, and observations supporting it extend well beyond the studies which we have discussed (see for example [23]). However, we would argue that much of this evidence, particularly with regard to HPV, is inferential, and there remain a number of important gaps in our knowledge which must be addressed in order for this hypothesis to be validated.

A final important consideration is that all of the experimental approaches available for the experimental manipulation of the sulfide concentration in biological systems, which have played a pivotal role in defining its cellular effects appear to have potentially severe drawbacks. The sulfide synthesis blockers BCA, HA, and AOA, which have been used widely in studies designed to examine the role of sulfide in hypoxia-induced changes in vascular tension, have powerful non-selective effects on contraction in PA even at concentrations much lower than those that have been used in these arteries to block sulfide synthesis [27]. The CSE knockout mouse introduced by Yang et al. [11] and used in studies of glomus cell O_2 sensing (e.g., [19]) demonstrates a marked increase in plasma levels of homocysteine to ~20 μM, which may be sufficient to inhibit the activity of BK_{Ca} channels [55] and thereby potentially bias the CB response to hypoxia. This lack of good methods for controlling and monitoring cellular H_2S concentrations, taken together with

the remarkable complexity of the interactions of sulfide with other signaling pathways and cellular systems, remains a significant barrier to resolving the role of sulfide in O_2 sensing. However, in light of the enormous current interest in the biological actions of sulfide, improved experimental techniques are likely to emerge in the near future and should allow its involvement in O_2 sensing to be convincingly elucidated.

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Redox Signaling and Persistent Pulmonary Hypertension of the Newborn

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Abbreviations

ADAM	Asymmetric dimethyl arginine
AKT	Protein kinase B
BH4	Tetrahydrobiopterin
ECMO	Extracorporeal membrane oxygenation
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
ET1	Endothelin 1
GCH1	GTP cyclohydrolase 1
H_2O_2	Hydrogen peroxide
HIFs	Hypoxia-inducible factors
HOCl	Hypochloric acid
hsp70 and 90	Heat shock protein 70 and 90
iNO	Inhaled nitric oxide
KEAP1	Kelch-like ECH-associated protein 1
NO	Nitric oxide
Noxes	NADPH oxidases
NRF2	Nuclear factor erythroid 2-related factor 2
O_2^-	Superoxide
$ONOO^-$	Peroxylnitrite
PA	Pulmonary artery
PASMCs	Pulmonary artery smooth muscle cells
PDE3 and PDE5	Phosphodiesterase-3 and -5
PI3K	Phosphoinositol-3 kinase
PPHN	Persistent pulmonary hypertension of the newborn
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
SOD	Superoxide dismutase
VSMCs	Vascular smooth muscle cells

1 Introduction

Newborn lungs undergo dramatic changes as it transitions from fetal to neonatal life. The fetus, who has grown and is accustomed to the relative hypoxic intrauterine environment, now must quickly adapt within minutes to a new and often challenging postnatal environment, where the oxygen concentration is five times higher than that in utero [1, 2]. As such, the fetal lung prepares for this transition by increasing the antioxidant defense in late gestations. Alterations in antioxidant defense mechanisms can potentially increase cellular oxidative stress, which has been implicated as a critical physiopathologic factor in the pathogenesis of in persistent pulmonary hypertension in the newborn (PPHN) [3, 4]. A successful postnatal transition, therefore, requires

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proper adaptive mechanisms to regulate the oxidant–antioxidant balance. In this chapter, we focus on the sources of ROS generation, mechanisms regulating ROS homeostasis, as well as the role of redox signaling in the development of PPHN.

2 Overview

Reactive oxygen species (ROS) are a group of free radicals containing oxygen or nitrogen-based unpaired electrons on their outer orbital or compounds that are not free radicals themselves, but have oxidizing properties [5, 6]. In addition to their well-known damaging effects, ROS have important signaling roles in modulating activities of diverse signaling pathways that can impact functions of endothelial cells. At low levels, ROS induce activities of multiple protein kinases, such as MAP kinase, PI3-K/AKT kinases, and transcription factors, and positively affect endothelial cell functions [7–11]. But, when produced in excess, ROS can potentiate the oxidation of numbers of molecules, including RNA, DNA, proteins, and lipids, and cause damage to the mitochondria, promoting a vicious cycle leading to tissue injury and potentially cell deaths (Fig. 1) [12, 13]. All the vascular wall components, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and adventitia, produce ROS. Among the free radicals, superoxide (O_2^-), hydroxyl ($OH^{\bullet}$), nitric oxide (NO), and lipid radicals ($LO^{\bullet}$, $LOO^{\bullet}$) are the most toxic. Non-radical ROS, such as hydrogen peroxide (H_2O_2), hypochloric acid (HOCl), and peroxinitrite ($ONOO^-$) are compounds that may be formed at higher rates under stress conditions and mediate cellular injury [14–16]. Generation of ROS often begins with one- or two-electron reduction of molecular oxygen to form O_2^-. Because O_2^- is very toxic, it is rapidly converted to H_2O_2 by enzymatic and nonenzymatic processes [17]. Neonatal lungs are enriched with an array of antioxidants that protects them from ROS-mediated injury, including the superoxide dismutase family of antioxidants (SOD) [18, 19]. The SODs are extremely efficient antioxidants that catalyze the dismutation of O_2^- into H_2O_2, which is then converted into water by catalase,

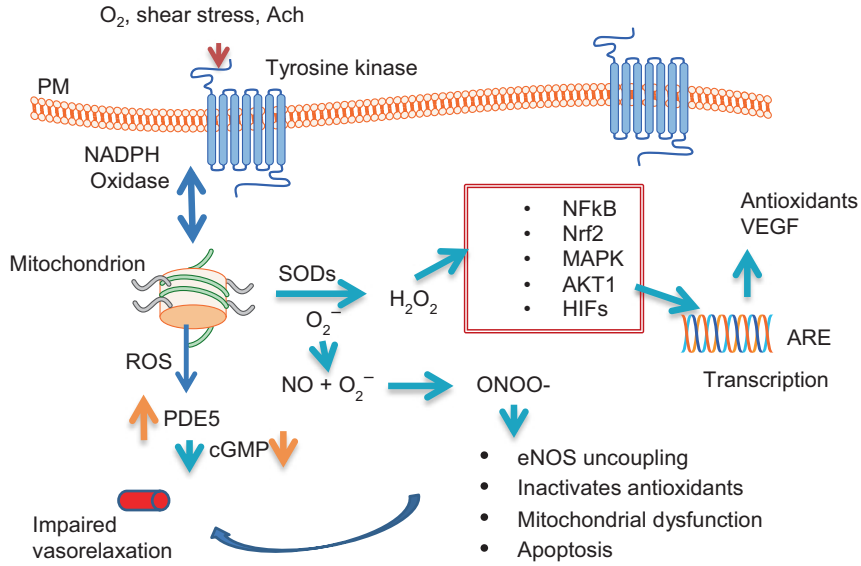

Fig. 1 A balance between reactive oxygen species (ROS) and antioxidants is critical for normal transition of pulmonary circulation during postnatal exposure to oxygen-rich environment that occurs at birth. ROS, particularly hydrogen peroxide serve a critical signaling role that can positively impact endothelial cell functions. Excess superoxide radical production can cause deleterious effects by inactivating antioxidants, uncoupling eNOS, and cause mitochondrial damage, which ultimately lead to impaired relaxation of the pulmonary arteries

glutathione, and thioredoxines [20]. H_2O_2 rapidly diffuses into the cytosol and extracellular space, and when produced in large amounts can undergo a Fenton reaction in the presence of heavy metals, such as iron to form the highly reactive OH [16, 21].

The reactivity of free radicals, which depends on the configuration of electrons around the oxygen atom, determines the toxicity of ROS and their ability mediate signaling processes. The balance of reactivity and stability of different ROS molecules determine their suitability for signaling. H_2O_2 is the most common and potent signaling molecule, as it has characterstics of regulated synthesis and breakdown, specific molecular targets, and measurable biologic effects. O_2^- and OH are more reactive and less stable but still play some roles in cell signaling [10, 22, 23]. NO is very stable and hence serves almost exclusively a signaling role but becomes damaging only after reacting with O_2^- to form $ONOO^-$. In the pulmonary blood vessels, sources of ROS include NADPH oxidase, mitochondria, xanthine oxidase, and endothelial nitric oxide synthase (eNOS) and several mechanisms are responsible for ROS formation from these sources.

3 Sources of ROS-Mitochondria

Mitochondria are the major sources of O_2^-, producing approximately five to ten times more O_2^- than the cytosolic sources [9, 15]. Approximately 2% of O_2 consumed by mitochondria is converted into $O_2^{\bullet-}$ [2]. Electron leaks from the electron transport chains (ETC) especially in complexes I and III, during oxidative phosphorylation accounts for most of O_2^- generation in the mitochondria [24, 25]. Hyperoxia can increase O_2^- formation in the mitochondria, which in turn plays a major role in the toxicity in that setting [11, 26–29]. Newborns are particularly at increased risk of experiencing oxidative stress because O_2 concentration at postnatal environment is several times higher than that in utero. Adaptive increases in cellular antioxidant defenses, particularly superoxide dismutase-2 (SOD2), during this transition protect endothelial cells from the potential increases in O_2^- and oxidative stress [18, 28, 30]. Mitochondria are not only the major sources of ROS but are important sources of redox signaling in pulmonary circulation, particularly in the context of low O_2 or hypoxia [24, 31–33]. At low O_2 level, the production of O_2^- in the mitochondria is increased. O_2^- is rapidly converted into H_2O_2 in the mitochondrial matrix and diffuses into the cytosol, where it stabilizes the highly conserved transcription complex, hypoxia-inducible factor-1 alfa (HIF1α). HIF-1α, when stabilized in hypoxic conditions, upregulates several genes to promote survival in low-oxygen conditions. These include glycolysis enzymes, which allow ATP synthesis in an oxygen-independent manner, and vascular endothelial growth factor (VEGF), which promotes angiogenesis that enables cells to adapt to prolonged hypoxia [25, 34]. HIFs are vital to development and loss of HIF-1α or HIF-1β gene results in severe cardiovascular malformations and fetal death at Embryonic Day 10 [16, 19, 35] In contrast, loss of HIF-2α leads to fetal death in approximately 50% of embryos, with the remaining offsprings exhibiting impaired lung development, respiratory distress syndrome (RDS) and neonatal deaths [36].

The relevance of mitochondria-derived ROS, particularly in hypoxic pulmonary vasoconstriction in the newborn, has recently received a greater attention. Increased mitochondrial oxidative stress and reduced mitochondrial biogenesis and efficiency have been demonstrated in the ductal ligation model of PPHN; and this phenomenon persists in pulmonary artery endothelial cells culture [4]. Recent studies have demonstrated increased expression and activity of HIF-1α in the lungs and pulmonary artery smooth muscle cells (PASMC) in PPHN fetal lambs during normoxic conditions, which may contribute to the mitochondrial metabolic dysfunction in this context [4, 37]. Increased mitochondrial oxidative stress has also been shown to cause an upregulation of phosphodiesterase type 5 (PDE5) activity and reduced cGMP levels in PASMC in PPHN fetal lambs. Inhibition of PDE5 activity by

sildenefil and hydrocortisone improves PA vasodilation in PPHN [38–40]. Accumulating evidence suggests that ROS produced by the mitochondria induce the activation of NADPH oxidase, which is a major cytosolic source of ROS, and leads to a viscous cycle of ROS induced ROS formation and oxidative stress [41, 42]. The resulting increase in ROS levels combine with free NO, forming $ONOO^-$, a potent radical that can oxidize tetrahydrobiopterin (BH_4) and uncouples eNOS and further increases oxidative stress [5, 43, 44].

4 NADPH Oxidase

NADPH oxidase is recognized the major source of O_2^- in the vascular endothelium, and has been implicated in the pathogenesis of oxidative stress underlying PPHN [6, 45–50]. The NADPH enzyme is a multi-subunit membrane bound enzyme complex that catalyzes O_2^- production by the one electron reduction of molecular oxygen using NADPH or NADH as the electron donor [51–53]. To date, seven isoforms of the NADPH oxidases have been described and designated by the common name Nox. Nox1 was initially identified in 1999, 20 years after NOx2 was identified [54, 55]. NOx1 is mainly found in the vascular wall and its expression induces intracellular O_2^- production [4]. Murine experiments demonstrated that NOx1 expression is increased in lung microvascular endothelial cells that are exposed to hyperoxia and indeed lung injury is significantly prevented in NOx1-deficient mice [56]. Nox2 isoform was the first NOx identified and is primarily expressed in neutrophils and produces O_2^- associated with pulmonary vasoconstriction [46, 57]. NOx2 knockout mice (without endothelial $gp91^{phox}$ containing NADPH oxidase) have markedly reduced hypoxia-induced O_2^- production and vasoconstrictor activity [51]. NOx3 is found in the fetal kidney, NOx4 is ubiquitous, while NOx5 is found in the spleen, sperm, mammary glands and brain [13, 48]. Nox4 was originally thought to share structural and functional homology with gp91phox; however, it has since become clear that Nox4 has a different structure from other NOxes. NOx4 is required for hyperplasia stimulated by hypoxia and TGF-β, and NOx4 silencing reduces proliferation of pulmonary vascular smooth muscle cells [48]. Recent studies showed that PPHN increases $p22^{phox}$ and Nox4 expression and activity resulting in elevated H_2O_2 levels in PPHN PA. Increased H_2O_2 induces vasoconstriction via mechanisms involving extracellular (ec)-SOD inactivation, and stimulates vascular remodeling via NFkB activation and increased cyclin D1 expression [58]. A new Nox4 pharmacologic inhibitor, GKT137831, which is under investigation, prevents hypoxia-induced pulmonary hypertension (PH) in mice [49]. This shows that Nox4 plays a critical role in modulating proliferative responses of pulmonary vascular wall cells. Targeting Nox4 with GKT137831 provides a novel strategy to attenuate hypoxia-induced alterations in pulmonary vascular wall cells that contribute to vascular remodeling and right ventricular hypertrophy. The amount of O_2^- produced by the endothelial NADPH oxidase enzyme is significantly lower than the one caused by the oxidative burst of phagocytosis. The oxidase components exist in preassembled cytosolic components including $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ that associate with the cytoskeleton [46, 54, 57] and are rapidly brought together and modulated by a variety of stimuli [59]. For example, in the vascular system, ROS production by the NADPH oxidase is triggered by stimulation of neuro-humoral vasoconstrictor agents, such as endothelin-1 (ET-1). ET-1 is a 21-amino acid peptide produced by endothelial cells that exerts a powerful vasoconstrictor effect [60, 61]. The effects of ET-1 are mediated by two isoforms of receptors, ET_A and ET_B. The vasoconstriction effect of ET1 is mediated via activation of ETA receptors, whereas activation of ET_B receptors leads to relaxation of vessels. ET-1 is capable of causing strong and sustained constriction of the vascular smooth muscle. ET-1 has a marked vasoconstrictor action on pulmonary arterial tissues and studies on human pulmonary tissue demonstrate a potent sustained contraction of both pulmonary arterial and bronchial smooth muscle. Recent studies demonstrated increased plasma levels of ET-1 in

patients with severe PPHN, and ET-1 levels correlate with disease severity [60].

5 Uncoupling of eNOS

Endothelial nitric oxide synthase (eNOS) plays a central role in pulmonary vasodilation during transition that occurs at birth [1, 2]. The reduction in NO production by the endothelium, which is the hallmark of endothelial dysfunction, is thought to be a key event in the development of PPHN. Intrinsic to endothelial dysfunction is endothelial oxidative stress, due to the production of damaging free radicals that combine with NO to form $ONOO^-$, a potent oxidant that can propagate deleterious reactions and cellular injury [6, 7, 26, 62]. Under physiological conditions, eNOS, in the presence of substrates L-arginine and cofactor, tetrahydrobiopterin (BH_4), produces NO. BH_4 is a critical cofactor, which facilitates coupled eNOS activity and eNOS dimer formation, necessary for NO production [43, 63, 64]. Factors that regulate cellular BH_4 levels include the GTP cyclohydrolase 1 (GCH-1), the rate-limiting enzyme for BH_4 biosynthesis and conversion of BH_4 to its oxidized product dihydrobiopterin (BH_2). The cofactor BH_4 is highly sensitive to oxidation by $ONOO^-$ and reduced levels of BH_4 promote O_2^- production by eNOS (referred to as eNOS uncoupling). The transformation of eNOS from a protective enzyme to a contributor to oxidative stress has been reported in the ductal ligation model of PPHN [64]. In that study, Sepiapterin supplementation increases BH_4 levels and restored coupled eNOS activity in the pulmonary artery endothelial cells [64]. Another recognized mechanism for eNOS uncoupling is elevated amino acid asymmetric dimethyl arginine (ADAM) levels. In cultured endothelial cells both from rat and humans, oxidative stress has been shown to increase the activity of protein arginine methyltransferases (PRMTs) and decrease that of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that degrades 80% of ADMA thereby, leading to increased ADAM concentrations [65, 66]. Thus, increased production of ROS could be the reason for increased ADAM levels. Elevated ADAM may inhibit NO synthesis by eNOS or could even uncouple the enzyme, which would enhance oxidative stress. More recently, impaired interactions between heat shock protein 90 (HSP90) and eNOS has been demonstrated to induce eNOS mediated O_2^- rather than NO production [67]. In bovine aortic endothelial cells, inhibition of HSP90-eNOS associations leads to uncoupling of eNOS and increased O_2^- [51, 68, 69]. Posttranslational modification of HSP90 as a result of oxidative stress accounted for the decreased HSP90–eNOS interactions and impaired eNOS function in PPHN lambs.

6 Biologically Important Redox Signaling in Pulmonary Vasculature

While in the past, ROS were known for their damaging effects, it is now clear that some ROS, such as H_2O_2 act as signaling molecules [10, 21, 22]. ROS particularly H_2O_2 and NO, act as secondary messengers by oxidizing thiol groups on cysteine residues on a target protein [70, 71]. This process alters the activity or binding affinity of the proteins. In response to H_2O_2, the thiol groups initially form a sulfenic acid (−SOH), and once formed, the sulphenic acid reacts with a glutathione (GSH) to form a glutathionylated protein. The effects of protein glutathionylation include inhibition of signaling proteins such as STAT3, but can also induce enzyme activities as seen HSP70 chaperone activity [20].

S-nitrosylation (SNO) of cysteine residues, i.e., the replacement of the hydrogen atom in the thiol group of cysteine residues by a NO moiety, is another dynamic posttranslational modification of eNOS. The mechanism of SNO formation remains unclear but it seems that eNOS itself provides the NO required for its own S-nitrosylation. Studies indicate that this nitrosylation reaction decreases eNOS activity and upon cells stimulation, and upon cells stimulation by physiologic stimuli, the nitrosylation reaction is quickly reversed to favor increases in

eNOS activity and NO release [68, 72–75]. Functions of a target protein can also be modified irreversibly by ROS through the alkylation of thiols. This is exemplified in the cytosolic pathway of nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (KEAP1) (NRF2–KEAP1 pathway), in which one of the KEAP1 thiol groups can react irreversibly with electrophiles to release the NRF2 transcription factor. NRF2 then translocate into the nucleus where it induces transcription of genes under the control of promoters that contain the antioxidant response element (ARE). Proteins encoded by these target genes in turn mediate cellular consequences leading to upregulation of antioxidants involved in antioxidant defense [66, 76].

Another classic redox signaling mechanism is phosphorylation of protein kinases including the mitogen-activated protein kinases (MAPKs), tyrosine kinases, and phosphoinositol-3-kinase/Akt kinase (PI3K/AKT). MAPKs are involved in controlling cellular responses to a diverse array of stimuli, such as oxidative stress and pro inflammatory cytokines. They regulate cell functions including proliferation, gene expression, cell survival, and apoptosis [44, 77–81]. The MAPK family includes serine–threonine protein kinases that play an important role in the transmission of extracellular signals from cell membrane to the nucleus. Three main subgroups of MAPKs have been identified in the vascular system: (1) extracellular signal-regulated kinases (ERKs), (2) c-Jun N-terminal kinases (JNKs), and (3) p38-MAPK. Studies have shown that increased production of ROS is responsible for activation of redox-sensitive p38-MAPK, which might be involved in the pathogenesis of hypertension [44]. In vascular smooth muscle cells and other cells, the ROS activate non-receptor tyrosine kinases such as Janus kinase 2 (JAK2), Src tyrosine kinase, or the receptor tyrosine kinases, such as the receptor for epidermal growth factor (EGF) [79], which is necessary for activation of the ERKs that induces growth of SMC [80]. Phosphorylation and activation AKT plays an important role in regulating eNOS, a critical regulator of endothelial function. Extracellular stimuli, including platelet derived growth factor (PDGF) and shear stress, activate AKT via PI3K-PDK1 signaling pathway. Studies indicate that intracellular H_2O_2 can also induce phosphorylation and activation of AKT in endothelial cells [82]. As such, impaired conversion of O_2^- to H_2O_2 will have a great impact on endothelial functions, demonstrating the critical role of the SODs-mediated conversion of O_2^- to H_2O_2.

7 Antioxidant Enzymes and ROS Destruction

Cellular homeostasis depends on the delicate balance between oxidants and antioxidants. The superoxide dismutases (SODs), expressed in both the intra and extracellular compartments, constitute the first line of defense against cellular oxidative stress [83–85]. There are three SOD isoforms, namely the cytosolic copper-zinc SOD (CuZnSOD, or SOD1), mitochondrial manganese SOD (MnSOD, or SOD2), and the extracellular SOD (ecSOD, SOD3). The SODs react with O_2^- by alternately adding or removing an electron from the superoxide molecules it encounters to form diatomic O_2 and H_2O_2. H_2O_2 is then degraded by catalase and glutathione peroxidase (GPx). Among the three isoforms of SOD, MnSOD is the important for cellular respiration. Mice homozygous for SOD2 gene disruption die within 10 days of life [83]. SOD2 is a nuclear encoded gene, synthesized in the cytosol and must be imported into the mitochondria, where it is activated and function as the antioxidant [86]. Recent studies demonstrated that impaired interactions between inducible heat shock protein 70 (hsp70), a cytosolic molecular chaperone and SOD2 contribute to decrease SOD2 activity and mitochondrial oxidative stress in the PPHN lambs [87, 88]. The indispensable roles of ROS in vascular signaling and injury show promise for antioxidant therapy in treatment of PPHN. Overexpression of SOD2 or administration of mitochondrial-targeted antioxidant, mitoTEMPO has been shown to reduce the pulmonary artery pressures in PPHN lambs [87, 89]. SOD3 is localized in the extracellular

compartment and binds to the cell membrane through a heparin-binding domain. It is the second most abundant SOD isoform in blood vessels and is produced mainly by smooth muscle cells. SOD3 is very important in endothelium-mediated vasodilation because of its extracellular localization, which facilitates diffusion of NO from endothelial cells into the smooth muscle cells and induces vasorelaxation via cGMP signaling pathways [90, 91]. EcSOD-deficient mice exhibited higher degree of hypertrophy, ventricular dilation, and myocardial fibrosis [88]. Overexpression of SOD3 reduces O_2^- levels in the blood vessels and decreases arterial pressure in spontaneous hypertensive rats [91].

8 Conclusions

Oxidative stress has been implicated in the pathogenesis of PPHN as depicted in Fig. 1. For more than a decade, attention has been focused on the use of vasodilators therapies including inhaled nitric oxide (iNO). While this therapy may have reduced rates of extracorporeal membrane oxygenation (ECMO) the mortality of this condition remains unchanged. Other treatment modalities may be necessary to improve survival, and redox signaling offers several potential strategies of modulating ROS homeostasis in the management of PPHN. Individual SOD isoforms can be influenced using SOD mimetics. As delivery of antioxidants directly into the mitochondria may be challenging, a detailed understanding of the post-translational regulation of SOD2 will provide unique therapeutic opportunities to influence SOD2 function. Other strategies include inhibition of NADPH oxidase, BH4 or sepiapterin supplementations, modulation of MAPK, AKT kinase activities, as well as RhoA/ROCK signaling pathways. The use of these strategies has been shown to attenuate oxidative stress in experimental models of hypertension. All these strategies should be further studied in the search for new therapeutic approaches in the management of PPHN.

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Cross Talk Between Mitochondrial Reactive Oxygen Species and Sarcoplasmic Reticulum Calcium in Pulmonary Arterial Smooth Muscle Cells

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Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
$[Ca^{2+}]_{mito}$	Intramitochondrial Ca^{2+} concentration
$[ROS]_i$	Intracellular ROS concentration
$[ROS]_{mito}$	Intramitochondrial ROS concentration
BSO	Buthionine sulfoximine
CIRP	Ca^{2+}-induced ROS production
COPD	Chronic obstructive pulmonary disease
DAG	Diacylglycerol
ER/SR	Endoplasmic reticulum/sarcoplasmic reticulum
ETC	Electron transport chain
HPV	Hypoxic pulmonary vasoconstriction
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
MCU	Mitochondrial Ca^{2+} uniporter
MEFs	Mouse embryonic fibroblasts
NOX	NADPH oxidase
PASMCs	Pulmonary arterial smooth muscle cells
PKC	Protein kinase C
RICR	ROS-induced Ca^{2+} release
RIPR	ROS-induced ROS production
RISP	Rieske iron–sulfur protein
ROCK	Rho kinase
ROS	Reactive oxygen species
RyR2	Ryanodine receptor 2
SOCC	Store operated Ca^{2+} channel
SOD	Superoxide dismutase
STIM	Stromal interaction molecule
TRPC	Canonical transient receptor potential
TRPV	Vanilloid transient receptor potential

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1 Introduction

Hypoxia is a common process where tissues or organs are deficient in oxygen. Arteries in the systemic circulation dilate in response to hypoxia, leading to increased blood flow to reconcile the oxygen deficiency and maintain cellular metabolism. In contrary, pulmonary arteries constrict and shunt the blood flow to well-oxygenated areas in order to preserve the sufficient matching of ventilation and perfusion in the lungs, thereby increasing oxygen saturation in the blood. This unique phenomenon is termed hypoxic pulmonary vasoconstriction (HPV).

The molecular mechanisms for HPV remain incompletely understood. However, extensive studies demonstrate that hypoxia results in a

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large increase in intracellular reactive oxygen species concentration ($[ROS]_i$) specifically in pulmonary artery smooth muscle cells (PASMCs). The increased $[ROS]_i$ is principally generated by the mitochondrial electron-transport chain (ETC) and plasmalemmal NADPH oxidase (NOX) [1–9]. Noticeably, a few reports have shown that hypoxia may decrease $[ROS]_i$. Regardless, the increased $[ROS]_i$ causes an increase in $[Ca^{2+}]_i$ and contraction in PASMCs, leading to HPV and pulmonary hypertension. Multiple ion channels may contribute to the hypoxia-induced, ROS-mediated increase in $[Ca^{2+}]_i$; however, ryanodine receptor-2 (RyR2)/Ca^{2+} release channel on the sarcoplasmic reticulum may serve as a most valuable player (MVP) as a result of ROS-induced Ca^{2+} release (RICR) process. Interestingly, our data reveal that hypoxia-produced mitochondrial ROS result in protein kinase C-ε (PKCε) activation and increased NOX activity, producing more ROS; this novel process is termed ROS-induced ROS production (RIRP) [1, 7]. Our recent findings indicate that hypoxia-induced, ROS-mediated, RyR2-released SR Ca^{2+} (ROS-induced Ca^{2+} release, RICR) can be taken up by mitochondria, in which more ROS are produced, i.e., Ca^{2+}-induced ROS production (CIRP). This newly recognized CIRP may also make a significant contribution to HPV.

In this chapter, we provide an in-depth review of recent findings with respect to the interaction of hypoxia-induced ROS with ion channels (particularly RyR2/Ca^{2+} release channel) to increase $[Ca^{2+}]_i$ in PASMCs, leading to HPV and associated pulmonary hypertension. We also comprehensively discuss how hypoxia causes the production of mitochondrial ROS and how these radicals cross talk with plasmalemmal NOX to mediate RIRP in PASMCs. Finally, we propose a novel model to indicate how hypoxia-induced, RyR2-released Ca^{2+} communicate with mitochondrial ETC to trigger CIRP, thereby making a potentially significant contribution to HPV and pulmonary hypertension.

2 Hypoxic Pulmonary Vasoconstriction: An Innate Unique Phenomenon

During the course of fetal development, the oxygenated placental blood is a primary source of oxygen for every fetal cell. Interestingly, the fetal lung is uniquely developing under a hypoxic condition, whereas the other parts of the fetus are exerting their best to grasp oxygen. Mechanistically, HPV plays a significant role in both the prenatal and postnatal period. Before birth, the function of pulmonary circulation is replaced by oxygenated placental blood flow in fulfilling the cellular metabolism; after birth, HPV provides a feedback response to the hypoxic condition (i.e., atelectasis, pneumonia, and COPD) because it shunts more blood to the well-ventilated alveolar in order to preserve pulmonary function.

3 Hypoxia Induces Mitochondrial ROS Production and Can Elevate $[Ca^{2+}]_i$ in PASMCs

HPV was first reported in 1894. Since then, investigators have continually unraveled the molecular mechanisms for this unique phenomenon [10]. We and other scientists have provided significant evidence showing that hypoxia-induced surges in $[ROS]_i$ in PASMCs can lead to pulmonary vessel contraction. Among all major cellular resources of $[ROS]_i$, mitochondrial ETC serves as a primary site for the hypoxic ROS generation. The ETC complex I, II, and III are all involved in the hypoxic ROS production; however, complex III is the major location [11].

For the first time we have reported that Rieske iron–sulfur protein (RISP) in complex III is an essential molecule in mediating hypoxic ROS production [12]. RISP acts by binding an ubiquinol or plastoquinol anion, transferring an electron to the 2Fe-2S cluster, and then releasing the

electron to the cytochrome c or cytochrome f; as such, ROS are produced [13]. Our data further reveals that gene knockdown and overexpression of RISP, respectively, blocks and increases the hypoxia-induced increase of ROS in isolated PASM complex III, mitochondria, and cells. Moreover, RISP protein expression levels are well correlated with hypoxic increases in $[ROS]_i$ and $[Ca^{2+}]_i$ in PASMCs. In this section, we summarize and explain singling pathway involved in $[ROS]_i$ induced Ca^{2+} release (RICR) which have provided important evidences for HPV.

Ryanodine receptor (RyR)/Ca^{2+} release channel in sarcoplasmic reticulum (SR) is well-believed in mediating $[Ca^{2+}]_i$ upon various stimulations [14]. Our previous study showed that RyR3 mediates hypoxia-induced Ca^{2+} release and contraction in rat and mouse PAMSCs. RyR3 gene knockout significantly inhibits hypoxia-, but not submaximal noradrenaline-induced Ca^{2+} and contractile responses in PASMCs [15]. Our recent findings also demonstrate that RyR2 can be activated by $[ROS]_i$, which causes Ca^{2+} release from the sarcoplasmic reticulum (SR) and subsequently increases $[Ca^{2+}]_i$ in PASMCs [16]. Supportively, Waypa et al. have shown that hypoxia-induced increase in $[Ca^{2+}]_i$ in pulmonary arteries and right ventricular systolic pressure are both abolished in smooth muscle-specific RISP knockout mice [17]. Taken together, both in vitro and in vivo studies unveil that RISP is a novel and key molecule in mediating hypoxic ROS production in PASMCs, HPV, and pulmonary hypertension.

Inositol 1,4,5-trisphosphate receptor (IP_3R) is another important Ca^{2+} release channel on the SR in PASMCs. Our findings provide novel evidence that hypoxia activates phospholipase C-γ1 (PLCγ1) by increasing RISP-dependent mitochondrial ROS production, which causes IP_3 production, IP_3R channel opening, and Ca^{2+} release, thus playing an important role in hypoxic Ca^{2+} and contractile responses in PASMCs [18]. In support of our findings, a previous study has also found that exogenous ROS activate IP_3Rs in many cell lines from multiple species using various cell lines [19].

In addition to RyR2 channels, ROS may also affect a number of other ion channels to increase $[Ca^{2+}]_i$ in PASMCs. Canonical transient receptor potential (TRPC) channel has been reported to mediate $[ROS]_i$-induced Ca^{2+} influx. Homo- and hetero-multimerization of different TRPC channel proteins within a tetrameric complex has been demonstrated for TRPC1/4/5 or TRPC3/6/7 channels [20]. TRPC1, 2, 4, and 5 channels have been associated with a store operated Ca^{2+} channel (SOCC). Inversely, TRPC3, 6, and 7 channels are rather associated with a receptor-operated Ca^{2+} channel (ROCC); therefore, they are activated following activation of a receptor and are sensitive to DAG and other components of G-protein receptor signaling pathways. Among the members of TRPC channel family, TRPC6 channel plays an important role. TRPC6 channel protein expression is shown to be higher in the distal (resistant) pulmonary artery than in proximal (conduit) pulmonary artery, correlating with changes in $[Ca^{2+}]_i$ during HPV [21]. More importantly, TRPC6 channel knockout mice show no acute phase of HPV. Furthermore, our published data, supported by other groups, demonstrate that PLCγ is activated by $[ROS]_{mito}$, subsequently increases diacylglycerol (DAG) production [18, 22]. DAG, a well-studied TRPC6 channel agonist, eventually enhances TRPC6 channel-induced Ca^{2+} influx in PASMCs. Recently vanilloid TRP channel-4 (TRPV4), another member of the TRP family, may also play a role in HPV, as TRPV4-deficient mouse models attenuated HPV and inhibition of TRPV4 blocked the hypoxia-induced Ca^{2+} influx and myosin light chain phosphorylation in human PASMCs [23]. More evidence is required in order to elucidate the relationship between $[ROS]_{mito}$ and TRPV4.

SOCC also plays an important role in mediating Ca^{2+} entry triggered by $[ROS]_{mito}$. Intracellular calcium release from RyR-sensitive calcium stores can cause the activation of stromal interaction molecule (STIM), subsequently couples and opens Ca^{2+}-selective Orai channels in the cell membrane. Studies have shown that oxidant stress such as buthionine sulfoximine (BSO) and hydrogen peroxide (H_2O_2) causes store-independent

Ca^{2+} entry in mouse embryonic fibroblasts (MEFs) but was ineffective in STIM1 deficient MEFs, establishing STIM1 as an important oxidative stress sensor [24]. Interestingly, as another groups of well-known SOCCs, TRPC1 and TRPC6 protein expression levels are associated with NADPH oxidase 4 (NOX4) activity in PASMCs [25]. Although lacking of direct evidences on hypoxia-induced mitochondrial ROS regulates SOCC expression and activity, the importance of other types of ROS (BSO and H_2O_2)-induced SOCC activation could shed light on the relationship between mitochondrial ROS and SOCC.

Protein kinase C (PKC) is a well-known kinase which participates in numerous signaling pathways. Many studies have proven that PKC plays a critical role in increasing $[Ca^{2+}]_i$ in PASMCs. Our lab has successfully demonstrated that mitochondrial ROS can directly activate PKCε, contributing to the hypoxic increase in $[Ca^{2+}]_i$ by inhibiting Kv channel and activating L-type Ca^{2+} channel [1, 26]. Recently, series of studies have shown that sphingomyelinase induced ceramide can further induce PKCε-dependent NOX activation, which elevates $[ROS]_i$ in PASMCs [27, 28]; ceramide can also inhibit Kv channel which contributes to pulmonary vasoconstriction [29].

4 Hypoxia-Caused Decrease in $[ROS]_i$ Can Elevate $[Ca^{2+}]_i$ in PASMCs

Interestingly, based on other investigations, conflicting results have been reported that increased hypoxic ROS production originates from the mitochondria. Under normoxic conditions, mitochondrial complex I and III generate superoxide radicals ($O_2^{\bullet-}$) converted to H_2O_2 by superoxide dismutase 2 (SOD_2). Other oxidized redox couples such as NAD^+, $NADP^+$, and FAD^{2+} have increased. During hypoxia, the limited presence of oxygen prevents generation of H_2O_2 and NAD^+, $NADP^+$, and FAD^{2+}, thus decreasing ROS production in hypoxic conditions [30]. Moreover, studies suggest decreased $[ROS]_i$ levels can lead to HPV, which was primarily caused by activation of L-type Ca^{2+} channels via cellular membrane depolarization caused by potassium channel inhibition [31]. In addition to decreased oxygen level during hypoxia, limited presentation of oxidized redox couples reduces sulfhydryl groups on Kv1.5 channels causing them to close. Subsequently, intracellular $[K^+]$ buildup causes cell membrane depolarization, which activates voltage-sensitive, L-type Ca^{2+} channels. Eventually the influx of Ca^{2+} will activate cellular contraction signal pathways [32]. Hypoxia-induced inhibition of K^+ channels is uniquely seen in PASMCs, and is not seen in systemic SMCs. Supplementary to Kv1.5, Kv2.1 plays an important role in mediating hypoxia-induced pulmonary hypertension. It has been demonstrated that anti-Kv1.5 or Kv2.1 antibodies substantially depolarize resistance in PASMCs as does the Kv blocker 4-aminopyridine, which also causes vasoconstriction [33].

5 Hypoxia-Induced Changes in $[ROS]_i$ Production Can Increase Ca^{2+} Sensitivity

It is well accepted that the Ca^{2+} sensitization mechanism involves the RhoA/Rho kinase (ROCK) pathway [34]. Activated RhoA will bind to ROCK, which then inhibits Myosin light-chain phosphatase (MLCP) and its activity on MLC20. Consequently, MLC20 cannot be dephosphorylated and thus remained in its phosphorylated form, allowing myosin–actin interaction responsible for smooth muscle contraction. Therefore, enhanced RhoA/ROCK activity leads to increased Ca^{2+} sensitivity to maintain SMCs contraction. Recent investigation pointed out that RhoA/ROCK mediates TGF-β1-induced kidney myofibroblast activation through Poldip2/NOX4-derived reactive oxygen species [35]. Supported by previous publication, $[ROS]_i$ can activate RhoA/ROCK-induced Ca^{2+} sensitization in PASMCs [36]. Although it does not show direct evidence, it suggests a reasonable direction to study the relationship between $[ROS]_{mito}$ and RhoA/ROCK activity in PASMCs because it is an important source of $[ROS]_i$. Another

publication demonstrated that hypoxia participated in RhoA/ROCK activation where RhoA/ROCK activity was inhibited by decreased RISP (by inhibiting mitochondrial complex III) in A549 cells [37]. This evidence can provide important suggestions on $[ROS]_{mito}$ could regulate hypoxia-induced RhoA/ROCK activation, which could further explain hypoxia mediated increased muscle contraction in pulmonary artery tissue.

6 Elevated Intracellular Calcium Concentration Can Increase $[ROS]_{mito}$

It is well known that hypoxia causes an increase in $[Ca^{2+}]_i$ and contraction in PASMCs. A recent study has pointed out that in cardiac myocytes, increasing intramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) leads to enhanced $[ROS]_{mito}$ production, consequently causing heart failure [38]. Our unpublished data supported this report and we have summarized this phenomenon as Ca^{2+}-induced ROS production (CIRP). Other than generating contraction forces in PASMCs, the downstream of increased $[Ca^{2+}]_i$ can be an increase in $[Ca^{2+}]_{mito}$, which further leads to increased production of $[ROS]_{mito}$. Previous publications indicated that cell stimulation causes elevated local concentrations of Ca^{2+} transience (20–40 μM), while the remaining areas in the cell resulted in lower concentration of Ca^{2+} transience (1–2 μM) [39]. In order to clarify the effects of Ca^{2+} on mitochondrial ROS generation, we designed different concentration of buffered Ca^{2+} to mimic local high Ca^{2+} transience and lower Ca^{2+} transience. Shown in Fig. 1, freshly isolated mitochondria from PASMCs were incubated with different concentrations of buffered Ca^{2+}. Interestingly, detected by DCF, 1, 3, 10, and 30 μM Ca^{2+} significantly changed mitochondrial ROS generation. Moreover, in agreement with our and other groups' publications, we found that ROS generated in Complex III are also enhanced by different concentrations of Ca^{2+}.

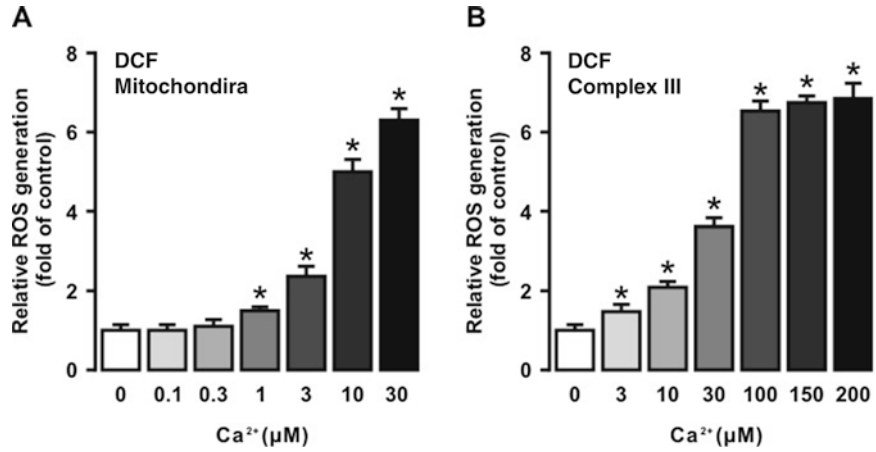

Fig. 1 Different concentrations of Ca^{2+} cause ROS generation from isolated mitochondria and Complex III. (**a**) Freshly isolated mitochondria from PASMCs were treated with different concentrations of Ca^{2+}. Mitochondrial ROS were determined by DCF as labeled. (**b**) Freshly isolated complex III proteins were treated with different concentrations of Ca^{2+}. Upon stimulation, $[Ca^{2+}]_i$ can vary from 1 to 40 μM because local Ca^{2+} release from Ca^{2+} store could induce 20–40 μM whereas the rest of cytosol could be 1–2 μM Ca^{2+}. We applied different concentrations of Ca^{2+} (0–30 μM) trying to test the effects of both local-high and cytosol low concentration of Ca^{2+} on mitochondrial/complex III ROS generation. Complex III ROS were determined by DCF. Data were obtained from three separate experiments. $^*P < 0.05$ compared with no Ca^{2+} treated group

7 Mitochondrial Calcium Uniporter (MCU) Plays a Significant Role in Regulating Ca^{2+}-Evoked $[ROS]_{mito}$ Generation

Mitochondria have been reported to play an important role in regulating $[Ca^{2+}]_i$ in SMCs [40]. MCU is one of the primary sources of mitochondrial uptake of Ca^{2+}, and its activity is largely dependent on the inner mitochondrial membrane potential and the difference in Ca^{2+} concentration between the mitochondria and cytosol. Interestingly, reports pointed out that relatively high concentrations of Ca^{2+} (5–10 μM) are able to fully activate MCU, which suggests its critical role in regulating Ca^{2+} transportation in ER/SR-mitochondria microdomain [41]. The importance of MCU in mediating PAH has been reported recently. Impaired MCU function associated with PAH development due to decreased $[Ca^{2+}]_{mito}$ and increased $[Ca^{2+}]_i$, thus inhibiting glucose oxidation and promote cell proliferation and migration [42].

We assumed that caffeine-induced Ca^{2+} release from the SR via RyRs might cause mitochondrial Ca^{2+} uptake and accordingly mediate Ca^{2+}-dependent $[ROS]_{mito}$ generation. To test this assumption, we assessed the effect of mitochondrial calcium uniporter (MCU) inhibitor Ru360. As displayed in Fig. 2a, treatment with Ru360 attenuated caffeine-elicited ROS production in PASMCs. More specifically, in the presence of Ca^{2+}, $[ROS]_{mito}$ was also prevented by Ru360 treated isolated mitochondria (Fig. 2b).

8 Role of NADPH Oxidase (NOX) in Hypoxic Pulmonary Vasoconstriction

NOX was first recognized as an enzyme playing a role in generating ROS in leukocytes for defending against bacteria [43]. Marshall et al. first reported that hypoxia activates NOX to cause ROS generation in PAMSCs. They found out that cytochrome b245, which contains NOX, is the key element in generating superoxide [6]. Followed by a series of studies, the functional importance of NOX in mediating HPV has been strengthened. Mittal et al. have pointed out that NOX-produced ROS can inhibit Kv channel in PASMCs, which explains vasocontraction in hypoxia condition [44]. Other signaling pathways, such as PKC mediated ROS production [45], especially the role of PKCε in NOX-induced

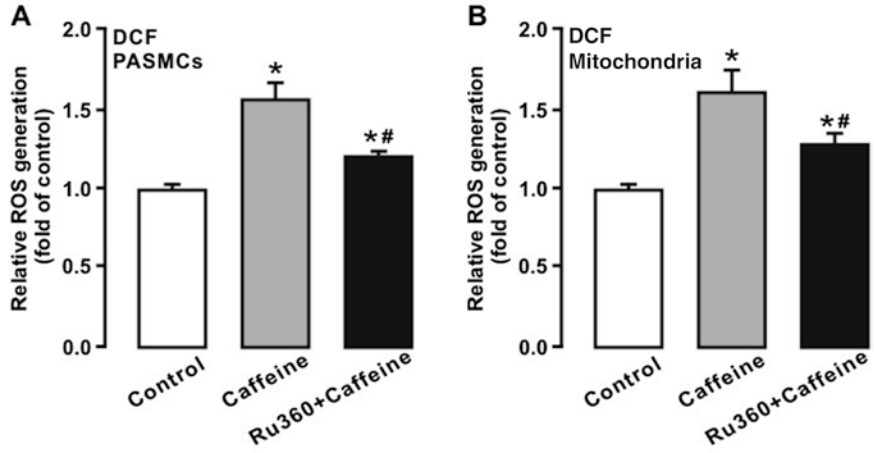

Fig. 2 Treatment with Ru360 prevents caffeine-induced ROS production in PASMCs and mitochondria. Ca^{2+}-evoked ROS generation is inhibited by Ru360 in mitochondria. Cells were treated with Ru360 (10 μM) for 5 min, exposed to caffeine (20 mM) for 5 min, and then subjected to ROS measurement (**a**) and isolation of mitochondria (**b**). Data were obtained from three separate experiments. $*P < 0.05$ compared with control cells, and $\#P < 0.05$ compared with cells treated with caffeine alone

ROS generation in PASMCs has been illustrated in a series of studies. Data has shown that PKCε activity is dependent on NOX activation by $[ROS]_{mito}$; PKCε deficiency, as well as PKC pharmacological inhibitor blocked hypoxia-induced NOX activation and ROS production [1, 2, 7, 26]. The different roles of $[ROS]_{mito}$ and NOX-induced ROS have been discussed and indicate that NADPH oxidase activity predominates in the acute phase, while a strong dependence on mitochondrial participation was observed for the second phase [9]. Another important signaling molecule, Src protein tyrosine kinase, could also participate in $[ROS]_{mito}$-induced NOX activation. It has been reported in human leukocyte and rat vascular artery that inhibiting Src by pharmacological inhibitor could decrease NOX activation [46–48].

Interestingly, NOX also participates in $[ROS]_{mito}$ production in vascular artery. A recent study points out that mitochondrial oxidants are involved in angiotensin II-NOX signaling pathway-mediated vascular smooth muscle senescence [49]. It has been reported that NOX can be activated by inflammatory and aging processes, and that this initially generated ROS will increase mitochondrial free radical formation via PKCε or MAPK-mediated mitochondrial alkalization, impaired Ca^{2+} homeostasis, and a change in mitochondrial membrane potential [50, 51]. Mechanistically, a group of studies have shown that mitochondrial potassium ATP channel ($mitoK_{ATP}$) was involved in $[ROS]_{mito}$ increase, and $[ROS]_{mito}$ can be diminished by pharmacological inhibitor 5-HD and it can also be enhanced by $mitoK_{ATP}$ agonist such as diazoxide [52–54]. Interestingly, mitoKATP can be regulated by Src tyrosine kinase and Src activity can be enhanced by NOX1 [55]. Thus, NOX activity could play a role in mediating $[ROS]_{mito}$ increase through $mitoK_{ATP}$.

9 Conclusion

RISP in the mitochondrial ETC complex III may function as a hypoxic sensor in PASMCs, by which hypoxia increases $[ROS]_{mito}$ in PASMCs (Fig. 3). The increased $[ROS]_{mito}$ results in the

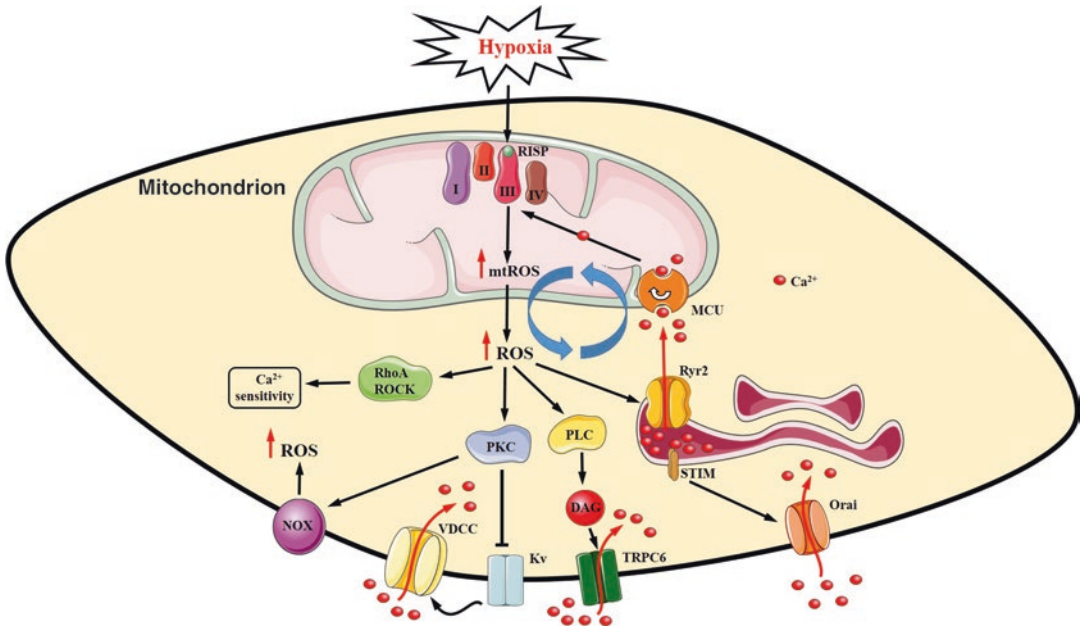

Fig. 3 Proposed model for cross talk between ROS and Ca^{2+} signaling in PASMCs. This schematic diagram summarizes the signaling pathways mentioned in this chapter. Hypoxia-induced increase in $[ROS]_{mito}$ causes elevation of cytosolic ROS and then initiates multiple signaling pathways in mediating increase of $[Ca^{2+}]_i$. It is worth mentioning that ROS-caused RyR2 activation can increase $[Ca^{2+}]_{mito}$ and then promote ROS production from Complex III (*blue arrow loop*).

activation of PKCε and then NOX, leading to RIRP and then $[ROS]_i$. Different opinions indicate that hypoxia could decrease production of $[ROS]_i$ in PASMCs. Although conflicting statements have been made on this topic, interestingly, both statements agree that the downstream changes lead to an elevation of $[Ca^{2+}]_i$. On one hand, RyR2 and IP_3R elicited-Ca^{2+} store release intensively associate with increasing of $[ROS]_{mito}$; DAG activated-TRPC6 channel also participates in this signaling pathway; $[ROS]_{mito}$ induced-PKCε and NOX activation and then Kv channel-related L-type Ca^{2+} channel activation has been well studied in our group. On the other hand, hypoxia-caused decrease in production $[ROS]_i$ can inhibit Kv channel and then activate L-type Ca^{2+} channel, and further elevate $[Ca^{2+}]_i$. In addition to previous studies, our group has recently discovered that RyR2-mediated increase in $[Ca^{2+}]_i$ can cause an increase in $[Ca^{2+}]_{mito}$, which promotes an increase in $[ROS]_{mito}$ in PASMCs, providing a positive reciprocal loop mechanism to further increase the hypoxic generation of intracellular ROS.

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Endothelial Cell Reactive Oxygen Species and Ca^{2+} Signaling in Pulmonary Hypertension

Karthik Suresh and Larissa A. Shimoda

1 Introduction

Within the lung vasculature, reactive oxygen species (ROS) are critical signaling intermediates in both health and disease. ROS are thought to play a particularly important role in the pathobiology of pulmonary hypertension (PH) [1, 2]. Alterations in ROS homeostasis in the pulmonary vasculature, specifically in pulmonary smooth muscle (SMC) and endothelial (EC) cells, lead to a variety of signaling events that promote enhanced vasoreactivity, increased cellular migration and proliferation, and vascular remodeling of the pulmonary arteries. For instance increased ROS activate multiple kinase pathways and, of particular interest to this review, has been shown to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in various vascular beds [3]. It is now appreciated that pulmonary vascular ECs exhibit considerable phenotypic heterogeneity based on whether they reside in the large, conduit vessels, i.e., the pulmonary artery, or the smaller vessels in the microvasculature [4]. Although a significant amount is known regarding the role of ROS in abnormal EC function in the systemic circulation, it is not clear which of these pathways may also play a role in promoting changes in EC phenotype in different types of lung ECs. While more recent studies have begun utilizing ECs isolated from specific sites within the lung (i.e., conduit lung vessels versus microvasculature) in order to better understand the specific pathways underlying lung EC dysfunction in PH [5–7], significant gaps in our understanding of the specific molecular mechanisms governing lung EC function remain. In this chapter, we will review the role played by elevations in ROS, and in particular ROS-induced changes $[Ca^{2+}]_i$, in promoting abnormal vasoreactivity and EC function in PH.

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2 Pulmonary Hypertension

PH refers to a heterogeneous condition defined as increased (≥25 mmHg at rest) mean pulmonary arterial pressure (mPAP), leading to progressive right ventricular overload and, in some cases, failure [8]. PH is diagnosed based on clinical examination and right heart catheterization, and is classified into five main groups based on hemodynamic and pathologic differences (Table 1).

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Table 1 Classification of PH (adapted from Galie et al., *Eur Heart J* 2015)

Classification of PH
Group 1: Pulmonary Arterial Hypertension (PAH)
Idiopathic PAH
Heritable PAH due to known (e.g., BMPR2) or other mutations
Drug and toxin induced
PAH associated with systemic disease
Connective tissue disease
HIV
Portal hypertension (liver disease)
Congenital heart disease
Schistosomiasis
Group 1′: Pulmonary veno-occlusive disease Pulmonary capillary hemangiomatosis
Idiopathic
Heritable (EIF2AK4 or other mutations)
Drugs, toxins and radiation-induced
Associated with connective tissue disease or HIV infection
Group 1″: Persistent PH of the newborn
Group 2: PH due to left heart disease
Left ventricular systolic or diastolic dysfunction
Valvular disease
Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
Congenital/acquired pulmonary vein stenosis
Group 3: PH due to lung diseases and/or hypoxia
PH due to chronic hypoxia (COPD, interstitial lung disease)
PH due to sleep-disordered breathing
Alveolar hypoventilation disorders
Chronic high altitude exposure
Developmental lung disease
Group 4: PH due to chronic thromboembolic disease and other pulmonary artery obstructions
Chronic thromboembolic pulmonary hypertension
Other pulmonary artery obstructions (e.g., Angiosarcoma, arteritis, parasites)
Group 5: PH due to unclear mechanism
Hematologic disorders (e.g., chronic hemolytic anemia)
Systemic disorders (e.g., sarcoidosis, LAM)
Metabolic disorders (e.g., glycogen storage disease)
Other (e.g., tumoral thrombotic microangiopathy, fibrosing mediastinitis. Chronic renal failure, segmental PH)

Group 1 PH, also called pulmonary arterial hypertension (PAH), is characterized by very high right-sided pressures and development of occlusive lesions in the pulmonary vasculature. The etiology of PH in Group 1 includes inherited and idiopathic causes as well as PAH due to drugs and toxins, connective tissue disease, HIV, and chronic liver disease (portal hypertension). Group 2 refers to PH that occurs as a consequence of left heart dysfunction, while Group 3 is defined as PH occurring in the context of chronic hypoxia, often in the setting of parenchymal lung disease. Group 4 and Group 5 refer to elevated P_{PA} due to chronic thromboembolic and systemic diseases, respectively.

While each group in the PH classification arises from varied etiologies, in each case the underlying pulmonary vascular abnormalities fall into three main categories: (1) increased vascular tone due to sustained contraction of the arterial tree, (2) vascular wall thickening due to uncontrolled EC, SMC, and fibroblast migration and proliferation, and in the case of PAH, (3) development of occlusive lesions in the distal arterioles that obstruct blood flow. These occlusive lesions of PAH comprise multiple cell types, including ECs that are monoclonal, apoptosis-resistant and hyperproliferative. The fundamental cell signaling abnormalities that lead to this aberrant EC phenotype are still not clear, with many potential pathways remaining under investigation. Over the past decade, there has been continued interest in the role of ROS in regulating both abnormal pulmonary vascular tone and promoting migration and proliferation.

3 ROS in Pulmonary Hypertension

Accumulating evidence supports a role for increased ROS in the development and progression of PH [1, 9]. Urinary and plasma levels of oxidative stress markers have been detected in PAH patients [10, 11] while histological examination of lung sections from patients displayed increased nitrotyrosine and 8-hydroxyguanosine residues, which are by-products of oxidative stress [12]. Experimentally decreasing ROS levels with antioxidants augmented pulmonary

arterial responsiveness to the vasodilator, nitric oxide (NO), in rat pulmonary arterial rings [13, 14]. Similarly, inhibition of xanthine oxidase or treatment with the ROS scavenger TEMPOL, attenuated development of chronic hypoxic PH. These data, collectively suggest that elevated ROS are involved in the pathobiology of PH, particularly by influencing Ca^{2+}-dependent pulmonary vasoreactivity and increased migration and proliferation of ECs.

4 Sources of ROS in PH

ROS can arise from multiple extracellular (i.e., infiltrating inflammatory cells) and intracellular sources [1]. Generation of intracellular ROS in the lung vasculature occurs via mechanisms similar to those described in other cell types. For example, membrane bound NOX produce ROS in response to external agonists, while non membrane-bound enzymes, such as xanthine oxidase, aid in generation of additional cytosolic ROS [2]. Perhaps the most common source of ROS generation is mitochondria [15].

NAPDH Oxidase. NOX is a complex enzyme comprising several protein subunits [16]. It catalyzes the transfer of two electrons from NADPH to two successive oxygen molecules, generating superoxide anions. In a variety of cell types, including ECs, NOX generates superoxide that is quickly converted to hydrogen peroxide (H_2O_2), allowing the enzyme to exert significant signaling effects on ROS-sensitive targets. In phagocytic cells, NOX-derived ROS forms part of the defense mechanism against pathogens whereas in non-phagocytic cells NOX is thought to serve primarily in a signaling capacity. The catalytic subunit has several isoforms (NOX1–5, DUOX1–2) and associates with multiple binding partners that enhance enzymatic activity (reviewed in [2, 16, 17]). Some of these binding partners are isoform specific; for instance, NOX2 associates with several "phox" proteins, including p47 and p67, and Rac1 while NOX4 associates with Poldip2 and p22 [18]. NOX1–4 are expressed in ECs, whereas both NOX2 and NOX4 have both been shown to contribute to EC proliferation [19]. NOX4 may be particularly important to EC function, as specific deletion of NOX4 inhibits microvascular and umbilical vein EC migration and proliferation. Conversely, NOX4 overexpression is sufficient to increase migration and proliferation [20].

Evidence suggests that NOX expression and activity is in fact increased in PH, which leads to pulmonary vascular hyperreactivity. For example, studies performed on homogenates of endothelium-intact pulmonary arteries isolated from a model of fetal/newborn PH demonstrated that increased NOX expression and uncoupling of endothelial nitric oxide synthase (eNOS; see Fig. 1) leads to elevated ROS production [21, 22]. Moreover, lung tissue expression of NOX subunits was increased in a murine model of chronic intermittent hypoxia (modeling PH due to obstructive sleep apnea) [23]. PH induced by either 3 or 10 days of hypoxia also increased NOX1 expression in pulmonary resistance arteries of newborn piglets [24]. On the other hand, mice with deficiency of the gp91phox (i.e., NOX2) subunit failed to develop PH with chronic sustained [25] or intermittent hypoxia [23]. Loss of gp91phox also attenuated hypoxia-induced, NO-dependent relaxation in isolated pulmonary arteries [26]. While all of these studies suggest a role for increased NOX expression/activity in PH, a caveat with the use of whole tissue homogenates or global knockouts is that the exact contribution of ECs (vs. SMCs) cannot be fully ascertained.

NOX is activated by ligation of VEGF receptor-2 (VEGFR2) via Rac1 [27]. In addition, NOX can be activated by a myriad other injurious stimuli including angiotensin II [27], tumor necrosis factor-alpha (TNF-α) [28] and shear stress [29]. The functional consequences of increased NOX-derived ROS in ECs are incompletely understood. Based on work performed in systemic ECs, basal ROS generation by NOX likely contributes to EC proliferation and angiogenesis [19, 20], although these effects are mild. The growth response is enhanced when NOX activity is stimulated by agonists known to be involved in PH, including hypoxia [30] and vascular endothelial growth factor (VEGF) [31].

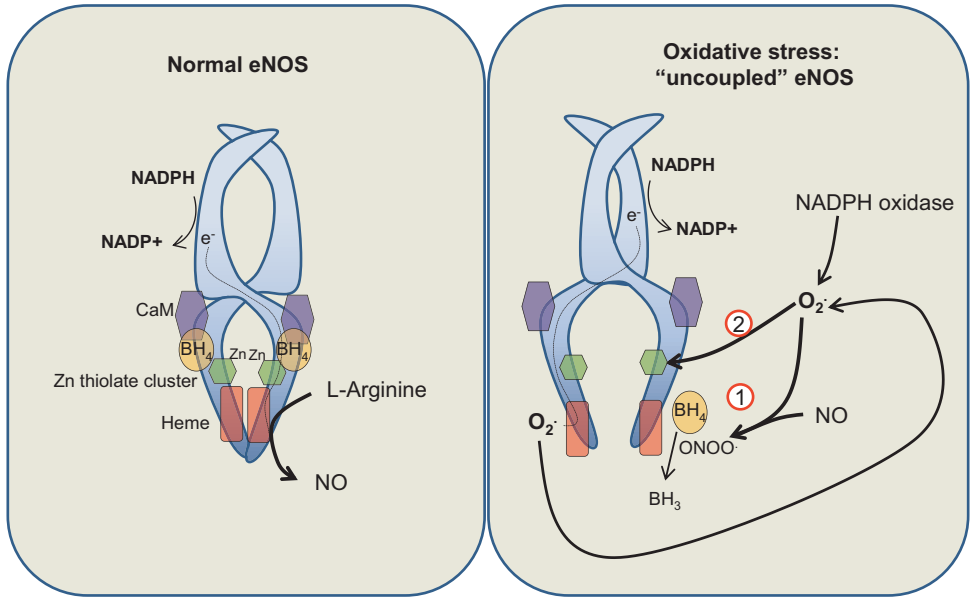

Fig. 1 Reactive oxygen species (ROS) and endothelial nitric oxide synthase (eNOS) uncoupling. Under normal circumstances (*left panel*), eNOS utilizes various cofactors including L-arginine, molecular O_2 and (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) to catalyze nitric oxide (NO) formation (at the heme site). The eNOS enzyme includes various regulatory subunits including a calmodulin (CaM) binding site. In situations of oxidative stress (*right panel*), local increases in superoxide ($O_2^{\bullet}$) lead to: (1) formation of peroxinitrites ($ONOO^-$) due to reactions with NO, causing oxidation of BH_4 (to BH_3) and inactivation of this critical cofactor and (2) oxidative modification of the Zn-thiolate cluster. Together, these events lead to decreased catalytic efficiency of eNOS and generation of $O_2^{\bullet}$ instead of NO. Increased $O_2^{\bullet}$ in turn can augment further uncoupling of eNOS via a feed-forward mechanism

Regarding the latter, NOX-generated ROS may serve as a signal for VEGF-induced changes in EC growth [31, 32].

Once generated, NOX-derived ROS activate a variety of targets, including signaling molecules (e.g., Src, Akt, and MAP kinases) and transcription factors (e.g., NF-kB and AP-1) [28]. ROS serve as an activation signal by increasing leukocyte recruitment to the endothelium [33, 34] and initiating and propagating EC migration [35, 36]. In response to VEGF, localized production of ROS by NOX mediates interactions with TRAF4 and PAK1, two cytoskeletal proteins involved in cell motility [37, 38]. NOX-derived ROS are also critical for EC proliferation [39] via activation of Ras/ERK and JNK pathways in response to the viral protein, Tat, in a HIV-induced model of EC migration and proliferation. These data suggest that ROS play a critical role in EC movement and division, especially in response to injurious stimuli.

*Mitochondrial ROS (mtROS)***.** Mitochondria are major sources of ROS in all cell types, and have recently come under increased scrutiny as drivers of ROS-induced EC dysfunction in PH. Complexes I and III of the electron transport chain are known mitochondrial sites of ROS generation [15, 40, 41]. Superoxide produced by complex I is released into the mitochondrial matrix, while superoxide produced by complex III can be released into either the intermembrane space or the mitochondrial matrix. Upon release, superoxide released into the intermembrane space or matrix is converted to H_2O_2 by superoxide dismutase (SOD)-1 or SOD-2, respectively. The site-specific superoxide release in mitochondria can modulate different signaling pathways [40]. Since mitochondria are thought to function as cellular oxygen sensors, mtROS may link changes in oxygen tension and hypoxia-induced alterations in cell signaling and function. In part due to the limitations surrounding ROS-measurement methods, difficulty of measuring mitochondrial O_2

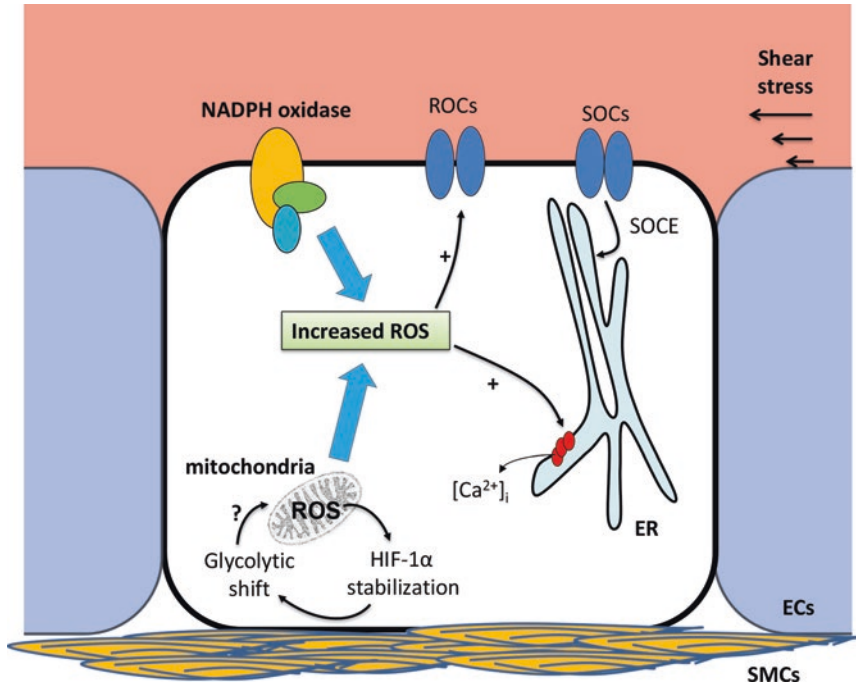

Fig. 2 Mechanisms of reactive oxygen species (ROS)-induced Ca^{2+} entry in endothelial cells (ECs) in pulmonary arterial hypertension (PAH). Increases in ROS from cytosolic (primarily NADPH oxidase) and mitochondrial sources can lead to both release of Ca^{2+} from internal endoplasmic reticulum (ER) stores as well as influx through receptor-operated (ROC) and store-operated (SOC) channels on the cell membrane. Release of Ca^{2+} from the ER can activate SOCs in order to replete ER Ca^{2+} stores via store-operated Ca^{2+} entry (SOCE). The sources of increased ROS in ECs include: NADPH oxidase, mitochondria and physical stimuli like shear stress. Based on data from both ECs and smooth muscle cells (SMCs) in PAH, mitochondrial ROS generation may be linked to abnormal HIF-1α stabilization and glycolytic shift

in vivo and the differential effects of superoxide due to compartmentalization, the exact relationship between hypoxia and mtROS remains an area of active investigation, with lines of evidence pointing towards both decreased [42–44] and increased ROS [45, 46] in the setting of acute hypoxia (also reviewed in [1, 45, 47]).

With respect to the role of mtROS in prolonged hypoxia, which is more relevant to PH, most studies have been performed in pulmonary arterial SMCs, where one of the main consequences of altered mtROS following hypoxia is stabilization of hypoxia-inducible factor-1α (HIF-1α), the oxygen-sensitive subunit of the HIF-1 transcription factor (Fig. 2). HIF-1, a master regulator of adaptive cell responses to hypoxia [48], is constitutively ubiquitinated and degraded due to hydroxylation by proline hydroxylase domain (PHD) proteins. Since this reaction requires O_2, hypoxia leads to loss of hydroxylation secondary to decreased substrate availability and stabilization of HIF-1α [49]. HIF-1α/HIF-1β heterodimers transcriptionally regulate a variety of cellular responses to hypoxia [50]. Although the mechanisms are not yet fully clear, regulation of HIF-1α by mtROS likely involves inhibition of enzymes (i.e., PHDs) that normally aid in HIF-1α degradation [51]. For instance, quenching to H_2O_2 in the intermembrane space of the mitochondrion before it is able to reach the cytosol attenuates hypoxia induced HIF-1α stabilization [52]. Less is known regarding the relationship between hypoxia and mtROS in pulmonary ECs. However, Al-Mehdi et al. [53] reported that, in PA ECs, acute hypoxia increased mtROS and mobilized mitochondria to the perinuclear space, leading to diffusion of mtROS into the nucleus. Taken together with reports

describing induction of HIF-1α [54] expression in response to hypoxia in PA ECs, it is likely that signaling pathways similar to those described in SMCs, involving mtROS generation and HIF-1α stabilization, exist in PA ECs as well.

Main transcriptional targets of HIF-1 include metabolic enzymes that control cellular shifts from oxidative phosphorylation towards glycolysis [44]. This shift, called the Warburg effect, was initially described in cancer cells and, while less efficient in terms of ATP production, allows for increased production of other macromolecules required for cellular growth and proliferation. Under conditions of tissue hypoxia, glycolysis is a normal response to cellular stress (i.e., the Pasteur effect). However, as increasingly seen in the context of PAH, abnormal HIF stabilization during normoxic conditions may induce a metabolic shift and consequent changes in cell function. Histologic sections of lungs from patients with PAH that stain positive for markers of oxidative stress also exhibit increased HIF-1α expression [55], while pulmonary ECs from patients with PAH display glycolytic shift [56]. These data indicate that HIF is abnormally stabilized in PAH and ROS, most likely mitochondrial in origin, are probably involved. It is likely that mtROS, maladaptive HIF stabilization and glycolytic shift are related processes that participate in a feed-forward mechanism such that changes in mtROS may enhance HIF stabilization, which in turn promotes glycolytic shift, altering mitochondrial redox state. However, much remains unknown with regards to the specifics of mtROS in PAH, particularly in ECs.

While little is known about mtROS levels in lung endothelium in the context of changes in mitochondrial respiration, data collected in systemic ECs may provide important clues [41]. In general, vascular ECs do not use oxidative phosphorylation as primary source of ATP [57]. Agonists, such as glucose, Ang II, and VEGF, increase EC mtROS production [41] and also activate NOX, arguing for the possibility that EC ROS production in PH may occur at multiple sites and may actually be linked, as significant cross talk likely exists between these pathways. As an example, mitochondrial biogenesis [58] and ROS production induced by VEGF, may in turn increase NOX expression/activity, further promoting oxidative stress [59].

Cyclic stretch provides one model where the interplay between mtROS and HIF-1α stabilization has been recently investigated. Unlike shear stress, which results from flow of fluid along (or parallel to) the endothelium in a single direction, stretch is primarily determined by the intraluminal vascular pressure [60], and exerts force perpendicular to the endothelial surface. Most often used in the context of ventilator-associated lung injury, in vitro models of stretch may mimic pressure-related changes in the PA that occur during PH. While the effect of increased strain has not been evaluated in pulmonary ECs, PASMCs undergoing cyclic stretch exhibit increased mtROS and HIF-1α activity; interestingly, these findings mirror those found in SMCs isolated from newborn lambs in a pediatric PH model induced by in utero ligation of the ductus arteriosus [61, 62]. Finally, HUVECs undergoing cyclic strain generated ROS, a response that was absent in cells depleted of mitochondria [63].

Although much remains unknown regarding the role of mtROS in promoting functional changes in the pulmonary vasculature in PH, a recent report noted that induction of mtROS promoted pulmonary arterial EC migration and proliferation in a process that involved p38 MAP kinases [64]. In the systemic circulation, the role of mtROS in EC-mediated vascular dysfunction has been studied more in more detail, with mtROS promoting EC migration and proliferation directly [40, 65]. Moreover, H_2O_2 generated by mitochondria was required for EC-mediated flow-induced coronary resistance artery dilation [66]. Taken together, these data suggest that EC mtROS are likely to play a role in ROS-induced EC dysfunction during PH.

5 EC $[Ca^{2+}]_i$ in Pulmonary Hypertension

In non-excitable cells, Ca^{2+} homeostasis is maintained via several mechanisms. Ca^{2+} can enter cells from the extracellular space through plasma

membrane channels or be pumped from the cell by Ca^{2+}-ATPases. Ca^{2+} is also sequestered within, and released from, organelles. In the endoplasmic reticulum (ER) , Ca^{2+} is tightly regulated by release channels and Ca^{2+} transporters on the ER membrane, while similar channels control mitochondrial Ca^{2+} influx and efflux (Fig. 2). $[Ca^{2+}]_i$ homeostasis between the cytosol and ER can be pharmacologically disrupted using agents such as thapsigargin (Tg), an agent that inhibits Ca^{2+} uptake into the ER and depletes ER stores. A full listing of endothelial Ca^{2+} channels/transporters is beyond the scope of this chapter; however, in general, plasmalemmal Ca^{2+} channels can be receptor-operated (e.g., transient receptor potential [TRP]), voltage-gated (e.g., T-type) or activated by depletion of ER Ca^{2+} stores (store-operated) [67–69], while ER release channels belong to inositol triphosphate or ryanodine receptor families.

At baseline, lung EC cytosolic Ca^{2+} levels are tightly controlled, typically averaging 100 nM [70]. Stimuli can increase $[Ca^{2+}]_i$ by directly binding receptor-operated channels on the cell membrane, activating metabolites that subsequently agonize channels, or stimulating Ca^{2+} efflux from the ER. Limited information is available regarding changes in, and the specific role of, basal EC $[Ca^{2+}]_i$ in PH. Globally, whole genome profiling approaches showed that genes encoding Ca^{2+} signaling pathways were significantly dysregulated in ECs in a model of PH induced by the diet drug, fenfluramine [71]. Exposure of pulmonary ECs to either acute [72] or chronic hypoxia [73] increased $[Ca^{2+}]_i$, with the latter mediated via upregulation of store-operated TRP channels. Together, these data suggest a role for increased $[Ca^{2+}]_i$ in PAH EC pathobiology, but as detailed below, the specific source of $[Ca^{2+}]_i$ and the channel involved remain under investigation.

One Ca^{2+} channel that has recently come into focus in PH studies is a member of the transient receptor potential (canonical) family, TRPC4. A store-operated Ca^{2+} channel, TRPC4 plays a key role in increasing endothelial permeability in the large vessels of the lung [74], leading to the formation of perivascular cuffing [75–77]. Interestingly, rats subjected to the SU5416 plus hypoxia (SuHx) protocol, which creates a severe experimental model of PAH, displayed increased EC Ca^{2+} transients through TRPC4 [78] and perivascular edema in response to Tg compared to normoxic controls, suggesting a higher sensitivity to induction of EC hyperpermeability through activation of SOCE [79]. These responses were attenuated in *TRPC4*$^{-/-}$ rats even though PA pressures were similar to wild-type animals [79]. Loss of TRPC4 also conferred a survival benefit in SuHx rats [80], although the exact cell type (EC or SMC) responsible for this benefit remains to be determined. These data suggest that changes in SOCE mediated changes in EC permeability may contribute to mortality in PAH, though it is unclear whether the same calcium channels also contribute to other aspects of PAH pathogenesis such as formation of vaso-occlusive lesions.

6 Links Between ROS and $[Ca^{2+}]_i$ in the Lung Endothelium

ROS-induced Ca^{2+} entry. Elevations in ROS have been shown to increase $[Ca^{2+}]_i$ and, conversely, increased $[Ca^{2+}]_i$ participates in ROS generation [3]. With respect to the former, ROS can activate Ca^{2+} channels by two mechanisms [81–83]: via direct activation through redox sensitive cysteine residues on the channels (i.e., TRPA1, TRPV4, TRPM4) or via activation by metabolites generated by elevations in ROS (i.e., TRPM2) [81, 84–86, 87]. In addition to modulating the activity of plasma membrane channels, ROS can also induce release from internal stores, presumably through an effect on ER Ca^{2+} transporters [88–91]. The proteins responsible for replenishing ER Ca^{2+} stores following depletion (i.e., STIM and Orai) are also redox sensitive [92]. It should be noted, however, that the specific radical generated and/or compartmentalization may result in different Ca^{2+} responses [93]. Further complicating ROS-mediated Ca^{2+} responses is the fact that the increase in $[Ca^{2+}]_i$ likely represents a summation of multiple, temporally distinct Ca^{2+} release

phenomena, with a mix of release and influx events.

mtROS and [Ca^{2+}]: Mitochondria also possess distinct Ca^{2+} channels that regulate Ca^{2+} uptake and release [94]. The mitochondrial Ca^{2+} uniporter (MCU) is responsible for Ca^{2+} transport into the mitochondria while various other pumps are responsible for Ca^{2+} efflux. In addition to buffering cytosolic Ca^{2+} levels, proximity of mitochondria to the ER allows for Ca^{2+} ion transfer between these organelles [95]. Several enzymatic reactions that occur in the mitochondria, including the portions of the Krebs cycle, require Ca^{2+} and large increases in mitochondrial Ca^{2+} can also act as a signal for impending cell failure and activate cell death [94]. Increased mitochondrial Ca^{2+} levels and mtROS are often observed under similar conditions, and mice deficient for UCP2, a mitochondrial Ca^{2+} transport protein, developed PH, suggesting a common pathway between mtROS and $[Ca^{2+}]_i$. Furthermore, pulmonary SMCs isolated from UCP2 mice exhibit stabilization of HIF under normoxic conditions. While these changes mimic those observed with hypoxia [96] or in ECs from PAH patients [97] and animal models [98], it is not clear if similar HIF stabilization was also present in ECs of UCP2-deficient mice. Thus, the role for EC UCP2 in modulating maladaptive mitochondrial function in PH remains unknown.

ROS and [Ca^{2+}]$_i$ in EC migration and proliferation. Migration and proliferation are dependent on both ROS and $[Ca^{2+}]_i$ in a variety of tissue types [3]. In PH, increased migration and proliferation occur in both SMCs and ECs, and the anatomic site for these functional changes is at the level of the resistance arteries [68]. Unlike SMCs, where significantly more is known regarding migration and proliferation following ROS-generating stimuli, considerably less is known about the interplay of ROS and Ca^{2+} in EC, particularly lung EC. Data from systemic ECs demonstrated that exposure to VEGF increased NADPH-derived ROS, leading to posttranslational modification of SERCA2b (S-glutathiolation), increased $[Ca^{2+}]_i$, and migration. Notably, removal of a reactive cysteine on SERCA2b attenuated increases in migration by either VEGF or exogenous H_2O_2 [91, 99]. Interplay between ECs and SMCs exists as well; for example, endothelial store-operated $[Ca^{2+}]_i$ entry is important for transcriptional activation of various inflammatory factors that in turn drive pulmonary smooth muscle proliferation and vascular remodeling [73].

ROS and [Ca^{2+}]$_i$ in EC shear stress responses. A phenomenon of particular interest in PH is shear stress. Recent studies using magnetic resonance imaging elegantly demonstrated the presence of complex flow patterns in the pulmonary arteries of patients with PAH [100, 101]. ECs are uniquely positioned to serve as sensors of changes in adjacent blood flow, with EC dysfunction occurring as a consequence of both changes in laminar flow and formation of non-laminar flow patterns. Changes in shear stress cause a wide variety of responses in ECs, from alterations in gene expression [102] to cytoskeletal reorganization [103]. With respect to ROS, both increased and abrupt cessation of blood flow triggered radical production [104–106] and increased $[Ca^{2+}]_i$ [107–109], suggesting the possibility of cross talk between these signaling pathways. Supporting this notion, flow-adapted microvascular ECs subjected to sudden disruption in flow exhibited augmented baseline expression of T-type Ca^{2+} channels and NOX-dependent ROS generation [110, 111] that was followed sequentially by an increase $[Ca^{2+}]_i$ [112, 113] that was inhibited by depletion of ER stores. However, the effect of changes in shear stress due to the complex flow patterns observed in PH has not been fully elucidated. Interestingly, high shear stress increased $[Ca^{2+}]_i$ to a greater extent in SMC from PAH patients compared to normal controls [114] through a mechanism involving increased expression of TRPM7 and TRPV4 channels. Further studies will be required to determine whether a similar response occurs through the same mechanisms in ECs.

ROS and Ca^{2+} in pulmonary vasoreactivity. Interactions between ROS and Ca^{2+} have been implicated in several stimulus-specific mechanisms of increased vasoreactivity including: (1) hypoxic pulmonary vasoconstriction (HPV), a well-described pulmonary vascular response to

acute hypoxia, (2) nitric oxide production, a key step in endothelium-mediated pulmonary vascular vasodilation, and (3) pulmonary arterial constriction in chronic hypoxia, a known mediator of PH, particularly in Class III disease.

(a) *Hypoxic Vasoconstriction*: It is well documented that decreased oxygen tension causes HPV in an effort to shunt blood away from poorly oxygenated alveoli. HPV may also play some role in the development of PH, although the exact cellular mechanisms underlying contraction in response to acute and chronic hypoxia may differ depending on the length of hypoxic exposure. Nonetheless, both ROS and increased $[Ca^{2+}]_i$ are involved in HPV [115–117]. ROS are sufficient to induce pulmonary vasoconstriction; in studies utilizing endothelium-intact pulmonary arterial rings, contraction induced by provisioning exogenous ROS was not attenuated by removal of extracellular Ca^{2+} or blocking of membrane channels, arguing for store release as the mechanism of ROS-induced Ca^{2+} influx mediating PA contraction [118–120] (Fig. 3). However, an important caveat to these studies is that it is not possible to delineate which effects were mediated by the endothelium and which might have occurred in SMCs.

(b) *Nitric Oxide Production*: Nitric oxide (NO) production by ECs is a key defense mechanism against vascular dysfunction across multiple vascular beds, including the pulmonary circulation. In ECs, NO production occurs primarily via endothelial nitric oxide synthase (eNOS). Dimeric units of eNOS catalyze electron transfer from NADPH to various electron carrying intermediates to eventually produce NO from L-arginine in a process that requires molecular O_2, L-arginine and (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4). This process occurs in two steps. First, electrons are transferred from NAPDH to a heme group within eNOS where they are stabilized with the aid of O_2. Next, the electrons are used to catalyze NO production from L-arginine in a process that requires BH_4. eNOS activity is regulated by a variety of posttranslational modifications, including phosphorylation and oxidative modification of Cys residues. NO production is also determined by the availability of the critical cofactors such that when BH_4 is unavailable, eNOS cannot successfully complete electron transfer from NADPH to L-arginine, resulting in formation of superoxide radicals. Under these conditions, BH_4 deficiency uncouples the first half of the reaction (electron transfer from NADPH to the heme group) from the second half (electron transfer from the heme group to L-arginine).

Increases in cytosolic ROS decrease the ability of eNOS to efficiently produce NO primarily by decreasing BH_4 availability [121]. NOX-generated superoxide can react with NO to produce peroxinitrites ($ONOO^{\bullet}$) which can oxidize BH_4 to a product (BH_3) that cannot serve as a eNOS cofactor [122]. NOX-generated ROS can also oxidize cysteine residues on eNOS to decrease BH_4 binding [121] and/or oxidize a moiety that is critical for NO generation (i.e., the Zn-thiolate cluster) [123]. Through these mechanisms, increased cytosolic ROS facilitate uncoupling of eNOS, leading to a feed-forward mechanism of ROS-induced ROS generation in ECs.

While increases in ROS disrupt eNOS function via oxidative modification of critical motifs and cofactors, increases in $[Ca^{2+}]_i$ modulate eNOS function via calmodulin (CaM). As reviewed in detail elsewhere [124, 125], the sensitivity of NOS to changes in $[Ca^{2+}]_i$ is isoform-dependent. For the purposes of this review, we will limit our discussion to the calcium-sensitive, constitutively expressed eNOS; however, inducible NOS (iNOS), which is not Ca^{2+} dependent, has also been implicated in oxidative stress. Although controversy remains regarding the specifics of eNOS subcellular localization and the role of various posttranslational modifications on eNOS activity, it is generally accepted that increased $[Ca^{2+}]_i$ leads to CaM binding which in turn facilitates electron transfer towards the

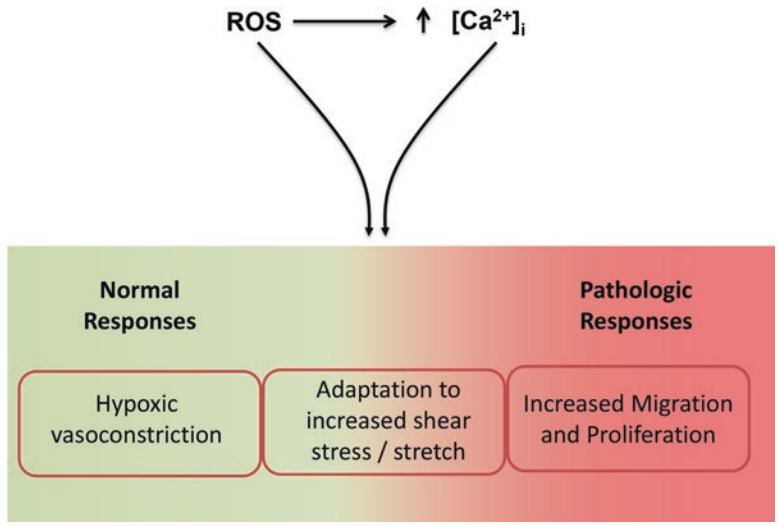

Fig. 3 Functional consequences of elevated reactive oxygen species (ROS) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in pulmonary endothelial cells (ECs). Elevations of both ROS and $[Ca^{2+}]_i$ promote normal physiologic responses, such as hypoxic vasoconstriction, maladaptive responses such as EC adaptation to shear stress and stretch and lastly, pathologic responses such as increased migration and proliferation

heme moiety where NO synthesis occurs. Thus, while increased ROS leads to uncoupling, increases in $[Ca^{2+}]_i$ activate NO production. The net result of increases in both ROS and $[Ca^{2+}]_i$ on NO production in pulmonary ECs may vary with source of ROS, and compartmentalization; studies conducted in ECs from PAH patients show both oxidant stress [12] and increased $[Ca^{2+}]_i$ [73], with decreased NO production [126], suggesting that in PH the net effect shifts towards uncoupling/decreased NO production. Consistent with these findings, Ghosh et al. [127] recently showed that abnormal phosphorylation of eNOS contributed to decreased NO production in PAH ECs and may contribute to eNOS uncoupling [128], but further work will be needed to elucidate whether this mechanism is related to ROS and/or Ca^{2+} levels.

Once produced, NO acts within the vessel lumen, with anti-inflammatory effects on leukocytes and platelets to decrease adherence of inflammatory cells to the endothelium and intracellularly, to induce production of cyclic guanosine monophosphate (cGMP). In SMCs, cGMP promotes vasodilation. Indeed, targeting cGMP availability, by either inhibiting degradation or stimulating production, are two treatment avenues currently utilized in the treatment of PAH.

(c) *Chronic Hypoxia*: Increased $[Ca^{2+}]_i$ is responsible for enhanced pulmonary vasoreactivity and development of abnormal EC function that occur with chronic hypoxia [129, 130]. For instance, chronic hypoxia increased agonist-induced vasoconstriction via both ROS generation and enhancement of Ca^{2+}-sensitivity of the contractile apparatus due to ROS-induced activation of RhoA/ROCK [131]. However, most of these studies were performed either in SMCs or preparations that included ECs and SMC; thus, the EC-specific roles of ROS and Ca^{2+} in regulating vasoreactivity during chronic hypoxia remain unclear.

In summary, it is evident that inhibition of either ROS or Ca^{2+} entry invokes largely similar responses with regards to vasoreactivity in the pulmonary vasculature. However, significant gaps still exist in our understanding as to whether ROS directly activates Ca^{2+} entry in pulmonary ECs, and if so, the exact channels that are involved in this response.

7 Conclusions

Based on available evidence, elevations in ROS and $[Ca^{2+}]_i$ play key roles in the pathobiology of PH, mainly because increased ROS generation elevates EC $[Ca^{2+}]_i$. While it seems clear that elevations in ROS from NOX and mitochondria are important for migration, proliferation, and vasoreactive responses, the role played by ROS-induced Ca^{2+} influx on these pathways, particularly in ECs, remains to be determined. Continued investigation is necessary to better understand the role of both ROS and $[Ca^{2+}]_i$ in promoting vascular reactivity, migration, and proliferation in pulmonary ECs. The hope is that this will lead to targeted therapies aimed at reversing or slowing progression of this devastating disease.

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Redox Signaling in the Right Ventricle

Yuichiro J. Suzuki and Nataliia V. Shults

1 Introduction

Pulmonary hypertension is a condition of increased pulmonary vascular pressure. Increased pulmonary vascular resistance directly influences the right ventricle (RV) that pumps the blood to the pulmonary circulation. The heart ventricles are capable of adapting to chronic conditions of increased resistance by elevating the force of muscle contraction by increasing the sarcomere units, which results in thickening of the single cardiomyocytes in the process of concentric cardiac hypertrophy. This compensatory process, however, eventually leads to decompensation if the condition persists. In the case of pulmonary hypertension, this decompensation results in the inability of the RV to sustain a satisfactory muscle contraction, leading to right heart failure. Right heart failure is a major cause of death among patients with various forms of pulmonary hypertension.

Compared with the left ventricle (LV), understanding of the RV pathophysiology has been delayed [1]. Moreover, the RV and LV can be viewed as fundamentally different entities because these two ventricles are developed from distinct progenitor cells [2]. The RV is expected to possess more plastic properties since it is subjected to a wide range of blood pressure throughout life (a level of systemic blood pressure in utero and much lower pulmonary circulation pressure after birth). Remarkably, patients with Eisenmenger syndrome can live with the RV pumping blood against systemic blood pressure throughout adulthood [3]. Thus, understanding the biology of the RV is scientifically exciting and undoubtedly important for developing right heart-specific therapeutic strategies to manage patients with pulmonary hypertension to increase their survival.

Our bodies are influenced by reactive oxygen species (ROS) and other biological oxidants in a variety of ways. Exogenously generated oxidants as well as those produced in our bodies can harm various biological molecules and biological processes. Further, it appears that biological oxidants can regulate cell signal transduction [4]. In particular, oxidants can promote both the growth and the death of cells. This is also important in regulating the fate of RV cardiomyocytes in patients with pulmonary hypertension. However, neither the exact mechanism of how biological oxidants regulate cell signaling nor the role of oxidant signaling in the RV are well understood. This book chapter compiles information on the mechanisms

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of redox signaling in the RV, which were mainly obtained through experiments performed in our laboratory.

2 Redox Regulation of Serotonin Signaling in the RV

Serotonin plays an important role in the pathogenesis of pulmonary hypertension by causing vasoconstriction and the growth of pulmonary artery smooth muscle cells [5, 6]. ROS have been reported to serve as second messengers in pulmonary artery smooth muscle cells [7–9]. Serotonin has also been reported to produce ROS in the heart [10, 11]. However, these results were obtained in isolated cardiac myocytes obtained from both the RV and LV. Thus, the difference between the RV and LV in terms of serotonin-mediated ROS generation was unknown when Dr. Lingling Liu started to perform her experiments in our laboratory.

Protein carbonylation is an important consequence of ROS-mediated protein oxidation [12]. Dr. Liu's experiments revealed that perfusing isolated rat hearts with serotonin caused protein carbonylation in the RV, but not in the LV [13]. As shown in Fig. 1, RV and LV homogenates derivatized with 2,4-dinitrophenylhydrazine (DNPH) exhibited multiple carbonylated proteins in SDS-PAGE gels. A 10-min treatment of isolated rat hearts perfused through a modified Langendorff system, in which vena carvas were also tied to perfuse the RV and large pulmonary arteries, resulted in the significantly increased carbonylation of various proteins in the RV (Fig. 1a). By contrast, the difference in the intensity of carbonylated bands between control and serotonin-treated LVs was minimal and not statistically significant (Fig. 1b).

The effort to identify the mechanism of this RV/LV difference led to our finding that the monoamine oxidase-A level is lower in the RV compared with the LV [13]. This event was confirmed by monitoring monoamine oxidase-A protein expression (Fig. 2a), monoamine oxidase-A enzymatic activity (Fig. 2b) and *monoamine oxidase-A* mRNA expression (Fig. 2c). Monoamine oxidase-A, uses serotonin as a substrate to produce hydrogen peroxide; thus, ROS produced by monoamine oxidase-A may mediate serotonin signaling. However, from our observations, we proposed that the ability of monoamine oxidase-A to degrade intracellular serotonin, rather than the production of ROS, mediates serotonin-induced protein carbonylation in the RV [13].

3 Carbonylation-Degradation Pathway of Signal Transduction in RV Hypertrophy

The GATA4 transcription factor plays a critical role in the development of cardiac hypertrophy [14]. In the LV, GATA4 is activated through post-translational modification mechanisms [15]. We studied the mechanism of GATA4 activation in the RV by using the chronic hypoxia model of pulmonary hypertension and RV hypertrophy in rats [16]. As expected, GATA4 DNA binding activity was increased in response to chronic hypoxia-induced pulmonary hypertension in the RV. Surprisingly, however, this was also associated with the increased gene expression of GATA4. Since the mechanism of *Gata4* gene transcription as well as the promoter sequences of the *Gata4* gene were unknown, Hiroko Nagase in our laboratory cloned the mouse *Gata4* promoter [17, 18]. Detailed analyses of the *Gata4* promoter region revealed that DNA binding to the CCAAT box is important for the chronic hypoxia-induced pulmonary hypertension-mediated activation of *Gata4* gene transcription. Dr. Ah-Mee Park in our laboratory discovered that the transcription factor that binds to the CCAAT box during this process is CBF/NF-Y [16].

Since the mechanism of CBF/NF-Y activation is unknown, we screened for proteins that can interact with CBF/NF-Y. One protein we found to interact with CBF/NF-Y during the hypoxic pulmonary hypertension-mediated development of RV hypertrophy is annexin A1. The binding of CBF/NF-Y to the CCAAT box was inhibited by the addition of recombinant annexin A1 in the

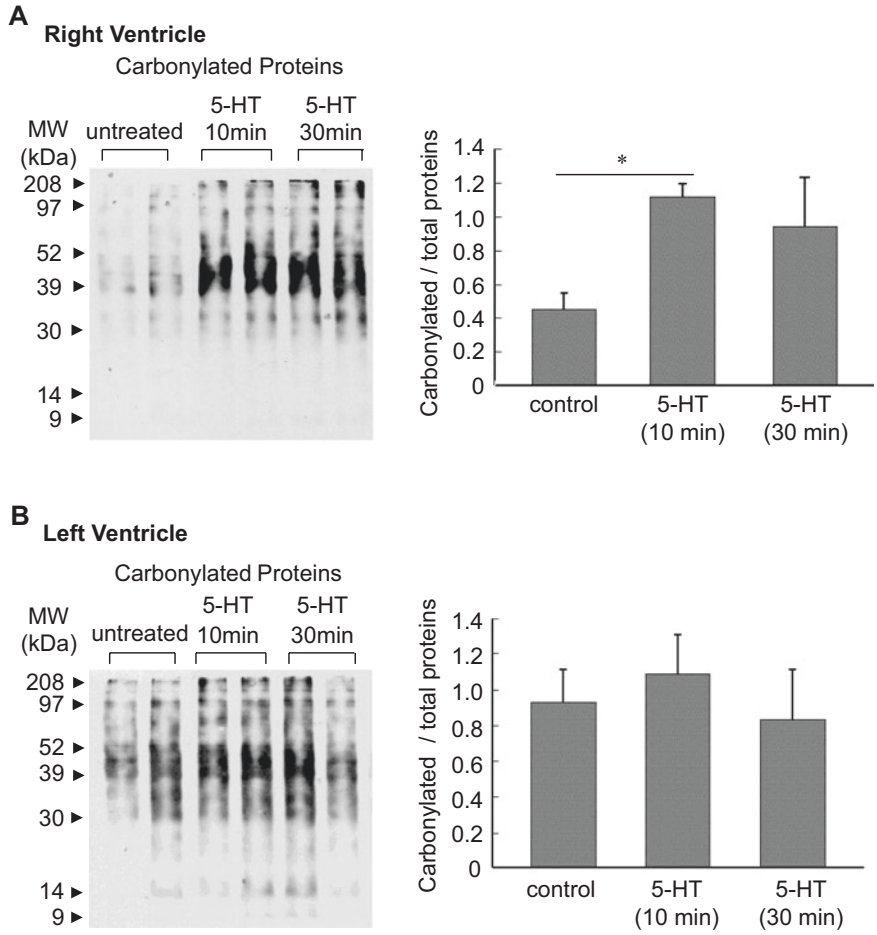

Fig. 1 Effects of serotonin (5-HT) on protein carbonylation in the RV and LV. Isolated rat hearts were subjected to retrograde perfusion on a Langendorff apparatus. After equilibration, the solution containing 5-HT was perfused for 10 or 30 min. (**a**) RV and (**b**) LV homogenates were derivatized with DNPH to label carbonyl moieties and subjected to SDS-PAGE and immunoblotting with the antibody, which interacts with DNPH-derivatized proteins. Total protein levels were visualized by Coomassie Blue staining. *Bar graphs* represent means ± SEM ($n = 3$). (*) denotes values significantly different from each other at $P < 0.05$ (Reproduced from [13] with permission)

reaction mixture for band-shift assays, suggesting that annexin A1 is a negative regulator of CBF/NF-Y DNA binding.

The finding that annexin A1 is involved in this mechanism of RV hypertrophy was of great interest to us because we previously reported a novel oxidant signaling mechanism, which involves protein carbonylation and the subsequent proteasome-dependent degradation of annexin A1 [19]. In this study, Dr. Chi-Ming Wong in our laboratory demonstrated that protein carbonylation is promoted in response to ligand/receptor-mediated cell growth signaling in pulmonary artery smooth muscle cells [19]. One protein identified to be carbonylated was annexin A1, using a proteomic approach and mass spectrometry. Further, this carbonylated annexin A1 protein is degraded by proteasomes. From these studies, we proposed the "oxidation/degradation pathway of signal transduction," which involves the carbonylation of annexin A1 and subsequent degradation of this oxidized protein by proteasomes. At that time, when we were studying pulmonary artery smooth muscle cells, we did not know exactly how the degradation of annexin A1 would promote cell signaling. It was exciting that

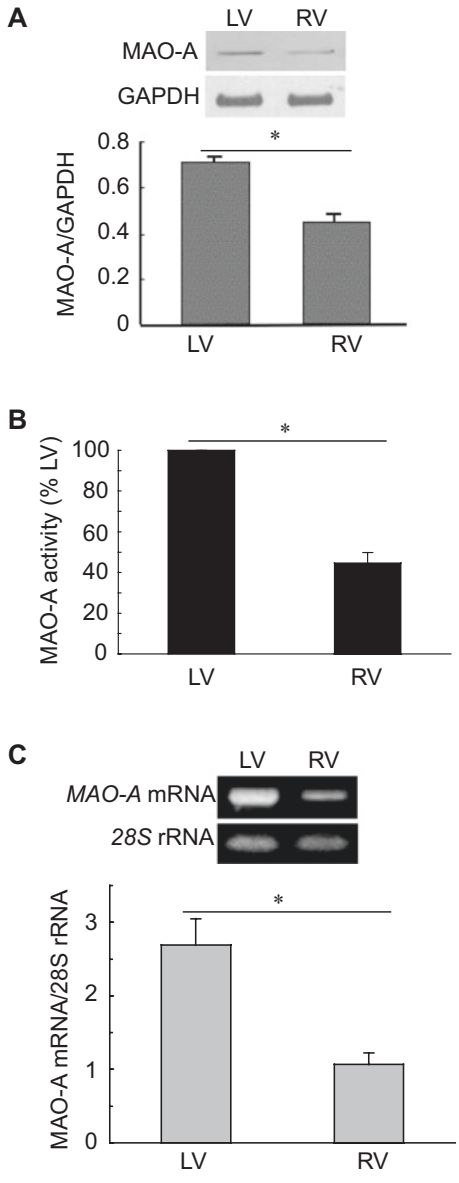

Fig. 2 Expression and activity of monoamine oxidase A (MAO-A) in the RV and LV. (**a**) Western blotting of MAO-A protein (61 kDa) and a loading control GAPDH. (**b**) MAO-A activity was measured fluorometrically with octopamine as a substrate. (**c**) Total RNA was isolated and *MAO-A* mRNA and *28S* rRNA levels were monitored by RT-PCR. The *bar graph* represents means ± SEM. (*) denotes values significantly different from each other at $P < 0.05$ (Reproduced from [13] with permission)

our investigations of the mechanism of cardiac hypertrophy revealed one mechanism, in which annexin A1 degradation can elicit cell signaling and gene transcription by regulating CBF/NF-Y in the RV [16].

We tested if this annexin A1 carbonylation/degradation cascade may regulate RV hypertrophy. Our experiments showed that hypoxic pulmonary hypertension indeed caused the carbonylation of annexin A1 within 2 h (Fig. 3a) and annexin A1 was downregulated within 6 h (Fig. 3b). Further, the treatment of an inhibitor of metal-catalyzed protein carbonylation, deferoxamine, inhibited hypoxic pulmonary hypertension-induced RV hypertrophy [16].

In the RVs of rats subjected to hypoxic PH, annexin A1 is carbonylated and its expression is reduced. This reduced annexin A1 expression should liberate CBF/NF-Y, promoting its DNA binding activity and thus *Gata4* gene transcription (Fig. 4).

We proposed two possible mechanisms by which the signaling pathway described in this study may preferentially occur in the RV compared with the LV. We found that the expression of CBF/NF-Y is higher in the RV compared with the LV (Fig. 5). This may increase the sensitivity of the RV to activate CBF/NF-Y by being liberated from annexin A1 as this protein is degraded. As discussed in the previous section of this chapter, we also found that protein carbonylation mediated by serotonin is more pronounced in the RV than in the LV, indicating that the sensitivity to carbonylation signaling might be higher in the RV. We attributed this finding to the differential expressions of monoamine oxidase between the RV and LV. Thus, this is another possible mechanism through which the activation of *Gata4* gene transcription in response to pressure overload preferentially occurs in the RV.

4 Oxidative Modifications in the RV of the SU5416/Ovalbumin Model of Pulmonary Arterial Hypertension

In rats, the injection of SU5416, an inhibitor of the VEGF receptor, plus some stimuli such as hypoxia and inflammation can trigger pulmonary arterial

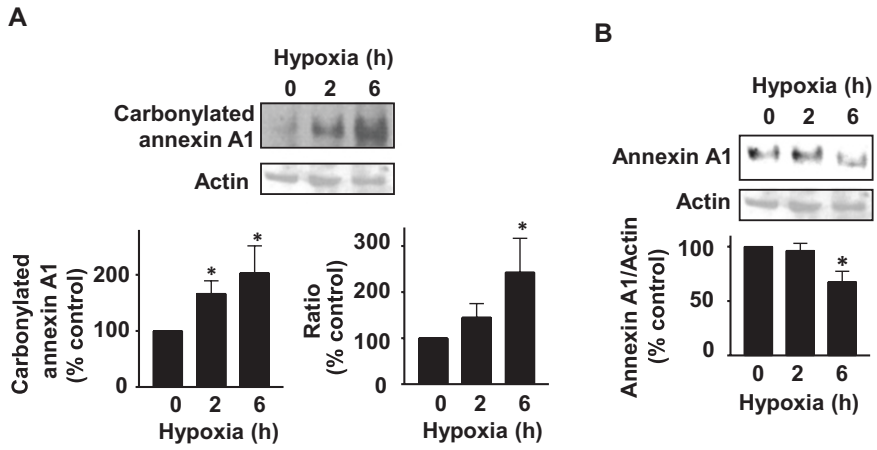

Fig. 3 A trigger of RV hypertrophy promotes annexin A1 carbonylation and degradation. Rats were subjected to hypoxia, and RV homogenates were prepared. (**a**) Carbonylated annexin A1 was monitored by labeling with DNPH, immunoprecipitated with the antibody for DNPH-derivatized proteins, and Western blotting with annexin A1 antibody. (**b**) Annexin A1 protein expression was monitored by Western blotting. Values in *bar graphs* represent means ± SEM. *Asterisks* indicate significant difference from control (Reproduced from [16] with permission)

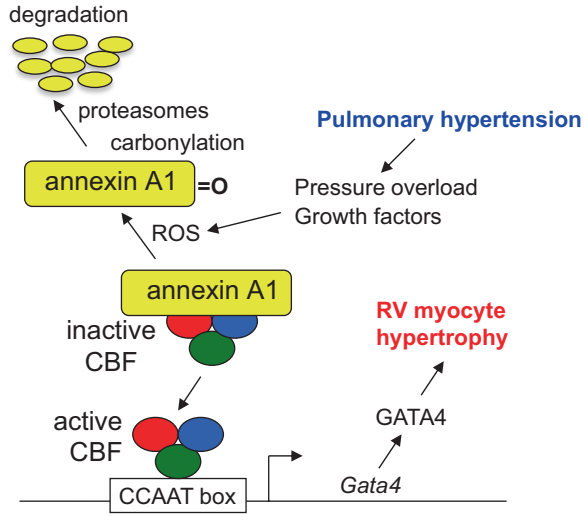

Fig. 4 Proposed mechanism for GATA4 activation in the RV in response to pulmonary hypertension. Pulmonary hypertension exerts pressure overload on the RV, resulting in the generation of ROS, which in turn carbonylate annexin A1 that is bound to the CBF/NF-Y transcription factor. Carbonylated annexin A1 is degraded, resulting in liberated CBF/NF-Y that can bind to the CCAAT box within the *Gata4* promoter. CBF/NF-Y binding enhances the gene transcription of *Gata4* and increases the level of the GATA4 transcription factor, which in turn promotes the gene expression of hypertrophic regulators [16]

hypertension that resembles the human disease [20, 21]. In these models, a severe increase in pulmonary arterial pressure seems to cause right heart failure [22]. The RVs of rats treated with the SU5416 injection and ovalbumin immunization in our laboratory in the same way as described by Mizuno et al. [20] were concentrically hypertrophied and severely fibrotic (collagen volume fraction between 0.15 and 0.20). We found that such RVs are subjected to biological oxidation as

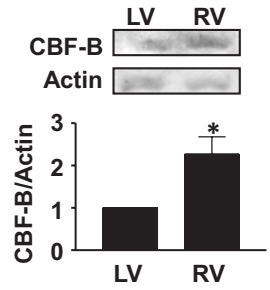

Fig. 5 CBF/NF-Y expression in the LV and RV. CBF-B protein expression was monitored in the LV and RV homogenates of normal rats. *Asterisks* indicate significant difference. (Reproduced from [16] with permission)

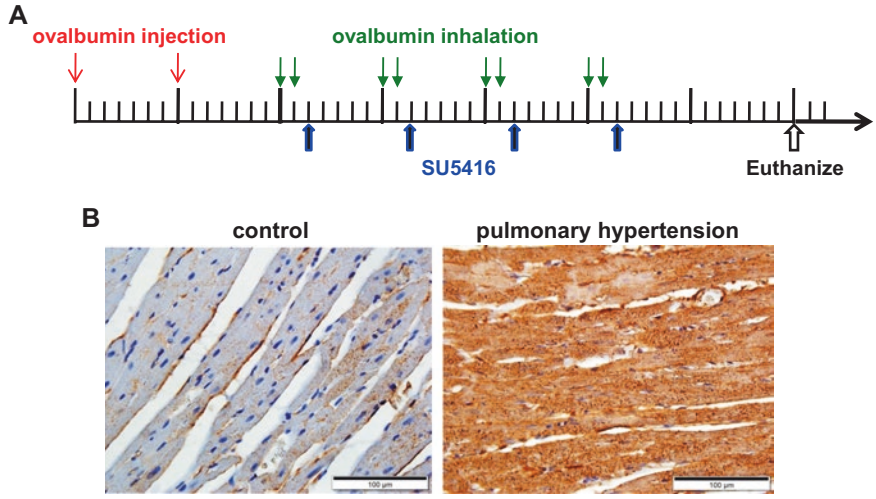

Fig. 6 Increased nitrotyrosine in the RV of rats treated with SU5416 and ovalbumin to promote pulmonary arterial hypertension. Rats were subjected to ovalbumin immunization and SU5416 injection as depicted in Panel **a**. At the end of the treatment, animals were euthanized and the heart tissues were immersed in formalin and embedded in paraffin. Paraffin-embedded tissues were cut and mounted on glass slides. Tissue sections were subjected to immunohistochemistry using the nitrotyrosine antibody (Santa Cruz Biotechnology). Magnification ×400

assessed by monitoring the nitrotyrosine formation by immunohistochemistry (Fig. 6). By using a proteomics approach, Dr. Xinhong Wang in our laboratory identified some of these nitrotyrosinylated proteins to be heat shock protein-90 and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2). Further, Dr. Wang found that S-glutathionylation of heat shock protein-90 and NADH-ubiquinone oxidoreductase was promoted in the RVs of rats with pulmonary hypertension.

Total protein carbonylation as detected by derivatizing the carbonyl groups with DNPH was not found to be altered in the RVs of rats with pulmonary hypertension compared with the controls in this model. Moreover, it was interesting to note that peptides that contain susceptible amino acids to be carbonylated are preferentially decreased in the RVs of pulmonary hypertensive rats compared with the controls. Dr. Earl Stadtman and coworkers defined that arginine, lysine, proline, and threonine (20% of amino acid types) are susceptible amino acids to be carbonylated and converted into glutamic semialdehyde, α-aminoadipic semialdehyde, glutamic semialdehyde, and 2-amino-3-ketobutyric acid, respectively [23].

Table 1 Peptides that are differentially expressed between the RVs of rats with pulmonary arterial hypertension and controls

	Peptide	Fold changes	Total number of amino acids	Number of susceptible amino acids
1	Glu Ile **Lys pro**	0.209	4	2
2	Asp **Lys Lys pro**	0.395	4	3
3	**Lys Arg Thr Thr**	0.458	4	4
4	Phe Gly **Arg Arg**	0.220	4	2
5	Ser Val **Lys Arg**	0.393	4	2
6	**Lys** Trp **Lys**	0.495	3	2
7	**Lys** Tyr Ile Glu	0.364	4	1
8	Ser Leu Leu Ser Phe	0.449	5	0
9	Asp Leu Phe **Arg**	0.412	4	1
10	**Thr Thr** Gly Leu Ile	0.357	5	2
11	**Lys** Tyr **Thr Arg**	0.402	4	3
12	**Arg** Ser **Lys Arg**	0.332	4	3
13	Trp Phe Trp	0.438	3	0
14	Asn **Arg** Phe **Lys**	0.351	4	2
15	His Ile Ile Val	0.323	4	0
16	**Arg Lys Lys** Cys	0.332	4	3
17	Asn **Arg** Phe **Lys**	0.313	4	2
18	Phe Ile Gln **Lys**	0.329	4	1
19	Ala **Arg** Tyr **Arg**	0.379	4	2
20	Ala ala Ile **Lys**	0.210	4	1
21	Glu Phe **pro** Trp	0.426	4	1
22	Phe **Thr Thr Thr**	0.445	4	3
23	Val **Arg** his **Arg**	0.385	4	2
24	Ile Ile Val Tyr	0.456	4	0
25	**Pro** Gln **Arg Thr**	0.336	4	3
26	Phe **Lys Lys**	0.024	4	2
27	**Thr Thr** Gly Leu Ile	0.406	4	2
28	Glu **Lys** ala **Arg**	0.477	4	2

Metabolomics analysis using a ultra-performance liquid chromatography and a quadrupole-time-of-flight mass spectrometer identified molecules differentially expressed between the RVs of rats with pulmonary arterial hypertension and

As shown in Table 1, metabolomics analysis revealed that 28 peptides were significantly modulated at least twofold. All of them were decreased in the RVs of pulmonary hypertensive rats compared with the controls. Notably, Phe-Lys-Lys peptide expression was 42-fold lower in the RVs of rats with pulmonary hypertension. All other peptides were two to five-fold lower. Among these 28 peptides, 24 (85.7%) contained at least one of the carbonylation-susceptible amino acids defined by Dr. Stadtman [23]. These 28 peptides contain 112 amino acids. Among them, 51 amino acids are carbonylation-susceptible amino acids (45.5%). This realization may lead to a potentially important area of research. From these results, one could hypothesize that the carbonylation of specific peptides and subsequent degradation may either mediate or be associated with the development of right heart failure. The latter event may be useful to be used as biomarkers of RV failure.

5 Conclusions and Future Perspectives

The survival of patients with pulmonary hypertension remains low, and right heart failure is the major cause of death among these patients. Drugs designed to treat left heart failure are not effective against right heart failure, and no right heart-specific therapeutic agents are available. Moreover, RV pathophysiology is still relatively under-studied and not well understood. In particular, the research field of the signal transduction biology of the RV is in its infancy. Understanding the molecular signaling mechanisms for the growth and death of RV cardiomyocytes is critical to developing useful therapeutic strategies to treat right heart failure in pulmonary hypertension patients.

Redox signaling has been shown to play important roles in a variety of diseases, and our laboratory has shown that the RV also utilizes redox signaling mechanisms for cell regulation. However, redox regulatory mechanisms are complex, and it is not likely that simply administering antioxidants to globally eliminate ROS will solve health problems.

In the future, we need to first confirm whether the results found in experimental animals can be translated to humans. Once animal models are confirmed to provide useful information, these systems will be useful to study the complex mechanisms of redox signaling. It is likely that different types of oxidative modifications need to be investigated in concert.

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Hypoxia and Local Inflammation in Pulmonary Artery Structure and Function

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1 Introduction

Pulmonary vascular diseases such as pulmonary hypertension (PH) [1–5], represent major healthcare burdens, and further detrimentally impact right ventricular structure and function. Given that the PA operates in a low-pressure environment compared to the systemic vasculature, the mechanisms underlying PH typically differ from that of coronary and systemic vascular disease. However, some common triggering/exacerbating factors such as inflammation [2, 4, 6–8], and common vascular structural components such as endothelium, smooth muscle, and fibroblasts with common influences of disease such as remodeling and fibrosis [4, 6, 8–12] and finally common functional features of enhanced vasoconstriction (with associated impairment of vasodilation) are present between pulmonary and systemic arteries. Thus, it would not be unusual to imagine that triggering or exacerbating factors such as inflammation, known to affect the systemic vasculature, also influence the pulmonary artery (PA) and contribute to PH pathophysiology. Here, a compounding factor (also common to several aspects of systemic vascular disease) is hypoxia. Accordingly, understanding the interactions between hypoxia, inflammation, and pulmonary vascular structure and function becomes important.

2 Hypoxia and Inflammation

Different types of PH differ in etiology, risk factors, and presentation. However, two factors, hypoxia and inflammation, are recognized to be important across multiple groups of PH (as classified by WHO). Hypoxia is certainly a key aspect of Group III PH, and can also contribute to Group IV [13, 14]. Inflammation plays a significant role in PH pathogenesis and exacerbation not only in Group III, but also Group I and IV [15–20]. Hypoxia can influence inflammatory responses in the context of PA structure and function [4, 10, 21]. High-altitude mountain sickness results in increased circulating levels of cytokines [22, 23]. Even in healthy

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individuals, hypoxia increases circulating IL-6 and CRP [24–26]. PH is also associated with inflammatory conditions such as rheumatoid arthritis, lupus, and collagen vascular diseases [20, 27, 28].

While hypoxia can certainly induce systemic inflammation with resultant "indirect" effects on the pulmonary vasculature, what may be particularly relevant in the context of PH is the effect of hypoxia on local inflammation, i.e., within the PA itself. In this regard, understanding the effect of hypoxia on production of pro-inflammatory factors by resident cells of the PA, i.e., endothelial cells (PAECs) and smooth muscle (PASMCs) becomes relevant. There is substantial interest in such locally produced growth factors (e.g., vascular endothelial growth factor) released within the PA in response to hypoxia with autocrine/paracrine effects on PAECs as well as PASMCs [29]. We focus on two such potential factors: brain-derived neurotrophic factor (BDNF) and thymic stromal lymphopoetin (TSLP) that may play such a role in the context of PA contractility and remodeling (Figs. 1 and 2).

3 Brain Derived Neurotrophic Factor

The family of neurotrophins are well known in the nervous system [30, 31], and include nerve growth factor, BDNF, neurotrophin-4, and neurotrophin-3. Neurotrophins are generated intracellular in a pro-form and secreted as such into the extracellular space where they are cleaved by factors such as matrix metalloproteinases, or tissue plasminogen activator into the active form. Neurotrophins act via both high-affinity tropomyosin related kinase (Trk) and low-affinity p75NTR receptors. Functional heterogeneity of neurotrophin action involve selectivity of different neurotrophin ligands for Trk receptors: NGF activates TrkA; both BDNF and NT4 activate TrkB, while NT3 activates TrkC. Full-length Trk receptor expression is required for binding of extracellular ligand and downstream activation of the many signaling cascades. Truncated receptors are expressed intracellularly and may serve to chelate ligand, and thus limit neurotrophin action. All neurotrophins, including the pro-form, activate the low-affinity p75NTR. Neurotrophins can

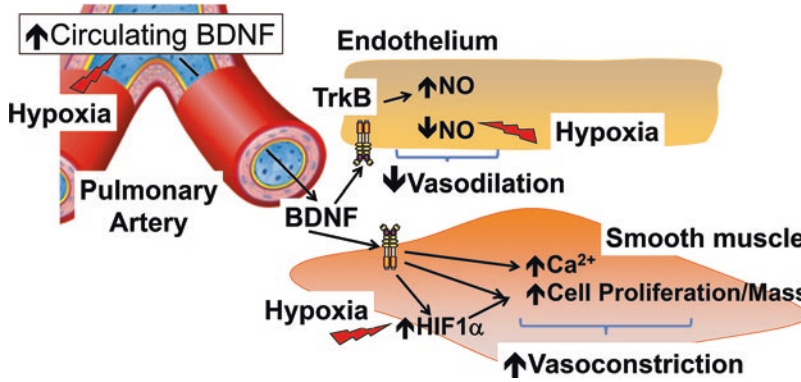

Fig. 1 BDNF expression and effect in pulmonary artery in the context of pulmonary hypertension. BDNF can be derived largely from endothelial cells (PAECs) with autocrine effect of inducing nitric oxide (NO). Thus BDNF could potentially modulate vasodilation. However, BDNF can also influence smooth muscle (PASMCs), acting via its receptor (TrkB). Hypoxia enhances PAEC-derived BDNF, and should increase NO, and thus promote vasodilation. However, hypoxia also enhances BDNF/TrkB signaling in PASMCs with enhanced cell proliferation and calcium responses to agonist (and thus enhanced contractility). Thus, the balance of PAEC vs. PASMC effects of BDNF in hypoxia may be relevant to diseases such as pulmonary hypertension

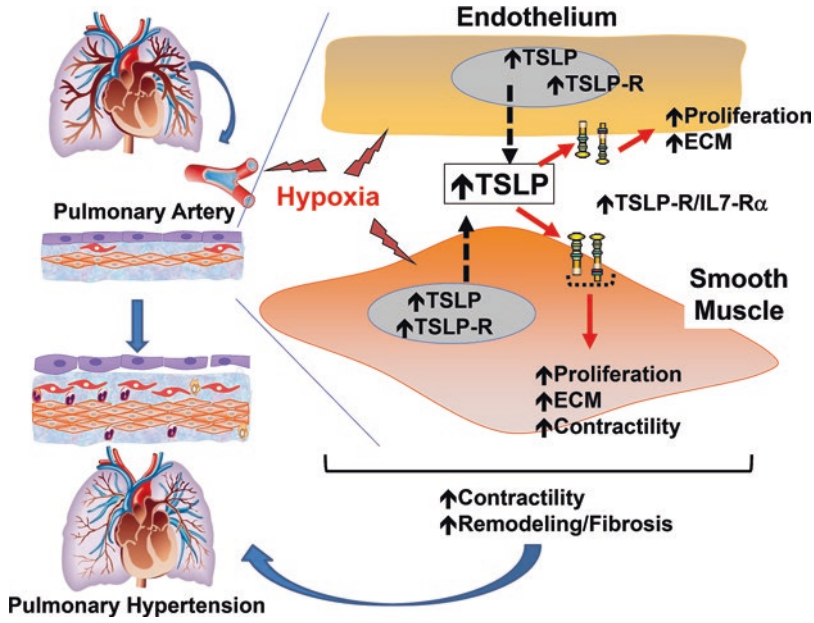

Fig. 2 TSLP expression and effect in pulmonary artery in the context of pulmonary hypertension. TSLP can be derived largely from endothelial cells (PAECs) and smooth muscle (PASMCs), with influence on receptor (TSLPR) expressed by both cell types. Hypoxia enhances PAEC-derived TSLP as well as TSLP-R in both cell types, thus enhancing the potential for TSLP effect. TSLP acts on PASMCs to enhance cell proliferation and calcium responses to agonist (and thus contractility). TSLP may interact with hypoxia by promoting HIF1-a signaling: effects relevant to diseases such as pulmonary hypertension

genomically and non-genomically alter Ca^{2+} as well as cell proliferation, survival and migration [32, 33]. In doing so, neurotrophins activate a multitude of pathways including phospholipase C, PI3 kinase, mitogen-activated protein kinases (MAPK), and NFκB.

There is now increasing evidence that neurotrophins such as BDNF as well as Trks and p75NTR are expressed within the airways and pulmonary vasculature [34–38]. In the airway, where much more is known, BDNF-TrkB effects appear to be important in several aspects relevant to asthma pathophysiology. BDNF is expressed and secreted by both bronchial epithelium and airway smooth muscle cells [36–39]. Baseline BDNF expression is low, but is substantially increased in the presence of inflammation and other stresses, and in ASM of asthmatics [40, 41]. Secretion of pro-BDNF occurs and is enhanced by cytokines, via Ca^{2+} influx pathways such as TRPC3 and Orai1 [42], with secretion involving the classical vesicular transport mechanisms more typical of neurons. Such secreted pro-BDNF is cleaved locally by MMPs (which can also be secreted by airway cells) resulting in active BDNF which can act in an autocrine/paracrine fashion on ASM itself. Indeed, TrkB is highly expressed by smooth muscle [42–44]. BDNF/TrkB interactions result in acute elevation of $[Ca^{2+}]_i$ and potentiation of agonist responses [45], with further enhancement in the presence of cytokines [43]. Furthermore, chronic exposure to BDNF promotes ASM cell proliferation, working largely via TrkB (interestingly with no observable role for p75NTR) and activating a number of cascades relevant to asthma including the MAPKs, PI3/Akt, and NFkB [46]. In mouse models of allergic asthma (e.g., ovalbumin and mixed allergens), BDNF levels are increased within airways, as evidenced by immunostaining and laser-capture microdissection based analysis of mRNA expression within smooth muscle and epithelial layers (Thompson, Pabelick, and Prakash; unpublished results). Importantly,

BDNF potentiates airway hyperresponsiveness in such models (Thompson, Pabelick, and Prakash; unpublished results), making this system relevant to asthma pathophysiology. More recent data suggest that BDNF expression is increased in epithelium of patients with severe asthma [39], while our own data suggest increases in ASM of asthmatics [41, 47, 48].

Emerging data, including from our group, suggest that the BDNF/TrkB system may also be important in the PA. Previous studies reported that the presence of BDNF in the adventitia and intima of human PA [49, 50], thus suggesting a potential local role for endothelium-derived BDNF. In this context, we showed that exogenous BDNF can induce generation of nitric oxide (NO) in human PAECs using the NO-sensitive fluorescent dye diaminofluorescein-2 [51]. Indeed, both BDNF and NT3 in the pM range that is commensurate to circulating levels of ~20 ng/mL acutely and substantially increased NO production, to levels comparable to that induced by 1 μM ACh. BDNF increased phosphorylation of both Akt and eNOS. BDNF-induced NO was blunted by tyrosine kinase inhibitor K252a or by TrkB siRNA, and by inhibiting NOS using N(G)-nitro-L-arginine methyl ester, as well as a Ca^{2+} chelator [51]. Interestingly, unlike in ASM, functional blockade of p75NTR was also effective in preventing BDNF-induced NO in PAECs. In endothelium-intact PA rings, BDNF increased cGMP and induced vasodilation in precontracted arteries, demonstrating a potentially functional role for BDNF in the pulmonary vasculature [51].

The above data did not show whether BDNF is actually produced by PAECs, or how disease states influence it. We recently reported using ELISA that circulating BDNF in healthy humans is increased under high altitude conditions (alveolar PO2 of 100 mmHg) compared to sea level [52]. Limited studies in cerebral arteries suggest that endothelium does produce BDNF [53] and that upregulated production occurs in neurons in response to hypoxia [54]. In neuroblastoma cells [55], TrkB expression is mediated by hypoxia. We recently explored the effect of hypoxia on BDNF secretion and possible autocrine effects in human PAECs [52]. In human PAECs exposed to normoxia vs. <5% hypoxia, ELISA showed increased extracellular BDNF as well as enhanced BDNF mRNA in PAECs [52]. BDNF-induced NO production was significantly higher under hypoxic conditions, an effect also noted with the TrkB agonist 7,8-DHF. In the context of autrocrine function, hypoxia-induced NO was blunted when secreted BDNF was neutralized using a chimeric TrkB-Fc. Interestingly, hypoxia increased iNOS mRNA expression, and BDNF enhancement of NO in hypoxia was blunted by iNOS inhibition. Overall, these limited data underlined the expression and potential autocrine function of PAEC-derived BDNF in the context of hypoxia.

Responsiveness of PASMCs to hypoxia is an important part of normal PA structure and function, as well as to the pathogenesis of diseases such as PH. There are currently limited data on BDNF expression and functionality in PASMCs. We recently reported that exposure of human PASMCs to <5% hypoxia enhances BDNF and TrkB expression, as well as extracellular release of BDNF. Importantly, in arteries of patients with PH, BDNF expression and release was found to be higher at baseline (although the relative roles of PAECs and PASMCs in this population remain to be determined).

In terms of hypoxia, an obvious consideration is the transcription factor hypoxia-inducible factor (HIF-1), that is known to be involved regulating multiple proteins and cellular functions [56, 57]. The human TrkB gene is known to have HIF-1 binding elements [58] although HIF-1 modulation of BDNF as well is not known. In our study on PAECs, we found that hypoxia and BDNF can both induce HIF-1α expression. In PASMCs, hypoxia-induced BDNF increased $[Ca^{2+}]_i$ responses to vasoconstrictors such as serotonin: effects prevented by HIF1α inhibition or by neutralization of extracellular BDNF using chimeric TrkB-Fc [59]. Furthermore, chronic exposure to BDNF increased PASMC survival and proliferation, and decreased apoptosis in the presence of hypoxia. Such effects involved induction of anti-apoptotic proteins and promotion of pro-proliferative pathways [59].

Overall, these limited data demonstrate a link between hypoxia and local neurotrophin production and effects in the PA with downstream effects relevant to PA contractility and remodeling. A potential role for BDNF promotion of the HIF1α pathway is suggested, and conversely, BDNF upregulation of a number of pathways involved in inflammatory signaling and remodeling such as the MAPKs and PI3/Akt. However, much more work is needed to better understand the relative roles of PAECs and PASMCs in the context of hypoxia–BDNF interactions in the PA, and more importantly, how these interactions are altered in different forms or severity of PH.

4 Thymic Stromal Lymphopoietin

TSLP is an IL-7-like cytokine first identified in the thymus as a factor in T and B cell development [60–62], but has been increasingly localized to a variety of non-thymic cell types including epithelial cells of the lung, gut and skin, fibroblasts, and circulating and tissue immune cells, particularly dendritic cells [61, 63, 64]. TSLP acts via a heterodimeric complex of its receptor TSLP-R and of IL7Rα. An important role of TSLP is to interface between environmental triggers and body responses to skew the immune response towards a Th2 phenotype, especially early in response to allergic and other stimuli [65, 66]. This feature has resulted in high interest in TSLP in conditions such as atopic dermatitis, allergic asthma, and noninfectious GI disorders [62, 67–70]. Given the increasing recognition that TSLP is an early inflammatory mediator in other organ systems, the exciting possibility exists that TSLP/TSLP-R signaling is important in development of PH, especially given the potential that TSLP may have pleiotropic effects relevant to PH pathophysiology.

Heterogeneity of TSLP expression and signaling patterns in species-, cell-, and context-dependent fashions is still being established, but may be fundamental to the diversity of roles played by TSLP in different organ systems and tissue beds [66, 71–74]. TSLP production can be induced by a number of factors such as respiratory viruses, bacterial products, dsRNA, by cytokines such as IL-1, TNFα, IL-4, and IL-13, and a variety of allergens. Such induction can involve a multitude of pathways specific to the trigger. However, downstream, TSLP transcription depends on Ca^{2+}, NFAT NFκB, and similar factors. Interestingly, there are three HIF1α binding sites on the human TSLP gene. Accordingly, upstream factors such as hypoxia and inflammation may converge to promote TSLP.

TSLP is known to be produced by airway epithelial cells, particularly in the context of allergic stimulation and asthma [68]. However, we have previously shown that human ASM can also produce TSLP, with enhanced production in response to cigarette smoke exposure [64]. Thus, TSLP produced either by epithelium or ASM could act on ASM. Indeed, ASM-derived TSLP has autocrine effects, acting via TSLPR to enhance $[Ca^{2+}]_i$ responses to agonist stimulation [64] and increase cell proliferation [68, 75–78], but blunts apoptosis [79, 80]. TSLP enhances smooth muscle contractility, particularly in the presence of factors such as inflammation [81]. Furthermore, TSLP promotes ECM formation [82] and can thus promote fibrosis. TSLPR itself has low affinity for TSLP, but the heterodimer of TSLPR/IL-7Rα enhances affinity and triggers signaling largely through the JAK and/or STAT pathways, with STAT1, STAT3, STAT5, and JAK1 and JAK2 being relevant [66, 72–74].

There are currently limited data on TSLP expression or signaling in pulmonary or systemic vasculature. Interestingly, systemic administration of TSLP can slow progression of atherosclerosis in ApoE-deficient mice through augmentation of tolerogenic immune responses [83], i.e., an indirect, chronic effect. In contrast, lifelong genetic inactivation of TSLPR accelerates progression of atherosclerosis via Th17/Treg imbalance in ApoE-deficient mice [84], again an indirect effect via the immune system. Platelets induce endothelial TSLP and promote clot [85, 86], while vasculature of hypercholesteromic patients show increased TSLP immunoreactivity [87] and therefore, if anything, upstream triggers

that contribute to coronary and systemic vascular disease likely directly enhance TSLP in the vasculature. However, there are no studies on the role of local TSLP signaling in endothelium or vascular smooth muscle cells.

Any effects of hypoxia on endothelial TSLP have interesting parallels to BDNF in the PA. In ongoing studies, we have found that TSLP is expressed by both human PAECs and PASMCs, although the PAEC appears to be the major producer of TSLP. In contrast, both PAECs and PASMCs express TSLPR. Interestingly, exposure to hypoxia enhances PAEC (but not PASMC) TSLP levels, while PASMC TSLPR levels are increased by hypoxia. These hypoxia effects involve HIF1α. In the context of function, what is not known is whether TSLP acts on the PAEC to promote NO and result in vasodilation as for BDNF. However, in PASMCs, TSLP appears to parallel BDNF, in promoting $[Ca^{2+}]_i$ responses of PASMCs to serotonin, enhancing cell proliferation, inhibiting apoptosis, and increasing ECM formation. Here, activation of the STAT3 and STAT5 pathways appears to be important. JAKs are involved in such mitogen-induced signaling in human PA [88, 89] ERK and PI3K pathways are also important in PA cell proliferation induced by mitogens [89–91]. These signaling pathways involved in mitogen-induced PA cell proliferation are also involved in TSLP signaling, at least in other nonvascular systems.

Overall, these emerging data point to TSLP as a hypoxia-induced mediator of PA structure and function which may be relevant to PH pathophysiology. Although much information is still to be obtained regarding the role of TSLP in the PA, the similarities between neurotrophins such as BDNF and cytokines such as TSLP in the context of local production of hypoxia mediators in the vasculature is novel. Here, the functional equivalence of BDNF as a cytokine and TSLP as a growth factor is interesting, and underlines the overall idea that the local milieu within the PA may play a key role in PH pathophysiology. In this regard, such factors may be important in maintaining the effects of hypoxia due to their autocrine/paracrine function, even after hypoxia is reversed.

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From Physiological Redox Signalling to Oxidant Stress

Jeremy P.T. Ward

1 Introduction

Oxidant stress and excessive production of reactive oxygen species (ROS) are important contributors to many pathological conditions, including pulmonary hypertension [1–4]. However, numerous studies have reported that antioxidant therapies are ineffective or even detrimental, which in part reflects a lack of understanding of the key roles that oxidant signalling plays in normal cell function [5]. The balance between production of oxidants and their removal by cellular antioxidant mechanisms is a dynamic equilibrium and determines redox state. Although cellular redox state has long been known to affect cell function, it is only relatively recently that the concept of ROS as important physiological signalling molecules has become widely accepted [6, 7].

Apart from their potential to cause pathological changes and sometimes irreversible damage under conditions of oxidant stress, ROS at more physiological levels reversibly affect the function of numerous protein kinases, phosphatases, ion channels, Ca^{2+} handling mechanisms and transcription factors in the systemic and pulmonary vasculatures, most often via thiol switches involving oxidation or (in the presence of NO) S-nitrosylation of protein cysteine and methionine residues [6, 8, 9]. It has also become clear that physiological ROS signalling must be constrained and focussed by spatiotemporal aspects analogous to those for Ca^{2+}, precise control of which are essential for normal cell function [10–12]. When such constraints dysfunction or are overwhelmed, promiscuous and uncoordinated signalling is inevitable, with overt oxidant stress and pathological consequences. This article considers the processes which regulate physiological ROS signalling and how they may become dysregulated in disease, with important implications for development of targeted anti-oxidant therapies.

2 Types and Sources of ROS

We should start by considering the types of ROS and their suitability as signalling mediators compared to their propensity to cause oxidant damage. Any signalling molecule must be produced (and/or removed) by mechanisms that are readily controllable in response to a physiological stimulus, should have (or be constrained to) a specific target or targets, and have effects on such targets that are reversible (except in specific processes such as apoptosis and ubiquitination for protein degradation). Uncontrolled production,

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promiscuous or irreversible effects predicate against a role in normal cell signalling, and if significant are likely to be pathological. Whilst this definition does not exclude pathological consequences of dysfunctional regulation or loss of spatiotemporal constraints, it does limit which ROS and sources of ROS might be considered suitable as part of a physiological signalling pathway.

The progenitor of most ROS is the superoxide radical ($SO^{\bullet-}$), formed by transfer of an electron to the outer shell of molecular oxygen (i.e. reduction) (Fig. 1). Many oxidoreductase and oxygenase enzymes generate $SO^{\bullet-}$, including NADPH oxidases (NOX), constituents of the mitochondrial electron transport chain (ETC), cytochrome p450, lipoxygenases and xanthine oxidase; uncoupled endothelial nitric oxide synthase (eNOS) is also an important source in cardiovascular disease [6, 14]. In the healthy vasculature the predominant sources are NOX and mitochondria, which exhibit significant crosstalk with implications for ROS signalling [6, 14–16].

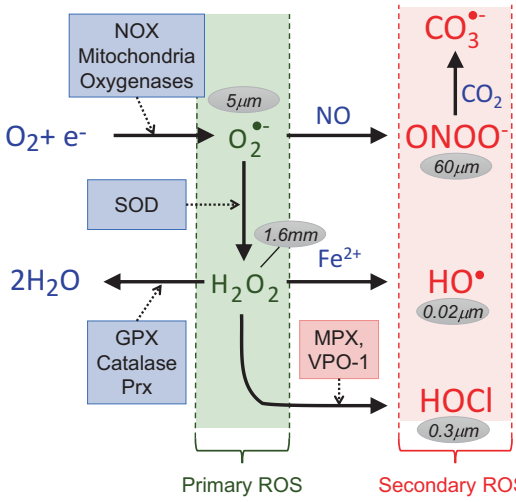

Fig. 1 Origin of reactive oxygen species (ROS) involved in physiological signalling (primary ROS) and oxidant damage or pathophysiological signalling (secondary ROS). The numbers appearing in an *oval* by each species represents estimated diffusion distances for a tenfold decrease in concentration [13], and are shown for comparison of suitability for signalling and reactivity. *GPX* glutathione peroxidase, *MPX* myeloperoxidase, *NO* nitric oxide, *NOX* NADPH oxidase, *Prx* peroxiredoxins, *SOD* superoxide dismutase, *VPO-1* vascular peroxidase 1

As a polar molecule $SO^{\bullet-}$ cannot cross cells membranes except via channels or transporters, and its reactivity and short biological half-life suggest that it could only act as a signalling molecule if source and target are closely approximated. $SO^{\bullet-}$ is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD); H_2O_2 is also generated directly by NOX4, though $SO^{\bullet-}$ may be an intermediate within the enzyme complex. H_2O_2 is more stable and has a longer half-life than $SO^{\bullet-}$; it is also more mobile and can cross membranes relatively easily. It is reduced to water by catalase, glutathione peroxidase (GPX) and peroxiredoxins, but in the presence of ferrous ions can also be converted by the Fenton reaction to the extremely reactive and short lived hydroxyl radical ($HO^{\bullet}$). Other secondary and highly reactive ROS of known biological significance are hypochlorous acid (HOCl), generated by myeloperoxidase and vascular peroxidase 1 (VPO-1) from H_2O_2 and Cl^- [17], and the less reactive peroxynitrite ($ONOO^-$), formed by the extremely rapid and avid combination of $SO^{\bullet-}$ with nitric oxide (NO) [10] (Fig. 1). The latter has important consequences for cardiovascular function apart from any actions of $ONOO^-$, as it reduces NO bioavailability. $ONOO^-$ can also react with CO_2 to form the highly oxidising and promiscuously damaging carbonate radical [13]. It is worth noting that the end products of such ROS cascades tend to be more highly reactive than the original species.

Due to its stability and mobility, H_2O_2 is generally believed to mediate downstream ROS signalling, both within and between cells [10, 14]. Nevertheless, $SO^{\bullet-}$ reacts with haem groups and FeS complexes, and is capable of modifying protein thiols, though via a different reaction to that for H_2O_2; there is significant evidence that it can act directly as a signalling mediator [9, 13, 18]. Interestingly, some studies suggest that $SO^{\bullet-}$ can have differential actions to H_2O_2. For example, a study in SOD knockout mice, where cytosolic $SO^{\bullet-}$ increases but H_2O_2 decreases, reported increased myogenic tone in renal arterioles independent of NO scavenging [19], and in pulmonary arterial smooth muscle cells (PASMC) $SO^{\bullet-}$ has been reported to promote, but H_2O_2 to

inhibit, activation of the transcription factor NFATc3 [20]. Also in pulmonary artery, we have demonstrated that $SO^{\bullet-}$ activates the Rho kinase pathway independently of H_2O_2 [21], whereas H_2O_2 elicits Ca^{2+} mobilisation but does not activate Rho kinase [22].

Whilst both $SO^{\bullet-}$ and H_2O_2 are undoubtedly increased in oxidant stress, it has been proposed that it is primarily the quality (i.e. species) rather than the quantity of ROS that leads to oxidant damage [18]. This is partly based on evidence that whilst primary ROS (i.e. $SO^{\bullet-}$, H_2O_2) may have a role in pathophysiological but still reversible signalling, the more highly reactive secondary ROS ($HO^{\bullet}$, HOCl, and $ONOO^-$), which do not have specific degradation mechanisms akin to SOD, catalase and GPX, tend to have more profound, indiscriminate and irreversible actions. Although this is an intriguing concept, $ONOO^-$, HOCl and even $HO^{\bullet}$ have been implicated in signalling, at least in the pathophysiological context. For example, S-nitrosylation of cysteine residues is an important NO-mediated mechanism for dynamic regulation of a wide variety of proteins, and it has been shown that both H_2O_2 and $SO^{\bullet-}$ can induce S-nitrosylation via $ONOO^-$ [8]. There is also evidence that a novel NOX, H_2O_2, VPO-1 and HOCl pathway contributes to regulation of vascular smooth muscle proliferation and potentially vascular remodelling in hypertension [17]. Furthermore, it has been shown that protein carbonylation mediated by a Fenton reaction (thus $HO^{\bullet}$)-dependent process can act as a potentially important (and reversible) signal transduction mechanism in pulmonary and systemic vascular smooth muscle and airways [23]. In all these examples, it is actually a secondary ROS that acts as a signalling intermediate, though considering the reactivity of $HO^{\bullet}$ and HOCl it must be supposed that their actions are highly localised.

3 Regulation of ROS

The cellular mechanisms involved in regulating ROS production, degradation and reversal of their actions are manifold and complex, with multiple interactions both between these mechanisms and those of other signalling pathways, importantly those involving Ca^{2+} [24]. Whilst SOD, catalase and GPX provide rapid enzymatic mechanisms for degrading ROS, overall cell redox state is controlled by glutathione, the main cellular redox buffer (GSH ⇔ GSSG), and thioredoxins [6, 25]. Thioredoxins and peroxiredoxins (thioredoxin peroxidases) also reduce and detoxify H_2O_2, peroxynitrite and hydroperoxides (ROOH), and reduce proteins by cysteine thiol-disulphide exchange, reversing the disulphide bridge formed by oxidation by ROS [13, 25]. Expression of the above antioxidant mechanisms is controlled via the transcription factor Nrf2, which is retained in the cytosol by binding to the redox sensitive Keap1; oxidation of Keap1 allows Nrf2 to translocate to the nucleus and bind gene promoters containing the antioxidant response element [26].

Physiological control of ROS production is primarily mediated via NOX, the only mechanism whose purpose is to generate ROS [27]; in the pulmonary vasculature the response to hypoxia is a special case (see below) (Fig. 2). The pulmonary vasculature expresses NOX1, NOX2 and NOX4 (and possibly NOX5) [14]; there is little evidence for NOX3 or Duox1/2. The classical isoform NOX2 ($gp91^{phox}$) activates on forming a complex with the cytosolic subunits $p47^{phox}$ and $p67^{phox}$, which translocate to the membrane following protein kinase C (PKC)-mediated phosphorylation of $p47^{phox}$; $p67^{phox}$ itself is activated and tethered to the membrane by the small Rho GTPase Rac1 [14, 31]. NOX1 is similar, but can also utilise the $p47^{phox}$ and $p67^{phox}$ analogues NOXO1 and NOXA1. NOX4 does not require cytosolic subunits and is believed to be constitutively active and regulated primarily via altered expression, though there is some evidence for regulation via Rac1 [14, 27].

Regulation of NOX1 and NOX2 activity is thus primarily under the control of PKC and Rac1, but as both of the latter have multiple and diverse targets this implies there must be compartmentalisation of signal transduction. Activation of PKC depends on the isoform. Conventional PKCs (α and β) are activated by

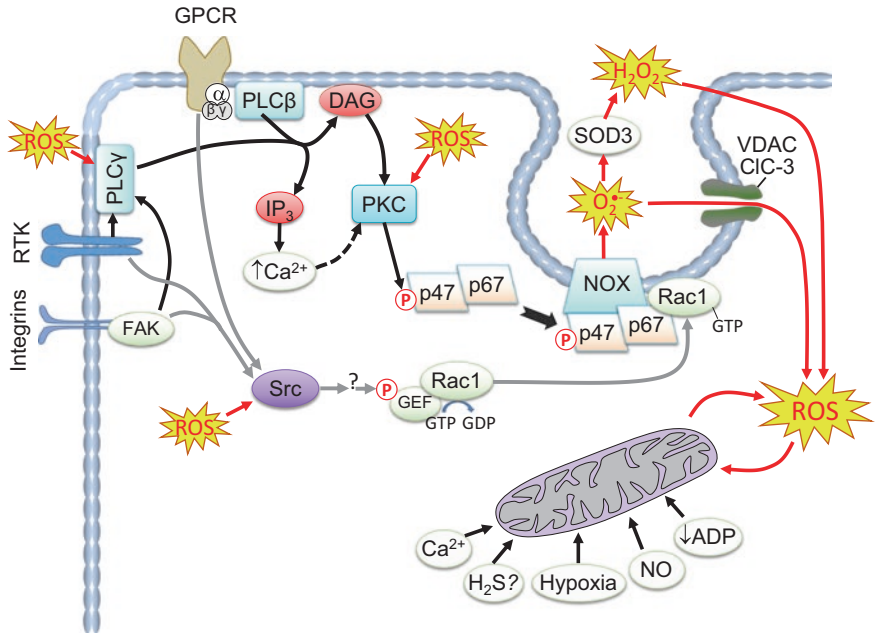

Fig. 2 Simplified scheme for physiological regulation of ROS production. Applies for NOX1 and NOX2, and ignores probable microsignalling domains and compartmentalisation except caveolae. Activation of PLCβ by a GPCR or PLCγ by a RTK or FAK leads to activation of PKC by DAG and Ca^{2+} mobilisation via IP_3; conventional PKCs but not novel isoforms (e.g. PKCε) can also be activated by Ca^{2+}. GPCRs, RTKs and FAK also activate Src, which indirectly leads to phosphorylation of a GEF (possibly VAV) so promoting exchange of GTP for GDP on Rac1; note however that Src is not the only means of regulating Rac1 activity [28]. PKC phosphorylates $p47^{phox}$, initiating translocation of the cytosolic subunit complex to NOX in the membrane, and Rac1 activates and membrane-tethers the activator subunit $P67^{phox}$ ($p22^{phox}$ and $p40^{phox}$ not shown for clarity). $SO^{\bullet-}$ generated by NOX is expelled on the extracellular side, but can enter the cell through anion channels (VDAC, ClC-3) or by diffusion once converted to H_2O_2 by SOD3. Factors that cause the mitochondrial ETC to become more reduced increase ROS generation, including increased cytosolic Ca^{2+}, hypoxia, a fall in ADP as substrate for the ATP synthase, and inhibitors of cytochrome oxidase such as NO and potentially H_2S [29, 30]. The diagram also shows where ROS can activate upstream mechanisms, potentially leading to positive feedback (see text). Although ROS promotes Src activity, this could either involve direct oxidation or inhibition of a phosphatase; conventional and novel PKCs are all ROS-sensitive, but PKCε is particularly so [9]

Ca^{2+} and diacylglycerol (DAG), novel PKCs (γ, δ and ε) by DAG alone, and atypical by neither. Many G-protein coupled receptors (GPCR) activate phospholipase Cβ (PLCβ), whereas various receptor tyrosine kinases (RTK) and focal adhesion kinase (FAK) activate PLCγ; both PLCβ and PLCγ initiate IP_3-mediated Ca^{2+} mobilisation and production of DAG [32–34]. Rac1 becomes active when phosphorylation of a guanine nucleotide exchange factor (GEF) such as VAV facilitates GTP binding in exchange for GDP, and Rac1 translocates to the membrane. Activation of GPCRs, RTKs and FAK can all initiate GEF phosphorylation and thus activation of Rac1; regulation of Rho GTPases such as Rac1 is however complex and can involve several pathways, though in the case of NOX1 activation in vascular smooth muscle the non-receptor tyrosine kinase Src (or other family member) appears to be a key intermediate [28, 35–37]. Consistent with the above, numerous studies have shown that GPCR vasoconstrictor agonists acutely activate NOX and increase ROS generation, and we have demonstrated essential roles for PLC, PKCε, Src and NOX1 [37–39].

Note that all NOX are transmembrane structures, and their orientation is such that $SO^{\bullet-}$ (or H_2O_2 in the case of NOX4) is expelled on the opposite side of the membrane to the cytosolic NADPH binding site on the COOH terminus,

i.e. into the extracellular fluid [27], or membrane-enclosed organelles [40]. However, numerous studies have shown that activation of NOX commonly leads to an elevation of intracellular ROS. Whilst extracellular SOD (SOD3) can dismute $SO^{\bullet-}$ to H_2O_2 which can then diffuse back into the cytosol, $SO^{\bullet-}$ can also pass through anion channels such as ClC-3 and other voltage-gated anion channels (VDAC), a process that may be facilitated by occurring within a constrained area such as caveolae (Fig. 2; and see Compartmentalisation section). ClC-3 and VDACs are expressed in the plasmalemma, mitochondria, endosomes and sarcoplasmic reticulum, all of which have been shown to express NOX isoforms [10, 41–43]. This potentially provides another level of control of cytosolic ROS signalling mediated via membrane potential and expression of VDACs, and also supports the concept of intracellular ROS signalling via redoxosomes (redox-active endosomes), and other organelles including the nuclear envelope [40].

The mitochondrial ETC constitutively generates $SO^{\bullet-}$ from complex 1 and 3, though most enters the matrix and is normally rapidly degraded there by powerful antioxidant mechanisms. Nevertheless, sufficient $SO^{\bullet-}$ exits from complex 3 into the intermembrane space and then cytosol to play a significant role in cell signalling. Mitochondrial ROS production is affected by cytosolic Ca^{2+}, NO and factors that influence oxidation phosphorylation, amongst others [24, 44]. There is also strong evidence that in the pulmonary vasculature hypoxia causes an increase in ROS generation from complex 3 of the ETC, which then act as a cytosolic second messenger to elicit the Ca^{2+} mobilisation and Rho kinase-dependent Ca^{2+} sensitisation that underlies hypoxic pulmonary vasoconstriction (HPV), and also contribute to chronic hypoxia-induced pulmonary hypertension [16, 30, 45, 46].

There is evidence for significant ROS-mediated crosstalk between mitochondria and NOX, leading to the supposition that mitochondria are key players and modulators of oxidant signalling and stress [15]. For example, it has been shown for HPV that mitochondrial-derived ROS activate PKCε and thus generation of ROS by NOX, which then promotes Ca^{2+} entry via L-type channels [16]. Notably, not just PKCε but also Src and PLCγ are activated by ROS [9, 47, 48], and any concomitant elevation in cytosolic Ca^{2+} would also promote mitochondrial and NOX ROS generation [14, 24] (Fig. 2). This arrangement therefore has an inherent and potentially dangerous propensity for positive feedback, with obvious consequences for pathology considering the possibly central role of Src in mediating multiple effects of ROS in pulmonary hypertension [47]. However, significant or at least uncontrolled positive feedback clearly does not occur under physiological conditions, as reflected by the fact that increases in ROS and their sequelae initiated by for example hypoxia or GPCR ligand binding can be rapidly reversed on removal of the stimulus. Whilst ROS-induced ROS release is now thought to be a common mechanism, the lack of apparent positive feedback implies there must be a degree of separation between ROS signalling domains due to regional and/or functional compartmentalisation [49].

4 Compartmentalisation and Microsignalling Domains

The concept of compartmentalisation of Ca^{2+} signalling and signalling microdomains in the vasculature is far from new, but the concept has only relatively recently been extended to ROS and redox signalling, although the distribution of ROS sources and antioxidant systems within the cell is known to be heterogeneous [10, 11, 50]. For example, NOX1 (and possibly NOX2) associates with plasmalemma caveolae (Fig. 2) whereas NOX4 is associated with focal adhesions and mitochondria; both NOX1 and NOX2 have been associated with redoxosomes and endoplasmic reticulum [11, 43, 51, 52]. Localised mitochondrial, sarcolemma and sarcoplasmic reticulum signalling microdomains have long been recognised as key to vascular Ca^{2+} signalling, but are also likely to be both affected by, and contribute to, ROS signalling [10, 50]. This has recently been affirmed in an elegant study in cere-

bral artery, which demonstrated that angiotensin II-elicited activation of L-type Ca^{2+} channels involves a H_2O_2 microsignalling domain involving amplification of ROS from NOX1 by closely approximated sub-sarcolemmal mitochondria [53]. The same group have previously demonstrated membrane clustering of angiotensin II receptors, NOX1, PKC and L-type channels in cerebral arteries [54], which is consistent with the operation of a similar pathway activated by another GPCR agonist in pulmonary and mesenteric arteries [37]. It is notable that although NOX1-derived ROS was able to influence Ca^{2+} entry in pulmonary artery under the latter conditions, it did not activate Rho kinase or Ca^{2+} release from stores [37, 55], although these are activated by hypoxia-induced mitochondrial-derived and exogenous ROS [21, 30].

5 Implications for Pathology

Pulmonary hypertension, whatever the type or experimental model, is associated with altered redox status, increased expression or activity of NOX1, NOX4 and/or xanthine oxidase, and altered expression of antioxidant mechanisms including Nrf2, SOD and thioredoxins [2–4]. It has also been associated with mitochondrial dysfunction, and though there is still discussion as to whether this is a cause or result of the disease process it is clear that such dysfunction will have a significant effect on ROS signalling and redox state [56]. Moreover, hypoxia and most probably other stimuli induce redistribution of mitochondria to the perinuclear region of the cell, a process believed to contribute to redox signalling by enhancing expression of hypoxia-sensitive genes [57]. Whilst initially such changes may be appropriate to the stimulus (i.e. adaptive), as they progress in severity they are likely to disrupt the checks and balances and tight compartmentalisation of physiological ROS signalling that are described above. This could lead to promiscuous signalling, and ultimately initiate uncontrolled positive feedback between ROS generating mechanisms, overload of antioxidant mechanisms and generation of high quantities of damaging secondary ROS, including $HO^{\bullet}$, HOCl, and $ONOO^{-}$. In other words, the condition could become self-perpetuating. Recognition of the importance of physiological ROS signalling and the role of oxidant stress in pulmonary hypertension has stimulated considerable interest in developing targeted antioxidant therapies, mostly directed to the mitochondria or NOX [1, 2]. However the complex interrelationships between the various ROS signalling pathways and their interaction with Ca^{2+} signalling makes this a potentially daunting task.

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Emerging Role of MicroRNAs and Long Noncoding RNAs in Healthy and Diseased Lung

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Abbreviation

Ago2	Argonaute
BMPR-II	Bone morphogenetic protein receptor type II
ceRNA	Competing endogenous RNA
CETPH	Chronic thromboembolic pulmonary hypertension
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribose nucleic acid
EGFR	Epidermal growth factor receptor
Fendrr	lncRNA fetal-lethal noncoding development regulatory RNA
HOTAIR	Hox antisense intergenic RNA
IPF	Idiopathic pulmonary fibrosis
KLF4–5	Krüppel-like factors 4 and 5
linc-MD1	Long intergenic noncoding RNA muscle differentiation 1
lncRNAs	Long noncoding RNAs
LPS	Lipopolysaccharide
MALAT1	Metastasis associated in lung adenocarcinoma transcript 1
MDM3	Transformed mouse 3 T3 cell double minute 3
MEG3	Maternally expressed gene 3
miRNAs	microRNAs
ncRNAs	Noncoding RNAs
NEAT2	Noncoding nuclear-enriched abundant transcript 2
NSCLC	Non-small cell lung cancer
nt	Nucleotides
PAH	Pulmonary arterial hypertension
PANDA	lncRNA p21-associated ncRNA DNA damage-activated
PARP-1	Poly (ADP-ribose) polymerase 1
PASMCs	Pulmonary arterial smooth muscle cells
PH	Pulmonary hypertension
PPARγ	Peroxisome proliferator-activated receptors γ
PRC	Polycomb repressive complex
pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species

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SCAL1	Smoke and cancer-associated lncRNA-1
SCLC	Small cell lung cancer
Sna1	Snail family zinc finger 1
TERRA	Telomeric repeat-containing RNA
trpm3	Transient receptor potentialmelastatin 3
VEGF	Vascular endothelial growth factor
WDR5	WD repeat domain 5
XBP1	X box-binding protein 1

1 Introduction

The pulmonary vasculature differs from the systemic vasculature in that it is thin-walled and operates with low-pressure arteries. Under physiological conditions, the pulmonary circulation carries oxygen-depleted blood away from the right ventricle to the lungs where gaseous exchange occurs between the pulmonary capillaries and air-filled alveolar sacs, and returns oxygenated blood back to the left atrium. Pulmonary hypertension (PH) is a hemodynamic and pathophysiologic state characterized by vasoconstriction and remodeling of the pulmonary vasculature and a rise in right ventricular pressure, resulting in the right heart failure and eventual death [1–4]. The pathogenesis of PH involves multiple vascular and non-vascular cell types and depends on several pathogenic events (i.e., genetic susceptibility, hypoxia, inflammation, viral infection, DNA damage, shear stress, and others) for the disease manifestation and progression [4]. Current medications for PH may target the three vasodilatory pathways including prostacyclin, endothelin, and nitric oxide, with the poorly understood upstream molecular origins of PH and inability to actively reverse pulmonary vascular remodeling [4–6]. The difficulty to fully unravel these molecular complexities has led to clinical challenges in developing new therapies for this disease [4]. There is an urgent need to identify the upstream molecular triggers of the disease and apply those discoveries to clinical benefit.

Recent work has identified the dysregulation of noncoding RNAs (ncRNAs) as integral events that shape the development and progression of PH [4, 7]. Over the past decade, a number of key publications have implicated noncoding RNAs in a wide range of pulmonary vascular processes, spanning pulmonary vascular development, physiology, and disease, which has led to speculation of their utility in diagnosis and prognosis of various diseases [4]. In this chapter, we will discuss the emerging role of ncRNAs in PH as well as other respiratory diseases.

2 ncRNAs

ncRNAs are a category of transcripts without proteins coding potentials. These non-protein-coding sequences increasingly dominate the genomes of multicellular organisms in contrast to protein-coding genes. The amount of ncRNA transcripts is up to 98% of the whole human genome's transcripts [8]. The difference in molecular structure and expressive pattern from other kinds of RNA endows them specific biological functions, although the exact functions of most ncRNAs are not well known. ncRNAs are expressed in a cell/tissue-specific or in a developmental stage-specific manner [9, 10]. Mounting evidence has demonstrated that ncRNAs are involved in the pathogenesis of human diseases such as cancer, respiratory, cardiovascular, developmental, and other ailments [11].

ncRNAs can be divided into infrastructural and regulatory ncRNAs. Constitutively expressed infrastructural ncRNAs include ribosomal, transfer, small nuclear, and small nucleolar RNAs. Regulatory ncRNAs can be classified into microRNAs (miRNAs), Piwi-interacting RNAs, small interfering RNAs, long noncoding RNAs (lncRNAs), promoter-associated RNAs, enhancer RNAs, and circular RNAs [12]. Many ncRNAs with a gene regulatory function have been identified to date and, similarly to proteins, found to act at various steps along the protein biosynthetic process, including transcription, RNA maturation, translation, and protein degradation. These discoveries are fueling a new era in pathophysiology [8].

3 miRNAs

MiRNAs are small/short noncoding double-stranded RNAs and approximately 16–29 nucleotides (nt) long. In 1993, the first miRNA lin-4 gene in *Caenorhabditis elegans* was discovered [13]. In 2000, the second miRNA let-7 was discovered [14], and since then thousands more miRNAs have been identified. To date, around 2000 human miRNAs have been catalogued; these are thought to negatively regulate gene expression via degradation or translational inhibition of their target mRNAs [15, 16]. Current estimates suggest that at least half of all human genes are regulated by miRNAs, leading to diverse effects on multiple cellular processes. Based on genomic location, miRNAs can be classified into (1) intronic miRNA encoded in noncoding transcriptional units, (2) exonic miRNA encoded in noncoding transcriptional units, (3) intronic miRNA encoded in protein-coding transcript units, and (4) exonic miRNAs encoded in protein-coding transcripts [17]. Many miRNAs have tissue-specific and developmental stage-specific patterns of expression [9]. Around half of the known human miRNAs are found in clusters, which contain 2–7 genes [18, 19]. Human miRNAs are located on all chromosomes except the Y chromosome. MiRNAs can localize to P-bodies-organelles that fuse with endolysosomes and be released into the extracellular space and circulation within exosomes [8, 20].

4 Biogenesis of miRNAs

miRNAs are synthesized and processed through a series of cleavage steps (Fig. 1) [2, 21–24]. A primary miRNA (pri-miRNA) transcript is encoded in the cell's DNA, transcribed in the nucleus, usually has several kilobases long, and possesses 5′cap and a poly-A tail, and cleaved in the nucleus by Drosha enzyme to 70 nt hairpin transcript (pre-miRNA). Transported to the cytoplasm by exportin 5 through nuclear pores, and further cleaved by dicer (RNase III enzyme) into around 22 nt double-stranded-transcripts in the cytoplasm. After strand separation by the helicase enzyme, the guide strand of the mature miRNA is loaded with argonaute (Ago2) proteins and incorporated into the RNA-induced silencing complex (RISC). Mature miRNA can target mRNAs via Watson-Crick base pairing, leading to translational repression/activation or degradation of its target mRNAs and repression of protein translation [25].

5 miRNAs in Healthy Lungs

In general, analysis of multiple organs and cells indicates that miRNAs have a dual role as both regulators of development in non-differentiated cells and in the maintenance of homeostasis in differentiated cells [26, 27]. In the respiratory system, their importance in the former is underlined by the wide-spread changes in miRNA expression observed during lung development and lung growth [28, 29]. Our understanding of the role of individual miRNAs during lung development is limited, although profiling studies have shown downregulation of the miR-154 family and the miR-17 to about 92 cluster (miR-17, -18a, -19a, -20a, 19b-1, and -92-1) and increases in the miR-29 family and miR-127 [28, 30]. Blocking the reduction in expression of the miR-17 to about 92 cluster using transgenic mice resulted in abnormal (and lethal) lung development that was characterized by continued proliferation and impaired differentiation of epithelial cells [31].

The centrality of miRNAs in the maintenance of homeostasis in the adult (differentiated) lung is supported by expression studies that demonstrate no change in miRNA expression during the process of lung aging [32]. Examination of the profile of expression in the adult lung shows that this is dominated by a small number of miRNAs, with miR-21 being the most highly expressed at about 30% of the total. Indeed, when added together, it was found that the 10 and 50 highest expressed miRNAs represent approximately 68% and 97% of the total, respectively. Interestingly, although some miRNAs are expressed in a cell-specific manner, many of these highly expressed miRNAs, including let-7 and miR-21, are com-

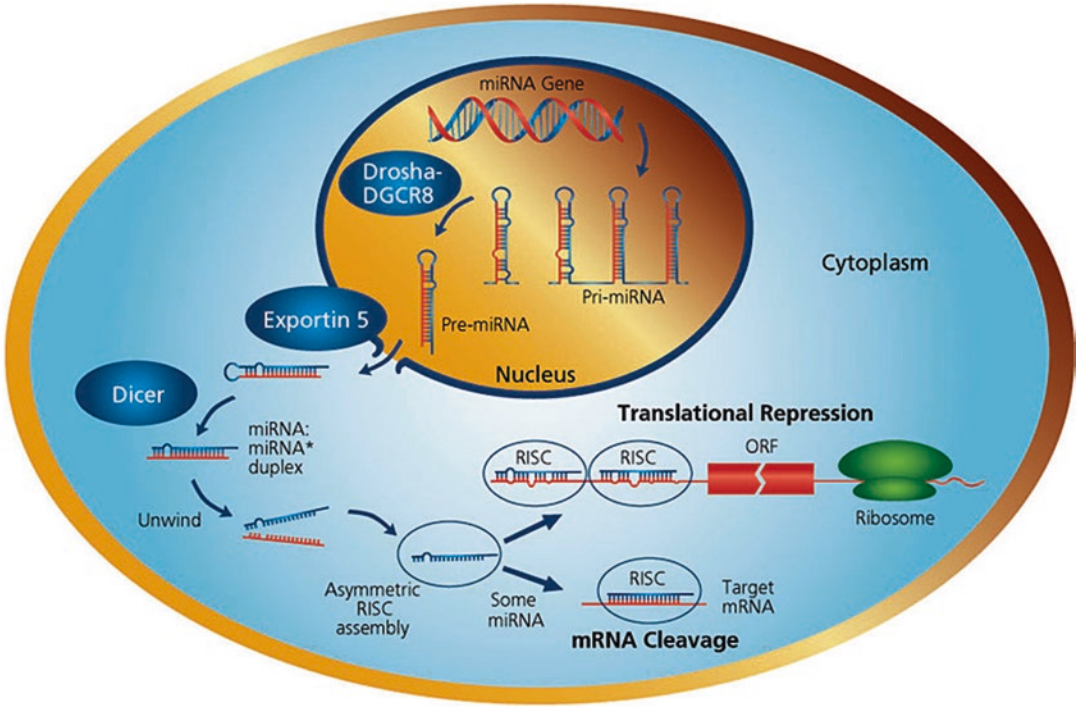

Fig. 1 miRNA biogenesis pathway [2]. *pri-miRNA* primary miRNAs, *pre-miRNA* 70-nt hairpin transcript, *RISC* RNA-induced silencing complex

mon among all differentiated cells/tissues (miRNA expression in specific cells and tissues can be found at http://www.mirbase.org). This has led to speculation that they regulate common cellular phenotypes such as proliferation and apoptosis, and that the changes in their expressions are associated with cancer. Attempts to elucidate the role of miRNAs have primarily involved the measurement of their differential expression in cells/tissues obtained from patients with the relevant respiratory disease, whereas subsequent functional and mechanistic studies have involved overexpression and inhibition of these miRNAs in cell and animal models [33].

6 miRNAs in PH

miR-204 was first shown to be a mechanistic link between miRNA dysregulation and signaling pathways involved in the pathogenesis of PH. The miR-204 gene is located intronic region of the transient receptor potentialmelastatin 3 (trpm3). The decreased expression levels of miR-204 were found in total lung and plexiform lesions from patients with PH, as well as animal PH models (chronic hypoxia mouse model and monocrotaline rat model). The expression level of miR-204 also found correlated with PAH severity. In addition, in vivo study showed that intratracheal nebulization of miR-204 by administration with miR-204 mimics decreased the pulmonary artery pressure, right ventricular wall thickness and reduced medial hypertrophy of pulmonary arteries in the monocrotaline-induced rat PH model [34, 35]. These findings suggest miR-204 is important in the pathogenesis of PH and can be served as a potential clinical biomarker of PH disease.

MiR-21 is a very extensive studied miRNA in human diseases and has been found to be upregulated in many pathological conditions including cancer and cardiovascular diseases [36]. The complex role of miR-21 in the development of PH has been highlighted by conflicting results, both in in vitro cell culture and in vivo animal PH

models. The variation among different studies may due to the different experimental conditions such as animal strain and species difference, gender difference, age, different PH models and the different pharmacological manipulation strategies of miR-21 in vivo [37–41]. The true potential of miR-21 in the development of PH deserves further research attention.

MiR-17-92 cluster is one of the best-characterized miRNA families located on human chromosome 13 and consists of six distinct mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1, each of which has a specific set of target genes that exert their functions [42]. MiR-17-92 cluster expression is upregulated in PH animal models and patients. Antagomir of miR-17, decreased expression levels of miR-17-92 cluster and SMC-specific knockout of miR-17-92 can attenuate chronic hypoxia induced PH in mice [2, 26, 43, 44].

MiR-130/301, miR-145, miR-190, miR-29b, miR-125, miR-210, miR-138, and miR-25 are upregulated in PH pulmonary arterial smooth muscle cells and endothelial cells [45]. These miRs regulate the following pathways that are relevant to PAH pathophysiology: PPARγ [46], BMPR-II [47], KLF4–5 [48], voltage gated potassium channels [49], and mitochondrial calcium uniporter [45].

An additional screening on deregulated miRNAs in circulating macrovesicles of PAH patients and rat models found that the down modulation of miR-150 correlate with the severity and prognosis of PAH [26, 50].

7 miRNAs in Lung Cancer

miRNAs are involved in many pathways related to lung cancer. ErbB2 (HER2/Neu), ErbB3 and ErbB4 belong to the family of receptor tyrosine kinases. These kinases initiate signal cascades leading to DNA synthesis and cell proliferation, and their overexpression is often critically involved in tumorigenesis and cancer cell proliferation [51]. The epidermal growth factor receptor (EGFR)/ErbB1 regulates cell proliferation, apoptosis, angiogenesis and tumor invasion in non-small cell lung carcinoma [52]. Recent investigations have demonstrated that miRNAs are likely to be involved in the regulation of EGFR in lung cancer. An inhibitor of miR-128b resulted in upregulated EGFR expression in an EGFR-expressing NSCLC cell line, and treatment with a miR-128b mimic resulted in a concomitant reduction of EGFR expression [53]. Inhibition of miR-21 was recently shown to enhance the anti-apoptotic potential of an anti-EGFR tyrosine kinase inhibitor in an EGFR-mutant lung adenocarcinoma cell line [54].

The proto-oncogene RAS is the central molecule of the RAS-Raf-MAPK cascade which is the downstream cytoplasmic effector of the growth factor receptors. Approximately 20% of tumors in general and 30% of lung tumors have activating mutations in one of the RAS genes [55]. One of the downstream effectors of the RAS signaling pathway is the MYC oncogene. MYC amplification and overexpression have been detected in different histologic subtypes of lung cancer [56]. Both RAS and MYC are targets of the let-7/miR-98 family of miRNAs. Johnson et al. showed that members of the let-7 family of miRNAs bind to the 3′UTR of RAS, downregulating RAS expression in human cells [57]. It is seen that let-7 is poorly expressed in lung cancer compared to normal lung tissue, and that the expression of let-7 is inversely correlated with the expression of RAS in lung tumor samples [58]. miR-29 is downregulated in a number of human cancers including lung cancers, and its ability to repress Mcl-1 coupled with its role in regulating epigenetic DNA methylation means that enhancing expression of this regulatory element could be an effective therapeutic strategy [59–62].

Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis during tumor development. VEGF and its receptor (VEGFR) have been primary targets of therapies designed to target pathological angiogenic signaling. Liu et al. have investigated the involvement of miR-126 in the regulation of angiogenic processes in a lung cancer model [63]. They found decreased expression of miR-126 and increased expression of VEGF-A in various lung cancer cell lines and showed that

introduction of miR-126 using a lentiviral vector could down-regulate the expression of VEGF-A and inhibit growth. Inactivation of tumor suppressor genes plays important role in lung carcinogenesis.

The p53 tumor suppressor gene, located on chromosome 17p, is affected in 60–75% of lung cancer including both NSCLC and SCLC while Rb is more likely inactivated in SCLC [64]. In lung cancer cells, induction of miR-34 results in apoptosis and miRNA profiling shows that the expression of miR-34a, miR-34b, and miR-34c are directly correlated with expression of the p53 tumor suppressor [65–67], suggesting that miR-34 is involved in regulating apoptosis as a regulatory target of p53. Evidence is emerging that tumor suppressors are likely to be regulated by miRNA activity. FUS1/TUSC2 is a tumor suppressor gene located on 3p21.3 that has been shown to be negatively regulated by the activities of miR-197, miR-93, miR-98, and miR-378 [68, 69]. Reduced or complete loss of Fus1 expression was found in 82% and 100% of non-small cell and small cell lung cancer cell lines respectively [70], and elevated levels of miR-93 and miR-197 have been shown to correlate with reduced Fus1 expression in NSCLC tumor specimens. Another important tumor suppressor gene is LKB1, whose loss-of-function mutation/deletion is observed in 30% lung adenocarcinomas and 20% of squamous cell l carcinomas [71, 72]. An investigation of miR-126 in the regulation of invasive potential in lung cancer revealed an inverse relationship between Crk and miR-126 expression in squamous cell tumors [73]. MiR-21 also targets PDCD4, a pro-apoptotic gene that inhibits tumorigenesis and whose downregulation has been linked to poor survival in colon and lung cancer patients [74–76]. The let-7 family has been shown to inhibit the expression of several oncogenes including RAS, MYC, and HMGA2 [77–79]. Several studies have implicated the important regulatory function of the miR-17-92 cluster of miRNAs as an actual oncogene in mediating the pathologic tumor cell proliferation in various tissues including the breast and lung [80, 81].

8 miRNAs in Other Respiratory Diseases

COPD present as the reduction of airflow due to abnormalities in the small airways compartment and associated infiltration of inflammatory cells in the alveolar wall and ultimately damage of the lung parenchymal and epithelium [26]. A study found five deregulated circulating miRNAs in serum of COPD patients, four of them downregulated (miR-20, miR-28-3p, miR-100, and miR-34c-5p) and one upregulated (miR-7) in comparison to healthy individuals [82]. Finally, similar attempts to apply miRNA expression as a diagnostic tool suggest the possibility of distinguishing COPD patients from lung cancer patients by measuring their miRNA expression pattern [83, 84].

Asthma is a common long-term inflammatory disease of the airways of the lungs triggered by genetic and environmental factors. Several aspects of asthma have been linked to the deregulation of miRNA. Of particular interest is the involvement of miRNA in the regulation of immune response and inflammation by targeting several immune receptors and cytokines [26].

Cystic fibrosis (CF) is a monogenic lung disease [85]. The first work describing miRNAs involvement in CF was published by Oglesby et al. In this work miR-126 has been shown to target TOM 1 protein a known regulator of endosomal trafficking of ubiquinated proteins [86], and is also a negative regulator of 1 L-1Beta, TNF-alpha and LPS signaling pathway [87]. miR126 which is highly expressed in the lungs is down-modulated in the epithelium of CF patients. This leads to increased expression of TOM 1and decreased NF-KB induced cytokines such as IL-8 [88]. miR-101 and miR-494 are elevated in CF patients and their alteration has been linked to other changes in proinflammatory cytokines, cell cycle progression and carcinogenesis [26, 89–91].

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive fibrotic intestinal pneumonia [92]. Evaluation of levels of miRNAs in RNA samples from biopsies of IPF patient with varying speeds of disease progression [93] revealed

down regulation of several miRNAs compared to normal tissue. And specific miRNAs can provide predictive clues about the progression rate of the disease [26, 94].

9 miRNAs as Biomarkers in Respiratory Diseases

In addition to their role in disease, there is now a growing literature to suggest that miRNAs obtained from either blood, tissue, or exhaled breath may provide reliable biomarkers for the diagnosis and prognosis of respiratory disease. A recent comparison of exhaled condensates showed consistent reduction in miRNA expression in asthmatic patients (miR-1248, let-7a, miR-155, miR-21, miR-328, and miR-133a) and patients with COPD (miR-21 and miR-328) compared with control subjects [95]. Examination of circulating exosomes, which are vesicular structures released into the blood stream by cells, has also demonstrated differential expression of miRNAs, including let-7a and miR-21, in mild asthma and increased expression of muscle-specific miRNA species (miR-1, miR-133, miR-206, and miR-499) in patients with COPD that was suggestive of muscle breakdown [96, 97]. As may be expected, there is a considerable body of biomarker work in NSCLC [98, 99], including reports showing that tissue and plasma miRNA signatures can be correlated with prognosis and disease classification [100]. As a specific example, laser capture from bronchial brushings was able to identify two miRNAs that could discriminate between small cell lung cancer and NSCLC (miR-29a and miR-375) and adenocarcinoma and squamous cell carcinoma (miR-205 and miR-34a) [101].

10 lncRNAs

lncRNAs are defined as all transcribed RNA molecules greater than 200 nt in length with no potential to encode for functional proteins of more than 30 amino acids, which separate them from miRNAs and from protein-coding genes [102]. Since 1980s to1990s, individual lncRNAs Xist and H19 were discovered through traditional gene mapping approaches. So far, more than 100,000 lncRNA genes have been defined in human genome due to the development of next generation sequencing techniques. In comparison to 20,345 annotated protein-coding genes found in the human genome, lncRNA genes have a dominant role in mammalian genome [103]. lncRNAs can be classified into five subtypes based on their genomic location: (1) sense lncRNAs (when they overlap one or more exons of another transcript on the same strand), (2) antisense lncRNAs (when they overlap one or more exons of another transcript on the opposite strand), (3) bidirectional lncRNAs (when their expression and that of a neighboring coding transcript on the opposite strand are initiated in close genomic proximity), (4) intronic lncRNAs (when they are derived from an intron of a second transcript) and (5) intergenic lncRNAs, when found as an independent unit within the genomic interval between two genes [8].

11 Biogenesis of lncRNAs

Biogenesis of lncRNAs is quite complicated. In general, lncRNAs can be transcribed from intergenic regions, promoter regions or be interleaved, overlapping or antisense to annotated protein-coding genes and display remarkable similarity to classical mRNA in that they are generally translated by RNA polymerase II. Following RNA polymerase production, the individual lncRNA is subjected to 5′-capped (m7G), alternative splicing, RNA editing, and 3′-polyadenylated. Final lncRNA developments essentially involve the formation of a stable secondary (and tertiary) structure, which confer the individual lncRNA with its unique function roles. In most cases, they lack any biochemical distinction from mRNAs besides the absence of translated open reading frame [103, 104]. There are other more general features of lncRNAs that distinguish them from mRNAs, including their shorter length, having fewer but longer exons, and being expressed at relatively low levels with poor primary sequence conservation.

In general, lncRNAs can be distributed into nucleus and cytoplasm. In nucleus, lncRNAs guide chromatin modifiers such as DNA or histone methyltransferases and polycomb repressive complex (PRC) 2 to specific genomic loci. In cytoplasm, lncRNAs modulate gene expression either positively or negatively at the translational level by binding to targeted mRNAs or act as competing endogenous RNAs that sequester miRNAs and, thus, prevent them from effecting translational repression of their targets [8, 105, 106]. Based on their molecular mechanisms of action, lncRNAs can be classified into four main categories as follows [8, 107].

1. Signal lncRNAs: lncRNAs are expressed in cell type-, tissue-, developmental stage or disease state-specific manner and respond to diverse stimuli, suggesting that lncRNA expression is under considerable transcriptional control. Thus, lncRNA can serve as molecular signals due to the temporal and spatial restriction of their transcription to integrate developmental cues and interpret cellular context or as a response to specific stimuli. lncRNA-RoR, Air (antisense Igf2r RNA), and Kcnq1ot1 (potassium voltage-gated channel subfamily Q member 1 overlapping transcript 1) are examples [8, 107, 108].
2. Decoy lncRNAs: The central role of lncRNAs is regulating transcription both positively and negatively via diverse mechanisms, a major one of which is to act molecular decoys. A decoy lncRNA may bind and then titrate away a protein, sponging up transcription factors and chromatin modifiers to produce broad changes in the transcriptome. NEAT2, lncRNA p21-associated ncRNA DNA damage-activated (PANDA) are examples [8, 109, 110].
3. Guide lncRNAs: They act as molecular chaperons, localizing ribonucleoproteins to specific chromatin targets. This activity can guide changes in the gene expression of neighboring (cis) or distantly located (trans) genes that cannot be easily predicted by just the lncRNA sequence itself. Hox antisense intergenic RNA (HOTAIR), lateral mesoderm-specific lncRNA fetal-lethal noncoding development regulatory RNA (Fendrr), and lncRNA-p21 are examples [111–114].
4. Scaffold lncRNAs: They have multiple domains, enabling them to bind distinct proteins to form complexes with functions such as transcriptional activation or repression. Thus, the lncRNA serves as an adaptor to form the functional protein complex. The lncRNA HOTAIR and telomeric repeat-containing RNA (TERRA) are examples [111, 115].

12 Biological Functions of lncRNA

Thousands of eukaryotic lncRNAs have been identified, with many found to be species specific but less conserved than protein-coding genes. Moreover, the expression profile of lncRNAs seems to be more cell-type specific than that of protein-coding genes and changes with differentiation and the developmental stage of an organism. Accumulating evidence suggests that lncRNAs play a significant role in a wide variety of important biological processes including regulating gene transcription, splicing, translation, cell cycle and apoptosis, cell differentiation, stem cell pluripotency and reprogramming, and heat shock response [103, 116]. For example, the muscle-specific lncRNA (long intergenic noncoding RNA muscle differentiation 1) (linc-MD1) is involved in muscle cell differentiation, acting as a competing endogenous RNA in mouse and human myoblasts [117, 118]. Kcnq1ot1, Air, and HOTAIR promote the formation of repressive chromatin across large areas of the genome by recruiting epigenetic enzymes [8, 119, 120]. NcRNA-a7 was found to induce transcriptional activation of snail family zinc finger 1 (Sna1) [8]. HOTTIP promotes the transcription of homeotic genes through the binding of WDR5 (WD repeat domain 5)/mixed-lineage leukemia [8, 121].

13 Role of lncRNAs in PH

In lung tissue from rats following hypoxia-mediated induction of PH, microarray analysis and qRT-PCR target validation revealed 362 lncRNAs that were differentially expressed in hypoxic animals compared to normoxic controls, among which 86 were upregulated and 276 were downregulated with fold-changes ≥2.0 [122]. A microarray study using endothelial tissues from the pulmonary arteries of chronic thromboembolic pulmonary hypertension (CETPH) patients and healthy controls has identified 185 differentially expressed lncRNAs observed in the CTEPH tissues compared with healthy controls. Gene ontology and pathway analysis suggested that these lncRNAs might play a role in the regulation of the inflammatory response, responses to endogenous stimuli and antigen processing and presentation [123]. In addition, RNA-sequencing analysis indicates the expression of lncRNAs in human heart failure, which may be a primary cause of death in patients with PH [124]. There are 105 differentially expressed lncRNAs in heart failure heart compared with donor hearts. Based on a case-control study with a total of 587 PH patients and 736 healthy controls in southern Chinese, a recent study reported that rs619586A to G in MALAT1 could directly upregulate X box-binding protein 1 (XBP1) expression via functioning as the competing endogenous RNA (ceRNA) for miR-214, and consequentially inhibiting the vascular endothelial cells proliferation and migration in vitro by shortening S–M phase transition [125]. The expression profiles of lncRNAs in animal models and human tissues show that lncRNAs may participate in the pathogenesis of PH.

14 Contributions of lncRNAs in Other Respiratory Diseases

Emerging evidence suggests that changes in lncRNA expression are associated with the development of various types of cancer [102, 107, 126]. It is, therefore, unsurprising that, as with miRNAs, most studies of the role of lncRNAs in respiratory disease have been performed in NSCLC. Indeed, one of the first lncRNAs was discovered in a screen of genes associated with lung adenocarcinoma and was named metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) [127]. Interestingly, this large noncoding transcript (>8 kb) is expressed at high levels in most cells, and initial mechanistic studies indicated that the nuclear-localized MALAT1 regulated alternative splicing through an interaction with serine/arginine splicing factors [109, 128]. However, more recent examinations of knockdown in cell and animal models have shown that MALAT1 regulates gene expression and is not splicing; moreover, those genes are specifically involved in cell migration, colony formation, and metastasis [129–131]. Two well-characterized and highly expressed lncRNAs whose levels are also increased in NSCLC are HOTAIR and H19 [132, 133]. HOTAIR, which is known to regulate chromatin structure through an interaction with the polycomb response complex 2, has been shown to upregulate cell migration and anchorage-independent cell growth [132]. Interestingly, a possible interaction between lncRNA and miRNAs has been uncovered from studies showing that the increased expression of the paternally imprinted H19 lncRNA may act as a sponge for let-7, thereby explaining the downregulation of this miRNA in NSCLC [134]. Expression changes in four additional lncRNAs have also been linked to NSCLC and/or COPD, including smoke and cancer-associated lncRNA-1 (SCAL1), GAS6-antisense 1, maternally expressed gene 3 (MEG3), and lncRNA low expression in tumor [135–138]. SCAL1, whose expression is driven by nuclear factor erythroid 2-related factor, is increased in NSCLC and following exposure to cigarette smoke in vivo and in vitro [135]. A protective role for this lncRNA was indicated in studies that demonstrated increased cigarette smoke-induced toxicity following SCAL1 knockdown [135]. In contrast, MEG3 expression was reduced in NSCLC, and overexpression of MEG3 increased MDM3 and p53 expression, resulting in increased proliferation and reduced apoptosis [137]. Interestingly,

we have also shown reduced expression of MEG3 in the circulating CD8+ T cells of patients with severe asthma, as well as the differential expression of an additional 18 lncRNAs [139].

15 ROS and ncRNA in PH

Reactive oxygen species (ROS) are unavoidable products of aerobic metabolism and act as signaling molecules in a variety of physiologic conditions including cell proliferation and apoptosis [140]. Increasing data demonstrate a reciprocal connection between ROS signaling and microRNA pathway, resulting in diverse biological effects, especially in cancer cells. To date, miR-27a, miR-20a, miR-181a, miR-205, miR-1, miR-21, miR-24, miR-25, miR-185, and miR-214 are found as ROS-responsive microRNAs. And miR-133, miR-145 and miR-495 can modulate ROS [140, 141].

In PH, DNA damage-dependent activation of poly (ADP-ribose) polymerase 1 (PARP-1) was responsible for enhanced pulmonary arterial smooth muscle cells (PASMCs) proliferation and suppressed apoptosis [142, 143], and miR-223 downregulation results in increased PARP-1 expression in PASMCs [144]. Indeed, ROS have been shown to activate PARP-1, and the increased DNA damage seen in PH patients is associated with increased oxidative stress [143, 145, 146]. Recent studies have demonstrated that DNA damage can alter the expression profile of miRNAs and that miRNAs are involved in the DNA damage response [146]. Hereby, complex relationships among ROS, PARP-1, and miRNAs together activate signaling pathways that promote proliferation and suppressed apoptosis and so contribute to the development of PH.

16 Conclusion

There is increasing evidence that the expression of miRNAs is altered in the lungs of patients with a broad range of respiratory diseases. Whether these are simply a reflection of the disease or an important mediator of the underlying pathology has yet to be determined. However, knockdown studies in animal models of PH, asthma and IPF suggest that miRNAs are important in the etiology of many diseases and may offer potential therapeutic targets using approaches such as antisense oligonucleotides and miRNA mimics. Although not related to respiratory disease, the targeting of the liver-specific miR-122 using an antisense-based approach is currently undergoing clinical trials for the treatment of the hepatitis C virus [147]. Given the accessibility of the lungs to topical administration, miRNA inhibitors and mimics may also be of use in tackling respiratory diseases. In contrast to miRNAs, much less is known regarding the role of the many thousands of ncRNAs, and this is likely to provide an exciting and fertile new area of scientific discovery. However, given that the development of therapeutic modulation of ncRNAs may span a medium to long prospect (5–10 years), it is likely that their short-term applications will be as biomarkers in various disease classifications and/or the assessment of novel drugs.

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Techniques for Detecting Reactive Oxygen Species in Pulmonary Vasculature Redox Signaling

Yi Xu and Steven Qian

1 Redox Signaling and Reactive Oxygen Species in Pulmonary Vasculature

A large body of evidence suggested that redox signaling play important roles in regulating pulmonary vasculature function, during which various reactive oxygen species (ROS) are involved [1–15]. As important cellular signaling molecules, the ROS, e.g., superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), lipid peroxyl radical ($LOO^{\bullet}$), and peroxynitrite ($ONOO^-$), could be generated from a variety of sources in cells including NADPH oxidase, uncoupled endothelial nitric oxide synthase, xanthine oxidase, cyclooxygenase, lipoxygenase, cytochrome P450, and the mitochondrial respiratory chain [1, 2, 11–15].

Aberrant redox signaling, e.g., overproduction of reactive oxygen species that exceeds the capability of cellular antioxidant mechanisms, has been found to alter normal vasculature function and remodel blood vessel structure, thus contributes to pathological processes of pulmonary vasculature [1–15]. In fact, increase of ROS and related enzyme expression levels have been commonly found in pulmonary arterial hypoxia and hypertension models, and the mechanism by which ROS influence on pulmonary vasculature function could vary in many aspects [1–15]. For example, evidence showed that hypoxic exposure led to an increase of mitochondria-derived ROS, which further triggered a rise of intracellular Ca^{2+} level and caused pulmonary vasoconstriction, thereby resulting in an increase in pulmonary vascular resistance [3–6]; production of ROS such as $O_2^{\bullet -}$ and H_2O_2 were found to contribute to blood vessel remodeling by affecting pulmonary smooth muscular cell proliferation and apoptosis [7, 8]; the excessive production of $O_2^{\bullet -}$ also led to a decrease of levels of nitric oxide and prostacyclin, two major vasodilating factors, which consequently induced endothelial dysfunction [9, 10]. Therefore, the regulation of pulmonary vasculature via redox signaling, especially through the production of ROS, is a very complicated process with various biological events involved, and technical advance in the study of ROS will result in a better understanding on how to maintain normal pulmonary vasculature functions under oxidative stress.

Although substantial evidence suggested that redox signaling is tightly implicated in the physiological and pathological processes of pulmonary vasculature, the specific effect of individual ROS and the underlying mechanism

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still remain unclear. This is mainly because of the extremely short lifetime of ROS, which makes it very difficult to monitor the free radicals and investigate their bioactivity. Therefore, developing specific and sensitive methods to detect ROS in complex biological system is essential for us to advance our knowledge in pulmonary vasculature regulation.

Here we introduced and summarized commonly used techniques for the detection of ROS in vitro and in vivo, including chemiluminescence-based assay, fluorescence-based assay, cytochrome c reduction, genetically encoded fluorescent probes, as well as ESR/spin trapping technique which has great potential and requires additional attention in future ROS study. Their individual advantages and limitations as well as recent technical advance were also discussed.

2 Techniques for Detecting Reactive Oxygen Species in Pulmonary Vasculature

2.1 Chemiluminescence Assay

Chemiluminescence-based assay is a commonly used method for the detection of cellular production of ROS. During a chemiluminescence assay, the ROS in cell or tissue samples are allowed to react with certain chemiluminescent probes, such as lucigenin, luminol, and diogenes, to produce oxidative end products, which could lead to emission of light that can be measured and quantified by a luminometer [16–25]. For instance, during lucigenin-enhanced chemiluminescence assay, ROS such as $O_2^{\bullet-}$ could firstly reduce lucigenin (LUC^{2+}) to lucigenin cation radical ($LUC^{\bullet+}$), then a second $O_2^{\bullet-}$ turns $LUC^{\bullet+}$ to lucigenin dioxetane ($LUCO_2$) which further breakdown to N-methylacridinium followed by emission of a photon (Scheme 1) [16, 24, 25]. The production of light could thus be monitored and quantified as a measurement of the $O_2^{\bullet-}$ level in the system.

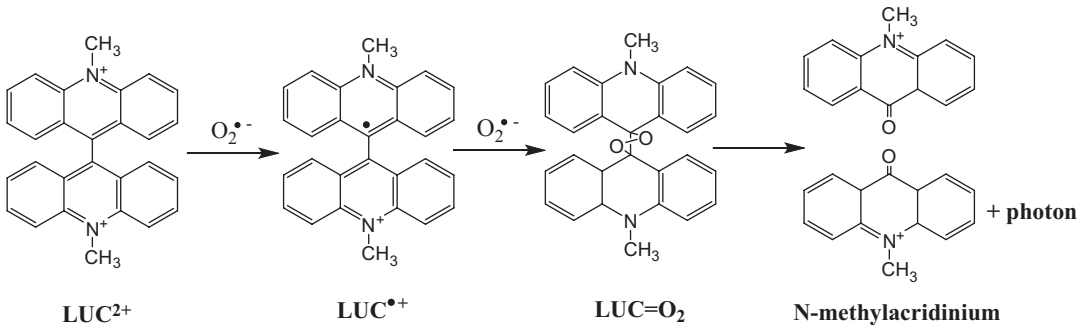
Among all of the chemiluminescent probes, lucigenin is the most commonly used probe for ROS detection in pulmonary vasculature study due to its specificity to superoxide. For example, to investigate the contribution of ROS in altered pulmonary vascular responses in piglets with pulmonary hypertension, Fike et al. employed lucigenin-enhanced chemiluminescence to detect and quantify production of ROS in dissected piglet pulmonary arteries with chronic hypoxia-induced hypertension [18]; Liu et al. used lucigenin-enhanced chemiluminescence to measure $O_2^{\bullet-}$ levels in isolated mouse intrapulmonary arteries [19]; Tickner et al. also used lucigenin chemiluminescence to measure the $O_2^{\bullet-}$ production in homogenized mouse lung tissue, in which inhibitors for various $O_2^{\bullet-}$-producing enzymes was employed to identify the enzymatic sources of $O_2^{\bullet-}$ [20].

Although it has been widely applied in various studies for ROS detection, lucigenin-enhanced chemiluminescence also suffers from a critical problem. During the reaction between lucigenin and $O_2^{\bullet-}$, the intermediate lucigenin radical $LUC^{\bullet+}$ could also react with oxygen molecule to produce additional $O_2^{\bullet-}$ [16, 17]. This side reaction leads to artificial overestimation of the superoxide in the system, making the results of this assay problematic.

In addition to lucigenin, there are also other chemiluminescent probes such as luminol, diogenes, and cypridina luciferin analog, which can react with a variety of ROS, including $O_2^{\bullet-}$, H_2O_2, hydroxyl radical and peroxynitrite [16, 17, 21–23]. By comparing their various reaction specificities, one may choose suitable probes for different detection purposes. In addition, instead of using a single probe in the experiment, Yamazaki et al. showed that combination of two chemiluminescent probes, e.g., luminol and diogenes, could work together synergistically to detect ROS in suspended neutrophils with a higher sensitivity [22].

2.2 Fluorescence-Based Methods for ROS Detection

During a fluorescence-based assay for ROS detection, the cell or tissue samples are incubated with various fluorescence probes to produce fluorescent end products, which could be detected and quantified by fluorescent microscopy or plate



Scheme 1 Reaction between lucigenin and $O_2^{\bullet-}$ during lucigenin-enhanced chemiluminescence assay

reader. There are many types of fluorescence probes with specificities towards different types of ROS as well as particular subcellular locations. For example, dihydroethidium (DHE) is commonly used for the detection of cellular production of $O_2^{\bullet-}$ because it can react with $O_2^{\bullet-}$ to form a specific fluorescent adduct 2-hydroxyethidium, which can be detected at 605 nm when excited at 518 nm [7, 17, 26, 27]. Dichlorodihydrofluorescein diacetate (DCFH-DA) is a commonly used fluorescence probe for detecting intracellular ROS due to its ability to rapidly diffuse across cell membranes. Once uptaken into cells, DCFH-DA can be hydrolyzed by intracellular esterase to form dichlorodihydrofluorescein (DCFH), which is retained inside of cells and oxidized by the intracellular ROS, e.g., H_2O_2, $O_2^{\bullet-}$, $ONOO^-$, and lipid hydroperoxides, to produce a fluorescent product dichlorofluorescein DCF [7, 17, 20, 28, 29]. Dihydrorhodamine (DHR) is another commonly used fluorescence probe for detection of ROS, especially peroxide and $ONOO^-$. It can be oxidized by ROS to produce a two-electron oxidized fluorescent product, rhodamine, which can be monitored by fluorescence spectroscopy with excitation and emission wavelengths of 500 and 536 nm, respectively [17, 30, 31]. Another commonly used fluorescence-based method is Amplex Red assay, which is a specific and sensitive method for detecting extracellular ROS, especially H_2O_2. During the assay, Amplex Red (*N*-acetyl-3, 7-dihydroxyphenoxazine) will be oxidized by H_2O_2 to form resorufin at the presence of horseradish peroxidase. When excited at 530 nm, the fluorescence of resorufin could be detected at 590 nm as a measurement of H_2O_2 level [17, 32–34].

By choosing proper type of fluorescence probes, researchers can selectively detect the production of various ROS in cells. In fact, fluorescence probes have been widely used to detect ROS production in dissected lungs in animal studies [7, 20, 29]. For example, Nijmeh et al. employed DHE and DCFH-DA method to detect and quantify the ROS level in mouse lung with ischemia induced by pulmonary artery ligation, and results showed that left pulmonary artery ligation led to transient ROS upregulation which promoted ischemia-induced angiogenesis [29]; Wedgwood et al. employed DHE and DCFH-DA to quantify ROS level in fetal pulmonary arterial smooth muscle cells (FPASMCs) in order to investigate the contribution of ROS in the proliferation of FPASMCs [7]; Tickner et al. measured ROS generation in the mouse lung tissues in situ via DHE fluorescence in order to investigate the effect of Nox2-derived ROS in PPARγ signaling and cell-cycle progression [20]. All these studies suggested that fluorescence-based assay is a powerful tool for ROS detection in the study of pulmonary vasculature.

However, there are also many limitations for fluorescence-based ROS detection. During DHE assay, DHE can undergo nonspecific redox reactions and produce another fluorescent product ethidium, whose fluorescent spectra overlap with that of 2-hydroxyethidium, therefore interfering the experimental outcome [17, 26, 35]. In the DCFH-DA method, the redox reaction of DCFH and DCF can form a DCF radical, which could

then react with oxygen to produce $O_2^{\bullet-}$, thereby leading to an overestimation of ROS level. In addition, oxidation of DCFH by ROS is not a direct reaction, it requires peroxidase activity, transition metals, and heme enzymes, hence changes in these cellular conditions could affect the experimental outcome independent of ROS levels [16, 17, 36]. In DHR assay, the oxidation of DHR by $ONOO^-$ is mediated by an intermediate DHR radical, however, the DHR radical can be reduced by reductant in cellular system, thereby leading to an artificial underestimate of the $ONOO^-$ production [17, 31]. These limitations must be taken into consideration during experiment design and data interpretation when fluorescence-based assay is used for ROS detection.

In order to circumvent abovementioned drawbacks, many efforts have been made to improve fluorescence-based ROS detection. For instance, to avoid the interference from nonspecific redox reaction in DHE-based method, Dikalov et al. and other groups employed HPLC analysis to separate the specific fluorescent adduct 2-hydroxyethidium from the nonspecific byproduct ethidium [16, 17, 26, 35]. This adapted DHE/HPLC method thus became a specific method for the detection of cellular $O_2^{\bullet-}$ level. In addition, DHE was also chemically modified to offer specificity to detect ROS in specific subcellular location. For example, mitoSOX, an analog of DHE, was developed with preference to accumulate in mitochondria, thus became a relatively specific probe to selectively detect mitochondria superoxide [16, 17, 35, 37].

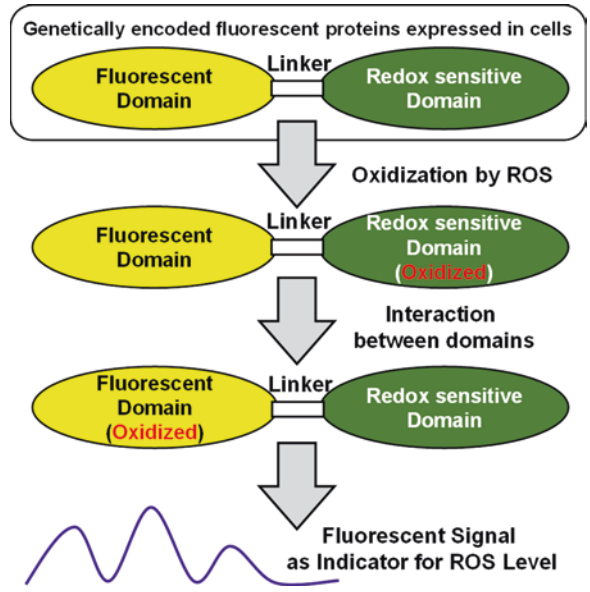
2.3 Cytochrome c Reduction

Cytochrome c reduction assay represents a classic method for detection of extracellular $O_2^{\bullet-}$ [16, 17, 38–44]. During the assay, cytochrome c-Fe^{3+} is reduced to cytochrome c-Fe^{2+} by receiving an electron from $O_2^{\bullet-}$, which can be observed by monitoring the changes in its spectrophotometric absorbance at 550 nm. At the meantime, the absorption at neighboring wavelength 540 and 560 nm remains same which could serve as isosbestic points. However, cytochrome c-Fe^{3+} can also be reduced by electrons donated from other enzymes and molecules in the cellular environment, which could interfere with the experiment outcome. To overcome this problem, the experiment must be performed in the presence and absence of superoxide dismutase (SOD), and the amount of $O_2^{\bullet-}$ should thus be calculated by comparing their difference, e.g., the SOD-inhibitable signals. The SOD/cytochrome c reduction method represents one of the most reliable method for specific detection of extracellular $O_2^{\bullet-}$ release, which has been applied in the study of pulmonary vasculature. For example, to investigate the contribution of bicarbonate-dependent superoxide release in pulmonary artery tone, Nozik-Grayck et al. used the SOD-inhibitable reduction of cytochrome c method to detect the extracellular $O_2^{\bullet-}$ in isolated rat pulmonary artery segments [44].

Although cytochrome c reduction is a well-recognized method for ROS detection, its relatively poor sensitivity may limit its application in the study of pulmonary vessels where the production of ROS is very low [16]. In addition, the presence of NO, $ONOO^-$, and peroxynitrous acid (ONOOH) in the system could oxidize cytochrome c-Fe^{2+} to cytochrome c-Fe^{3+}, thereby leading to underestimation of $O_2^{\bullet-}$ production [45].

2.4 Genetically Encoded Fluorescent Protein as Redox Indicators

In recent studies, genetically encoded fluorescent probes have been developed in which a fluorescent protein was linked to a ROS sensing protein to detect ROS inside living cells (Scheme 2) [17, 46–50]. For instance, Belousov et al. genetically inserted circularly permuted yellow fluorescent protein (cpYFP) into the regulatory domain of OxyR, a prokaryotic H_2O_2-sensing protein, to create a sensitive genetically encoded fluorescent probe, e.g., HyPer. The Hyper was found to be able to detect H_2O_2 production at the single cell level in the cytoplasm and mitochondria of HeLa cells [46]. Later on, Amit



Scheme 2 General principle for genetically encoded fluorescent protein as redox indicators [46–49]. The proteins containing a fluorescence domain and a redox sensitive domain are genetically encoded and expressed in cells. When the redox sensitive domain is oxidized by ROS, the fluorescence domain will undergo structural change or be oxidized by redox sensitive domain, thereby generating fluorescence signal

S et al. employed pHyPer-dMito (a modified version of HyPer which could exclusively target to mitochondrial inner membrane) to detect mitochondrial intermembrane space ROS generation in isolated pulmonary arterial smooth muscle (PASMCs) [47]. Gregory et al. linked enhanced cyan (CFP) and yellow (YFP) fluorescent protein motifs to the redox-dependent regulatory domain of bacterial heat shock protein HSP-33 to create a redox-sensitive fluorescence resonance energy transfer probe. After expressed in PASMCs, the oxidation of the thiols by ROS in HSP-33 domain led to a structural change in the optical coupling of CFP and YFP and a change in fluorescence signal, thus resulting in a sensitive, real-time assessment of changes in redox conditions in the cytosol [48]. Gutscher et al. incorporated a redox-sensitive green fluorescent protein roGFP2 into Orp1 protein, a seleno-independent member of the glutathione peroxidase family, to form orp1-roGFP2 fusion protein. The fusion protein could thus serve as a probe for H_2O_2 detection because H_2O_2-oxidized Orp1 could accept an electron from cysteine residues on the roGFP2, which then emits fluorescence [49]. Gregory et al. also directly expressed roGFP2 in different compartments of PASMCs, including cytosol, mitochondrial matrix and mitochondrial intermembrane space, in order to assess oxidant signaling in distinct intracellular compartments [50]. The genetically encoded fluorescent proteins have been demonstrated to result in specific, sensitive, and real-time measurement of intracellular ROS under physiologically relevant conditions, thus becoming a powerful tool in the study of pulmonary vasculature redox signaling.

2.5 Electron Spin Resonance

Electron spin resonance (ESR), or electron paramagnetic resonance (EPR), is a technique that detects the signals responding to the transition of unpaired electrons between different energy levels. When provided with certain value of energy from a magnetic cavity, the unpaired electrons in a compound of interest, e.g., a free radical species, will move between two energy states, and the ESR could monitor the absorption of energy and convert it into a spectrum [51, 52]. This characteristic makes ESR analysis a unique and conventional technique for the detection of free radical species.

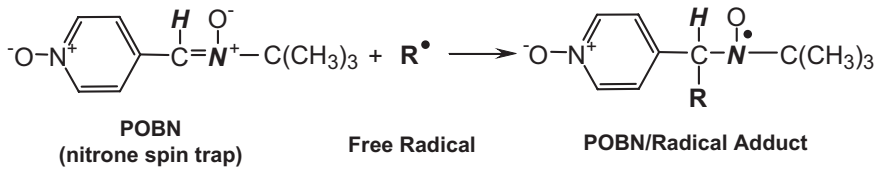
However, due to the high reactivity and short lifetimes in biological systems, the free radical species are usually undetectable by direct ESR analysis with only a few exceptions, such as ascorbyl,

tocopheroxyl and semiquinone radicals [51–53]. To overcome this shortcoming, spin probes, e.g., cyclic hydroxylamines, have been developed and applied to assist the ESR detection of free radical species [16, 17, 54–57]. These spin probes can be oxidized by free radical species to become ESR-active form with longer lifetime. For example, l-hydroxy-3-carboxy-2, 2, 5, 5-tetramethylpyrrolidine (CPH), a commonly used cyclic hydroxylamine spin probe, can be oxidized by superoxide to become a radical form $CP^{\bullet}$ and give ESR active signal [56]. The cyclic hydroxylamines spin probes can react with ROS with high rate constants, thus can be applied at low concentrations and result in high detection sensitivity and efficiency in ESR analysis [54, 55]. This characteristic makes cyclic hydroxylamines suitable for detecting ROS in complex biological system, including pulmonary vasculature. For instance, using ESR/spin probe technique, Weissmann et al. have made the effort to detect reactive oxygen species, mainly superoxide, in isolated rabbit and mouse lungs [56]. In this study, the isolated and ventilated rabbit and mouse lungs were perfused with CPH as a spin probe, which was oxidized by ROS to form ESR-active $CP^{\bullet}$ radicals for the following ESR analysis. Using this method, the researchers demonstrated that chemical-induced pulmonary vasoconstriction is caused by superoxide generated from NADPH oxidases. In a more recent study, ESR analysis was also used to detect and quantify the superoxide levels in isolated mouse pulmonary arterial smooth muscle cells using another cyclic hydroxylamine spin probe 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) [57]. Using this method, the authors demonstrated that the productionn of ROS is involved in pulmonary vascular remodeling during pulmonary hypertension. These studies suggested that perfusing isolated lungs from animal model with spin probes is a practical way for the detection of intravascular ROS release by ESR analysis.

In addition to spin probes, a spin-trapping technique was developed in the early 1970s, in which the primary free radicals are allowed to react with spin-trapping agents to form radical spin-trapping adducts prior to direct ESR detection [58–62]. The spin trapped adducts are newly formed radicals but are much more stable with longer lifetime that could thus be detected by ESR analysis. For example, POBN (α-[4-pyridyl 1-oxide]-*N*-tert-butyl nitrone) is a widely used spin trap agent that can react with various carbon-centered radicals to form POBN/radical adducts (Scheme 3) [63–71]. Sato et al. used ESR and POBN spin trapping to detect free radicals formed during lung injury caused by *Pseudomonas aeruginosa* and lipopolysaccharide in mouse and rat model [72, 73]; Arimoto et al. also employed ESR/spin trapping to detect radical production during diesel exhaust particles and lipopolysaccharide-induced lung injury [74]. In addition to POBN, there are also many other types of spin trap agents, mainly nitrone or nitroso compounds, including PBN (α-phenyl-tert-butylnitrone), MNP (2-methyl-2-nitrosopropane), TMPO (3,3,5,5-tetramethylpyrroline-N-oxide) and DMPO (5,5-dimethyl-1-pyrroline N-oxide) etc. They have also been widely used to detect free radicals generated during in physiological and pathological conditions [58–62, 72–76].

Although the ESR/spin trapping technique has become a common and powerful tool for the study of free radical species, it also suffers from some critical limitations. First of all, the ESR spectrum of spin trapping adducts lacks of specificity which makes unambiguous identification of primary free radicals impossible. This is because after adding spin traps, the ESR signals will be originated from the radical/spin trap adducts, instead of the primary free radicals. As long as the same spin trap is used, the ESR spectrum will be nearly identical independent of the type of primary free radicals. Secondly, the reaction rate between spin traps and free radicals are usually very low, and thus a high concentration of spin trap agents is required in order to generate ESR detectable signals, which limited the application of spin trap in biological study. In addition, the spin traps may undergo background reaction with various components in complex biological system, which interferes with the experiment outcome.

To overcome the limitation of nonspecificity of ESR-spin trap technique, Qian's group made a breakthrough in combining ESR/spin trapping



Scheme 3 Reaction between POBN (a nitrone spin trap reagent) with free radical species to form a spin trapping adduct for ESR analysis

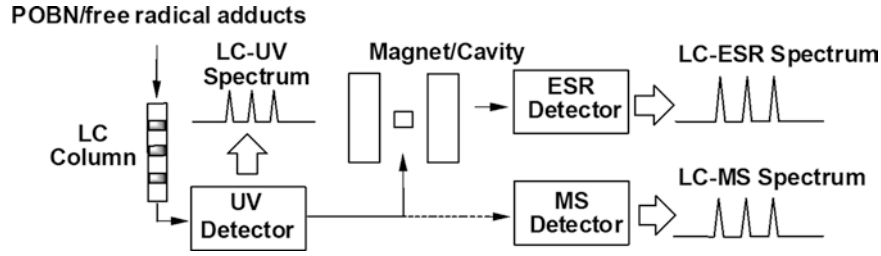
technique with HPLC and MS analysis in which individual free radical species are trapped with POBN, separated in HPLC column according to their distinct chromatographic behaviors, followed by ESR and MS detection to obtain detailed structural information from ESR spectrum and MS fragmentation (Scheme 4) [63–71]. Using the refined ESR-spin trapping/HPLC/MS combined technique, various free radical species produced from cyclooxygenase-catalyzed lipid peroxidation have been successfully detected and identified [70, 71, 77]. Furthermore, those lipid-derived radical by-products were recently found to play important roles in inhibiting cancer growth, including colon cancer and pancreatic cancer [77–80]. These studies suggested that the ESR-spin trapping/HPLC/MS combined technique has a huge potential to be employed for detecting free radical species in various disease models, including the pulmonary vasculature dysfunction.

In addition to ESR/spin trapping analysis, an immuno-spin trapping technique has also been developed in which protein or DNA-centered radicals are trapped by DMPO and allowed to react with anti-DMPO antibody followed by biological measurement such as western blots and immunofluorescence. This method does not require expensive ESR equipment, and provides unique specificity for the in situ and real time detection of protein or DNA-centered radicals in biological studies [81–86]. Recently, the immuno-spin trapping technique has been applied in the study of various disease models including ischemia–reperfusion injury, asthma, diabetes, obesity as well as endotoxin-induced experimental acute respiratory distress [87–93]. Therefore, the immuno-spin trapping technique shows a great potential for the study of redox signaling in physiological and pathological conditions.

3 Summary

Redox signaling and the production of ROS play important roles in regulating pulmonary vasculature constriction, structural remodeling, and cell proliferation during various physiological and pathological processes. The development and improvement of state-of-the-art techniques for ROS detection thus provide us with powerful tools for investigating the implications of redox signaling in pulmonary vasculature disorders. Thus, it is very important for us to understand the principles, individual advantages as well as limitations of various ROS detection methods in order to select proper methods for the study of specific ROS of our interest.

In this chapter, we review currently commonly used methods and techniques for the detection of ROS produced in vitro and in vivo, including chemiluminescence-based and fluorescence-based assay, cytochrome c reduction, genetically encoded fluorescent probes, and ESR/spin trapping technique. As demanded for the study in complex biological systems, many of these methods have been modified to more sensitive and target-specific versions, making them more powerful tools in the study of redox signaling in pathological conditions, including pulmonary vasculature dysfunction. In fact, many of these methods have been successfully applied in the study of redox signaling in pulmonary vasculature as we discussed above, which greatly advanced our knowledge in this field. However, more attention still need to be paid on those advanced techniques with great potential in ROS study, such as



Scheme 4 ESR-spin trapping, LC/ESR/MS combination technique for free radical detection

genetically encoded fluorescent probes and ESR/spin trapping technique. Continuous improvement of these techniques on their sensitivity and species/location-specificity is essential, as it will lead to a more comprehensive understanding on the diverse and complex functions of ROS as important signaling molecules in living system.

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Mitochondrial and Metabolic Drivers of Pulmonary Vascular Endothelial Dysfunction in Pulmonary Hypertension

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1 Introduction

The pulmonary vascular endothelium comprises a single layer of endothelial cells (ECs) on the inner layer of the vessel wall and controls a variety of vessel functions. These ECs participate in the regulation of vascular tone and barrier, leukocyte trafficking, blood coagulation, nutrient and electrolyte uptake, and neovascularization of hypoxic tissue [1]. Pulmonary hypertension (PH) describes a heterogeneous and deadly set of vascular disease conditions, defined by increased pulmonary arterial pressure and lung vasculopathy, triggered by varied and often disparate stimuli [2]. Although PH has been a historically neglected disease, its significance and documented prevalence worldwide are growing due to improvement in awareness and diagnostic capabilities for detection [3]. Current treatment modalities of PH focus primarily on vasomotor tone and limited efficacy or specificity on other cellular processes [4]. At the molecular level, while many cell types within and outside the pulmonary arterial wall are crucial to PH development, endothelial dysfunction is thought to be a major contributor to overall pathogenesis. Yet endothelial dysfunction in PH remains incompletely defined and is thought to be marked by both altered apoptosis and proliferation via a precisely regulated spatiotemporal molecular orchestration [5]. These processes are tightly linked to the development of oxidative stress, inflammatory response, and adverse remodeling of the pulmonary vasculature and are recognized as some of the initiating events of PH under various genetic and exogenous stresses [2]. Importantly, alterations in endothelial mitochondrial and metabolic functions in the pulmonary vasculature are emerging as prominent regulators of endothelial dysfunction and consequently of PH pathogenesis. In contrast to numerous cell types, endothelium has historically been thought to function relatively independent of the mitochondrial pathway of energy supply, and synthesis of ATP in endothelium occurs mainly via a glycolytic pathway [6]. However, as evident from recent studies, mitochondrial modulation of free radicals, calcium homeostasis, and iron–sulfur clusters in endothelial cells can control responses to inflammation, oxidative stress, and apoptotic stimulus. This chapter reviews the roles of mitochondria in pulmonary ECs, with a particular

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focus on how these organelles can modulate endothelial dysfunction in PH. We propose that the study and in-depth understanding of pulmonary EC metabolism may offer powerful therapeutic targets for the next generation of drugs designed to reverse or prevent PH.

2 Mitochondria in Endothelial Metabolism and Energy Reserve

Mitochondria are essential to cellular energy production in all higher organisms adapted to an oxygen-containing environment. Yet the endothelium is not considered to be a major energy-requiring tissue. In comparison with other cell types with higher energy requirements, mitochondria content in ECs is modest at best. In rat ECs, for example, mitochondria compose 2–6% of the cell volume as opposed to 28% in hepatocytes and 32% in cardiac myocytes [7, 8]. Despite their close proximity to oxygenated blood, ECs rely mainly on anaerobic glycolysis instead of mitochondrial oxidative metabolism for adenosine triphosphate (ATP) production. In fact, under physiological conditions, ECs produce over 75% of their ATP from the conversion of glucose to lactate. Less than 1% of glucose-derived pyruvate enters the tricarboxylic acid cycle (TCA) and hence the low mitochondrial electron transport chain (ETC) respiration dedicated for oxidative metabolism [9]. Because ECs rely much more on glycolytic metabolism than other cell types, the potential pathophysiological role of their mitochondria has been, to some degree, neglected. However, ECs retain an extensive mitochondrial network and the ability to switch to oxidative metabolism of glucose, amino acids and fatty acids in case of reduced glycolytic rates [10]. For example, bovine aortic ECs have been shown to use only approximately 35% of their maximal oxygen consumption at basal conditions, and mitochondria in these endothelial cells are highly coupled and possess a considerable bioenergetic reserve. The presence of a reserved respiratory capacity that is available for ECs when bioenergetic demand is increased suggests that mitochondria may function as stress sensors and signaling initiators in ECs [11].

3 Repression of Endothelial Oxidative Phosphorylation in PH

In contrast to the baseline state, the diseased state of the endothelium highlights the importance of mitochondrial metabolism to overall cellular (patho)phenotype. EC dysfunction and apoptosis appear to be an early event in PH. In PH, a widely cited theory exists that initial EC apoptosis gives rise to a separate population of hyperproliferative and pathogenic ECs that drive later stages of disease. This was originally described by Voelkel and colleagues [12] in 2005, which has been echoed by others since then [5, 13]. Interestingly, the hyperproliferative and apoptosis-resistant ECs exhibit a metabolic profile strikingly similar to that of cancer cells [7]. Cancer cells show reliance upon glycolysis and shift away from oxidative phosphorylation for cellular energy production, a phenomenon known as the Warburg effect [14]. Yet, aerobic glycolysis is not just a unique feature of malignancy; it is also observed in nontransformed proliferating cells when glucose is sufficient [15].

Studies in human, rodent, avian and lamb PH have demonstrated that ECs in PH also produce energy primarily from glycolytic metabolism [16–19], which is analogous to the alterations in cancer cell metabolism [20]. In normal ECs, ATP is generated nearly equivalently by glycolysis and mitochondrial respiration [9], representing a relatively high glycolytic activity compared to other cell types and increased tolerance to hypoxia because of low oxygen demand. Indeed, cellular ATP content of ECs of PH models is similar to control cells under normoxia; however, under hypoxia, cellular ATP remains static in PH endothelial cells but decreases significantly in control endothelial cells [21]. The greater tolerance to hypoxia suggests a predominant anaerobic metabolism in PH ECs and a lesser dependence upon mitochondrial respiration.

A number of metabolic control points have been identified in the repression of endothelial

oxidative phosphorylation observed in PH. Overall, as we describe in Fig. 1, these include: (1) activation of hypoxia-induced factor-1 (HIF-1), which upregulates pyruvate dehydrogenase kinase (PDK), hence suppressing mitochondrial oxidative phosphorylation [22]; (2) diversion of pyruvate into anabolic pathways [23]; (3) inhibition of voltage-gated K^+ channels (Kv channels), thereby increasing cytosolic Ca^{2+}, inducing the hyperproliferative transcription factor nuclear factor of activated T cells (NFAT), and activating glycolytic enzymes [24]; and (4) suppression of apoptosis by hyperpolarized mitochondrial membrane potential ($\Delta\psi_m$) [25].

Notably, it has been speculated that decreased mitochondrial function may be a primary stimulus to glycolysis [26]. There is evidence supporting intrinsic deficiencies in mitochondrial function in the metabolic shift to glycolysis in PH [16, 19] as well as secondary mitochondrial changes following endothelial dysfunction. Site-specific defects in the ETC within complexes I and III have been identified

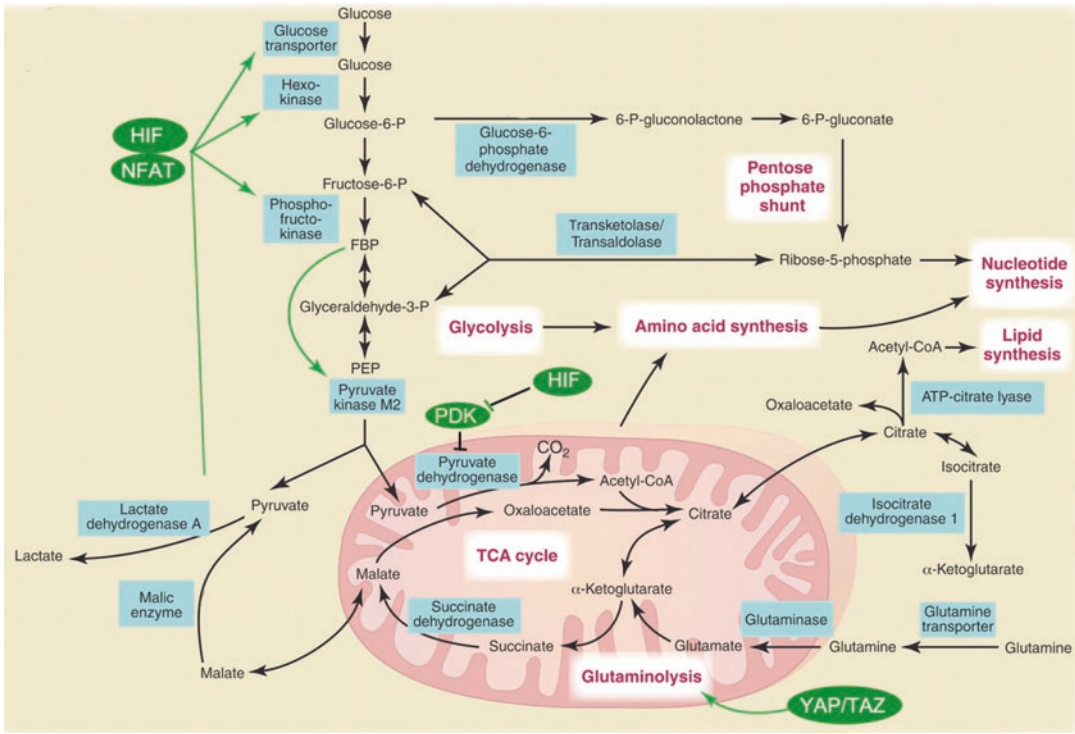

Fig. 1 Relationships between metabolome, proteome, and genome in metabolic reprogramming of endothelial cells in PH. In typical cells under normal oxygen levels, a majority of pyruvate derived from glucose enters the mitochondria where it is oxidized in the TCA cycle to generate ATP to meet the cell's energy demands. However, in PH endothelial cells, pyruvate is directed away from the mitochondria toward glycolysis in order to create lactate through the action of lactate dehydrogenase (LDH)—a process typically activated by low oxygen exposure. Lactate production in the presence of oxygen is termed "aerobic glycolysis" or the Warburg effect, a phenomenon common in cancer cells. In aerobic glycolysis, excess glucose is diverted through the pentose phosphate shunt (PPS) and serine/glycine biosynthesis pathway to create nucleotides. Fatty acids are critical for new membrane production and are synthesized from citrate in the cytosol by ATP-citrate lyase (ACL) to generate acetyl-CoA. Signals impacting levels of hypoxia inducible factor (HIF) and nuclear factor of activated T cells (NFAT) can increase expression of enzymes such as LDH to promote lactate production, as well as pyruvate dehydrogenase kinase to inhibit the action of pyruvate dehydrogenase and limit entry of pyruvate into TCA cycle. Highly proliferative endothelial cells need to produce excess lipid, nucleotide, and amino acids for the creation of new biomass. There is also increased use of glutamine as another fuel source, which enters the mitochondria and can be used to replenish TCA intermediates or to produce more pyruvate through the action of malic enzyme. Adapted with permission from *Science* [66]

in avian spontaneous idiopathic PH, where the lower respiratory chain coupling, inefficient use of oxygen, and increased ROS generation are directly related to the development of PH [17]. Similarly, fawn-hooded rats, a spontaneously PH rodent strain, present dysmorphic mitochondria with reduced expression of ETC components (complexes I, III, and IX), reduced expression of manganese superoxide dismutase (MnSOD), depressed mitochondrial reactive oxygen species (ROS) production, and activated PDK which shift metabolism away from oxidative phosphorylation toward glycolysis. The abnormalities in mitochondrial function have been found to activate the master transcription factor of hypoxia, HIF-1α, which then can inhibit expression of oxygen-sensitive Kv channels, analogous to the pathophysiology of chronic hypoxic exposure [16]. Strikingly, dichloroacetate (DCA), a mitochondrial PDK inhibitor, was found to reverse PH in fawn-hooded rats, providing a mechanistic relationship between mitochondrial function and PH pathogenesis [18]. The decreased expression and activity of mitochondrial enzymes required for carnitine metabolism in lambs also lead to mitochondrial dysfunction, via decreased MnSOD expression and increased uncoupling protein-2 expression, thus increasing glycolysis, endothelial dysfunction, and ultimately PH [19]. Similarly, pulmonary artery endothelial cells from human idiopathic PH lungs have revealed decreased mitochondrial dehydrogenase activity, mitochondrial number and mitochondrial DNA content per cell as well as higher glycolytic rate than in control cells [27]. In vitro study of human PAH ECs demonstrated that they have lower than normal oxygen consumption for state 3 and state 4 respiration with glutamate-malate or succinate as substrate [27]. It was also observed that activity of complex IV, the terminal enzyme complex of the respiratory chain that catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen, is significantly lower, while complex III activity is similar among control and PH ECs. Notably, the coupling between oxygen consumption and ATP production, the respiratory control index of mitochondrial function, was also found to be similar between PH and control cells. Thus, human PH ECs appear to display lower numbers of active mitochondria, which result in greater reliance upon glycolysis [13].

4 Balancing Endothelial Proliferation and Glycolysis in PH: The Role of Anaplerosis

Glycolysis is likely advantageous to rapidly growing cells, as it renders them less dependent on oxygen, thereby improving cell survival in an environment that may become hypoxic as cell number increases. Furthermore, the transition to glycolysis in proliferating cells minimizes exposure to reactive oxygen species (ROS) [28]. Yet glycolysis is far less effective in meeting the enhanced ATP demand of proliferating cells. Indeed, increased pulmonary arterial EC (PAEC) proliferation has been noted by multiple groups and linked to PH pathogenesis. Historically, this has included the study of plexiform lesions (previously described as "disorganized endothelial proliferation" [29]) which are pathognomonic signs of this disease. Moreover, recent studies such as Kim et al. [30] have demonstrated that cultured PAECs isolated from human PAH patients display increased proliferation compared with non-diseased control cells. Other studies have isolated highly proliferative endothelial cells from PAH patients as well [31]. Studies in other animal models of PH (to name a few) such as transgenic mice expressing interleukin-6 [32], long-term inhibition of the potassium channel KCNK3 (Potassium Two Pore Domain Channel Subfamily K Member 3) in rats [33], and monocrotaline-exposed rats [30] all display increased PAEC proliferation in diseased arterioles. However, in PH as in cancer, the metabolic needs of rapid proliferation cannot be met by glycolysis alone.

In that context, even in the setting of the glycolytic state, the TCA cycle can serve as a primary source of energy production via the replenishment of carbon intermediates. This process known as anaplerosis is accomplished via two major pathways: glutaminolysis (deamidation of glutamine via the enzyme glutaminase [GLS1]) and carboxylation of pyruvate to oxaloacetate via ATP-dependent pyruvate carboxylase. Specifically, glutaminolysis

via GLS1 activity contributes to anaplerosis by allowing for mobilization of cellular energy, carbon, and nitrogen, particularly in rapidly proliferating cells [34] and serves as a crucial process in transformed cells that have switched their metabolism from oxidative phosphorylation to glycolysis in order to maintain cell growth and viability [35]. Recently, our group reported a novel link between vascular stiffness and EC glutaminolysis that drives the proliferative vascular phenotype in PH [36]. Namely, in cultured pulmonary vascular cells and in PH samples, including primate and human instances of human immunodeficiency virus-induced PH, we found that ECM stiffening mechanoactivated the transcriptional co-activators YAP (Yes-associated protein 1) and TAZ (Transcriptional activator with PDZ binding motif 1) to modulate metabolic enzymes including GLS1 and thus coordinate glutaminolysis and glycolysis (Fig. 2). Glutaminolysis replenished aspartate for anabolic biosynthesis (anaplerosis), sustaining proliferation and migration within stiff extracellular matrix. In vivo, pharmacologic modulation of pulmonary vascular stiffness and YAP-dependent mechanotransduction altered glutaminolysis, pulmonary vascular proliferation, and manifestations of PH. Correspondingly, using two separate GLS1 inhibitors, pharmacologic targeting of GLS1 ameliorated PH progression. Thus, our data demonstrate that ECM stiffening sustains vascular cell growth and migration through

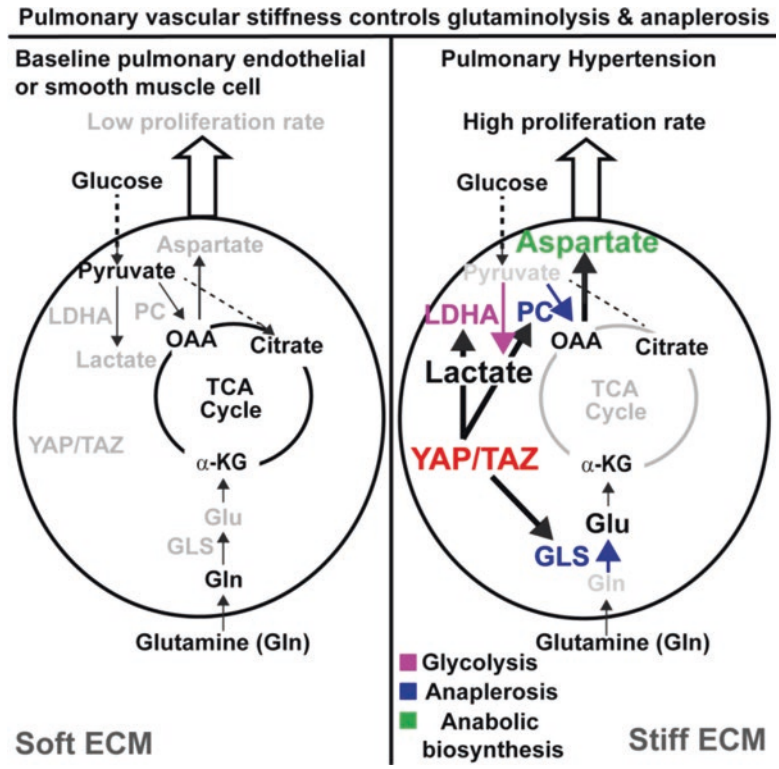

Fig. 2 Pulmonary vascular stiffness controls major metabolites in anaplerosis and glycolysis in cultured pulmonary vascular cells and in PH-diseased primate and human samples. Glutaminase (GLS1) and pyruvate carboxylase (PC) generate the major anaplerotic metabolites (*blue*), feeding into the TCA cycle (*black*) and supporting the anabolic demand for biosynthesis (*green*). Lactate dehydrogenase A (LDHA) modulates glycolysis (*red*). As compared with soft ECM (*left panel*), ECM stiffening (stiff ECM, *right panel*) mechanoactivates the transcriptional co-activators YAP/TAZ to modulate metabolic enzymes including LDHA, GLS1, and PC—implicated in both glycolysis (LDHA) and anaplerosis (GLS1 and PC). As a result, lactate production increases, while intracellular glutamine declines accompanied by a robust increase of glutamate and aspartate, thus driving anaplerosis during accelerated glycolysis in endothelial dysfunction in PH. Adapted with permission from the *Journal of Clinical Investigation* [36]

YAP/TAZ-dependent glutaminolysis and anaplerosis—a paradigm that advances our understanding of the connections of mechanical stimuli to dysregulated endothelial metabolism. These results also indicated the possibility of novel glutaminolytic targets in ECs for the treatment of PH. Additional triggers of this EC metabolic shift likely exist and potentially are regulated by both protein factors as well as other noncanonical effectors (i.e., microRNAs [37]), but these await further investigation.

5 Endothelial Mitochondria as Stress Sensors in PH

Mitochondria critically determine cell survival and death by regulating ATP synthesis.Besides oxidative ATP production, however, mitochondria hold other essential regulatory roles in heme synthesis, β-oxidation of free fatty acids, metabolism of certain amino acids, production of free radical species, formation and export of iron–sulfur clusters, iron metabolism, and calcium homeostasis [6]. Specific mitochondrial abnormalities related to clinical PH and experimental PH include induction of a pseudohypoxic state via activation of HIF1α, impaired mitochondrial–ER interaction, and mitochondrial fission [38]. Dysmorphic mitochondria with defects in the ETC, decreased respiratory chain coupling, and inefficient use of oxygen have also been reported [27]. A recent study revealed that mice with endothelial deletion of bone morphogenetic protein receptor 2 (BMPR2) develop PH following hypoxia-reoxygenation by increasing mitochondrial ROS production, reducing mitochondrial membrane potential, and inhibiting mitochondrial biogenesis [39]. These findings suggested a critical role of endothelial mitochondria in the pathogenesis of BMPR2 deficiency—a heterozygous genetic state found in approximately 70% of familial PAH and in 20% of sporadic cases of idiopathic PAH [40]. Another example is the connection between dasatinib-induced PH and endothelial mitochondrial dysfunction. Dasatinib, a dual Src and BCR-ABL tyrosine kinase inhibitor used to treat chronic myelogenous leukemia, causes pulmonary vascular damage by inducing ER stress and mitochondrial ROS production in the endothelial cells, which leads to increased susceptibility to PH development [41].

5.1 Mitochondrial ROS in Pulmonary ECs

Beyond the production of ATP, the generation of ROS as secondary messengers allows endothelial mitochondria to act as a primary signaling organelle in the cell. In the hypoxic lung, mitochondrial ROS production has been localized predominantly to complex II [42], although complexes I and III are recognized to produce most of the ROS in other occasions. In a monocrotaline-induced rat model of PH, selective increases in the expression and activity of complex II were observed as well as complex II-derived ROS [43]. Exposure of PAECs to hypoxia triggers the perinuclear clustering of mitochondria and accumulation of ROS in the nucleus, which can be attenuated by nocodazole to destabilize microtubules and inhibit retrograde mitochondrial movement [44]. Evidence also supports the critical roles of increased mitochondrial ROS production and reduced MnSOD activity in the pathogenesis of PH, events that also contribute, via consumptive reactions, to the loss of the vasodilator nitric oxide (NO) often observed in PH. For example, mitochondrial derived ROS have been shown to induce pulmonary vascular remodeling in PH by increasing intracellular Ca^{2+}, inducing $\Delta\Psi m$ depolarization/hyperpolarization, and PAEC apoptosis and pulmonary artery smooth muscle cell proliferation [45]. The increase in mitochondrial ROS in PH has been linked to elevated circulating levels of asymmetric dimethylarginine (ADMA), which induces mitochondrial dysfunction by increasing uncoupling protein-2 (UCP-2) protein levels and reduction in cellular ATP levels [46]. Furthermore, supplementation of ECs with mitochondria-targeted antioxidants inhibits peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis [47]. Antioxidants such as dimethylthiourea [48], recombinant human SOD [49] and *N*-acetyl-l-cysteine [50], a

precursor to the nonenzymatic antioxidant glutathione, have all been shown to significantly abrogate the pulmonary hypertensive response and consequent right ventricular dysfunction. However, reduced mitochondrial ROS have also been found in hypoxia and monocrotaline-induced PH, as well as in the fawn-hooded rats [16, 51]. Other studies have also demonstrated that hypoxia attenuates ROS production in the rabbit lung [52], and inhibition of the mitochondrial ETC with rotenone and antimycin A results in reduced ROS production and pulmonary vasoconstriction [53]. These discrepancies may be due to the inherent differences in the models employed or the progression of the disease. Thus, the role of mitochondria-derived ROS in PH is complex, and much work lies ahead to clarify these complex issues.

5.2 Mitochondrial Ca^{2+} in Pulmonary ECs

Agonist stimulated increase in cytosolic Ca^{2+} in endothelial cells has been repeatedly linked to accumulation of Ca^{2+} in the mitochondria [54]. This is achieved by voltage-dependent anion channel (VDAC) in the outer mitochondrial membranes and mitochondrial Ca^{2+} uniporter (MCU) in the inner mitochondrial membranes [55]. Mitochondrial Ca^{2+} and its buffering of the cytosolic Ca^{2+} serve as important orchestrators of mitochondrial biogenesis and many downstream aspects of mitochondrial functions including mitochondrial ROS production, aerobic metabolism, dynamics and biogenesis [8]. Such processes cooperate with the endoplasmic reticulum (ER) to maintain cellular Ca^{2+} homeostasis. Indeed, the distance between the ER and mitochondria has been associated with intramitochondrial Ca^{2+}, mitochondrial membrane potential ($\Delta\psi_m$) and mitochondria-dependent apoptosis in pulmonary arterial cells in PH [56]. The accumulation of Ca^{2+} in the mitochondrial matrix ($[Ca^{2+}]_{mt}$) leads to the activation of several Ca^{2+}-sensitive effectors, including key metabolic enzymes such as pyruvate dehydrogenase phosphate phosphatase and NAD^+-isocitrate dehydrogenase [57], as well as mitochondrial nitric oxide synthase, thus producing nitric oxide which relaxes smooth muscle cells by increasing cytosolic cGMP [58]. Additional work will be necessary to determine whether pharmacologic modulation of calcium handling specifically in PAECs independent of smooth muscle cells may be effective for more precise endothelial therapies in PH.

5.3 Mitochondrial Iron–Sulfur (Fe-S) Clusters in Pulmonary ECs

Recently, the importance of iron–sulfur (Fe–S) clusters to endothelial metabolism in PH has been described. Fe–S clusters ([4Fe-4S] and [2Fe-2S]) are critical bioinorganic prosthetic groups that promote electron transport and oxidation–reduction reactions integral to numerous cellular processes ranging from ribosome biogenesis, purine catabolism, heme biosynthesis, DNA repair, and iron metabolism, among others [59]. In particular, [Fe-S] clusters are essential components of enzymes involved in the maturation of subunits of complexes I, II, and III, which facilitate electron transport. Fe-S clusters also participate in the synthesis of the enzyme-bound cofactor lipoate present in the 2-oxoacid dehydrogenases, including pyruvate dehydrogenase and oxoglutarate dehydrogenase complexes, which are integral to TCA cycle function [59]. Depending upon the level of ambient oxygen exposure, alteration of these and other iron–sulfur-dependent mitochondrial activities can lead to distinct downstream consequences on ROS production and cellular survival [60]. Despite their established importance in these cellular redox reactions, regulation and function of these critical prosthetic groups in hypoxic mammalian cells are poorly characterized. The formation of Fe-S clusters is controlled by a conserved set of assembly and scaffold proteins. Current knowledge regarding the importance of these proteins in human disease has been derived largely through investigation of genetic mutations [61]. For example, mutations in Fe-S cluster scaffold genes NFU1 and BOLA3 have been

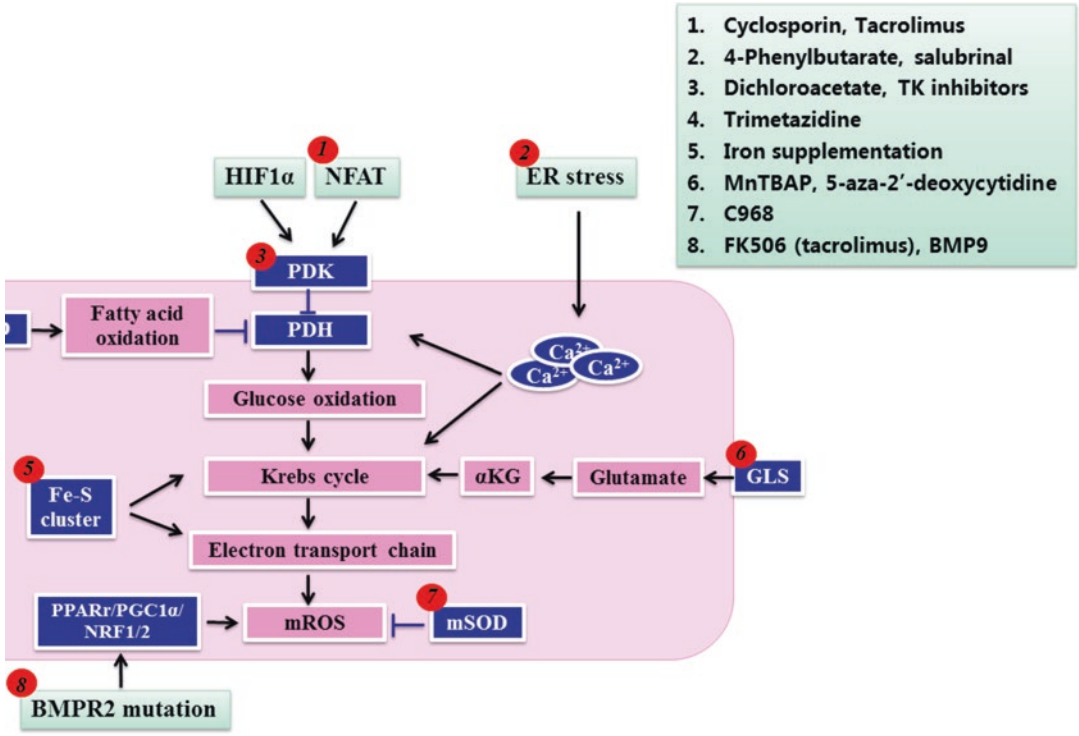

Fig. 3 Potential therapeutic targets in PH based on mitochondrial and metabolic dysfunction of endothelial cells. This schematic of endothelial metabolic dysfunction in PH suggests several therapeutic targets (shown in *red circles* and listed on *top* of the figure) that have shown preclinical promise and in several cases are currently being tested in early-phase clinical trials. *αKG* α-ketoglutarate, *ER* endoplasmic reticulum, *GLS* glutaminase, *HIF* hypoxia-inducible factor, *MCD* malonyl-CoA decarboxylase, *MnSOD* manganese superoxide dismutase, *MnTBAP* Mn(III)tetrakis(4-benzoic acid)porphyrin chloride, *mROS* mitochondria-derived reactive oxygen species, *NFAT* nuclear factor of activated T cells, *PDH* pyruvate dehydrogenase, *PDK* pyruvate dehydrogenase kinase, *TK* tyrosine kinase. Adapted with permission from *Circulation Research* [67]

linked to the multiple mitochondrial dysfunction syndrome (MMDS), a fatal and rare autosomal recessive disease characterized by deficiency of complexes I, II, and III in the mitochondrial respiratory chain and 2-oxoacid dehydrogenase enzymes [62]. Notably, histologic analysis of deceased infants carrying NFU1-mutations and suffering from MMDS has revealed substantial pulmonary vascular remodeling [63]. Correspondingly, in PAECs from PH mice, downregulation of a separate Fe-S biogenesis gene, ISCU, in PAECs was observed, compromising integrity of Fe-S clusters [64], repressing mitochondrial oxidative phosphorylation, increasing ROS, and promoting PH in mice. Importantly, cardiopulmonary exercise testing of a woman with homozygous ISCU mutations also has revealed exercise-induced pulmonary vascular dysfunction [65]. Thus, Fe-S deficiency may represent an overarching point of regulation promoting PH via metabolic dysregulation, and it remains to be seen whether other Fe-S biogenesis genes can similarly influence PH inception or progression.

6 Conclusions

In summary, it is increasingly clear that dysregulated endothelial metabolism and mitochondrial function drive primary aspects of PH from inception to end-stage disease. Yet the molecular details of this activity are not fully defined. Currently, it is thought that endothelial mitochondria may

participate in PH through two main mechanisms: (1) alterations of glycolysis, oxidative phosphorylation, and anaplerosis to provide substrates for proliferation and (2) generation of secondary signaling messengers (i.e., ROS, Ca^{2+}) to promote downstream endothelial pathophenotypes. Future work will entail further definition of the molecular triggers and regulatory checkpoints of these processes (Fig. 3). In doing so, it is hoped that these discoveries could be translated rapidly into clinical applications for introducing a new era of metabolic diagnostics and therapeutics for PH and its devastating consequences.

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Subcellular Redox Signaling

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1 Introduction

Increase of oxidative free radicals and reactive oxygen species (ROS) disturbs the balance of redox status, resulting in oxidative stress. As an important intracellular second messenger, it can trigger a multiple of signaling transduction pathways and mediate pathological processes, including inflammation, proliferation, differentiation, apoptosis, and gene expressions [1, 2]. Up to now, the mechanisms of how ROS produce, how ROS act on target signaling protein and mediate downstream effects, and how the signaling protein inversely regulate the level of ROS have been explored widely. Especially with the development of new methods for detecting ROS compartmentalization, the studies of subcellular ROS have become available [3, 4]. More and more advancements revealed that ROS generated in specific subcellular organelles seems to be crucial for regulating cell (patho-)physiology.

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2 Redox Signaling of Mitochondria

2.1 Generation of Mitochondrial-Derived ROS (mtROS)

Mitochondria are the main source of intracellular ROS. The production of superoxide radicals in the electron transport chains is catalyzed by several enzyme complexes located in the inner membrane of mitochondria including complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c oxidase), which are oxidized by coenzyme Q [5, 6]. In vitro experiments, isolated mitochondria produced H_2O_2 when high concentrations of succinate were used as oxidative substrate. This reaction was catalyzed by succinate-ubiquinone oxidoreductase, Complex II [7]. Under normoxic conditions, succinate level is low and proportion of ROS produced by Complex II can be neglected [7] and complex II may not contribute to ROS production [8, 9]. In hypoxic status, tissue level of succinate may rise to about 2 mmol [10–12], suggesting a potential role of Complex II in the ROS production. For hypoxic pulmonary vasoconstriction, complex I, II, and III pre-ubisemiquinone site are possibly responsible for ROS production in pulmonary arterial smooth muscle cells (PASMCs) [13, 14]. Wang's group further showed that Rieske iron–sulfur protein on complex III played a

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pivotal role in hypoxia-induced ROS production [13], and that hypoxia-induced ROS production occurred earlier from mitochondria than from any other subcellular region [15]. The in-depth understanding is that hypoxia induced mitochondrial ROS production as the first step, the mitochondrial ROS then activated PKCε, which in turn upregulated NADPH-oxidase (NOX) and finally triggered more ROS production in PASMCs [15].

In cancer cells, pyruvate and glucose deprivation also induces oxidative stress driven by mitochondria and NOX [16]. Several subtypes of NOX have been identified with distinct catalytic domains [17]. NOX4 is proved to have mitochondrial localization [17, 18] and it is related with production of H_2O_2 [19]. Whether the subcellular localization and or level of NOX determines the spatiotemporal feature of ROS production remains unknown.

Other components of mitochondria including monoamine oxidase (MAO), glycerol phosphate dehydrogenase and $p66^{shc}$ contribute to the formation of ROS [6, 20]. MAO located in the outer membrane of mitochondria catalyzes the oxidation of biogenic amines to generate ammonium, acetaldehyde and H_2O_2 [21]. MAO produces high level of H_2O_2 under ischemia–reperfusion condition in heart, kidney and brain [22–25]. Another potential candidate for mtROS may be α-glycerophosphate dehydrogenase that located in the outer surface of the inner mitochondrial membrane [26]. Compared with complexes I or II, higher production efficiency of ROS can be detected in isolated mitochondria supplemented with α-glycerophosphate dehydrogenase and treated with antimycin A [26, 27].

ROS reduced and life span extended in $p66^{shc}$ deficient mice demonstrating the importance of $p66^{shc}$ in production of ROS [28–30]. P66shc belongs to the Shc A protein family, which contains another two isoforms, $p46^{shc}$ and $p52^{shc}$ [31]. They have the same structure including an SH2 region, a CH1 region, and a PTB region. $p66^{shc}$ is a target of receptor tyrosine kinases [31, 32]. Most $p66^{shc}$ exists in the cytosol normally, in ischemia/reperfusion states $p66^{shc}$ migrates into mitochondria where it catalyzes electron transfer from cytochrome c to oxygen generating H_2O_2 [16, 31, 33]. A small amount of $p66^{shc}$ exists in the mitochondrial intermembrane space (IMC) and mediates generation of ROS [6, 31]. Mammalian $p66^{shc}$ is also a ligand of ZAP-70 (zata chain associated protein-70) involved in cell apoptosis and the production of mtROS [31, 32].

2.2 Biological Effects of mtROS

New evidence supports that mtROS signaling is important in physiology and adaptive response [34–36]. Although increased production of ROS were harmful as previously thought, recent studies suggested that mtROS production delays worm aging [37]. Investigations proposed that an increased mtROS caused "mitohormesis," which in turn promoted additional longevity [35, 36, 38]. mtROS reduced Insulin/IGF signaling and D-glucosamine supplementation, which resulted in antiaging effect [39, 40]. The increased mtROS production in damaged mitochondrial electron transport chain (ETC) appeared to increase worm lifespan [41]. mtROS was also reported to achieve protection, rather than apoptosis in stress responses [42]. Lifespan caused by mtROS may be linked with HIF1 and AMP kinase cascades [43, 44] and TORC1 signaling [45–47].

The similar effect of mtROS on longevity effect was found in mice [48, 49]. mtROS influences not only on aging but also on wound healing [50], hypoxia response [51–53], pH homeostasis [54], cell differentiation [34, 55, 56], and immunity [57].

2.3 mtROS Microdomains

Microdomains of ROS formation exist in mitochondria; ROS accumulation occurs on the outer or inner side of the outer mitochondrial membrane (OMM), intermembrane space (IMS) or in the matrix of mitochondria [4]. Studies using different probes like roGFPs and HyPer identified that different stimuli can lead to ROS accumulating in different regions of mitochondria; for example, MPP^+ resulted in oxidation in the mitochondrial matrix, but did not induce

oxidative stress in IMS [58]. Rotenone led to formation of H_2O_2 in the IMS earlier than mitochondrial matrix [59]. Hypoxia led to ROS changes in the IMS and cytosol, not in mitochondrial matrix [60]. Different species with different dissemination abilities like membrane-permeable H_2O_2 and membrane-impermeable O_2^- are found in ROS microdomains.

3 Redox Signaling of Endoplasmic Reticulum (ER)

3.1 Generation of ER ROS

The enzymes of ER, including cytochrome p-450, b5 enzymes, and diamine oxidase enzyme all contribute to the production of ROS [6, 61]. As a thiol oxidase enzyme, Erop1p participates in the process of electron transfer to molecular oxygen, which results in the formation of H_2O_2 [62].

3.2 Biological Effects of ER-Derived ROS

The Aggregation ROS-associated small protein can occur in the ER and impair the ubiquitin–proteasome pathway, because they can bind the TATA-binding protein and cAMP response element-binding protein (CREB) and impair the ubiquitin–proteasome pathway [63, 64]. However, aggregation of larger protein like TCP1-ring complex (TRiC) and heat shock protein 70 (HSP70) can be protective through suppression of small protein aggregate formation [65, 66]. Under normal conditions, Nrf2 locates in cytoplasm associated with protein Keap 1 [67]. It translocates to the nucleus under stress condition [68, 69] and affect functions of cells, including ER stress and unfolded protein response [70]. Nrf2 can reduce ROS levels, increase antioxidant activities and change the cell metabolish from aerobic to anaerobic respiration [71, 72]. External stimulation such as hypoxia, acidosis, and glucose deprivation can break the balance of redox signaling and induce aggregation of oxidized proteins in the ER. This provides a molecular mechanism of occurrence of diseases, including Parkinson's disease, diabetes mellitus, and atherosclerosis [73].

3.3 The ER–Mitochondrial Interface: Redox–Ca^{2+} Interactions

The ER is a major source of intracellular calcium ions. Mitochondrial respiration and ROS production, the internal environment homeostasis [74], and ER can establish links with mitochondria through membrane contact sites (MCSs), including mitochondria-associated membranes (MAMs) [75]. MAMs are ER membranes, which possess similar double membrane structure like mitochondria [76]. The MAM is the location where ER and mitochondria associated proteins exist, including grp75, Ero1α, PERK and MFN-1/2 [76–78], but also calcium related protein, such as the inositol 1,4,5-trisphosphate receptor(IP_3R) [79, 80]. Both MAM and IMS are influenced by redox reactions [81] and IMS may be more intensely involved [82].When the exchange of oxidized products between IMS and cytosol is suppressed, the IMS become oxidized [83]. Some studies suggested that OMM contain voltage-dependent anion channel (VDAC) [84, 85], and it controls Ca^{2+} transport through mitochondria. La^{3+} can influence Ca^{2+} binding sites of VDAC and inhibit mitochondrial Ca^{2+} uptake [84]. Mitochondrial VDAC is able to form a complex with grp75 and IP_3R [3], this may indicate a communication between ER and mitochondria. ROS in the interface between mitochondria and ER can stimulate IP_3R, cause Ca^{2+} release, and increase mitochondrial Ca^{2+} uptake, which induce an additional production of ROS through oxidation of thiols and activation of ERO-lα and ERp44 [3, 86].

The mechanism of redox-regulated IP_3Rs has been linked to ROS-induced thiols oxidation and the association with ERp44 and ERO-1α expressed in ER luminal [86, 87]. The interesting evidence is that ERp44 and ERO-1α are also enriched in the MAM interface [88, 89] and

cytosol-exposed thiols modification of ER, other thanintraluminal of ER is more critical for modulation of IP_3R [3, 89]. Effective flow of Ca^{2+} released through the IP_3R to mitochondria was proved by Rizzuto, illuminating that ER was linked with mitochondria and elevated $[Ca^{2+}]_c$ in microdomain mediated Ca^{2+} transfer into mitochondria [90].

4 ROS Signaling of Nucleus

4.1 Mechanisms of Nuclear ROS Signaling

Many studies supported that the nuclear membrane, like the plasma membrane, possesses channels, pumps and exchangers, so the nuclei is considered as a cell within a cell [91]. Nucleus can also generate its own ROS signaling via its membrane receptors, transducers and others, independence of cytoplasmic ROS transportation to nuclei [91, 92].

4.1.1 Nuclear ROS Transmitted from Cytoplasm

It is reported that 50 μmol/L H_2O_2 induced an increase of ROS in isolated nuclei, suggesting ROS in the nuclei increase along with the elevation of the cytoplasmic H_2O_2 [92]. Cytoplamic ROS signaling transmits to the nuclei most likely via nuclear pore complex (NPC), since the NPC was the most important gateway for molecular exchange between cytoplasm and nucleus, and it was demonstrated that NPC can permit molecule up to 80 MDa into nuclei in less than 1 s [93]. The major functions of NPC were divided into two aspects, NPC permit the molecules of smaller than 40 kDa or 9–10 nm diameter to diffuse passively, and as for the larger molecules, they were transported selectively via NPC [94, 95].

4.1.2 Nuclear Membrane Receptor and ROS

Previous work showed that G protein-coupled receptors (GPCRs) such as angiotension II (Ang II) receptor, endothelin-1 (ET-1) receptor, bradykinin (BK) receptor, and prostaglandin E2 (PEG2) receptor existed in nuclear envelope membranes [91, 92, 96–98]. Activation of these nuclear membrane receptors and their downstream signaling pathways can result in production of nuclear ROS. Ang II has been demonstrated to stimulate ROS production in isolated nuclei [99]. The underlying mechanism is that ANG II binds to the ANG II type 1 receptor (AT1R) localized on nuclear envelope membrane and then stimulates phosphoinositol 3 kinase (PI3K) and PKC, activates the NAD(P)H oxidase within the nuclear, results in nuclear ROS increase [100–102]. Moreover, the ANG II type 2 receptor (AT2R) also was found to be localized on the nuclei although it did not possess a canonical nuclear localization sequence [103]. Further, researchers found that the AT2 antagonist, blocking the binding of AT2 to AT2R, exacerbated ANG II induced-ROS increase [103]. This finding suggested that the AT2R may play a potential role in buffering ROS in the nuclei. Provost et al. proved that extracelluar ET-1 (10^{-9} mol/L) induced both cytoplasmic and nuclear ROS increase in intact cells. More importantly, ROS production in isolated nuclei could be activated at a much lower concentration of ET-1 (10^{-11} mol/L) and the addition of H_2O_2 did not further increase nuclear ROS levels [92]. These results suggested that cytoplasmic ET-1 induced nuclear ROS increase independent of cytoplasmic ROS. However, the in-depth mechanism of ET-1induced increase in nuclear ROS remains unclear. It is hypothesized that the regulatory mechanism may involve the activation of NADPH oxidase and endothelial nitric oxide synthase (eNOS) by ET-1 binding to its receptor, which is similar to ET-1 mediated cytoplasmic ROS production. Previous study found bradykinin-elevated cytoplasmic ROS through B2 receptor and subsequent PLC-Ca^{2+} signaling [104]. Moreover, there were a few findings that indicated bradykinin B1 and B2 receptors both localized in the nucleus [96, 105, 106]. So, we speculate that cytoplasmic bradykinin can induce an increase of ROS in the nuclei through B2 receptor, although this has not been confirmed experimentally. Recently, Wang et al. reported that ANG II induced endogenous PEG2

production, activation of cyclooxygenase 1 (COX-1) and NADPH oxidase subunits NOX2, and a significant increase of ROS in wild type SFO cells and this response was inhibited in PEG2 receptor type 1 (EP1R) knockout cells [107]. These results indicate that ANG II increased cytoplasmic ROS through PEG2 binding with EP1R. Although the above mechanism has not been identified in nuclear ROS production, because PEG2 receptor exists in nuclear membrane, we speculate that ANG II also could promote PEG2 binding with its receptor in nuclear membrane, and induce nuclear ROS production. These could offer clues on exploring the mechanism of nuclear ROS increase.

4.1.3 NAD(P)H Oxidase and Nuclear ROS

Although the source of nuclear ROS has not been explored thoroughly, the important role of NAD(P)H oxidase system were verified in many studies. Previous studies found that Nox4, NAD(P)H oxidase isoform, was localized in nuclear compartment of different cells, such as smooth muscle cells and endothelial cells [108, 109]. In the nuclei of umbilical vein endothelial cells, Nox4 was further found to form a heterodimer with $p22^{phox}$ [109]. The NADH-induced ROS increase was significantly inhibited in the nuclei treated with Nox4-siRNA, not $gp91^{phox}$/Nox2-siRNA, which indicated that Nox4 may be involved in the nuclear ROS production in umbilical vein endothelial cells [109]. Also, a recent study found that NADPH oxidase subunits Nox2, and $p47^{phox}$was co-localized on nuclear envelope with the NPC (nuclear pore complex), but $p22^{phox}$ was absent in the control cardiomyocytes. However, in the ischemic cells, the expression of nuclear Nox2, $p47^{phox}$ and $p22^{phox}$ were upregulated, and $p22^{phox}$ co-localized with Nox2 and $p47^{phox}$ at the NPC [110]. And the authors further proved the NAPDH oxidase inhibitors reduced ischemia-induced nuclear ROS increase, suggesting Nox2, $p47^{phox}$, and $p22^{phox}$ were important molecules in generating nuclear ROS. A more recent a study reported the levels and distributions of different NADPH oxidase subunits in the nuclei of three types of cells, including vascular endothelial cells, smooth muscle cells, and endocardial endothelial cells [111]. The results indicated that Nox1, Nox2, Nox3, Nox4, and Nox5 were all existed in the nuclei of three cell types, but the levels of these five subunits in the nuclei of three cell types were different. The specific effects of these subunits in producing nuclear ROS under the different pathophysiological conditions are still elusive.

4.2 Biological Significances of Nuclear ROS Signaling

ROS as an important molecular signaling can regulate a variety of proteins functions and then mediate pathological processes, including inflammation, proliferation, differentiation, apoptosis, and gene expressions [1, 2]. However, whether the ROS in the nuclei possess the similar ability as the cytoplamic ROS to regulate (patho-)physiologic events has been studied in recent years.

4.2.1 Transcriptional Regulation Dependent on Nuclear ROS

Transcription factors, like NF-κB, p53, HIF, AP-1, and others are sensitive to redox status. These transcription factors translocate into the nuclei when cells received an original stimulus and can be activated as long as their cysteine residues in DNA binding region remains in reduction status. The reduction of cysteine residue is controlled by the redox status in the nuclei and it is a prerequisite for transcription factor–DNA binding and subsequent transcriptional activation [4, 112–114], indicating ROS in the nuclei may block transcription factor binding to DNA and inhibit transcriptional activation [4]. Therefore, this result seems to be inconsistent with that the study of cytoplasmic ROS activating transcription factors, such as NF-κB. How can we understand this contradiction? For the study of cytoplasmic ROS activating NF-κB, a major viewpoint is that ROS can increase the phosphorylation of IκBα, IκBα degredation result in NF-κB translocation into the nuclei, and then activate NF-κB [115–117]. Subsequently, a question is raised whether NF-κB is activated or

inhibited when cytoplasmic and nuclear ROS increase simultaneously. Based on previous studies, it may be concluded that cytoplasmic ROS increase could induce NF-κB translocate into the nuclei. At the same time, if nuclear ROS is too high to decrease the reduction of cysteine residues, the degree of NF-κB activation might be inhibited. The reduction of transcription factor critical cysteine residues is controlled by the nuclear redox system, such as glutathione (GSH) and thioredoxin (Trx) [112]. Previous studies have reported that GSH/GSSH and Trx are more reduced in nuclei than in cytoplasm [118, 119] and H_2O_2 increase in cytosol did not affect cytoplasmic GSH and Trx redox state [120], suggesting ROS-regulated redox state of GSH and Trx in cytosol might be different from nuclei. Another possible reason is that the level of H_2O_2 in cytosol is not enough to change GSH and Trx redox state.

4.2.2 DNA Damage Dependent on Nuclear ROS

It is well known that DNA is sensitive to the oxidative stress, and is a major cause of bases and phosphodiester backbone damage. More extensive investigations found that the lesion of 8-oxoguanine (8-oxoG) and 8-oxo-2′-deoxyguanosine (8-oxodG), oxidative base modifying DNA bases, resulted in mismatches of the DNA strands [121–123]. 8-oxodG accumulated on the nuclear DNA under the oxidant stress has been reported and Oka et al. further verified that the accumulation of 8-oxoG resulted in nuclear translocation of apoptosis-inducing factor in poly-ADP-ribose polymerase (PARP)-dependent manner, which contributed to cell death [123]. Undoubtedly, oxidant stress-induced the accumulation of 8-oxodG and 8-oxoG in the nuclei result in nuclear DNA damage and subsequent cell death. A recent review discussed the effects of the major sensors of oxidative DNA damage, 8-oxoguanine DNA glycosylase 1 (OGG1) and ataxia telangiectasial mutated (ATM) on nuclear ROS-controlled pulmonary innate immunity [124]. This review described that ATM and OGG1 can serve as nuclear ROS sensors and transmit nuclear signaling, finally regulate pulmonary innate immunity [124], suggesting DNA damage response to nuclear ROS can mediate the generation of diseases. Furthermore, recent reports of ROS derived from nuclei inducing nuclear DNA damage has been published [125, 126].

4.2.3 Cell Proliferation and Others Dependence on Nuclear ROS

There existed a strong likelihood that cell proliferation is associated with the nuclear redox status [112, 127–129]. Previous studies reported the effects of redox system in the nuclei, such as GSH and thioredoxin on regulating cell proliferation [127–129]. All the above studies found that nuclear environment remaining in reduction status could promote proliferation. Guida et al. reported that inhibition of nuclear ROS increase through decrease of Nox4 activity can improve stem cells proliferation [130], suggesting that the increase of nuclear ROS might inhibit cells proliferation. Additionally, this group continuously explored the important role of nuclear Nox4-derived ROS in myelodysplastic syndromes [125], stemness regulation and proliferation potential of stem cells [126]. They identified that Nox4 was highly expressed in nuclei in sample and cell lines of myelodysplastic syndromes, and nuclear ROS decreased when Nox4 was silenced [125]. Moreover, Nox4 interaction with Akt and ERK in nuclei was demonstrated, suggesting that Nox4 play an important role in dysregulation of nuclear signaling associated with myelodysplastic syndromes [125]. The markers of pluripotent stem cells such as Oct4, Sox2, and redox-sensitive transcription factors such as NF-κB and Nrf2 were proved to co-localize with Nox4 in nuclei of stem cells, suggesting that nuclear Nox4 may involve in stemness regulation and differentiation potential [126]. In these studies, investigators have fully identified that Nox4 mediated nuclear ROS production, however, whether the effect of Nox4 on proliferation, differentiation, and stemness regulation is regulated by nuclear ROS still needs further investigation.

5 ROS Signaling in Cytoplasm

5.1 Mechanisms of Cytoplasmic ROS Production

In recent decades, numerous investigators have found that ROS participate in physiological and pathological processes [34, 131]. ROS are produced from a cascade of reactions that starts with the production of superoxide, generated by mitochondrial respiration [132], xanthine oxidase [133], uncoupled NO synthase [134], or via reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) [135, 136].

5.2 Biological Significances of Cytoplasmic ROS Signaling

During pathological processes, production of ROS in excessive amounts or generation of ROS in the wrong place or generation of ROS at the wrong time results in oxidative stress leading to cellular dysfunction and apoptosis which contributes to atherosclerosis [137], heart failure [138], hypertension [139], and ischemia–reperfusion injury [140]. Recent studies have paid close attention to subcellular distribution of ROS and redox state.

A lot of studies have demonstrated that changes in ROS generation play key roles in hypoxic pulmonary vasoconstriction (HPV) [52, 141–143], but controversy exists regarding whether hypoxia increases or decreases ROS generation. Waypa et al. [60] have tested that hypoxia induces redox changes that differ among subcellular compartments in pulmonary (PASMC) and systemic (SASMC) smooth muscle cells. They employed a novel, redox-sensitive, ratiometric fluorescent protein sensor (RoGFP) [144–148] to assess the effects of hypoxia on redox signaling in cultured PASMC and SASMC. Using genetic targeting sequences, RoGFP was expressed in the cytosol (Cyto-RoGFP), the mitochondrial matrix (Mito-RoGFP), or the mitochondrial inter-membrane space (IMS-RoGFP), allowing assessment of oxidant signaling in distinct intracellular compartments. Superfusion of PASMC or SASMC with hypoxic media increased oxidation of both Cyto-RoGFP and IMS-RoGFP. However, hypoxia decreased oxidation of Mito-RoGFP in both cell types. The hypoxia-induced oxidation of Cyto-RoGFP was attenuated through the overexpression of cytosolic catalase in PASMC. These results indicate that hypoxia causes a decrease in nonspecific ROS generation in the matrix compartment, while it increases ROS production in the IMS, which diffuses to the cytosol of both PASMC and SASMC.

More recently, to reveal whether mitochondrial heterogeneity controls the distinct responses of pulmonary versus systemic artery smooth muscle cells to hypoxia, Zhou et al. [53, 149] transplanted intact mitochondria into Sprague-Dawley rat pulmonary artery smooth muscle cells in culture and pulmonary arteries in vitro and in vivo. Mitochondria retained functional after transplantation. They have proved that the difference between mitochondria of PASMCs and FASMCs lies in their capability to generate ROS in response to hypoxia and the levels of ROS determine SMC behaviors in the pattern of cell membrane potential depolarization, $[Ca^{2+}]_i$ elevation and constriction or cell membrane potential hyperpolarization, $[Ca^{2+}]_i$ decline and relaxation. For detecting the distribution of ROS production in cytoplasm, RoGFP was employed to evaluate hypoxia-altered generation of ROS after mitochondria transplantation in three different areas of cytoplasm including the areas close to cell membrane, the areas close to cell nucleus, and the areas in middle of membrane and nucleus. They have certified that the most prominent alteration of hypoxia-stimulated ROS seemed localized at subcellular areas close to cell membrane [53] (Fig. 1).

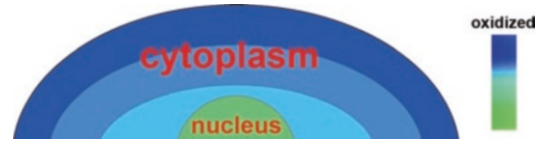

Fig. 1 The diagram of subcellular ROS levels in PASMC exposed to hypoxia.

The overall understanding and or speculation may be that "The intracellular distribution of mitochondria is not uniform and profoundly controls cellular behavior, including $[Ca^{2+}]_i$ signaling. The mitochondria localized in areas close to cytoplasma membrane were suggested to sense hypoxia and mediate downstream events in PASMCs" [53], and that "The transplanted mitochondria were found to be primarily localized in the areas close to cytoplasma membrane and this can partially explain why the hypoxia-induced cellular signaling and vascular responses were changed by exogenous mitochondria while endogenous mitochondria still retained in the cells" [149].

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Reactive Oxygen Species in COPD-Related Vascular Remodeling

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1 Introduction

Chronic obstructive pulmonary disease (COPD) is a global health issue and a progressive disease characterized by the persistent airflow limitation, inflammation, and airway obstruction [1]. COPD has been shown to associate with pulmonary vascular remodeling, an alteration of vasculature in the lungs as a result of environmental and/or cellular stimuli [1]. The subsequent vasoconstriction and pulmonary arterial resistance can exacerbate disease progression by limiting gas exchange [2]. Under physiological conditions, structural changes in the vasculature can be actively triggered by cellular processes such as cell growth and migration [3]. However, dysregulated vascular remodeling due to prolonged agitation in the lungs results in endothelial dysfunction and pulmonary hypertension (PH), both of which are manifested in several pulmonary diseases [4]. In fact, irritants such as air pollution and cigarette smoke are known to induce chronic inflammation in both pulmonary and systemic circulation, potentially contributing to atherosclerotic plaque progression and other cardiovascular events [4]. Smoking also interfere the revascularization [5]. In addition to smoking, animal studies showed that the administration of vascular endothelial growth factor (VEGF) receptor blocker can lead to lung cell apoptosis and significant airspace enlargement [6, 7]. VEGF is widely known for its role in angiogenesis [8]. It is suggested that lung microvascular endothelial structures such as alveolar septal capillary cells are particularly sensitive to the alteration of VEGF expression [1]. The loss of capillaries in the lungs due to decreased VEGF expression and endothelial cell apoptosis subsequently can impede efficient pulmonary gas exchange [9].

Oxidative stress is a predisposing factor in the pathogenesis of COPD [10]. The overproduction of reactive oxygen species (ROS) under agitated or diseased lung environments can result in endothelial cell damage, leading to vascular remodeling, as ROS are essential mediators of vascular homeostasis [11, 12]. Elevated ROS generation in COPD lungs are mainly attributed to external factors such as cigarette smoke and pollutant [13]. However, upon cessation of smoking or pollutant inhalation, a large amount of oxidative damage remains in the lungs [14, 15]. Increased mitochondrial-derived ROS stimulate intracellular Ca^{2+} production and muscle contraction,

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contributing to pulmonary vascular remodeling [11, 16]. The presence of oxidative stress in COPD promotes vascular smooth muscle cell (VSMC) proliferation along with extracellular matrix (ECM) inflammation and migration [17]. While the exact mechanisms remain unknown, the resultant dedifferentiated cells show less contractile function and demonstrate higher rates of migration, ECM synthesis, and proliferation. The process works in a vicious cycle continually sustaining and prompting excessive vascular remodeling [11, 18, 19]. Thus, this chapter particularly aims to elucidate the roles of vascular remodeling and ROS as well as their interactions in the pulmonary system in COPD conditions. While there are currently no sound strategy to reverse the adverse effects of pulmonary vascular remodeling and nor any substantial cure for COPD, several treatment options have been well proposed to alleviate symptoms related to vascular alteration in COPD, of which will be discussed as well.

2 Driving Forces of Pulmonary Vascular Remodeling

Morphological change in the vasculature is an active process. At cellular levels, vascular remodeling is mediated by various factors such as cell growth/death, cell migration, vasoactive forces, hemodynamic forces, and ECM fluctuations (Fig. 2) [3]. Vascular cell growth and apoptosis in response to the levels of growth factors are essential processes leading to angiogenesis and remodeling [1, 20]. Common vasoactive factors including angiotensin-II, endothelin-1, and thromboxane A_2 also play key roles in vascular remodeling by altering vascular tone and affecting the growth of smooth muscle [11]. Excessive vascular remodeling can be induced by stress (Fig. 2) [21]. Stress-induced dedifferentiation in VSMCs leads to dysregulation of vascular tone, blood pressure and blood flow [18, 19, 22]. Increased VSMC proliferation and migration contribute directly to the decreased diameter in small pulmonary arteries [23, 24]. The malfunction of ECM components in the vascular walls, including collagen and elastin, can further exacerbate vascular remodeling by promoting VSMC migration and proliferation [21]. For example, COPD-induced hypoxia suppresses elastin expression resulting in the increased vascular resistance [25]. The dynamic characteristics of vasculature are necessary to accommodate environmental fluctuations over time; yet prolonged changes in the hemodynamic conditions may lead to concerns.

Pulmonary vasculature is commonly subjected to remodeling in response to stresses such as inflammation and hypoxia, and sustained stresses may impede pulmonary function [26]. For instance, the degree of inflammatory cell infiltration in small airways is highly correlated with the severity of pulmonary vascular remodeling [27]. Chronic hypoxia contributes to vascular remodeling by promoting mammalian target of rapamycin complex 1 (mTORC1) activation and VEGF upregulation, accounting for a critical trigger for PH [23, 28]. Under hypoxic condition, pulmonary artery adventitial fibroblasts undergo active proliferation, stimulating VSMC proliferation and altering vascular wall structure [29]. Moreover, hypoxia can upregulate the generation of vasoconstrictive mediators, such as endothelin-1, thereby leading to increased vascular tone [23]. Dysregulated vascular remodeling has been implicated in various lung diseases [30]. Indeed, increased inflammatory cell infiltration in the airways and the adventitia of muscular pulmonary arterioles are observed in patients with COPD [27]. PH, a common condition that is comorbid with COPD, is characterized by various degrees of vessel wall thickening and pulmonary arterial resistance [31]. Oxidative stress is an essential pathogenic mechanism related to such abnormal vascular remodeling in pulmonary system. Thus, their interaction in the pathogenesis and progression of COPD and PH are prominent concerns, in which details will be discussed in the following sections.

3 Roles of ROS in Pulmonary Vascular Remodeling

ROS are byproducts of cellular metabolism that act as intracellular signaling molecules to modulate vascular function (Fig. 1) [32]. ROS can alter vascular tones by triggering the contraction of SMC or depleting endothelial-derived nitric oxide (NO) [28, 29]. The major ROS sources in the lungs include NADPH oxidase (NOX), xanthine oxidase (XO), and nitric oxide synthase [33]. NOXs are regarded as main ROS generators involved in the lungs' immune defense (Fig. 1). For example, deposition of high levels of NOX2-induced ROS into phagosomes, a process known as respiratory burst, is an effective immune response against microbial infection [34]. Similar to NOX, XO is an essential ROS generator in the pulmonary vasculature. Pulmonary vasculature is particularly susceptible to oxidative damage [11, 35]. Hyperoxia, or excess oxygen, can induce ROS generation and initiate associated oxidative damage in the alveoli. For example, mouse tissues have been shown to generate higher ROS levels when exposed to hyperoxic conditions in comparison to normoxic and hypoxic conditions [36]. However, hypoxia still stimulates ROS formation as a result of impaired mitochondrial complexes [37–39].

Chronic hypoxic vasoconstriction is associated with sustained inflammation and ROS accumulation in the lung parenchyma [28]. ROS can modulate several downstream signaling molecules including mitogen-activated protein kinases (MAPKs) and tyrosine kinases/phosphatases. The subsequent activation of signaling pathways that involve redox-sensitive transcription factor upregulation eventually leads to VSMC growth and migration and ECM deposition, both of which are essential determinants of vascular remodeling [11, 32]. ROS also elevate intracellular concentration of free Ca^{2+} thereby altering vascular reactivity [32]. In addition, the transcriptional regulation of several target genes is important for vascular remodeling under hypoxia [40]. For example, chronic hypoxia-induced ROS have been shown to mediate the expressions of multiple growth factors such as transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF), contributing to vascular alterations [41–43]. Individuals who were exposed to decreasing levels of hypobaric

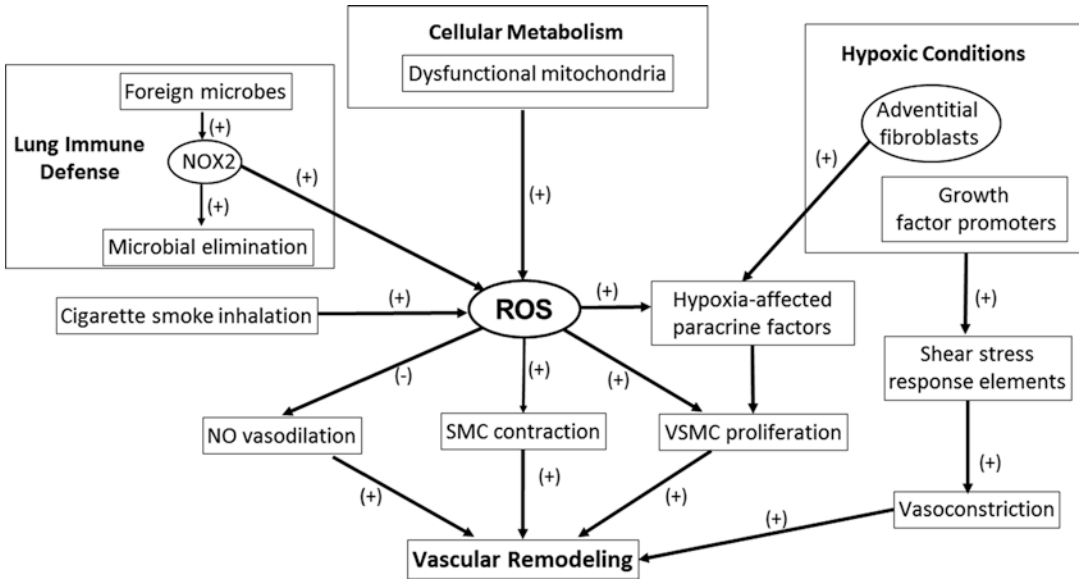

Fig. 1 This schematic illustrates potential sources of reactive oxygen species (ROS) production in vascular remodeling. *NO* nitric oxide, *NOX2* NADPH oxidase 2, *ROS* reactive oxygen species, *SMC* smooth muscle cell, *VSMC* vascular smooth muscle cell

hypoxia for a 6-week period have shown significantly higher pulmonary pressure than levels observed in response to acute hypoxia [44]. Hypoxia-activated pulmonary artery adventitial fibroblasts can generate ROS, which serve as paracrine factors to induce medial vascular wall hypertrophy and vasoconstriction [45]. Additionally, other paracrine growth factors can be released in the presence of ROS, resulting in VSMC proliferation [46]. For example, cyclophilin A, a molecular chaperone that regulate the acceleration of protein folding, can be induced by ROS and ERK1/2 in vascular SMC, stimulating VSMC growth in oxidative condition [46].

4 Pulmonary Vasculature Alterations in COPD and Their Association with ROS

Alterations in the structure of pulmonary vessels are indicative of COPD at all stages [47]. Intimal enlargement of pulmonary arteries in precapillary vessels can be found in patients with either advanced or mild COPD [48]. This arterial wall thickening causes a reduction in vessel diameter, restricting gas exchange and inducing PH, a known contributor to the decreased survival rates of COPD patients [49]. While typically associated with advanced COPD, arterial structural changes have also been observed in patients with mild COPD (without hypoxemia or low blood oxygen content) and smokers with baseline pulmonary functions. It is suggested that tobacco smoke exposure, a major risk factor of COPD, may be responsible for pulmonary vasculopathy in COPD [50]. Vascular changes found in patients with severe COPD in postmortem studies were also observed in patients with mild COPD, as well as smokers without obstructed airways [48]. In addition to vascular changes, Wright and Churg demonstrated that guinea pigs exposed to cigarette smoke developed PH before any damage from emphysema could even occur [51]. Further studies demonstrated that damage to the endothelial lining of pulmonary vessels through the inhibition of VEGF receptor 2 could induce signaling cascades ultimately resulting in pulmonary cell death and emphysema in rat models [6, 52]. Moreover, cigarette smoke constitutes various toxic chemicals that may be released in pulmonary microvasculature, leading to localized sequestration of neutrophils and inflammation [53]. While there are other possible causes for PH and emphysema in patients with COPD, such as genetic variation [54], the connection between oxidative stress and endothelial damage caused by tobacco use should be further studied.

The PH induced by the aforementioned arterial wall thickening may be irreversible and results in an increase in the distal muscularization of arteries and arterioles [55, 56]. Muscularization occurs when the VEGF is released by endothelial cells in response to increased blood pressure. As mentioned previously, VEGF can induce proliferation and migration of VSMCs, thereby altering the phenotype of vascular SMCs [57, 58]. Accordingly, Garcia-Lucio et al. and Zanini et al. both observed the association between pulmonary vascular remodeling and VEGF in human lung biopsies [59, 60]. The vascular transformation stems from the fact that VEGF, an angiogenic factor, can contribute to high blood pressure and resistance levels if not regulated properly (Fig. 2) [61]. To combat this, the body undergoes angiogenesis, allowing blood to be diverted to reduce the blood pressure. Studies have shown that dysregulated angiogenesis can also cause increased peripheral resistance [61]. However, VEGF expression is not the only indicator to determine the severity of a patient's COPD. Another potential indicator involves the amount of inflammatory cell infiltration of pulmonary cells. In particular, the presence of large amounts of cytotoxic T cells (CD8+ T cells) has been shown to correlate to the progression of a patient's COPD pathogenesis (Fig. 2). A study performed by Cornwell et al. showed that CD8+ cells secrete Tc1 cytokines that upregulate the production of interferon (IFN)-gamma, which is a known cause of autoinflammatory and autoimmune diseases [62, 63]. While these add insight into the pathogenesis of COPD, it is crucial to understand the consequences

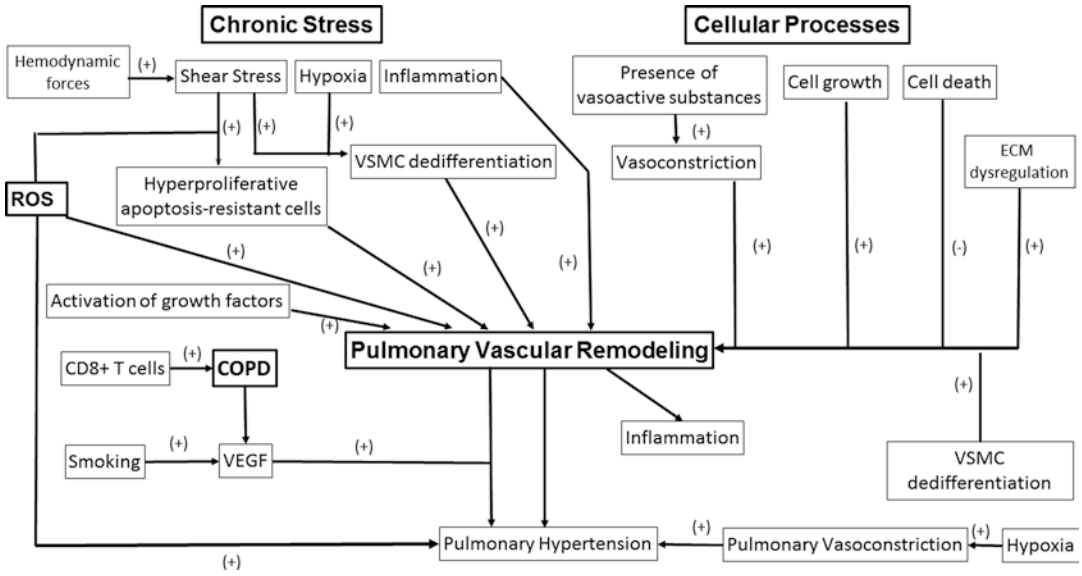

Fig. 2 This schematic illustrates cellular alterations leading to vascular remodeling. *COPD* chronic obstructive pulmonary isease, *ROS* reactive oxygen species, *VEGF* vascular endothelial growth factor, *VSMC* vascular smooth muscle cell

of COPD severity at various levels. For instance, in more severe cases of COPD, vascular endothelial cells show reduced vasorelaxation which stems from a lack of production of endothelium-derived hyperpolarizing factor (EDHF), leading to hypertension and angiogenesis [64]. The damage to endothelial cell function leads to a reduced sensitivity of pulmonary arteries to hypoxia induced by COPD. When this occurs, the body fails to regulate the ventilation/perfusion ratio, which relates the amount of air reaching the alveoli to the amount of blood diffusing from the capillaries into the alveoli. A divergence from this ratio propagates arterial hypoxemia and can ultimately lead to respiratory failure [65].

PH is an important factor in the overall prognosis of patients with COPD (Fig. 2). Due to the lack of precision of noninvasive detection methods, such as Doppler echocardiography, in documented severe COPD patients, the exact prevalence of PH has ranged from 30 to 70% [54, 66]. However, it is essential to focus on this group of patients as PH can have a strong negative effect on long-term survival. One study found that COPD patients with median pulmonary artery pressure of >20 mmHg had a 49% 4-year survival rate, while those without PH had a 4-year survival rate of 72% [67]. Multiple factors contribute to the development of PH including destruction of capillary beds from emphysema, thromboembolic disease, and pulmonary vascular remodeling [68, 69]. PH is strongly correlated to the muscularization of arterioles, shown by increased numbers of small pulmonary vessels with double elastic lamina [48, 51]. A key factor in the increase of pulmonary vascular resistance, which manifests itself as elevated pulmonary arterial pressure, is hypoxic pulmonary vasoconstriction [68, 69]. Studies also observed greater radical-initiated lipid peroxidation and DNA/protein oxidation in PH patients, indicating the important involvement of ROS in the pathophysiology of PH [70, 71]. While the extent of pulmonary vasoconstriction development appears to be based on length and severity of hypoxia, the mechanism is not fully understood [68]. A connection does appear to exist between direct and indirect effects that lowered oxygen levels have on smooth muscles in pulmonary vasculature [68]. In addition to the pulmonary vascular reconstruction and hypoxia-induced vasoconstriction, both inflammation and oxidative stress contribute

to PH in patients with COPD, whether working independently or concurrently [69, 71–73]. Excess ROS promote janus kinase 2 (JAK2) signaling pathways and result in enhanced endothelial dysfunction in hypertension [71]. The correlation between inflammation, COPD, and hypoxia-induced oxidative stress remains a fairly controversial topic. However, the fact that inflammation and hypoxia cause atherosclerosis indicates they may be contributing factors to PH [74].

5 Intervention for Prolonged Life in COPD Patients

As COPD becomes one of the major causes of death in the world, it is of the utmost importance to develop treatments that prolong the lives of COPD patients [69]. Standard treatments of COPD and/or COPD-associated PH include smoking cessation, medications, pulmonary rehabilitation, and long-term oxygen therapy (LTOT), all of which aim to alleviate specific COPD symptoms.

5.1 Smoking Cessation

Attempting to quit smoking may be a simple and effective way of lowering COPD morbidity and mortality, as cigarette smoke is a main risk factor for COPD [53]. As a complement to smoking cessation, nicotine replacement therapy (NRT) medications can both lower the discomfort associated with tobacco withdrawal and promote successful quitting [53]. However, the application of NRT requires further clinical considerations as nicotine is known to stimulate ROS production by damaging epithelial cells and/or increasing NOX expressions in macrophages [75]. Nicotine-induced ROS can potentially deplete antioxidant levels in the lungs [76]. In addition, nicotine alone is sufficient to modulate fibroblast activation, potentiating fibrosis via enhanced collagen production [75]. Despite the pathophysiological roles of nicotine, NRT is considered as a safe aid for smoking cessation. This is likely due to the avoidance of other potent components (e.g., oxidants) in cigarette smoke that may induce and sustain severe oxidative stress. Thus, additional studies should be performed to evaluate the long-term usage of NRT [75].

5.2 LTOT

Oxygen therapy is a noninvasive treatment option that has been widely used in various pulmonary diseases or conditions. Increased tissue oxygenation can stimulate angiogenesis and anti-inflammatory responses [77]. In particular, LTOT is found to increase the life span of COPD patients with hypoxemia and is the only treatment available that slows the progression of PH [50]. Hypoxemia arises in patients with COPD due to limitations in airflow as well as emphysema-related damage in the pulmonary capillary beds [69]. LTOT reduces pulmonary artery pressure and vascular resistance, thereby increasing blood flow and subsequently lowering the severity of hypoxemia and PH in patients with moderate to severe COPD [50, 69]. In two major randomized clinical trials of LTOT (British Medical Research Council trial and Nocturnal oxygen therapy trial), the survival rates of COPD patients with hypoxemia were shown to increase proportionally with the duration of oxygen therapy. However, there are not sufficient correlations between the treatment of hemodynamic factors, PAT and VR, and the increased survival rate of COPD patients. In the Nocturnal Oxygen Therapy trial, patients with high levels of pulmonary artery pressure experienced slight improvement in the hemodynamic levels but not increased survival rate [78, 79]. Furthermore, autopsy suggests that there is no significant difference between pulmonary vascularity in LTOT users vs. nonusers. This indicates that patients experience hemodynamic stabilization and that no permanent structural changes occur as a result of LTOT treatment [50]. LTOT has also been shown to alleviate dyspnea and increase exercise tolerance in COPD patients when compared to the results of compressed air administration [80]. Diminished occurrences of dyspnea may be attributed to a decrease in dynamic hyperinflation

and ventilation rate per minute [80]. Dynamic hyperinflation, described by a new breath occurring before static lung equilibrium has been reached, is commonly caused by increased airway resistance and decreased alveolar attachments in COPD [80, 81]. Therefore, the decreased dynamic hyperinflation and minute ventilation after LTOT contribute to the attenuation of dyspnea by inducing the return of normal breathing patterns.

Despite the aforementioned beneficial effects of LTOT in COPD, prolonged tissue oxygenation may inevitably induce ROS production and exacerbate the existing oxidative stress [82]. Accordingly, Kaplan et al. observed an accumulation of bityrosines, a marker of protein modifications, and mitochondrial oxidative damages in guinea pigs subjected to long-term oxygenation [77]. Other studies reported that supplemental oxygen enhances airway inflammation and the expressions of biomarkers associated with oxidative stress (e.g., breath methylated alkane contour) [82–84]. Interestingly, long-term inhalation of partially ionized oxygen (positively charged) has been shown to greatly lower the adverse effects and oxidative stress observed in molecular oxygen treatment, indicating a potential of optimizing oxygen therapy [77, 85]. Moreover, LTOT can accentuate hypercapnia through diminished ventilator drive, increased ventilation, or perfusion mismatch [69, 80]. Regardless of potential toxicities and side effects, LTOT remains to be one of the most favorable COPD treatments due to its noninvasiveness and unequivocal survival benefits in COPD patients. However, LTOT requires further refinement, in particular, since oxidative stress-related injury is well implicated in COPD pathophysiology [82].

5.3 Endothelium-Targeted Treatment

Despite the effectiveness of LTOT, it only provides temporary therapeutic effects in regard to hemodynamic levels or arterial damage. Other treatment options are therefore being considered [49]. Studies have suggested that treating pulmonary hypertension can alleviate COPD symptoms, as PAH is commonly comorbid with COPD [49]. Drugs for PAH, belonging to the classes of ET-1 receptor antagonists, prostanoids, and phosphodiesterase-5 inhibitors, are vasodilators and anti-proliferators. Both have been shown to reduce arterial tension and vascular resistance in COPD patients thereby lowering hypoxemia [86]. However, the improvements in COPD-related PH were only observed in severe cases of COPD [86]. An endothenin-1 receptor antagonist, bosentan, has shown promises in preclinical trials in lowering pulmonary pressure but was not successful in clinical trials [87]. Further controlled studies are needed to define the relationship between PH treatment drugs and their potential use in relieving symptoms of COPD [49]. The administration of traditional vasodilators such as angiotensin-II antagonists and calcium channel blockers is not recommended due to the potential for adverse alterations to gas exchange and hypoxic vasoconstriction ability [49].

5.4 Pharmacologic Treatments

5.4.1 Statins Therapy Improves Pulmonary Hemodynamics

Currently, statins are administered to COPD patients as a mean to prevent secondary development of cardiovascular diseases. In addition to the main effect of lowering lipid levels, statins are responsible for modulating aortic stiffness and attenuating inflammation [88]. Several studies have demonstrated that statins decrease the amount of inflammatory mediators, such as C-reactive proteins, which are major indicators of morbidity and mortality in COPD [89]. Additional studies are necessary to determine if the reduction in mortality of COPD patients is due to the induced anti-inflammatory environment or by reducing any potentially unknown comorbid cardiovascular disease [90]. Moreover, statins treatments increase effectiveness in individuals with high baseline aorta stiffness compared to those with normal stiffness levels [88]. Statins also reduce pulmonary artery wedge

pressure and mean pulmonary arterial pressure in COPD patients, thereby decreasing the severity of symptoms [91]. Additionally, statins are known for its antioxidant activities. Statins therapy has been shown to downregulate NOX expressions and subsequently reduces ROS production in the vasculature [92, 93]. Similarly, Shishehbor et al. reported that statins suppress the formation of NO-derived and myeloperoxidase-derived oxidants, both of which are implicated in atherogenesis, and promote systemic antioxidant activities [94]. Decreased ROS release by statins is also essential in attenuating endothelial dysfunction [93]. It is likely that statins confer both anti-inflammatory and antioxidative activities in alleviating oxidative damage and vascular alteration in COPD. However, limited studies have focused on the antioxidative effects of statins using COPD models [92, 95]. Taken together, statins provide promising therapeutic results for patients with COPD, but further studies are required to better understand how statins directly affect COPD and improve pulmonary hemodynamics, especially in the context of its antioxidative ability.

5.4.2 Antioxidant Therapeutics Reduce Pulmonary Inflammation and Remodeling

It is well understood that ROS produced from chronic cigarette smoke and inflammation, along with altered antioxidant expressions, are tightly associated with COPD pathophysiology [53]. Therefore, it is plausible to consider antioxidants as directed treatments against ROS-related vascular remodeling in COPD patients. Indeed, antioxidant *N*-acetylcysteine (NAC) has been shown to lower c-jun n-terminal kinase (JNK) activity, one of the redox-sensitive signaling proteins involved in vascular remodeling [32]. Superoxide dismutase (SOD), which is widely expressed in the lungs, demonstrates vasodilator effects by scavenging excess ROS and increasing NO bioavailability. Catalase further eliminates H_2O_2 to counterbalance oxidizing environment in COPD lungs [11, 71]. Both SOD and catalase also reduce inflammation through inhibiting the activation of nuclear factor kappa B (NFκB), thus serving as effective candidates for antioxidant therapy in COPD or PH [11, 32]. In addition, a diet with cruciferous vegetables, which consist of natural antioxidants, can help restoring endogenous oxidative balance by enhancing intracellular antioxidant defenses via nuclear factor erythroid 2-derived factor 2 (NRF2) activation [53]. Collectively, antioxidant therapies for mitigating inflammation and vascular remodeling in COPD are promising areas of studies. Further understanding of pharmacokinetics and pharmacodynamics of exogenous antioxidants, endogenous antioxidant activators, and redox-sensitive transcription factor targets will provide valuable and consistent results in similar studies.

6 Conclusion

Vascular remolding is one of the main characteristics of COPD. Stresses such as hypoxia and cigarette smoke can induce pulmonary vascular remodeling by generating oxidative stress and cellular alterations. Due to the predisposed tendency towards oxidation in lung diseases, VSMC can proliferate and lower vascular function, negatively affecting pulmonary hemodynamics and exacerbating the severity of COPD. Elevated ROS in the diseased lungs also damage vascular endothelial cells and promote ECM deposition, all of which are closely associated with vascular remodeling. ROS are essential mediators of vascular homeostasis. As ROS overproduction and antioxidant dysregulation are manifested in COPD, it is reasonable to infer the interrelationship between pulmonary vascular remodeling and ROS and their roles in COPD pathogenesis. Although no cure is currently available for COPD, treatments to lessen the effect of vascular remodeling by restoring oxidative balance in the lungs have been developed with varying degrees of success. LTOT, endothelium targeted medications, and pharmacological antioxidant treatments have shown promises in alleviating COPD symptoms and extending the life span of patients.

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