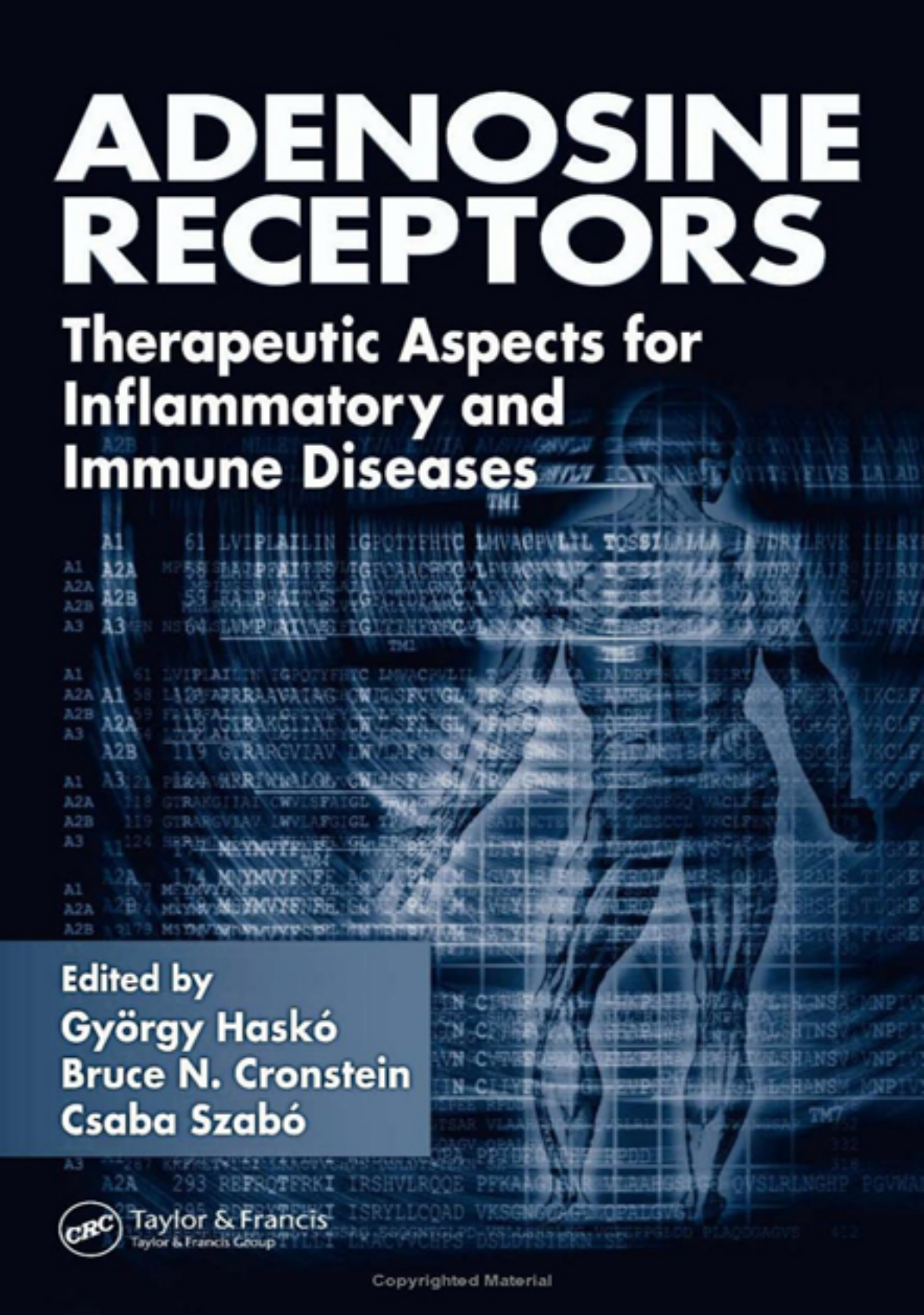
ADENOSINE RECEPTORS

Therapeutic Aspects for Inflammatory and Immune Diseases



Edited by
György Haskó
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Csaba Szabó

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Preface

ADENOSINE: 100 YEARS OLD AND GOING STRONG!

The purine nucleoside adenosine is a modulatory substance that has been intensively studied for about a century by investigators from different biomedical areas because of the plethora of its actions on organs and tissues. Several lines of evidence now indicate that the ability of adenosine to control inflammatory cells plays a key role in the modulatory effects of adenosine in both health and disease. Adenosine indeed has a major impact on the functions of the inflammatory and immune systems. There are many promising emerging therapeutic approaches that focus on the modulation of adenosine, including compounds that interfere with the breakdown of adenosine, as well as specific agonists and antagonists of various adenosine subtypes. Some of these compounds are in intense preclinical investigations, whereas others have already entered clinical trials. In recent years it also became apparent that adenosine is responsible for the anti-inflammatory effect of some compounds that are in therapeutic use, including the antirheumatic disease-modifying agent methotrexate.

This recent interest and emerging widespread awareness of the effect of adenosine in the control of the inflammatory and immune systems covers some of the most active areas of biomedical research (heart, vessels, lung, intestine, kidney, skin, and brain). It is expected that this basic knowledge will generate new therapeutic modalities in the very near future for pathologies with large incidence and of major socioeconomic impact such as ischemia and reperfusion (vascular injury and transplants), atrial fibrillation, heart disease, wound healing, tumors, atherosclerosis, pain, and a variety of central nervous system diseases (Parkinson's and Alzheimer's diseases, epilepsy, mood disorders, and sleep disorders).

The data on the role of adenosine in inflammatory and immune responses are being published in a large number of generalist and specialist scientific journals and have not yet been compiled comprehensively into a single publication. This book contains a compilation of reviews on how adenosine, acting at its cellular receptors, regulates immune responses. The book provides the reader with a general overview of adenosine receptors, covering aspects of molecular biology, cell biology, and pharmacology. Separate chapters focus on the role of adenosine receptors in regulating the function of the various cell types that are involved in immune responses. Further chapters delineate the role of purinergic signaling in the pathophysiology of a variety of disease states that are associated with an overzealous or insufficient immune response. These include autoimmune diseases, asthma, atherosclerosis, ischemia-reperfusion injury, and cancer. The current book is intended to serve as a useful starting point for investigators entering the field of adenosine and inflammatory disease, and also as a handy reference for those already active in the field.

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Karl-Norbert Klotz

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1.1 INTRODUCTION

Adenosine is a building block of RNA and many small biomolecules such as ATP and NADH, and at the same time it serves as a regulator of tissue function in practically every cell of the human body. The main source of adenosine is from degradation of adenine nucleotides[1,2] and, therefore, its regulatory functions are intricately linked to the energy balance of organs with a high energy demand. A large family of purinergic receptors may respond to ATP, ADP, and adenosine, and thereby orchestrate the respective metabolic requirements of a cell (Figure 1.1).

The first documented physiological function of adenosine was the inhibition of AV conduction in mammalian heart and was discovered almost 80 yr ago.[3] This and all other known effects of adenosine are mediated via four subtypes of G-protein-coupled receptors named A_1, A_{2A}, A_{2B}, and A_3 (for a recent review see Reference 4). Adenosine receptors are also classified as P1 receptors in order to distinguish them from the large P2 subfamily of purinergic receptors specific for ATP, ADP, and other nucleotides.[5,6] Adenosine plays the most important regulatory roles in the cardiovascular system, kidney, and CNS, which are briefly discussed in this introductory chapter.

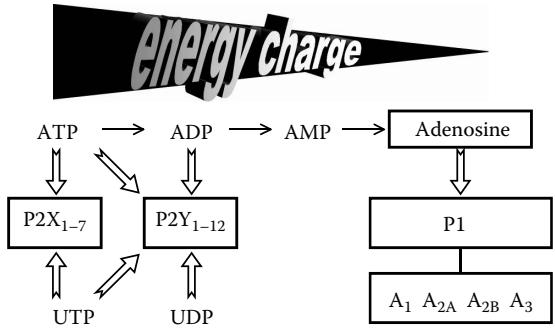

FIGURE 1.1 Adenosine receptors and the energy balance. Adenosine production goes up as increasing amounts of energy in the form of ATP are consumed. An intricate network of receptors for adenosine and adenosine nucleotides is stimulated by different amounts of agonists whose concentrations reflect the metabolic state of a tissue. P2 receptors are also stimulated by additional nucleotides; therefore, UTP/UDP is given as an example. The picture for P2 receptors is simplified, and many other ligands such as diadenosinepolyphosphates or UDP-glucose have been identified as ligands for P2X and P2Y receptors, respectively.

Owing to these profound actions, adenosine receptors are promising drug targets in these and other organ systems for the treatment of numerous diseases.[7–10]

Large amounts of adenosine may be produced in pathophysiological conditions, making adenosine receptors an interesting target also in situations that are associated with cell damage, including hypoxia or inflammatory diseases, which are the subject of this book. Recent evidence shows that adenosine may also have a potential role in cell growth and proliferation and may therefore play an important role in conditions leading to the development of cancer (for a review see Reference 11).

1.2 ADENOSINE RECEPTOR SUBTYPES

1.2.1 Signaling

The primary effector of all four subtypes of adenosine receptors is adenylyl cyclase, whose activity is either stimulated or inhibited depending on the receptor subtype present on a cell. A_1 and A_3 receptors mediate an inhibitory signal via $G_{i/o}$, resulting in decreasing levels of cAMP, whereas the A_{2A} and A_{2B} subtypes stimulate adenylyl cyclase by activation of G_s with a consequent increase of cAMP. Signaling pathways in addition to the modulation of cAMP levels have been described for all four receptor subtypes (for an overview see Reference 4). The inhibitory A_1 and A_3 subtypes may induce a $G_{\beta\gamma}$-dependent activation of PLC with a consecutive Ca^{2+} signal[12–14] and activation of MAP-kinase signaling.[15–17] They have also been shown to trigger opening of K^+ channels[18,19] and inhibition of Ca^{2+} channels, which leads to inhibition of neurotransmitter release via A_1 receptors.[19] These effects are in analogy to other G_i-coupled receptors such as M_2 acetylcholine receptors or α_2 adrenergic receptors. Both A_{2A} and A_{2B} adenosine receptors may also mediate a Ca^{2+} response. The actual pathway leading to an intracellular increase of Ca^{2+} seems to

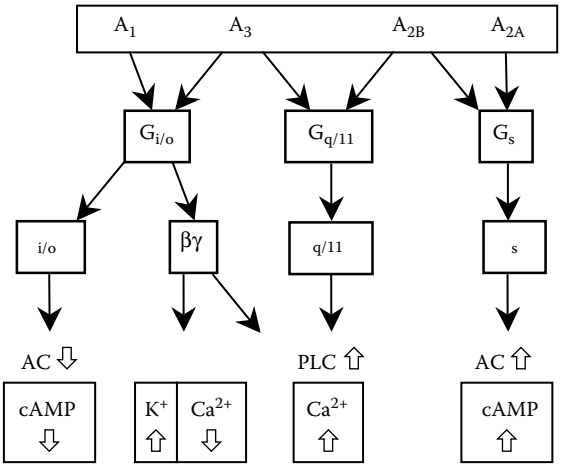

FIGURE 1.2 Signaling pathways for adenosine receptor subtypes.

be dependent on the cell type, as in the case of A_{2B} both coupling via G_q[20,21] as well as signaling independent of G_q[22] have been described (Figure 1.2). For both A_2 subtypes, coupling to the MAP-kinase pathway was demonstrated.[15]

1.2.2 Structure

The human A_1, A_{2B}, and A_3 adenosine receptors are very similar in length (326, 332, and 318 amino acids, respectively), whereas the A_{2A} adenosine receptor has 412 amino acids on account of a C-terminal extension. Figure 1.3 shows an amino acid sequence alignment and reveals a high sequence identity in the seven transmembrane domains of up to 88% and a homology reaching 100% if pairs of receptor subtypes are compared. The overall identity between pairs of the human subtypes is about 50%. All subtypes possess a pair of conserved cysteines (C80 and C169 in the A_1 sequence), which most likely form a disulfide bond. All but the A_{2A} subtype bear a C-terminal cysteine (C309 in A_1, C311 in A_{2B}, and C303 in A_3) that may serve as a site for a potential lipid modification.

Comparison between species reveals the largest variation for the A_3 adenosine receptor, which plays an important role in ligand recognition. It has been shown that some high-affinity antagonists at the human A_3 subtype do not bind at all to the rat A_3 receptor.[23,24] Also, the selectivity of some agonists for A_3 receptors is highly dependent on the species. This is of critical importance for the interpretation of pharmacological experiments that attempt to identify the receptor subtypes involved in an adenosine-mediated physiological or pathophysiological event. Such pharmacological characterization with binding and functional studies is still the most important method for unambiguous classification of adenosine receptor subtypes.

Human adenosine receptors are well characterized *in vitro*,[25] and a large panel of ligands suitable for subtype characterization is available. Nevertheless, it is still a challenge to unambiguously characterize a subtype involved in a specific response

```
A1   1      MPPSISAFQy AYIGIEVLIA LVSVPGNVLV IWAVKVNQAL RDATFCFIVS LAVADVAVGA    60
A2A  1        MPIMGSS VYITVELAIA VLAILGNVLV CWAVWLNSNL QNVTNYFVVS LAAADIAVGV    57
A2B  1        MLLETQDA LYVALELVIA ALSVAGNVLV CAAVGTANTL QTPTNYFLVS LAAADVAVGL    58
A3   1 MPN NSTALSLANV TYITMEIFIG LCAIVGNVLV ICVVKLNPSL QTTTFYFIVS LALADIAVGV    63
                                 TM1                                 TM2

A1      61 LVIPLAILIN IGPQTYFHTC LMVACPVLIL TQSSILALLA IAVDRYLRVK IPLRYKMVVT   120
A2A     58 LAIPFAITIS TGFCAACHGC LFIACFVLVL TQSSIFSLLA IAIDRYIAIR IPLRYNGLVT   117
A2B     59 FAIPFAITIS LGFCTDFYGC LFLACFVLVL TQSSIFSLLA VAVDRYLAIC VPLRYKSLVT   118
A3      64 LVMPLAIVVS LGITIHFYSC LFMTCLLLIF THASIMSLLA IAVDRYLRVK LTVRYKRVTT   123
                                                 TM3

A1     121 PRRAAVAIAG CWILSFVVGL TPMFGWNNLS AVER----AW AANGSMGEPV IKCEFEKVIS   176
A2A    118 GTRAKGIIAI CWVLSFAIGL TPMLGWNNCG QPKE----GK NHSQGCGEGQ VACLFEDVVP   173
A2B    119 GTRARGVIAV LWVLAFGIGL TPFLGWNSKD SATNNCTEPW DGTTNESCCL VKCLFENVVP   178
A3     124 HRRIWLALGL CWLVSFLVGL TPMFGWNNMKL TSEY----HR NVT------F LSCQFVSVMR   173
                        TM4

A1     177 MEYMVYFNFF VWVLPPLLLM VLIYLEVFYL IRKQLNKKVS A--SSGDPQK YYGKELKIAK   234
A2A    174 MNYMVYFNFF ACVLVPLLLM LGVYLRIFLA ARRQLKQMES QPLPGERARS TLQKEVHAAK   233
A2B    179 MSYMVYFNFF GCVLPPLLIM LVIYIKIFLV ACRQLQR--T E-L-MDHSRT TLQREIHAAK   234
A3     174 MDYMVYFSFL TWIFIPLVVM CAIYLDIFYI IRNKLSLNLS ---NSKETGA FYGREFKTAK   230
                        TM5

A1     235 SLALILFLFA LSWLPLHILN CITLFCPSC- -HKPSILTYI AIFLTHGNSA MNPIVYAFRI   292
A2A    234 SLAIIVGLFA LCWLPLHIIN CFTFFCPDC- SHAPLWLMYL AIVLSHTNSV VNPFIYAYRI   292
A2B    235 SLAMIVGIFA LCWLPVHAVN CVTLFQPAQG KNKPKWAMNM AILLSHANSV VNPIVYAYRN   294
A3     231 SLFLVLFLFA LSWLPLSIIN CIIYFN---G -EVPQLVLYM GILLSHANSM MNPIVYAYKI   286
                        TM6                                 TM7

A1     293 QKFRVTFLKI WNDHFRCQPA PPIDEDLPEE RPDD                              326
A2A    293 REFRQTFRKI IRSHVLRQQE PFKAAGTSAR VLAAHGSDGE QVSLRLNGHP PGVWANGSAP   352
A2B    295 RDFRYTFHKI ISRYLLCQAD VKSGNGQAGV QPALGVGL                          332
A3     287 KKFKETYLLI LKACVVCHPS DSLDTSIEKN SE                                318


A2A    353 HPERRPNGYA LGLVSGGSAQ ESQGNTGLPD VELLSHELKG VCPEPPGLDD PLAQDGAGVS   412
```

FIGURE 1.3 Sequence comparison of the human adenosine receptor subtypes. Amino acids are shown in bold if identical in all four subtypes. The seven transmembrane domains are marked with TM1–TM7.

owing to limitations in selectivity of some of the most commonly used agonists and antagonists (see the following text).

1.3 ADENOSINE RECEPTOR LIGANDS

1.3.1 Agonists

Adenosine receptor agonists are all structurally derived from adenosine (for an exception, see the following text). For preservation of agonistic activity of adenosine, modifications are tolerated only in the 5′-position (and some minor modifications of the 2′-position) of the ribose and substitutions at the 2- and N^6-positions of the purine structure (Figure 1.4). Other modifications of the parent molecule result in complete loss of affinity. Some examples of antagonists derived from the adenosine structure are also known.[26–28] For each subtype, more or less selective agonists are known. Some of the most common structures are summarized in Figure 1.5.

The prototypical A_1 selective agonists are CCPA, CPA, and R-PIA. Generally, the N^6-substitution results in excellent selectivity toward the A_1 subtypes and limited selectivity toward A_3. All these compounds were developed before the A_3 receptor was known and thus appeared to be excellent A_1 agonists. With the discovery of the

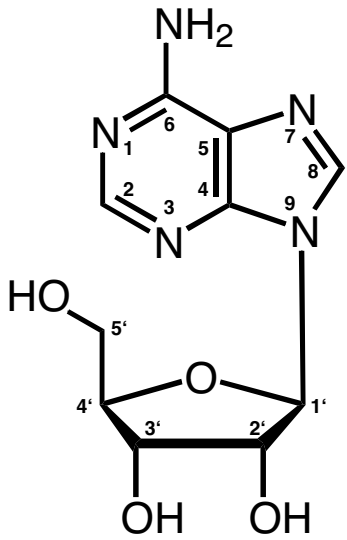

FIGURE 1.4 Structure of adenosine.

A_3 subtype, they lost some of their glory, but better agonists with selectivity, in particular, for the human A_1 receptor have not been found since. An exception seems to be a novel class of agonists that are not derivatives of adenosine.[29] These new agonists are based on structures that were found in high-throughput screening of compound libraries and described in a patent by Bayer.[30]

Although the 2-substituted derivative CGS 21680 is considered to be an A_{2A}-selective agonist, it turned out that in the case of human adenosine receptor subtypes it distinguishes A_{2A} well from A_{2B} receptors but not from the other subtypes.[25] Truly A_{2A}-selective agonists are still to be developed. However, as both A_{2A} and A_3 receptors seem to be involved in regulation of inflammatory processes,[31–33] mixed A_{2A}–A_3 agonists may be of therapeutic interest.

The availability of agonists for A_{2B} adenosine receptors is unsatisfactory as no high-affinity agonist is known. The most potent compounds are nonselective agonists such as NECA or PHPNECA with potencies around 1 μM.[34] It seems that adenosine has an equally low potency at the A_{2B} subtye,[35] which would mean that only pathophysiologically high adenosine levels are able to stimulate a response through this receptor. This has led to the speculation that the A_{2B} receptor is operative only in such pathological states, making it an even more interesting candidate for drug therapy. There is evidence, however, that not all responses to adenosine occur with low potency, opening the possibility that the activation of a specific signaling pathway is dependent on the concentration of adenosine. It was shown by Schulte and Fredholm[15] that A_{2B}-mediated ERK1/2 phosphorylation occurs at much lower concentrations than an increase in cAMP.

A large number of agonists with selectivity for A_3 adenosine receptors has been developed since the discovery of this subtype in 1992.[36] In general, it appears that 5′-modification with an *N*-methyl or *N*-ethyl carboxamide is beneficial for A_3 potency.

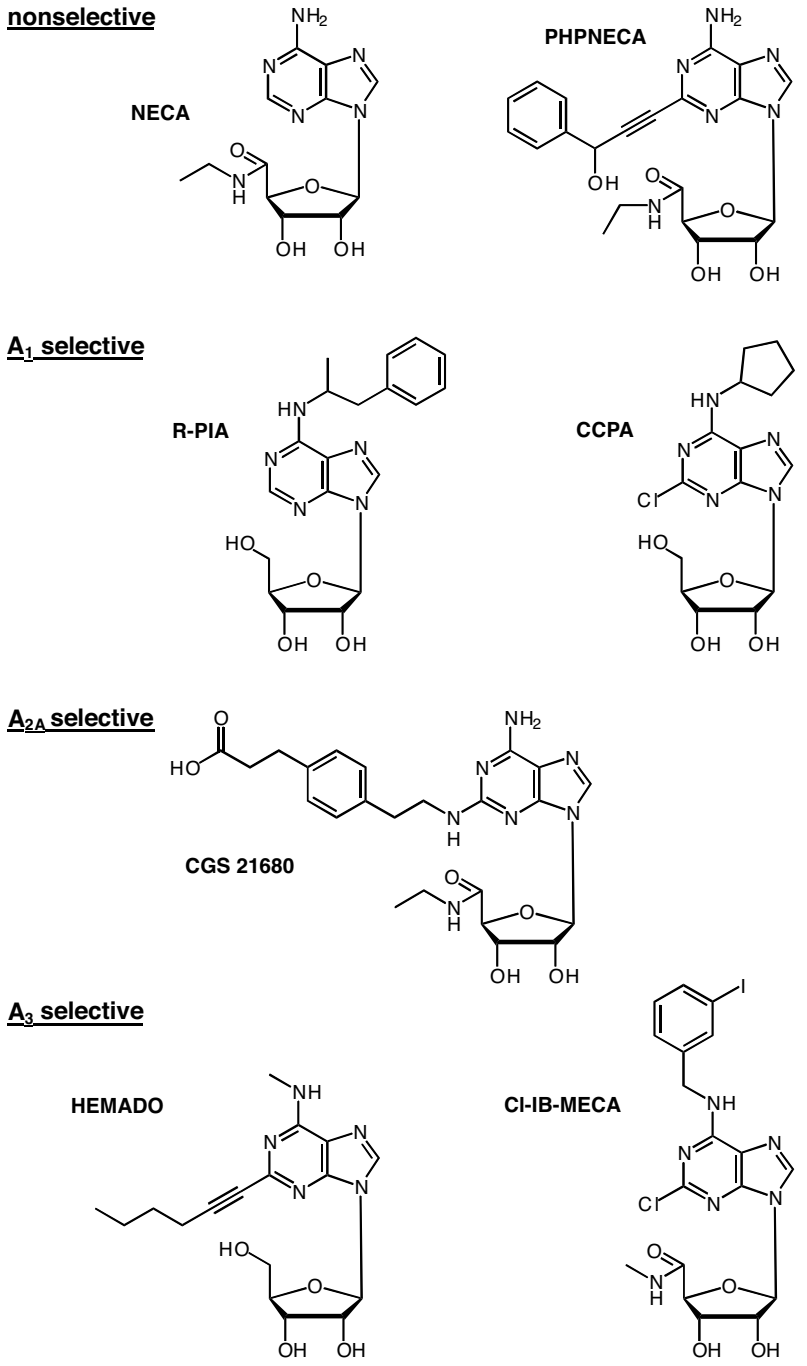

FIGURE 1.5 Adenosine receptor agonists.

One of the first agonists introduced as a selective agonist was the N^6-substituted compound Cl-IB-MECA,[37] which shows good A_3 selectivity in the case of rat receptors but is less selective for the human A_3 compared to the human A_1 receptor.[34] PENECA is another adenosine derivative bearing a 5′-modification, whereas the purine moiety is modified in the 2-position. In contrast to the 2-substituted nonselective compound PHPNECA, PENECA shows good A_3 selectivity.[34] Recently, a number of compounds with an unmodified ribose and high A_3 affinity and selectivity were found.[38] Compound 8a (HEMADO, 2-hexyn-1-yl-N^6-methyladenosine) from this study was also tritiated and shown to be a useful high-affinity radioligand for the human A_3 adenosine receptor with a K_D value of 1 n*M* (Klotz et al., 2006 manuscript in preparation).

1.3.2 Antagonists

Classical adenosine receptor antagonists are derived from the naturally occurring methyl xanthines caffeine and theophylline. This structure was modified extensively to yield ligands with high affinity and selectivity for specific receptor subtypes. Figure 1.6 shows representatives of this class of compounds.

The efforts to identify nonxanthine stuructures were initiated before most subtype-selective xanthines were known. The first compound was the nonselective triazoloquinazoline CGS 15943.[39] Many of the nonxanthines developed in recent years are structurally related to this compound (Figure 1.6). Over the years numerous nonxanthine antagonists were characterized, including compounds not related to CGS 15943 such as dihydropyridines,[40] adenine derivatives,[41–43] or also some adenosine derivatives.[26–28]

One of the most A_1-selective xanthine derivatives is DPCPX.[44,45] Unfortunately, the affinity for the human A_1 is lower than for the rat counterpart.[25] Another drawback of this compound is its relatively high affinity for A_{2B} receptors,[46] which makes it only about tenfold A_1 selective compared to this subtype.

The most prominent A_{2A}-selective antagonists are the xanthines BS-DMPX[47] and MSX-2,[48] and the nonxanthine SCH 58261.[49] These ligands show high affinity and selectivity and are valuable tools for blocking A_{2A} receptors without affecting the function of the other subtypes.

Recently, several reports presented antagonists with selectivity for the A_{2B} receptor.[50–53] Currently, blocking the action of nonselective agonists with subtype-selective antagonists is the most reliable approach to identifying A_{2B}-receptor-mediated signaling.

Initially, the A_3 adenosine receptor was thought to be insensitive to xanthines. Although it binds some of the classical nonselective xanthines such as theophylline only with very low affinity,[25] xanthines such as I-ABOPX (3-(4-amino-3-iodophenyl)-8-(4-oxyacetate)phenyl-1-propylxanthine) with higher affinity for the human A_3 have been found in recent years.[20,54,55] The vast majority of A_3-selective antagonists, however, are derived from the triazoloquinazoline and similar structures. Some of the most prominent examples are MRS 1220[56] or MRE 3002F20.[57] In addition, numerous structurally diverse antagonists have been developed for A_3 receptors that do not show appreciable affinity for the other adenosine receptor subtypes.[24,54,55]

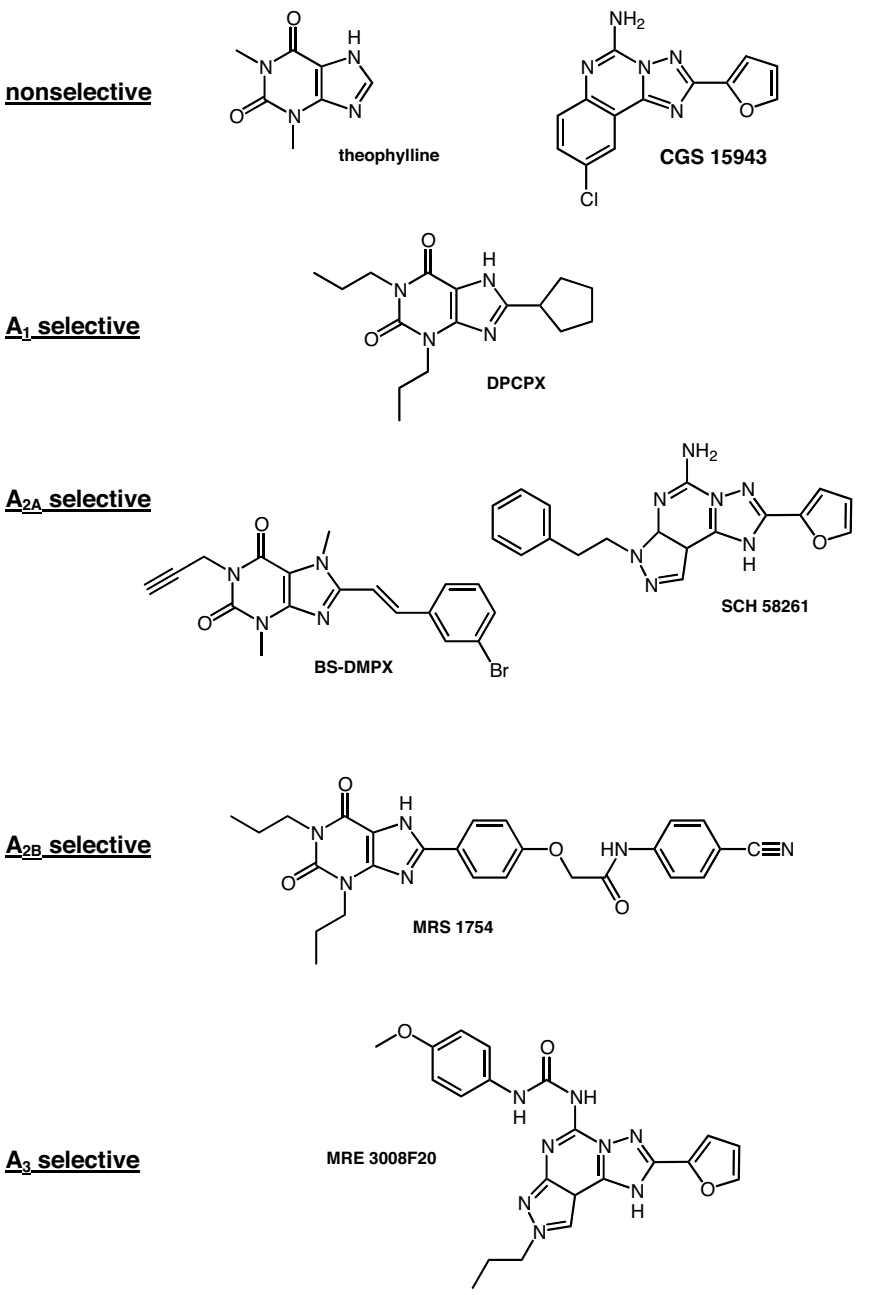

FIGURE 1.6 Adenosine receptor antagonists.

1.4 PHYSIOLOGICAL FUNCTIONS OF ADENOSINE

1.4.1 Cardiovascular Actions

The effects of adenosine on heart function were denoted as antiadrenergic[58] because the A_1-receptor-mediated actions on cardiomyocytes are indeed opposite to the effects induced by activation of the sympathetic nervous system and stimulation of β_1-adrenergic receptors by noradrenaline and are not seen in unstimulated tissue. Obviously, this is the expected response as the G_i-coupled A_1 receptor will counteract the adrenergic response mediated via the G_s-coupled β_1-receptor. In addition to this inhibitory response, adenosine acts on vascular tone and is the most important vasodilator in coronary arteries.[59] This effect on vascular smooth muscles is mediated via A_{2A} adenosine receptors[60] and provides important evidence of adenosine's role in maintaining the energy balance of the heart: As energy and ATP consumption increase, more adenosine is produced, which then reduces energy demand via its antiadrenergic effects mentioned earlier and at the same time improves oxygen supply via vasodilation of the coronaries. Figure 1.1 shows an overview of the relationship between adenosine production and regulatory aspects via its receptors and the large family of P2 receptors, which will not be discussed further (for a review see Reference 6).

The effect of adenosine on conduction is therapeutically exploited in the treatment of supraventricular tachyarrythmias. Adenosine is also used as a vasodilator in myocardial scintigraphy. Currently, these are the only established therapeutic and diagnostic regimens targeting adenosine receptors.

1.4.2 Adenosine and the Kidney

As in the heart, the role of adenosine in the kidney is important as a regulator of energy balance. By limiting the glomerular filtration, adenosine prevents energy-consuming reabsorption of relevant constituents of the filtrate. The nonselective adenosine receptor antagonist caffeine has, therefore, diuretic activity, which is well known to caffeine consumers. In particular, A_1 receptors are an interesting target for a novel class of diuretic drugs. Several compounds have reached phase II of clinical trials and may be used in acute renal failure and chronic heart failure. For an overview of renal effects of A_1 antagonists, see Reference 10.

1.4.3 CNS Actions of Adenosine

The effects of adenosine on the CNS are antagonized by the most used drug in the world, caffeine.[61] The central stimulation that is the sought-after effect of caffeine is mediated by blockade of central effects of adenosine mediated by both A_1 and A_{2A} receptors.[19] This is elegantly confirmed by knockout mice with targeted disruption of A_1[62] and A_{2A} receptors.[63]

Adenosine receptors have also been proposed as a target for neuroprotection. The role of A_1 receptors relates to their inhibition of neurotransmitter release, which may help prevent the neurotoxic effects of glutamate release under certain pathological conditions.[19] The role of A_3 adenosine receptors is not as clear and depends on the time course of the neurodegenerative events.[64]

1.4.4 Adenosine Receptors and Cancer

Tumor tissue is often characterized by hypoxia and consequently contains high levels of adenosine. It can be speculated, therefore, that adenosine receptors present in tumor tissue might be important regulators of growth, differentiation, and proliferation of cancer cells. As recently summarized by Merighi et al.,[11] there is evidence for the involvement of all four receptor subtypes in cancer cell growth and proliferation. By far the most data are available for the A_3 receptor, lending support to the idea of a role for this subtype as a therapeutic target for the treatment of certain types of cancer.[65–67] In a study with stably transfected CHO cells, it was also shown that the human A_3 adenosine receptor may play a critical role in cell cycle regulation and growth.[68]

Recent evidence has shown that certain cancer cells express high levels of A_{2B} adenosine receptors.[24] In addition, these receptors are often not only coupled to adenylyl cyclase but mediate an increase of intracellular Ca^{2+},[22,24,69] which could act as a key player in the regulation of proliferation.[70]

1.5 CONCLUSION

Four subtypes of adenosine receptors comprising the subgroup of P1 purinergic receptors have been identified and characterized in great detail in many tissues as well as in transfected cell systems. They show a distinct distribution pattern in most, if not all, cell types, and they are involved in the regulation of vital functions of tissues and organs. This widespread but specific occurrence makes them interesting targets for pharmacological intervention in many diseases. The availability of structurally diverse agonists and antagonists should be a good starting point for future successful exploitation of adenosine-based therapies for a large variety of indications ranging from inflammatory conditions to neurological diseases. Even more therapeutic opportunities may emerge in the purinergic field with the large family of P2 receptors, although ligand development for nucleotide receptors is still a major challenge.

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2 Medicinal Chemistry of Adenosine A_3 Receptors

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CONTENTS

2.1 INTRODUCTION

There are four subtypes of adenosine receptors (ARs), specifically, A_1, A_{2A}, A_{2B}, and A_3, all of which are members of the Class A (rhodopsin-like) family of G-protein-coupled receptors (GPCRs).[1] The most recently deorphanized subtype of ARs,[2] the A_3AR, has been the focus of intense efforts in government, industry, and academic research laboratories over the past 15 yr. The availability of selective ligands, both agonists and antagonists of the A_3AR, made it possible to study the physiological role of this receptor. Clinical targets have become apparent, and several clinical trials using selective agonists have been initiated. This chapter will outline the development of selective A_3AR agonists and antagonists.

The structure–activity relationships (SARs) at A_3ARs have been extensively probed.[3,4] Although analysis of the SAR of known adenosine agonists has provided the necessary insights to design A_3AR-selective agonists early in this process, the identification of selective antagonists was initially slower. The classical AR antagonists (e.g., the xanthines caffeine and theophylline) have not provided fruitful leads for A_3AR-selective antagonists. Thus, the search for such antagonists has mainly involved screening of chemically diverse structures and optimizing the resulting "hits."

Typically, SAR studies in pursuit of these goals compare effects in binding and function at the A_3AR and other AR subtypes heterologously expressed in mammalian cells such as Chinese hamster ovary (CHO) cells. Selectivity of binding often differs between rat and human ARs.[4,5] Functional effects may be studied using standard assays of adenylate cyclase; the A_3AR is coupled to G_i protein and, thus, agonists inhibit adenylate cyclase that has been stimulated either by agents such as forskolin that activates the enzyme or isoproterenol that produce activation of G_s protein.

2.2 A_3AR AGONISTS

Analogs of the purine nucleoside adenosine **1** modified in the N^6-or 2-position of the adenine moiety and in the 3′- or 5′-positions of the ribose moiety have been most useful as A_3 agonists (Figure 2.1 and Table 2.1).[3] Highly selective agonists have been designed through both empirical approaches and a semirational approach based on molecular modeling. Key structural features that have been systematically probed in the interaction with the A_3AR include the N^6-substituent (such as substituted benzyl groups), 2-position substitution (such as halo), and substitution of ribose (e.g., the (*N*)-methanocarba ring system, various 2′- and 3′-substitutions, and 4′-thio substitution of oxygen).[6]

2.2.1 Optimization of N^6-Substitution of Adenosine in Binding to the A_3AR

Binding to the human A_3AR was studied for a wide range of N^6-substituted derivatives of adenosine **1**. Among N^6-alkyl substitutions, small N^6-alkyl groups were associated with selectivity for human vs. rat A_3ARs.[5] Mogensen et al.[7] replaced the typically bulky purine 6-amino substitutent with the smaller *N*-methoxy group.

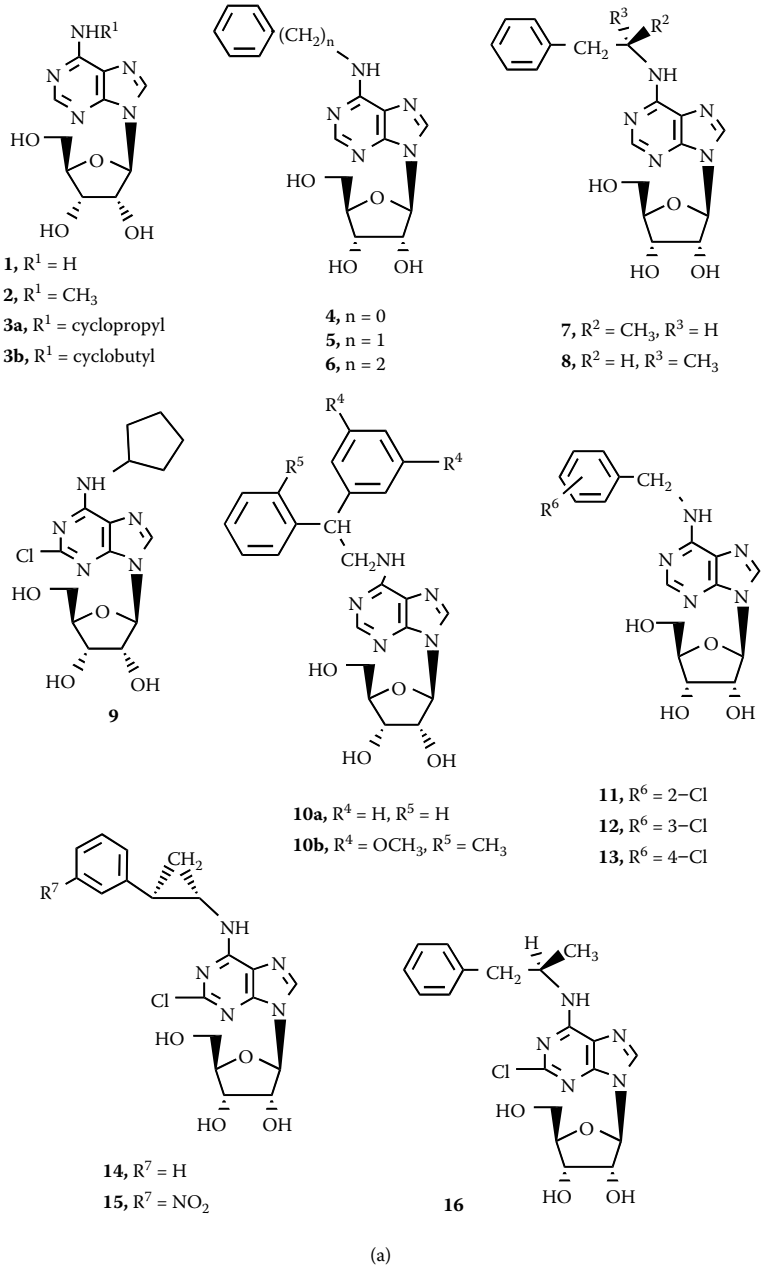
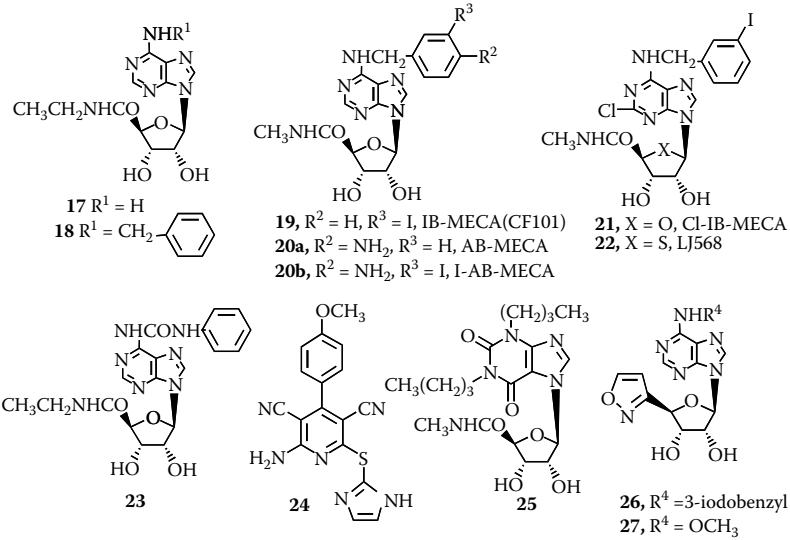
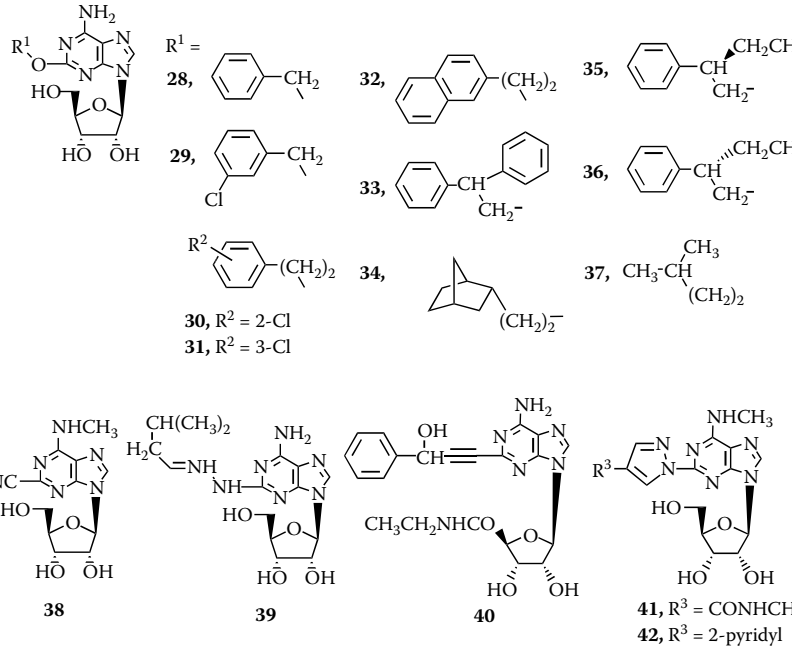


FIGURE 2.1 Adenosine **1** and various adenosine derivatives that are potent and/or selective in binding to the A_3AR. (a) Adenosine-like 5′-CH_2OH derivatives containing 2- and N^6-modifications, (b) adenine and 5′-modified analogs and an atypical agonist (**24**), (c) 2-Ethers (**28**–**37**) and other 2-position modified analogs, (d) ribose-modified analogs.

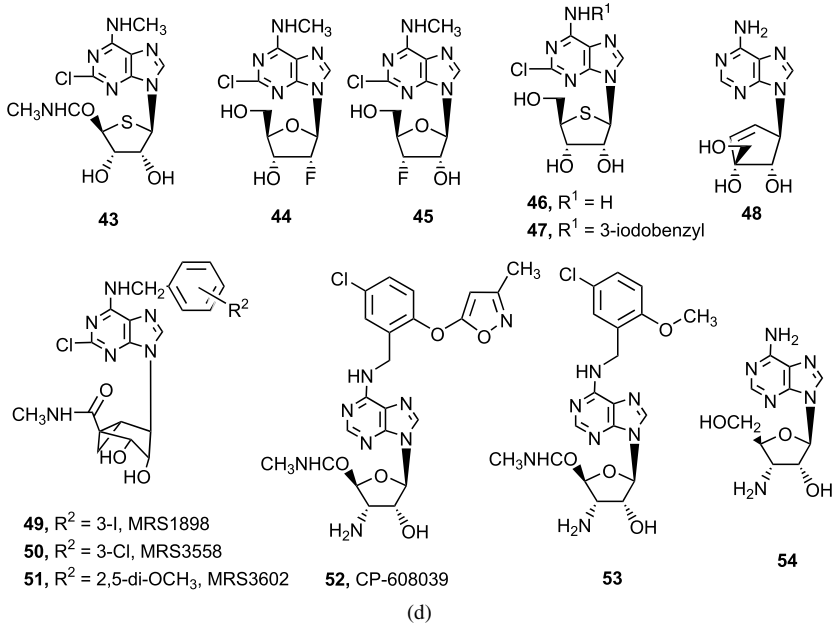


(b)



(c)

FIGURE 2.1 (Continued)



(d)

FIGURE 2.1 (Continued)

High-affinity agonists at the human A_3AR have also been reported to be associated with N^6-methyladenosine **2** and its analogs.[5]

Among N^6-cycloalkyl-substituted adenosines,[8] such as **3** and higher homologues, the optimal affinity at the human A_3AR was observed for the cyclobutyl group **3b**, although this compound displayed higher affinity at the A_1AR (K_i 0.7 n*M*, rat) than at the A_3AR (K_i 6.4 n*M*, human). The cyclopentyl and cyclohexyl analogs displayed considerable affinity at the human A_3AR (K_i < 100 n*M*); however, N^6-(*endo*-norbornyl) adenosine was inactive at both rat and human A_3ARs and was therefore highly selective for the A_1AR.[5]

In the series of N^6-arylalkyl analogs, the order of affinity at the human A_3AR was 2-phenylethyl **6** > phenyl **4** > benzyl **5** (K_i values of 2.1, 14.9, and 41.3 nM, respectively). Nevertheless, the N^6-benzyl moiety provided deselection of high affinity at the A_1 and A_{2A} receptors, thereby favorably increasing the A_3 receptor selectivity. Furthermore, N^6-benzyl-substituted adenosine derivatives tend to have similar potency for human and rat A_3ARs, although N^6-methyl substitution as in **2** increased affinity only for the human A_3AR.[5] Numerous N^6-arylmethyl analogs, including substituted benzyl derivatives, tended to display the following order of affinity in binding to $A_3 \geq A_1 \gg A_{2A}$ARs (with varying degrees of partial to full A_3AR agonism). Stereoselectivity of binding was demonstrated, which favored the N^6-(*R*-1-phenyl-2-propyl)adenosine **7** diastereomer (K_i 8.7 n*M*) over the corresponding *S*-isomer **8** (K_i 68 n*M*).[5]

The effects of nucleoside structure on efficacy at the human A_3AR have been systematically explored. Multiple points of branching of simple N^6-alkyl substituents were associated with decreased human A_3AR efficacy. N^6-Cycloalkyl-substituted

TABLE 2.1
Affinity of Selected A_3 Adenosine Receptor Ligands at Three Receptor Subtypes

			K_i Values (n*M*) or Percentage Inhibition, Where Indicated		
No.	**Compound**	**Reference**	**A_1AR[a]**	**$hA_{2A}AR$[a]**	**hA_3AR[a]**
2	N^6-methyl	5	60[b]	>10,000[b]	9.3
3a	N^6-cycloproyl	10	6.9	7,860	100
3b	N^6-cyclobutyl	5	0.7[b]	1,740[b]	6.4
4	N^6-phenyl	5	3.3[b]	663[b]	14.9
5	N^6-benzyl	5	175[b]	285[b]	41.3
6	N^6-(2-phenylethyl)	5	24.0[b]	161[b]	2.1
7	N^6-(R-phenylisopropyl)	5	1.2[b]	124[b]	8.7
9	CCPA	9,18	0.83	2270	38
10a	N^6-(2,2-diphenylethyl)	10	49.9	510	3.9
10b	DPMA	10,34	168	153	106
14	N^6-(2-phenyl-1-cyclopropyl)	5,10	124	2530	0.86
17	NECA	20,34	6.8	2.2	16.0
18	Bn-NECA	34	87.3[b]	95.3[b]	6.8[b]
19	IB-MECA	11,27	51	2,900	1.8
21	Cl-IB-MECA	12,27	1,240	5,360	1.4
22	LJ-568	24	193	223	0.38
23	N^6-urea derivative	15	110[b]	5,360[b]	39[b]
25	DBXRM	17	37,300[b]	19%[b]	816
26	NNC 53-0083	7	—	—	7.8
27	NNC 53-0082	7	—	—	31
28	2-benzyl-O	18	642	585	117
29	2-(3-chlorobenzyl)-O	18	27.4	228	71.6
30	2-(2-(2-chlorophenyl)ethyl))-O	18	366	17.9	144
31	2-(2-(3-chlorophenyl)ethyl))-O	18	372	11.5	41.0
32	2-(2-(2-naphthyl)ethyl)-O	18	220	3.8	205
33	2-(2,2-diphenylethyl)-O	18	38%	310	53.6
34	2-(2-(2-norbornyl)ethyl)-O	18	3,590	137	149
35	2-(R-2-phenylbutyl)-O	18	28%	503	201
36	2-(S-2-phenylbutyl)-O	18	4,780	26.9	175
37	2-((4-methyl)pentyl)-O	18	3,700	77.8	105
38	2-cyano-N^6-Me Ado	19	69.8	23%	3.4
39	2-hydrazino derivative	21	—	92	24
40	PHP-NECA	20	2.7	3.1	0.42
41	N^6-Me-2-pyrazole analog	22	>6,000	>6,000	73.0
42	N^6-Me-2-pyrazole analog	22	3,800	>5,000	2.0
43	4′-thio N^6-Me analog	24	1,330	20%	0.28
48	MRS1898	26,27	136	784	1.5
50	MRS3558	27	260	2,300	0.2
51	MRS3602	27	1,600	52%	1.4
52	CP608039	28	7,200		5.8
53	3′-deoxy-3′-NH_2 analog	29	8,190	49%	27
55	MRS1191	36	—	—	2,750
56	MRS1334	37	—	—	2.69

TABLE 2.1 (CONTINUED)
Affinity of Selected A_3 Adenosine Receptor Ligands at Three Receptor Subtypes

			K_i Values (n*M*) or Percentage Inhibition, Where Indicated		
No.	**Compound**	**Reference**	**A_1AR[a]**	**hA_{2A}AR[a]**	**hA_3AR[a]**
57	MRS1523	38	—	—	18.9
58	MRS1067	39	—	—	561
59	CGS15943	40	—	—	13.8
60	MRS1220	40	305[b]	52.0[b]	0.65
61a	Triazoloquinoxaline analog	41	31%	21%	0.6
61b	Quinoxaline analog	42	127	0%	0.5
62	OT-7999	43	>10,000	>10,000	0.61
63	MRE 3008-F20	46	1,200	141	0.82
64a	Phenyl carbamate	47	594	381	0.16
64b	Pyridyl carbamate	48	350	100	0.01
65	Sulfophenyl carbamate	47	>10,000	594	25
66	Quinolin-4-one, p-methyl	50	>1,000	>1,000	9
67	Quinolin-4-one, p-methoxy	50	>1,000	>1,000	16
68	Quinolin-4-one, p-chloro	50	>1,000	>1,000	19
69	VUF 5574	51	52%	43%	4.03
70	VUF 5455	52	—	—	1680
71	VUF 8502	52	—	—	96
72	VUF 8504	52	37%	19%	17
73	VUF 8507	51	0%	2%	495
74	DU 124,183	77	—	—	820
75	LUF 5417	53	32	2,300	82
76	Thiadiazole analog	54	24%	28%	0.79
77a	Aminothiazole analog	55	33	>10,000	0.21
77b	Thiazole analog	55	0.29	>10,000	0.11
78	Aminothiazole analog	55	>10,000	>10,000	0.4
79	Thiazole analog	56	197	—	10
80	KF-26777	57	1,800	—	0.20
81	PSB-11	59	1,640	1,280	2.3
82	Pyridopurine-2,4-dione analog	60	50	—	4.0
83	Pyrrolopurinone analog	61	>1,000	>1,000	8.0
84	Imidazopurinone analog	61	>1,000	>1,000	0.8
85	Azaadenine analog	62	430	8,050	6
86	MRS3777	63	26%	16%	47
87	MRS1292	14,74	12,100[b]	29,800[b]	29.3
88	3′-F-3′-deoxy-Cl-IB-MECA	76	6%	0%	460
89	LJ314	76	110	47%	4.3

Note: The compound numbers correspond to numbers used in the text and schemes.

[a] Binding experiments at recombinant human A_1, A_{2A}, and A_3ARs, unless noted. Values expressed as Ki (n*M*) ± SEM, except when a percentage is indicated, which means inhibition percentage of binding at 10 μM.

[b] Binding and functional experiments at rat ARs.

adenosines were full (4 or 5 carbons) or partial (6 carbons) hA_3AR agonists. Combination of the N^6-cyclopentyl and 2-chloro substituents in **9** abolished efficacy.[5,9] Similarly, N^6-[2,2-diphenylethyl]adenosine **10a** and DPMA (N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenylethyl)]adenosine) **10b**, potent agonists at the rat $A_{2A}AR$ (K_i = 75 and 4.4 n*M*, respectively), were identified as potent antagonists for the human A_3AR (K_i = 3.9 and 106 n*M*, respectively). Thus, the loss of efficacy upon certain structural modifications may be complete, thereby providing an entry to novel nucleoside-based A_3AR antagonists (see Section 2.3). Among N^6-benzyl analogs **11**–**13**, a chloro ring-substituent on an N^6-benzyl ring decreased the efficacy depending on its position, with the 3-chloro displaying the highest affinity (K_i = 4.4 n*M*) but least efficacy (80%) at the human A_3AR.[5]

N^6-substitution was also used to probe interactions between the ligand and the receptor that were distal to the binding site of the principal pharmacolophore.[10] The A_3AR affinity and efficacy of N^6-arylethyl adenosines depend highly on stereochemistry, steric bulk, and ring constraints. The sterically constrained analog, N^6-(1*S*,2*R*)-2-phenyl-1-cyclopropyladenosine **14**, was highly potent in binding to the human (K_i = 0.63 n*M*) but not rat A_3AR. The dependence of the AR affinity and selectivity on phenyl ring substitution of N^6-(1*S*,2*R*)-(2-phenyl-1-cyclopropyl)adenosine analogs were analyzed. A 3-nitrophenyl analog was resolved chromatographically into pure diastereomers, which displayed tenfold stereoselectivity in A_3AR binding in favor of the 1*S*,2*R* isomer **15**. A heteroaromatic group (3-thienyl) could substitute for the phenyl moiety of **14** with retention of high affinity of A_3AR binding. Also, the N^6-(2-phenyl-1-cyclopropyl) derivatives were identified as full A_3AR agonists, whereas several other derivatives had greatly reduced efficacy. Although N^6-cyclopropyladenosine **3a** was an A_3AR antagonist, adding either one or two phenyl rings at the 2-position of the cyclopropyl moiety restored efficacy. Similarly, N^6-(2,2-diphenylethyl)adenosine **10a** was an A_3AR antagonist, yet adding a bond between the two phenyl rings (N^6-9-fluorenylmethyl) or shortening the ethyl moiety in N^6-diphenylmethyladenosine restored efficacy.[5,9]

The following adenosine derivatives were found to be dual-acting A_1/A_3 agonists at human, but not rat, receptors: N^6-3-chlorobenzyl-**12**, N^6-(*S*-1-phenylethyl)-**7**, and 2-chloro-N^6-(*R*-phenylisopropyl)adenosine **16.**[5,9] Agonists of such combined selectivity might be useful for cardioprotection, because selective agonists for either A_1 or A_3ARs precondition cardiac myocytes in parallel pathways.

Quantitative SAR (QSAR) studies of the N^6 region provided a ligand pharmacophoric model that was complementary to the putative A_3AR binding site in a rhodopsin-based homology model. A molecular model defined a hydrophobic region (Phe168) in the putative A_3AR binding site around the phenyl moiety of N^6-(2-phenylethyl)adenosine derivatives.[6]

2.2.2 Combination of N^6-Substitution of Adenosine with 5′-Uronamide Modification to Achieve A_3AR Selectivity

The 5′-alkyluronamide nucleoside NECA **17** is a nonselective, highly potent adenosine agonist, which was initially found to be among the most potent in binding to the receptor. NECA was used as an agonist in the initial deorphanization and

characterization of the A_3 receptor and was also used subsequently as a radioligand of moderate affinity for the human A_3AR.

The first entry into A_3AR-selective agonists was accomplished with adenosine derivatives modified at the 5′-position as a uronamide followed by substitution of the amino functionality with benzyl substituents.[11] Thus, the combination of the N^6-benzyl and NECA-like 5′-*N*-alkyluronamido groups generates Bn-NECA **18**, which was slightly selective for the rat A_3 receptor (seven-fold in comparison to rat A_1 and A_{2A}ARs). The prototypical A_3 agonist IB-MECA **19** was identified in that study as being approximately 50-fold selective for the rat A_3 in comparison to the rat A_1 and A_{2A}ARs. IB-MECA and the more selective Cl-IB-MECA **21**[12] have been used widely as pharmacological probes in the elucidation of the physiological role of the A_3AR. The related 4-aminobenzyl derivative, AB-MECA **20a**, is also selective for the A_3AR. The **20a** may be radioiodinated by virtue of the electron-rich aniline ring, giving rise to [^{125}I]I-AB-MECA **20b**, which is widely used as a high-affinity radioligand for A_3AR (although binding to the A_1AR has also been observed at higher concentrations).[13]

A 5′-uronamide group was shown to restore full efficacy in adenosine derivatives, even if the efficacy was diminished as a result of substitution at the N^6 and/or C2 positions.[14] Thus, a flexible 5′-uronamide group was able to override the reduction of efficacy upon certain adenine modifications, but not 3′-ribose modification.

Acylation at the N^6-position (e.g., urea derivative **23**) of adenosine 5′-uronamides enhanced agonist potency at the A_3AR, but with only moderate selectivity in comparison to other subtypes.[15]

Up to this point, the effects of substitution of the adenine moiety have been described; however, nonadenine nucleoside and even nonnucleoside derivatives have been found to activate the A_3AR. For example, derivatives of pyridine (e.g., **24**, K_i = 224 n*M* at the human A_3AR)[16] and xanthine-7-ribosides[17] are such agonists. The replacement of adenine by an appropriately substituted xanthine moiety, leading to 1,3-dialkylxanthine 7-riboside analogs modified at 1-, 3-, and 8-purine positions as well as at the ribose 5′-position, have provided A_3AR agonists. One such agonist is DBXRM (*N*-methyl 1,3-dibutylxanthine 7-β-D-ribofuronamide) **25**, which is a moderately selective agonist of the A_3AR.

2.2.3 Other 5′-Position Modifications

Several novel 5′-isoxazole derivatives were reported to be selective human A_3AR agonists, including NNC 53-0083 (**26**, K_i = 7.8 n*M*) and NNC 53-0082 (**27**, K_i = 31 n*M*).[7]

2.2.4 Optimization of the 2-Position of Adenosine in Binding to the A_3AR

The affinity and efficacy of a wide range of 2-substituted ethers and other derivatives of adenosine were evaluated at the four subtypes of human ARs.[18] Similar to previous studies of the N^6-position, several 2-substituents were found to be critical structural determinants for activation of the A_3AR. The following adenosine 2-ethers were moderately potent partial agonists (K_i, n*M*): benzyloxy **28** (117), 3-chlorobenzyloxy

29 (72), 2-(3-chlorophenyl) ethyloxy **31** (41), and 2-(2-naphthyl)ethyloxy **32** (130). The following adenosine 2-ethers were A_3AR antagonists: 2,2-diphenylethyloxy **33**, 2-(2-norbornyl)ethyloxy **34**, *R*- and *S*-2-phenylbutyloxy **35** and **36**, and 2-(2-chlorophenyl)ethyloxy **30**. An A_3AR antagonist, 2-(*S*-2-phenylbutyloxy)adenosine **36**, right-shifted the concentration–response curve for the inhibition by NECA of cyclic AMP accumulation with a K_B value of 212 n*M*, which was similar to its binding affinity (K_i = 175 n*M*). Generally, these 2-substituted adenosine derivatives were less potent at the A_1AR in comparison to the A_3AR, but fully efficacious at that subtype, with binding K_i values over 100 n*M*. The 2-phenylethyl moiety resulted in higher A_3AR affinity (K_i in n*M*) when linked to the 2-position of adenosine through an ether group (54) than when linked through an amine (310) or thioether (>10,000).

Typically, 2-ethers of adenosine are A_{2A}-selective agonists; thus, the compounds mentioned earlier are somewhat exceptional. For example, the closely related 2-[2-(1-naphthyl) ethyloxy]adenosine was found to be the most potent and selective (>50-fold) A_{2A} agonist (K_i = 3.8 n*M*) in this series. Given the tendency for A_{2A} selectivity, mixed A_{2A}/A_3AR agonists, such as MEBA (2-[(3-methylbutyl)oxy]adenosine) **37,** have been identified in this series of 2-ethers. Most of these compounds were extremely weak at the A_{2B}AR.

Carbon substituents at the 2-position of adenosine have been combined with N^6-substitutions known to enhance human A_3AR affinity. Sterically small carbon substituents at the 2-position were found to modulate both the affinity and intrinsic efficacy at all subtypes. The 2-cyano group decreased hA_3AR affinity and efficacy in the cases of N^6-(3-iodobenzyl) and N^6-(2-phenylcyclopropyl). In the latter case, a full A_3AR agonist was converted to a selective antagonist, whereas the 2-cyano N^6-methyl analog **38** was a full A_3AR agonist.[19] The combination of N^6-benzyl and various 2-substitutions (chloro, trifluoromethyl, and cyano) resulted in reduced efficacy at the A_1AR.

Tilburg et al.[20] prepared and characterized a 2-(*N*-3-methyl-1-butylidenehydrazino)-adenosine **39**, a 2-substituted derivative with an intact 5′-hydroxyl group, which showed partial agonism toward the A_3 as well as the A_{2A}AR.

Larger 2-carbon substituents, including alkynyl groups, have been studied by Klotz et al.[21] They have prepared a number of 2-alkynyl derivatives of 5′-*N*-ethylcarboxamidoadenosine (NECA) **17**, which were investigated for their affinity and selectivity at the human A_3AR. In binding studies, they found that the most potent compound, 2-(3-hydroxy-3-phenyl)propyn-1-yl-NECA (PHPNECA) **40**, exhibited subnanomolar affinity for the human A_3AR with a K_i value of 0.4 n*M*.

Recently, a novel class of 2-pyrazolyl-N^6-substituted adenosine analogs was reported to be high-affinity and selective A_3 AR agonists.[22] The N^6-methyl analog **41** showed relatively high binding affinity at the human A_3AR, with a K_i value of 73 n*M*. The replacement of the carboxamide group in **41** by various heteroaryl groups resulted in several analogs with high binding affinities and selectivity for A_3AR. For example, compound **42** (K_i = 2 n*M*), with a 2-pyridyl substitution on the pyrazoyl ring, showed extremely high selectivity for the A_3AR vs. A_1 and A_{2A}ARs.[22]

By using rhodopsin-based homology modeling and ligand docking, the environment surrounding the 2-position within the putative A_3AR binding site was explored.

Mutagenesis of the second extracellular loop (EL2) of the human A_3AR suggested its involvement in the process of nucleoside recognition.[23]

2.2.5 Optimization of the Ribose Moiety of Adenosine in Binding to the A_3AR

The 4′-thio modification of adenosine derivatives, explored for its effect on AR selectivity, has produced several highly potent and selective A_3 agonists, such as LJ568 **22**.[24] One of the most potent compounds at the human, but not rat, A_3AR was 2-chloro-N^6-methyl-4′-thioadenosine-5′-methyluronamide **43**, which had a K_i value of 0.28 n*M*. Novel 4′-thio analogs of large and structurally diverse 5′-uronamides have been explored for their effects on AR selectivity.[24]

As described previously, structural features of adenosine derivatives, particularly at the N^6- and 2-positions of adenine, determine their intrinsic efficacy as A_3AR agonists. This phenomenon has also been probed with respect to the ribose moiety using a series of ribose-modified adenosine derivatives, examining binding affinity and activation of the human A_3AR expressed in CHO cells. Both 2′- and 3′-hydroxyl groups in the ribose moiety contribute to A_3AR binding and activation, with 2′-OH being more essential. Thus, the 2′-fluoro substitution, as in **44**, eliminated both binding and activation, whereas a 3′-fluoro substitution, as in **45**, led to only a partial reduction of potency and efficacy at the A_3AR. A 5′-uronamide group, known to restore full efficacy in other derivatives, failed to overcome the diminished efficacy of 3′-fluoro derivatives.[9]

The 4′-thio substitution, which generally enhanced A_3AR potency and selectivity, resulted in 5′-CH_2OH analogs (**46** and **47**) that were partial agonists of the A_3AR. A novel apio analog (**48**) of neplanocin A was a full A_3AR agonist. When the affinities of specific, novel analogs at rat ARs were examined, species differences were revealed.

Conformational studies of the ribose moiety and its equivalents revealed two key features when binding to the A_3AR: (1) The ring oxygen is not required, and (2) the North (N) ring conformation is preferred over the South (S) conformation. One means of locking the riboselike ring in either the N- or S-conformation is through the use of the bicyclo[3.1.0]hexane ring system. When the bicyclic fusion point is at the 4′- and 6-position carbons, the ring assumes an (N)-envelope conformation.[25] Highly selective A_3AR agonists in the series of (N)-methanocarba 5′-uronamide derivatives were reported by Lee et al.,[26] and the list was greatly expanded recently by Tchilibon et al.[27] These agonists include the 5′-*N*-methyluronamide derivatives MRS1898 **49** and MRS3558 **50**, which displayed a K_i value at the human A_3AR of 0.29 n*M*.[27] Another highly selective A_3AR agonist in this series was the 2,5-dimethoxybenzyl analog, MRS3602 **51**. Based on these observations, many of the previously known groups that enhance A_3AR affinity in the 9-riboside series, including those that reduce intrinsic efficacy, may be adapted to the (N)-methanocarba 5′-uronamide nucleoside series of full agonists.[27]

Other replacements of the 3′-hydroxyl group have been utilized successfully in A_3AR agonists. For analogs that are otherwise optimized at the N^6- and the 5′-positions, the 3′-hydroxyl group may be substituted with an amino group.[28]

CP608039 **52**, for example, displayed full agonist activity at the human A_3AR with an EC_{50} of 3.4 n*M* and is intended for use in perioperative cardioprotection. The 3′-amino group, which is mainly charged at physiological pH, also enhances water solubility when formulated in acidic buffers.

In a similar fashion, Van Rompaey et al.[29] synthesized a series of adenosine derivatives having the 3′-amino-3′-deoxy or 3′-aminomethyl-3′-deoxy modification, with both associated with moderate selectivity in binding to the human A_3AR. They concluded that the 3′-amino function resulted in partial agonist activity, whereas introduction of a methylene spacer between amino functionality and the ribofuranose ring reduced both efficacy and affinity. Thus, compound **53** was a partial agonist (~half efficacy) with selectivity for the human A_3AR (K_i 27 n*M*).

In summary, critical structural determinants for human A_3AR activation have been identified, which should prove useful for further understanding the mechanism of receptor activation and development of more potent and selective full agonists, partial agonists, and antagonists for A_3ARs.

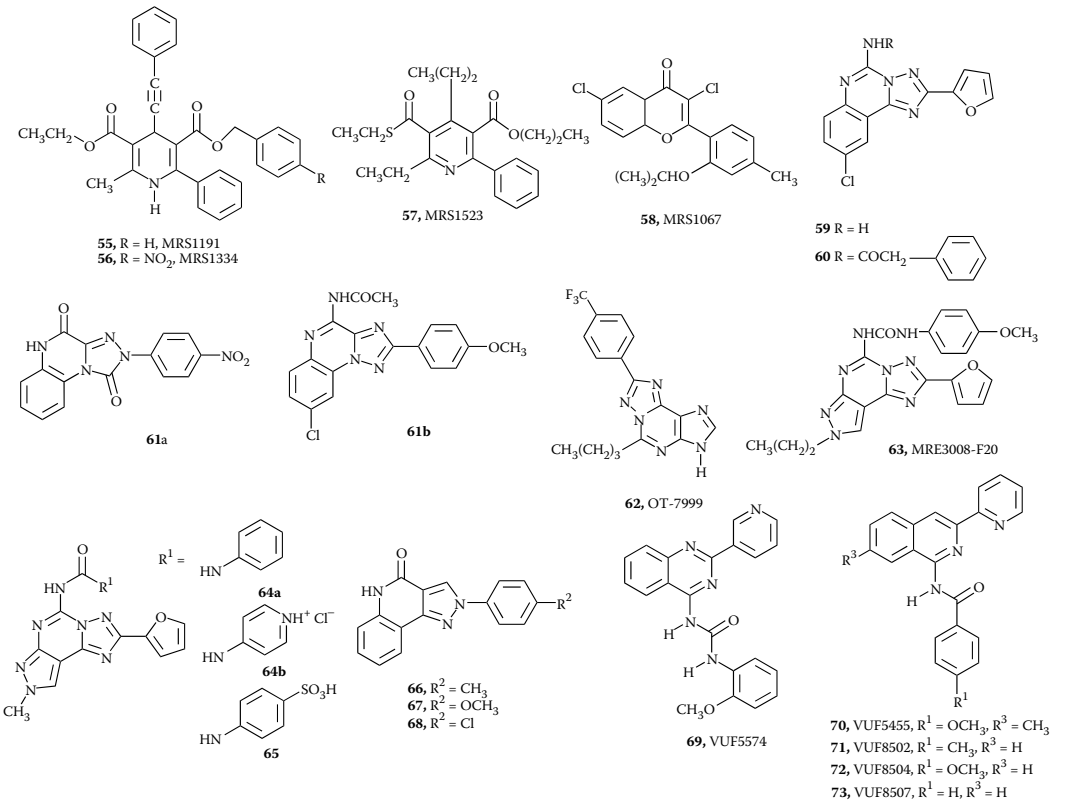
2.2.6 Agonists for Reengineered A_3ARs: Neoceptors

In general, agonist therapy often carries the disadvantage of side effects, especially if the targeted receptor is widely distributed throughout the body. For this reason, and to devise a general solution to the problem, the A_3AR was reengineered into a neoceptor that could recognize nucleosides modified in a complementary fashion that were inactive at the native A_3AR.[30–32] This neoceptor strategy, intended for eventual use in organ-targeted gene therapy, is enabled by rhodopsin-based modeling of the receptor and its putative ligand-binding site, supported by receptor mutagenesis results. This allows the incorporation of a complementary functional group in a synthetic agonist, or "neoligand," which is recognized as agonist exclusively by the reengineered receptor.

The neoceptor concept was initially applied to the human A_3AR.[30] The putative ribose-binding region of the receptor, in the vicinity of TM3 and TM7, was strategically mutated to introduce a negative charge that would serve as a recognition element for a tailor-made positively charged adenosine derivative. One amine-bearing derivative of adenosine, 3′-amino-3′-deoxyadenosine **54**, showed selective affinity enhancement at the mutated receptor H272E. A novel electrostatic pair formed between the A_3AR-derived neoceptor containing the H272E mutation in TM7 and the neoligand **54** allowed selective activation of the neoceptor at concentrations below the K_i value of the nucleoside determined at the wild-type A_3AR. Thus, in theory, compound **54** could be administered *in vivo* to an animal expressing the neoceptor gene in a given tissue, such as the heart, to effect cardioprotection, whereas the native A_3AR would not respond to the presence of **54** at the appropriate concentration. This would consequently avoid side effects of the activation of the naturally expressed A_3AR by the nucleoside.

2.3 A_3AR ANTAGONISTS

Numerous classes of fused heterocyclic compounds have been characterized as adenosine antagonists selective for the A_3AR (Figure 2.2).[4] A modeling approach



(a)

FIGURE 2.2 Selective AR antagonists (affinities at human AR unless noted, r = rat).

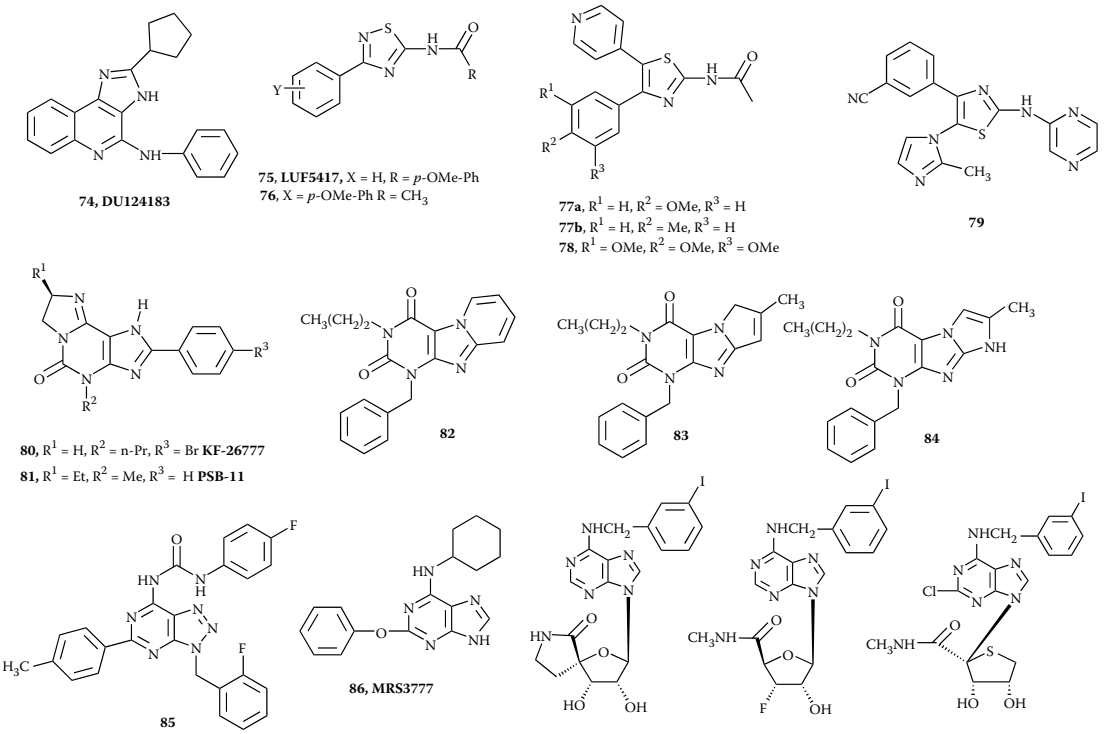


FIGURE 2.2 (Continued)

combining quantitative models of receptor and ligand demonstrated predictability in antagonist design.[33] Species differences in A_3AR affinity that are present for agonists are especially pronounced for A_3AR antagonists.

At A_3ARs, the search for antagonists began with the discouraging observation that xanthines, such as caffeine and theophylline, the classical adenosine antagonists of A_1, A_{2A}, and A_{2B}ARs, typically bound very weakly to the A_3AR.[34] The initial observation was made for the rat A_3AR (prior to the availability of the cloned human homologue), at which the common xanthines bound only in the range of 100 μM, but the cloning of the receptor from other species was a positive development.[35] At the sheep and human A_3ARs, the xanthines displayed intermediate affinity (typically 100 nM for 8-phenylxanthine analogs). A marked species dependence of antagonist affinity is present at the A_3AR, to a greater degree than at other ARs. The affinity of xanthine and nonpurine A_3 antagonists at the human A_3AR nearly always greatly exceeds that at the rat A_3AR.[34,35] Thus, the search for A_3 antagonists turned toward more novel heterocyclic systems.

The screening of diverse chemical libraries resulted in the identification of new "hits" for high-affinity antagonism at the human A_3AR, including the following:

1. Dihydropyridines and pyridines
2. Flavonoids
3. Fused heterocycles containing triazole or pyrazole (triazoloquinazolines, triazoloquinoxalines, pyrazolotriazolopyrimidine, pyrazoloquinolines, and triazolopurines)
4. Isoquinolines and quinazolines
5. Thiazoles and thiadiazoles
6. Fused xanthines (purinones and purinediones)
7. Adenine and adenosine derivatives; and others, which were then structurally optimized

2.3.1 Dihydropyridines and Pyridines

The 1,4-dihydropyridines (DHPs) are well known as antagonists at L-type calcium channels. From a screening of chemically diverse libraries, several DHPs were found to inhibit binding at the human A_3AR. Upon detailed SAR analysis, it was determined that substitution of the 4-aryl group of typical L-type calcium channel blockers with a 4-phenylethynyl group and replacement of the small alkyl group at the 6-position by a 6-phenyl group increased A_3AR affinity and completely abolished binding to the L-type calcium channel. Thus, the DHP derivatives MRS1191 **55** and MRS1334 **56** were not active at L-type calcium channels and displayed K_i values at the human A_3AR of 31 and 2.7 nM, respectively. However, the affinity at the rat A_3AR was considerably lower.[36,37]

In a parallel study of related compounds, the SAR of the oxidized pyridine derivatives (derived from the DHP series) was studied. The SAR differed mainly in the lack of tolerance of large substituents at the 4-position; for example, the 4-phenylethynyl group precluded binding to the A_3AR. Instead, 4-*n*-propyl and *n*-butyl groups were favored. This finding is also explainable on the basis of receptor

docking of the energetically optimized models of the ligands. One advantage of the pyridine series was increased affinity at the rat A_3AR. Thus, the pyridine derivative MRS1523 **57** was a moderately selective A_3AR antagonist in the rat and human.[38]

2.3.2 Flavonoids

Flavonoids were identified as the lead compounds for A_3AR antagonists from a screening of natural products of medicinal plants showing micromolar affinity. Further SAR and QSAR studies of various flavonoid analogs enabled the optimization of the parent structure, leading to the discovery of a selective submicromolar-affinity antagonist with a K_i value of 561 n*M* at the human A_3AR, MRS1067 **58**, which also antagonized rat A_3AR.[39]

2.3.3 Triazoloquinazolines

The first subnanomolar-affinity A_3AR antagonist, the triazoloquinazoline analog MRS1220 **60** (K_i value at the human A_3AR of 0.65 n*M*), was developed from the SAR study of a nonselective AR antagonist, CGS15943 **59**, with diverse derivatization at the exocyclic N^5 position. The study showed that substitutions with hydrophobic chains, such as aromatic acetyl groups, resulted in significant increases in affinity and selectivity, suggesting that a hydrophobic binding pocket in the receptor might provide an additional interaction site.[40]

2.3.4 Triazoloquinoxalines

The SAR study of triazoloquinoxaline derivatives showed that the substitutions at the *p*-position of the phenyl ring were critical either in the probing A_3AR affinity or selectivity, because of the different hydrogen-bonding interactions and steric effects in this region of the binding pocket of each receptor subtype. Thus, an analog with a *p*-nitro substitution **61a**, which has strong hydrogen bond acceptors, was developed as the most potent A_3AR antagonist with a K_i value of 0.60 n*M* and more than 16,600-fold selectivity in comparison to A_1 and A_{2A}ARs. In contrast, when the nitro group was reduced to NH_2, which is more polar and possesses hydrogen bond donor ability, high affinity and selectivity at A_1AR, with a K_i value of 8.7 n*M*, was observed. The nitro compound also showed a functional antagonism of the stimulation of GTP binding to G proteins.[41]

The SAR study of another series of triazoloquinoxaline derivatives revealed that the 4-acetylamino group was important to distinguish between the A_1 and A_3ARs as well as provide the most potent analog for A_3AR antagonism, **61b**. Most of the analogs of this series were inactive at A_{2A}AR.[42]

2.3.5 Triazolopurines

The selective A_3 antagonist OT-7999 **62**, a triazolopurine analog, was evaluated in a monkey model of intraocular pressure by topical administration to the eye and found to significantly decrease intraocular pressure in this *in vivo* model. This study, based on earlier work by Civan and colleagues,[75] indicated that a selective and potent A_3AR antagonist could be applied for the therapeutic intervention to treat glaucoma.[43]

2.3.6 Pyrazolotriazolopyrimidines

Among the pyrazolo-containing fused heterocyclic derivatives, i.e., pyrazolotriazolopyrimidines, some were designed into potent and selective A_3AR antagonists from a combination of an N^6-substituted phenyl carbamoyl group, a pharmacophore of A_3AR agonists, and a pyrazolotriazolopyrimidine skeleton previously reported as a template for A_{2A}AR antagonists. Variations at the phenyl carbamoyl ring and pyrazole nitrogen (N8) resulted in the development of subnanomolar-affinity A_3AR antagonists having several-thousand-fold selectivity in comparison to other receptor subtypes.[44,45] The *p*-methoxyphenyl carbamoyl and *N*8-*n*-propyl analog MRE3008-F20 **63** was regioselectively radiolabeled through catalytic hydrogenation of the corresponding *N*8-allyl analog with tritium gas.[46]

Another potent and selective A_3AR antagonist in this series was an analog, **64a,** containing an unsubstituted phenyl carbamoyl group, which displayed a K_i value of 0.16 n*M* and high selectivity. The series of pyrazolotriazolopyrimidine derivatives were also tested for functional antagonism in a cAMP assay, showing antagonistic potency similar to those of receptor-binding affinities.[47]

In an effort to develop water-soluble A_3AR antagonists, a pyridyl carbamoyl derivative, **64b,** was discovered as the most potent and selective A_3AR antagonist, displaying high water solubility (15 m*M*) and the highest affinity (K_i = 0.01 n*M*) and selectivity for the hA_3AR vs. the A_1, A_{2A}, and A_{2B} receptors (>10,000-fold) ever reported. In contrast, the oppositely charged analog, a sulfophenyl carbamoyl compound, **65**, showed a significant decrease in A_3AR affinity and selectivity.[48]

Through extensive SAR studies, additional structural requirements for A_3 receptor recognition were identified (e.g., the intact form of a 2-furanyl group as an essential pharmacophore and steric sensitivities around the pyrazole ring system).[49]

2.3.7 Pyrazoloquinolines

The 2-aryl-pyrazolo [4,3-c] quinolin-4-one skeleton was recently reported as a template for selective A_3AR antagonists. Most of this series of compounds, including those substituted with *p*-methyl **66**, methoxy **67**, or chlorine **68** groups at the 2-phenyl ring, displayed nanomolar affinities (9, 16, and 19 n*M*, respectively) and more than 1000-fold selectivity in comparison to all other AR subtypes.[50]

2.3.8 Isoquinolines and Quinazolines

Several isoquinoline and quinazoline urea derivatives were found to bind to human A_3AR, and the optimization of the SAR of this skeleton resulted in VUF5574 **69** as a potent and selective human A_3AR antagonist, with a K_i value of 4.0 n*M*. However, this compound is species selective and does not act at the rat A_3AR.[51]

In a study of allosteric modulation of the A_3AR with 3-(2-pyridinyl)isoquinoline derivatives, including VUF5455 **70**, VUF8502 **71**, VUF8504 **72**, and VUF8507 **73**, the compounds tended to decrease the dissociation rate of the agonist radioligand [125I]I-AB-MECA in a concentration-dependent manner, with no effect on the dissociation rate of an antagonist radioligand, suggesting an allosteric interaction.[52,77] Other allosteric modulators (e.g., the imidazoquinoline derivative DU124,183 **74**)

were also identified in a library of compounds described earlier as a source of A_3AR antagonists.

2.3.9 Thiazoles and Thiadiazoles

Thiazole and thiadiazole analogs **75–79** were first identified as a promising class of compounds after the screening of several 5- and 6-membered heterocyclic core templates to replace isoquinoline and quinazoline skeletons for new adenosine receptor antagonists. Initial SAR studies showed that the thiadiazole analog (X = H, R = *p*-MeO-Ph) LUF5417 **75** afforded relatively high affinity for the A_3AR compared to other analogs, although it lacked selectivity.[53]

Another series of thiazole and thiadiazole analogs substituted with various functional groups at the Y and R positions were evaluated. Compound **76**, having a *p*-methoxy group at the Y position, a short acyl group such as acetyl (R = CH_3), and a thiadiazole (X = N) ring system, showed subnanomolar affinity and high selectivity for the A_3AR with a K_i value of 0.79 n*M*.[54]

Pyridine substitution of acetamino-thiazole analogs were found to provide high-affinity but nonselective A_3AR antagonists with K_i values of 0.21 and 0.11 n*M* for the compounds containing *p*-OMe **77a** and *p*-Me **77b** as Y groups, respectively. Further optimization identified the compound **78**, which had a 3,4,5-trimethoxy substitution at the Y position and more than 100-fold selectivity as an A_3AR antagonist with a K_i value of 0.4 n*M*.[55]

In an application of the thiazole template toward dual antagonists for the A_{2B} and A_3ARs with oral bioavailability, the analog **79**, with substituted heterocyclic rings such as 2-aminopyridazine and 5-imidazole, showed a suitable *in vivo* profile for animal tests to evaluate the effects in allergic disease models.[56]

2.3.10 Fused Xanthines: Purinones and Purinediones

Several imidazopurinones were reported as potent and selective A_3AR antagonists. The analog KF-26777 **80** (R_1 = H, R_2 = n-Pr, R_3 = Br) displayed a K_i value of 0.2 n*M* with several-thousand-fold selectivity in comparison to other receptor subtypes and functional antagonisms either in guanine nucleotide binding or Ca^{2+} mobilization in activated HL-60 cells.[57]

An inverse A_3AR agonist, PSB-11 **81** (R_1 = (*R*)-ethyl, R_2 = Me, R_3 = H, K_i = 2.3 n*M* at human A_3AR), was tritiated for characterization of the human A_3AR, showing an extraordinarily low degree of nonspecific binding with high affinity at the human A_3AR (K_D = 4.9 n*M*, B_{max} = 3.5 pmol/mg of protein).[58,59]

Among a series of pyridopurine-2,4-diones, the lead analog **82** (X = n-Pr, Y = Bn, Z = H) showed a K_i value of 4.0 n*M* at human A_3AR with 10- to 30-fold selectivity.[60]

By changing the pyrido-fused ring system to pyrrolopurinones **83** or imidazopurinones **84**, the selectivity problem was solved with retention of the A_3AR antagonistic potency. One of the representative imidazopurinone analogs, **84** (R = H, R_1 = CH_3), showed a K_i value of 0.8 n*M* with more than 1000-fold selectivity.[61]

2.3.11 Adenine and Adenosine Derivatives

Although the agonists, which are generally adenosine derivatives, and adenine share a common ring system, the SAR for adenine substitution does not appear to be parallel in the two series. Nevertheless, a variety of adenine-like molecules have been identified as A_3AR antagonists.

One example is that of the 8-azaadenine ring system. The phenyl carbamoyl pharmacophores used previously in pyrazolotriazolopyrimidienes were incorporated into the N^6-position of an 8-azaadenine template, of which derivatives have demonstrated selective A_1AR antagonistic behavior. By 2-substitution with a *p*-methylphenyl group in **85,** the A_3AR affinity increased to single-digit nanomolar K_i values.[62]

Although numerous adenine derivatives have been studied as selective antagonists for A_1 or A_{2A}ARs, high selectivity for the human A_3AR was only recently reported for MRS3777 **86**.[63]

An alternate approach to designing A_3 antagonists is to start with high-affinity adenosine derivatives and simply truncate the molecule in stages to remove its ability to activate the receptor without losing its high affinity of binding. An initial attempt to find adenine derivatives, such as 9-alkyl-N^6-iodobenzyladenines, that displayed these characteristics was unsuccessful.[64] More successful approaches either added substituents to adenosine derivatives or rigidified the nucleosides to reduce their intrinsic efficacy.[9,14] With more systematic studies of structure–efficacy relationships upon substitution of adenosine at the N^6, ribose, and C2 adenine positions, it became apparent that the efficacy at A_3ARs was more easily diminished by structural modification than at the other subtypes.[5,9,14,18] In some cases, N^6-substitution of adenosine 5′-OH derivatives with large groups (e.g., substituted benzyl groups or large cycloalkyl rings) reduced the maximal efficacy, leading to reduced efficacy at the A_3AR. For example, CCPA and DPMA, full agonists at A_1 and A_{2A}ARs, respectively, were A_3 antagonists. This structural insight was used to obtain the conformationally constrained nucleoside MRS1292 **87**, which proved to be a selective A_3AR antagonist in both the rat and human.[14] Other modifications of the ribose ring reduced the efficacy of the nucleoside derivatives at the A_3AR. For example, the 3′-deoxy-3′-fluoro analog **88** of Cl-IB-MECA, in which a hydrogen bond donor/acceptor was replaced solely by an acceptor, also appeared to be an A_3AR antagonist.[76]

Interestingly, the shifting of the N^6-(3-iodobenzyl)adenine moiety of agonist **22** from the 1′- to the 4′-position had a minor influence on A_3AR selectivity, but significantly effected the transformation into a potent antagonist (**89**) (K_i = 4.3 n*M*). Compound **89** antagonized human A_3AR agonist-induced inhibition of cyclic AMP with a K_B value of 3.0 n*M*.[76]

2.4 USE OF SELECTIVE AGONISTS AND ANTAGONISTS AS PHARMACOLOGICAL PROBES TO DISCERN THE ROLE OF THE A_3AR

Selective A_3AR agonists, such as IB-MECA **19** and Cl-IB-MECA **21**, have been widely used to study the cardioprotective role of the A_3AR.[65–67] Unlike the A_1AR, A_3AR-mediated cardioprotection is achieved *in vivo* in the absence of hemodynamic

side effects and is therefore therapeutically more promising.[66] Stimulation of A_3ARs may also be advantageous over A_1AR activation because of a lack of contractile side effects.[68] The A_3AR agonist CP608039 **52** is under development to limit the damage of cardiac ischemia.[67] Also, the A_3 agonist Cl-IB-MECA **21** has also been shown to protect rat cardiac myocytes from toxicity induced by the cancer chemotherapeutic agent doxorubicin.[65]

Although A_3AR expression levels were low in all regions of the brain, the A_3AR agonist IB-MECA **19** depressed locomotor activity in mice,[69] suggesting a role for the A_3AR in depression of motor activity. Chronic administration of the same agonist was highly effective in a gerbil model of cerebroprotection against global ischemia.[70]

The A_3AR also has been implicated in mediating allergic responses, which facilitates the release of allergic mediators, such as histamine, in mast cells (Ramkumar et al., 1993). Systemic infusion of IB-MECA **19** has been shown to cause scratching in mice, which was prevented by coadministration of histamine antagonists.[69] The same A_3 agonist also demonstrated protection against lung injury and apoptosis in cats following reperfusion.[71] This protection was antagonized by the selective A_3 antagonist MRS1191 **55**.[64] A thiazole derivative that was bioavailable and acted as a mixed antagonist at A_{2B} and A_3ARs was reported and suggested for potential application in asthma.[56]

A_3AR activation has been implicated in inhibition of tumor growth both *in vitro* and *in vivo*.[72] IB-MECA (designated CF101) **19** is in clinical trials for treatment of rheumatoid arthritis and colon carcinoma. The novel anticancer effect discovered by Fishman and colleagues is caused by a cytostatic effect on tumors related to the Wnt pathway, rather than by induction of apoptosis. Recently, it was shown that the A_3AR was more highly expressed in tumor than in normal cells, which may justify A_3AR as a potential target for tumor growth inhibition.[73]

The selective A_3 antagonist OT-7999 **62** reduced intraocular pressure in a monkey glaucoma model.[43] Because most A_3 antagonists reported are selective only at the human A_3AR, OT-7999 **62** and other heterocyclic derivatives are not suitable for use in rodent models. The cross-species A_3 antagonist MRS1292 **87** was recently found to reduce mouse intraocular pressure and also inhibited adenosine-triggered human fluid release into the aqueous humor through action on the nonpigmented ciliary epithelial cells.[74]

2.5 CONCLUSIONS

The medicinal chemistry of the A_3AR is well developed, with selective agonists and antagonists for most of the subtypes. It is hoped that new agents in development will avoid the undesirable side effects that impeded the progression of AR ligands into the clinical pipeline in the past. Selective agonists are in clinical trials for the treatment of rheumatoid arthritis and cancer. Selective antagonists are in preclinical testing for the treatment of glaucoma. In addition to the potential of directly acting orthosteric ligands, modulation of the ARs allosterically is a promising line of inquiry. The approach of genetic therapy with neoceptors could further achieve organ or tissue selectivity in the future.

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3 The Role of CD73 in the Generation of Extracellular Adenosine for Adenosine Receptor Signaling

Masahide Takedachi, Sean P. Colgan, and Linda F. Thompson

CONTENTS

3.1 INTRODUCTION

Adenosine (Ado) modulates a variety of biological functions such as heart rate, smooth muscle contraction, neurotransmission, neutrophil activation, cytokine secretion, and chemotaxis[1–6] through the activation of adenosine receptors (AdoRs) that are ubiquitously expressed on the cell surface. AdoRs have been classified into four subtypes: A_1, A_{2A}, A_{2B}, and A_3, according to their affinities for Ado and coupling to G-proteins.[7] The development of effective agonists, antagonists and, in addition, gene-targeted mice deficient in the expression of each AdoR has made it possible to clarify the functions of the various AdoRs with respect to intracellular signaling in both physiological and pathophysiological conditions. These issues are addressed in various chapters in this volume. The purpose of this chapter is to discuss the role of CD73 in regulating AdoR signaling through its ability to generate extracellular Ado.

CD73 is a 70-kDa glycosyl phosphatidylinositol (GPI)-anchored cell surface protein with ecto-5′-nucleotidase enzyme activity that catalyzes the dephosphorylation of 5′-AMP to Ado.[8] Therefore, this molecule has the potential to play a key role in the generation of extracellular Ado by catalyzing the last step in the cascade of ATP breakdown initiated by another cell surface enzyme, CD39 (ATP-diphosphohydrolase)[9] (see Figure 3.1). After uptake into the cytosol through the action of a nucleoside transporter, Ado is metabolized by either adenosine deaminase (ADA) or adenosine kinase (AK).[10] In humans ADA can be anchored on the cell surface via CD26[11–13] and catalyze the conversion of extracellular Ado to inosine. One group has reported that ADA can also be anchored to cell surface A_1R[14] or $A_{2B}R$,[15,16] but these findings are yet to be replicated by other investigators.

Although Inbe et al. suggested that the orphan receptor GPR80/99 may function as an AMP receptor,[17] there is now convincing evidence that GPR80/99 binds α-ketoglutarate, thereby functioning as a receptor for citric acid cycle intermediates.[18] In addition, other studies demonstrated that GPR80/99 does not bind Ado or AMP.[19] Therefore, there is now consensus that GPR80/99 is not a nucleotide receptor.[20] Furthermore, there is no unequivocal evidence that extracellular AMP itself can function as a signaling molecule.

The ability of endogenously produced Ado to engage AdoR can be modulated by the enzymes that metabolize it. For example, treatment of mice with an AK inhibitor resulted in attenuation of inflammatory responses with accumulation of Ado and enhancement of AdoR activation in various inflammation models such as LPS-induced septic shock,[21] dextran sodium sulfate (DSS)-induced colitis,[22] adjuvant

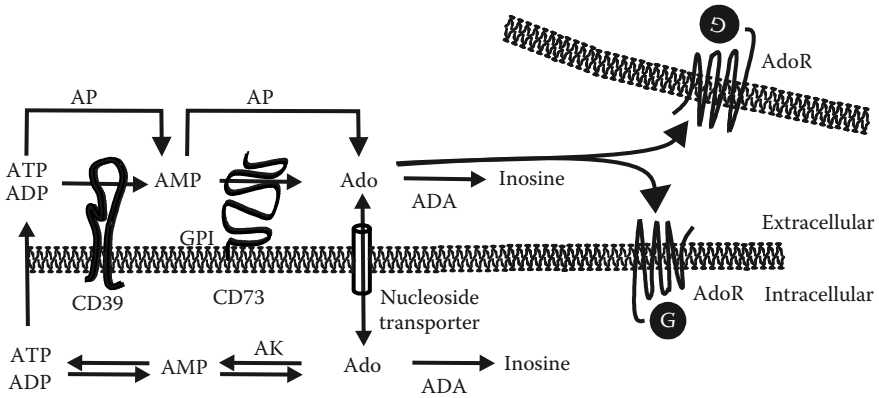

FIGURE 3.1 Generation of adenosine to engage adenosine receptors. Extracellular adenosine can be generated through the extracellular breakdown of ATP by the sequential action of CD39 and CD73 or via the action of alkaline phosphatase (AP). Alternatively, adenosine can be formed intracellularly and then exported to the outside of the cell via the action of a nucleoside transporter. The concentration of extracellular adenosine can be increased by blocking the action of the nucleoside transporter or by inhibiting the action of two enzymes that metabolize adenosine, adenosine kinase (AK), and adenosine deaminase (ADA). Extracellular adenosine can engage adenosine receptors in either an autocrine or paracrine fashion.

arthritis,[23] and carrageenan-induced inflammation in air pouches.[24] Similarly, treatment with an ADA inhibitor inhibited the production of proinflammatory cytokines and decreased mortality in LPS- or fecal-matter-induced endotoxemia and sepsis models.[25] In contrast, ADA anchored to the cell surface by CD26 inhibited AdoR activation and cAMP accumulation in Jurkat cells treated with exogenous Ado.[26] Together, these reports suggest that suppression of Ado metabolism increases AdoR activation and that AK and ADA can modulate AdoR signaling. Furthermore, Noji et al.[27] reported that an Ado uptake inhibitor can also attenuate LPS-induced TNF-α production and leukopenia by promoting AdoR activation. This finding is particularly interesting because such drugs not only inhibit the uptake of extracellular Ado but also its release from the cytosol to the extracellular milieu. These observations suggest that the anti-inflammatory action of endogenous Ado derives from its extracellular production in inflammatory sites. To clarify the role of CD73 in the production of extracellular Ado and to evaluate the contribution of CD73-generated Ado in the modulation of various physiological responses mediated by AdoR, *cd73*-deficient mice were generated.

3.2 GENERATION OF CD73 GENE-TARGETED MICE

Three groups have generated *cd73*-deficient mice: our group in Oklahoma City,[28] Koszalka and colleagues in Dusseldorf,[29] and Castrop et al. at The National Institutes of Health.[30] All three groups used a similar strategy, that of deleting one or more exons required for the ecto-5′-nucleotidase enzyme activity of CD73. Earlier work by Gutensohn et al. showed that both exons 2 and 3 contain highly conserved histidine residues that are essential for catalytic activity.[31] We deleted exon 3, Koszalka et al. deleted exon 2, and Castrop et al. deleted both exons 2 and 3. As expected, all three strains of CD73 gene-targeted mice are markedly deficient in ecto-5′-nucleotidase enzyme activity in every tissue examined. Heterozygous mice have intermediate levels of enzyme activity, consistent with a gene dosage effect. Cell-surface-expressed CD73 as assessed by immunofluorescence is also undetectable on the lymphoid cells of our mice.

Cd73-deficient mice appear normal and are healthy when maintained in conventional housing. They breed normally and have normal-sized litters. Thus, the significance of highly regulated CD73 expression in the primary decidua of pregnant mice,[32,33] appearing coordinately with the $A_{2B}R$ on day 5 of gestation, peaking on day 7, and disappearing again on day 9, remains unexplained. *Cd73*$^{+/+}$, *cd73*$^{+/-}$, and *cd73*$^{-/-}$ pups are born to heterozygous parents in the expected 1:2:1 ratio. *Cd73*-deficient pups cannot be distinguished from their *cd73*$^{+/-}$ and *cd73*$^{+/+}$ littermates based on size or behavior. Even though CD73 first came to the attention of immunologists because its expression on the surface of lymphocytes from patients with a variety of primary immunodeficiency diseases is markedly reduced,[34] the immune system of *cd73*-deficient mice appears to develop normally.[28] Their lymphoid organs are of normal size and contain normal proportions of T and B lymphocytes. *Cd73*-deficient mice have normal serum immunoglobulin levels and exhibit normal T and B cell responses to immunization with ovalbumin.

3.3 PHENOTYPES OF CD73-DEFICIENT MICE

3.3.1 Hypoxia-Induced Vascular Leak and Neutrophil-Endothelial Adhesion

CD73 expression is markedly upregulated under conditions of hypoxia.[9,35,36] Examination of the promoter region of the *cd73* gene revealed the presence of an HIF-1α binding site, and transfection studies with truncated promoter-luciferase constructs demonstrated that this binding site is functionally active.[37] Thus, it is not surprising that *cd73*-deficient mice exhibited a striking phenotype when exposed to hypoxic conditions. We examined the role of CD73-generated Ado in modulating transendothelial migration of polymorphonuclear leukocyte (PMN) initiated by PMN-endothelial adhesion and endothelial barrier function during hypoxia *in vivo* by utilizing *cd73*-deficient mice.

Previous *in vitro* studies suggested that activated PMN can be a source of extracellular ATP and that the ecto-enzymes CD39 and CD73 on endothelium can catalyze the conversion of ATP to Ado.[9] Furthermore, this endogenously generated Ado can regulate transendothelial migration of PMN through the activation of AdoR on both PMN and endothelial cells. It is well known that hypoxia induces tissue PMN accumulation, so the production of extracellular Ado through the coordinated action of hypoxia-induced CD39 and CD73 may be an important part of the mechanism. As such, given the strong evidence that AdoR activation "reseals" vascular endothelial leaks[38] (see later text), it appears that Ado generated at points of endothelial cell–PMN contact may function to "close the door" as PMNs exit the vasculature.

Eltzschig et al.[39] demonstrated the role of CD73 in PMN trafficking *in vivo* using *cd73* gene-targeted mice. They showed that much higher myeloperoxidase activity (caused by infiltrating PMN) was observed in the lung, colon, and liver of *cd73*-deficient mice subjected to normobaric hypoxia (8% O_2 and 92% N_2) for 4 h compared with wild-type mice. The increase in myeloperoxidase could be corrected by administration of exogenous purified snake venom 5′-nucleotidase. These results were verified in studies using the ecto-5′-nucleotidase inhibitor α,β-methylene adenosine 5′-diphosphate (APCP).[40] APCP treatment resulted in an increase in PMN adhesion to posthypoxic endothelia *in vitro*, and wild-type mice treated with APCP exhibited similar accumulations of PMN in tissues as *cd73*-deficient mice after hypoxia exposure *in vivo*. Thus, these results suggested that the enzyme activity of CD73 catalyzes the final critical step for Ado production and attenuates PMN accumulation in tissues under hypoxic conditions.

Thompson et al.[28] reported a key role for CD73 in controlling vascular leakage during hypoxia. It has been reported that Ado generated by CD73 can modulate endothelial barrier function *in vitro*.[38,41] In order to examine whether *cd73*-deficient mice have any defects in endothelial barrier function, these mice were injected with Evan's blue dye intravenously and exposed to normobaric hypoxia or room air for 4 h. The amount of dye that leaked into several organs was then examined. Vascular leakage was significantly increased in lung, liver, and skeletal muscle in *cd73*-deficient mice even in normoxic conditions. Furthermore, under hypoxic conditions, all the organs examined (lung, liver, skeletal muscle, colon, heart, and kidney), except for the brain,

showed significantly higher permeability in *cd73*-deficient mice compared with wild-type mice. Treatment of wild-type mice with APCP also enhanced hypoxia-induced vascular leakage. Vascular leakage in *cd73*-deficient mice was rectified by administration of either purified 5′-nucleotidase or the general AdoR agonist 5′-(*N*-ethylcarboxamido)-adenosine (NECA). These findings clearly demonstrated that Ado generated by CD73 protects most tissues from hypoxia-induced vascular leakage.

3.3.2 Coronary Blood Flow, Platelet Aggregation, and Leukocyte-Endothelial Adhesion after Ischemia Reperfusion

It is well known that Ado can modulate platelet aggregation, vascular tone, and adhesion of leukocytes to the vascular endothelium. In tissues that are well oxygenated, it is believed that the majority of Ado is generated via the action of cytosolic 5′-nucleotidase. Therefore, the contribution of CD73 to Ado-mediated regulation of vascular biology was investigated by Koszalka et al.[29] with their *cd73*-deficient mice. Systolic blood pressure, ejection fraction, and cardiac output were found to be normal in *cd73*$^{-/-}$ mice. However, in the isolated perfused heart, basal coronary flow was 14% lower in *cd73*-deficient animals than in control littermates, and this difference was statistically significant. This could not be attributed to any differences in $A_{2A}R$ expression or signaling, as the dose response of wild-type and *cd73*-deficient animals to Ado administered intracoronarily was similar. As expected, the dose response to AMP was shifted to the right by an order of magnitude in *cd73*-deficient hearts, showing that AMP must be hydrolyzed to Ado for dilation to occur.

Two approaches were undertaken to evaluate the importance of CD73-generated Ado in platelet thrombosis *in vivo*: time to vessel occlusion after $FeCl_3$-induced free radical injury and bleeding time after tail tip resection. In each case, time to response was reduced in the *cd73*-deficient animals (20% after topical application of $FeCl_3$ to the carotid artery and 35% after tail tip resection). The concentration of platelet cAMP was also reduced in *cd73*$^{-/-}$ mice, suggesting that plasma Ado levels are lower in the gene-targeted animals. However, platelet aggregation *in vitro* in response to various doses of ADP was similar between control and *cd73*$^{-/-}$ animals, suggesting that platelet function is intrinsically normal.

To investigate the role of CD73-generated Ado in the vascular inflammatory response, the cremaster muscle ischemia-reperfusion model was used. Reperfusion after 30 min of ischemia resulted in a 2.5-fold increase in the number of leukocytes adhering to the vessel walls of *cd73*$^{-/-}$ compared to wild-type mice, and the effect was greatest after 10 to 15 min of reperfusion. This difference could not be explained by variations in blood flow in microvessels, as this parameter was very similar in both strains of mice. Because histochemistry revealed a very strong expression of CD73 in carotid and coronary arteries, additional experiments were performed to evaluate leukocyte adhesion in these vessels. In this case, isolated carotid arteries were perfused with the monocytic cell line Mono Mac 6 *ex vivo*. Adhesion to wild-type vessels was negligible, but significant numbers of monocytes adhered to the vessel walls of *cd73*$^{-/-}$ mice after 10 min of perfusion. Together, these results suggest that CD73-generated Ado plays a significant role in anti-inflammatory vasoprotection.

3.3.3 Tubuloglomerular Feedback

In the kidney, Ado is an important regulator of the glomerular filtration rate, as it mediates vascular responses induced by changes in NaCl concentration. Tubuloglomerular feedback involves a functional interaction between the tubular epithelium in the environment of the macula densa and the underlying smooth muscle cells of the afferent and efferent glomerular arterioles. When NaCl concentrations increase in the luminal fluid around the macula densa, smooth muscle cells are activated, resulting in vasoconstriction of the arterioles. As a result, both the glomerular filtration pressure and filtration rates decrease. Experiments with A1R gene-targeted animals suggest that these phenomena are dependent on the A1R. However, conflicting data suggested that ATP rather than Ado might play a dominant role in the tubuloglomerular feedback response. To assess the contribution of CD73-generated Ado in local hemodynamic control mechanisms in the kidney, Castrop and colleagues[30] studied tubuloglomerular feedback in the kidneys of *cd73*$^{-/-}$ and wild-type mice.

The kidney function of *cd73*-deficient mice was carefully evaluated and found to be normal with respect to the following parameters: renal blood flow, renal vascular resistance, stimulation of renin secretion by furosemide, plasma osmolarity, and plasma concentrations of Na^+, Cl^-, BUN, creatinine, uric acid, and total protein. Therefore, under basal conditions, kidney function of *cd73*$^{-/-}$ mice appears to be normal. However, in response to saturating increases in tubular perfusion flow, the gene-targeted animals demonstrated significantly decreased reductions in stop flow pressure and superficial nephron glomerular filtration rates compared to those in wild-type mice. Furthermore, although wild-type mice showed relatively constant tubuloglomerular feedback responses during prolonged perfusion of the loop of Henle, in *cd73*$^{-/-}$ mice, over a period of 10 min of perfusion, there was a complete disappearance of the residual feedback response. Nevertheless, isolated afferent arterioles from *cd73*$^{-/-}$ mice showed normal contractile responses to exogenous Ado, suggesting that the observed deficient tubuloglomerular feedback responses were due to decreased concentrations of extracellular Ado, rather than any defects in AdoR responsiveness. Taken as a whole, these findings suggest that CD73-generated Ado is needed for optimal signaling between macula densa cells and the underlying vascular smooth muscle cells. The CD73 substrate 5′-AMP is most likely derived from ATP released from macula densa cells.

3.4 FUTURE STUDIES

From published studies using the ecto-5′-nucleotidase inhibitor APCP, it is highly likely that CD73 plays an important role in regulating several other AdoR-mediated physiological responses. For example, Morabito and colleagues[42] reported that APCP prevented the methotrexate-mediated increases in Ado observed in carrageenan-treated air pouches in mice. Similarly, Kitakaze et al.[43] showed that CD73-generated Ado is at least partially responsible for the phenomenon of ischemic preconditioning in dogs. Studies to evaluate the role of CD73-generated Ado in the anti-inflammatory action of methotrexate and in ischemic preconditioning are already underway.

Other models that have good potential to be informative are inflammatory lung disease in ADA-deficient mice,[44] DSS-induced colitis,[22] LPS-[21] and cecal ligation and puncture-induced septic shock,[45,46] and wound healing.[47,48] Experiments evaluating *cd73*-deficient mice in these models are also in progress.

Although the role of Ado and AdoR signaling in mediating anti-inflammatory responses is well known in several experimental systems, CD73 may not always be the source of extracellular Ado. This will depend on the level of CD73 expression, which is highly variable from tissue to tissue, and the relative abundance of other AMP dephosphorylating enzymes such as alkaline phosphatases and cytosolic 5′-nucleotidases. As part of one study addressing the phenotype of *cd73*$^{-/-}$ animals,[28] assessment of CD73 enzyme activity (i.e., AMP hydrolyzing activity that can be inhibited by APCP) in a broad range of tissues produced several important findings. First, there was a nearly 50-fold variation in CD73 ecto-5′-nucleotidase enzyme activity in different tissues from wild-type animals. Second, the colon showed the highest level of enzyme activity among the tissues surveyed, a somewhat surprising result as a number of previous studies suggested that the greatest activity in any tissue was in the kidney. Instead, it was found that the colon exhibits nearly twice as much activity as the kidney, with the order of tissue activity ranked as follows: colon > kidney = brain > liver > lung > heart >> muscle. Furthermore, AMP-hydrolyzing enzyme activity that was not attributed to CD73 (i.e., nucleotidase or phosphatase activity that cannot be inhibited by APCP) also varied widely among individual tissues (up to 20-fold among different tissues). Why such seemingly disparate enzyme expression patterns exist is not clear at present. However, the data suggest tissue-specific patterns of extracellular nucleotide metabolism and differences in the relative contribution of CD73 to extracellular Ado generation among individual tissues. Understanding the source of extracellular Ado is important if pharmacological approaches to modulate Ado levels at sites of inflammation are to be devised. *Cd73*-deficient mice have been, and will continue to be, an important tool to accomplish this goal.

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4 Regulation of Monocyte/Macrophage Function by Adenosine Receptors

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CONTENTS

4.1 INTRODUCTION

Adenosine is an endogenous purine nucleoside that, subsequent to its release from cells or after being generated extracellularly, diffuses to the membrane of surrounding cells, in which it binds to specific cell surface structures that recognize it, termed *adenosine receptors*. There are four types of adenosine receptors, all of which are members of the G-protein-coupled family of receptors. They are the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors.[1–4] Although adenosine is present at low concentrations in the extracellular space, metabolically stressful conditions increase its extracellular levels dramatically. Adenosine receptors are expressed abundantly on cells of the monocyte/macrophage lineage, and through these receptors adenosine exerts substantial modulatory effects on monocyte/macrophage function.[5–7] Because macrophages are activated by stressful stimuli such as inflammation, hypoxia, and ischemia, the same stimuli that can result in accumulation of extracellular adenosine, adenosine receptors expressed on macrophages represent a sensor system providing macrophages with essential information about the inflammatory or ischemic environment.[6] This mechanism allows macrophages to fine-tune their responses to stressful stimuli. In this chapter, we focus on the consequences of adenosine receptor engagement on monocyte and macrophage function. We will delineate the effect of stimulating the various adenosine receptor subtypes on macrophage differentiation/proliferation, phagocytosis, membrane protein expression, and mediator production. A more complete understanding of the regulation of monocyte/macrophage function by adenosine receptor engagement should help develop novel therapeutic ways of manipulating disorders that are associated with exuberant or insufficient macrophage activation.

4.2 FUNCTIONAL HETEROGENEITY OF MACROPHAGES AND ENVIRONMENTAL REGULATION

Macrophages are a heterogeneous population of ubiquitously distributed mononuclear phagocytes responsible for diverse homeostatic, immunological, and inflammatory processes.[8,9] Their wide tissue distribution makes these cells ideally suited to providing an immediate defense against foreign elements prior to the migration of neutrophils and, thus, macrophages are major factors in the body's immune surveillance. Newly formed macrophages, termed *monocytes*, arise from progenitor cells in the bone marrow.[10,11] Following differentiation caused by local cytokines such as interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF), they leave the unique environment of the bone marrow and enter the blood. In the blood, they are exposed to a plethora of agents (including cytokines, chemokines, hormones, fatty acids, and immunoglobulins), which are capable of impacting their functional and phenotypic characteristics. Circulating monocytes provide a mobile source of functionally competent immunocytes capable of infiltrating the tissue, in which homing process occurs in response to chemokines and other tissue-specific homing factors.[12] Upon migration into tissues, monocytes undergo further differentiation to become macrophages. Contrary to the previously held view that the distinct and unique functional patterns displayed by macrophages in various tissues represent distinct lineages, it is becoming

increasingly appreciated that these distinct phenotypes of macrophages in different tissues reflect an impact of the actual tissue environment, rather than originating from lineage specificity.[9]

The biological functions of activated monocytes and macrophages are myriad and well documented.[8,9,13] These cells, which are one of the key constituents of innate immune responses, participate in the initial capture, processing, and nonspecific killing of antigens, and then in the activation of specific lymphocyte effector mechanisms. The process of lymphocyte activation is dependent on the expression of macrophage cell surface molecules such as major histocompatibility complex and costimulatory molecules, as well as production of a wide variety of mediators, which include cytokines and free radicals.[14] These activated lymphocytes, representing the specific arm of the immune response, in turn cooperate with macrophages to enhance destruction of pathogens, as well as virus-infected cells, tumor cells, and apoptotic host cells. Adenosine has the potential to regulate all aspects of macrophage activation and thereby to modify the inflammatory process. Generally speaking, adenosine favors an anti-inflammatory macrophage phenotype that is characterized by decreased production of proinflammatory mediators and phagocytosis, as well as increased levels of the anti-inflammatory cytokine IL-10.

Once the inflammatory response has neutralized an injurious stimulus, the process of inflammatory resolution ensues, which is a highly coordinated and active process that is controlled by endogenous "pro-resolving" mediators.[15–17] These factors switch off leukocyte trafficking to the inflamed site, reverse vasodilation and vascular permeability, and bring about the safe disposal of inflammatory neutrophils, exudate and fibrin, thereby leading to the restoration of the inflamed tissue to its prior physiological function. Macrophages have central roles in mediating the resolution of inflammation, and there is evidence that adenosine can interfere with features of the inflammatory resolution. The significance of this process is that successful resolution will limit excessive tissue injury and give little opportunity for the development of chronic, immune-mediated inflammation.[16]

4.3 ADENOSINE METABOLISM INTRA- AND EXTRACELLULARLY

Physiological actions of adenosine almost exclusively result from its stimulation of cell surface adenosine receptors and the activation of downstream second messenger pathways. Processes related to its generation, liberation from cells, cellular reuptake, and metabolism determine the bioavailability of adenosine at receptor sites. These processes are closely interconnected and tightly regulated. One good example of this interdependence is the concerted action of intracellular purinergic metabolic pathways in increasing intracellular adenosine concentrations during tissue hypoxia or ischemia. Under these conditions, the increased dephosphorylation of ATP to adenosine by the metabolic enzyme 5′-nucleotidase is accompanied by a suppression of the activity of the salvage enzyme adenosine kinase, which prevents the rephosphorylation of adenosine.[18] Once adenosine reaches high concentrations inside the cell, it is then liberated into the extracellular space through nucleoside transporters.[19,20] The other major, and probably dominant, pathway that contributes to high extracellular adenosine

concentrations during metabolic stress is release of precursor adenine nucleotides (ATP, ADP, and AMP) from the cell. This is followed by extracellular catabolism to adenosine by a cascade of ectonucleotidases, which include CD39 (nucleoside triphosphate dephosphorylase [NTPD]) and CD73 (5′-ectonucleotidase).[21–25] Adenosine bioavailabilty is limited by its catabolism to inosine by adenosine deaminase. Inosine is finally degraded to the stable end product uric acid.[26,27]

It is not entirely clear which cell types are the most important producers of extracellular adenosine. Neutrophils and endothelial cells have both consistently been reported to release high levels of adenosine at sites of metabolic distress, inflammation, and infection.[28–31] In addition, our group recently documented that nerve terminals are a major source of extracellular adenosine in spleen that has been subjected to ischemia.[32] ADP released by platelets at sites of injury can also be dephosphorylated to adenosine.[33] Macrophages themselves can be the source of extracellular adenosine via ATP production. We recently found that bacterial lipopolysaccharide (LPS) was able to promote the release of ATP from macrophages,[34] and because the modulatory effects of exogenously added ATP on macrophage responsiveness can be prevented by adenosine deaminase,[35] this indicates that endogenously released ATP is also metabolized to adenosine by macrophages.

It is generally thought that the concentrations of extracellular adenosine are less than 1 μM in unstressed tissues, whereas adenosine levels in inflamed or ischemic tissues can be as high as 100 μM. For example, a recent study has documented that systemic (plasma) adenosine levels reach 4 to 10 μM in patients with sepsis, a condition associated with both ischemia and inflammation, whereas adenosine concentrations in healthy individuals are less than 1 μM.[36] Even higher adenosine concentrations, in the 10 to 100 μM range, were recently found in the synovial fluid of patients with rheumatoid arthritis.[37] Lower concentrations of adenosine activate the high-affinity A_1 and A_{2A} receptors, and high adenosine concentrations stimulate the low-affinity A_{2B} and A_3 receptors. Thus, because the degree of metabolic distress can dictate the release and thereby the concentration of extracellular adenosine, the higher the metabolic distress, the more probable it is that A_{2B} and A_3 receptors are activated. Other factors that determine the net effect of adenosine on macrophage function are adenosine receptor expression and coupling efficacy to intracellular signaling pathways, both being very dynamically regulated.

Finally, it needs to be emphasized that adenosine signaling in the immune system should be viewed in the context of signaling initiated by both upstream and downstream metabolites of adenosine. It is well established that adenine nucleotides have powerful immunoregulatory effects, which are mediated by P_2-purinoceptors.[38–41] Furthermore, recent evidence indicates that the adenosine breakdown products inosine and uric acid can influence many features of the innate immune response.[42–44] Conventional thinking is that inosine does not possess biological activity; however, recent studies document that it has potent immunomodulatory and macrophage-deactivating effects, some of which are mediated through adenosine receptor binding. For information on the effects of inosine on the immune system, the reader is referred to Chapter 15, which details the immunomodulatory properties of this adenosine metabolite.

4.4 EFFECT OF ADENOSINE ON MONOCYTE/MACROPHAGE PROLIFERATION AND DEVELOPMENT

As discussed earlier, macrophage differentiation, maturation, and proliferation are tightly regulated processes that determine the nature and magnitude of macrophage responsiveness to activating stimuli. There is some evidence available that adenosine can interfere with the course of macrophage proliferation and differentiation. Indirect evidence that endogenous adenosine promotes human monocyte maturation came by the demonstration that adenosine deaminase activity is increased during early monocyte differentiation and that blockade of adenosine deaminase during this period retarded the maturation process.[45] In contrast, high concentrations of exogenous adenosine appear to prevent monocytes from proceeding into macrophages and arrest monocytes in a phase with high accessory function having a phenotype similar to dendritic cells (Reference 46; Figure 4.1). An interesting aspect of adenosine function with regard to monocyte maturation is that whereas A_1 receptor stimulation promotes the formation of multinucleated giant cells, A_2 receptor activation prevents it (Figure 4.1).[47] The precise mechanisms by which adenosine receptor signaling affects monocyte maturation are unclear and remain an important area for future investigation.

Only a few studies have assessed the impact of adenosine receptor occupancy on macrophage proliferation. M-CSF-induced proliferation of mouse bone marrow macrophages is inhibited by adenosine, an effect that is mediated through A_{2B} receptors (Reference 48; Figure 4.1). The mechanism of adenosine's action involves induction, in a PKA-dependent manner, of the expression of $p27^{kip-1}$, a cyclin-dependent kinase inhibitor that causes growth arrest at the G_1 phase of the cell cycle.

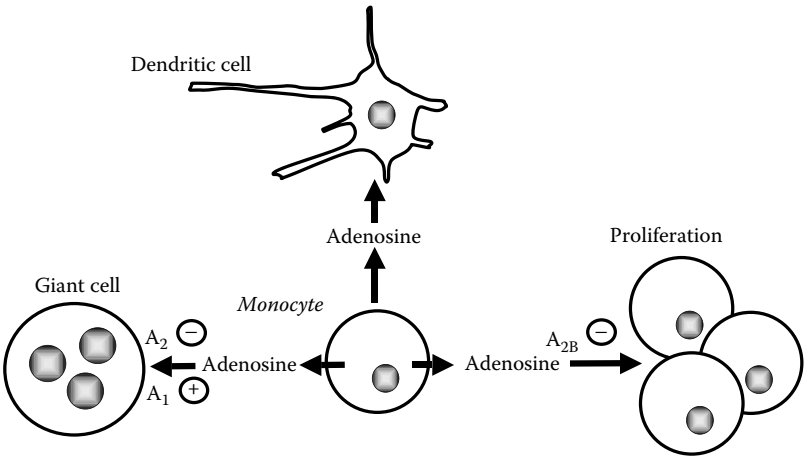

FIGURE 4.1 Adenosine inhibits macrophage proliferation via A_{2B} receptors. Adenosine promotes multinucleated giant cell formation through activation of A_1 receptors and inhibits it through A_2 receptors. Adenosine drives monocyte differentiation toward a phenotype that resembles dendritic cells.

In human monocytes, the nonselective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA), but not 2-p-(2-carboxyethyl)phenethylamino-5-*N*′-ethyl-carboxamidoadenosine (CGS-21680), a selective agonist of A_{2A} receptors, inhibited the proliferation of human peripheral blood mononuclear cells from healthy subjects, whereas the selective A_1 receptor agonist N^6-cyclopentyladenosine (CPA), but not other agonists, suppressed cell proliferation in asthmatic patients.[49] Because these effects appeared only at high, nonselective drug concentrations (10 μM), the authors concluded that their results did not support a major role for adenosine in mononuclear cell proliferation. Additional studies to examine the effect of extracellular adenosine on macrophage proliferation are clearly warranted.

4.5 OPPOSING ROLES FOR A_1 AND A_2 RECEPTORS IN MODULATING FC-RECEPTOR-MEDIATED PHAGOCYTOSIS

Phagocytosis is the mechanism used by macrophages to internalize, degrade, and eventually present peptides derived from particulate antigens. When a small particle is coated (opsonized) with IgG, the Fc regions of the IgG molecules bind to Fc receptors in the macrophage plasma membrane and initiate a phagocytic response.[50] The regulation of Fcγ-receptor-mediated phagocytosis by adenosine was among the first actions described for this nucleoside in monocytes/macrophages.[51] Adenosine, in the presence but not in the absence of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), caused a dose-dependent enhancement of Fcγ-mediated phagocytosis by undifferentiated monocytes of antibody-coated sheep erythrocytes.[51] The fact that the presence of EHNA was necessary to show an impact of adenosine on monocyte function indicates that monocytes contain high levels of adenosine deaminase, which degrades adenosine resulting in loss of its activity. In contrast to the observed augmentation of phagocytosis in human monocytes, adenosine in the presence of EHNA inhibited Fcγ-mediated ingestion of erythrocytes by mouse peritoneal macrophages,[52,53] suggesting that the effects of adenosine can be different on the same cell type from varying sources. Subsequent studies have provided clues to explain the opposing effects of adenosine on phagocytosis by undifferentiated monocytes and peritoneal macrophages, which represent a differentiated macrophage population. Eppell and coworkers[54] confirmed that adenosine in the absence of EHNA fails to affect phagocytosis by undifferentiated monocytes; however, these investigators demonstrated that monocytes that were in culture for 2 d or more showed a decreased phagocytic response to adenosine. The kinetics of this change in effect closely paralleled the appearance of specific adenosine binding to the cells, suggesting that certain adenosine receptors appear on monocytes during *in vitro* differentiation into macrophages. The inhibitory effect of adenosine on monocyte phagocytosis was attributable to A_2 receptor occupancy, because the order of potency of agonists was NECA > adenosine > N^6-R-phenylisopropyladenosine (R-PIA, an A_1 receptor agonist) as measured after 2 d of culture. In addition, the suppressive effect of adenosine on monocyte phagocytosis was reversed using a selective protein kinase A (PKA) inhibitor, further implicating A_2 receptors, because A_2, but not other adenosine receptors, are positively coupled to the cAMP–PKA signaling pathway. Although these

findings could explain why adenosine inhibits phagocytosis in mature macrophages,[52,53] it was still unclear why adenosine in the presence of EHNA enhances phagocytosis by fresh, undifferentiated cells.[51] A subsequent study using selective, nondegradable adenosine receptor analogs in freshly isolated monocytes appeared to resolve this issue. Salmon and coworkers[55] demonstrated that in these undifferentiated monocytes the A_1 receptor agonist CPA was able to enhance phagocytosis, revealing a role for A_1 receptors in promoting Fcγ-receptor-mediated phagocytosis. These pharmacological results were supported by data obtained using flow cytometry, which indicated that the A_1 receptor was expressed on the surface of immature monocytes. Thus, the major issue here appears to be the fact that when the degradation of adenosine is blocked by using either EHNA or a nondegradable adenosine analog, the stimulatory effect of A_1 receptor stimulation on monocyte phagocytosis is unmasked. At the same time, the selective A_2 receptor agonist NECA decreased phagocytosis even in these fresh monocytes, which appears to contradict the earlier conclusion of Eppell et al.[54] that A_2 adenosine receptors are not expressed on these immature cells. Again, a plausible explanation for this observation is that by using a nondegradable adenosine receptor agonist, NECA, the inhibitory effect of A_2 receptors could be unveiled even in these immature monocytes. A_2 receptor expression increases as a function of time, because the degree of inhibition in fresh monocytes by NECA is smaller than that observed in more mature cells. In summary, monocyte/macrophage phagocytosis is regulated in an opposing and temporal manner by both A_1 and A_2 receptors, where in fresh monocytes the stimulatory effect of A_1 receptors appears to dominate, giving way, however, to an A_2-receptor-mediated suppression of phagocytosis in more mature macrophages. These studies, which were carried out more than 10 yr ago, provided some insight into how adenosine regulates monocyte/macrophage phagocytosis. Nonetheless, it is important to note that the selectivity of the agents used is highly questionable (for example, NECA can bind to all four adenosine receptor subtypes), which implies that additional studies on the involvement of the various adenosine receptor subtypes are warranted.

4.6 ADENOSINE-INHIBITED MONOCYTE/MACROPHAGE OXIDATIVE BURST

Monocytes/macrophages produce reactive oxygen species (mainly superoxide and H_2O_2) during phagocytosis or stimulation with a wide variety of agents through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase, which is critical for the bactericidal activation of macrophages.[56] Several independent studies have shown that adenosine and adenosine analogs are potent inhibitors of monocyte/macrophage respiratory burst. Almost two decades ago, Leonard et al.[57] reported that adenosine-treated human monocytes exhibited diminished responsiveness to f-Met-Leu-Phe- (fMLP) but not phorbol myristate acetate (PMA). Because adenosine failed to moderate the PMA-induced respiratory burst, it was concluded that the mechanism of adenosine's action was independent of, and had no effect on, protein kinase C (PKC). Two subsequent studies, one using fMLP[58] and the other LPS[59] to stimulate oxidative burst, confirmed that the effect of adenosine

could be attributed primarily to A_3 receptor stimulation. This conclusion was based on the observations that the A_3 receptor agonist N^6-(3-iodobenzyl)adenosine-5′-*N*-methyuromide (IB-MECA) was more potent than the A_{2A} agonist CGS-21680 or A_1 receptor agonist R-PIA. For all four adenosine receptor subtypes, mRNA was expressed in freshly isolated monocytes, and although the expression of A_1, A_{2B}, and A_3 receptors did not change dramatically during a 7-d cultivation period, the mRNA for A_{2A} receptors was upregulated after day 1 and disappeared by day 7 of culture.[59] Consistent with the pharmacological data that IB-MECA was more potent than CGS-21680, which triggers its cellular effects mainly via the cAMP–PKA pathway,[60] the cAMP-dependent protein kinase inhibitor KT5720 was unable to prevent the inhibitory effect of adenosine on fMLP-triggered monocyte respiratory burst.[58] The inhibitory effect of adenosine on human monocyte/macrophage respiratory burst was reproduced using both mouse[61] and rat[62] peritoneal macrophages; however, there was no detailed analysis of the adenosine receptors involved in either study.

4.7 ADENOSINE-MODULATED NITRIC OXIDE (NO) PRODUCTION BY MONOCYTE/MACROPHAGES

In addition to H_2O_2 and superoxide, NO is another critical factor that contributes to the bactericidal activity of macrophages. NO synthases (NOSs) catalyze the oxidation of one of the guanidino nitrogens of l-arginine to NO.[63] Of the multiple NOS isoforms that can catalyze NO synthesis, iNOS is most associated with antimicrobial activity. Host expression of iNOS is primarily regulated at the transcriptional level and can be stimulated following interaction with microbial products or in response to cytokines such as IL-1, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ).[64] In 1996, our group provided evidence that both the selective A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) and A_{2A} agonist CGS-21680 decreased NO production by LPS-stimulated RAW264.7 macrophages, both agents being efficacious at high concentrations (300 μM) only.[65] In subsequent studies, it was found that several adenosine receptor agonists (0 to 100 μM) increased NO production under very similar conditions,[66,67] which is difficult to reconcile with our results showing a lack of effect of adenosine receptor agonists at these concentrations. On the other hand, treatment of IFN-γ-activated bone marrow macrophages with NECA reduced both iNOS induction and NO production in these cells.[68] Studies with radioligands and antibodies indicated the presence of both A_{2B} and A_3 receptors on these bone marrow macrophages; however, a detailed pharmacological analysis was not performed to identify the receptor type responsible for the downregulation of NO production. Although the receptor type that downregulated iNOS expression was not addressed directly in this study, the A_{2B} receptor appears to be the primary receptor modulating IFN-γ-induced responses in bone-marrow-derived macrophages. The reason for this hypothesis is that NECA, but not selective A_1, A_{2A}, or A_3 agonists, inhibited the IFN-γ-induced upregulation of major histocompatibility complex II expression on these cells.[68] Taken together, further research will be necessary to define the effect of adenosine on NO production by macrophages as well as to determine whether any modulatory effect results in altered bactericidal activity by these cells.

4.8 REGULATION OF PATTERN RECOGNITION RECEPTOR (PRR)-MEDIATED CYTOKINE PRODUCTION BY ADENOSINE RECEPTOR SIGNALING

Probably the most important recent development in the field of macrophage immunology is the discovery of PRRs, which have evolved to permit recognition of conserved repetitive microbial elements (e.g., LPS, CpG DNA, and viral RNA). These include the Toll-like receptors (TLRs), mannose-binding lectins, and scavenger receptors.[69,70] Recent studies have made it abundantly clear that signals initiated by adenosine receptor occupancy can interfere with the intracellular pathways activated by PRRs. The best-studied aspect of the interaction between the two pathways is the effect of adenosine receptor stimulation on cytokine production by monocytes/macrophages.

4.8.1 Adenosine-Modulated TNF-α Production by Monocytes and Macrophages

TNF-α is a pleiotropic cytokine, which is produced principally by monocytes and macrophages.[71] This proinflammatory mediator has a diverse range of biological activities. Systemic exposure to recombinant TNF-α causes a syndrome of shock and tissue injury indistinguishable from septic shock syndrome. In addition to its well-known role in endotoxemic shock, TNF-α has been implicated in the pathogenesis of chronic processes such as autoimmunity, graft-versus-host disease (GVHD), rheumatoid arthritis (RA), Crohn's disease, and the cachexia accompanying cancer and acquired immunodeficiency syndrome (AIDS). Given the importance of TNF-α in mediating inflammation, it is not surprising that the regulation of TNF-α production by adenosine receptors has been the major focus of studies that examine the impact of adenosine on monocyte/macrophage activation.

Le Vraux et al.[72] published the first report on the ability of adenosine receptor ligands to suppress TNF-α production by monocytes. These authors found that adenosine receptor agonists decreased TNF-α production by LPS (TLR4 ligand)-stimulated human monocytes with a rank order of potency: NECA > R-PIA = CGS-21680 > 2-CADO (2-chloroadenosine) = CHA (N^6-cyclohexyladenosine). It was concluded that this order of potency is not characteristic of either A_1 or A_2 receptors, suggesting the involvement of another subtype. Studies using the mouse macrophage cell line RAW264.7 revealed a similar picture, in which CCPA and CGS-21680 were equally potent in suppressing LPS-induced TNF-α secretion.[65] In another study, NECA was somewhat more potent than CPA, allowing the conclusion that A_2 receptors were responsible for the inhibitory effect of adenosine on TNF-α secretion.[73] In yet another study, CGS-21680 was most potent and the selective A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX), but not A_1/A_3 antagonist 1,3-dipropyl-8-phenylxanthine amine congener (XAC), reversed the effect of CGS-21680, supporting the conclusion that A_2 receptors mediated the inhibition of TNF-α production by LPS-activated human monocytes.[74]

Shortly after the discovery of A_3 receptors and relatively selective A_3 receptor ligands,[75] attention shifted to the investigation of these receptors in modulating

TNF-α production by monocytes. Sajjadi and coworkers,[76] using PMA-differentiated U937 (human monocyte) cells, found the order of agonist potency in inhibiting LPS-induced TNF-α production to be IB-MECA > 2-CADO = I-ABA (N^6-(4-amino-3-iodobenzyl)adenosine) > N^6-benzyl NECA > NECA > CGS-21680 > CHA, which is indicative of a principal role for A_3 receptors. Further support for this conclusion came with the demonstration that the selective A_1/A_3 receptor antagonist XAC, but not A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or A_2 agonist DMPX, reversed the action of the selective A_3 agonist I-ABA. Northern blot analysis detected A_3 receptors in these cells, although A_1 and A_2 receptors were also present. Also, the inhibitory effect of I-ABA on TNF-α secretion was accompanied by a decrease in TNF-α mRNA accumulation. A detailed analysis of multiple signaling pathways indicated that the decrease in TNF-α production following I-ABA treatment was associated with changes in activation of the activator protein-1 (AP-1) transcription factor system, whereas the action of I-ABA appeared to be independent of mitogen-activated protein (MAP) kinases and nuclear factor-κB (NF-κB), as well as PKA, PKC, and PLC. The inability of adenosine, at least at physiological concentrations, to suppress LPS-induced NF-κB activation was confirmed by other studies.[77–79]

Studies with the murine macrophage cell line J774.1 reinforced the idea that A_3 receptors may contribute to the reduction of TNF-α production as the A_3 receptor agonist N^6-(2-(4-aminophenyl)ethyl)-adenosine (APNEA) was more potent than various A_1 and A_2 agonists, and a selective A_3 antagonist reversed the effect of APNEA.[80] Also, A_3 receptor mRNA was expressed at levels ten times higher than A_1 or A_{2A} receptor mRNA. Recent studies using knockout mice for the A_{2A} and A_3 receptors have illustrated that both these receptors contribute to the adenosine suppression of TNF-α production following TLR stimulation.[81–83]

Although, as illustrated in the preceding studies, A_1 receptors do not appear to have a role in diminishing TNF-α production in LPS-stimulated human monocytes, this may not be the case when other stimuli are used. For example, upon stimulation with a combination of PMA and phytohemagglutinin (PHA), peripheral blood mononuclear cell TNF-α release is suppressed by A_1 receptor activation.[84] Evidence to support this conclusion was based on the observation that the selective A_1 receptor antagonist DMPX counteracted the inhibitory effect of the A_1 agonist R-PIA on TNF-α secretion. Interestingly, this mechanism was operational in healthy patients but not in individuals afflicted with multiple sclerosis, pointing to a possible role of dysregulated A_1-receptor-mediated cytokine modulation as an etiological factor in multiple sclerosis. In addition to A_1 receptors, A_{2A} receptors might also regulate the TNF-α response to PMA/PHA, because the inhibitory effect of CGS-21680 was prevented by the selective A_{2A} antagonist in U937 cells.[85] Interestingly, the mechanism of action by which CGS-21680 decreased TNF-α production was posttranscriptional because this drug decreased the stability of TNF-α mRNA, an effect that was closely associated with a reduction in p38 MAPK activation, an important regulator of TNF-α mRNA stability.[86]

Taken collectively, TNF-α production by monocytes/macrophages can be subject to inhibition by A_1, A_{2A}, and A_3 receptors, and the receptor subtype involved depends on many factors, which include the source of cell and the inflammatory stimulus used.

Remarkably, the inhibitory effect of adenosine on TNF-α production by macrophages is not confined to TLR4-mediated induction of this cytokine because adenosine downregulates TNF-α production when induced by agonists of TLR2, TLR3, TLR4, TLR7, and TLR9.[77] Although it is probable that adenosine receptor ligation targets a common major intracellular pathway in suppressing TNF-α production following TLR stimulation, the nature of this intracellular target is unclear at this juncture. Nevertheless, the fact that the various adenosine receptors are all coupled to TNF-α production in an inhibitory fashion suggests that endogenous adenosine may represent a pivotal negative feedback signal on inflammatory processes.

4.8.2 Adenosine Suppression of IL-12 Production by Monocytes and Macrophages

IL-12 is a heterodimeric cytokine that is secreted mainly by activated antigen-presenting cells, including macrophages, and plays a key role in determining the nature of the immune response to exogenous or endogenous antigens. Adenosine receptor ligation on monocytes and macrophages strongly suppresses LPS-induced IL-12 production. Using A_{2A} receptor knockout mice, our group[81] showed that the adenosine suppression of IL-12 production by LPS-stimulated mouse peritoneal macrophages is dependent, in part, on A_{2A} receptors. Further proof implicating A_{2A} receptors came from studies with human monocytes, in which CGS-21680 potently blunted LPS-induced IL-12 production, which effect was reversible by A_{2A} antagonists and dependent on cAMP (Figure 4.2).[87,88] Furthermore, A_{2A} receptor stimulation

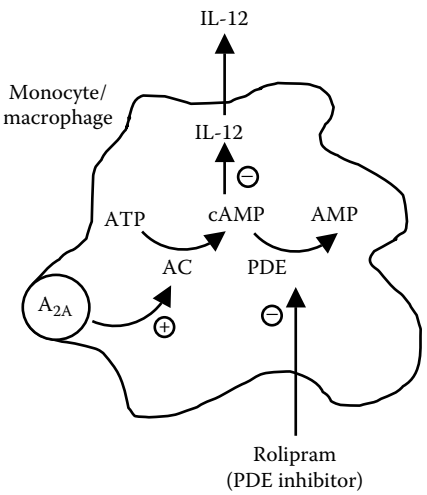

FIGURE 4.2 A_{2A} receptor stimulation inhibits IL-12 production by monocytes/macrophages via a cyclic adenosine monophosphate (cAMP)-mediated mechanism. A_{2A} receptor stimulation activates adenylate cyclase (AC), leading to increased intracellular cAMP levels. Phosphodiesterase (PDE) inhibition with rolipram can mimic the effect of A_{2A} receptor stimulation in suppressing IL-12 production.

led to decreased levels of IL-12 p40 mRNA.[87] Khoa and coworkers[88] reported recently that the degree of downregulation of IL-12 production by macrophages following A_{2A} receptor stimulation is profoundly affected by the inflammatory milieu that the cells encounter. The proinflammatory cytokines TNF-α and IL-1 enhanced responsiveness to A_{2A} receptor stimulation, resulting in a more impaired IL-12 production following treatment with CGS-21680. On the other hand, the presence of IFN-γ attenuated the suppressive effect of A_{2A} receptor stimulation on IL-12 production. In parallel with the observed functional changes following treatment with proinflammatory cytokines or IFN-γ, expression of A_{2A} receptor mRNA and protein was found to be increased or decreased, respectively.

A_3 receptor activation can also suppress IL-12 production, because the selective A_3 receptor agonist IB-MECA moderates IL-12 production both in LPS-treated mice[89] and by human monocytes.[90] IB-MECA was shown to activate the phosophatidyl inositol-3-kinase (PI3K) pathway, and the activity of both PI3K and Akt was required for its suppressive effect.[90]

Because IL-12 is instrumental in directing a strong inflammatory response, the adenosine suppression of IL-12 production is probably one of the central mechanisms whereby adenosine receptor occupancy prevents inflammation-induced tissue injury.

4.8.3 Adenosine-Augmented IL-10 Release by Monocytes and Macrophages

IL-10 is an important immunomodulatory cytokine, which has attracted much attention because of its anti-inflammatory and immunosuppressive properties. IL-10 was initially described as a T helper 2 product that reduced the production of cytokines by T helper 1 T cell clones.[91] Subsequently, it has become clear that IL-10 is also produced by cells of the monocyte/macrophage lineage,[92] and monocytes/macrophages appear to be the predominant cell type secreting IL-10 following LPS administration *in vivo*.[93] Macrophages exposed to LPS secrete IL-10 with a later onset when compared to proinflammatory cytokines. This delayed production of IL-10 represents an essential autoregulatory mechanism that limits excessive production of the proinflammatory cytokines TNF-α, IL-1, and IL-12,[94] thereby contributing to both the limitation and resolution of inflammation.[16]

There is good evidence that adenosine receptor stimulation can augment IL-10 production by monocytes and macrophages. In human monocytes stimulated with TNF-α, H_2O_2, or LPS, adenosine upregulated IL-10 production, an effect that was not reproducible using NECA, 2-CADO, or R-PIA.[95] Additionally, the nonselective adenosine receptor antagonist theophylline was unable to block the stimulatory effect of adenosine. The authors concluded that the effect of adenosine was not receptor mediated. Despite these observations, it is important to note that a role of cell surface adenosine receptors cannot be ruled out for the following reasons: First, selective A_{2A} or A_3 agonists were not tested in this study. Second, theophylline at the concentration used (100 μM) might well have inhibited phosphodiesterases,[96] which effect has been shown to increase IL-10 production,[97] thereby potentially masking its adenosine receptor antagonistic property. Finally, the adenosine uptake blocker

dipyridamole was also ineffective in preventing the stimulatory effect of adenosine on IL-10 production, which observation precludes an intracellular receptor-independent effect of adenosine. These results, that adenosine receptor agonists were ineffective at increasing IL-10 production in human monocytes, were later reproduced by another group.[87] In sharp contrast, when human whole blood was incubated with various adenosine receptor agonists, there was a substantial stimulation of LPS-induced IL-10 production, and the agonist order of potency was indicative of a primary role for A_{2A} receptors.[87] In addition, the A_{2A} antagonist (8-(3-chlorostyryl)caffeine) CSC, but not antagonists of the other adenosine receptors, prevented the stimulatory effect of CGS-21680. Importantly, adenosine receptor stimulation increased IL-10 production when this cytokine was induced using *Staphylococcus aureus*, a TLR2 ligand. Thus, similar to the observations with TNF-α, the modulatory effect of adenosine on IL-10 is not limited to TLR4-induced IL-10 production. In a study utilizing murine peritoneal macrophages, adenosine augmented IL-10 production[81] to a similar degree (approximately by 100%) as seen in human monocytes. We recently found that the stimulatory effect of adenosine on IL-10 production in RAW264.7 mouse macrophages was mediated through the A_{2B} receptor, because the order of potency of selective agonists was NECA > IB-MECA > CCPA = CGS-21680.[98] Also, the selective A_{2B} antagonist, alloxazine, prevented the effect of adenosine. The possible role of the A_{2B} receptor was further highlighted by the fact that RAW264.7 cells expressed the A_{2B} receptor, which was further increased following LPS treatment. With regard to cellular signaling, our data indicate that although LPS induces both IL-10 promoter activity and IL-10 mRNA accumulation, adenosine fails to alter the magnitude of these responses, attesting to a translational rather than a transcriptional effect of adenosine on the stimulation of IL-10 production. In addition, the 3′-untranslated region (UTR) of IL-10 mRNA has a substantial inhibitory effect on the translation, but not transcription, of a reporter construct, and this repressive effect is partially relieved by adenosine. The IL-10 3′-UTR forms specific complexes with proteins present in macrophage extracts, and adenosine enhances the formation of these complexes.[98]

4.9 ADENOSINE-INHIBITED TISSUE FACTOR EXPRESSION

Tissue factor is an integral membrane protein constitutively expressed in many cell types outside the vasculature, but it is not normally expressed on cells that are in contact with blood flow. Exposure of active tissue factor to blood triggers blood coagulation and thrombosis.[99] Blood monocytes do not constitutively express functional tissue factor; however, they are capable of tissue factor synthesis and expression when stimulated with LPS or certain proinflammatory cytokines.[100]

The antiplatelet agent dilazep exerts its antithrombotic effects by increasing the adenosine level in the extracellular fluid via blocking adenosine transporters.[101] In addition to its direct antiplatelet effects that occur via binding A_{2A} receptors expressed on platelets,[102] dilazep was reported to also block tissue factor expression on monocytes and thereby decrease blood coagulation.[103] The inhibitory action of dilazep on monocyte tissue factor expression was recently demonstrated to occur

via an adenosine-receptor-dependent mechanism, as the dilazep suppression of tissue factor expression was reversed by theophyilline, a nonspecific adenosine receptor antagonist.[104] Adenosine inhibits tissue factor expression on LPS-stimulated human monocytes through activation of A_3 receptors, because IB-MECA is the most potent agonist to block tissue factor expression, and the mixed A_1/A_3 antagonist XAC (but not selective A_1, A_{2A}, or A_{2B} antagonists) prevents the effect of adenosine.[105]

4.10 REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) PRODUCTION BY ADENOSINE: IMPLICATIONS FOR THE RESOLUTION OF INFLAMMATION

VEGF is an important inducer of angiogenesis, acting through specific receptors on endothelial cells to induce neovascularization.[106] VEGF is also an important mediator of vascular permeability, modulating the exchange of solutes from the plasma to the extravascular tissues. VEGF is thus an important mediator of both inflammation and repair, and is critical for the resolution of injury by the process of wound healing. Macrophages are a major source of VEGF in wound healing, as well as in chronic inflammation and cancer.[107]

Adenosine plays an important role in the promotion of angiogenesis. Adenosine applied to the chick chorioallantoic membrane induces growth of new microvascular blood vessels.[108] Regulation of expression of VEGF via adenosine receptors has been demonstrated in several cell types, including endothelial cells, smooth muscle cells, and macrophages. Stimulation of A_2 receptors in U-937 cells induces VEGF mRNA accumulation.[109] Moreover, hypoxia, which is a major trigger of VEGF expression in most cell types,[106] increased VEGF mRNA expression in U-937 cells in a manner inhibitable by adenosine deaminase and DMPX, indicating that hypoxia acts through the release of adenosine to increase VEGF mRNA. In contrast, although both hypoxia and A_{2A} stimulation acted as potent inducers of VEGF production by murine peritoneal macrophages, the effect of hypoxia was independent of adenosine receptors.[110] Interestingly, A_{2A} receptor stimulation and LPS via TLR4 synergistically enhanced the production of VEGF.[110] In a recent study, our group reported that this synergistic interaction in inducing VEGF production was not limited to TLR4, but occurred also following the stimulation of TLR2, TLR7, and TLR9.[77] It remains to be determined which intracellular signaling mechanisms account for this synergistic impact of A_{2A} receptor and TLR stimulation on VEGF expression.

4.11 FUTURE PERSPECTIVES AND THERAPEUTIC IMPLICATIONS

A large body of evidence supports the view that adenosine receptors might be targets for drug development to treat several disease states, in which macrophage activation has an important role. Protective effects of adenosine receptor stimulation have been observed in models of ischemia-reperfusion,[111–113] as well as autoimmune diseases, such as rheumatoid arthritis,[114] multiple sclerosis,[115] colitis,[116] and hepatitis.[83]

Although bone-marrow-derived immune cells have been shown to contribute to tissue protection in some of these models,[111–113] there is no direct evidence that the beneficial effects of adenosine receptor stimulation are caused by altering macrophage function. Nevertheless, given the profound effect that adenosine and adenosine receptor ligands have on the course of inflammatory diseases, it appears likely that targeting adenosine receptors in inflammatory and immune diseases has enormous potential for pharmaceutical development for the therapeutic management of patients with ischemic, autoimmune, and inflammatory diseases.

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5 Impaired Lymphocyte Activation in the Presence of Adenosine: Mechanisms and Physiologic Relevance

Jonathan Blay and David W. Hoskin

CONTENTS

5.1 INTRODUCTION

Adenosine is a purine nucleoside that is present in a free form in both intracellular and extracellular compartments.[1] Concentrations of adenosine in biological fluids such as plasma and cerebrospinal fluid are typically in the range 0.4 to 2.4 μM.[1,2] The interstitial (extracellular) fluid concentrations of adenosine are somewhat lower in normal tissues that are fully oxygenated, ranging from 0.02 to 0.1 μM in connective tissues[1,3] to 0.4 to 0.9 μM in myocardial and skeletal muscle tissues.[2,4] Interstitial adenosine levels become elevated in tissues that are rendered experimentally acutely

hypoxic[5,6] and in tissues such as those of solid cancers that are chronically deprived of an adequate oxygen supply.[3]

The increase in extracellular levels of adenosine in hypoxic tissues is the consequence of changes in adenosine metabolism as well as transport out of the cell through bidirectional equilibrative nucleoside transporters.[7–9] However, the concentration of adenosine that is locally available to act on cell surface receptors is further modulated by a series of ectoenzymes that mediate adenosine production or destruction at the cell surface. These play a particularly important role in the supply and availability of adenosine at the lymphocyte surface and include NTPDase 1 (CD39), ecto-5′-nucleotidase (CD73), and the binding protein for adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4), which is CD26.[10–12] A further level of control is evident in that ADA also binds directly to A_{2B} adenosine receptors on lymphocytes[13] and to A_1 receptors on nonlymphoid cells.[14]

5.2 ADENOSINE RECEPTOR SUBTYPES EXPRESSED ON LYMPHOCYTES

5.2.1 Adenosine Receptor Subtypes

The molecular, functional, and pharmacological properties of adenosine receptors have been extensively reviewed elsewhere.[15–20] The four clearly defined adenosine receptor subtypes are all G-protein-coupled receptors that are linked to G_i/G_o (A_1), G_s/G_{olf} (A_{2A}), G_s/G_q (A_{2B}), or G_i/G_q (A_3) G protein family members. Relative affinities of the different receptor subtypes for adenosine are highest (~10^{-8} to 10^{-7} *M*) for A_1 and A_{2A} receptors and substantially lower (~10^{-6} to 10^{-5} *M*) for the A_{2B} and A_3 receptors.[15,20]

5.2.2 T Lymphocytes

Resting mouse T lymphocytes and the CTLL-2 T cell line express mRNA coding for A_{2A}, A_{2B}, and A_3 receptors, but little if any A_1-receptor-encoding mRNA.[21,22] A_{2A} mRNA is particularly abundant in mouse T lymphocytes.[23] Interestingly, A_{2A} mRNA levels in mouse $CD4^+$ T lymphocytes undergo a transient tenfold increase following T cell receptor (TCR) stimulation,[24] which is consistent with a role for A_{2A} receptor signaling in feedback inhibition of T lymphocyte activation.[25] It is noteworthy that there is no significant compensatory increase in A_1, A_{2B}, or A_3 receptor expression in mutant mice that lack A_{2A} receptors.[23] An inability to regulate cell-mediated immune responses via increased signaling through other inhibitory adenosine receptor subtypes may contribute to the accumulation of proinflammatory cytokines and excessive inflammation observed in A_{2A}-receptor-deficient animals.[25] Unactivated human T lymphocytes also express A_{2A}, A_{2B}, and A_3 receptors, all of which exhibit increased expression following T lymphocyte activation.[26–28] Again, the capacity for increased adenosine receptor signaling by activated human T lymphocytes suggests a possible feedback role for adenosine in controlling cell-mediated immune responses. A_{2A} receptors are present on $CD4^+$ (Th1 and Th2) and $CD8^+$ T lymphocytes, although the proportion of A_{2A}-receptor-bearing $CD8^+$ T

lymphocytes is less than that of A_{2A}-receptor-bearing $CD4^+$ T lymphocytes.[26] In contrast, a similar percentage of $CD4^+$ and $CD8^+$ T lymphocytes express A_{2B} receptors.[27] $CD4^+$ and $CD8^+$ T lymphocytes also express similar amounts of A_3 receptor, but only $CD4^+$ T lymphocytes show increased A_3 receptor expression after activation with phytohemagglutinin.[28] It is not yet known whether human T lymphocytes express A_1 receptors.

5.2.3 B Lymphocytes

Adenosine-mediated inhibition of B lymphocyte function is a consequence of signaling through cell surface adenosine receptors. Mouse B lymphocytes express abundant mRNA coding for the A_{2A} receptor (in fact, several times more than in T lymphocytes or macrophages) but very little A_{2B} or A_3 receptor mRNA and virtually no A_1 receptor mRNA.[23] It is therefore likely that adenosine exerts its effects on murine B lymphocytes primarily through A_{2A} receptors. Interestingly, A_{2A} receptors are not detectable on human peripheral B lymphocytes when these cells are stained with a monoclonal anti-A_{2A} receptor antibody and examined by flow cytometry.[26] However, this result does not rule out A_{2A} receptor expression by human B lymphocytes at a level that lies below the threshold for detection by flow cytometry. At present, there is no information on A_1, A_{2B}, and A_3 receptor expression by human B lymphocytes. Thus, further investigation is needed to clarify the mechanism by which adenosine might affect the function of human B lymphocytes.

5.2.4 Natural Killer Cells

A formal analysis of cell surface adenosine receptor expression by either mouse or human natural killer (NK) cells has not yet been performed. However, studies with adenosine receptor agonists that are selective for each adenosine receptor subtype led Priebe et al. to conclude that A_1 and A_2 receptors are present on murine NK cells and that these receptor subtypes are involved in regulating the cytolytic activity of mouse NK cells.[29] Selective stimulation of A_1 receptors causes increased cytotoxic activity by NK cells, whereas treatment of NK cells with A_2 receptor agonists has a potent inhibitory effect on cytotoxic effector function. Intracellular cAMP levels are known to modulate NK cell function,[30] suggesting that the opposing effects of A_1 and A_2 receptor ligation on NK-cell-mediated cytotoxicity most likely involve an A_1-receptor-induced decrease in intracellular cAMP content that stimulates NK cell lytic activity and an A_2-receptor-induced increase in intracellular cAMP content that inhibits cytotoxic activity by NK cells. A recent study demonstrates that oral administration of the A_3 receptor agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (Cl-IB-MECA) potentiates mouse NK cell activity, increases serum interleukin (IL)-12, and causes a reduction in the *in vivo* growth of B16-F10 melanoma cells.[31] However, it is not clear at this time whether enhanced NK cell activity is the result of direct stimulation of NK-cell-associated A_3 receptors or a consequence of an A_3 receptor agonist-induced increase in IL-12 synthesis by antigen-presenting cells. The latter scenario seems more likely because IL-12 produced by accessory cells is known to potently stimulate NK-cell-mediated cytotoxicity.[32]

5.3 ADENOSINE EFFECTS ON LYMPHOCYTE SUBSET ACTIVATION

5.3.1 T Lymphocytes

T lymphocyte subsets include T helper cells, cytotoxic T lympocytes (CTLs), and regulatory T cells, all of which are critical for proper immune function. T helper cells consist of $CD4^+$ Th1 and Th2 cell subsets that are an important source of cytokines that drive predominantly cell-mediated and humoral immune responses, respectively.[33] $CD8^+$ CTLs are essential for the elimination of virus-infected and transformed cells,[34] whereas $CD4^+CD25^+$ regulatory T cells regulate T cell responses to foreign antigens and mediate peripheral tolerance to self-antigens.[35] Dysregulated T cell function can result in excessive tissue damage during inflammatory responses.[36] Adenosine released from metabolically active or stressed cells is believed to be an important endogenous regulator of inflammatory responses because adenosine potently inhibits both innate immune responses[37] and T lymphocyte function, including TCR-driven proliferation,[38] synthesis of IL-2 and proinflammatory interferon-γ,[24,39] CTL induction,[21] CTL adhesion to target cells,[40,41] and granule exocytosis.[42] Additional evidence that favors an important role for adenosine as a negative feedback regulator of inflammatory responses is provided by the observation that A_{2A}-receptor-deficient mice exhibit excessive liver damage in response to *in vivo* T cell stimulation with concanavalin A.[25] Elevated levels of extracellular adenosine associated with chronic hypoxia in solid tumors have also been proposed to contribute to tumor progression by suppressing cell-mediated antitumor immune responses.[3,43]

As indicated above, T cells themselves may express each of the adenosine receptor subtypes except A_1. Adenosine-mediated inhibition of T lymphocyte activation and effector function has been shown to involve signaling through A_{2A}, A_{2B}, and A_3 receptors (Figure 5.1). However, to date virtually all functional studies have been performed in the mouse system. Signal transduction through the TCR initiates the transcription of genes that are required for T cell proliferation and differentiation, as well as for activation-induced cell death. In this regard, adenosine has been shown to block TCR-induced apoptosis and Ca^{2+} mobilization in murine thymocytes.[44] Adenosine also interferes with TCR-associated signal transduction in peripheral mouse T cells because CD25 (IL-2 receptor α chain) upregulation in response to TCR triggering is impaired in the presence of adenosine.[38] Adenosine appears to affect some of the earliest events in TCR signal transduction in that tyrosine phosphorylation of intracellular proteins with molecular weights corresponding to the protein tyrosine kinases $p56^{lck}$ and ZAP-70 is reduced in response to TCR stimulation of murine T cells in the presence of adenosine.[39] Autophosphorylation of tyrosine residues on the Src-family protein tyrosine kinase $p56^{lck}$ in response to TCR and coreceptor clustering leads to $p56^{lck}$ activation and phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 ζ chain.[45] Tyrosine-phosphorylated ITAMs then form docking sites for the protein tyrosine kinase ZAP-70 that is, in turn, activated by $p56^{lck}$-mediated tyrosine phosphorylation. Adenosine inhibition of $p56^{lck}$ activation in T lymphocytes seems a likely mechanism by which adenosine could interfere with proximal events during

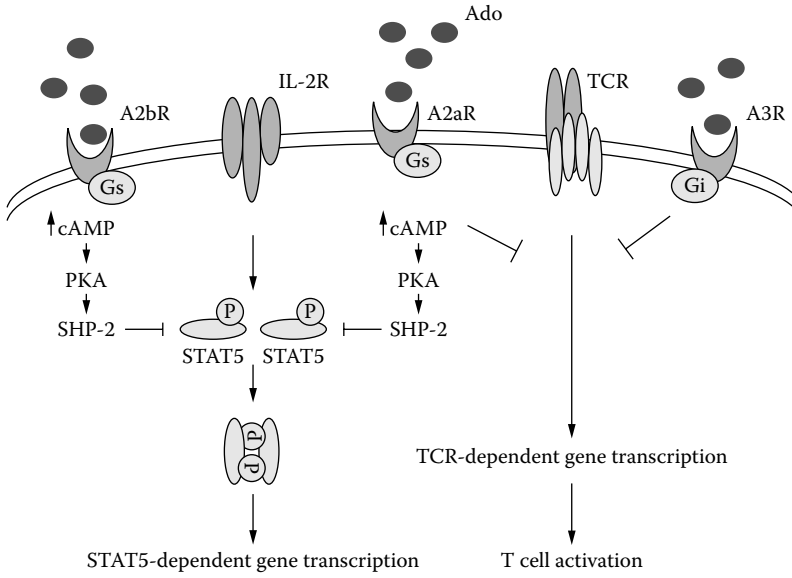

FIGURE 5.1 Adenosine inhibition of TCR and IL-2 receptor signaling in T lymphocytes. Adenosine (Ado) acts through A_{2A} and A_3 receptors to block TCR signal transduction. A_{2A}-receptor-mediated inhibition involves the activation of adenylyl cyclase, whereas the mechanism of A_3-receptor-mediated inhibition is not well understood. Adenosine blocks IL-2 receptor (IL-2R) signaling by activating A_{2A}-receptor- and A_{2B}-receptor-associated adenylyl cyclase. Elevated intracellular cAMP and the resulting induction of protein kinase A (PKA) cause activation of the protein tyrosine phosphatase SHP-2, which dephosphorylates the transcription factor STAT5, thereby preventing STAT5 homodimerization and translocation to the nucleus.

TCR signal transduction and is consistent with reduced CD3 ζ chain phosphorylation in thymocytes from adenosine deaminase (ADA)-deficient mice.[44] Although an A_{2A}-receptor-triggered increase in intracellular cAMP has been implicated in the inhibition of TCR-induced CD25 expression and interferon-γ synthesis,[24,38] adenosine inhibition of TCR- and IL-2-dependent upregulation of costimulatory CD2 and CD28 molecules by mouse T lymphocytes is not mediated through A_{2A} receptor stimulation.[39] In this regard, adenosine signaling through the A_3 receptor has been shown to suppress TCR-driven T lymphocyte proliferation,[21] as well as the adhesion of activated T lymphocytes to syngeneic adenocarcinoma cells.[40,41]

Consistent with the inhibitory effect of A_3 receptor signaling in mouse T lymphocytes, adenosine has also been shown to act through A_3 receptors to inhibit tumor necrosis factor (TNF)-α synthesis by lipopolysaccharide-stimulated murine and human macrophages,[46,47] as well as the migration of human eosinophils.[48] However, the mechanism by which A_3 receptor signaling mediates these inhibitory effects is unclear and remains an important area for future investigation.

We have recently shown that adenosine also inhibits signal transduction through the IL-2 receptor of mouse T lymphocytes.[22] IL-2 binding to the IL-2 receptor results

in the phosphorylation, activation, and translocation of the transcription factor STAT5 to the T cell nucleus. STAT5 is important for IL-2-induced cell cycle progression and the expression of effector molecules such as perforin by T lymphocytes.[49,50] Adenosine stimulates T-lymphocyte-associated A_{2A} and A_{2B} receptors, which induce cAMP- and protein-kinase-A-dependent signaling pathways that activate the protein tyrosine phosphatase SHP-2.[22] Activation of SHP-2 results in the dephosphorylation of STAT5, which prevents STAT5 homodimerization and translocation to the nucleus. This finding reveals a novel mechanism by which adenosine might impair other cytokine-dependent T lymphocyte responses that are initiated by tyrosine phosphorylation of the STAT5 transcription factor.

Adenosine also indirectly interferes with the induction of T-cell-mediated immune responses by reducing the capacity of antigen-presenting cells to promote T lymphocyte activation. Dendritic cells are an important class of antigen-presenting cells that are uniquely suited to activate naïve T lymphocytes.[51] Although immature dendritic cells express mRNA transcripts for A_1, A_{2A}, and A_3 receptors, only A_{2A} receptor mRNA is expressed by mature dendritic cells.[52] Moreover, lipopolysaccharide maturation of human-monocyte-derived dendritic cells leads to a substantial increase in A_{2A} receptor mRNA transcripts.[53] Adenosine acts through A_1 and A_3 receptors on immature dendritic cells to induce chemotaxis, whereas A_{2A} receptor stimulation suppresses the ability of mature dendritic cells to synthesize IL-12.[52] Because IL-12 functions to link innate and adaptive immunity[54] and is an essential cytokine for the development of Th1 cells,[33] reduced IL-12 synthesis by dendritic cells in the presence of adenosine is predicted to interfere with the induction of cell-mediated immune responses. Furthermore, adenosine enhances the secretion of IL-10 by mature dendritic cells in such a manner that dendritic cells matured in the presence of adenosine show a reduced capacity to promote Th1 cell differentiation.[55] Adenosine also inhibits the expression of molecules involved in dendritic cell migration (CCR5, MIP-3β/CCL19, and MDR-1), and slows the *in vitro* and *in vivo* migration of mature dendritic cells,[56] which might impair the initiation of T-cell-mediated immune responses.

In addition to serving as a phagocytic barrier to microbial infection, macrophages also function as antigen-presenting cells for T lymphocytes.[57] Although the effect of adenosine on antigen presentation by macrophages has not yet been determined, several lines of evidence suggest that adenosine may interfere with the capacity of macrophages to promote the activation of T lymphocytes by modulating macrophage production of cytokines. For example, the J774.1 mouse macrophage line expresses mRNA transcripts coding for A_1, A_2, and A_3 receptors, although A_3 receptor mRNA is the most abundant.[46] A_3 receptors are involved in regulating the macrophage cytokine milieu because A_3 receptor agonists block lipopolysaccharide-induced synthesis of TNF-α by mouse and human macrophage cell lines.[46,47] In addition, pretreatment of the RAW 264.7 macrophage cell line with A_1 or A_{2A} receptor agonists inhibits endotoxin-induced production of TNF-α, IL-10, and nitric oxide.[58] Although decreased IL-10 synthesis by macrophages activated in the presence of adenosine should favor the development of Th1 cells,[33] the concomitant reduction in TNF-α synthesis likely has a negative impact on overall T lymphocyte activation because TNF-α promotes macrophage function and enhances T cell proliferation in response

to mitogens and antigens.[59,60] In addition to inhibiting TNF-α synthesis, adenosine acts via both A_{2A}-receptor-dependent and A_{2A}-receptor-independent mechanisms to inhibit IL-12 production by murine macrophages,[61] suggesting that macrophages may not be able to effectively promote the development of Th1 cells in the presence of adenosine. Collectively, these findings suggest that adenosine exerts an inhibitory effect on T cell activation at the level of antigen-presenting cells.

5.3.2 B Lymphocytes

Early studies revealed that a high concentration of adenosine, as well as the adenosine analog 2-chloroadenosine, suppressed cellular proliferation and antibody synthesis in mitogen- and antigen-stimulated cultures of murine B lymphocytes.[62,63] Consistent with these *in vitro* findings, lymphocytes from ADA-deficient mice, which exhibit increased levels of adenosine and deoxyadenosine in all tissues examined,[64] show a marked reduction in their ability to proliferate in response to stimulation with anti-IgM antibodies or lipopolysaccharide.[65] Although deoxyadenosine, which does not act through cell surface adenosine receptors, has been suggested to exclusively mediate immune suppression associated with ADA deficiency, recent findings indicate that adenosine also contributes to immune dysfunction associated with a failure to express ADA.[66] Taken together, these findings suggest that the accumulation of extracellular adenosine in inflamed, damaged, or hypoxic tissues may under some circumstances function to regulate the humoral arm of the immune response.

The B cell antigen receptor (BCR) consists of a disulfide-linked Ig-α and Ig-β heterodimer that forms the signal-transducing element and is noncovalently associated with a membrane immunoglobulin molecule that binds antigen.[67] BCR cross-linking by multivalent antigen results in Src-family protein tyrosine kinase-mediated phosphorylation of tyrosine residues contained within the two ITAMs of the Ig-α and Ig-β heterodimer, followed by the recruitment of Syk protein tyrosine kinase and the initiation of downstream signaling processes that result in the activation and translocation of transcription factors such as NF-κB to the nucleus.[68–70] During B cell activation, the CD19–CD21 complex functions as a coreceptor and lowers the signaling threshold for B lymphocyte activation by enhancing Src-family protein tyrosine kinase activation.[71] Several lines of evidence suggest that adenosine affects distal rather than proximal portions of the signal transduction cascades involved in B lymphocyte activation. Our own unpublished data show that low micromolar concentrations of adenosine (in the presence of coformycin to inhibit endogenous ADA activity) potently suppress mouse B lymphocyte proliferation induced *in vitro* with phorbol ester and ionophore, suggesting an inhibitory effect by adenosine that lies downstream of Ca^{2+} mobilization and protein kinase C activation.[72] Recently, adenosine treatment has been shown to block antigen- and lipopolysaccharide-driven activation of the transcription factor NF-κB in chicken B cell lymphoma cells and primary mouse B lymphocytes by inhibiting BCR- and toll-like receptor (TLR) 4-induced IκBα phosphorylation and degradation (Figure 5.2).[73] A similar effect, which is prevented by inhibiting protein kinase A, is obtained when intracellular cAMP levels are elevated in B lymphocytes by treatment with forskolin and 3-isobutyl-1-methylxanthine, suggesting that adenosine blocks NF-κB activation by

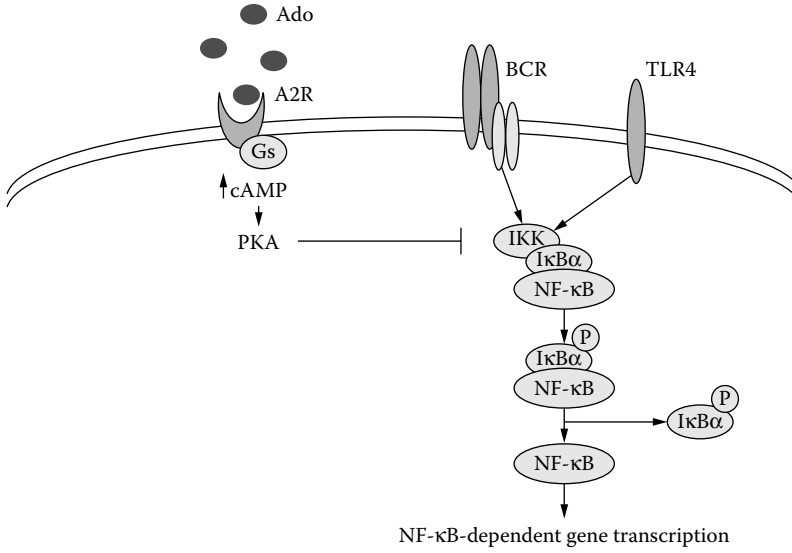

FIGURE 5.2 Adenosine inhibition of BCR and TLR4 signaling in B lymphocytes. Adenosine (Ado) acts through A_{2A} and A_{2B} receptors to block BCR and TLR4 signal transduction. Activation of receptor-associated adenylyl cyclase leads to an increase in intracellular cAMP and the activation of protein kinase A (PKA), which prevents the activation and nuclear translocation of the transcription factor NF-κB.

acting through adenylyl-cyclase-coupled A_2 receptors to activate protein kinase A. Adenosine suppression of NF-κB activity in mouse B lymphocytes is most likely mediated through A_{2A} receptors because A_{2A} mRNA is abundant whereas A_{2B} mRNA is scarce in this lymphocyte population.[23] Interestingly, adenosine has also been shown to act through A_2 receptors to inhibit TNF-α-induced NF-κB activation in human myeloid leukemia cells, although in this instance adenosine blocked the DNA-binding activity of NF-κB rather than IκB phosphorylation and degradation.[74] Adenosine therefore interferes with NF-κB activation by multiple mechanisms.

It is noteworthy that adenosine does not prevent BCR downregulation or inhibit tyrosine phosphorylation of intracellular proteins in response to BCR stimulation,[73] suggesting that adenosine does not interfere with the activity of protein tyrosine kinases involved in the more proximal portions of BCR- and coreceptor-associated signal transduction pathways. However, the finding that A_{2A}-receptor-induced SHP-2 protein tyrosine phosphatase activity impairs STAT5-dependent signaling through the IL-2 receptor of mouse T cells[22] suggests the intriguing possibility that adenosine may also act through A_{2A} receptors on B lymphocytes to inhibit STAT5-dependent IL-4, IL-5, and IL-13 receptor signaling involved in driving B lymphocyte proliferation and differentiation.[75,76] Additional studies to assess the effect of extracellular adenosine on cytokine receptor signaling in B lymphocytes are clearly warranted.

It is important to note that adenosine has direct and indirect effects on B lymphocytes that extend beyond simple inhibition of BCR-driven proliferation and

antibody synthesis. Adenosine treatment upregulates expression of the activation marker CD69 on primary mouse B cells, most likely via A_2 receptor signaling because a similar effect is obtained when B lymphocytes are treated with forskolin and 3-isobutyl-1-methylxanthine (IBMX).[73] In addition, A_2 receptor stimulation and activation of intracellular cAMP have also been shown to rescue human B lymphocytes from BCR-induced apoptosis by preventing the activation of caspase-3.[77] Interestingly, human B lymphoblasts release significant amounts of adenosine under conditions that induce ATP catabolism,[78] suggesting that adenosine may function in an autocrine fashion to promote B lymphocyte survival following antigenic stimulation. Finally, adenosine acts through mast-cell-associated A_{2B} receptors to cause increased IL-4 and IL-13 synthesis, thereby indirectly promoting IgE synthesis by human B lymphocytes.[79] Adenosine is therefore postulated to amplify allergic inflammatory responses that are responsible for the pathology of asthma.

5.3.3 Natural Killer Cells

NK cells have the unique ability to kill virus-infected and neoplastic cells in a manner that is not restricted by histocompatibility molecules, as well as secreting proinflammatory cytokines such as interferon-γ and TNF-α.[80] As such, these cytotoxic effector cells are critical elements of both the innate and acquired immune response. Several independent studies have shown that adenosine and adenosine analogs are potent inhibitors of both human and murine NK cell activity.[29,81,82] Over a decade ago, low micromolar concentrations of adenosine were found to impair the ability of mouse splenic NK cells to lyse YAC-1 lymphoma cells.[29] The molecular basis for A_1-receptor- and A_2-receptor-induced stimulation and inhibition, respectively, of NK cell lytic function postulated by Priebe and colleagues is not known but may involve a perturbation of the balance of protein tyrosine kinase and phosphatase activity that govern signaling through NK cell stimulatory and inhibitory receptors.[83] Accumulation of intracellular cAMP is also responsible for adenosine inhibition of IL-2-induced cellular proliferation and TNF-α synthesis in cultures of human NK cells.[82] Given that adenosine causes a cAMP- and protein-kinase-A-dependent inhibition of NF-κB activity in B lymphocytes[73] and that NF-κB is important for the transcription of many inflammatory cytokine genes, including TNF-α,[84] it is reasonable to conclude that adenosine acts via A_2 receptors on human NK cells to suppress NF-κB-dependent expression of TNF-α.

More recently, we have shown that adenosine-mediated inhibition of mouse NK cell function is at least in part caused by defective granule exocytosis in the presence of adenosine.[81] The inhibitory effect appears to be mediated through a cell surface receptor that does not belong to the known family of adenosine receptor subtypes, because neither adenosine uptake inhibitors (dilazep and *S*-(4-nitrobenzyl)-6-thioinosine [NBTI]) nor A_1 or A_2 receptor antagonists (8-cyclopentyl-1,3-dipropylxanthine [DPCPX], and 3,7-dimethyl-1-propargylxanthine [DMPX]) are able to restore NK cell granule exocytosis to normal levels in the presence of adenosine, and the A_3 receptor agonist aminophenylethyladenosine (APNEA) does not reproduce the inhibitory effect of adenosine. Effective granule exocytosis is critical for NK-cell-mediated cytotoxity because the cytoplasmic granules of NK cells contain several

different cytotoxic effector molecules, including granzymes and perforin.[85] Adenosine also suppresses the IL-2-driven proliferation of human NK cells, as well as decreasing TNF-α production by NK cells in response to IL-2.[82] In contrast, IL-2-induced synthesis of interferon-γ by NK cells is unaffected by adenosine treatment, indicating that adenosine acts on specific cellular pathways rather than causing generalized NK cell inhibition. Surprisingly, the same study shows that the cytolytic activity of human NK cells against K562 leukemia cells is intact in the presence of adenosine. However, the addition of 1000 units/ml of IL-2 to human NK cell cultures at the same time as adenosine may have obscured an inhibitory effect of adenosine by potently upregulating both granule-dependent and granule-independent cytotoxicity and/or enhancing NK cell adhesion to tumor target cells.[86–88] Adenosine is postulated to play an important role in negative feedback regulation of NK cell function *in vivo* because adenosine is among the purine metabolites that are released when target cells are lysed by NK cells in inflamed tissues or at sites of tumor destruction.[82] In addition, we have proposed that elevated levels of adenosine that are present in the microenvironment of solid tumors may promote tumor progression by interfering with the cytotoxic effector function of tumor-infiltrating lymphocytes such as NK cells.[3]

Interestingly, the adenosine analog 2-chloroadenosine triggers granule exocytosis by mouse NK cells by stimulating a cell surface receptor that does not correspond to conventional adenosine receptors and may therefore represent a novel 2-chloroadenosine-selective receptor.[89] PC12 pheochromocytoma cells have also been reported to express a cell surface receptor that binds 2-chloroadenosine but not other A_1 or A_2 receptor agonists,[90] and nonlymphoid cells such as rheumatoid synovial fibroblasts have been shown to respond to 2-chloroadenosine but not adenosine through a mechanism that is independent of adenosine receptors.[91] Results such as these underscore the importance of confirming all experimental results obtained with adenosine analogs by showing that the effect can be reproduced with adenosine.

5.4 CYTOTOXIC EFFECT OF ADENOSINE ON LYMPHOCYTE POPULATIONS

Although low micromolar concentrations of adenosine typically suppress the function of immune effector cells, higher concentrations of adenosine are cytotoxic for certain lymphocyte populations. Mouse thymocytes cultured in the presence of 1-m*M* adenosine or 100-μM 2-chloroadenosine exhibit internucleosomal DNA cleavage,[92,93] which is characteristic of apoptosis.[94] A more recent study shows that adenosine preferentially induces apoptosis in $CD4^+CD8^+$ thymocytes and does not substantially affect the viability of $CD4^-CD8^-$, $CD4^+CD8^-$, or $CD4CD8^+$ thymocytes.[95] Adenosine also triggers apoptosis in peripheral mouse T lymphocytes, albeit to a lesser extent than in murine thymocytes.[96] Kizaki and colleagues have suggested that adenosine-induced apoptosis in both thymocytes and mature T lymphocytes involves the accumulation of intracellular cAMP, implying that adenosine signals through adenylyl-cyclase-coupled A_2 receptors to activate endonucleases and cause internucleosomal DNA fragmentation. The involvement of A_{2A} receptor signaling in adenosine-mediated apoptosis of mouse thymocytes was recently confirmed in a study that compared the cytotoxic effect of the A_{2A}-receptor-selective agonist CGS

21680 on thymocytes from A_{2A}-receptor-expressing mice vs. A_{2A}-receptor-deficient mice.[97] Moreover, elevated extracellular adenosine in ADA-deficient mice also protects thymocytes from TCR-induced apoptosis by blocking TCR signal transduction, suggesting that high levels of systemic adenosine may have a negative impact on intrathymic T lymphocyte development and TCR-dependent T lymphocyte function in patients with ADA deficiency.[44] In contrast, B lymphocytes appear to be refractory to the toxic effect of elevated extracellular adenosine because normal B lymphocyte development occurs in the bone marrow of ADA-deficient mice, although there is an increased tendency for B lymphocytes from these mutant mice to undergo activation-induced apoptosis.[65] NK cells are also highly resistant to the toxic effect of high micromolar concentrations of adenosine.[82]

Taken together, these studies suggest that although elevated deoxyadenosine is likely to be the major cause of T lymphocyte, B lymphocyte, and NK cell depletion in patients with severe combined immunodeficiency due to ADA deficiency, the accumulation of extracellular adenosine in these patients may also contribute to T lymphocyte depletion by direct cytotoxicity and by compromising TCR-dependent T cell development.[66] It is also possible that the accumulation of extracellular adenosine in the microenvironment of solid tumors might also cause tumor-infiltrating T cells such as CTL to undergo apoptosis, thereby promoting tumor progression.

5.5 LYMPHOCYTE-ASSOCIATED ADENOSINE DEAMINASE AND ITS IMPACT ON ADENOSINE RECEPTOR STIMULATION

The importance of adenosine deaminase (ADA) for the development of the immune system and its proper function is evident from the profound combined immunodeficiency state that occurs with a genetic deficiency of ADA.[98] A substantial advance in our understanding of the role of ADA in lymphocyte function came with the realization that the major cellular binding protein for ADA—hitherto known as the ADA binding protein (ADAbp)[99] or ADA complexing protein (ADCP)[100] in other tissues—was identical to the CD26 activation marker on lymphocytes.[12,101,102] ADA is therefore closely associated with the lymphocyte surface through its interaction with CD26. CD26 is also identical to the membrane-associated peptidase dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5), and has proved to play multiple roles, depending on the cell type and context in which it is expressed.[103]

Levels of CD26 (referred to as thymocyte-activating molecule [THAM] in earlier papers) increase during thymocyte maturation,[104] in which CD26 may play a role in negative selection[105] and during T cell activation either *in vitro* or *in vivo*.[106,107] CD26 is upregulated by activation of both $CD8^+$ and $CD4^+$ T lymphocytes[108,109] and on NK cells activated in response to IL-2, IL-12, or IL-15.[110] CD26 shows distinct expression levels on different T cell subsets. Within human $CD4^+$ T cells, CD26 is strongly expressed only on the $CD45RO^+/CD29^+$ population, implying that CD26 itself plays a role in memory T lymphocyte function.[111] Human Th1 cells express three- to sixfold more CD26 protein than Th2 cells, consistent with a role for CD26 primarily in Th1-cell-mediated responses.[112,113] Much of the interest in CD26 regulation in these contexts,[104,106,107] or in certain T cell leukemias and lymphomas in which altered

CD26 expression is of interest,[114,115] has focused on regulation of its dipeptidase activity. This is reasonable in the context of understanding immune function, because the type IV dipeptidyl peptidase activity inherent in CD26 involves cleavage of molecules having a proline (or alanine) residue at the penultimate N-terminal position, which is the boundary of a terminal dipeptide motif found in many chemokines. CD26/DPPIV removes the chemokine N-terminal dipeptide, typically resulting in a loss or reduction in chemokine function or alteration of receptor specificity. Thus, CD26 acts to modify SDF-1 (CXCL12),[116] RANTES,[117,118] and MIP-1α/LD78α,[119,120] with significant implications for the evolving immune response. However, the concomitant alteration in bound ecto-ADA as the expression of CD26 is altered during T cell ontogeny or activation is also likely to affect lymphocyte responsiveness to adenosine.

The amount of ADA that can bind to lymphocytes increases with increasing CD26 expression.[11,108,121] Furthermore, broad activation stimuli such as anti-CD3 antibody or phorbol esters, or cytokines such as IL-2 and IL-12, that can upregulate CD26 levels also increase the amount of ecto-ADA at the T cell surface.[11,122] This is consistent with a view that the capacity to deaminate adenosine (and therefore reduce its capability to influence lymphocyte function through adenosine receptors) increases in response to lymphocyte activation. However, there are several caveats to this view. First, CD26 may not work as an ADA-binding protein in all species.[123] Second, cytokines such as IL-4 can alter the level of lymphocyte ecto-ADA independently of changes in CD26.[122] Third, the molecular structure, cellular localization, and functional properties of CD26 may change during T cell activation.[124] Finally, CD26 itself is able to provide a second costimulatory signal that contributes to T cell activation due to anti-CD3 or anti-CD2 antibodies, as well as enhancing T cell activation in response to mitogenic lectins.[125,126] However, notwithstanding the complexity of what may be a sophisticated regulatory network, there is strong evidence that the effect of adenosine on the lymphocyte is modulated by the ecto-ADA that is bound to CD26. In particular, Morimoto's group has shown that the ADA–CD26 complex on T cells confers resistance to the inhibitory effects of adenosine on T cell proliferation and IL-2 production.[127] Furthermore, CD26-transfected Jurkat cells with CD26 mutated within the ADA-binding domain are much more sensitive to the inhibitory effects of adenosine than wild-type transfectants that retain the ability to bind ADA.[128]

In addition to its ability to degrade adenosine, ADA may function in cell adhesion. Although CD26 itself in other cell types is known to be able to also bind to the extracellular matrix proteins collagen and fibronectin,[129,130] lymphocytes appear able to bind through CD26 to ADA displayed at the surface of epithelial cells.[121] This phenomenon may be somewhat restricted because human T lymphocytes have been reported not to bind through CD26 to endothelial cells or fibroblasts.[131] Furthermore, adhesion does not take place when T cell CD26 is already saturated with ADA.[121]

The ability of ADA to bind to A_1 and A_{2B} receptors,[13,14] as well as to CD26, has already been mentioned. Receptor-bound ADA is likely less important than CD26-bound ADA in its ability to locally degrade adenosine because in most cells CD26 is substantially more abundant than adenosine receptors and therefore constitutes the major ecto-ADA binding site. The significance of A_{2B}-receptor-bound ADA on

lymphocytes may be in terms of modulating the signal that is delivered through the receptor. In the case of the A_1 receptor on nonlymphoid cells, receptor-bound ADA promotes both adenosine binding and G-protein coupling of the receptor.[14]

5.6 ADENOSINE SIGNALING IN DISEASE PROCESSES

Adenosine and adenine nucleotides play a broad role in different pathophysiologic processes.[16,19,132] Adenosine itself may act on a diverse range of cells in addition to those of the immune system, including cells of the nervous system, muscle cells, and cells of epithelial tissues. Adenosine signaling has now been implicated in a number of different disease processes, acting beneficially or otherwise on these different cells.

Many disease processes involve both an immune response and inflammation. The involvement of adenosine as a suppressor of both the immune and inflammatory responses has been appreciated for more than a decade.[133] In recent years, the mechanisms of adenosine action and the interplay between the cells involved have become better understood.[37,66] The realizations that adenosine pathways play a part in the action of certain anti-inflammatory drugs[134] and that adenosine is a modulator of NF-κB, a major potential target in the field of anti-inflammatory therapies,[66,73] have focused attention on these pathways in the area of new drug development. The view has evolved that adenosine acts as a "retaliatory metabolite" that is released under conditions of stress or an active immune response and acts to regulate the innate immune and inflammatory responses so as to prevent tissue damage.[37,135] In that regard, attention has become focused on how adenosine pathways might be manipulated by pharmacologic means to ensure tissue protection.[19] Although adenosine may play a more direct role in cytoprotection, as in modulation of ischemic reperfusion injury in the myocardium,[136] it is likely that in most contexts the ability of adenosine to limit excessive immunological and inflammatory responses is the more important factor in tissue protection. However, this is unlikely to be a universal solution to controlling tissue damage because at the higher concentrations attained in chronic diseases, adenosine can exacerbate or amplify the disease process.[137]

5.7 CONCLUSIONS

Although immunosuppressive and anti-inflammatory effects of adenosine have attracted much attention in recent years and adenosine has become viewed as an important factor in protecting tissue from potential injury due to overactive host defence mechanisms, adenosine may also act in an opposing fashion to provoke the inflammatory response and trigger apoptosis in normal tissues. This paradox can be explained by understanding the complex network of players that participate in determining the adenosine response. In addition to the suite of enzymes—both intracellular and extracellular, membrane-bound and soluble—that regulate adenosine concentrations, the various adenosine receptors differ in their affinities for the ligand and are linked (in some cases oppositely) to diverse signaling pathways. Coupled with the differences in receptor expression between leukocyte subsets and

their modulation by other factors, this means that the adenosine system underlies a rich network of regulatory interactions, a role that is at first glance unexpected of this humble metabolite.

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6 Adenosine and Neutrophil Functions

Marc Pouliot

CONTENTS

6.1 INTRODUCTION

Adenosine has multiple anti-inflammatory effects on human neutrophils: inhibition of phagocytosis, generation of cytotoxic oxygen metabolites, and adhesion. Several excellent reviews have been written on the immunomodulatory effects that adenosine has on neutrophils. This chapter will provide a brief overview of these effects and will focus in a little more detail on recent developments in this field, especially aspects regarding the impact of adenosine on the profile of inflammatory mediators generated by neutrophils. Possible consequences on the inflammatory response are discussed.

6.1.1 The Neutrophil: A Role in Orchestrating the Inflammatory Response

Neutrophils constitute the majority of circulating leukocytes and are generally the first cells to migrate toward inflammatory lesions, where they initiate host defense

functions including the phagocytosis of cell debris and invading microorganisms, the generation of oxygen-derived reactive agents, and the release of proteolytic enzymes through a process termed *degranulation*.[1] These cells are generally regarded as short-lived and terminally differentiated white blood cells. However, in response to specific stimuli, neutrophils have the capacity to synthesize an array of factors such as antimicrobicidal proteins and extracellular matrix proteins, in addition to several cytokines and chemokines that can contribute in regulating the inflammatory response.[2,3] Although frequent and severe infections seen in patients with defects in neutrophil function confirm their importance in host defense against infection, they also have an enormous destructive capacity and can elicit significant tissue damage; their unchecked activation has been associated with disease states such as ischemia, Gram-negative bacterial sepsis, and rheumatoid arthritis.[4,5] Elevated numbers of neutrophils in the synovial fluid of patients with arthritis support a role for these cells in joint destruction.[6] Unraveling endogenous mechanisms that regulate and specifically limit neutrophil activation is, therefore, of interest in the context of identifying new and better therapeutic targets in the treatment of inflammatory diseases associated with unrepressed neutrophil activation.

Neutrophils stimulated with inflammatory agonists can generate and secrete IL-1β (as well as IL-1 receptor antagonist; IL-1RA), TNF-α, IL-8, macrophage inflammatory peptide (MIP)-1α, MIP-1β, MIP-2α, and MIP-3α. In turn, they have the potential to influence the activation levels of most inflammatory cells, including monocytes and macrophages, lymphocytes, platelets, endothelial cells, mastocytes, and fibroblasts, in addition to neutrophils themselves. Moreover, neutrophils can rapidly generate a selection of arachidonic acid (AA)-derived lipid mediators of inflammation; they are well known to be a substantial source of leukotriene (LT)B_4, which is a potent chemoattractant and agonist for leukocytes and important in asthma, allergies, and inflammation.[7] More recently, neutrophils have been shown to generate prostaglandin (PG)E_2 and tromboxane (TX)A_2 via the inducible cyclooxygenase (COX-2) pathway.[8–11] Whereas TXA_2 is recognized for stimulating platelet aggregation and activation, PGE_2 has both pro- and anti-inflammatory properties; it participates in regulating blood flow and vascular permeability, bronchial airway contraction, nociceptor activation, and hyperresponsiveness.[12] On the other hand, PGE_2 inhibits many of the leukocyte's effector functions by raising intracellular cAMP above basal levels.[13–18]

In view of the distinct and sometimes divergent activities of inflammatory mediators derived from neutrophils, factors that can modulate the overall profile of these mediators are likely to alter in a definitive manner the participation of neutrophils in the orchestration of an inflammatory response.

6.1.2 Adenosine Receptor Expression in Neutrophils

Regarding adenosine receptor expression in neutrophils, reports exist for all of the four known subtypes. To date, however, activities mediated by A_1 and A_{2A} receptors (R) have received most of the attention. Activation of A_1R by low concentrations of adenosine or by compounds showing specificity for this receptor subtype actually promotes neutrophil functions such as adhesion to endothelial cells or phagocytosis

of immunoglobulin-coated red blood cells. On the other hand, higher adenosine concentrations, as well as compounds specific for the $A_{2A}R$ subtype, inhibit adhesion, superoxide anion generation, and several other neutrophil activities through engagement of $A_{2A}R$.[19–22] Adenosine inhibits VEGF production by neutrophils with a pharmacological profile consistent with an $A_{2B}R$-mediated event.[23] A recent study using Western blots, radioligand binding techniques, and functional endpoints has reported reduced activity of $A_{2B}R$ in neutrophils from patients with systemic sclerosis, which may well contribute in part to the anti-inflammatory events mediated by the A_2R subtype.[24] The A_3R subtype is now being considered for its adenosine-mediated anti-inflammatory effects. Its stimulation has been shown to mediate adenylyl cyclase inhibition and phospholipase C activation.[25–28] A_3R may exert its anti-inflammatory properties by inhibiting specific cell functions in different systems. The expression of A_3R has been reported in human neutrophils. Its engagement inhibits degranulation and has been linked with increased intracellular Ca^{2+} concentrations through a combination of Ca^{2+} release from intracellular stores and influx from the extracellular space, and with a relative involvement in inhibition of superoxide anion generation. However, the pharmacological, biochemical, and functional properties of adenosine on neutrophil A_3R subtype remain incompletely documented at the present time. Further studies will be required in order to understand its relative contribution to anti-inflammatory activities of adenosine in neutrophils.[29]

6.2 IMPACT OF ADENOSINE ON NEUTROPHIL FUNCTIONS

One of the first reports to document the impact of adenosine on neutrophil functions was published more than 20 yr ago by Cronstein et al., in which adenosine was found to inhibit superoxide production in response to inflammatory stimuli.[30] The interest in the anti-inflammatory properties of adenosine in general, and in neutrophils in particular, has since continuously grown and triggered major efforts that have contributed toward appreciating the importance of this autacoid in controlling inflammation.

The mechanism by which adenosine inhibits neutrophil functions is not fully understood; however, it is clear that these inhibitory effects of adenosine involve $A_{2A}R$ and that the engagement of this receptor results in increased levels of intracellular cyclic AMP.[31] The elevation of intracellular cyclic AMP levels has long been recognized as a cellular event leading to inhibition of functional responses in neutrophils. Other hypotheses for the inhibitory effect of adenosine on neutrophil functions include uncoupling of chemoattractant receptors from their signal transduction mechanisms, and the stimulation of a serine and threonine phosphatase.[32,33] Another consequence of adenosine stimulation of human neutrophils is the inhibition of agonist-induced Ca^{2+} influx.[34] The following sections provide an overview of the known effects of adenosine on major neutrophil functions.

6.2.1 Chemotaxis and Transmigration

Considerable evidence now indicates that $A_{2A}R$ engagement has profound inhibitory consequences on neutrophil functions; however, its impact on cell movement

appears marginal. Indeed, results from a recent study showed that adenosine does not significantly affect IL-8—the main chemokine produced by neutrophils—either at the expression or release levels.[35] Also, the number of neutrophils recruited in the LPS-injected dorsal air pouches was comparable in $A_{2A}R$-knocked-out ($A_{2A}R^{-/-}$) and wild-type ($A_{2A}R^{+/+}$) mice, in line with a previous study which reported that, in carrageenan-injected air pouches, absence of a functional $A_{2A}R$ did not result in higher cell numbers.[36] In a series of *in vitro* chemotaxis experiments in which fMLP, IL-8, and LTB_4 were used as chemotactic agents, neither adenosine nor $A_{2A}R$ activation affected neutrophils movement toward these factors (M. Pouliot, unpublished observations). On the other hand, it has been reported that engagement of $A_{2A}R$ uniquely modulates neutrophil function so as to promote migration of neutrophils to sites of tissue damage while preventing the neutrophils from injuring healthy tissues en route.[37] Thus, the anti-inflammatory activities of adenosine do not appear to essentially rely on a reduction of neutrophil movement.

6.2.2 Superoxide Generation and Degranulation

Adenosine inhibits superoxide anion generation by chemoattractants and by Ca^{2+}-ionophore-stimulated neutrophils.[38] Pharmacological profiles are consistent with an $A_{2A}R$-mediated event.[39] Depending on the dose, adenosine also inhibits the LPS- and TNF-α-induced release of the azurophilic granule components, bactericidal/permeability-increasing protein, elastase, and defensins to approximately the same extent, with an IC_{50} in the 10 to 25 μM range. The inhibitory effects of adenosine were partially blocked by the A_2R antagonist 3,7-dimethyl-1-propargylxanthine, the A_1R/A_2R antagonist 8(p-sulfophenyl)theophyline, and the A_1R/A_3R antagonist xanthine amine congener, but not by the A_1R antagonist 1,3-dipropyl-8-cyclopentylxanthine. The highly selective A_3R agonist N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide and the nonselective agonist 2-chloroadenosine can also reduce degranulation more potently than the A_1R agonist N6-cyclopentyladenosine, indicating that adenosine acts via A_2R as well as A_3R to inhibit neutrophil degranulation.[40] However, several studies were unable to demonstrate that adenosine inhibits degranulation, and some controversy still exists regarding the identity of the implicated receptor subtypes and their potential to inhibit degranulation in neutrophils.[39]

6.2.3 Generation of Lipid Mediators

6.2.3.1 The 5-Lipoxygenase Pathway

Leukotriene generation is a tightly regulated process in which each metabolic step potentially represents a regulatory checkpoint. Its biosynthesis is initiated by the release of AA from membrane phospholipids by a phospholipase (PL)A_2 enzyme, in a Ca^{2+}-dependent fashion.[41] In a number of cell types—including neutrophils—type IV cytosolic (c)PLA_2 has been identified as the preeminent PLA_2 isoform responsible for the release of endogenous AA committed for transformation in LTs.[42,43]

6.2.3.1.1 Adenosine and Inhibition of Arachidonic Acid Release

AA availability is a limiting factor in the biosynthesis of eicosanoids in cells and tissues; it has already been observed in several cells (including neutrophils) producing LTs that elevation of intracellular cyclic AMP levels results in inhibition of AA release.[44] The release of AA by neutrophils activated with PAF can be almost completely abolished by treatment with the $A_{2A}R$-specific agonist CGS-21680. CGS-21680 also inhibits AA release in thapsigargin-activated neutrophils. Because the release of AA in activated neutrophils is a Ca^{2+}-dependent process, it is possible that the inhibitory effect of adenosine on agonist-induced Ca^{2+} influx in neutrophils might account for the inhibition of AA release observed in these experiments.[34,45] However, experiments demonstrating the ability of human neutrophils to produce LTB_4 in the presence or absence of extracellular Ca^{2+} rules out this hypothesis.

6.2.3.1.2 Adenosine and Inhibition of 5-Lipoxygenase Translocation

The translocation of the cytosolic 5-LO to the nuclear envelope upon neutrophil stimulation is recognized as a crucial step in leukotriene biosynthesis. Both CGS-21680 and the cAMP-elevating mixture forskolin/RO-1724 efficiently prevent the ligand-induced 5-LO translocation to neutrophil nuclei. Of interest, the inhibitory effect can be reversed by the PKA inhibitor H89, further supporting a role of cAMP and PKA in the subcellular localization of 5-LO.[46]

6.2.3.1.3 Adenosine and Suppression of Leukotriene Synthesis in a Cyclic AMP-Dependent Process

Studies have demonstrated that CGS-21680 (and other adenosine analogs) are potent inhibitors of LTB_4 biosynthesis, both in heparinized whole blood stimulated with fMLP and in isolated neutrophils also stimulated with agonists.[47] Studies with analogs of adenosine selective for the A_1R or $A_{2A}R$, as well as with selective $A_{2A}R$ antagonists, have clearly established that the inhibitory effect of adenosine on LT biosynthesis involves engagement of the $A_{2A}R$ on neutrophils. Interestingly, heparinized whole blood and isolated neutrophils were found to be most sensitive to inhibition of LTB_4 synthesis by CGS-21680. Blood monocytes and alveolar macrophages were much less sensitive, with a median inhibitory concentration (IC_{50}) of approximately 1 m*M*, and eosinophils and platelets were even more resistant to the inhibitory effect of CGS-21680, with IC_{50} values above 1 m*M*. The reason for these marked differences in the sensitivity of various cell types to eicosanoid biosynthesis inhibition by CGS-21680 is not clear; it may be explained by differences in the level of expression and function of the $A_{2A}R$ between these various cell types.[48] Positive coupling of the $A_{2A}R$ with the adenylate cyclase pathway is well documented in human neutrophils. Indeed, $A_{2A}R$ occupancy induces a rise in cAMP concentration in these cells.[49] Preincubation of neutrophils with the PKA inhibitor H89 prior to the incubation with CSG-21680 leads to a significant reversal of leukotriene synthesis inhibition, again supporting an important role for PKA in the inhibition of leukotriene biosynthesis.[50]

6.2.3.2 The Cyclooxygenase Pathway

The COX enzyme catalyzes two reactions by which AA is transformed to PGH_2, the common precursor of all prostanoids. PGH_2 can be isomerized to PGE_2, either

nonenzymatically or by one or more of several PGE_2 synthase isoforms, whereas TXA_2 formation results from the activity of TXA_2 synthase. In humans, two distinct genes are known to encode COX isoforms.[51,52] Both isoforms contribute to the inflammatory process, but COX-2 has attracted special attention as it is specifically induced during acute and chronic inflammation. Regulation of COX-2 expression is highly cell specific; in inflammatory neutrophils, COX-2 prevails over COX-1 for the generation of prostanoids, and appears to already have some level of basal expression in resting cells.[15,53,54]

6.2.3.2.1 Adenosine and Potentiation of the COX-2 Pathway in Human Neutrophils

In sharp contrast to the observed inhibitory impact of adenosine on the 5-lipoxygenase pathway, activation of the $A_{2A}R$ potentiates the expression of COX-2 in neutrophils, both at the mRNA and protein levels.[15] For virtually all agonists having the capacity to upregulate COX-2 in neutrophils, including LPS, fMLP, and opsonized particles, engagement of the $A_{2A}R$ results in increased COX-2 expression.[55] Extensive studies of the signal transduction pathways involved in this process point to an elevation in intracellular cAMP partially linked to phospho-CREB, and to an activation of the p38 MAPK, ERK-1/2, and PI-3K pathways. These results were corroborated *in vivo* using the dorsal air pouch model of leukocyte infiltration with $A_{2A}R^{+/+}$ or $A_{2A}R^{-/-}$ mice. In these experiments, air pouches were injected with LPS for a period of 4 h; migrated neutrophils were harvested and COX-2 mRNA and protein levels were determined. Neutrophils elicited from $A_{2A}R^{-/-}$ mice displayed COX-2 levels that were approximately half those found in wild-type animals. Significantly, the expression of COX-2 in lining tissues surrounding the air cavity was similar in $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice, once more emphasizing the particular responsiveness of neutrophils to $A_{2A}R$ engagement.

Although neutrophils clearly express higher levels of COX-2 as a result of $A_{2A}R$ occupancy, they do not automatically biosynthesize more PGE_2. As noted earlier, activation of $A_{2A}R$ efficiently blocks AA release, thus preventing eicosanoid production. On the other hand, increased PGE_2 biosynthesis can be obtained in response to exogenous AA.[11,15] In an inflammatory milieu, AA can be released from damaged cell membranes and be made available as a result of cell–cell interactions; in this situation, inflammatory neutrophils metabolize available AA through the COX-2 pathway—rather than through the 5-LO pathway—and generate PGE_2. In view of the potent immunomodulatory actions of PGE_2 in inflammatory cells, which include inhibition of chemotaxis, aggregation, superoxide production, lysosomal release, and LTB_4 and cytokine generation, an augmentation in COX-2 expression in neutrophils can be regarded as an anti-inflammatory event and may reflect a potentially important role for these cells in limiting inflammation.[15]

In turn, high concentrations of adenosine, which can be found at sites of inflammation, would tend to inhibit the generation of leukotriene by neutrophils, and to potentiate the COX-2 pathway. As neutrophils migrate out of the circulation and accumulate toward inflamed tissues, adenosine may gradually transform them into PGE_2-producing cells. This shift in the profile of eicosanoids generated from LTB_4 to PGE_2, promoted by activation of the $A_{2A}R$, could represent a key event for the

limitation—in amplitude and in duration—of an inflammatory response, and might constitute one of the most important anti-inflammatory activities of adenosine.

6.2.4 Generation of Cytokines and Chemokines

6.2.4.1 Adenosine's Role in Prevention of Production and Secretion of TNF-α and MIPs

Neutrophils have the specific capacity of generating selected subsets of cytokines and chemokines. For example, when stimulated with LPS (a ligand of the toll-like receptor-4), neutrophils release cytokines such as TNF-α and IL-1β, and several chemokines including IL-8/CXCL8, MIP-1β/CCL4, MIP-1α/CCL3, MIP-2α/CXCL2, MIP-3α/CCL20, and MIP-3β/CCL19; of these, IL-8 is by far the most abundant chemokine released by neutrophils and acts as a potent chemoattractant for this cell type in particular.[3]

A recent study has shown that engagement of $A_{2A}R$ with CGS-21680 resulted in the near-complete inhibition of TNF-α release from LPS-stimulated neutrophils. $A_{2A}R$ activation also had a potent inhibitory impact on the release of MIP-1α, MIP-1β, MIP-2α, and MIP-3α, with inhibitions ranging from 50 to 90%. The inhibitory effect of $A_{2A}R$ on these inflammatory mediators could also be observed at the mRNA level, largely preventing upregulation of their respective mRNA.[35] In contrast, levels of IL-8 were not modulated by $A_{2A}R$ activation, both at the mRNA and secretory levels, a fact that likely relates to the lack of effect that adenosine has on neutrophil recruitment. Collectively, these results identify TNF-α and the MIP family as gene products whose expression is crucially regulated by $A_{2A}R$ in toll-like receptor-4-activated human neutrophils.

These results found confirmation *in vivo*, where the expression of chemokines expressed by neutrophils was exacerbated in $A_{2A}R^{-/-}$ mice. Using the dorsal air pouch model of inflammation and leukocyte recruitment, injection of LPS into the cavity elicited, within hours, migration of neutrophils both in $A_{2A}R^{+/+}$ and in $A_{2A}R^{-/-}$ animals. Comparison of the expression of chemokine mRNA levels from $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice was performed by real-time PCR. Absence of a functional $A_{2A}R$ was accompanied by an increase in TNF-α, MIP-1α, and MIP-1β mRNA levels in migrated neutrophils. Interestingly, no significant difference between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice was observed in lining tissues of the air cavity for any of the genes tested. Secreted protein levels of TNF-α, MIP-1α, and MIP-1β were assessed in cell-free exudates and were significantly increased in samples obtained from $A_{2A}R^{-/-}$ animals, approximately twice those observed in wild-type mice. These results strongly support a role *in vivo* for $A_{2A}R$ in regulating the expression of important inflammatory cytokines and chemokines from inflammatory neutrophils, particularly that of TNF-α, MIP-1α, and MIP-1β.

As in the normal course of inflammation and wound healing, neutrophils are the first cell type to migrate into the air pouch model following injection of LPS. Within days, mononuclear cells replace neutrophils and become the most numerous leukocytes present in exudates.[56,57] Real-time PCR comparison of mRNA levels in mononuclear cells collected from the air pouch 72 h following LPS injection from $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice revealed an increased expression of TNF-α, IL-6, MCP-2, IL-1β, and, to a lesser extent, IL-1RA. Although it cannot be established at this time whether

the exacerbated chemokine profile observed in mononuclear cells from $A_{2A}R^{-/-}$ mice is the direct result of a nonfunctional $A_{2A}R$ in these cells, or rather a consequence of increased activation by neutrophil-elicited cytokines, these results nonetheless show that the $A_{2A}R$ signaling pathway has profound immunomodulatory activities in leukocytes, namely, by regulating the expression of pivotal inflammatory cytokines and chemokines produced. They also contribute to identify the neutrophil as an early and responsive target that can mediate adenosine's anti-inflammatory activities, in particular through activation of the $A_{2A}R$ subtype.

6.3 SUMMARY AND CONCLUDING REMARKS

Adenosine receptors play an increasingly recognized modulatory role in inflammatory responses. Neutrophils appear to be particularly responsive to $A_{2A}R$ activation. In stimulated neutrophils, adenosine has been shown to inhibit several of their primary functions, including superoxide production, adherence, and degranulation. Recent studies have revealed a major impact for $A_{2A}R$ occupancy on the profile of eicosanoids generated by neutrophils, namely, by inhibiting LTB_4 generation and by potentiating the COX-2 pathway. Moreover, adenosine has a pivotal inhibitory effect on the generation of selected cytokines and chemokines. Thus, it is clear that adenosine, through $A_{2A}R$ activation, exerts a profound control over the inflammatory functions of neutrophils, and accumulating evidence supports a role for the neutrophil as an early and important mediator of adenosine's anti-inflammatory actions.

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7 Dendritic Cells Regulated by Nucleotides and Nucleosides

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CONTENTS

7.1 NUCLEOTIDES AS ENDOGENOUS MODIFIER SIGNALS

Dendritic cells (DCs) are characterized by their unique ability to initiate adaptive immunity. They are heavily equipped with surface receptors for the recognition of many different types of pathogens. However, these cells can also directly react to tissue damage on recognizing constitutive or inducible endogenous "danger signals" provided by surrounding insulated cells.[1,2] Classically known endogenous-inducible danger signals are TNF-α, IL-1, or CD40L. All these molecules are upregulated during inflammation and deliver an activation stimulus to DCs.

DCs are a heterogeneous group of cells that circulate in the bloodstream, or reside in the peripheral tissues in an immature state in which they are specialized in the uptake of potential antigens. After maturation, they migrate to the lymph nodes in order to activate naïve T lymphocytes and initiate primary immune responses.[3,4,5] The circulation of DCs through tissues is strongly regulated by the level of chemotaxin receptor expression or the functional coupling of the receptors to signal pathways; e.g., immature DCs express receptors for inflammatory chemokines (CXCR1, CCR1, CCR2, and CCR5), which account for their capacity to migrate from blood to inflamed tissues, where their cognate ligands are produced and where danger signals and immune modifiers are likely to occur.

Constitutive molecules can signal danger and modify immune responses as well. Substances that are normally confined and hidden inside cells can be released into the extracellular space following tissue damage. Therefore, their increased extracellular concentration can be a simple sign of cell damage. However, for constitutive molecules to function as immune modifiers, they must be recognized by antigen-presenting cells. Nucleotides fulfill these requirements, as they occur in high concentrations (5 to 10 m*M*) in the cytoplasm of all cells. However, in the extracellular compartment, their concentration is in the nanomolar range. Therefore, nucleotides that have reached the extracellular milieu can serve this function.

Importantly, nucleotides are also released via nonlytic mechanisms through regulated transport: ATP is reportedly secreted by different cell types under a broad variety of conditions such as shear stress, endotoxin stimulation, or at sites of platelet aggregation.[6,7,8] In the extracellular microenvironment, nucleotides are recognized by P2 purinergic receptors (P2Rs) that are ubiquitously expressed on cell membrane throughout the human body, and a growing body of data indicates that activation of P2Rs can have a profound impact on immune cell functions.

7.2 NUCLEOTIDE AND NUCLEOSIDE RECEPTORS

P2Rs are subdivided into P2XR and P2YR families. The former are multimeric ligand-gated plasma membrane ion channels, and the latter belong to the family of seven membrane-spanning G-protein-coupled receptors.[9] The activation of P2XRs by extracellular ATP leads to increased plasma membrane permeability of ions (Na^+, K^+, and Ca^{2+}). The only known physiological ligand for P2XR is ATP, whereas the P2YR subtypes are differentially sensitive to various nucleotides: At $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, the preferred agonist is adenosine 5′-diphosphate (ADP); at $P2Y_2$, ATP and uridine 5′-triphosphate (UTP) are equipotent; at $P2Y_4$, UTP is preferred; at $P2Y_6$, uridine 5′-diphosphate; whereas $P2Y_{11}$ is the only P2YR selective for ATP.[10] P2R-mediated responses are regulated by the local nucleotide concentration: $P2X_{1-6}$ have EC_{50} in the low micromolar range and $P2X_7$ in the hundred micromolar range, whereas P2YRs display a higher affinity, responding to nanomolar concentrations of the ligand.

Another factor that modulates P2R-mediated responses is the extracellular nucleotide metabolism: ATP can be hydrolyzed to ADP by ecto-ATP/ADPase (CD39) and NTPDase2 (CD39L1).[11] CD39 can also hydrolyze ADP to adenosine 5′-monophosphate (AMP). CD39 is expressed on the membrane of a wide variety of cells such as DCs, macrophages, natural killer (NK) cells, activated B lymphocytes, and endothelial cells. In addition, AMP is the substrate for 5′-ectonucleotidase (CD73), leading to the generation of adenosine, which can activate P1 adenosine receptors.[12] P1 adenosine receptors also belong to the family of seven membrane-spanning G-protein-coupled receptors.

To date, four adenosine receptor subtypes—A_1, A_{2a}, A_{2b}, and A_3—have been identified. A_1 and A_3 receptors are coupled to G_i-, G_0-, and G_q-proteins and mediate inhibition of adenylate cyclase and activation of phospholipase C.[13] Phospholipase C cleaves phosphoinositide into diacylglycerol and inositol 1,4,5-trisphosphate, the latter mobilizing Ca^{2+} from intracellular stores.[14,15,16] Studies with various leukocytes revealed that activation of pertussis-toxin-sensitive G_i-proteins, followed by intracellular Ca^{2+} transients and actin reorganization, is a prerequisite for the migration

response.[14,15,16] Adenosine A_{2a} receptors interact with G_s-proteins, which activate adenylate cyclase, thus generating the second messenger cAMP.[17,18,19,20] Recent experiments reveal that DCs express A_1, A_{2a}, and A_3 P1 adenosine receptors.

7.3 ADENOSINE BREAKDOWN

Inosine is another naturally occurring purine, which is formed during adenosine breakdown by adenosine deaminidase. It is further metabolized in tissue to hypoxanthine and ribose-1-phosphate by purine nucleoside phosphorylase.[21,22] Therefore, significant amounts of inosine and hypoxanthine can be generated extracellularly from adenosine. In the past, the degradation products of adenosine were widely believed to have no biological functions. However, recent reports indicate that inosine regulates the inflammatory activity of mouse macrophages and spleen cells, suppressing the release of IL-12 and TNF-α.[21] Recently, we were able to demonstrate that inosine triggers intracellular Ca^{2+} transients, actin reorganization, and chemotaxis in immature DC.[23] In contrast, hypoxanthine and xanthine, the degradation products of inosine, do not show these biological effects.[23]

The local extracellular concentrations of inosine at inflammatory sites are not well characterized. In the extracellular space, an enzymatic cascade involving ecto-ATPases and adenosine deaminase regulates final degradation of ATP and adenosine to inosine.[24,25] Because of the high intracellular content of ATP ($2–5 \times 10^{-3}$ *M*), extracellular concentrations of nucleotides in the $10^{-4}M$ range have been detected in injured tissues.[26] In addition, about 20% of the intracellular ATP content can be released by prokaryotic cells using the type III secretion machinery,[27] and activated neutrophils can release AMP in the $10^{-5}M$ range.[28] Extracellular ATP, ADP, and AMP are degraded in tissue instantly to adenosine with a half-time of about 200 msec.[25,29] Infact, elegant measurements collected from rat heart and brain indicate that under hypoxia, extracellular adenosine concentration is around 2×10^{-5} *M*.[25,30] Using modern microdialysis techniques during the infusion of nucleotide and adenosine in rat intestine and heart, extracellular tissue concentrations of inosine in the 6×10^{-5} *M* range have been revealed.[24,31] These later findings are consistent with previous results that indicate extracellular inosine concentrations in the high μM and low *mM* range in ischemic tissue,[32,33,34] and increased extracellular inosine levels in various inflammatory diseases.[35,36] Based on this information and the reported concentration dependency, one can assume that the chemotactic activity of inosine in immature DCs is likely to be physiologically relevant. In mature DCs, inosine-induced $G_{i/o}$-protein-dependent migration and the analyzed signaling events are no longer present, although mature DCs are still responsive to other $G_{i/o}$-protein-dependent chemotactic agents such as CCL19.

7.4 EXTRACELLULAR NUCLEOTIDE–DC INTERACTION

To date, most information available on the effects of extracellular nucleotides, nucleosides, and inosine on DC biology has been provided by studies on human DCs. In this model, ATP, ADP, adenosine, and inosine act as chemoattractants for immature DCs.[37] Physiological concentrations of these molecules induce G_i-protein-dependent

calcium transients, actin polymerization, and chemotaxis in immature DCs, not mature ones, through the activation of P2YR[38], A1 and A3 receptors, and an unidentified inosine receptor. In these circumstances, it is interesting that CD39 null monocyte/macrophages, which have a lower capacity to metabolize extracellular ATP, display an impaired chemotactic response associated with P2YR-mediated signaling pathway desensitization.[39]

In the current paradigm, the maturation of DCs is induced by different stimuli, such as lipopolysaccharide (LPS), CpG oligonucleotides, viral RNA, and TNF-α. Maturing DCs migrate from peripheral tissues to lymph nodes, where they present the antigen captured at the time and site of activation, and deliver potent, instructive signals to naïve T lymphocytes, thus initiating an adaptive-immune response.[3] DC maturation encompasses a coordinated downregulation of inflammatory chemokine receptors (CCR1, 2, 5, and CXCR1) and induction of CCR7 and CXCR4. During maturation, functional downregulation of chemotaxis-regulating A_1 and A_3 P1 receptors occurs, and P2YR are uncoupled from chemotaxis-associated signal transduction pathways. As a result, DCs lose sensitivity to inflammatory chemokines, ATP, ADP, and adenosine, and acquire the ability to colocalize with naïve T cells in the lymph nodes. Moreover, ATP and ADP foster their differentiation processes and confer functional responsiveness on CCL19 and CXCL12, whereas chemotaxis to CCL4 is reduced, preparing DCs for enhanced lymph node localization.[40]

Maturing DCs on their way to lymph nodes upregulate membrane expression of peptide-loaded major histocompatibility complex (MHC) molecules as well as costimulatory molecules, and become effective in activating naïve T cells. At the initial stage of naïve T cell–DC interaction, the production of high levels of IL-12 by maturing DCs is pivotal in the preferential development of T helper cell type 1 (Th1) responses.[41] Moreover, IL-12 and TNF-α released by DCs activate NK cells, which in turn reinforce IL-12 production of DCs by releasing interferon-γ (IFN-γ).

The presence of extracellular ATP, ADP, and adenosine in peripheral tissue may profoundly modify this picture. Beside interaction with G_i-protein-coupled receptors, the nucleotides ATP and ADP, and as well as adenosine, are able to activate G_s-protein-dependent signal pathways and stimulate formation of cAMP (12). Now prototypical stimuli for DC maturation fail to induce production of TNF-α and IL-12, although the anti-inflammatory cytokine IL-10 is induced. As a result of blocked IL-12 production, DCs that have matured in the presence of ATP and adenosine have an impaired capacity to promote type 1 polarization of naïve T lymphocytes, favoring instead the development of type 2 lymphocytes.[42] In contrast to ATP, ADP, and adenosine, inosine did not affect cAMP levels and IL-12 secretion.

Besides representing highly mobile cells themselves, DCs are also critically involved in the guidance of trafficking pathways for other cells by the release of chemokines: immature DCs constitutively release CCL22 and CCL17.[43] During the early stages of maturation, DCs produce high levels of CCL2, CCL3, CCL4, CCL5, CXCL8, and CXCL10. These chemokines sustain the recruitment of circulating, immature DCs, monocytes, and T cells to inflamed tissue.[44] Lymphoid chemokines such as CCL19, CCL17, and CCL22 are produced or upregulated later during DC maturation,[44,45] providing chemotactic signals for mature DCs and for T cells in secondary lymphoid organs. CCL19 is chemotactic for naïve T cells expressing

CCR7, whereas CCL17 and CCL22 act on naïve and recently activated type 2 T cells expressing CCR4.[46,47] ATP and adenosine affect the pattern of chemokine release from DCs. They upregulate the constitutive production of CCL22 and inhibit LPS-induced secretion of CXCL10 and CCL5.[40] This causes selective impairment in the capacity of DCs to recruit type 1 but not type 2 lymphocytes.[40] In fact, CCR5 and CXCR3 (which can be activated by CCL5 and CXCL10, respectively) are preferentially expressed by Th1 cells, whereas CCR4 (which is activated by CCL22 and CCL17) is more prominent on Th2 cells.[47] By diminishing DC capacity to attract IFN-γ-producing lymphocytes, ATP and adenosine may potently impair the amplification of a type 1 response and favor type 2 immunity.

The *in vivo* relevance of P2R and adenosine A_2 receptors on immunological reaction patterns has recently attracted much attention. Mizumoto et al. describe the critical role of CD39 in the induction of T cell responses by skin Langerhans cells.[48] T-cell-mediated contact hypersensitivity is diminished in $CD39^{-/-}$ mice. In addition, T cells increase ATP pericellular concentration upon activation, and $CD39^{-/-}$ DCs display ATP unresponsiveness as a result of P2YR desensitization. These observations suggest that nucleotide-mediated communication between DCs and T cells involving P2YR represents an important event during antigen presentation *in vivo*.

7.5 CONCLUSION

In sum, recent studies have highlighted a more complex role for extracellular nucleotides and nucleosides in the biology of DCs: beyond activation, ATP exerts regulatory effects that influence the course of inflammatory reactions and the outcome of T-cell-mediated immune responses. Increasing the concentration gradient of extracellular ATP, adenosine, and inosine attracts immature DCs. In the proximity of damaged cells, where the concentration may be in the micromolar range, nucleotides such as ATP and ADP, as well as nucleosides, block the synthesis of proinflammatory cytokines by DCs and their capacity to promote a Th1 response, favoring the development of type 2 responses, which may be less harmful. Further studies on nucleotide- and nucleoside-regulating enzymes, as well as functional expression of P2R *in vivo* by different DC subsets, may help pave the way for the potential application of nucleotide and nucleoside analogs in the manipulation of inflammatory and immune responses.

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8 Adenosine and Endothelial Cell Function

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CONTENTS

8.1 INTRODUCTION

The vascular endothelium lines all blood vessels in the body and serves as a dynamic and selective barrier regulating the flow of nutrients, biologically active molecules, and cells across blood vessel walls. Quiescent endothelial cells also serve as an active antithrombotic surface, promoting the transit of plasma and cellular constituents throughout the vasculature. Conversely, activation of endothelial cells creates a prothrombotic and antifibrinolytic microenvironment, in which platelets and neutrophils attach to the endothelium and initiate an inflammatory cascade. The endothelium also plays an important role in regulating blood flow. Vasoactive substances released from endothelial cells modulate vascular tone in a paracrine manner by acting on smooth muscle cells. In addition, endothelial cells play a central role in the expansion, regression, and remodeling of preexisting blood vessels, a process commonly known as *angiogenesis*. Thus, endothelial cells function in close cooperation with other cell types as integrators, transducers, and effectors of local environmental signals to maintain homeostasis. When these homeostatic mechanisms fail, endothelial cells can participate in the pathogenesis of several disease processes.

Adenosine is one of many biologically active molecules released during pathological states. This nucleoside is generated by ATP catabolism at sites of tissue stress,

injury, and local hypoxia. Endothelial cells have the capacity to release adenosine,[1] and indirect evidence suggests that they are an important source of adenosine during ischemia in humans.[2] Adenosine can be derived from a variety of other cells within the vascular compartment. Blood cells contain adenosine and adenine nucleotides that can be a potential source of extracellular adenosine. For example, platelet activation elicits the release of ADP, which can then be converted to adenosine. Similarly, neutrophils, eosinophils, and erythrocytes release ATP and AMP,[3–6] which are rapidly converted to adenosine by apyrase and ecto-5′-nucleotidase.

Once generated, adenosine has the capacity to modulate endothelial functions via activation of cell surface adenosine receptors. Expression of adenosine A_{2A} and A_{2B} receptors on vascular endothelial cells is well documented.[7–11] Some studies also suggested the presence of A_1 and A_3 receptors on endothelial cells.[6,12,13] This, however, is based on detection of receptor mRNA and the use of rather high, and most likely nonselective, concentrations of ligands. It still remains unclear if A_1 and A_3 receptors are functionally expressed and what role, if any, they play in endothelial cells.

Thus, adenosine can act in an autocrine and paracrine fashion to modulate endothelial cell function. Historically, the regulation of vascular tone was the first function suggested for adenosine receptors.[14] Despite a large body of data, there is still no consensus about the relative importance of endothelial vs. smooth muscle adenosine receptors in the modulation of vascular tone, or about the intracellular mechanisms involved. We will summarize these still contradictory data on adenosine-dependent vasodilatation in this chapter. We will also highlight the potential role of adenosine receptors in the regulation of other important endothelial functions including barrier, coagulation, recruitment of inflammatory cells, and angiogenesis.

8.2 HETEROGENEITY OF ENDOTHELIAL CELLS AND EXPRESSION OF ADENOSINE RECEPTORS

Arteries, veins, and capillaries exhibit very distinct physiologic functions in the circulatory system, and endothelial cells are central to many of them. It is not surprising, therefore, that endothelium from a given vascular site has a distinctive appearance, specific functions, and a characteristic expression of cell surface receptors. The distinction between arterial and venous endothelial cells is observed early in embryonic angiogenesis. For example, ephrin-B2 is selectively expressed in arterial endothelial cells, whereas venous endothelial cells specifically express its receptor Eph-B4.[15] In adults, vascular endothelium heterogeneity can be revealed by wheat germ agglutinin, which binds to endothelial cells of arterioles and capillaries but not of postcapillary venules.[16] Even endothelium of capillaries is different depending on the tissue location. Capillaries in the brain are lined by continuous endothelial cells that maintain the blood–brain barrier; capillaries in the liver, spleen, and bone marrow are lined by discontinuous endothelial cells that allow cellular trafficking between intercellular gaps; the intestinal villi, endocrine glands, and kidneys are lined by fenestrated endothelial cells that facilitate selective permeability required for efficient absorption, secretion, and filtering.[17,18] Of relevance to inflammation, it has been reported that susceptibility to injury by activated neutrophils differs significantly between endothelial cells from different tissues, as well as between microvascular

and macrovascular endothelial cells from the same tissue.[19] Thus, accumulating evidence suggests that endothelial cells are differently regulated in space and time. All this adds to the complexity of studying endothelial cells. Current views on endothelial cell heterogeneity are summarized in a recent review by Aird.[20]

Differential expression of cell surface adenosine receptors is also part of the phenotypic heterogeneity of endothelial cells, and endothelial responses to adenosine can differ depending on the relative expression of adenosine receptor subtypes. For example, human umbilical vein endothelial cells express predominantly A_{2A} adenosine receptors, whereas human microvascular endothelial cells HMEC-1 express predominantly A_{2B} receptors. This expression pattern correlates with the ability of adenosine to stimulate production of angiogenic factors; adenosine upregulates expression of angiogenic factors in microvascular endothelial cells expressing mostly A_{2B} receptors, but not in human umbilical vein endothelial cells preferentially expressing A_{2A} receptors.[21]

Although it is always tempting to draw general conclusions about adenosine's actions in terms of arterial vs. venous or macrovascular vs. microvascular endothelial cells based on its effects on representative cells, one should remember that endothelial cells are heterogeneous even within these groups and may respond differently to adenosine. For example, it was initially suggested that adenosine increases permeability of microvascular endothelium but reduces permeability of endothelium in large vessels based on comparisons between rat coronary microvascular and porcine aorta endothelial cells.[22] We now know that adenosine reduces permeability of human dermal microvascular endothelial cells,[23,24] thus demonstrating that adenosine can have dissimilar effects on microvascular endothelial cells derived from different tissues or different species.

Furthermore, there are multiple factors that can modify an endothelial phenotype, including mechanical forces, biologically active compounds, the composition of extracellular matrix, and contact with circulating and tissue-based cells. Conditions present in inflammatory processes provide powerful stimuli for such phenotypic changes. The Th1 cytokines IL-1 and TNF-α increase expression of both A_{2A} and A_{2B} adenosine receptors in human dermal microvascular endothelial cells. IFN-γ treatment increases the expression of A_{2B} receptors, but decreases expression of A_{2A} receptors.[11] Hypoxia, a condition often present in inflamed tissues, also selectively increases A_{2B} expression in endothelial cells.[24] Hypoxia decreased A_{2A} and increased A_{2B} receptor expression in human umbilical vein endothelial cells. Consistent with these changes in receptor expression, adenosine stimulated VEGF release under hypoxic but not normoxic conditions, indicating that hypoxia increased the expression of A_{2B} receptors that were functionally coupled to upregulation of VEGF.[21] Because it is difficult to mimic *in vitro* the exact conditions present in inflammation, we can only speculate that inflammation would generally favor an increase in A_{2B} over A_{2A} receptors based on the effects of individual inflammatory cytokines and hypoxia. This hypothesis awaits experimental testing.

8.3 REGULATION OF VASCULAR TONE

Adenosine-induced vasodilation was among the first actions described for this nucleoside.[14] Adenosine is now used clinically to produce maximal coronary vasodilation to measure coronary reserve, as well as to unmask perfusion differences

between ischemic and nonischemic myocardium. One would assume, therefore, that the mechanisms of adenosine-induced vasodilation are completely understood. This process, however, is complicated by the variable contribution of endothelial cells, the involvement of more than one receptor subtype, and the participation of distinct cell-signaling pathways.[25] Furthermore, there are some vascular beds in which adenosine produces vasoconstriction rather than vasodilation. The renal circulation is the prime example. Adenosine decreases renal blood flow because of constriction of afferent arterioles in cortical glomeruli. This effect is mediated through adenosine A_1 receptors and involves a complex interaction with angiotensin II. Angiotensin II antagonists or genetic ablation of angiotensin AT_{1a} receptors attenuate the renal vasoconstriction produced by adenosine and, conversely, A_1 antagonists or ablation of A_1 adenosine receptors reduce the renal vasoconstrictor effect of angiotensin II.[26–32] Adenosine also constricts the sheep pulmonary[33] and rat liver[34] circulations via release of vasoconstrictive eicosanoids acting on thromboxane receptors, an effect that is presumably mediated by A_1 receptors.[35] Adenosine also constricts human chorionic arteries and veins through release of eicosanoids, but this effect is mediated via A_{2B} receptors located on endothelial cells and vascular smooth muscle cells.[36]

In most vascular beds, however, adenosine produces vasodilation. Adenosine receptors are expressed in vascular smooth muscle cells, and the vasoactive effects of adenosine could be accounted for solely by a direct action on these cells. However, it has been suggested that the endothelium contributes to, or is even essential for, the vasodilatory effects of intravascular adenosine. The half-life of adenosine in human blood is estimated to be less than 1 sec because of cellular uptake by a high-affinity nucleoside transporter.[37] Endothelial cells express this transporter[38] and may act as a barrier to intravascular adenosine, preventing it from reaching the underlying vascular smooth muscle. For example, it has been shown that most of the labeled adenosine administered intra-arterially is contained within endothelial cells, and very little escapes this endothelial trap to reach the underlying vascular smooth muscle.[1] In humans, intravascular adenosine does not reach the underlying interstitium unless the nucleoside transporter is blocked by dipyridamole.[39] Similarly, intravascular administration of adenosine and adenosine agonists, linked to macromolecules and therefore less likely to cross the endothelium, is still able to produce vasodilation.[40–43] This would imply that adenosine infused into the vasculature would need to interact with endothelial cells to induce vasodilation. Endothelial cells contribute to the regulation of vascular tone by releasing vasoactive autacoids that act in a paracrine fashion on vascular smooth muscle cells. These include vasodilators such as nitric oxide (NO), prostacyclin (PGI_2), and the less characterized endothelium-derived hyperpolarizing factor (EDHF), as well as vasoconstrictors such as endothelin (ET) and platelet-activating factor (PAF) (for a review see Reference 17).

Despite these theoretical considerations, it has not been easy to define the contribution of endothelial cells to the dilation induced by intravascular adenosine. *In vitro* studies have yielded conflicting results about whether the vasodilatory actions of adenosine are different in vascular preparation with intact or denuded endothelium.[44–75] Isolated vascular preparations are often used to study this phenomenon,

but this approach has limitations in evaluating a putative endothelium-dependent vasodilation by adenosine, because adenosine will dilate vessels with or without endothelium. This is particularly true when stable agonists are used, because they are not trapped by the endothelium, as adenosine is, and have more ready access to the underlying vascular smooth muscle. In contrast, other endothelium-dependent vasodilators will constrict vascular smooth muscle in the absence of endothelium,[76] making their distinction easier.

Adenosine has been reported to induce NO production in some but not all cultured endothelial cells.[13,77–80] Animal studies also provide contradictory data about the role of NO in the regulation of vascular tone by adenosine. Some studies show that adenosine-dependent vasodilation is dependent on NO production,[43,48,51–53,55,56,59,61,66,69,71,74,81–90] whereas others do not.[50,57,64,68,91,92] A handful of investigations have used nitric oxide synthase (NOS) inhibitors to determine whether release of NO contributes to adenosine-induced vasodilation in humans, but results have been contradictory. Studies in which adenosine and NOS inhibitors were directly infused into the forearm or coronary circulation have shown either NO-dependent[93] or NO-independent[94–96] adenosine vasodilation. Data obtained from human small coronary arteries suggested that adenosine-induced relaxations are independent of NO.[97] Furthermore, adenosine-induced vasodilation could conceivably release NO nonspecifically through a flow-related mechanism, giving the appearance of NO-mediated vasodilation.[98] In addition, it has been suggested that adenosine mediates vasodilation in part by the release of other endothelial factors, including prostaglandins[68,92,99] and endothelium-dependent hyperpolarizing factors.[56,64] It has been proposed that the actions of adenosine in endothelial cells, and particularly in smooth muscle cells, are mediated by the opening of adenosine-triphosphate-sensitive potassium (K_{ATP}) channels. Moreover, in this case studies both for and against this hypothesis have been reported.[57,65,69,70,89,97,100–109]

It is possible that permutations of all of these mechanisms contribute to adenosine-induced vasodilation and that the magnitude of their contributions varies depending on the vascular bed or species studied. Additionally, the variability in the mechanisms involved in adenosine-induced vasodilation could depend on the differential expression of adenosine receptor subtypes. Adenosine-induced vasodilation has been attributed to activation of A_{2A} receptors[49,50,56,57,59–62,64,65,69,74,89,106,107,110] However, there are vascular beds in which the nonselective agonist NECA produces profound vasodilation but the selective A_{2A} agonist CGS 21680 has little effect, suggesting that adenosine-induced vasodilation is mediated via A_{2B} receptors (for review, see Reference 111). This phenomenon is observed in guinea pig aorta, dog saphenous vein,[112] and dog coronary arteries.[113] A_{2B} receptors were also implicated in relaxation of coronary arteries in guinea pigs,[70] pigs,[108] and humans.[97] Furthermore, studies in A_{2A} knockout mice suggested that coronary blood flow is regulated by both A_{2A} and A_{2B} receptors[114] and that carotid artery dilatation is A_{2B} dependent.[115]

In summary, both A_{2A} and A_{2B} receptors mediate vasodilation. The relative contribution of these receptors to adenosine-induced vasodilation is still not well defined. There are also conflicting results about the importance of NO generation in adenosine-induced vasodilation. To complicate matters further, in some vascular beds, adenosine-induced vasodilation is endothelium dependent but does not appear

to be mediated by NO, because it is not blocked by inhibition of NOS, raising the possibility that other endothelial factors may be involved. The precise nature of the interaction between adenosine receptors and endothelial cells and their role in the regulation of vascular tone are areas in which more research is needed.

8.4 REGULATION OF ENDOTHELIAL BARRIER FUNCTION

Communication between blood and tissue requires the delivery of molecules and circulating substances across the endothelial barrier by directed transport either through or between cells. Regulated changes in permeability, therefore, is an important function of endothelial cells, and this appears to be modulated by adenosine.

In a comparative study of endothelial cells of different origin, activation of adenosine A_2 receptors reduced permeability to albumin across confluent monolayers of porcine aortic endothelial cells, but increased permeability of rat coronary microvascular endothelial cells. In both cases, adenosine apparently acted via cAMP increase, because stimulation of adenylate cyclase by isoproterenol or forskolin mimicked these opposite adenosine actions on endothelial permeability.[22,116] Adenosine decreased microvascular permeability to albumin in porcine coronary arterioles but not venules.[117] In another study using microvascular endothelial cells from bovine brain as an *in vitro* model of the blood–brain barrier, adenosine failed to alter endothelial permeability,[12] despite the fact that an elevation of cAMP in these cells was shown to reduce permeability. Most other studies, using endothelial cells of various origins, have shown that adenosine decreases endothelial permeability.[6,23,24,118–129] Furthermore, mice lacking either apyrase (CD39) or ecto-5′-nucleotidase (CD73), enzymes involved in the generation of extracellular adenosine, had a higher leakage of albumin through endothelium in various tissues, as measured by the Evans blue technique.[24,128]

Regulation of endothelial permeability is important in inflammatory processes. Inflammation promotes macromolecular transport by decreasing cell–cell and cell–matrix adhesion and by formation of intercellular gaps. Inflammation may also increase the selective transport of macromolecules through cells. During the inflammatory response, neutrophils represent a first-line defense of the organism against invading pathogens and produce oxygen-derived free radicals when activated by appropriate stimuli. However, reactive oxygen species released from neutrophils are thought to contribute to vascular injury by increasing endothelial permeability,[130] and adenosine is proposed to counteract this effect by inhibiting neutrophil activation[131] and sealing the endothelial monolayer.[132] Adenosine was shown to maintain endothelial barrier function, both in the absence and in the presence of oxygen radicals, in human umbilical vein endothelial cell monolayers exposed to xanthine plus xanthine oxidase as a model of oxidative injury.[132]

Proinflammatory cytokines and chemotactic factors signal the recruitment of neutrophils to sites of infection or injury. Under the influence of a chemotactic gradient, neutrophils penetrate the endothelial layer and migrate to sites of infection. Migration of neutrophils has the potential to disturb vascular barrier function and give rise to intravascular fluid extravasation and edema. However, generation of

extracellular adenosine at the sites of neutrophil–endothelial interactions provides a mechanism limiting vascular permeability. Activated neutrophils release ATP and AMP, which are then converted to adenosine by apyrase and nucleotidase located on the surface of endothelial cells.[6,23,24,128] Adenosine increases cAMP via stimulation of A_2 adenosine receptors on endothelial cells. Reduction of cellular permeability by adenosine seems to be mediated by the cAMP-protein kinase A-dependent pathway, because this effect was mimicked by reagents elevating cAMP or stimulating protein kinase A.[133] The exact mechanism downstream to these events remains largely unknown. It has been proposed that it can be explained in part by relaxation of actin cytoskeletal tension, as a result of phosphorylation by protein kinase A of actin-associated, vasodilator-stimulated phosphoprotein.[134] Inhibition of the Rho family GTPases RhoA and Rac-1 was also implicated in adenosine- and cAMP-dependent regulation of endothelial barrier function.[127,135] Stimulation of ERK via A_{2B} receptors was also proposed to promote barrier function through dephosphorylation of the myosin II regulatory light chains.[136]

Interactions between platelets and neutrophils are also important in inflammatory processes. Activated platelets can bind to neutrophils and stimulate the oxidative burst,[137] while themselves serving as sources of ADP that is converted to adenosine. Similar to neutrophils, activated platelets were shown to decrease macromolecular permeability of confluent bovine pulmonary artery endothelial monolayers. The importance of adenosine in transvascular water flux was examined in an isolated perfused guinea pig lung model and measured by the capillary filtration coefficient (Kfc). Platelets added to the perfusate reduced Kfc by 29%, but pretreatment of platelets with adenosine deaminase abolished this response, whereas addition of adenosine reduced Kfc by 11%. These results indicate that adenosine is a component in platelet-mediated decreases of albumin permeability across endothelial monolayers and of transcapillary water flux in isolated perfused lungs.[118] Not everybody, however, agrees that adenosine is the main mediator by which platelets affect endothelial permeability. For example, it has been proposed that protein, rather than adenosine, mediates the decrease in endothelial permeability induced by platelets.[138]

8.5 RECRUITMENT OF INFLAMMATORY CELLS

During inflammation, leukocytes tether to and roll on the endothelial surface. The cells then arrest, spread, and finally migrate between endothelial cells to reach the underlying tissues. In most circumstances, interactions with E- and P-selectins, transmembrane glycoproteins that recognize cell surface carbohydrate ligands found on leukocytes, initiate and mediate tethering and rolling of leukocytes on the endothelial surface.[139] Mononuclear cells and eosinophils initiate tethering and rolling by binding to the Ig ligands VCAM-1 and MAdCAM-1 on the endothelial surface.[140,141] The slow velocities of rolling cells favor encounters with chemokines and binding to Ig ligands such as ICAM-1 and ICAM-2.[142] Subsequently, inflammatory cells migrate between endothelial cells into tissues by mechanisms that are not completely understood but are affected by gradients of chemokines with restricted specificities.

Bouma et al. demonstrated that adenosine reduces the expression of E-selectin and VCAM-1 in activated human umbilical vein endothelial cells.[143] This effect is presumably mediated via A_{2A} adenosine receptors, the predominant receptor subtype expressed in these cells.[10] A_{2A} adenosine receptors have also been implicated in inhibition of ICAM-1 and E-selectin expression in human pulmonary endothelial cells.[144] The anti-inflammatory role of endothelial A_{2A} receptors was later confirmed in the murine carotid artery ligation model of early inflammatory response. Treatment of mice with a selective A_{2A} agonist significantly reduced neutrophil and macrophage recruitment, and also significantly reduced expression of P-selectin, VCAM-1, and ICAM-1 in carotid arteries.[145] Similarly, treatment of animals with a selective A_{2A} agonist in mouse and rat models of ischemia/reperfusion injury of the kidney significantly reduced neutrophil infiltration and endothelial expression of P-selectin and ICAM-1.[146] Thus, stimulation of endothelial A_{2A} receptor cells produces anti-inflammatory effects by downregulating expression of adhesion molecules and cytokines involved in the recruitment of inflammatory cells by activated endothelium.

8.6 THE ROLE OF ADENOSINE IN THE ANTICOAGULANT PROPERTIES OF ENDOTHELIUM

A crucial physiological function of the endothelium is to facilitate blood flow by providing an antithrombotic surface that inhibits platelet adhesion and clotting. However, when the endothelium is perturbed by physical forces or by specific chemical factors, the cells undergo biochemical changes that culminate in their transformation into a prothrombotic surface. A dynamic equilibrium exists between these two states, modulated both at the level of gene transcription and at the level of the intact cell, which often permits the injured endothelium to return to its unperturbed state once the procoagulant stimulus has dissipated.[17]

The pivotal step in transforming the endothelial cell membrane from an anticoagulant to a procoagulant surface is the induction of tissue factor. Adenosine downregulates the expression of tissue factor induced by TNF-α, thrombin, or phorbol ester on human umbilical vein endothelial cells,[147–149] presumably via activation of A_{2A} receptors. Adenosine also inhibited tissue-factor-dependent activity induced by adhesion of polymorphonuclear leukocytes to endothelial cells.[150] In addition, the pro-aggregatory effect of ADP, derived from activated platelets, can be countered by its conversion to adenosine via endothelial apyrase and nucleotidase, which then acts on A_{2A} receptors in platelets to inhibit aggregation.[151]

8.7 ROLE OF ADENOSINE IN ANGIOGENESIS

Adenosine has long been known to promote angiogenesis. Many studies demonstrated that chronic elevation of tissue adenosine concentrations, induced by the adenosine reuptake blocker dipyridamole[152–165] or long-term administration of adenosine and its analogs,[166–168] promoted capillary proliferation in the heart and skeletal muscles. The angiogenic properties of adenosine were demonstrated also in

the chick chorioallantoic membrane and embryo,[169–171] the optical tectum of *Xenopus leavis* tadpoles,[172] and the mouse retina.[173,174] Furthermore, administration of adenosine to humans increased plasma levels of vascular endothelial growth factor (VEGF).[175]

Several mechanisms have been proposed to explain the effects of adenosine on new blood vessel formation. Adenosine-induced changes in blood flow are believed to produce shear stress in capillaries and this, by itself, may stimulate angiogenesis.[166,167,176] Shear stress, in turn, may increase local adenosine concentrations because of ATP release from endothelial cells and its subsequent breakdown to adenosine.[177–182] Adenosine modulates release of angiogenic factors from various cells and tissues,[21,183–192] which may regulate capillary growth in a paracrine fashion. In addition, adenosine can modulate release of angiogenic factors from endothelial cells,[8,10,11,192–195] which may regulate capillary growth in an autocrine fashion. It is also possible that adenosine promotes endothelial cell growth directly.[196]

Recent reviews on the role of adenosine in angiogenesis extensively address all these potential mechanisms.[182,197–199] Adenosine reportedly modulates a number of steps involved in angiogenesis, including endothelial cell proliferation,[8,200–205] migration,[200,204–207] and capillary tube formation.[205,207] Several alternative mechanisms have been proposed to explain adenosine actions on endothelial cell proliferation. Very high (~1 m*M*) concentrations of adenosine induced apoptosis of human and bovine pulmonary artery endothelial cells. This adenosine-induced apoptosis appears to be mediated by an intracellular mechanism, rather than by cell surface receptors, because it is blocked by the adenosine uptake inhibitor dipyridamole.[208] The physiological relevance of this finding is unclear, given the high concentrations required to elicit this effect. In fact, most studies suggest that adenosine promotes endothelial cell proliferation, even though there is discrepancy about the mechanism of its action. Both receptor- and non-receptor-mediated actions have been reported. Meininger et al. found that adenosine stimulated the proliferation of bovine aortic and coronary venous endothelial cells via cell surface receptors, because the adenosine receptor antagonist 8-phenyltheophylline prevented these effects.[200] A similar receptor-mediated mitogenic response was reported in human umbilical vein endothelial cells.[209] In contradiction to these findings, it was concluded in other investigations in the same cells that adenosine-induced proliferation was mediated by a nonreceptor mechanism, because its effect was not mimicked by adenosine receptor agonists or inhibited by adenosine receptor antagonists.[202,210] In a recent review, Adair raised doubts about the relevance of nonreceptor mechanisms in mediating adenosine-induced proliferation. Studies that reached this conclusion, he pointed out, used [^{3}H] thymidine uptake as an estimate of cell proliferation in experiments carried out in a serum-free medium, a condition in which endothelial cells do not proliferate.[199]

Depending on the endothelial cell studied, either A_{2A} or A_{2B} receptors have been implicated in induction of angiogenesis. Adenosine A_{2B} receptors have been shown to mediate the proliferative actions of adenosine in human retinal microvascular endothelial cells,[8,173,174,205] porcine coronary artery, and rat aortic endothelial cells.[204] The proliferative effects of adenosine on endothelial cells are mediated at least partly by stimulating the production of growth factors that facilitate new blood vessel formation. Adenosine increased VEGF production in pig cerebral microvascular

endothelial cells,[193] but the adenosine receptor subtype involved in VEGF upregulation in these cells remains uncertain because of nonspecific concentrations of antagonists used in that study. Takagi and associates showed that adenosine upregulates VEGF mRNA in bovine retinal microvascular cells via A_{2A} receptors.[192] However, adenosine upregulated VEGF mRNA expression and protein secretion via A_{2B} receptors in human retinal endothelial cells.[8]

VEGF is not the only angiogenic factor modulated by adenosine in endothelial cells. In human retinal microvascular endothelial cells, A_{2B} receptor activation also upregulated basic fibroblast growth factor (bFGF) and insulin-like factor-1.[8] In immortalized human dermal microvascular endothelial cells HMEC-1, stimulation of A_{2B} adenosine receptors upregulated bFGF and IL-8 in addition to VEGF.[10] Of interest, stimulation of A_{2A} receptors in human dermal microvascular endothelial cells inhibited the release of the antiangiogenic factor trombospondin 1, providing yet another means by which adenosine may regulate angiogenesis.[195]

The intracellular mechanisms mediating proangiogenic effects of adenosine in endothelial cells remain uncertain. Both A_{2A} and A_{2B} receptors are coupled to G_s proteins and stimulate adenylate cyclase. Although some data suggest that cAMP may play a role in the proangiogenic effects of adenosine in certain cells,[192] most studies show that upregulation of angiogenic factors does not involve G_s-cAMP pathway, but is rather mediated via coupling to G_q, possibly involving MAPK pathways.[8,10,205]

Inflammation often causes local tissue hypoxia because of the disruption of microcirculation.[211] Oxygen deprivation, in turn, results in dramatic changes in cell metabolism and surface receptor expression. Progressive pulmonary inflammation in IL-13 transgenic mice results in increased total expression of A_1, A_{2B}, and A_3 adenosine receptor expression in the lung, whereas expression of A_{2A} subtype remains unchanged. The increased A_{2B} receptor expression was localized to pulmonary blood vessels.[212] Hypoxia-induced upregulation of A_{2B} receptors has been reported in human tumor cells,[190] rat hippocampus,[213] and human dermal microvascular endothelial cells.[24] Depending on the cell type studied, adenosine has been shown to upregulate VEGF under normoxic or hypoxic conditions. Some studies have suggested that this effect is more important in normoxia,[184] whereas others have shown that the effects of hypoxia and adenosine are additive.[185] However, even in cells lacking regulation of VEGF by adenosine under normoxic conditions, adenosine can upregulate VEGF under hypoxic conditions.[21] For example, in human umbilical vein endothelial cells expressing predominantly A_{2A} receptors, adenosine is unable to stimulate VEGF production under normoxic conditions.[10] Hypoxia decreased the expression of A_{2A} and increased the expression of A_{2B} adenosine receptors; with this predominance of A_{2B} receptors, adenosine was able to upregulate VEGF.[21] Even if endothelial cells produce relatively small quantities of VEGF in response to hypoxia or A_{2B} receptor stimulation, it is possible that modulation of VEGF release through these mechanisms provides an autocrine pathway regulating endothelial cell growth. Adenosine released during hypoxia can also act on cells located outside the vasculature to release VEGF, establishing a gradient that recruits new blood vessel formation toward the hypoxic focus. In this regard, adenosine A_{2B} receptors were also shown to gain prominence in nonvascular cells under hypoxic

conditions. For example, in human bronchial smooth muscle cells, adenosine does not stimulate VEGF secretion under normoxic conditions, but hypoxia increases expression of A_{2B} receptors, which are then able to stimulate VEGF release.[21] Thus, A_{2B} receptors can participate in autocrine and paracrine regulation of endothelial growth, particularly during hypoxia.

8.8 CONCLUDING REMARKS

The function of adenosine in endothelial cells generally fits the concept of the retaliatory metabolite proposed by Berne et al.[214,215] (Figure 8.1). In situations in which oxygen supply is decreased or energy consumption is increased, adenosine is released into the extracellular space and signals to restore the balance between energy supply and demand. In many tissues including brain, heart, and skeletal muscle, adenosine acutely increases blood flow by causing vasodilation. In addition to the immediate actions of adenosine originally proposed by Berne et al., it is now evident that adenosine can also modulate long-term blood supply by inducing angiogenesis. Thus, adenosine serves as a feedback signal to maintain tissue oxygenation within a normal range. Adenosine also plays a feedback role during platelet activation, in which adenosine generated from activated platelets promotes the anticoagulation properties of endothelium. Similarly, adenosine produced from the release of ATP and AMP from neutrophils during their migration through the vessel wall will inhibit endothelial permeability and downregulate the expression of endothelial adhesion molecules responsible for the attachment of inflammatory cells. Adenosine's effects on endothelium could be beneficial if it promotes cell and tissue survival, or may be detrimental if it leads, for example, to pathologic angiogenesis or exacerbation of pulmonary inflammation.

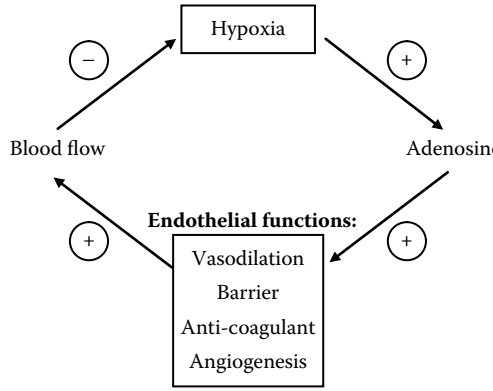

FIGURE 8.1 Adenosine-dependent regulation of endothelial functions generally fits the concept of a retaliatory metabolite.

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9 Adenosine A_{2B} Receptor in the Intestinal Epithelia

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CONTENTS

9.1 INTRODUCTION

In this chapter, we discuss the current knowledge on the intestinal A_{2B} receptor in terms of its expression, signaling, desensitization, function, and its potential role in colitis. There are a limited number of studies on the intestinal A_{2B} receptor, largely owing to problems related to generating an antibody to the receptor and the availability of specific agonists/antagonists. However, over the past 5 to 10 yr, using improved tools to study the A_{2B} receptor, we have learned a great deal, which we present in the following text.

9.2 ADENOSINE IN THE INTESTINE

Adenosine is a ubiquitous, endogenous nucleoside that has been established as an important modulator of intracellular signaling. Adenosine modulates various physiological processes such as muscle tone, neuronal firing, immune function, and

secretion of various hormones and cytokines.[1–3] In addition to its role in the regulation of these physiological processes, adenosine is released during inflammatory conditions and acts as an autocrine or paracrine factor with diverse effects on a variety of organ systems, including the cardiovascular, nervous, urogenital, respiratory, and digestive systems.[1,3–7]

In the intestine, adenosine is generated during inflammation by direct neutrophil–epithelial interaction in the intestinal lumen. Neutrophil transmigration into the intestinal lumen to form crypt abscesses is the pathologic hallmark of the active phase of many intestinal disorders such as acute infectious diarrhea or inflammatory bowel disease. The classic appearance of acute intestinal inflammation is the "crypt abscess" (Figure 9.1), a lesion characterized by marked migration of neutrophils across the crypt epithelium, with the subsequent collection of aggregated neutrophils in the crypt lumen. The term *active inflammation* refers to the presence of polymorphonuclear leukocytes (PMNs) or neutrophils. When present extravascularly, neutrophils almost invariably infiltrate the epithelial lining of the intestine. Upon stimulation with activating factors such as those normally present in the intestinal lumen, PMNs release 5′ AMP in a regulated fashion.[7,8] This classic intracellular metabolite interfaces, in a paracrine fashion, with the apical membrane of crypt cells.[7] There, it is converted by an epithelial ectoenzyme, 5′-ectonucleotidase (CD73), to adenosine, a bioactive molecule that activates apical adenosine receptors of the A_{2B} subtype. 5′-ectonucleotidase is apically polarized in native intestinal crypts[9] and 5′ AMP has been shown to stimulate freshly isolated crypt cells to secrete chloride.[9] Consistent with the *in vitro* studies, adenosine is indeed present in the luminal fluid of patients with active intestinal inflammation (Figure 9.2). Although epithelial–neutrophil interaction is an important source of adenosine during inflammation, adenosine may also be released from cells in response to metabolic stress[10] or from the sympathetic nervous system.[11]

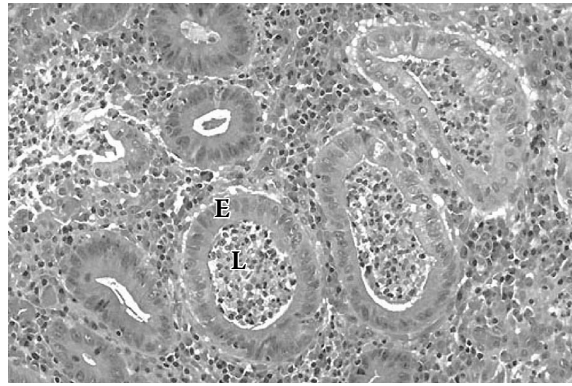

FIGURE 9.1 Light micrograph of intestinal epithelial crypt abscess in a patient with active inflammatory bowel disease. Numerous neutrophils have transmigrated across the crypt epithelium (E) and have collected within the crypt lumen (L).

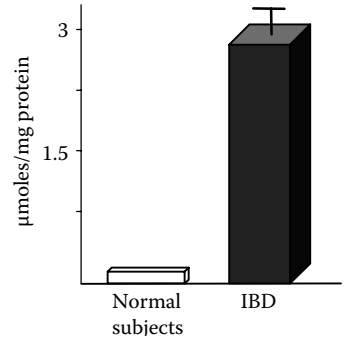

FIGURE 9.2 Adenosine levels are increased in the intestinal lumen during active inflammation. Adenosine was measured in the luminal lavage fluid from healthy subjects (n = 8) and patients with active inflammatory bowel disease (IBD) (n = 8) during colonoscopy.

9.3 DISTRIBUTION OF A_{2B} RECEPTORS IN THE INTESTINE

The generation of cDNA for A_{2B} receptors has facilitated the identification of the tissue distribution of this receptor subtype. The A_{2B} receptors show a ubiquitous distribution; the highest levels are present in the colon and bladder, followed by blood vessels, lung, eye, and mast cells.[12] In humans, the A_{2B} receptor has the highest expression in the colon and urinary bladder. Analysis of the mRNA expression of A_{2B} receptor along the longitudinal axis of the human gastrointestinal tract has shown that the A_{2B} receptor has the highest expression in the cecum and right colon, and is also detected in the esophagus, stomach, and jejunum but is absent in the ileum. In addition to the mucosa, the A_{2B} receptor is expressed in the glial cells and myenteric plexus of the jejunum, where it is thought to play a role in the motility of the gastrointestinal tract.[13] Interestingly, studies using degenerate primers to the conserved regions of the adenosine receptor and Northern blot analysis of mRNA from human colon have shown that the A_{2B} receptor is the only adenosine receptor present in the human colonic epithelia.[9,14] As in the native human colon, in a model intestinal epithelial cell line, T84 cells, the A_{2B} receptor is the only adenosine receptor.[14,15] Confocal imaging performed on frozen sections of the human colon showed that the A_{2B} receptor is expressed at both apical and basolateral membranes of epithelial cells. However, the A_{2B} receptor expression is at least 10-fold higher at the basolateral membrane compared to the apical membrane. As in human colonic epithelia, in both T84 cells and the stably transfected Caco2-BBE cell line, the A_{2B} receptor was predominantly at the basolateral membrane.[15] Interestingly, A_{2B} receptor expression is upregulated in human colitis as well as in murine models of colitis.[15a]

9.4 SIGNALING AND TRAFFICKING OF THE A_{2B} RECEPTOR IN THE INTESTINE

In the intestinal epithelial cells, the A_{2B} receptor positively couples to $G\alpha s$ and activates adenylate cyclase. Apical or basolateral stimulation of the A_{2B} receptor induces an increase in intracellular cyclic AMP (cAMP). Adenosine mediates

increased expression of genes such as IL-6 and fibronectin[16,17] through the activation of the cAMP response element (CRE)-binding protein (CREB), which belongs to a large superfamily of immediate early transcription factors.[18,19] The response begins with the cAMP-mediated dissociation of the inactive tetrameric protein kinase A (PKA) complex into active catalytic subunits and regulatory units. The catalytic subunits then migrate into the nucleus, where they phosphorylate and thus activate transcriptional activators, including CREB. This then interacts as a dimer with CRE and other regulatory sequences. Unlike in other tissues, adenosine does not activate G_q,[20,21] and cAMP appears to be the only signaling pathway triggered by A_{2B} receptors in the intestinal epithelial cells.[14]

In parallel with the distribution of the A_{2B} receptor, cAMP generated by the stimulation of the basolateral receptor is at least ten fold higher compared to that by apical stimulation. However, the subsequent signaling pathway, such as the activation of CREB and PKA, and the functional consequences (such as chloride, fibronectin and IL-6 secretion) of apical or basolateral A_{2B} receptor stimulation are superimposable.[14] This led us to hypothesize that the A_{2B} receptor and its signaling complex, including adenylate cyclase and protein kinase A, are compartmentalized in microdomains at the apical membrane. Such compartmentalization of signaling networks in a multiprotein complex results in the stabilization of constituent proteins at the cell surface, leading to enhanced efficiency of signaling.[22] One mechanism for establishing multiple protein complexes is via protein–protein interaction with submembrane scaffolding proteins,[23] which are now known as *PDZ domain proteins*. The name has been derived from their first three proteins: PSD-95/DLG/Z0-1. PSD-95/SAP-90 is a postsynaptic density protein, DLG is a *Drosophila* septate junction protein Disc-large, and Z0-1 is an epithelial tight junction protein zonula-occludens. PDZ domain proteins interact on the one hand with the membrane receptor and on the other hand with cytoskeletal linker proteins belonging to the family of ezrin-radixin-moesin (ERM) proteins. The cytoskeletal linker proteins can function as signaling molecules, or anchor signaling molecules such as PKA, in addition to their cytoskeletal association. Thus, the PDZ domain proteins localized to the membrane–cytoskeletal interface have emerged as important organizing centers for regulatory complexes, and these scaffold-based regulatory proteins are often polarized to specific sites in polarized epithelial cells.[22,24] Our studies have confirmed that, upon agonist stimulation, the A_{2B} receptor associates with ezrin (a cytoskeletal linker protein), PKA and the PDZ domain protein, and sodium hydrogen exchange regulatory factor-2 (NHERF-2/E3KARP; see Reference 15) to form a multiprotein signaling complex (Figure 9.3). Such multiprotein complexes of the A_{2B} receptor and its signaling molecules have also been reported in the lung.[25]

Typically, PDZ domain proteins such as NHERF-2 interact with the PDZ consensus sequence (D/E) (T/S) XV/L, where X represents any amino acid present in the C-terminus of the target membrane protein. Using computer-based homology modeling, it was found that the peptide sequence (**QRTEL**) that corresponds to the third intracellular loop of the A_{2B} receptor interacts with the PDZ-1 domain of NHERF-2. The docked peptide sequence makes several stabilizing interactions, which include van der Waals and hydrogen bonds. Further, the terminal ^{218}Leu residue of the A_{2B} receptor plays an important role in this interaction by inserting

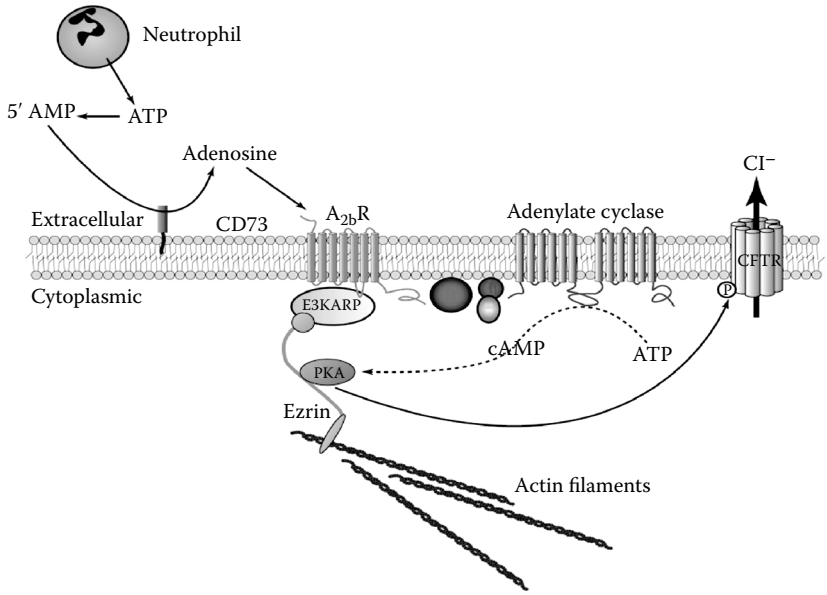

FIGURE 9.3 Schematic representation of A2B trafficking and its interaction with E3KARP. The figure shows that neutrophils release 5′-AMP, which is then converted into adenosine by epithelial ectonucleotidase (CD73). The adenosine then interacts with the A_{2B} receptor, a G-protein-coupled receptor, is coupled to $G\alpha_s$, and increases cAMP. Upon stimulation, the receptor is recruited to the apical plasma membrane via a SNARE-mediated mechanism involving VAMP-2 and SNAP-23. In the membrane, the A_{2B} receptor exists in a multiprotein complex with E3KARP, which in turn is associated with the cytoskeletal linker, ezrin. Ezrin on one hand interacts with the actin cytoskeleton and on the other hand acts as a protein kinase A anchoring protein. Protein kinase A is activated by A_{2B}-receptor-mediated increase in cAMP. PKA phosphorylates CFTR-activating chloride secretion.

itself into a hydrophobic pocket of PDZ-1, and mutation of this residue was predicted to abolish the interaction with NHERF-2. Consistent with the modeling data, cotransfection studies with FLAG-NHERF and the A_{2B} receptor, with point mutation of the terminal ^{218}Leu of the PDZ-consensus sequence to Ala, abolished the interaction of the A_{2B} receptor with NHERF-2.[25a] In addition, adenosine-induced cAMP was inhibited in cells cotransfected with NHERF-2 and the mutated A_{2B} receptor. Thus, the interaction of the third intracellular loop of the A_{2B} receptor with NHERF-2 is required for A_{2B} receptor signaling. The potential G-protein-binding site is also located in the third intracellular loop, and hence further studies are needed to clarify the effects of NHERF-2 binding and G-protein binding to the third intracellular loop.

A rather surprising finding in the trafficking studies of the intestinal epithelial A_{2B} receptor is that the greater part of the A_{2B} receptor is intracellular at rest. This was observed both in the native human colonic tissue[15a] as well as in the intestinal cell lines expressing endogenous A_{2B} receptor or intestinal cell lines transfected with A_{2B} receptor.[15] However, the A_{2B} receptor is recruited to the membrane upon agonist stimulation.

Interestingly, the recruitment of the A_{2B} receptor occurs in a polarized fashion to the apical membrane, and involves vesicle-mediated trafficking as described in the following text.[26] The apical membrane recruitment of the A_{2B} receptor may be relevant to the polarized secretion of IL-6, fibronectin, and chloride.

It is known that the apical sorting of proteins is mediated through the trans–golgi-network (TGN), a mechanism specific to polarized epithelial cells, as apical proteins are generally found only in polarized epithelial cells.[27] On the other hand, basolateral proteins are constitutive to all cell types and hence sorted by the default mechanism. Apical and basal-lateral membrane proteins that have been sorted into distinct subdomains of the TGN are packaged into transport vesicles. Delivery of transport vesicles from the TGN to different plasma membrane domains has a remarkably high fidelity. Such fidelity could be based on vesicle targeting along the cytoskeleton, and/or by specifying vesicle docking and fusion with the correct membrane domain.[28] Mechanisms that specify vesicle docking and fusion with the apical and basal-lateral membrane follow soluble *N*-ethylmaleimide (NEM)-sensitive factor attachment protein receptor (SNARE) hypothesis.[28] According to the hypothesis, correct pairing of addressing proteins on the transport vesicle (termed *v-SNARES*) with cognate receptors on the target membrane (termed *t-SNARES*) determines the specificity of vesicle docking and fusion. v-SNARE and t-SNARE interaction directs the vesicles to the correct membrane, with subsequent dissociation of the SNARE complex by ATPase activity of soluble NEM-sensitive factor (NSF) during membrane fusion. v-SNAREs are members of the synaptobrevin or vesicle-associated membrane protein (VAMP) family. t-SNAREs are comprised of syntaxins and soluble NEM-sensitive factor attachment protein (SNAP). Our studies show that vesicular trafficking involved in the recruitment of the A_{2B} receptor to the apical membrane is mediated by various SNARE proteins.

Upon agonist stimulation, the A_{2B} receptor associates with VAMP-2, a v-SNARE expressed in vesicles, and SNAP-23, a t-SNARE expressed in the apical plasma membrane, forming a molecular complex. This complex dissociates within 10 min after agonist stimulation. Together, these data suggest that VAMP-2/A_{2B}–receptor-containing vesicles dock with SNAP-23 at the apical plasma membrane.[26] The stimulus that initiates membrane recruitment of the A_{2B} receptor after its binding to the agonist is not yet known. Our data show that c-AMP is required for the A_{2B} receptor's trafficking to the apical membrane. One possibility is that the cAMP generated by the stimulation of existing membrane receptors initiates recruitment, leading to the generation of more cAMP and recruitment of additional receptors. Consistent with this notion is the data that show cAMP may modulate sialylation of proteins and vesicles budding from the TGN targeting them to the apical membrane.[29] These authors suggest that cAMP may direct secretion of proteins through the cystic fibrosis conductance regulator (CFTR) located at the apical membrane.

9.5 DESENSITIZATION OF THE A_{2B} RECEPTOR

A characteristic feature of the G-protein-coupled receptor is its ability to undergo desensitization upon prolonged exposure to agonist. As expected, adenosine induces desensitization of the A_{2B} receptor. Interestingly, the apical and basolateral A_{2B}

receptors differentially desensitize, based on the membrane domain on which they are expressed. *N*-ethyl carbamido adenosine (NECA), a nonmetabolizable analog of adenosine, added to either the apical or basolateral compartments rapidly desensitizes receptors on their respective domains. Whereas basolateral NECA desensitizes apical as well as basolateral receptors, apical NECA has no effect on cAMP response of basolateral receptor stimulation, indicating that cross-desensitization displays strict polarity. Moreover, the desensitization of the apical receptor by basolateral NECA is complete, delayed (desensitization begins around 3 h), and resensitization is incomplete even 6 h after NECA washout. Desensitization of the A_{2B} receptor does not affect short-circuit current (I_{sc}) induced by other protein-kinase-A-dependent chloride secretogogues such as vasoactive intestinal peptide, forskolin (a direct activator of adenylate cyclase), and cholera toxin, suggesting that the basic ability of cells to signal to chloride channels via cAMP is not affected by desensitization. Thus, the A_{2B} receptor exemplifies a novel concept that agonist exposure on one domain can result in desensitization of receptors on the opposite one and cross-domain desensitization can display strict polarity.[30]

9.6 BIOLOGICAL EFFECTS MEDIATED BY THE INTESTINAL A_{2B} RECEPTOR

9.6.1 Adenosine-Mediated Vectorial Chloride Secretion

It has long been known that adenosine regulates ion transport in a variety of epithelia.[31–33] In the colonic epithelium, the A_{2B} receptor mediates electrogenic chloride secretion through the activation of apical cystic fibrosis conductance regulator (CFTR).[14,34–37] Further, studies using human colonic biopsies have shown that adenosine (acting through A_2 receptors) mediates chloride secretion stimulated by neurotensin.[38] Neurotensin is a neuropeptide produced by the enteric neurons and is thought to be an important contributor to the ion secretion that accompanies inflammation.[39] In addition to the A_{2B} receptor, recent studies have shown that the A_1 receptor is involved in ion secretion in the jejunum.[40] In the colon, the chloride secretory pathway results in movement of isotonic fluid into the lumen, a process that naturally serves to hydrate the mucosal surface, thereby protecting the intestine by preventing the translocation of bacteria, bacterial products, and antigens to lamina propria.[41,42] Regulation of this secretory mucosal flush often parallels active inflammatory responses elicited by luminal pathogens. By reducing the duration of colonization by these pathogens, it serves as a crude form of mucosal defense.[43,44] However, upregulation of ion secretion during inflammation produces secretory diarrhea.[45, 46]

9.6.2 Adenosine-Induced Polarized Secretion of IL-6

Depending on the organ, the A_{2B} receptor activates pro- or anti-inflammatory pathways. For example, in joints and the cardiovascular system, the A_{2B} receptor is anti-inflammatory, whereas in lungs the A_{2B} receptor is a potent proinflammatory mediator. Indeed, A_{2B} receptor antagonists are becoming potential drugs for reactive

airway disease.[47] Adenosine induces IL-10 and suppresses IL-12 secretion in monocytes,[48] induces IL-8 release by mast cells,[4] and stimulates IL-6 secretion in astrocytes.[49] In the intestine, stimulation of A_{2B} receptors with either apical or basolateral adenosine causes a ninefold increase in IL-6 secretion, which is polarized to the apical (luminal) compartment.[16] IL-6 is an important proinflammatory cytokine that is consistently present in high levels in the serum and tissue of patients with active inflammatory bowel disease, and one of the sources for the IL-6 is the lamina propria cells, including macrophages and monocytes.[50–52] Interestingly, as in the epithelial cell lines, IL-6 is found in higher levels in the luminal fluid of patients with active inflammatory bowel disease compared to control patients (Figure 9.4). IL-6 secretion induced by apical or basolateral adenosine is abolished when cells are exposed to 8-(*p*-sulfophenyl theophylline) (8-SPT), an adenosine-receptor antagonist. Further, IL-6 secretion by adenosine is transcriptionally mediated by activating transcription factor (ATF) and CREB elements.[16]

The finding that adenosine induces IL-6 secretion to the luminal compartment is novel in that all known cytokines are released basolaterally, where they participate in immune regulation. Thus, the apical secretion of IL-6 may also contribute to immune regulation at the mucosal surface. Indeed, apically secreted IL-6 increases intracellular [Ca^{2++}] in neutrophils, a classic early signal in neutrophil activation. Such neutrophil signaling has been shown to induce elastase release, platelet activating factor (PAF) production, and production of oxygen-free radicals by neutrophils.[53,54] Other studies have shown that IL-6 triggered activation of neutrophil-mediated bacterial killing.[55,56] Thus, the activation of A_{2B} receptors may provide epithelial-derived paracrine signals to neutrophils positioned apically after transmigration. Paracrine signaling of luminal immune cells provides an additional means of regulation of inflammatory responses by intestinal epithelia.

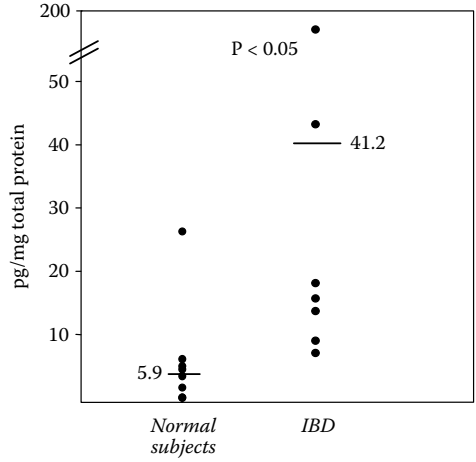

FIGURE 9.4 IL-6 is elevated in the luminal fluid of patients with IBD. IL-6 was measured in the luminal lavage fluid from healthy subjects (n = 8) and patients with active inflammatory bowel disease (n = 8) during colonoscopy.

9.6.3 Fibronectin Secretion

Cellular fibronectins are produced by a wide variety of cell types, including epithelial cells, which secrete them and often organize them into extensive extracellular matrices at their basal surfaces. Adenosine, whose levels are upregulated during inflammation, modulates fibronectin synthesis. As with IL-6 secretion, adenosine added to either the apical or basolateral surface of the colonic epithelial cells leads to the accumulation of fibronectin in a time- and dose-dependent manner to the apical compartment. Confocal microscopy demonstrates that fibronectin localizes to the apical domain of model intestinal epithelial cells stimulated with apical or basolateral adenosine. Moreover, adenosine increases fibronectin promoter activity, indicating that fibronectin induction is, in part, transcriptionally regulated.

The secretion of fibronectin, a matrix protein, to the apical surface is intriguing. Interestingly, adenosine-induced fibronectin significantly enhances the adherence and invasion of *Salmonella typhimurium* to cultured epithelial cells as well as consequent IL-8 secretion.[17] These data suggest that adenosine-induced fibronectin may serve as a critical host factor that modulates adherence and invasion of bacteria, thus playing a key role in mucosal immune responses during inflammation.[17] Taken together, adenosine-induced vectorial secretion of chloride, IL-6, and fibronectin may ultimately modulate bacterial–epithelial interaction and host immune responses, which are intimately involved in the pathogenesis of inflammatory bowel disease.[57,58]

9.7 Regulation of the Intestinal A_{2B} Receptor

Regulation of the A_{2B} receptor in intestine remains largely unknown. We have recently shown that IFN-γ and TNF-α affect the expression and function of A_{2B} receptors. These cytokines are highly upregulated during intestinal inflammation and play a central role in both barrier dysfunction as well as inflammatory response.

9.7.1 Effect of IFN-γ on the A_{2B} Receptor

Short-term exposure of intestinal epithelial cells to IFN-γ inhibits adenosine-induced short-circuit current (ion secretion) without affecting the transepithelial resistance (a measure of barrier function). Although IFN-γ does not directly affect protein expression and membrane recruitment of the A_{2B} receptor, it inhibits adenosine-induced cAMP levels and its downstream signaling pathway such as phosphorylation of cAMP response element-binding protein (CREB) and protein kinase A (PKA) activity.[59] Studies on the mechanism of IFN-γ-mediated inhibition of adenosine-induced functional responses have demonstrated that IFN-γ inhibits cAMP levels by inhibiting the expression of adenylate cyclase isoforms 5 and 6.[59b] Thus, IFN-γ downregulates adenosine-mediated signaling through the direct inhibition of adenylate cyclase expression and by affecting global cAMP-mediated responses in the intestinal epithelia, thereby decreasing secretory responses, which may consequently aggravate inflammatory processes. Therefore, it is possible that the early inhibitory effect of IFN-γ on adenylate cyclase may further aggravate the proinflammatory process in acute or chronic colitis.

9.7.2 Effect of TNF-α on the A_{2B} Receptor

TNF-α levels are significantly increased in intestinal mucosa, serum, and stools in Crohn's disease and, to a lesser extent in ulcerative colitis.[60–64] TNF-α is one of the most important proinflammatory cytokines. It mediates inflammatory and immune responses in IBD. Anti-TNF-α strategies are an effective treatment for this disease.[65,66] TNF-α is thought to play a central role in the pathogenesis of inflammation and diarrhea associated with these diseases.[67] We have recently shown that TNF-α is an important regulator of the A_{2B} receptor.[15a] Unlike INF-γ, TNF-α directly affects A_{2B} receptors by upregulating A_{2B} receptor mRNA, protein expression, and its membrane recruitment. Further, TNF-α potentiates A_{2B}-receptor-mediated cAMP and its downstream signaling, leading to an increased secretory response in a dose- and time-dependent manner. TNF-α also potentiates adenosine-induced fibronectin secretion. Interestingly, TNF-α alone had no effect on cAMP response, short-circuit current, or fibronectin secretion; nor did TNF-α potentiate forskolin-induced cAMP or short-circuit current. This suggests that during an inflammatory state in which TNF-α is abundantly secreted, mostly by monocytes and macrophages, the subsequent upregulation of A_{2B} receptor, along with the endogenous release of adenosine, constitutes a previously unexplored pathway of the inflammatory response. In addition, given the pleiotropic effect of adenosine on intestinal function, the upregulation of A_{2B} receptor by TNF-α constitutes a novel downstream effector pathway for TNF-α.

9.8 A_{2B} RECEPTOR EXPRESSION DURING COLITIS

A_{2B} receptors are upregulated during colitis in both murine models of colitis as well as during an active flare-up of inflammatory bowel disease in humans.[15a] Two models of murine colitis were used, namely, dextran-sodium-sulfate-induced colitis and IL-10 knock-out mice, which develop spontaneous colitis. In both these models, A_{2B} receptor expression was upregulated, as evidenced by Western blot and immunohistochemistry. Upregulation of the A_{2B} receptor in human colitis was confirmed by confocal microscopic imaging of biopsies obtained from patients with active Crohn's disease or ulcerative colitis. Patients undergoing screening colonoscopy with normal colon served as controls. This suggests that A_{2B} receptor antagonists may serve as potential therapeutic targets for treating intestinal inflammation. Such studies are now feasible with the development of highly specific inhibitors of A_{2B} receptors (Linden J, Personal communication),[68–71] some of which can be administered orally on luminal A_{2B} receptors.

9.9 SUMMARY

In summary, the A_{2B} receptor is the predominant adenosine receptor in human colonic epithelial cells. Here, the A_{2B} receptor is intracellular at rest and is recruited to the membrane upon agonist stimulation. The membrane recruitment of the A_{2B} receptor involves the SNARE proteins, VAMP-2, and SNAP-23. In the membrane, the A_{2B} receptor exists in a multiprotein complex with the PDZ-domain membrane anchor NHERF-2, cytoskeletal linker ezrin, and PKA. The A_{2B} receptor mediates chloride,

IL-6, and fibronectin secretion, all of which are polarized to the apical or luminal side. Such apical polarization of adenosine-induced secretory response mediates autocrine and paracrine signals to luminal immune cells, bacteria, and epithelial cells. Thus, adenosine may mediate bacterial–epithelial interaction and immune responses at the mucosal surface. Both adenosine and the A_{2B} receptor are upregulated during intestinal inflammation, and A_{2B} receptor expression, or signaling, and function are regulated by the inflammatory cytokines, INF-γ and TNF-α. In conclusion, the study of the intestinal A_{2B} receptor, in regard to both structural and biochemical aspects as well as biological aspects such as those found in colitis, has an exciting future with the development of highly specific antagonists and, possibly, agonists.

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10 Adenosine, Adenosine Receptors, and the Regulation of Glial Cells in Neuronal Damage

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CONTENTS

10.1 ADENOSINE'S ROLE IN ISCHEMIC BRAIN DAMAGE: REEVALUATION OF THE "NEUROCENTRIC" VIEW

Even though it would be interesting to cover many of the effects of adenosine on glial cells, we will, for reasons of space, focus on actions that are relevant in the context of neuronal damage following ischemia and related events. It is becoming increasingly clear that glial cells are very important in the events that determine whether an insult to the central nervous system will lead to major lasting damage or not. If glia play a prominent role in ischemia, and adenosine plays a role in modulating the outcome after ischemia, then at least some of its actions are likely to take place in glial cells.

Two recent studies using adenosine receptor knockout mice have highlighted a need to change the current neurocentric emphasis when examining the roles of adenosine in ischemia. Adenosine A_1 receptors have been assumed to play a major role to protect against postischemic damage.[1,2] However, damage after middle cerebral artery (MCA) occlusion was not significantly altered by a targeted deletion of

the adenosine A_1 receptor.[3] Nevertheless, the glutamatergic neurotransmission in hypoxia and simulated ischemia was markedly affected in these mice. Therefore, the results imply that glutamatergic neurotransmission is not of central importance in damage caused by this type of ischemia.

In earlier studies, Jiang-Fan Chen, Michael Schwarzschild, and their colleagues showed that mice lacking adenosine A_{2A} receptors or those given an adenosine A_{2A} receptor antagonist suffered less damage in, for example, hippocampus and cortex.[4] Because these brain regions do not have high expression of adenosine A_{2A} receptors in neurons, the results were somewhat difficult to interpret. Recently, the same group showed that the protective effect of adenosine A_{2A} receptor elimination was mimicked if only the bone-marrow-derived cells lacked adenosine A_{2A} receptors.[5] Furthermore, mice lacking adenosine A_{2A} receptors in all cells except those derived from bone marrow exhibited the same level of brain damage as in wild-type mice. The major brain-related consequence of the adenosine A_{2A} receptor deletion in bone-marrow-derived cells was that glial expression of cytokines was altered. This result clearly shows that communication between blood and brain parenchyma is of critical importance in ischemia. Glial cells are important in this communication.

10.2 GLIAL CELLS AND MODULATION BY EXTRACELLULAR ADENOSINE

10.2.1 Astrocytes

Astrocytes are endowed with all the known subtypes of adenosine receptors, and they control carbohydrate metabolism, astrogliosis, and the release of neuroactive substances.[6] However, not all effects are receptor mediated. For example, adenosine- and inosine-mediated reduction of cell death in glucose-deprived astrocyte cultures[7] appears to be due to intracellular formation of ribose-1-phosphate, which is able to fuel intracellular ATP production. Even in those cases in which adenosine's effects are receptor mediated, the use of pharmacological agents with unclear specificity precludes the establishment of the type of receptor involved. Thus, a great deal remains to be understood about the role of adenosine receptors in astrocyte functions.

It is known that changes in astrocytes are among the earliest events following ischemia.[8] Astrocytes have numerous very fine processes (so fine that they can be difficult to detect) and have been estimated to contact more than 100,000 synapses. Because astrocytes also form contacts with one another via gap junctions, they can potentially react to a huge number of synaptic events.[8] In addition, astrocytic end feet surround capillaries and are thus indirectly in contact with the blood compartment and the rest of the body.

Swelling in the perivascular part of astrocytes occurs early in ischemia. This swelling is enhanced by adenosine.[9,10] It is still unclear which receptors are involved and whether this effect of adenosine is beneficial or detrimental. Swelling and other local processes after ischemia may be very important as they could compromise the blood–brain barrier (BBB), and failure of the BBB could lead to vascular edema. Several mechanisms probably contribute to astrocytic swelling, and swelling in turn affects several processes.[11] One is activation of the volume-regulated anion channel,

which is further stimulated by ATP and peroxynitrite.[12] This can contribute to glutamate release (but see the following text also). In fact, blockade of these channels by tamoxifen leads to a major decrease in glutamate levels after ischemia.[12]

Later on in ischemia, there is reactive astrogliosis, the significance of which is unclear. This process has been reported to be activated by adenosine acting at an adenosine A_2 receptor.[13] Besides adenosine A_2 receptors, adenosine A_3 receptors may mediate astrogliosis,[14] whereas adenosine A_1 receptors inhibit reactive astrogliosis.[15] Decreasing reactive gliosis by means of genetic deletion of the intermediary filaments, glial fibrillary acidic protein (GFAP), and/or vimentin, has been shown to reduce wound healing in the central nervous system, as well as increase infarct size after MCA occlusion.[16] However, one can probably not conclude that adenosine's effects on reactive gliosis are necessarily beneficial. Indeed, it has been argued that reactive glial cells are important in maintaining the K^+ level close to normal over the longer term.[17]

Glutamate is removed from the extracellular space by glutamate transporters;[18] astrocytes possess two forms of these transporters. Knocking out the predominant form of glial glutamate transporter in hippocampus does not affect glutamate release in early ischemia.[19] This is compatible with the view that in the first phase of ischemia, glutamate is released by reversal of neuronal glutamate transporters but is removed by glial transporters. Thus, in this phase, glial glutamate transport would protect against putative excitotoxic damage. However, in later stages of ischemia, release of glutamate from glial cells (by reversal of their transporters, too) may contribute to excitotoxic damage. As noted earlier, glia also contribute to glutamate release by the volume-regulated anion channels. Adenosine in the physiological concentration range (10 n*M*) inhibits glutamate uptake via GLT-1 transporters through activation of adenosine A_{2A} receptors *in vitro*.[20] It also stimulates glutamate release from astrocytes via adenosine A_{2A} receptors through a PKA-dependent pathway, independently of GLT-1. Thus, adenosine actions on glial cells, rather than neurons, might explain adenosine-A_{2A}-receptor-mediated potentiation of hippocampal glutamatergic transmission.[20–22]

The removal of glutamate released from neurons places an increased energetic demand on astrocytes.[22] This is compounded by the astrocytes' need to restore the K^+ balance. Even though astrocytes may be able to rely more on the anaerobic metabolism than neurons do, they still rely predominantly on oxidative metabolism to generate the necessary ATP.[22] Thus, the increased need for metabolic energy in astrocytes can exacerbate a precarious situation in the penumbral region of a focal ischemia.

Astrocytes also play an important role by breaking down glycogen and providing glucose for neurons.[23,24] Also, in numerous other ways, astrocytes can influence neuronal metabolism. All of these metabolic pathways may be influenced by adenosine, particularly via adenosine A_2 receptors (both A_{2A} and A_{2B}).

Of potentially greater importance is the ability of astrocytes to produce a multitude of factors that can influence neuronal survival.[24] They include cytokines and chemokines, such as interleukins and tumor necrosis factor (TNF)-α as well as growth factors such as brain-derived neurotrophic factor (BDNF) and neurotrophins, glia-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and erythropoietin.

Other important factors include nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS), metalloproteinases, and other proteinases as well as tissue plasminogen activator (tPA). Many of these are formed by and act upon microglial cells, thus establishing a complex paracrine network. There is currently great interest in investigating a possible role of adenosine in the regulation of these factors. A recent study[25] has demonstrated that stimulation of primary mouse astrocytes with the somewhat specific adenosine A_3 receptor agonist 2-chloro-*N*^{6}-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (Cl-IB-MECA) induces the release of the chemokine CCL2 through a G_i-protein-independent pathway. Thus, adenosine acting on the adenosine A_3 receptor appears to modulate the level of a chemokine that can exert both neuroprotective and detrimental effects. Adenosine is able to enhance the production of other important neuroprotective substances, such as NGF, transforming growth factor (TGF)-β1, and the calcium-binding protein S-100β, in cultured astrocytes via activation of adenosine A_1 receptors.[6,26,27] The increase in interleukin-6 (IL6 or CXCL6) synthesis and secretion induced by adenosine, on the other hand, is inhibited by the nonselective adenosine receptor antagonist 8-(p-sulfophenyl)theophylline, but not by specific adenosine A_1 or A_{2A} antagonists.[26] Furthermore, signaling via adenosine A_{2B} receptors is influenced by interleukins in parallel with the generation of NGF.[26]

10.2.2 Oligodendrocytes

Perinatal hypoxia is prevalent and can lead to permanent damage, often related to white matter loss. In rodents perinatal hypoxia can lead to periventricular leukomalacia.[28] This can be mimicked by increases in adenosine brought about by genetic targeting of adenosine deaminase.[29] Furthermore, the increase in the size of the cerebral ventricles is abolished in mice lacking adenosine A_1 receptors and is smaller in mice with only one copy of the adenosine A_1 receptor gene.[29] This indicates that in this particular situation, adenosine exerts detrimental effects by influencing a glial cell or its precursor.

10.2.3 Microglia

Microglia cells are brain-specific macrophages that represent the immunocompetent cells of the central nervous systems. They are capable of macrophage-typical innate defense and adaptive immune reactions such as phagocytosis and antigen presentation.[30] Under resting conditions, these cells are assumed to play an important role in the surveillance of brain tissue.[31] Under physiological conditions, microglia cells are necessary for the maturation, support, and homeostasis of the central nervous system by providing soluble factors and growth factors. Furthermore, microglia cells continuously monitor their surroundings and are able to react rapidly to changes in the environment such as increased neuronal activity, infection, tissue damage, or hypoxia.[31,32] Imbalance in tissue homeostasis will lead to activation of microglia, resulting in their transition from a resting, ramified state into activated microglia. This activation process is accompanied by morphological changes and expression of a K^+ outward current.[32] During the activation process, microglia proliferate, migrate, and can release a broad spectrum of inflammatory (e.g., NO) and anti-inflammatory substances such as neurotrophic factors (BDNF, NGF, and NT-3).[32]

The degree of microglial activation and its impact on the surrounding cells is dependent on the intensity of the insult. Mediators of microglial activation are numerous[33] and include ions, such as increasing K^+ upon neuronal activity, compounds from pathogens, such as the bacterial cell wall compound lipopolysaccharide (LPS), factors from the complement system, immunoglobulins, chemokines and nerve growth factors, serum factors, neurotransmitters, and neuromodulators to name but a few. It has become clear that microglial activation might not always be beneficial but, depending on its intensity, can become detrimental to the surrounding cells. Although moderate microglial activation is thought to mediate tissue-protective signals, strong and persistent microglial reactions might aggravate neuronal damage.[33]

Our interest in microglia originates from the fact that their activation occurs under exactly the same conditions as those that evoke a dramatic increase of extracellular adenosine levels. General energy imbalance (the cells consume more ATP than they produce) is mirrored by increased levels of extracellular adenosine that can bind and activate its cognate receptors. Extracellular adenosine levels normally lie between 10 and 300 n*M* but can increase to 10 μM or even higher, depending on the severity of the insult. Furthermore, several lines of evidence support a causal relationship between the increase in extracellular adenosine and the modulation of the activity state and/or function of microglia cells (see also Table 10.1). First, ATP is believed to play a particularly important role in mediating rapid microglial response to local brain injury[31] such as the characteristic purinergic current response to passing astrocytic calcium waves[34] or induction of microglial migration.[31,35] Second, microglia present a particularly elaborate enzymatic machinery on their extracellular side for very rapid, specific, and efficient ATP breakdown to adenosine. This group of enzymes comprises enzymes such as nucleoside triphosphatase (NTPase), nucleoside diphosphatase (NDPase), and 5′-nucleotidase activity,[36] which successively hydrolyze ATP to ADP to AMP to adenosine. The enzymes involved in hydrolysis of ATP/ADP are mainly the ATP-preferring ecto-ATPase and the related ecto-ATP diphosphohydrolase (ecto-apyrase, CD39), which hydrolyzes both substrates equally well.[37] Microglia thus contribute to a general increase in adenosine levels in the extracellular fluid not only by releasing adenosine via an equilibrative transporter, as any cell does under stress, but also by producing adenosine through ATP breakdown. Moreover, microglia express different adenosine receptors,[38] rendering them highly responsive to adenosine in an autocrine/paracrine manner.

Apparently, adenosine receptor expression profiles—as described in the literature—depend on the nature of the microglial preparation. Functional and pharmacological data have shown that adenosine receptors are expressed on microglia cells; however, results vary concerning the expression of the different adenosine receptor subtypes (see Reference 39 and Reference 40 for examples, as well as Reference 38 for a detailed summary). We have shown by RT-PCR that the adenosine A_{2A}, A_{2B}, and A_3, but not adenosine A_1, receptors are expressed in the mouse microglia-like cell lines BV-2 and N13. In our hands, the adenosine A_1 receptor appeared to be absent from mouse primary microglia cells.[38,41] However, others could detect adenosine A_1 receptors in rat, as well as in mouse microglia.[25,42,43] It will clearly be very interesting to determine if adenosine A_1 receptor expression is dependent on the functional state of the microglial cell.

TABLE 10.1
Role of Different Microglial Adenosine Receptors

Receptor	Detected by	Functional Readout	Ligand	Reference
A_1	RT-PCR	No effect on CCL2 release	CPA	25
	Pharmacology	Microglial proliferation	NECA, CPA	52
	IHC	Upregulation of microglia in $A_1R^{-/-}$	—	43
A_{2A}	RT-PCR (upon LPS stimulation)	No effect on CCL2 release	CGS21680	25
	RT-PCR	n.d.	—	39
	Pharmacology	↑ K^+ channel (Kv1.3) mRNA	CGS21680/ZM241385	42
	RT-PCR/ELISA	↑ NGF expression/ release	NECA/ CGS21680	53
	Pharmacology	↑ COX2 expression; PGE_2 production	CGS21680/NECA/ KF17837	54
	Pharmacology	Microglial proliferation	CGS21680/CPA/NECA	52
	Pharmacology	↑ Microglial activation; OX-42 expression	DMPX	55
A_{2B}	RT-PCR	n.d.	—	25
	RT-PCR	n.d.	—	39
A_3	RT-PCR	No effect on CCL2 release	Cl-IB-MECA	25
	RT-PCR	ERK1/2 phosphorylation	Cl-IB-MECA	39
	Pharmacology	p38, ERK1/2 phosphorylation	Cl-IB-MECA	41
Unspecified	Pharmacology	Cytokine release	NECA	40
	Pharmacology	Apoptosis	2-Cl-adenosine	56

Notes: n.d.—not determined; CCL2—chemokine; COX2—cyclooxygenase 2; ERK1/2-extracellular signal-regulated kinase1/2; NGF—nerve growth factor; p38—stress-activated protein kinase p38; PGE_2—prostaglandin E_2.

Pharmacological tools: CGS21680—2-[*p*-(2-carbonyl-ethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine;Cl-IB-MECA—2-chloro-N^6-(3-iodobenzyl-*N*-methyl-5′-carbamoyladenosine); CPA—N^6-cyclopentyladenosine; DMPX—3,7-dimethyl-1-propargylxanthine; KF17837—1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine;NECA—5′-*N*-ethyl-carboxamidoadenosine; ZM241385—4-(2-[7-amino-2-[2-furyl]-[1,2,4]triazolo[2,3-*a*]{1,3,5}triazin-5-yl-amino]ethyl)phenol.

The role of adenosine A_{2A} receptors in cAMP-mediated regulation of nerve growth factor, cyclooxygenase and K^+ channel expression, and microglial proliferation has been described in fairly great detail (see Table 10.1). Expression of this adenosine receptor subtype seems to be transcriptionally upregulated in LPS-activated mouse microglia.[25] The adenosine A_{2B} receptor, on the other hand, has only been detected by RT-PCR.[25,39] We have found no published reports of the presence of functional adenosine A_{2B} receptors and their role in microglia cells. Our recent report of functional adenosine A_3 receptors in immortalized murine microglia cell lines and primary microglia cells isolated from mice is the first evidence for this receptor subtype on microglia.[39] Its presence could be confirmed by RT-PCR;[25] however, the adenosine A_3-receptor-specific agonist Cl-IB-MECA had no functional effects on the parameters measured in that study. The adenosine A_3 receptor has been notoriously difficult to detect and localize in the central nervous system[44] by either radioligand binding, *in situ* hybridization, or immunohistochemistry. The fact that the adenosine A_3 receptor is hard to detect in normal brain and that both mRNA and functional protein are detectable in N13 and primary microglia (which represent models for microglia cells with different degrees of activation*) might suggest a regulated expression of the adenosine A_3 receptor with microglia activity state. This hypothesis is, however, still under investigation.

The consequences of microglial activation appear to include both beneficial and detrimental effects on brain tissue, dependent on the severity of the insult (for a review see Reference 33). These findings parallel the variable and confusing findings concerning the A_3 receptor obtained in studies of different biological systems. Stimulation with low agonist concentrations was reported to protect cells from apoptosis,[45–47] whereas high agonist concentrations would rather induce adenosine A_3-receptor-mediated apoptosis.[45,46] Furthermore, not only the strength but also the timing of adenosine A_3 receptor stimulation has been suggested to influence tissue survival upon ischemic damage.[48] Indeed, the ischemia-induced microglial infiltration was completely abolished by adenosine A_3 receptor stimulation after injury, whereas receptor stimulation prior to the injury strongly augmented microglial presence in the injured region compared to untreated control.[49] In view of the opposing effects of adenosine A_3 receptor stimulation and its role in modulating cell/tissue fate in pathophysiological conditions,[50] it is not surprising that unconventional dose-dependent effects have been shown on the intracellular level. The adenosine A_3-receptor-mediated phosphorylation of the extracellular signal-regulated protein kinase1/2[39] in microglia-like cell lines as well as in primary microglia did not follow a sigmoid dose–effect curve as previously shown in adenosine A_3-receptor-expressing CHO cells. On the contrary, low concentrations of Cl-IB-MECA, resembling normal adenosine levels, led to an increase in ERK1/2 phosphorylation, whereas increasing concentrations of the agonist resulted in smaller or even negligible responses. Even though this phenomenon has not yet been linked to a physiological readout such as

* The normal primary cell culture represents so-called resting microglia in culture. Their activation in culture can be triggered by LPS. Microglia in culture are in the first stage of activation (big K^+ inward current) in contrast to the resting microglia in the normal healthy brain (no K^+ inward current), whereas activated microglia in culture and in brain slices show the characteristic K^+ outward current. Depending on the culture conditions and handling, microglia in culture might be activated to various degrees.

an effect on, for example, microglial activity state, it is likely to be of importance because the family of MAPK appears to be crucial for microglial regulation.[51]

An important tool for investigation of an adenosine receptor's role in the regulation of microglial function is genetically modified animals lacking the receptor. In the case of Cl-IB-MECA-induced ERK1/2 phosphorylation we could use adenosine A_3 receptor knockout animals to confirm the involvement of this specific receptor subtype.[39] In addition, we have investigated various injury models in animals lacking the gene for the adenosine A_3 receptor. However, even though a clear activation of microglia was apparent in models such as trypanosoma infection and ischemic brain injury, no difference in microglial activation, number, infiltration, or morphology were observed in the adenosine A_3 receptor knockout animals compared to wild-type littermates (unpublished results, Fredholm, Schulte, Ådén, Robertson, and Dalmau, 2004).

10.3 GENERAL CONCLUSION

The general understanding of adenosine as a neuromodulator is that it has neuroprotective effects brought about by inhibition of neuronal function through, for example, the adenosine A_1 receptor and by inhibition of excitatory transmitter release. However, glial cell reaction is clearly modulated as well and is apparently also important for the adenosine-induced neuroprotective effects. Even though the increase in extracellular adenosine parallels the activation of glial cells and even though we have evidence that glial cells react to increased adenosine levels, the causal relationship between adenosine, adenosine receptor activation, and the modulation of glial cell activity remains obscure. Further research will be required to decipher the glia-modulatory function of adenosine and its role in the effects of adenosine on tissue survival, cellular fate, and the immune system.

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11 Adenosine Receptors, Wound Healing, and Angiogenesis

Aneesh Sheth and Bruce Cronstein

CONTENTS

11.1 INTRODUCTION

Rapid and complete repair of cell and tissue injury is critical to restoration of homeostasis. The organ in which wound healing is most visible is the skin; rapid restoration of the skin's protective function maintains the integrity of the largest barrier between external pathogens and tissues. Wound healing in the skin is characterized by three nonexclusive phases: inflammation, tissue regeneration, and tissue reorganization. Inflammatory cells eliminate debris and release the signals required to promote tissue regeneration. These events occur rapidly (days to weeks), as might be expected in a process required to maintain a barrier. Tissue reorganization (scar formation) takes place over a longer period (months to years).

The finding that adenosine A_{2A} receptor agonists promote wound healing was first reported in 1997,[1] an observation suggesting that A_{2A} receptors also may play a role in normal wound healing. Adenosine and its receptors were first reported to regulate inflammation in 1983,[2] and numerous subsequent studies have demonstrated a role for all four adenosine receptors in the regulation of inflammation. Although adenosine's role in regulating vascular tone has been known since 1929,[3] it was not until 1987 that specific adenosine receptors were observed on endothelial cells,[4] and their role in promoting angiogenesis and vascular permeability was rapidly established.

Similarly, although adenosine receptors had been demonstrated on fibroblasts, their role in regulating matrix production was not established until it was found that adenosine A_{2A} receptor agonists promoted matrix production in wounds.[1]

In the skin, tissue regeneration involves reepithelialization and granulation tissue formation; granulation tissue consists of the matrix, cells secreting the matrix substance, and blood vessels of the microvasculature being regenerated. Here, we will review adenosine's role in regulating the processes so critical to wound healing and the evidence of adenosine receptors' involvement in wound healing.

11.2 ADENOSINE A_{2A} RECEPTOR AGONISTS AND WOUND HEALING

In 1997, Montesinos and colleagues reported that the application of an adenosine A_{2A} receptor agonist to full-thickness wounds increased the rate at which wounds close and heal,[1] an effect that was reversed by adenosine A_{2A}-selective antagonists. The enhanced rate of wound healing was associated with accelerated matrix and blood vessel formation in these studies. A subsequent study by Sun et al.[5] reported that an A_1 receptor agonist promotes wound healing. However, because a high concentration of the agonist was applied and receptor-selective antagonists were not studied, it is unclear whether, in fact, the A_1 receptor was involved in this process. Nonetheless, this study confirms the initial report that topical application of an adenosine receptor agonist could promote wound healing. Later studies using adenosine A_{2A} receptor knockout mice and more selective adenosine receptor agonists established that the A_{2A} was the adenosine receptor most prominently involved in promoting wound healing.[6–8] No less significant, these studies established adenosine A_{2A} receptor agonists as superior to recombinant platelet-derived growth factor (PDGF, becaplermin) in promoting wound healing. Interestingly, disorganized granulation tissue formation was noted in adenosine A_{2A} receptor knockout mice, suggesting that endogenous adenosine, acting at these receptors, plays a significant role in normal wound healing as well as in the pharmacological effects described earlier. Currently, a selective adenosine A_{2A} receptor agonist (MRE0094) is in clinical trials for the promotion of wound healing in patients with diabetic foot ulcers.

11.3 ADENOSINE-MEDIATED TRANSFORMATION OF INFLAMMATORY RESPONSE TO REGENERATION RESPONSE

Adenosine is a potent suppressor of inflammation, whether endogenously released or exogenously applied, and a number of new therapies are being developed based on this hypothesis.[9,10] The inflammatory cells most commonly observed at the site of a wound are neutrophils and macrophages, although lymphocytes are present in large numbers in chronic wounds. Activation of adenosine A_{2A} and A_3 receptors suppresses the proinflammatory functions of all of these cell types and diminishes their production of inflammatory mediators, including cytokines and leukotrienes.[9,11–36] Adenosine diminishes stimulated neutrophil production of oxidants, leukotrienes,

TNF, and other injurious and inflammatory mediators, primarily via interaction with A_{2A} receptors. Similarly, adenosine A_{2A} receptor activation diminishes production of inflammatory mediators by monocyte/macrophages. Thus, the application of an adenosine receptor agonist to the healing wound could suppress the deleterious effects of inflammatory cells recruited to eliminate cellular debris. Just as important is the potential role of inflammatory cells in stimulating regeneration of the tissue at the wound site. Leibovich and colleagues[37] have recently reported that stimulation of adenosine A_{2A} receptors on endotoxin-activated macrophages stimulates the production of the angiogenic peptide vascular endothelial growth factor (VEGF). Thus, adenosine A_{2A} receptor activation can convert a potentially destructive inflammatory response to a constructive stimulus for granulation tissue formation.

11.4 ADENOSINE RECEPTORS AND MATRIX PRODUCTION

Application of adenosine A_{2A} receptor agonists to wounds increases the rate of matrix formation as part of the overall wound healing effect. Although the effect of adenosine receptor agonists on fibroblasts, the cells that synthesize and secrete the extracellular matrix, has not been fully established, dermal fibroblasts do express messages for at least adenosine A_{2A} and A_{2B} receptors.[1] The role of these receptors in promoting tissue regeneration has not been fully established either, but evidence from a number of laboratories suggests that adenosine receptors do modulate extracellular matrix production by fibroblasts.

Adenosine A_{2B} receptors are present on fibroblasts from a variety of different tissues. However, the functional effects of these receptors vary markedly depending upon what tissue they were derived from. Synovial fibroblasts express adenosine A_{2B} receptors that when occupied diminish the release of matrix metalloproteases.[38] Similarly, A_{2B} receptors on pulmonary fibroblasts increase collagen production, diminish metalloprotease production, and promote the formation of myofibroblasts. In contrast, adenosine A_{2B} receptors diminish collagen production and proliferation of cardiac fibroblasts.[39–46] In human dermal fibroblasts, definitive studies have not been carried out, but preliminary studies indicate that adenosine A_{2B} receptors are not involved in regulation of collagen production. These studies indicate that in the skin and liver, adenosine A_{2A} receptors promote collagen synthesis and diminish matrix metalloprotease secretion.[47,48]

11.5 ADENOSINE RECEPTORS AND ANGIOGENESIS

Results of early studies indicated that adenosine A_{2A} receptor agonists promote new vessel formation in healing wounds.[1] Numerous prior studies had demonstrated the presence of adenosine A_2 receptors on vascular endothelial cells and the specific effects of these receptors on endothelial cell proliferation and function, although the specific receptors involved in stimulating these effects were not identified. Adenosine receptor agonists were shown, among other things, to stimulate endothelial cell proliferation, migration, NO production, and VEGF production, as well as to inhibit tissue factor production.[4,19,49–61]

Interestingly, as with the effect of adenosine on fibroblast function, the effects of different adenosine receptors appear to vary depending on the source of endothelial cells. Adenosine A_{2B} receptors also appear to promote angiogenesis and related processes in the retina.[52,58,59,62–66] In contrast, adenosine A_{2A} receptors stimulate new blood vessel formation in healing skin wounds[1,6–8,37,67] by increasing both endothelial and macrophage production of VEGF and inhibiting production of the antiangiogenic matrix protein thrombospondin I.

11.6 ADENOSINE RECEPTORS AND EPITHELIAL REGENERATION

The expression and regulation of dermal epithelial cell function by adenosine receptors have not been directly studied. Thus, it is not clear whether topical application of adenosine receptors to dermal wounds affects the epithelium directly or indirectly via cells of the regenerating dermis.

11.7 CONCLUSION

Topical application of adenosine A_{2A} receptor agonists to wounds in the skin increases the rate at which these wounds close, an effect currently being studied in the treatment of diabetic foot ulcers. Nearly all the cells present in the granulation tissue of healing skin ulcers are affected by adenosine receptors, and it is likely that all the functional effects of A_{2A} receptor agonists on these cells contribute to the enhanced rate of granulation tissue formation. It is interesting to note that the effects of adenosine receptors on fibroblasts and endothelial cells from various tissues differ; different receptors are more important for altering functions in cells from various tissues, and the functional effects of adenosine receptor occupancy move in opposite directions depending on the tissue being studied. It is thus difficult to extrapolate the effects of adenosine receptor agonists and antagonists on promotion of tissue regeneration and repair outside the skin.

Although adenosine A_{2A} receptor agonists are currently in trials for promotion of diabetic foot ulcers, their full effect on wound healing has not been established. One advantage of topical application of A_{2A} receptor agonists is that the potential for adenosine A_{2A}-receptor-mediated toxicity is greatly reduced.

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12 A_{2A} Adenosine Receptors and Ischemia Reperfusion Injury

Courtney M. Lappas and Joel Linden

CONTENTS

12.1 ISCHEMIA REPERFUSION INJURY

It has long been recognized that reduced delivery of oxygen to tissue results in cellular dysfunction, tissue injury, and subsequent cell death. Ischemia results in oxygen deprivation due to reduced blood flow caused by diseases such as myocardial infarction, stroke, or tumor; or it may be a by-product of a medical intervention such as tissue transplantation or surgery accompanied by hypoperfusion of one or more tissues. Based on the observation that the duration of oxygen deprivation correlates directly with the degree of cellular injury, it was thought that tissue injury resulted primarily from ischemia *per se*, and the primary aim of physicians throughout history has been to restore blood flow to the affected tissue as rapidly as possible. Although the restoration of blood flow to occluded large blood vessels, or reperfusion, is

necessary for the recovery of cell function, it is now known that restored blood flow is often accompanied by secondary injury that occurs as a result of inflammation during reperfusion, referred to as *reperfusion injury* or *ischemia-reperfusion injury* (IRI). Reperfusion injury results in part from the occlusion of microvessels with adherent platelets and leukocytes. The extent of injury that occurs as a consequence is partly due to the activation of endothelial adhesion molecules and the release of cytokines from tissue-resident inflammatory cells. Hence, IRI can be divided into an initial phase that occurs during ischemia and a secondary phase that occurs during reperfusion and is associated with inflammation.

The elucidation of the mechanisms underlying IRI has proved to be no simple task. It was initially believed that the cellular damage observed after restoration of blood flow to ischemic tissue was caused solely by reactive oxygen species (ROS, defined as any species derived from molecular oxygen containing one or more unpaired electrons).[1] Although studies have indicated that ROS do play a role in IRI, it has since been demonstrated that there are several other mediators involved in the pathogenesis of IRI, which include nitric oxide (NO), inflammatory cells, cytokines, eicosanoids, the complement cascade, endothelial cells, and adhesion molecules. The interactions among these mediators are complex and are yet to be fully revealed. An additional complication in the discussion of IRI is the observation that the mechanism and manifestation of injury vary somewhat with tissue. Although a complete review of the mechanisms underlying IRI is beyond the scope of this chapter, we will address some of the principal mediators of injury and focus on recent evidence that the activation of A_{2A} adenosine receptors (ARs) during reperfusion offers clinical promise as a new therapeutic intervention to limit IRI.

12.2 MEDIATORS OF IRI

12.2.1 Reactive Oxygen Species

IRI is a clinically significant phenomenon that is characterized by the production of oxygen-derived free radicals during the initial phase of reperfusion. ROS, by definition, react with numerous signaling molecules and are of great interest as the possible primary initiators of IR-induced tissue injury.[1] Although the activity of ROS can sometimes be beneficial to organisms (e.g., to kill invading microorganisms), excessive or unregulated ROS activity is injurious to host cells, causing lipid and protein peroxidation and DNA mutation.[2–4] There are several mechanisms by which IRI may trigger the production of ROS. Periods of ischemia drive both the degradation of ATP (which results in an increase in the concentration of the purine metabolite, hypoxanthine) as well as the conversion of xanthine dehydrogenase to xanthine oxidase. The enzyme xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine, and xanthine to uric acid. These reactions utilize molecular oxygen as an electron donor and produce both hydrogen peroxide and superoxide anion as by-products. During periods of ischemia, the activity of the enzyme is inhibited because of a lack of the molecular oxygen substrate. Upon reperfusion, however, the system becomes rapidly activated, driving the oxidation of the accumulated hypoxanthine and resulting in an accompanying burst of ROS

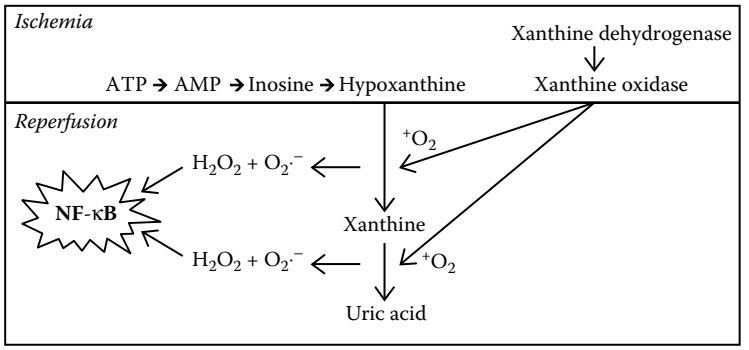

FIGURE 12.1 The pathway of NF-κB activation by xanthine-oxidase-produced ROS.

(Figure 12.1).[5–7] Studies indicating that the inhibition or inactivation of xanthine oxidase protects against IRI lend support to the hypothesis that this enzyme contributes to the pathogenesis of injury.[8] However, several pieces of data suggest that this system is not the sole mediator of IR-induced ROS or injury.

12.2.2 Formation of Xanthine Oxidase

The conversion of xanthine dehydrogenase to xanthine oxidase is driven by proteases (such as the neutrophil protease elastase), cytokines, and complement.[7] This raises the possibility that these compounds may themselves be by-products of ischemic injury. Also, although the rate at which xanthine dehydrogenase is converted to xanthine oxidase varies by tissue, it has been noted in many instances that the kinetics of this conversion do not correspond well with the kinetics of cellular damage observed after IR.[9] There are two potential explanations for these observations. First, the reactive oxygen metabolites produced by xanthine oxidase may trigger the activation of secondary mediators of injury. In this manner, xanthine oxidase may play an integral role in the facilitation of reperfusion injury, but may not be the only factor involved. Second, there may be additional sources from which ROS are produced in response to reperfusion. These two possibilities are not mutually exclusive, and evidence suggests that both may be correct.

12.2.3 NADPH-Dependent Oxidase in Macrophages and Neutrophils

Although much attention has been focused on xanthine oxidase, it must be remembered that there are multiple other soluble and plasma-membrane-associated oxidases that produce ROS. Specifically, the NADPH-dependent oxidase found on the surface of macrophages and neutrophils drives the conversion of molecular oxygen to hydrogen peroxide and superoxide anion when activated by a number of stimuli.[10] Studies in multiple models of IRI have shown that reperfusion triggers the activation of tissue-resident macrophages and, as a result, these inflammatory cells become sources of ROS. In fact, Kupffer cells (tissue-resident macrophages found in the liver) isolated

from liver post-IRI demonstrate increased superoxide generation *in vitro*.[11] Furthermore, treatments with gadolinium chloride and methyl palmitate, inhibitors of superoxide generation by Kupffer cells, have protective effects on hepatic IRI.[12–14] Activated macrophages release cytokines such as TNF-α that are chemotactic to neutrophils. The involvement of neutrophils in IRI is indicated by the observations that polymorphonuclear leukocytes (PMNs, i.e., neutrophils) infiltrate postischemic tissue, and agents that block this infiltration or deplete these cells from circulation have a protective effect in several models of IRI.[15–19] The events leading to neutrophil extravasation into interstitial space are threefold: selectin-mediated rolling of the neutrophil along the endothelium, β2 integrin-dependent firm adhesion of the neutrophil to the endothelium, and transmigration. The adhesion of neutrophils to the vascular endothelium after reperfusion is accompanied by a characteristic response of activated neutrophils, the so-called oxidative burst.[20,21] An oxidative, or respiratory, burst is a primary mechanism by which neutrophils induce tissue damage and is characterized by the release of myeloperoxidase (MPO), which converts hydrogen peroxide to hypochlorous acid, and the production of superoxide anion by the NADPH-dependent oxidase. Activated neutrophils may also release the proteases, elastase, gelatinase, and collagenase.[22,23] So, ROS are produced in response to reperfusion by at least three distinct mechanisms: xanthine oxidase, the NADPH-dependent oxidase on macrophages, and the oxidative burst of neutrophils. Much work has been directed toward understanding the interactions between these three systems.

12.2.4 NF-κB

Underlying the relationship between ROS and inflammatory cells is evidence indicating that the transcription factor NF-κB is activated in response to oxidative stress (defined as an environment in which excessive production of ROS has overwhelmed the compensatory mechanisms of antioxidants).[24–28] It is generally observed that levels of proinflammatory cytokines are elevated after reperfusion, and this relationship provides a mechanism by which this upregulation may occur. NF-κB is a member of the early immediate gene family and is a primary activator of proinflammatory cytokine transcription (including TNF-α, IL-1, IL-6, IL-8, PAF, and IFN-γ). Additionally, NF-κB can facilitate the transcription of the endothelial adhesion glycoprotein, ICAM-1. In a self-propagating cycle, TNF-α can itself further upregulate the expression by various inflammatory cells of these same factors.[29,30] This link between the activity of xanthine oxidase (i.e., ROS production) and the activation of inflammatory mediators is significant because it indicates a mechanism by which the activity of multiple factors may be coordinated to drive the pathogenesis of IRI. In contrast to the stimulatory effect of ROS on NF-κB activity, cAMP-elevating drugs such as forskolin and IBMX can block both basal and antigen receptor or toll-like receptor-induced activity of NF-κB.[31] Data indicate that the cAMP effector PKA negatively regulates NF-κB activity via the alteration of its DNA binding ability or concentration.[32,33] However, conflicting reports show an increase in NF-κB DNA-binding and subsequent transcriptional activity facilitated by direct phosphorylation by PKA.[34,35] It is therefore evident that the role of PKA in the regulation of NF-κB remains somewhat controversial.

12.2.5 Role of Cytokines in IRI

The functions of proinflammatory cytokines are both varied and complex. These polypeptide or glycoprotein mediators influence the differentiation, proliferation, and activation state of many cell types, and they also interact with and regulate the activity of other cytokines.[36] Although we will not present a complete review of proinflammatory cytokine activity, we will point out some key features as they pertain to IRI. TNF-α is an early-response gene that is involved in many facets of inflammatory responses. TNF-α mRNA is elevated within 15 min of ischemia, and this macrophage- and neutrophil-derived cytokine can both directly (via the inhibition of nitric oxide production by the endothelium and the activation of the neutrophil oxidative burst) and indirectly (via the upregulation of cytokine and adhesion molecule expression) contribute to IR-induced tissue dysfunction.[37–39] The upregulation of IL-1 and TNF-α by NF-κB is significant in that these cytokines are chemoattractant, drawing neutrophils out of circulation by facilitating their rolling and firm adhesion along the endothelium via the upregulation of E-selectin and ICAM-1.[39–41] Additionally, both TNF-α and IL-1 stimulate the production of IL-8, which along with PAF also stimulates neutrophil accumulation and activation in ischemic tissue.[42] The activity of these proinflammatory cytokines is redundant and interrelated, and they serve to propagate the initial xanthine-oxidase-driven ROS response and an accompanying increase in vascular permeability and cell death.

12.2.6 Role of T Cells in IRI

In addition to the aforementioned cytokines, the expression of the T cell chemotactic factors, RANTES, MCP-1, and IP-10, are upregulated after IRI.[43] Multiple studies have demonstrated that in addition to neutrophils and macrophages, T lymphocytes are also intimately involved in the pathogenesis of IRI. When mice lacking T lymphocytes are utilized in models of renal or hepatic IRI, there is a resulting decrease in neutrophil recruitment and inflammation after reperfusion, and reduced injury.[44–48] Because large numbers of T lymphocytes are not detected in affected tissue until 48 to 72 h after the initiation of reperfusion, it was initially believed that these cells were involved only in the late stage of reperfusion injury. However, evidence is accumulating to suggest that T lymphocytes play a role in subacute phases of IRI.[49–51] The production of chemokines such as IP-10 and MCP-1 by activated macrophages may serve to recruit both T lymphocytes and neutrophils to ischemic tissue, whereupon both cell types are likely to play a role in the propagation of injury; neutrophils via the release of MPO, the generation of superoxide anion, and the release of proteases, and T cells via the production and release of proinflammatory cytokines (particularly IFN-γ) that may serve to further activate macrophages.

12.3 STRATEGIES FOR LIMITING IRI

Although the pathogenesis of IRI is extremely complex and many aspects are still unclear, a preponderance of evidence indicates that ROS and leukocytes play integral roles in the pathophysiology of this disorder. It is even possible that ROS production

by xanthine oxidase or other oxidases in response to reperfusion could initiate a cascade of inflammatory events leading ultimately to cellular damage and death. Although the onset of an ischemic episode cannot generally be predicted, it may be possible to control further injury due to the subsequent reperfusion phase of the phenomenon. Several attempts have been made to achieve this goal; treatments with free radical scavengers, antioxidants, and neutralizing antibodies have been used in several models of IRI with varying degrees of success. The inflammatory component of IRI provides a clear target at which therapeutic agents may be directed. Although the inhibition of the reperfusion-induced inflammatory cascade may not provide complete protection from injury, data indicate that this approach shows significant promise.

12.4 ADENOSINE AND IRI

Periods of ischemia are accompanied by a significant outpouring of adenosine from injured tissue, and this endogenously released adenosine participates in a phenomenon known as *ischemic preconditioning*, defined as the protection conferred during a sustained ischemic period by a preceding brief ischemic episode.[52–54] The role of adenosine in ischemic preconditioning was first identified in the myocardium, and although the mechanism by which preconditioning protects the heart has not yet been fully elucidated, evidence indicates that the activation of the A_1AR or A_3AR on cardiomocytes activates a signaling cascade involving K^+-ATP channels and alterations in mitochondrial function.[55,56] These observations suggest that the administration of exogenous adenosine either before or during ischemia or reperfusion may mimic (or supplement) this protection. The therapeutic relevancy of adenosine, however, is limited by its short half-life and nonselective activation of all four AR subtypes.[57] Thus, the administration of more stable, subtype-selective AR agonists is an attractive alternative. Although the A_1AR and A_3AR are presumably involved in preconditioning, the activation of A_1 receptors can produce heart block, and the activation of A_3 receptors is sometimes proinflammatory. In contrast to A_1AR and A_3AR agonists that appear to block ischemic injury, A_{2A} agonists block inflammation and protect against reperfusion injury. A_{2A}AR receptor activation inhibits inflammation by effects on virtually all cells of the immune system including, but not limited to, T lymphocytes, macrophages, monocytes, platelets, and neutrophils.[58–60] Multiple manifestations of inflammatory cell activation, including proinflammatory cytokine production and adhesion molecule expression, are inhibited by A_{2A}AR activation.[61–64] One mechanism by which the anti-inflammatory effects of A_{2A}AR agonists may be mediated is via the inhibition of NF-κB signaling. Evidence indicates that A_{2A}AR activation blocks the NF-κB pathway downstream of immunoreceptors by interfering with the activation of the IKK complex or by hindering the IKK-IκB interaction; this activity is cAMP and PKA dependent.[31] It has also been shown in murine models that adenosine suppresses IκBα degradation induced by cardiac ischemia.[65] Additionally, the ability of NF-κB to bind DNA after TNF-α-induced activation is inhibited by adenosine in a variety of cell types including T lymphocytes, epithelial cells, and myeloid cells.[32] In addition to their effects as anti-inflammatory agents, A_{2A}AR agonists are also vasodilators. It is notable, however, that inhibition of inflammation

is observed at much lower doses than are needed to elicit cardiovascular effects. Although this is a relatively new field, a growing body of work demonstrates that administration of newly available A_{2A}AR selective agonists protects from IRI in multiple organs by inhibiting the inflammatory cascade via multiple mechanisms. We will discuss the effects of A_{2A}AR activation in hepatic, renal, spinal cord, and cardiac IRI, and we will present what is known of the mechanisms by which these effects are mediated.

12.4.1 Hepatic IRI

Hepatic IRI occurs as a result of transplantation, tumor, trauma, or surgery. Chronic hepatic hypoxia is also associated with heart failure and alcoholism.[66] IRI of the liver can be classified as either cold IRI (which occurs during organ transplantation) or warm IRI (which generally occurs as a by-product of surgery, tumor, or trauma). Interestingly, injuries as a result of cold or warm IRI differ in their characteristics. Cold IRI causes injury primarily to vascular endothelial cells, resulting in massive Kupffer cell activation and the activation of glycolysis.[67–69] Conversely, warm IRI damages mainly hepatocytes and results in an accompanying increase in serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as a decrease in bile output.[67] Several experimental animal models of warm hepatic IRI have been developed. Most commonly, the caudate lobe, left lobe, and right-upper and lower lobes are separated to allow visualization of the hepatic triad that is clamped above the bifurcation to block the flow of blood through the hepatic artery, portal vein, and bile duct. This model of partial hepatic ischemia allows venous return and normal function of the right liver lobe, while preventing intestinal congestion, sepsis, and peritonitis as a result of bacterial leakage from the intestine.[43,71–74]

In models of hepatic IRI utilizing Wistar rats, serum levels of ALT and AST have been found to increase significantly following 1 h of ischemia and 1 h of reperfusion, reaching peak levels after 12 h of reperfusion.[75] The effect of continuous IV administration of adenosine or the selective A_{2A}AR agonists CGS21680C or YT-146,[76] on serum enzyme levels in these rats after 12 h of reperfusion has been investigated. When an IV infusion of adenosine, CGS21680C, or YT-146 is initiated at the onset of ischemia and continued for 6 h after reperfusion, serum levels of ALT are reduced in a dose-dependent manner to as low as half those for vehicle-infused control animals, and serum levels of AST are reduced to as low as one third of those for controls. Although still considerably above the levels of serum ALT and AST in uninjured rats, significant protection is observed.[72]

Similar but more dramatic results have been obtained via the treatment of C57BL/6 mice with the potent and selective A_{2A}AR agonist ATL146e.[77] In these studies, an initial loading dose of drug is administered immediately after the onset of reperfusion (following 1 h of ischemia), and subsequent continuous delivery (for 24 h) of ATL146e is facilitated by the implantation of osmotic pumps. Mice treated with ATL146e have significantly reduced levels of serum ALT after 3 h of reperfusion, with the protective effect peaking after 24 h of reperfusion with a 90% reduction in serum ALT compared to vehicle-treated controls. The protective effect of ATL146e is absent in C57BL/6 mice with a disrupted A_{2A}AR gene, and is abolished in WT

C57BL/6 mice by cotreatment with equimolar concentrations of the selective A_{2A}AR antagonist ZM241385. Additionally, hematoxylin and eosin (H and E) staining of liver after 24 h of reperfusion demonstrates that the enzymatic findings are well correlated with histological evidence of liver injury.[43] The discrepancies in the magnitude of protection observed with YT-146 and CGS214680 treatment of Wistar rats as compared to ATL146e treatment of C57BL/6 mice may be attributable to species differences or, more likely, to the increased potency and selectivity of ATL146e for the A_{2A}AR over the proinflammatory A_3AR.[78]

The observation that A_{2A}AR activation protects from hepatic IRI is significant, but the mechanisms facilitating this protection are perhaps of greater consequence. The protection from IRI-induced hepatocyte damage elicited in Wistar rats by YT-146 and CGS21680C is accompanied by an inhibition in the expression of TNF-α and CINC (a member of the IL-8 family) following reperfusion. A_{2A}AR agonist treatment also reduces hepatic MPO activity and neutrophil accumulation in reperfused liver and results in enhanced hepatic tissue blood flow by preventing the occlusion of hepatic microvessels.[72] Similarly, ATL146e treatment significantly inhibits the increases in MPO activity, tissue edema, and cytokine expression (most notably RANTES, MCP-1, and IL-10) observed after 24 h of reperfusion in vehicle-treated animals. These data suggest that activation of the A_{2A}AR protects from reperfusion-induced hepatocyte damage via the inhibition of proinflammatory cytokine production, resulting in a corresponding reduction of inflammatory cell accumulation and activation.[43]

As mentioned previously, the activity of endogenous adenosine as an agent of ischemic preconditioning was first identified in models of cardiac IRI, and subsequent studies have shown that exogenous adenosine is also a pharmacological preconditioning agent. Various data show that this function is conserved in models of hepatic IRI. The administration of exogenous adenosine via carotid artery cannula for 20 min prior to a period of partial hepatic IRI in Wistar rats results in an approximate sixfold reduction in serum ALT and fourfold reduction in serum AST after 6 h of reperfusion as compared to vehicle-treated animals. H and E staining indicates that these decreases in liver enzyme levels are correlated with a reduction in hepatocyte necrosis, and immunohistochemical studies indicate that the downregulation of eNOS expression by sinusoidal endothelial cells observed in untreated animals is significantly attenuated by adenosine pretreatment. When the eNOS inhibitor N^G-Nitro-L-arginine (L-NAME) is administered 3 min prior to adenosine preconditioning, liver enzyme levels and hepatocyte necrosis are not significantly different from those in untreated animals. These data suggest that adenosine may precondition the liver against IRI by inhibiting the downregulation of eNOS, thereby helping to maintain NO production.[73]

Although A_1 and A_3 receptors have been implicated in myocardial preconditioning, substantial evidence supports a role for the A_{2A}AR in hepatic preconditioning. Administration of an A_{2A}AR, not A_1AR, selective antagonist before periods of warm ischemic preconditioning abrogates the protective effect of preconditioning on sinusoidal endothelial cell death after 30 h of cold ischemia. Moreover, preconditioning with the selective A_{2A}AR agonist CGS21680 or dibuteryl-cAMP (but not the selective A_1AR agonist CCPA) mimics the effects of ischemic preconditioning. These observations,

coupled with evidence that CG21680 and adenosine stimulate intracellular cAMP accumulation in cultured rat sinusoidal endothelial cells, indicate that protective preconditioning of the liver by endogenous or exogenous adenosine is mediated by the A_{2A}AR-driven elevation of cAMP.[71] Furthermore, recent experiments with bone marrow chimeric mice indicate that protection of liver from IRI is mediated predominantly by A_{2A} receptor activation on bone-marrow-derived cells.[79]

12.4.2 Renal IRI

Renal IRI is characterized by ROS production and the onset of an inflammatory cascade leading to neutrophil accumulation and activation.[80,81] Clinically, renal IRI often results from iatrogenic procedures and can be predicted.[82] IRI of the kidneys occurs as a product of systemic hypotension, renal artery stenosis, or trauma, but it is predominantly induced by surgical procedures including renal transplantation, tumor removal, and the suprarenal aortic cross-clamping necessary for the repair of abdominal aortic aneurysms.[83,84] Although the cause of renal IRI varies, the primary results are consistent: a reduction in total renal blood flow (especially in the inner medulla), a decrease in glomerular filtration, an increase in plasma creatinine levels, and proximal tubule damage.[85–87] Models of IRI utilizing rats and mice generally differ slightly in their design. In experiments performed with rats, the right renal artery and vein are ligated and the right kidney removed. The left renal artery and vein are then cross-clamped.[88] In murine models of renal IRI, both renal pedicles are cross-clamped.[89]

In mouse (C57BL/6) and rat (Sprague-Dawley) models of renal ischemia, plasma creatinine levels are found to be maximally increased after 24 to 48 h of reperfusion, respectively. This increase is directly correlated to an increase in neutrophil accumulation as indicated by MPO activity.[88,89] Continuous administration via osmotic pump of ATL146e, beginning 5 h prior to ischemia and extending through 48 h of reperfusion, results in a significant reduction in plasma creatinine levels in both models of renal IRI after 24 and 48 h of reperfusion; plasma creatinine levels are reduced in C57BL/6 mice and Sprague-Dawley rats after 48 h of reperfusion by approximately 60% and 80% respectively. Similar levels of protection are observed when administration of ATL146e is delayed until immediately after the onset of reperfusion, and maximal protection is achieved when dosing is extended through 6 h of reperfusion; no additional benefit is garnered from further treatment. Histological examination confirms that renal IRI results in necrosis of the outer medulla and red cell pooling in the inner medulla, pathologies that are attenuated by ATL146e treatment. Cotreatment with equimolar concentrations of ZM241385 antagonizes this protection. Likewise, MPO activity and neutrophil accumulation in the cortex are attenuated approximately 30% by treatment with ATL146e, whereas cotreatment with ZM241385 results in MPO activity not significantly different from vehicle-treated controls. It is significant that the effects of ATL146e that protect from reperfusion-induced renal injury are elicited at doses that have no effect on blood pressure or heart rate.[88,89] Also of interest is the observation that infusion of the type IV phosphodiesterase inhibitor rolipram attenuates IRI-induced increases in plasma creatinine levels and MPO activity to a similar degree (approximately 60%), as does ATL146e.

Because ATL146e and rolipram increase intracellular cAMP via two distinct mechanisms, it would be expected that if cAMP elevation were involved in the protective mechanism of either drug, coadministration would result in synergism. In fact, co-infusion of ATL146e and rolipram produces a protective effect that is significantly greater (approximately 90% vs. 60%) than that elicited by either compound alone.[90]

As discussed previously, neutrophil transmigration must be preceded by adherence to the endothelium, an event that is dependent on the upregulation of adhesion molecule expression by endothelial cells. Immunohistochemical studies demonstrate that episodes of renal IRI induce an upregulation of P-selectin in endothelial layers of peritubular capillaries and interlobular arteries, and ICAM-1 in the outer medulla; these events contribute to the transmigration of PMNs into affected tissue. Treatment with ATL146e has an inhibitory effect on the IRI-induced expression of both of these adhesion molecules, an effect that is inhibited by ZM241385.[89] These data demonstrate the indirect modulation of neutrophil activity by adenosine, but they do not address the question of whether or not the activation of A_{2A}AR expressed on inflammatory cells directly modulates their activity as stimulated by renal IRI.

The creation of various chimeric mice has allowed for the contribution of A_{2A}ARs on bone-marrow-derived cells to be determined. To create bone-marrow-chimeras, recipient WT C57BL/6 mice were lethally irradiated and then immediately injected with 3 million bone marrow cells from WT C57BL/6 or A_{2A}AR KO C57BL/6 donor mice to result in WT→WT or A_{2A}AR KO→WT chimeras. The reconstitution efficacies of lymphocytes and granulocytes six weeks after bone marrow transplantation were approximately 84% and 98%, respectively.[91]

The rise in plasma creatinine levels observed in WT C57BL/6 mice after renal IRI is significantly less than the increase observed in age-and-sex-matched A_{2A}AR KO C57BL/6 mice, indicating that endogenous adenosine may exert a protective effect via A_{2A}AR activation. Because the A_{2A}AR is expressed by several cell types, this effect may be mediated by the modulation of endothelial cell, epithelial cell, smooth muscle cell, or bone-marrow-derived cell function. The observation that WT→WT chimeras also sustain notably less injury after renal IRI than do A_{2A}AR KO→WT chimeras suggests that adenosine has a direct effect on bone-marrow-derived cells. When WT→WT chimeras are treated with ATL146e before being subjected to renal IRI, a protective effect similar to that elicited in WT animals (an approximate 60% reduction in plasma creatinine levels compared to vehicle-treated controls) is observed; this effect is absent in A_{2A}AR KO→WT chimeras, in which plasma creatinine elevations after renal IRI are unaffected by ATL146e infusion. The isolation of total kidney RNA from WT→WT and A_{2A}AR KO→WT chimeras before ischemia and after 8 or 24 h of reperfusion indicates that IL-6 and TGF-β transcript expression are upregulated at both time points following reperfusion, and this induction is inhibited by ATL146e administration in WT→WT, but not A_{2A}AR KO→WT mice. Additionally, IL-1β and IL-1ra mRNA expression is enhanced after 8 and 24 h of reperfusion in WT→WT chimeras, and this upregulation is blocked by ATL146e treatment.[91] These results demonstrate that the protective effect of ATL146e in renal IRI is dependent upon the activation of the A_{2A}AR on bone-marrow-derived cells, which include neutrophils, T lymphocytes, and macrophages.

To further discern which cell type is responsible for mediating the effects of A_{2A}AR activation, C57BL/6 mice were infused with clodronate (Cl_2MBP) liposomes, which induce selective apoptosis of macrophages. This treatment results in a marked reduction of macrophage density in the spleen, kidney cortex, and outer medulla of treated animals. Clodronate-induced macrophage deletion reduces IRI-driven increases in plasma creatinine to approximately 26% of vehicle-treated controls, and treatment with ATL146e has no additional protective effect. The adoptive transfer into clodronate-treated animals of WT RAW 264.7 monocytic cells or RAW 264.7 cells subjected to A_{2A}AR deletion by small interfering RNA reconstitutes injury, and infusion of ATL146e protects against injury comparably in both cases. Additionally, the protective effect of ATL146e is blocked in A_{2A}AR KO C57BL/6 mice reconstituted with WT RAW 264.7 cells, suggesting that the protective effect of ATL146e is independent of activation of the A_{2A}AR on macrophages.[92]

12.4.3 Spinal Cord IRI

IRI of the spinal cord is a phenomenon resulting primarily from trauma or as a by-product of the surgical repair of thoracoabdominal aneurysm.[93,94] The manifestations of spinal cord IRI are quite severe, resulting potentially in pain, paralysis, and the loss of bowel or bladder function.[95] Historically, clinicians have been concerned primarily with limiting the damage sustained during the ischemic period of injury by cooling the ischemic segment of the spinal cord and restoring blood flow as rapidly as possible.[94] This approach has proved to be less than adequate, however, because of the major contribution of reperfusion injury to overall injury. More recently, several strategies have been employed to protect against spinal cord IRI, including preoperative administration of methylprednisolone or long-term IV treatment with high doses of steroids.[96–99] Limited success has been achieved with these therapies owing to the occurrence of severe adverse side effects and the inconsistent ability of these treatments to produce any sustained improvements in motor function or neuronal viability.[100,101] The observation that retrograde venous cooling of ischemic spinal cord with an adenosine-containing solution elicits some beneficial effects coupled with the fact that adenosine has a protective effect on IRI in various other tissues led to the hypothesis that the administration of exogenous adenosine may hold promise as a novel therapy for IRI of the spinal cord.[102]

In an experimental model utilizing New Zealand rabbits, reproducible IRI injury to the spinal cord is produced by clamping the abdominal aorta and inferior vena cava distal to the left renal artery and proximal to the aortoiliac bifurcation.[102,103] A 45-min episode of ischemia results in significant impairment of hind limb motor function after 48 h of reperfusion as assessed by the Tarlov scoring system. In the Tarlov system, the motor function of an animal is assigned a value ranging from zero (if no hind limb movement is observed) to five (if a normal gait is observed).[102,103] The IV infusion of ATL146e beginning 15 min before reperfusion and continuing for 3 h results in a significant increase in the Tarlov score after 48 h of reperfusion.[104] This protective effect is dependent on both time and dose. The administration of ATL146e for 1.5 h results in significantly less improvement than is observed after 3 h of treatment, and 1 h of treatment does not produce significant

spinal cord protection.[105] Animals subjected to sham surgeries remain neurologically intact. H and E staining of lumbar sections can be utilized as a means by which to assess neuronal viability after IRI. Neuronal injury in this case is characterized as the loss of neuronal nuclear structure, vacuolization, and an eosinophilic cytoplasm; viable neurons display prominent nucleoli, loose chromatin, and Nissl substance in the cytoplasm. The neuronal damage observed in vehicle-treated rabbits after 45 min of ischemia and 48 h of reperfusion is attenuated significantly by 3 h of treatment with ATL146e; a conservation of neuronal histological architecture is observed and an approximate threefold increase in neuronal viability index (defined as the number of viable neurons divided by the total neuronal count) is achieved.[104,105]

The protection of neuronal viability and motor function by ATL146e is accompanied by a notable decrease in neutrophil infiltration into the lumbar cord, again indicating that the protective effect of A_{2A}AR activation may be due to the regulation of leukocyte extravasation into reperfused tissue.[105] Because the upregulation of TNF-α expression is a key mediator of this phenomenon, the effect of ATL146e treatment on IRI-induced TNF-α production was investigated. Neither vehicle- nor ATL146e-treated rabbits subjected to 45 min of spinal cord ischemia have any detectable systemic TNF-α release (as measured in blood serum collected from an ear artery) after 1 h of reperfusion. TNF-α levels peak after 2 h of reperfusion in both vehicle- and drug-treated animals; however, there is an approximate 93% reduction in TNF-α production as a result of A_{2A}AR activation, resulting in serum levels of TNF-α not significantly different from those found in sham-surgery controls. By 3 h post ischemia, there is no longer any measurable TNF-α in the serum of rabbits receiving ATL146e infusion, but the cytokine remains significantly elevated in vehicle-treated animals.[106] One manifestation of TNF-α production is the activation of a cascade leading ultimately to the fragmentation of the DNA repair protein poly (ADP-ribose) polymerase (PARP) by caspases.[107] Tissue levels of PARP cleavage products (including the p85 subunit) have been shown to correlate well with the degree of neuronal apoptosis, such that increasing the duration of spinal cord IRI leads to decreasing levels of intact PARP.[108] Whereas no difference is noted between p85 PARP levels in ATL146e- or vehicle-treated, rabbits after 2.75 h of reperfusion, there is a 65% decrease in p85 fragment expression in spinal cord samples taken from drug-treated, as compared to vehicle-treated, controls after 48 h of reperfusion. This correlates with the observed inhibition of TNF-α production in these animals.[104] Along with increased cytokine production, the upregulation of adhesion molecule expression by the endothelium also plays a role in IRI-induced neutrophil accumulation. Immunohistochemical experiments demonstrate that the expression of PECAM-1 by lumbar spinal cord capillary endothelial cells is markedly increased by episodes of spinal cord IRI, and this induction is blocked by A_{2A}AR activation.[106]

The observation that ATL146e administration elicits similar protective effects in a porcine model of spinal cord IRI in which the descending aorta is cross-clamped for 30 min distal to the left subclavian artery is noteworthy. Tissue reperfusion in this model results in motor-function impairment, increased TNF-α production (both systemically and in lumbar spinal cord lysates), and elevated MPO activity; all of these effects are inhibited by IV infusion of ATL146e beginning 10 min prior to reperfusion and continuing for 3 h.[109] Additionally, the efficacy of ATL146e in

protecting against spinal cord injury is conserved in models of IRI resulting from blunt trauma rather than cross-clamping. A_{2A}AR activation beginning 10 min after injury provides protection from blunt spinal cord trauma produced in New Zealand rabbits by dropping a 10-g weight from 6 cm onto a brass impounder placed on the dura.[110,111] Neurological outcomes after injury as assessed by Tarlov scoring are significantly improved by both acute (3 h) and extended (additional bolus doses at 12 and 24 h) administration of ATL146e. Hind limb function in vehicle-treated rabbits subjected to blunt spinal cord trauma is significantly impaired after 12, 24, and 48 h as compared to animals receiving drug. Furthermore, after 2 d of reperfusion only 12.5% of the vehicle-treated controls recover sufficient function to allow for extended survival, whereas 71% of ATL146e-treated and 100% of sham-surgery controls achieve Tarlov functioning (a score of 3) great enough for survival. Hind limb motor function in these animals does not deteriorate over the course of 7 d.[111] Notably, the doses of ATL146e observed to elicit protection do not alter mean arterial pressure or heart rate, which is significant owing to the increased morbidity and mortality associated with hypotension in spinal cord injury patients.

12.4.4 Cardiac IRI

Worldwide, myocardial infarction is a leading cause of morbidity and mortality. The rapid reperfusion of coronary arteries following myocardial infarction is vital to the preservation of reversibly injured myocardium and results in improved short-term mortality rates. However, reperfusion injury contributes to long-term mortality, with risk of death directly correlated to the size of initial infarction.[112–114] Thus, therapies that limit infarct size or abrogate damage due to reperfusion would be of benefit. However, because myocardial ischemic episodes are generally unpredictable, any clinically relevant therapy must impart protection when administered after the onset of reperfusion.[115] There is extensive evidence indicating that endogenous adenosine released during periods of ischemic preconditioning plays a role in protecting against injury due to subsequent IR. Although somewhat controversial, it is generally accepted that this protection is mediated via the activation of the A_1AR or A_3AR.[55,56] The observation that reperfusion after myocardial infarction is accompanied by a rapid accumulation of neutrophils, generation of ROS, production of proinflammatory cytokines, and endothelial cell dysfunction suggests that activation of the A_{2A}AR at the onset of this inflammatory cascade may have therapeutic benefit in cardiac IRI.

Experimental models of myocardial infarction vary slightly by species and laboratory; however, the underlying design is generally consistent. The left anterior descending coronary artery (LAD) is occluded for 30 to 60 min by placing a snare just distal to the first diagonal branch, producing a region of ischemia in the left ventricle. After reperfusion, the heart is removed and perfused with Evans blue (or a variety of alternative stains including 0.1% zinc or cadmium sulfide particles or Unisperse blue) to distinguish unaffected myocardium from the area of risk. Quantification of infarction size is accomplished by perfusion with triphenyltetrzolium chloride, which stains viable myocardium bright red and necrotic tissue pale red.[118–122] The myocardial area of risk (or "risk zone") in this model is reproducible within a species, generally encompassing 20 to 40% of the left ventricular tissue.

The IV infusion of the A_1AR or A_{2A}AR agonist AMP579 has been shown to limit infarction size in a time- and dose-dependent manner. In a model of cardiac IRI utilizing New Zealand rabbits, the administration of AMP579 for 70 min, beginning either 10 min before ischemic episode or 10 min before reperfusion, results in an approximate 65% reduction in infarction size compared to vehicle-treated controls after 3 h and 72 h of reperfusion; H and E staining confirms this attenuation of injury.[121] However, if infusion of AMP579 is halted after 30 or 40 min, or delayed until 10 min after the onset of reperfusion, no protection is observed.[123] This indicates that the drug must be present at the beginning of reperfusion and for some critical length of time thereafter in order for protection to be imparted. Similar results are observed in a porcine model of cardiac IRI, with the protective effect of AMP579 abolished by pretreatment with the A_1AR or A_{2A}AR antagonist CGS15943.[120] Although these results do not distinguish between the activity of A_1AR and A_{2A}AR, the inability of the selective A_1AR agonists CCPA or GR79236 to limit infarction size argues against a role for this receptor in the protection of reperfusion-driven cardiac injury.[118,120] Furthermore, administration of the selective A_{2A}AR agonist CGS21680 shortly before reperfusion limits infarction size in pig, rabbit, and dog models of cardiac IRI to a degree similar to that of AMP579.[119,124–126] This observation, coupled with the fact that the protection imparted by AMP579 is blocked by ZM24135 in a dose-dependent manner, suggests that the activation of the A_{2A}AR is primarily responsible for the observed reduction in infarction size.[118]

Consistent with A_{2A}AR activity in other tissues, the protection elicited by AMP579 in cardiac IRI is correlated with a significant (up to 59%) dose-dependent decrease in MPO activity in the area of risk, such that a linear relationship exists between MPO activity and infarction size after 24 h of reperfusion. Additionally, treatment with AMP579 results in an increase in myocardial blood flow in the area of risk as compared to vehicle-treated controls after 24 h reperfusion.[127] Thus, a by-product of the inhibition of neutrophil accumulation and activation by AMP579 may be protection from the reperfusion-driven, neutrophil-facilitated, mechanical plugging of microvessels, the so-called no-reflow phenomenon.[128,129] Interestingly, New Zealand rabbits treated with CGS21680 exhibit increased PI3K activity during the first 15 min of reperfusion after LAD occlusion. Caspase-3 function is modulated by PI3K, and the CGS21680-mediated increase in PI3K activity during reperfusion facilitates the preservation of stable caspase-3 activity, as opposed to the rapidly increased activity observed in vehicle-treated controls. Furthermore, in the presence of the selective PI3K inhibitor LY294002, the favorable effects of CGS21680 on infarction size are abolished.[130] These data indicate that the myocardial tissue protection facilitated by A_{2A}AR activation is PI3K dependent and at least partly due to the inhibition of caspase-3 activity upregulation. Additionally, the reduction in infarction size achieved by treatment with AMP579 is blocked in a dose-dependent manner by the inhibitor of MEK1/2, PD098059, ultimately resulting in the complete abrogation of protection.[118] Thus, the attenuation of myocardial infarction by A_{2A}AR activation may be linked to the activation of multiple downstream-signaling modulators.

In a canine model of reperfused myocardial infarction, a nonvasodilating low dose of ATL146e given at the onset of reperfusion reduces infarct size by 45%.

When ATL146e is combined with a very low dose of rolipram, a marked reduction in P-selectin expression and neutrophil infiltration (approximately 51% of control) is seen in the myocardial infarct zone and the infarct size is reduced by 58%.[131] The additive protection observed by combining ATL146e with the PDE IV inhibitor rolipram may be due to the activity of both of these compounds inhibiting inflammation mediated by bone-marrow-derived cells.

12.5 CONCLUSIONS

IRI results in part from secondary damage that occurs during reperfusion. Clinicians have historically focused on reducing injury during ischemia, but recent advances in the understanding of reperfusion injury coupled with a growing appreciation of the sizable contribution of reperfusion injury to overall IRI suggest that therapies targeting this second phase of the phenomenon may be of equal or greater benefit. Although the pathophysiology of reperfusion injury is complex, ROS and leukocytes are key mediators; initial treatments aimed at limiting the activity of ROS (antioxidants) or leukocytes (high-dose steroids) have had limited success. In this chapter we summarize evidence that A_{2A}AR on bone-marrow-derived cells may be an attractive therapeutic target for the treatment of IRI (Figure 12.2). The activation of A_{2A}AR

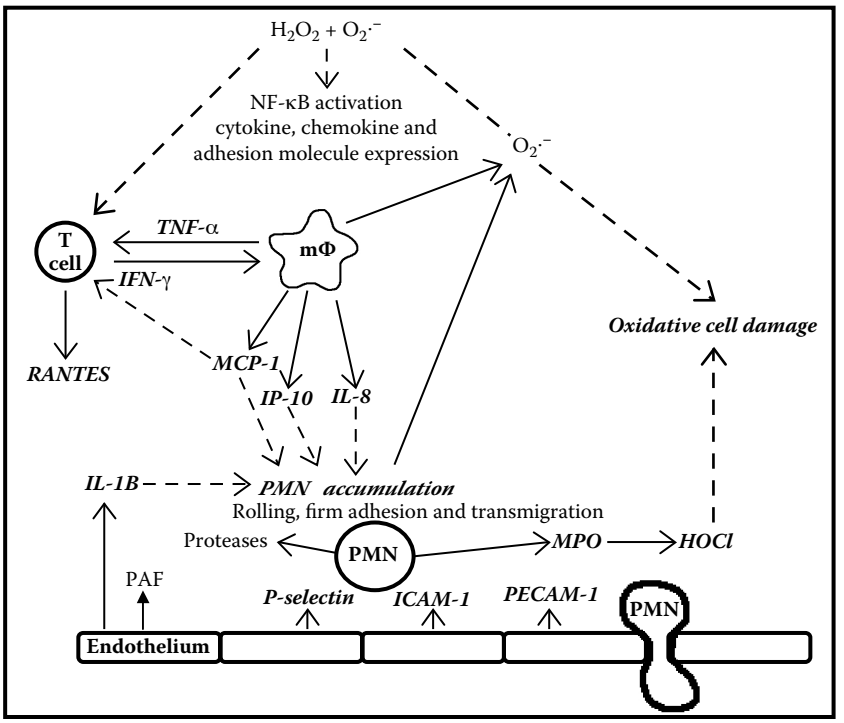

FIGURE 12.2 Key events in the reperfusion-induced inflammatory cascade. Events inhibited by A_{2A}AR activation are bold-faced and italicized.

has potent anti-inflammatory effects on multiple cells of the immune system. In fact, the selective activation of the A_{2A}AR has significant protective effects in various models of IRI via the inhibition of reperfusion-induced cytokine and chemokine production, adhesion molecule expression, and neutrophil accumulation and oxidative burst. The cellular and molecular actions of A_{2A}AR agonists occur more rapidly than steroids, which require transcription to mediate most of their anti-inflammatory effects. It will be of interesting to see if A_{2A}AR agonists prove to be more clinically effective than steroids at inhibiting IRI.

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13 Adenosine Signaling in Chronic Lung Disease

Michael R. Blackburn

CONTENTS

13.1 INTRODUCTION

Adenosine is a purine signaling nucleoside that is generated in excess during cellular stress and damage. Once produced, adenosine can engage specific G-protein-coupled receptors on the surface of cells. Four adenosine receptors (ARs) have been identified and are termed A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR.[1] The expression pattern of these receptors is widespread in tissues and cells, and their engagement can activate multiple cellular responses. Hence, physiologic functions of adenosine signaling are diverse, with actions attributed to regulation of the immune,[2] neurological,[3]

cardiovascular,[4] and renal systems.[5] In addition, this signaling pathway plays important roles in the regulation of tissue injury and repair.[6,7] Numerous studies have implicated adenosine signaling in promoting anti-inflammatory and tissue-protective effects in situations such as ischemia-reperfusion injury and acute inflammation. In contrast, adenosine signaling is associated with proinflammatory and tissue-destructive properties in chronic inflammatory diseases of the lung, including asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis.[8–11] Emerging information concerning the metabolism of adenosine and the regulation of adenosine signaling components suggests that this signaling pathway may provide the opportunity for novel treatment options for both acute and chronic inflammation and injury processes. This chapter is designed to provide an overview of the current findings investigating the role of adenosine signaling in aspects of chronic lung disease, including the regulation of pulmonary inflammation, fibrosis, and alveolar destruction.

13.2 CHRONIC LUNG DISEASE

Chronic lung disease is a term used to describe pulmonary disorders in which persistent inflammation and alteration in lung structure contribute to progressive loss of lung function. In contrast to most injury and repair responses, the inflammation and damage seen in these disorders is chronic and may last throughout the life of the affected individual. Examples of chronic lung disease include asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis. Together, these lung diseases afflict millions of individuals and result in billions of dollars in annual health care costs. Although these diseases are distinguished by the specific types of inflammation and damage that can occur (for example, eosinophils and mucus in asthma vs. macrophages and emphysema in COPD),[12] they share the common feature that they are chronic in nature. A large amount of information has been gathered concerning the regulatory pathways that can lead to the development of chronic lung disease. For example, the inflammation associated with asthma and COPD is driven in part by cytokine and chemokine signaling networks,[12] whereas the chronic remodeling and destruction of the airways is associated with the activation of growth factor signaling pathways and disruption of protease–antiprotease balances.[13,14] Although signaling pathways associated with the genesis of inflammation and the control of tissue remodeling have been described, little is known about signaling pathways that serve to regulate the chronic nature of these diseases.

Pulmonary fibrosis is a type of interstitial lung disease characterized by inflammation, aberrant fibroblast proliferation, and extracellular matrix deposition which results in pathogenic remodeling that eventually distorts pulmonary architecture and compromises pulmonary function.[15–17] There are many causes of pulmonary fibrosis, including exposure to fibrosis-inducing agents such as silica,[18] coal dust,[19] radiation,[20] and certain chemotherapeutic agents.[20] Pulmonary fibrosis is also a feature found in disorders such as scleroderma,[21] sarcoidosis,[22] and cystic fibrosis.[23] Idiopathic pulmonary fibrosis (IPF) is a particularly deadly form of pulmonary fibrosis with unknown causes.[16] In addition to these classical forms of pulmonary fibrosis, it is becoming increasingly evident that patients with severe asthma and COPD also

develop features of pulmonary fibrosis,[12,24,25] which greatly broadens the number of individuals afflicted with this disorder. Despite its prevalence, the pathogenesis of pulmonary fibrosis is not completely understood, because of a lack of knowledge of the molecular mechanisms governing its onset and progression. Moreover, treatment options for the resolution of pulmonary fibrosis are lacking. Knowledge of the cellular signaling pathways and events that govern the inflammatory and fibroproliferative aspects of pulmonary fibrosis is needed to aid in the development of novel therapies for its treatment.

Asthma, COPD, and pulmonary fibrosis are distinguishable pulmonary disorders with clear diagnostic differences. However, there is evidence that they share common features,[26] including the chronic nature of inflammation, airway remodeling, and progressive loss of lung function with repeated disease exacerbations.[12] What drives the chronic nature of these lung disorders is not known.

Adenosine is produced in response to cellular stress and damage, and elevations in adenosine are found in patients with chronic lung disease.[27,28] Emerging evidence suggests that adenosine signaling can activate features of chronic lung disease,[29] giving rise to the hypothesis that adenosine serves as a link between cell damage and ensuing exacerbation of tissue injury. This concept will be explored in detail in the following text, following a description of the basic concepts of adenosine metabolism, transport, and signaling.

13.3 ADENOSINE PRODUCTION, METABOLISM, AND SIGNALING

13.3.1 Adenosine Production, Metabolism, and Transport

Regulation of intracellular and extracellular adenosine concentrations is carried out by the concerted actions of proteins that regulate adenosine production, metabolism, and transport across the plasma membrane. Understanding the regulation of extracellular adenosine production is particularly important when considering that many of the autocrine and paracrine actions of adenosine are mediated by ligation of cell surface adenosine receptors.[30,31] ATP levels in the cell are high (approximately 5 m*M*[32]), whereas the levels of extracellular ATP are relatively low.[33] In response to cellular stress or damage, ATP is released into the extracellular space by mechanisms that are not fully understood.[33] Possible routes include the constitutive release of ATP through vesicle fusion with the plasma membrane and programmed release through membrane channels. With regard to the latter, increasing attention is being given to the ATP-binding cassette family of membrane transporters, including the CFTR[34] and multiple drug resistance[35] channels. ATP release has also been shown to occur through connexin hemichannels,[36] maxi-anion channels,[37] stretch-activated channels,[38] and voltage-dependent anion channels.[39] In addition, activated inflammatory cells such as mast cells, neutrophils, and eosinophils are able to release adenine nucleotides, and adenosine into the local environment.[40–42] Once released, ATP is itself a potent signaling molecule via its interaction with P_2 purinergic receptors.[43] Extracellular ATP is rapidly dephosphorylated by ectonucleoside triphosphate diphosphohydrolases such as CD39 to form ADP and AMP.[44] ADP also signals through P_2

purinergic receptors;[43] however, the ability of AMP to serve as a direct extracellular signal is controversial. Extracellular AMP is dephosphorylated to adenosine by the 5′-nucleotidase CD73 (also known as ecto-5′-nucleotidase).[45–49] Most evidence supports ATP release followed by CD39- and CD73-dependent formation of adenosine as the major route of extracellular adenosine production during situations of cellular stress and damage.

Adenosine levels are also regulated within the cell, where elevations can lead to the increased release of adenosine. Adenosine can be produced by the dephosphorylation of intracellular AMP through the action of a cytosolic form of 5′-nucleotidase.[50] In addition, adenosine is produced by the hydrolysis of *S*-adenosylhomocysteine via the enzyme *S*-adenosylhomocysteine hydrolase, which is part of a pathway that utilizes *S*-adenosylmethionine as a methyl donor for numerous cellular transmethylation reactions.[51] Adenosine can be phosphorylated back to AMP by the enzyme adenosine kinase[52] or it can be catabolized by the enzyme adenosine deaminase (ADA), which carries out the hydrolytic deamination of adenosine to inosine.[53] ADA is a cytosolic enzyme; however, it is also found in extracellular fluids, and in humans can interact with CD26 on the surface of certain cells.[54] Interactions of extracellular ADA with CD26 may play an important role in regulating adenosine signaling at the cell surface.[55,56]

In addition to adenine nucleotide release and metabolism, increases in extracellular adenosine levels can occur from the release of adenosine itself from cells. Evidence exists to suggest that adenosine release can be promoted by a number of stimuli including K^+, glutamate receptor agonists, glucose and oxygen deprivation, and electrical stimulation.[57] In mammals, adenosine is transported across the plasma membrane by both facilitated and active nucleoside transport mechanisms. The facilitated nucleoside transporters known as the equilibrative nucleoside transporters (ENTs) are bidirectional transporters.[58] There are four members of the ENT family (ENT1-4, also known as the SLC29 family of transporters) that all transport nucleosides and nucleobases. They are widely distributed in mammalian tissues and, given their bidirectional function, play a major role in transporting adenosine in and out of the cell. Active transport of adenosine into the cell occurs through concentrative nucleoside transporters (CNTs), which are Na^+-dependent concentrative transporters.[59]

There are three members of the CNT family (CNT1-3, also known as the SLC28 family of transporters) that transport purine and pyrimidine nucleosides with various efficiencies. The tissue distributions of the CNTs vary, with CNT1 localizing primarily to epithelial cells, whereas CNT2 and CNT3 are more widely distributed.

13.3.2 Adenosine Production and Metabolism in Chronic Lung Disease

Extracellular adenosine exerts its functions as an autocrine and paracrine signaling molecule by engaging cell surface adenosine receptors.[60–65] There is growing *in vivo* evidence that extracellular adenosine concentrations increase in human tissues during situations of cellular stress and damage, particularly where hypoxia and inflammation are evident.[31,66,67] With regard to chronic lung diseases in humans, adenosine

concentrations have been estimated to reach 100 μM in the bronchial alveolar lavage fluid of asthmatic and COPD patients.[27] In addition, a recent study has shown that elevations in adenosine levels are found in the exhaled breath condensate of asthmatics.[28] Interestingly, nitric oxide levels, which correlate with the degree of inflammation, were measured in conjunction with adenosine, and results suggest that adenosine accumulations correlate positively with the severity of inflammation. Local provocative challenges in the lungs of asthmatics with allergen leads to increased adenosine in the plasma,[68] and challenging asthmatics with exercise can lead to elevations in adenosine in plasma[69] and exhaled breath condensate.[70] Together, these findings demonstrate that adenosine concentrations are elevated in the inflamed and damaged human lung, and suggest that triggers of exacerbations, such as allergen and exercise, can induce adenosine elevations, at least in asthmatics. Adenosine levels have not been measured in the lungs of patients with pulmonary fibrosis; however, the degree of inflammation and damage seen in the lungs of patients with this disorder and evidence from animal models with pulmonary fibrosis (see later text) suggest that adenosine is likely elevated in human fibrotic disorders.

The production of adenosine in the lungs of asthmatics suggests that it may play a role in regulating aspects of the disease. There is substantial clinical and scientific evidence to support this hypothesis. Adenosine can directly influence cellular and physiological processes in the lungs of asthmatics and patients with COPD.[8,9] For example, exogenous adenosine can elicit acute bronchoconstriction in patients with asthma[71] or COPD[72] while having no effect on normal individuals, suggesting a fundamental difference in adenosine signaling between these patients. In addition, adenosine signaling can influence the activity of a number of cell types that play a central role in chronic lung disease, including mast cells,[73] eosinophils,[74] macrophages,[75] and neuronal,[76] epithelial,[77] and smooth muscle cells.[78]

Examination of adenosine levels in animal models that display pulmonary inflammation and injury supports the preceding findings in humans. Transgenic mice that overexpress the Th2 cytokine IL-13 develop progressive pulmonary inflammation and injury characterized by eosinophilic and monocytic infiltrates, mucus overproduction, airway fibrosis, and alveolar airway destruction.[79] Adenosine levels are greatly increased in the lungs of IL-13-overexpressing mice, with lung inflammation and damage.[80] Moreover, the accumulation of adenosine is progressive. As the inflammation and damage increase in these mice, so do the absolute levels of lung adenosine. Another interesting finding is that accumulations in adenosine are limited to the lung and are not found in the circulation or in the liver, suggesting that increases in adenosine production are limited to the site of injury. Similar findings are seen in mice that overexpress the Th2 cytokine IL-4 in the lung (Elias, J.A., unpublished data, 2005). In addition, we have recently shown that adenosine levels are elevated in the lungs of mice that have been sensitized and chronically challenged with the allergen ovalbumin, which leads to pronounced eosinophilic inflammation and airway remodeling associated with collagen deposition and mucus overproduction (Blackburn, M.R., unpublished data, 2005). Adenosine levels are also elevated in the lungs of mice exposed to the fibrosis-inducing agent bleomycin (Volmer, J.B., unpublished data, 2005). Lastly, there are correlations between the degree of inflammation and damage and the absolute levels of lung adenosine accumulations in

ADA-deficient mice.[81,82] Together, these findings support the hypothesis that lung inflammation and damage are associated with increases in endogenous adenosine levels. Furthermore, they demonstrate that the stimulus and nature of the inflammation and damage that promote such increases can be diverse.

13.4 ADENOSINE IN ANIMAL MODELS WITH CHRONIC LUNG DISEASE

13.4.1 ADA-Deficient Mice

Mice deficient in ADA have been useful in examining the consequences of endogenous adenosine accumulations in the lung.[83] ADA functions to regulate the levels of adenosine and deoxyadenosine in tissues and cells. Mice deficient in this enzyme exhibit pronounced accumulations of these substrates in many of their organs.[83] Deoxyadenosine accumulates to high levels in the thymus and spleen of ADA-deficient mice and is thought to account for the combined immunodeficiency seen in these animals. Adenosine accumulates to high levels in many organs, including the lung. The concentrations of adenosine in the lungs of ADA-deficient mice are estimated to reach 100 μM, which are comparable to those measured in fluid collected from the airways of asthmatics.[27] In association with these adenosine accumulations, ADA-deficient mice develop severe pulmonary inflammation and airway remodeling.[81] Features of the pulmonary phenotype noted include the following: mast cell degranulation,[84] a progressive increase in lung macrophages and eosinophils, mucus metaplasia, subepithelial and distal airway fibrosis, airway enlargement,[81] and airway hyperreactivity.[85] These animals die by approximately three weeks of age. The exact cause of death is not known; however, the extensive inflammation and damage seen in the lungs suggest that complications from respiratory insufficiency are likely involved. The finding that merely elevating endogenous adenosine levels can access pathways that lead to the promotion of lung inflammation and damage suggests that adenosine signaling plays active roles in the exacerbation of chronic lung disease.

Many of the pulmonary phenotypes in ADA-deficient mice are dependent on the accumulation of adenosine in the lungs. Purified ADA that is covalently linked to polyethylene glycol (PEG-ADA) is an effective means of lowering systemic and tissue levels of adenosine in both ADA-deficient humans[86] and mice.[87] Injecting ADA-deficient mice with PEG-ADA at a stage when lung disease is already established rapidly lowers the levels of adenosine in the lungs and reverses aspects of lung inflammation and airway remodeling. Most notable is the rapid reduction of lung eosinophilia and mucus production in the bronchial airway epithelium.[81] ADA enzyme therapy is also able to prevent mast cell degranulation in ADA-deficient lungs,[84] reverse airway hyperreactivity,[85] and prevent alveolar airway enlargement (emphysema).[82] One of the most striking features of the use of ADA enzyme therapy in these mice is its ability to promote survival.[81] Apparent respiratory insufficiency is rapidly relieved, and mice are able to survive as long as they are kept on enzyme therapy. When therapy is ceased, features of pulmonary disease and subsequent death occur within three weeks (Blackburn, M.R., unpublished data, 2005). These findings

demonstrate that there is a strong correlation between elevations in endogenous lung adenosine concentrations and the activation of pathways that can culminate in chronic lung disease.

ADA-deficient mice exhibit alterations in mediators characteristic of experimental asthma and COPD. Levels of the Th1 cytokine IFNγ are decreased in the lungs of ADA-deficient mice, whereas levels of the Th2 cytokines IL-13, IL-6, and IL-5 increase.[80,81] Similarly, increases in Th2-associated chemokines such as eotaxin, RANTES, TARC, and the MCP proteins are also found. In addition, serum IgE levels are significantly increased,[81] which is an interesting finding considering these animals are largely immunodeficient. Together, these findings demonstrate the development of a Th2 environment in the lungs of ADA-deficient mice that exhibit elevations in endogenous adenosine. Large-scale analysis of gene expression using cDNA microarrays was used to identify additional mediators that might play a role in the development of lung inflammation and remodeling in ADA-deficient lungs.[88] Mediators that were found to be increased include regulators of inflammation such as ICAM-1 and LFA-1, regulators of vascular growth (VEGF), molecules involved in remodeling (TGF-β1, collagen, fibronectin, and laminin), and regulators of airway destruction such as the matrix metaloproteinases and cathepsins. The potential importance of these molecules in regulating the phenotypes seen in ADA-deficient mice is strengthened by the observation that they are decreased in ADA-deficient lungs following the lowering of lung adenosine levels when using ADA enzyme therapy. Thus, regulation of cytokine and chemokine expression by adenosine might represent an important link between elevations in endogenous adenosine and the ensuing inflammation in this model.

13.4.2 Pulmonary Fibrosis in Partially ADA-Deficient Mice

Completely ADA-deficient mice die by three weeks of age,[81] preventing the investigation of adenosine's effects on chronic aspects of lung injury such as pulmonary fibrosis. ADA enzyme replacement therapy was utilized to regulate the levels of endogenous adenosine in ADA-deficient mice to extend the lifespan of these mice and determine the consequences of prolonged adenosine elevations in the lung. Completely ADA-deficient mice were maintained on low dosages of PEG-ADA for up to 4 months, which allowed for chronic but sublethal disturbances in purine metabolism.[11] Prolonged elevations in lung adenosine levels led to the development of severe pulmonary fibrosis.[11] Features of fibrosis seen in these mice included increased numbers of myofibroblasts throughout the distal airways, and increased production and deposition of collagen in both the distal airways and in bronchial vascular bundles. Treatment of ADA-deficient mice with a high dosage of PEG-ADA before the onset of pulmonary fibrosis prevented the development of fibrosis.[11] Remarkably, treatment of ADA-deficient mice with high dosages of PEG-ADA after fibrosis was established was able to reverse features of pulmonary fibrosis in association with lowering lung adenosine levels. These findings suggest that prolonged elevations of adenosine can activate pathways that lead to pulmonary fibrosis. The observation that PEG-ADA can reverse aspects of established pulmonary fibrosis is of particular importance, because most patients with pulmonary fibrosis present with

established disease and there are few treatment options available that will halt and resolve existing pulmonary fibrosis. Continued research is needed to promote the potential use of ADA enzyme therapy in the treatment of pulmonary fibrosis.

The development of pulmonary fibrosis in completely ADA-deficient mice treated with ADA enzyme therapy was recently confirmed using a genetic approach. A line of mice that expresses an ADA minigene in the gastrointestinal tract of otherwise ADA-deficient mice was developed.[85] These partially ADA-deficient mice live for up to 5 months and die from apparent respiratory distress. Detailed investigations of the lung revealed that partially ADA-deficient mice exhibit severe pulmonary fibrosis, as indicated by the increased presence of pulmonary myofibroblasts and increased collagen deposition.[89] Furthermore, important proinflammatory and profibrotic signaling pathways, including IL-13 and TGF-β1, were found to be activated. These changes occurred in association with pronounced elevation in lung adenosine concentrations, supporting the hypothesis that elevation in endogenous adenosine can serve as a proinflammatory and profibrotic signal. Interestingly, fibrosis was also noted in the liver and kidney of partially ADA-deficient mice, suggesting that systemic elevations of adenosine may access profibrotic pathways in multiple tissues. This implies that adenosine signaling may play a role in the pathogenesis of fibrosis in many disorders, including hepatic fibrosis and cirrhosis, and chronic renal scarring in patients with glomerulonephritis.

Insight into the mechanisms by which adenosine regulates fibrosis in ADA-deficient mice comes from the analysis of adenosine-dependent elevations of key regulators of pulmonary inflammation and fibrosis in the lungs of partially ADA-deficient mice. Numerous cellular mediators have been implicated in the ontogeny and maintenance of pulmonary fibrosis.[16,90,91] Amongst these, IL-1β appears to be an important proinflammatory signal,[92] whereas TGFβ1, Pai-1, OPN, and MMP-2 are involved in the regulation of the extracellular matrix environment and structural integrity of the airways.[93–96] These mediators are elevated in patients and animal models exhibiting pulmonary fibrosis,[97–101] and studies in transgenic and knockout mice demonstrate their importance in bleomycin-induced pulmonary fibrosis.[94,102,103] Similarly, these profibrotic mediators are substantially elevated in the lungs of partially ADA-deficient mice that exhibit chronic elevations of adenosine and fibrosis.[11] Moreover, lowering levels of endogenous adenosine in ADA-deficient mice with established pulmonary fibrosis can reverse elevations of these mediators, suggesting that adenosine can directly access these profibrotic pathways. Adenosine has been shown to regulate the secretion of MMP-2;[104] however, a direct relationship between adenosine and the regulation of IL-1β, TGFβ-1, Pai-1, or OPN has not been demonstrated. Additional research is needed to examine whether, and how, adenosine directly regulates mediators of fibrosis or activates upstream signaling molecules. Such research could provide important information into the mechanisms by which adenosine regulates fibrosis in the lung.

13.4.3 IL-13-Overexpressing Mice

IL-13 is a pleiotropic cytokine that is produced in abundance by Th2 cells. It is overproduced in asthma[105] and has been implicated in the regulation of Th2

inflammation in this and other disorders. In addition to regulating tissue inflammation, IL-13 is being increasingly found to be a major mediator of tissue remodeling.[106,107] Accordingly, IL-13 plays an important role in the pathogenesis of the remodeling responses in asthma, COPD, and pulmonary fibrosis.[12]

Mice engineered to overexpress IL-13 in the lung develop features of chronic lung disease that include progressive monocytic and eosinophilc inflammation, mucus metaplasia, airway fibrosis, and alveolar enlargement.[79] Adenosine levels increase in a lung-specific manner in IL-13-overexpressing mice, as the inflammation and damage get progressively worse.[80] Treatment of IL-13-overexpressing mice with PEG-ADA efficiently lowers adenosine levels in animals with established lung disease.[80] Furthermore, PEG-ADA treatments decrease pulmonary inflammation, collagen deposition, and alveolar destruction. Levels of key regulatory cytokines, chemokines, and proteases are also regulated by elevated adenosine in the lungs of IL-13-overexpressing mice.[80] Thus, adenosine appears to regulate features of IL-13-induced pulmonary pathologies. These studies strengthen the notion that adenosine serves as a profibrotic and tissue-destructive signaling molecule in certain aspects of chronic lung disease.

The pulmonary phenotypes of IL-13-overexpressing and ADA-deficient mice are remarkably similar, with each manifesting macrophage- and eosinophil-rich inflammation, mucus metaplasia, alveolar enlargement, and tissue fibrosis.[79,81] Interestingly, elevated levels of adenosine in ADA-deficient mice can stimulate IL-13 elaboration in a manner that is sensitive to both PEG-ADA treatment and treatment with a broad-spectrum adenosine receptor antagonist.[80] The ability of adenosine receptor signaling to promote the expression of IL-13 suggests that a positive feedback loop, or amplification pathway, may exist in which IL-13 increases adenosine accumulation (because of the damage it can inflict) and adenosine, in turn, can induce additional IL-13 elaboration. It is not yet clear which cells produce IL-13 in response to adenosine in the lung, nor are the receptors involved known. However, a recent study in a human mast cell line has demonstrated the ability of adenosine to directly induce IL-13 production through A_{2B}AR.[108] Given the numerous proinflammatory and tissue-destructive features of IL-13, understanding how adenosine regulates its production may provide useful avenues for the treatment of disorders in which this cytokine has been implicated.

13.4.4 Allergic Models of Lung Disease

Adenosine signaling also plays a role in allergic models of pulmonary inflammation. Allergen-sensitized and allergen-challenged Brown Norway rats have been used to examine the mechanisms of adenosine-induced bronchoconstriction and inflammation.[109–111] In this model, the bronchoconstrictive effects of adenosine are associated with mast cell degranulation, and are adenosine receptor dependent. The specific adenosine receptors involved in bronchoconstriction are not clear; however, A_3AR appears to account for mast cell degranulation[112] and treatment with an A_{2A}AR agonist can attenuate inflammation, suggesting this receptor may play an anti-inflammatory role in allergic lung disease.[113] Interestingly, treatment of sensitized and challenged rats with ADA enzyme therapy, or an ADA inhibitor, did not prevent

or enhance bronchoconstriction or inflammation, respectively,[111] suggesting that endogenous adenosine accumulation has little effect on allergen-induced responses in this model. The reason for this may be related to the absolute levels of adenosine that accumulate in the lungs under the specific challenge protocol used. Studies in a mouse model of allergic lung inflammation have also demonstrated that adenosine can promote bronchoconstriction[114] and inflammation, in a manner that appears to involve the degranulation of mast cells.[115] Again, the specific adenosine receptors involved in adenosine-mediated airway constriction and inflammation are not known. Continued efforts to subject mice deficient in the various adenosine receptors to allergen challenge protocols will help validate the involvement of these signaling pathways in allergic lung disease.

The aforementioned animal models suggest that adenosine signaling is playing a proinflammatory and profibrotic role in aspects of chronic lung disease. Although these models have been useful in examining novel mechanisms of lung inflammation and injury, it must be realized that they are models, and lack many components seen in human lung disease. The field is significantly lacking in the assessment of adenosine metabolism and adenosine signaling in aspects of human lung disease, especially the most chronic forms of lung disease such as COPD and pulmonary fibrosis, in which generation of this nucleoside may have significant impact on promoting the chronic nature of the lung inflammation and remodeling that is seen.

13.5 ADENOSINE RECEPTOR CONTRIBUTIONS IN ANIMAL MODELS WITH CHRONIC LUNG DISEASE

The ability to genetically and biochemically manipulate the levels of adenosine in the lungs of ADA-deficient mice has provided the opportunity to assess mechanisms involved in the adenosine-mediated lung histopathology within the context of the whole animal. The use of both genetic and pharmacologic approaches in ADA-deficient mice has begun to reveal specific functions of individual receptors in this model. These findings are summarized in the following text together with pertinent findings from studies in other model systems.

13.5.1 Contribution of the A_3AR

The A_3AR has received considerable attention in the context of pulmonary inflammation and its involvement in asthma and COPD. Walker and colleagues demonstrated that transcript levels for the A_3AR are elevated in lung biopsies of patients with asthma or COPD,[74] suggesting that increased signaling through this receptor may be a feature of these diseases. In addition, numerous studies suggest that A_3AR signaling can influence inflammatory cell types associated with asthma and COPD.[116–121] In rodents, airway mast cells express the A_3AR,[121] and engagement of this receptor can promote[121] or enhance[122,123] mediator release from mast cells. This mechanism may not hold true in humans, for whom increasing evidence suggests that the A_{2B}AR is responsible for adenosine-mediated mast cell degranulation.[124,125] Both human and mouse eosinophils express the A_3AR;[126,127] however, the function

of A_3AR signaling on this cell type remains controversial with both pro-[128] and anti-[117] inflammatory activities being reported.

Levels of the A_3AR are elevated in the lungs of ADA-deficient mice in which receptor transcripts are localized to mast cells,[121] eosinophils, and mucus-producing bronchial epithelial cells.[127] Treatment of ADA-deficient mice with the A_3AR antagonist MRS 1523 demonstrated that the adenosine-dependent mast cell degranulation is mediated through the A_3AR.[121] These findings were confirmed in A_3AR-deficient mice, in which engagement of the A_3AR promotes the release of histamine from airway mast cells through a mechanism that involves PI3 kinase and increases in intracellular Ca^{+2} levels.[121] Genetic removal of the A_3AR or treatment with an A_3AR antagonist has significant effects on airway inflammation and mucus production in ADA-deficient mice.[127] Blocking A_3AR signaling in ADA-deficient mice decreases the number of eosinophils infiltrating into the airways while not affecting the degree of circulating eosinophilia, suggesting that A_3AR signaling is important in eosinophil trafficking in this model. In addition, removal of A_3AR signaling results in decreased mucus metaplasia, suggesting that the A_3AR receptor contributes to airway mucus production. Interestingly, the removal of A_3AR signaling from ADA-deficient mice does not rescue these mice from death at 3 weeks of age, suggesting that other mechanisms may be involved, including the involvement of other adenosine receptors. Nonetheless, data generated thus far in this model suggest that A_3AR signaling contributes to airway inflammation and mucus production in an environment of elevated adenosine. Whether similar mechanisms hold true in human lung diseases in which A_3AR levels are elevated is yet to be determined.

13.5.2 Contribution of the A_1AR

The high-affinity A_1AR has been implicated in both pro- and anti-inflammatory aspects of disease processes. For example, A_1AR signaling can promote neutrophil[129] and monocyte activation,[130,131] as well as engage anti-inflammatory and protective pathways in neuroinflammation and injury,[132] and in cardiac[133] and renal[134,135] injury. In the lung, A_1AR activation regulates bronchoconstriction in allergic rabbit models,[78,136,137] suggesting a provocative role for A_1AR signaling in acute phases of asthma. Similarly, A_1AR receptor antagonism can attenuate ischemia reperfusion[138] and endotoxin-induced[139] lung injury, suggesting a proinflammatory role for A_1AR signaling in acute lung injury. Thus, as is often the case with putative adenosine receptor actions, both pro- and anti-inflammatory actions of the A_1AR likely exist.

Transcript levels for the A_1AR are elevated in the lungs of ADA-deficient mice at a stage when there is extensive pulmonary inflammation and remodeling.[82] Although the complete cellular localization of the A_1AR receptor in the lungs of ADA-deficient mice is not clear, activated alveolar macrophages appear to express fairly high levels of this receptor.[82] This is of particular interest because nonactivated mouse alveolar macrophages do not possess transcripts for the A_1AR, and elevations in this receptor may represent a novel specialized pathway in activated macrophages. The genetic removal of the A_1AR results in enhanced pulmonary inflammation, mucus metaplasia, alveolar destruction, and precocious death of ADA-deficient mice,

suggesting that A_1AR signaling plays an anti-inflammatory and tissue-protective role in the progression of chronic lung disease in this model.[82]

The precise anti-inflammatory mechanisms and tissue-protective effects of this receptor in this model are not clear, but may be mediated by the repression of mediators of airway inflammation and remodeling. Removal of the A_1AR from the lungs of ADA-deficient mice results in hyperelevations of IL-4, IL-13, chemokines, and matrix metalloproteinases,[82] which could account for the exacerbation of the pulmonary phenotypes seen. The mechanisms by which adenosine is regulating the levels of IL-13 and IL-4 may relate to the regulation of intracellular cAMP levels. Increased IL-4 and IL-13 production is associated with elevations in cAMP,[140–142] and recent findings have demonstrated that adenosine can promote the expression of IL-4 and IL-13 in mast cells by engaging the A_{2B}AR,[108] which can couple to G_{as} to activate adenylate cyclase to elevate cAMP.[1] The A_{2B}AR has a relatively low affinity for adenosine. Thus, in situations in which adenosine levels are elevated, A_{2B}AR engagement may serve to elevate the expression of IL-13 and IL-4 (Figure 13.1). The A_1AR couples to G_i, which inhibits adenylate cyclase to lower cAMP.[1] Engagement of this receptor may serve to dampen the levels of IL-13 and IL-4 produced in cells. Based on this model, as inflammation and damage increase in the lung and adenosine levels rise, A_{2B}AR-mediated effects would predominate over the anti-inflammatory effects of A_1AR signaling and thus contribute to the promotion of chronic lung injury (Figure 13.1). Thus, A_1AR and A_{2B}AR signaling pathways may work in opposition to each other in the regulation of Th2 cytokine production.

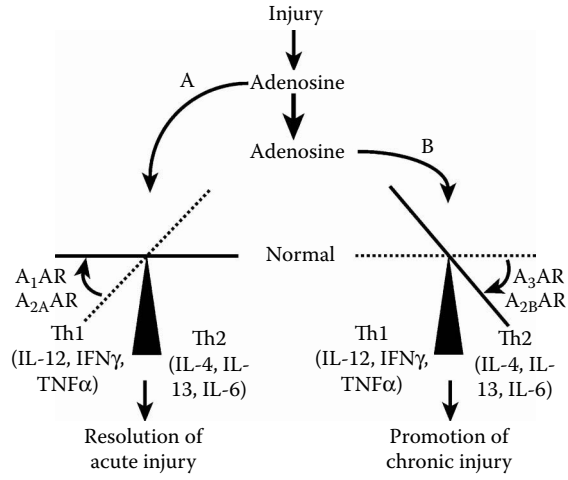

FIGURE 13.1 Model for adenosine regulation of Th1/Th2 balances. (A) Adenosine produced in response to acute injury could activate high-affinity anti-inflammatory adenosine receptors such as A_1AR and A_{2A}AR. Doing so could facilitate the downregulation of Th2 cytokine production and promote tissue protection and tissue repair. (B) Prolonged and heightened elevations in adenosine levels could promote the production of Th2 cytokines through engagement of low-affinity adenosine receptors such as A_3AR and A_{2B}AR. Tipping the scale toward a Th2 cytokine profile could in turn promote chronic tissue injury.

Understanding such mechanisms could help explain how adenosine regulates aspects of inflammation and damage in chronic lung disease.

13.5.3 Contribution of the A_{2A}AR

The A_{2A}AR is widely expressed in tissue and cells, and engagement of this receptor plays important functions in many physiological systems.[1] Some of the more prominent functions that have been noted include the regulation of vascular tone and blood pressure, mediating pain and the stimulant effects of caffeine, and inhibiting platelet aggregation.[143–145] In addition, the A_{2A}AR plays a major role in mediating the "retaliatory" effects of adenosine on inflammatory cells.[7,146] There is substantial evidence that activation of this receptor can promote anti-inflammatory pathways in a number of immune cells and in general protect tissues from the damage incurred by ischemia followed by reperfusion, or in response to certain pathogens. A_{2A}AR expression is regulated by cytokines, and its activation can down regulate the expression of largely Th1 cytokines and chemokines (reviewed in Reference 7). Engagement of this receptor by endogenous adenosine generated in response to hypoxia and inflammation is thought to serve as an immunosuppressive signal to dampen the immune response.[146] In the lung, A_{2A}AR activation can reduce lung reperfusion injury following transplantation[147] and can inhibit pulmonary inflammation in a rat model of allergic inflammation.[113] Interestingly, in brain injury models, the A_{2A}AR appears to play a detrimental role in the ensuing inflammation and damage.[148] Thus, though A_{2A}AR appears to be the major adenosine receptor subtype involved in the immunosuppressive effects of adenosine, there are context-specific effects that must be considered.

Transcript levels for the A_{2A}AR decrease in mouse models of chronic lung injury. In both the ADA-deficient model and the IL-13 overexpression model of chronic lung disease, transcript levels for the A_{2A}AR are lower in whole-lung RNA extracts.[80,82] The localization of A_{2A}AR in these models is not clear; nor is its function in these model systems understood yet. However, one can speculate that the apparent down regulation of these receptors in the chronically inflamed lung may represent a loss of A_{2A}AR-mediated immunosuppression. Experiments to examine the contribution of A_{2A}AR signaling to the pulmonary phenotypes seen in ADA-deficient and IL-13 transgenic mice will help to clarify whether this receptor, similar to the A_1AR, is playing a protective role in chronic lung disease.

13.5.4 Contribution of the A_{2B}AR

The A_{2B}AR is widely expressed in tissues and cells and has the lowest affinity for adenosine.[1] This feature has promoted the hypothesis that activation of the A_{2B}AR may be important in situations in which adenosine levels are elevated, such as is seen following hypoxia or in inflammation. Consistent with this hypothesis are observations that expression of this receptor is increased in response to hypoxia and inflammatory mediators.[65,149,150] However, the function of A_{2B}AR activation is not clear, with both pro- and anti-inflammatory actions likely existing. With regard to the regulation of chronic lung disease, several features make the A_{2B}AR a likely candidate for mediating the proinflammatory and profibrotic effects of adenosine.

Engagement of the A_{2B}AR can mediate the direct expression of proinflammatory cytokines such as IL-8, IL-4, and IL-13 from human HMC-1 mast cells.[108,151] In addition, the A_{2B}AR mediates expression of the proinflammatory cytokine IL-6 in numerous cell types, including pulmonary smooth muscle cells and pulmonary fibroblasts.[150,152] The aforementioned cytokines are all elevated in patients with chronic lung disease, and they can influence many of the remodeling responses seen in these patients.[12] Thus, elevations in adenosine levels may contribute to the pathogenesis of these conditions by engaging the A_{2B}AR and inducing the production of detrimental cytokines.

There is also compelling evidence to suggest that adenosine signaling through the A_{2B}AR can directly activate profibrotic pathways. A recent study in human pulmonary fibroblasts cultured *ex vivo* demonstrated that activation of the A_{2B}AR can promote the differentiation of pulmonary fibroblast into collagen, producing myofibroblast.[150] This response involved the release of IL-6 and was enhanced in the presence of hypoxia. The transformation of pulmonary fibroblast into myofibroblasts is considered a major mechanism underlying pulmonary fibrosis.[90,153] In addition, engagement of A_{2B}AR can directly upregulate the production of the profibrotic molecule fibronectin in alveolar epithelial cells and lung fibroblasts.[154] The demonstration that adenosine can directly promote these processes further strengthens the likelihood that this nucleoside is in itself profibrotic and strongly implicates A_{2B}AR as a profibrotic receptor. However, it must be noted that other studies have implicated adenosine, acting through A_{2B}AR, as an antifibrotic signal via its ability to inhibit cardiac fibroblast growth and collagen production.[155,156] Interestingly, in one of these studies, activation of A_{2B}AR with high levels of agonist actually promoted increased collagen production,[156] suggesting that there may be a threshold for the antifibrotic effects of A_{2B}AR signaling in cardiac fibroblasts. It is not yet known whether A_{2B}AR is involved in the chronic lung inflammation and fibrosis seen in ADA-deficient mice and IL-13-overexpressing mice. However, it is interesting to note that levels of this receptor are elevated in both these models.[80,85,89] The use of mice deficient in the A_{2B}AR and selective A_{2B}AR antagonists will help identify the contribution of this receptor in these models, which will in turn help guide our thinking of A_{2B}AR contributions to similar diseases in humans.

13.6 PURINERGIC REMODELING IN CHRONIC LUNG DISEASE

Whereas it appears clear that adenosine levels are elevated in humans and animal models that exhibit lung inflammation and injury, the mechanisms underlying these elevations are not well understood. Given that elevations in adenosine in the lung may serve important physiological functions, it will be important to understand the mechanisms of adenosine production, metabolism, and signaling in chronic lung inflammation and injury. Emerging evidence suggests that there may be an orchestrated, local alteration in the production, metabolism, and signaling capabilities of adenosine in the inflamed lung. Such "purinergic remodeling" may serve to regulate the levels and responses to adenosine, which may help dictate the balance between

anti-inflammatory and tissue-protective effects, and between proinflammatory and tissue-destructive effects of adenosine.

Recent observations in mice with Th2 chronic lung disease suggest that alterations in purine metabolism do occur in the inflamed and injured lung. Mice overexpressing IL-13 in the lungs not only exhibit elevations in lung adenosine, but also a downregulation of ADA levels.[80] Both mRNA levels and enzymatic activity of ADA are lower in the lungs of IL-13-overexpressing mice than in wild-type littermates. Moreover, these reductions in ADA are found only in the lung and not in peripheral tissues. Similar observations are seen in mice overexpressing IL-4 in the lung (Elias, J.A., unpublished data, 2005), and in response to bleomycin exposure (Chunn, J.C., unpublished data, 2005). These findings indicate that the local downregulation of ADA at the site of injury in the lung may contribute to the elevations in adenosine levels.

The association between elevations in adenosine levels and alterations in purine metabolism has long been noted.[30,32] For example, there is a well-established relationship between increased adenosine release and the inhibition of adenosine kinase.[30] Factors that can lead to adenosine kinase inhibition include hypoxia and elevations in adenosine levels itself.[157–159] It is not known whether adenosine kinase activity is affected in the inflamed and injured lung; however, one can speculate that decreased adenosine kinase activity may be part of an orchestrated purinergic remodeling response in the lung. Interestingly, recent studies from our laboratory suggest that CD73 activity is elevated in the lungs of various animal models exhibiting features of chronic lung disease (Volmer, J.B., unpublished data, 2005), further supporting an environment of adenosine accumulation.

Another factor to consider in the orchestrated elevations of extracellular adenosine is the regulation of adenosine transporters. Recent findings demonstrate that adenosine itself, signaling through the A_{2A}AR, may promote increased transporter activity though the ENTs.[160] Transporter activity is also affected by hypoxia.[157] Thus, regulation of adenosine transporter function may represent another mechanism for regulating adenosine elevations in the chronically injured lung. Understanding how the enzymes of adenosine metabolism and adenosine transport are regulated in chronic lung diseases will be important in understanding the role of adenosine signaling in these disorders.

There is emerging evidence that, in addition to mechanisms that lead to orchestrated elevations in extracellular adenosine levels, adenosine receptors are regulated in response to various stimuli, which may increase or decrease their availability in certain inflammatory or injurious environments. For example, hypoxia can increase expression of A_{2B}AR receptors in pulmonary fibroblasts[150] and endothelial cells.[149] In addition, the largely Th1-associated cytokine INFγ upregulates the expression of the A_{2B}AR in macrophages[161] and endothelial cells,[65] whereas IL-1 and TNFα promote increased A_{2A}AR and A_{2B}AR expression in endothelial cells.[65]

Alterations in adenosine receptor levels are also seen in animal models exhibiting features of chronic lung disease. There are increased levels of mRNA for the A_1AR, A_{2B}AR, and A_3AR in the lungs of IL-13-overexpressing mice[80] and ADA-deficient mice.[85] Interestingly, there is evidence that mRNA levels for the A_{2A}AR are decreased in the lungs of both models.[80,82] The alteration of receptor expression in these models

is associated with the influx of inflammatory cells expressing adenosine receptors, as well as the upregulation of receptors in structural components of the lung. In addition to altered expression of adenosine receptors, it is also likely that factors which can impact functional aspects of adenosine receptor signaling or downstream pathways are also dynamic in situations of chronic inflammation and injury. Regulation of adenosine receptor stabilization and desensitization/downregulation, as well as the efficiency of G-protein signaling, must be considered when trying to decipher the function of these pathways in situations in which adenosine levels are elevated for prolonged periods of time. Thus, alterations in adenosine receptor expression patterns and functions are likely an important aspect of local purinergic remodeling responses in environments in which chronic inflammation and tissue remodeling are evident.

13.7 TH1 VS. TH2 CYTOKINES, ADENOSINE, AND FIBROSIS

An emerging hypothesis in the regulation of fibrosis is that imbalances in Th1 and Th2 cytokine responses can lead to an abnormal response to injury and, hence, to fibrosis.[17,162] This hypothesis has emerged from studies demonstrating that Th1 cytokines such as INFγ are decreased in the lungs of patients with pulmonary fibrosis,[163] whereas levels of Th2 cytokines such as IL-4, IL-5, and IL-13 are elevated.[164,165] Furthermore, studies *in vitro* and in animal models have shown that these Th2 cytokines are able to activate features of fibrosis.[79,166,167] Among these Th2 cytokines, IL-13 is gaining particular attention because of its ability to promote pulmonary fibrosis. Thus, understanding the regulation of Th2 cytokine production in the lung will provide important insight into the regulation of pulmonary fibrosis.

Adenosine signaling may contribute to the induction or maintenance of pulmonary fibrosis by regulating the production of Th2 cytokines in the lung. Adenosine can directly promote the production of Th2 cytokines such as IL-13,[80,108] and elevated adenosine levels in the lung of partially ADA-deficient mice are associated with decreased levels of the Th1 cytokine INFγ and increased production of the Th2 cytokines IL-4, IL-5, IL-6, and IL-13.[89] The ability of adenosine to regulate the cytokine milieu may also represent a fundamental distinction between the anti- and proinflammatory effects of adenosine (Figure 13.1). Inhibition of IL-12 is known to be associated with not only diminished Th1 cytokine production, but also the increased production of Th2 cytokines.[162] Engagement of the high-affinity A_{2A}AR decreases the production of IL-12 as well as INFγ and TNFα in several immune cells.[7] Whereas these alterations most likely play important functions in stemming inflammatory responses to acute pathogens and injuries such as ischemia-reperfusion, they may also contribute to skewing the cytokine balance toward Th2 production. Furthermore, the balance toward increased Th2 production may be enhanced as adenosine levels increase in response to chronic injury, and engagement of the A_{2B}AR directly regulates the increased production of Th2 cytokines (Figure 13.1). In this proposed manner, adenosine may serve to amplify Th2 production, which can in turn contribute to features of chronic lung disease such as prolonged inflammation, fibrosis, and alveolar destruction.

13.8 CONCLUDING REMARKS

How cells and tissues respond to stress and damage determines their ability to survive various insults. The ability of cells to increase extracellular adenosine levels in response to various injurious states represents a fundamental cellular response in orchestrating cellular protection and tissue repair. This is clearly indicated by adenosine's ability to mount anti-inflammatory and tissue-protective actions in acute models of inflammation and injury. Thus, the ability to selectively activate such responses may offer a beneficial therapy for certain conditions. For example, selective A_{2A}AR activation may prove useful in the treatment of acute inflammation and damage resulting from ischemia-reperfusion injury or Th1-driven inflammation. In contrast, high and prolonged elevations in adenosine levels may activate proinflammatory and profibrotic processes. This seems to be the case in models of chronic lung disease in which Th2 environments predominate. Thus, selective inactivation of proinflammatory and profibrotic adenosine receptors, such as A_{2B}AR, may prove beneficial in treatment of chronic lung disease.

Aspects of chronic lung disease, such as fibrosis and airway remodeling, have been suggested to result from abnormal attempts of the lung to repair itself following injury.[168] Adenosine may represent an important link in such a hypothesis. When lower concentrations activate important repair pathways early and as adenosine concentrations increase, separate profibrotic and tissue-destructive pathways may predominate. Factors that may contribute to the balance of adenosine-mediated repair or destruction include the local cytokine milieu and the ability of adenosine to regulate Th1 and Th2 cytokine production, the ability of tissues to undergo "purinergic remodeling" to allow for prolonged elevations in adenosine, and the context-specific regulation of adenosine receptor function, including receptor stabilization and coupling efficiency. Understanding the interplay of these factors is a daunting task, but efforts to do so will help lead to the development of adenosine-based therapeutics for the treatment of numerous disorders.

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14 Role of Adenosine in the Control of Inflammatory Events Associated with Acute and Chronic Neurodegenerative Disorders

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CONTENTS

14.1 NEUROINFLAMMATION IN ACUTE AND CHRONIC NEURODEGENERATIVE DISEASES

Throughout the body, but mainly in the periphery, adaptive and innate immune systems operate to help protect against a variety of physical and biological insults. The brain, however, is in a slightly different situation because it is encased by the skull. For many years it was thought that the brain was an organ privileged against both adaptive and immune reactions, so that adaptive immune system mediated inflammatory swelling occurs rarely, especially when and where there is an intact blood–brain barrier. The concept that the brain is immunologically privileged does not hold, however, for the innate immune system, which has been implicated increasingly in a variety of acute and chronic neurodegenerative disorders. The cells that mainly constitute the innate immune system in brain are phagocytic microglia, and in addition, astrocytes, endothelial cells, and neurons; all contribute to neuroinflammatory reactions.[1] Indeed, when activated, microglia, astrocytes, and, to a somewhat lesser extent, neurons can produce and secrete a wide range of inflammatory and, in some cases, toxic substances including cytokines, chemokines, prostaglandins, leukotrienes, thromboxanes, coagulation factors, adhesion molecules, acute-phase proteins, complement and reactive oxygen species, as well as such destructive enzymes as cyclooxygenase-2, inducible nitric oxide synthase, and proteinases.[2–6] However, not all proinflammatory substances and events are mediated by brain-resident cells. Indeed, supplementing the microglia-derived compounds are proinflammatory substances released from monocytes, lymphocytes, and neutrophil granulocytes that traffic into brain parenchyma as a result of acute and chronic neurodegenerative insults.[7] Thus, inflammatory reactions are part of normal brain function and are linked to the pathogenesis of a variety of neuropathological conditions.

Multiple acute and chronic neurodegenerative disorders of the CNS, including such acute disorders as hemorrhagic stroke, ischemic stroke and traumatic brain injury, and such chronic disorders as multiple sclerosis, HIV-1-associated dementia, epilepsy, as well as Alzheimer's, Parkinson's, and Huntington's diseases, are accompanied by inflammatory events that contribute to brain injury. These inflammatory events in brain tend to be delayed and less pronounced, but can be rapid when underlying factors include activation of microglia, and the release of proinflammatory cytokines and chemokines. The contribution that proinflammatory cytokines make in neurodegenerative disorders comes from direct and indirect studies. Indirect evidence largely consists of comparing levels of protein and mRNA with the temporal profile of neural damage as well as determining the neurotoxic and neuroinflammatory effects of applying recombinant cytokine protein directly to brain. On the other hand, direct evidence is derived from studies in which neutralizing antibodies, soluble receptors, antisense oligonucleotides, siRNA, and dominant-negative technologies have been used. Regardless of the temporal and spatial profile, inflammation is an important component of neurodegenerative disorders, and multiple factors are involved.

Of the proinflammatory substances identified, the proinflammatory cytokines interleukin-1 (IL-1), IL-6, and TNF-α have been linked closely to the pathogenesis

of neurodegenerative disorders. Cytokines are proteins with pleiotropic actions. When proinflammatory, they are described as stimulatory and Th1-type. When anti-inflammatory, they are described as inhibitory and Th2-type. Cytokines in brain can originate peripherally and enter brain through specific transporters—through the blood–brain barrier—under normal but leaky as well as under conditions in which the barrier is damaged.[8] Alternatively, and especially under conditions of brain injury, cytokines can be produced by resident microglia and, to a lesser extent, by astrocytes and neurons.[9,10] Cytokine levels are carefully controlled by an array of neuroactive substances including the purine nucleoside adenosine, the topic of this chapter. Of the cytokines, TNF-α has been shown repeatedly to be a central mediator of neuroinflammation and has been a primary target for therapeutic intervention.[11] Of clear relevance therefore are findings that adenosine, regulators of endogenous adenosine levels, and adenosine receptor agonists and antagonists are effective regulators of the levels and actions of TNF-.

14.2 ANTI-INFLAMMATORY ACTIONS OF ADENOSINE RECEPTOR ACTIVATION

The adenosine system continues to be a target for pharmaceutical intervention. Adenosine is used clinically, and a wide range of adenosine receptor agonists and antagonists as well as regulators of endogenous adenosine levels, the so-called REAL agents,[12] are either used clinically or have been evaluated pharmacotherapeutically.[13,14] Adenosine is capable of affecting multiple physiological functions throughout the body, including acting as a neuroprotective agent in CNS.[12,15] Adenosine, when released from cells or formed following the release of adenine nucleotides, may afford neuroprotection through its ability to depress activation of macrophages and microglia, thereby reducing proinflammatory cytokines, chemokines, and nitric oxide.[15–17] These actions are complemented by its established roles in depressing neuronal depolarization and stimulation of glutamate uptake by astrocytes.[17,18]

Adenosine may act as a "metabolic switch" capable of sensing different degrees of inflammation and tissue damage, and subsequently triggering appropriate immune responses.[19] This proposal was based on findings that adenosine levels are increased during inflammatory responses and following tissue damage in a manner that reflects the severity of the insult or inflammation. Adenosine levels, known to increase as a result of bacterial infection, can exert anti-inflammatory actions without suppression of immune responses.[20] In acting as a sensor or indicator of inflammation, high levels of adenosine would be associated with more severe inflammation or tissue damage and would activate both high- and low-affinity adenosine receptors. A_2 and A_3 receptor activation is associated with decreased T lymphocyte proliferation, decreased CTL activity, decreased IL-2 production, and decreased IFN-γ production.[21–23] These effects appear to decrease the initiation of adaptive immunity. In addition, A_{2B} and A_3 receptor activation is associated with increased mast cell degranulation.[13,24] These effects may be critical for the role adenosine plays in immune responses because macrophages and mast cells already stationed in the tissue mediate the initiation of the response to either infection or tissue injury.[25]

Observations that adenosine and adenosine-related compounds possess anti-inflammatory properties have been demonstrated in various animal models including experimental adjuvant arthritis, ischemia-reperfusion, and carrageenin-induced inflammation.[26–29] Further, several studies have shown that adenosine potently inhibits its TNF-α production.[28,30–35] The ultimate effects of adenosine in the context of inflammatory events can be beneficial or harmful, depending on receptor expression, cell-signaling pathways activated by adenosine receptors, local adenosine concentrations, and the conditions studied.[36] Adenosine receptors have been classified into four subtypes, A_1, A_{2A}, A_{2B}, and A_3, and all four subtypes have been implicated in brain inflammation. A first level of evidence for involvement of adenosine receptors in inflammation comes from findings that proinflammatory cytokines, interleukin-1β and TNF-α, increased the expression and signaling of A_1 receptors in brain,[37] caused increased A_{2A} receptor density and mRNA in PC12 cells[38] and human monocytic THP-1 cells,[39] and increased A_{2B} receptor functioning without changes to levels of receptor protein or mRNA in human astroglial cells;[40] but when lipopolysaccharide was injected into the fourth ventricles of young rats, immunoreactivity for A_{2B} receptors was decreased.[41]

Certainly for A_1, A_{2A}, and A_3 receptors, agonists and, in some cases, antagonists of these receptors have been shown to regulate immune responses including neutrophil activation and proinflammatory cytokine production. For A_{2A} receptors, anti-inflammatory events have been described for receptor activation and blockade.[29] Subthreshold inflammatory stimuli caused tissue damage and increased cytokine production in mice deficient in adenosine A_{2A} and in wild-type mice injected with the adenosine A_{2A} receptor antagonist ZM 241385,[42] thus suggesting a beneficial and anti-inflammatory role for A_{2A} activation. A_{2A} activation is also protective in ischemia-reperfusion injury of kidney, skin, lung, blood vessels, brain, and spinal cord.[19,43] In other situations, adenosine receptor activation has been shown to inhibit proinflammatory cytokine production and mediate proinflammatory effects. For example, A_1 receptors promote chemotaxis of neutrophils and adherence of neutrophils to endothelium, A_3 receptors promote mast cell degranulation,[13] and adenosine-deaminase-deficient mice exhibit markedly increased levels of extracellular adenosine, inflammation, and decreased longevity that is improved with adenosine deaminase therapy.[36,44] Such pro- and anti-inflammatory actions of adenosine receptor activation highlight the complexity of this system as well as its potential importance in understanding healthy and disease states.

14.3 ANTI-INFLAMMATORY ACTIONS OF ADENOSINE RECEPTOR ACTIVATION IN ACUTE NEURODEGENERATIVE DISEASES

14.3.1 Ischemic Stroke

Inflammation clearly plays a role in ischemic neuronal injury.[45] As with all neurodegenerative conditions in which inflammation has been proposed as a pathogenic mechanism, the involved cells include trafficking neutrophils, monocytes, and macrophages as well as neurons, astrocytes, and microglia and

endothelial cells that reside in brain. At least for cells that are brain resident, microglial activation correlates best with degree of damage caused by ischemia, and their activation precedes or is concurrent with neuronal degeneration.[45] A number of factors have been implicated including cytokines, chemokines, adhesion molecules, reactive oxygen species, and a number of enzyme systems including those for nitric oxide, COX-2, and matrix metalloproteinases.[45] Relevant to the focus of this chapter, high levels of endogenous TNF-α are present in brain following ischemic stroke, and treatment with anti-TNF-α antibodies or soluble receptors reportedly decreased infarct size as a result of focal cerebral ischemia.[11,46–50]

Adenosine and adenosine receptor systems have been implicated in the pathogenesis of ischemic stroke for many years, and a large number of studies have been published demonstrating that adenosine levels increase after hypoxic or ischemic events and that mostly adenosine receptor activation protects against, but in some cases exacerbates, ischemic neuronal injury.[51–53] A_1 receptor activation appears to consistently yield neuroprotection in a variety of animal models of ischemic stroke and hypoxia;[54,55] however, the potential usefulness of A_1 receptor agonism as a therapeutic approach might be limited to acute neurodegenerative disorders because this receptor system is subject to desensitization under chronic brain insult conditions.[52,56]

A_3 receptors too have been shown to be involved in protecting against hypoxic or ischemic insults. In a transient middle cerebral artery occlusion model of focal ischemia, the A_3 agonist IB-MECA administered 20 min after ischemia was neuroprotective and almost totally prevented the appearance of activated microglia in the penumbra.[57] More recently, the neuroprotective actions of inosine against damage secondary to transient middle cerebral artery occlusion were shown to be mediated at least in part by activation of A_3 receptors.[58] However, activation of A_3 receptors under conditions of global ischemia appears to be damaging. Indeed, A_3 receptor activation worsened damage to neurons and increased animal mortality,[59] and antagonism of A_3 receptors was protective.[61] The role of A_3 receptor activation in stroke appears to depend on spatial, cellular, mechanistic, and temporal considerations, and as such remains unclear.

One avenue of study that does suggest that anti-inflammatory actions of adenosine might protect against stroke comes from work with the methylxanthine pentoxyfylline. In spontaneously hypertensive stroke-prone rats in which inflammation has been shown to precede neuronal damage, pentoxyfylline with its anti-inflammatory and immunomodulatory properties, possibly secondary to adenosine uptake inhibition, protected against brain abnormalities (as demonstrated with magnetic resonance imaging), decreased the numbers of macrophages and activated glia in brain, and decreased the levels of inflammatory proteins peripherally.[62]

Similar to A_3 receptors, the neuroprotective actions of A_{2A} receptor activation are unclear and complicated. Antagonists for A_{2A} receptors have been shown to protect against ischemic neuronal injury, and this may be due at least in part to decreased release of glutamate.[63–68] On the other hand, the neuroprotection shown by A_{2A} agonists may have been due to alternate mechanisms mediated peripherally including increased blood flow, decreased inflammatory cell infiltration into brain,

decreased platelet aggregation, and decreased glucose metabolism.[65,69–71] Consistent with studies implicating A_{2A} receptor activation in the pathogenesis of a number of acute and chronic neurodegenerative conditions, it has been shown using pharmacological and genetic approaches that inactivation of A_{2A} receptors decreased infarct size and levels of proinflammatory cytokines caused by transient focal ischemia,[72] but increased liver damage.[73] Thus, A_{2A} receptor activation might be protective or destructive depending on the insult, receptors, and cells involved.[74] However, unclear at present is the extent to which adenosine regulation of inflammatory responses is responsible for ischemia-induced changes to neuronal cell life and death.

14.3.2 Hemorrhagic Stroke

Hemorrhagic stroke accounts for approximately 10% of all strokes among North Americans and results in 30 to 50% mortality. Cerebral hemorrhage rapidly raises levels of proinflammatory cytokines, including TNF-α, in experimental animals[75] and in cerebrospinal fluid of human patients whose CSF levels of TNF-α correlate positively with extent of brain damage.[76–78] Similar to other neurodegenerative disorders, intracerebral hemorrhage-induced elevations in TNF-α may act on astrocytes to impair removal of extracellular glutamate and dysregulate intracellular calcium homeostasis, and on microglia and macrophages to increase proinflammatory cytokine production via activation of NF-κB,[79] and increase formation of reactive oxygen species and nitric oxide.[80] TNF-α likely plays a central and pathogenic role during intracerebral hemorrhage because antisense oligonucleotides that targeted TNF-α reduced TNF-α protein levels and neuronal cell death within the hematoma, as well as neurobehavioral deficits.[81]

A single report has appeared, testing the extent to which adenosine receptor activation blocks TNF-α production associated with intracerebral hemorrhage and inhibits proinflammatory responses.[43] In this study, it was reported that the A_{2A} receptor agonist CGS 21680 decreased TUNEL positive cells, and inhibited TNF-α mRNA production by greater than 95% in the immediate area of the hematoma, possibly through interactions with infiltrating neutrophils, mononuclear cells, resident microglia, astrocytes, and neurons—all cells that express A_{2A} receptors.[82,83] Presently unclear is the extent to which CGS 21680-induced decreases in levels of TNF-α reduced neutrophil infiltration, or CGS 21680 acted directly on A_{2A} receptors on neutrophils to inhibit neutrophil activation,[84] reduce reactive oxygen species,[15,85] block the adherence of stimulated neutrophils to endothelium,[85] and decrease degranulation.[86] As will be made clearer elsewhere in this chapter, A_{2A} receptor agonists and antagonists might be pro- or anti-inflammatory, depending on the cells involved, and therefore additional studies about adenosine and intracerebral hemorrhage are warranted.

14.3.3 Traumatic Brain Injury

Motor vehicle accidents, falls, assaults, and various sports-related activities result in traumatic brain injury (TBI), which affects millions of North Americans yearly. Traumatic and posttraumatic contributors to TBI-induced neuronal cell damage include inflammation, glutamate-mediated excitotoxicity, dysregulation of intracellular

calcium levels, formation of reactive oxygen intermediates, and lipid peroxidation;[87,88] these same factors are implicated in most neurodegenerative disorders. However, it is now clear that inflammation plays a role central to the pathological responses to TBI.[89] As with many of the neurodegenerative conditions considered in this chapter, the neuroinflammatory events following TBI are mediated by brain-resident cells including microglia, astrocytes, and neurons as well as chemokine-induced recruitment into brain of blood-derived leukocytes, and many of the same proinflammatory factors are implicated.

Similar to studies of ischemic and hemorrhagic stroke, adenosine levels increase, albeit transiently, following TBI in a time- and severity-dependent manner in animals and humans.[90–95] In at least one study on patients with severe TBI, the increased levels of adenosine correlated temporally with delayed cerebral swelling.[96] On the basis of previous work showing that adenosine might have both anti-inflammatory and neuroprotective effects, the preceding studies suggested that adenosine might be protective against TBI and stimulated studies on the effects of adenosine receptor agonists and antagonists.

A number of studies have been now published showing that adenosine receptor agonists protect against deleterious effects of TBI.[91,97–101] Using a fluid percussion-induced TBI model, it was shown that TBI decreased measures of neurological function, increased transiently extracellular adenosine levels, and decreased ATP and ADP ratios, and that the nonselective adenosine receptor agonist 2-chloroadenosine protected against the TBI-induced changes to measures of bioenergetic, neurological status, and irreversible injury.[91] Using a similar, controlled cortical impact model, it was noted that 2-chloroadenosine improved motor function, the A_1 selective agonist 2-chloro-N^6-cyclopentyladenosine decreased neuronal cell death in the CA3 hippocampal subregion, and the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine worsened behavioral measures and contusion volumes.[98] In rats subjected to controlled cortical contusion, 2-chloroadenosine and CGS 21680 both increased cerebral blood flow, whereas 2-chloroadenosine decreased posttraumatic hypoperfusion.[100] With an *in vitro* model of traumatic cell death, the A_1 receptor agonist N^6-cyclopentyladenosine decreased and 8-cyclopentyl-1,3-dimethylxanthine worsened excitotoxic neuronal cell death and the release of a metabolically unstable substance capable of inducing neurotoxicity in uninjured cultures.[101]

To our knowledge, there is only one study that addressed the hypothesis that adenosine receptor manipulation might affect TBI-induced inflammation and neuronal cell death. Indirect evidence for the involvement of adenosine receptors and inflammation in concussive brain injury comes from a study with the nonselective and nonspecific adenosine receptor antagonist caffeine.[99] In this study, they reported that caffeine increased mortality, neuronal degeneration, neutrophil infiltration, edema, disruption of the blood–brain barrier and lipid peroxidation, and decreased neurological status in rats exposed to concussive brain injury. Clearly, inflammation plays an important role in the deleterious effects of traumatic brain injury, and results suggest that adenosine is formed following such insults and adenosine receptor activation may attenuate the resulting injury. However, lacking currently are studies directly linking possible anti-inflammatory actions of adenosine receptor activation with the observed protection against TBI.

14.4 ANTI-INFLAMMATORY ACTIONS OF ADENOSINE RECEPTOR ACTIVATION IN CHRONIC NEURODEGENERATIVE DISEASES

14.4.1 Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory, degenerative disorder of the CNS that affects about 1 million people worldwide and has a financial burden of about $40,000 per person annually in the U.S. The pathological hallmarks of MS include lesions within brain white and gray matter that are associated with demyelination, local inflammation, loss of oligodendrocytes, axonal degeneration, and a secondary loss of neurons.[102] Although the causes of this disorder remain elusive, there is strong evidence for three exacerbation factors: autoimmunity, excitotoxicity, and viral infection.[103,104]

In MS, levels of TNF-α are elevated in brain, serum, and CSF and hence, TNF-α has been implicated in MS pathogenesis.[105,106] This hypothesis is supported by studies of experimental allergic encephalomyelitis (EAE) in which addition of anti-TNF-α neutralizing antibodies and soluble TNF-α receptors abrogated disease development.[107] In addition, overexpression of TNF-α in transgenic mice caused demyelination similar to inflammatory demyelination observed in MS patients.[108] However, TNF-α is not the only cytokine or mediator of MS, because TNF-α receptor knockout mice are capable of developing EAE.[109] The role of TNF-α in MS is made further unclear by findings that clinical trials with anti-TNF-α antibodies did not result in clinical benefit but rather resulted in exacerbation of the disease and more severe neurological deficit.[110] Therefore, TNF-α may play beneficial and destructive roles in MS,[111] and it is important to understand more fully the endogenous mechanisms that regulate its production and relevance to the development and progression of MS.

Results from work by others and us suggest strongly that purines are involved as regulators of immune responses and cytokine production, and the pathogenesis of MS. Elevated levels of inosine and uric acid, by-products of purine metabolism, may help alleviate pathophysiological aspects of MS, and a clear inverse relationship has been established between increased uric acid levels associated with gout and a diagnosis of MS.[112–114] It has also been shown that even brief activation of adenosine receptors on oligodendrocyte progenitor cells resulted in differentiation into mature oligodendrocytes and profound increases in myelination.[115]

Adenosine and its A_1 receptors may play particularly important roles in regulating levels of TNF-α as well as IL-6 in relapsing–remitting MS patients. This conclusion was supported by four observations:[116] First, serum levels of TNF-α were significantly higher and those of adenosine were significantly lower in MS patients. Second, stimulation of A_1 receptors in peripheral blood mononuclear cells from control subjects, but not relapsing-remitting MS patients, significantly inhibited TNF-α levels. Third, stimulation of A_1 receptors in peripheral blood mononuclear cells from relapsing-remitting MS patients, but not control subjects, significantly inhibited IL-6 levels. Fourth, significantly reduced levels of A_1 receptors were found in peripheral blood mononuclear cells from MS patients. Thus, the decreased plasma

adenosine levels, the reduced levels of A_1 receptors in peripheral blood mononuclear cells, and increased A_1 receptor-mediated inhibition of IL-6 may all contribute to elevated levels of TNF-α and the pathogenesis of MS.

These findings were later confirmed and extended in a study of brain- and blood-derived macrophages obtained from MS patients.[117] In blood- and brain-derived macrophages, A_1 receptor protein and mRNA levels were decreased by about 40 to 50% in samples obtained from MS patients; similar changes were not observed for A_{2A} and A_3 receptors. Moreover, findings that the A_1-beta transcript was decreased by about 50% suggested that the receptor levels were influenced by differential splicing. Further evidence implicating A_1 receptors in the pathogenesis of MS came from findings that A_1 receptor null mice develop, following induction of experimental allergic encephalomyelitis, a more pronounced progressive-relapsing form of the disorder, increased expression of proinflammatory genes, and a down-regulation of A_1 receptors compared to wild-type mice.[118] Furthermore, upregulation of adenosine receptors following chronic caffeine ingestion lessened the severity of the experimental allergic encephalomyelitis.[118] Some of the best evidence for involvement of the anti-inflammatory actions of adenosine and its receptors in the pathogenesis of neurodegenerative disorders comes from studies on MS. Nevertheless, a great deal more research on the involvement of adenosine receptor regulation of cytokines and inflammatory molecules in the occurrence and progression of MS needs to be done.

14.4.2 Alzheimer's Disease

Alzheimer's disease (AD) is the commonest cause of dementia in people 65 yr of age or older. Defining neuropathological features of AD, including neurofibrillary tangles and amyloid plaques, are observed both in less prevalent early-onset autosomal dominant forms and the far more common sporadic forms of AD. The progressive loss of neurons and synaptic connections results in memory loss, cognitive impairment, and eventually death; as such, this disease is very costly to society and has a devastating effect on patients and families. Epidemiological studies indicated initially that inflammation was part of the pathogenesis of AD and that people taking anti-inflammatory drugs had a reduced incidence and severity of the disease.[119] According to some, there is now indisputable evidence for the involvement of inflammation in the pathogenesis of AD, and some excellent reviews on this topic have been published.[3,120] In support of this, neuropathological studies have shown two defining features of the disease, neurofibrillary tangles and amyloid plaques as well as highly activated astrocytes and microglia, to be associated with the plaques and tangles from which increased levels of proinflammatory substances are released.[121] Moreover, the effectiveness of nonsteroidal anti-inflammatory drugs in delaying the onset and lessening the severity of AD suggests strongly that inflammatory processes contribute to AD pathogenesis.

Adenosine receptor systems have been implicated in the pathogenesis of AD and inflammatory processes associated with this disease. In AD brain, there are reduced numbers of A_1 receptors in hippocampus,[122] but this reduction was later found in patients with dementia not related to AD.[123] This receptor decrease might

contribute to AD pathogenesis because A_1 receptor activation is neuroprotective and anti-inflammatory. However, others showed that A_1 receptor protein, but not gene transcription, levels were slightly higher in AD hippocampus; A_1 receptor protein was markedly increased in degenerating neurons with neurofibrillary tangles, in pretangle neurons, and in dystrophic neurites associated with senile plaques.[124] These increases in adenosine receptors might be an attempt of the brain to protect itself because the A_1 receptor agonist R-N^6-phenylisopropyladenosine was shown in neuroblastoma (SH-SY5Y) cells to increase levels of the neuroprotective soluble form of amyloid precursor protein and promote ERK-mediated tau phosphorylation and translocation to particulate fractions.[124]

That inflammation itself, as is observed in AD brain, might affect adenosine receptor systems is supported by findings that AD modeled using chronic infusions of LPS into lateral ventricles resulted in activation of microglia, brain inflammation, and reduced immunoreactivity (downregulation) for A_{2B} receptors, possibly secondarily to inflammation-induced increases in adenosine levels.[34,41] The nitric-oxide-donating derivative of the nonsteroidal anti-inflammatory drug flurbiprofen reduced LPS-induced activation of microglia and prevented A_{2B} receptor downregulation.[41] Furthermore, in AD brain, A_{2A} receptor immunopositive cells in hippocampus were shown to be microglia, thus indicating that these receptors are present where they might be having an effect.[124]

Adenosine receptors have also been implicated in therapeutic approaches to AD. Relevant to humans is the discovery of an inverse correlation between caffeine consumption and development of AD,[125] a finding similar to a previous report of a lower incidence of Parkinson's disease in individuals consuming caffeine.[126] Furthermore, caffeine, the A_{2A} receptor antagonist ZM 241385, but not the A_1 antagonist 8-cyclopentyltheophylline, prevented amyloid-induced cell death in cultured cerebellar granule cells.[127] Although the mechanisms by which the antagonists confer protection remain unclear, implicated mechanisms include glutamate clearance by astrocytes, inflammatory reactions by microglia, and vascular changes.[128] However, even though the neuroprotection occurred in the absence of microglia, this still does not discount the possible involvement of proinflammatory substances originating from the neurons themselves. Thus, inflammation is an important component of AD, and adenosine receptor manipulations have been shown to regulate inflammatory events; but much more work is required to determine the extent to which adenosine receptors might positively or negatively affect AD pathogenesis through their actions on inflammatory processes.

14.4.3 Parkinson's Disease

Parkinson's disease (PD), which occurs mainly sporadically with 1 per 1000 individuals affected over the age of 55, results from degeneration of substantia nigra neurons, and is characterized by muscle rigidity, bradykinesia, and resting tremor. Increasingly, neuroinflammation has been implicated in the pathogenesis of PD.[129,130] In substantia nigra of PD brain, a region normally enriched in microglia,[131] there are highly elevated levels of human leukocyte antigen-positive reactive microglia,[132] increased levels of PK11195 binding to activated microglia,[133] increased numbers

of activated microglia in PD patients and animal models of PD,[129,134] and elevated levels of TNF-α in brain and CSF of PD patients.[135] Moreover, there appears to be a strong correlation between development of PD later in life and earlier inflammatory processes associated with brain injuries, exposure to viruses or other infectious agents, or with intrauterine exposure to viruses or endotoxins.[132,136,137] Similarly, brain inflammation and microglial activation have been observed in animal models of PD including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine.[130,136,138] Furthermore, although the mechanisms by which they might work are unclear, immunosuppressants and anti-inflammatory drugs have been shown to decrease neuronal degeneration in animal models of PD.[139–141] Thus, there is a strong link between PD and neuroinflammation.

Much work has been conducted on involvement of adenosine and adenosine receptors in the pathogenesis and possible treatment of PD; of the receptor subtypes, the main focus continues to be on A_{2A} receptors.[142] In part, A_{2A} receptors have been focused on because of findings that levels of mRNA for and binding of the antagonist [^{3}H]SCH 58261 to A_{2A} receptors were both selectively increased in putamen of PD brain from dyskinetic patients.[143] Furthermore, increased levels of A_{2A} receptors were observed in striatum of 6-hydroxydopamine mice treated intermittently with L-DOPA to induce motor dysfunction.[144] These studies suggest that A_{2A} receptors normally enriched in striatum are overexpressed and possibly overactive in PD.[145]

A number of studies have shown that A_{2A} antagonists are protective against PD,[138,145–148] and at least one report has appeared showing that A_{2A} receptor activation can protect against L-DOPA-induced behavioral sensitization and can improve L-DOPA therapeutics.[149] The most direct evidence that A_{2A} receptor inactivation might be protective against PD through anti-inflammatory actions comes from early findings that microglia express A_{2A} receptors.[150] There is also a single recent study showing that MPTP increased levels of activated microglia in substantia nigra and that blockade of A_{2A} receptors with the A_{2A} antagonist KW-6002 decreased microglial activation and decreased neuronal cell death.[138] However, it is not yet certain whether the decreased microglial activation resulted in the neuroprotection, and additional work is required to clarify the causality involved. Although the results of neuroprotective studies with A_{2A} receptor antagonists are exciting, caveats do exist, including the potential for such agents to act systemically in promoting inflammation.[145]

14.4.4 HIV-1 Associated Dementia

The HIV or AIDS epidemic has not yet been controlled; over 40 million people are infected worldwide and the infection and prevalence rates continue to increase. Partly because people infected with HIV-1 are living longer, the prevalence of neurological complications, including impairment of cognition and motor performance, is increasing. According to some, neurological impairments are observed in approximately 60% of HIV-infected individuals, neuropathological changes are observed in about 90% of autopsied brains, and as many as 30% of untreated people develop HIV-associated dementia.[151] Of the factors that have been implicated in the pathogenesis of the cognitive/motor complex and frank dementia, microglia/macrophage activation,

increased levels of inflammatory mediators, and infiltration of peripheral immune cells correlate most closely.[152,153]

Structural and nonstructural proteins associated with HIV have also been shown to directly and indirectly activate neurons and may cause neuronal cell death secondarily to proinflammatory events.[151,154] However, despite findings that inflammation underlies mechanistically HIV-1-associated dementia and that adenosine receptor activation can regulate inflammatory events, only a few reports have appeared implicating the adenosine system in the pathogenesis of HIV-associated dementia or suggesting the use of adenosine therapeutics for this condition. In terms of effects of HIV-1 infection on adenosine receptor expression, A_1 receptor gene expression was shown recently to be increased,[155] but protein levels as determined immunohistochemically were shown previously to be not affected in HIV-1 encephalopathy brain.[117] Nevertheless, adenosine receptor activation can play a role in regulating proinflammatory events associated with HIV-1 infection because in human monocytes, A_{2A} receptor activation with CGS 21680 almost completely blocked Tat-induced increases in TNF-α through an action mediated by protein phosphatases.[156] Further, although adenosine levels were not measured, adenine nucleotide (ATP/ADP ratio) levels were increased following treatment of cultured cortical neurons with Tat.[157] Thus, increased adenosine levels and receptors might provide feedback protection against proinflammatory stimuli associated with HIV-1.

14.4.5 Huntington's Disease

Huntington's disease (HD), a genetic neurodegenerative disorder with a prevalence rate of about 6 per 100,000 people, is characterized by motor and cognitive impairment due mainly to neuronal degeneration within striatum and cerebral cortex.[158,159] The mutation involved produces a polyglutamine expansion within the N-terminal part of the huntingtin protein, and this leads to excitotoxicity and mitochondrial metabolic impairment.[160] Chronic inflammation in the striatum and cortex plays a significant role in the progression of HD,[161] and microglial cells are highly activated in HD brain, especially in the vicinity of neuron inclusions containing huntingtin protein.

Adenosine A_1 and A_{2A} receptors, both of which are enriched in striatum in which the most severe neurodegeneration occurs in HD, have been implicated in the pathogenesis of HD and have been identified as possible therapeutic targets for this devastating chronic neurodegenerative disease. A_{2A} receptor binding levels were significantly decreased in striatopallidal neurons during early phases of 3-nitropropionic-acid-induced HD-like symptoms and were increased at later time periods,[162] and were reduced in striatum of R6/2 HD transgenic mice.[163] Similar early changes in A_{2A} receptor densities were noted in transgenic mice and in HD patients.[164–168] In a transgenic rat model for a slowly progressing phenotype of HD, it was reported recently that A_{2A}, but not A_1, receptors were decreased in caudate-putamen and nucleus accumbens of heterozygous and homozygous animals.[169] In striatal cells overexpressing a truncated form of huntingtin, A_{2A} mRNA was decreased, the affinity—but not the maximal number—of A_{2A} binding sites was increased, and A_{2A} receptor-induced increases in adenylate cyclase activity were markedly elevated.[170]

In platelets, lymphocytes, and neutrophils, adenosine A_{2A}, but not A_1 or A_3, receptor numbers (B_{max}), K_D values, and function (agonist-stimulated cAMP accumulation) were significantly increased in even presymptomatic subjects carrying the mutant huntingtin gene.[171] On the basis of such results, it was suggested that changes to adenosine receptors may be exploited not only for diagnostic purposes but also for design and evaluation of therapeutic approaches.[171]

Agonists and antagonists of adenosine receptors have been used in attempts to provide protection against HD modeled genetically or chemically in animals. However, although inflammation contributes to the pathogenesis of HD in patients and in animal models of HD, rarely have the anti-inflammatory aspects of adenosine receptor activation and deactivation been studied. Using the 3-nitropropionic acid model, acute but not chronic treatments with an adenosine A_1 agonist, adenosine amine congener, decreased the size of striatal lesions, decreased neurodegeneration in striatum, and decreased resulting behavioral disorders.[172] Using the R6/2 HD transgenic mouse model, chronic administration of the A_{2A} receptor agonist CGS 21680 protected against a variety of symptoms that develop in these mice, including motor coordination, choline/creatine ratios, ventricular enlargement, the formation of neuronal intranuclear inclusions, and abnormal metabolism as indicated by high levels of blood glucose and activation of 5′-AMP-activated protein kinase.[163] The preceding studies suggest strongly that activation of adenosine receptors is protective against HD.

On the other hand, numerous studies have shown that adenosine receptor antagonists are also protective against HD. Using a quinolinic acid model of HD and electrophysiological measurements, the A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine was shown to be protective against EEG abnormalities,[173] the A_{2A} antagonist SCH 58261 increased striatal levels of glutamate,[174] and the neuroprotective properties of low-dose SCH 58261 appeared to be due to inhibition of glutamate outflow from striatum.[175] Similarly, in R6/2 HD mice, SCH 58261 decreased the outflow of glutamate from striatum.[176] More directly relevant to inflammation are findings that SCH 58261 decreased quinolinic-acid-induced increases in astrocytic hyperplasia.[175] Further evidence suggestive that adenosine receptor antagonism might be a therapeutic approach against HD comes from findings that neither A_{2A} receptor knockout mice nor wild-type mice treated with the A_{2A} receptor antagonist 8-(3-chlorostyryl) caffeine developed 3-nitropropionic-induced striatal lesions.[177]

The neuroprotective actions of adenosine antagonists appear to be mediated mainly presynaptically[158] or, alternatively, by increasing glutamate reuptake by astrocytes.[178] However, targeting A_{2A} receptors therapeutically must be approached with considerable caution because postsynaptic activation of A_{2A} receptors can exacerbate striatal lesions.[158,175] For the inflammatory component, mRNA for macrophage antigen complex-1 was shown to be increased in 3-nitropropionic-acid-treated animals, but was not altered by CGS 21680 at a dose shown to increase the size of striatal lesions.[158] Nevertheless, the anti-inflammatory actions of A_{2A} receptor agonists may be mediated through nonneuronal cells,[43,179] and the role of adenosine receptor activation overall toward inflammation in HD remains unclear and in need of further study.[160]

14.4.6 Seizures and Epilepsy

Severe and repeated seizures result in neuronal cell death, astrogliosis, microglial activation, and increased levels of proinflammatory cytokines.[180] Evidence that enhanced levels of proinflammatory cytokines play an important role in seizure susceptibility comes from studies showing that anti-TNF-α antibodies prevent seizures induced with infection by *Shigella*,[181] that transgenic mice overexpressing TNF-α develop an inflammatory demyelinating disease characterized by seizures and early death,[108] that IL-6 null mice have reduced brain inflammation following kainic-acid-induced seizures,[182] and that audiogenic seizures increase levels of IL-1α.[183] Furthermore, increased immunostaining for IL-1α has been demonstrated in activated microglia in human epileptic brain.[184] In terms of linkage between seizure-induced inflammation and neuronal cell death, it was shown that status epilepticus induced with intraamygdala injections of kainic acid led to increased hippocampal levels of TNF-α 2 and 7 d posttreatment, and these increases correlated with neuronal cell death and probably originated from activated microglia.[185]

Adenosine and adenosine receptor agonists have strong anticonvulsant properties;[186,187] a number of anticonvulsant drugs have been shown to work at least in part by releasing adenosine,[188] and strategies aimed at increasing levels of endogenous adenosine suppress convulsive seizures.[187,189] As discussed earlier in this chapter, and because adenosine and adenosine receptor agonists can decrease the levels of proinflammatory cytokines that can affect seizure initiation and duration, it is now important to know the extent to which adenosine receptor activation can decrease seizures through cytokine-mediated mechanisms; we are unaware of any such reports.

14.5 SUMMARY

Clearly, neuroinflammation plays an important role in acute and chronic neurodegenerative diseases, inflammation affects adenosine receptor expression and signaling, and adenosine receptor modulation can be both neuroprotective and anti-inflammatory. What is not known in virtually all neurodegenerative conditions is the extent to which adenosine receptor modulation is neuroprotective by virtue of its effects on inflammatory processes. Current therapeutic strategies that have targeted only a single proinflammatory substance or mechanism have largely failed. Encouraging therefore are findings that adenosine, regulators of endogenous adenosine levels, and adenosine receptor modulators affect multiple inflammatory events simultaneously as well as affecting neuronal cell survival, possibly independently of their anti-inflammatory actions. Therefore, it is important to study further the anti-inflammatory actions of adenosine with the goal of better defining mechanisms involved and to identify possible therapeutic agents for acute and chronic neurodegenerative disorders.

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15 Anti-Inflammatory and Cytoprotective Effects of Inosine

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CONTENTS

15.1 THE FORMATION AND DEGRADATION OF INOSINE

Inosine can be formed intracellularly as well as extracellularly. The two major routes for the intracellular formation of inosine are the deamination of adenosine to inosine by intracellular adenosine deaminase and the dephosphorylation of inosine monophosphate to inosine by 5′-nucleotidase (Figure 15.1). This deamination of adenosine to inosine occurs mainly at high intracellular adenosine concentrations associated with hypoxia, ischemia, and other forms of cellular stress.[1] Once inosine reaches

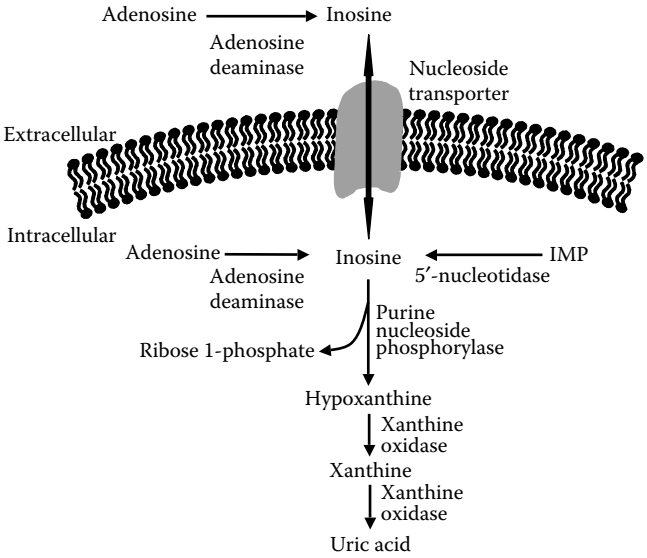

FIGURE 15.1 Schematic illustration of some of the major pathways involved in inosine metabolism. Inosine is formed from its precursor adenosine in both the intracellular and extracellular spaces. Inosine monophosphate is dephosphorylated to inosine by 5′-nucleotidase inside the cell. Intracellular inosine is shunted into the extracellular space through membrane nucleoside transporters. Inosine is degraded intracellularly to hypoxanthine and ribose 1-phosphate by purine nucleoside phosphorylase, which is further converted to uric acid by way of xanthine in a reaction catalyzed by xanthine oxidase. IMP—inosine monophosphate. (Reproduced from Hasko, G., Sitkovsky, M.V., and Szabo, C., Immunomodulatory and neuroprotective effects of inosine, *Trends Pharmacol. Sci.,* 25, 152, 2004. With permission of Elsevier Science Inc.)

high concentrations inside the cell, it is shunted into the extracellular space via the operation of bidirectional equilibrative nucleoside transporters (Figure 15.1).[2] Consistent with the fact that inosine exerts its most powerful regulatory actions in the immune system (see the following text), adenosine deaminase expression is highest in lymphoid tissues.[3] The extracellular formation of inosine is the result of conversion of adenosine to inosine by extracellular (plasma or cell surface) adenosine deaminase. Similar to inosine, adenosine is liberated from cells during metabolic stress.

The catabolic degradation of inosine takes place inside cells. Extracellular inosine gets access to the intracellular space through both equilibrative and concentrative nucleoside transporters.[2] Inosine is degraded to hypoxanthine and ribose 1-phosphate by purine nucleoside phosphorylase, which is further converted to uric acid by way of xanthine in a reaction catalyzed by xanthine oxidase (Figure 15.1). The reaction resulting in the formation of ribose 1-phosphate serves as a basis for some of the modulatory effects of inosine on cellular processes, because ribose 1-phosphate is an important source of energy under hypoxic/ischemic conditions. Three ribose 1-phosphate molecules are isomerized to two glucose 6-phosphates and one glyceraldehyde 3-phosphate, via transaldolases and transketolases of the pentose

phosphate pathway. These phosphorylated intermediates enter the glycolytic pathway, yielding a net production of eight molecules of ATP per three molecules of ribose 1-phosphate.

15.2 INOSINE-SENSITIVE CELL SURFACE RECEPTORS

It has become clear that many of the cellular actions of inosine occur through occupancy of G-protein-coupled adenosine receptors. As discussed in Chapter 1, four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3, have been identified.[4,5] The target cells of inosine express one or several of the four adenosine receptor subtypes at various levels. Inosine has been reported to interact with A_1, A_{2A}, and A_3 receptors;[6–13] however, little is known about the intracellular pathways triggered by inosine binding to adenosine receptors. A recent study implicates the ERK pathway in the cellular actions of inosine in Sertoli cells.[14] Consistent with the ability of A_1 and A_3 receptor engagement to activate $G_{i/o}$ proteins, occupancy of both of these receptors by inosine has been shown to prevent isoproterenol or forskolin-induced cAMP accumulation.[6,11] In addition, inosine has been recently demonstrated to trigger protein kinase B (PKB/Akt) phosphorylation via an A_3-receptor-mediated mechanism.[10]

15.3 INOSINE AND MAST CELL DEGRANULATION VIA ENGAGEMENT OF A_3 RECEPTORS

The first evidence of inosine's immunomodulatory effects came from the observation that inosine stimulated mast cell degranulation, an effect mediated by selective binding to A_3 receptors.[6] It was later reported that the inosine-induced enhancement of mast cell degranulation was lost in A_3 receptor null mice.[8] Inosine's potentiating effect on mast cell degranulation via A_3 receptors provides a probable explanation for the controversial observation that adenosine can both enhance and decrease mast cell degranulation.[6] According to the concept introduced by Jin et al,[6] adenosine must be converted to inosine to be able to bind to A_3 receptors and facilitate mast cell degranulation. This concept is further supported by a previous finding that adenosine deaminase inhibitors decrease the anti-IgE-induced mast cell degranulation.[15] Thus, it may be the ratio of local concentrations of adenosine to its product inosine that determines the outcome of the mast cell response to adenosine, because inosine appears to preferentially bind to A_3 receptors in mast cells and thus enhance mast cell degranulation, although adenosine can also bind to A_{2A} receptors, through which mast cell degranulation is decreased.

This concept describing the importance of adenosine-to-inosine ratio also explains why adenosine produces a complex and inconsistent vascular response with both vasodilatory and vasoconstrictor effects in some vascular preparations.[16] The vasodilatory component of adenosine's effect is likely to be secondary to occupancy of A_{2A} receptors on smooth muscle cells resulting in smooth muscle relaxation. On the other hand, the vasoconstrictor effect of adenosine is probably due to a mechanism that involves conversion to inosine and its binding to A_3 receptors on perivascular mast cells with a concomitant release of histamine, causing smooth muscle constriction in an indirect manner.

15.4 INOSINE'S ROLE IN SUPPRESSION OF MACROPHAGE, LYMPHOCYTE, AND NEUTROPHIL ACTIVATION *IN VITRO*

In contrast to its proinflammatory effects on mast cells, inosine acts as a powerful anti-inflammatory agent in macrophages and lymphocytes *in vitro*.[7] Inosine reduces the production of a number of proinflammatory cytokines, including TNF-α, IL-1, and MIP-1α, in immunostimulated mouse peritoneal macrophages. This anti-inflammatory effect of inosine does not require cellular uptake, suggesting a role for a cell surface receptor.[7] The inhibitory effect of inosine on proinflammatory cytokine production is posttranscriptional, as inosine fails to reduce steady-state mRNA levels for these cytokines. In addition to preventing the production of these soluble macrophage products, inosine also attenuates IFN-γ-induced expression of cell surface major histocompatibility complex II.[17] Finally, the anti-inflammatory effects of inosine are not confined to macrophages, because inosine attenuates IFN-γ production in mouse spleen cells stimulated with the specific lymphocyte stimulus anti-CD3 antibody.[7]

In a follow-up study, the anti-inflammatory effects of inosine were confirmed using human cells.[18] Inosine attenuated TNF-α production by immunostimulated whole blood and blocked the fMLP-induced superoxide generation by neutrophils. One important difference between the mouse and human studies is that inosine was approximately ten times less potent in exerting its anti-inflammatory effects in human cells. This difference in potency between effects observed in the two species can readily be explained by the fact that there are wide species differences in adenosine receptor expression, especially with regard to A_3 and A_{2B} receptors.[4,5]

15.5 INOSINE AND PROINFLAMMATORY EFFECTS OF ENDOTOXIN *IN VIVO*

Endotoxin (bacterial lipopolysaccharide, LPS), the major proinflammatory component of the cell wall of Gram-negative bacteria, is a central contributor to many of the pathophysiological events characteristic of sepsis and septic shock.[19] This proinflammatory effect of endotoxin can be ascribed to its ability to stimulate macrophage inflammatory mediator production via toll-like receptor 4.[19] *In vivo* evidence was provided that inosine can blunt the macrophage-mediated inflammatory response to endotoxin.[7] Intraperitoneal (systemic) treatment of mice with inosine prior to injection with endotoxin substantially ameliorated the proinflammatory cytokine storm characteristic of *in vivo* endotoxin administration. Inosine reduced the systemic levels of the proinflammatory cytokines TNF-α, IL-12, MIP-1α, and IFN-γ, whereas it increased the production of IL-10, a major anti-inflammatory cytokine. Consistent with this protective anti-inflammatory cytokine profile following inosine administration, inosine protected the mice from the lethal effect of endotoxin (Figure 15.2).[7] The endotoxin-induced cytokine storm leads to lethality by injuring a number of organs, including the gut, liver, lung, and cardiovascular system.[19] A study by Garcia Soriano and coworkers[20] revealed that inosine prevented both gut and lung injury as well as liver and vascular failure secondary to systemic endotoxin administration

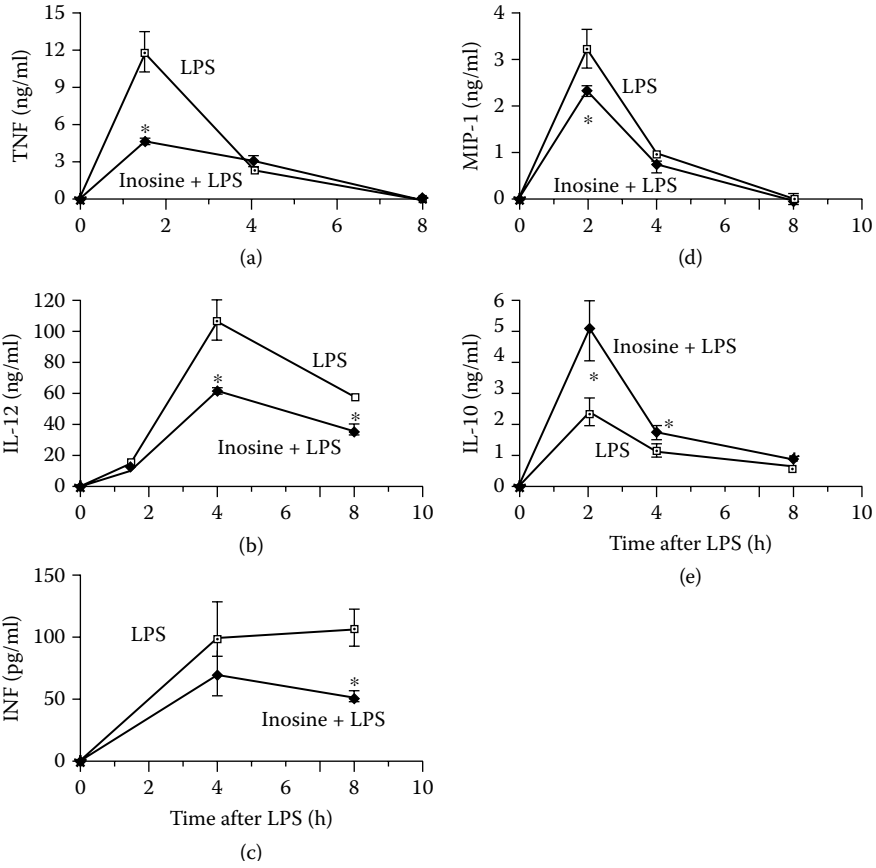

FIGURE 15.2 Inosine suppresses the production of: (a) TNF-α, (b) IL-12, (c) IFN-γ, and (d) MIP-1α, but augments (e) IL-10 formation in endotoxemic mice. Male BALB/c mice were pretreated with inosine (100 mg/kg; i.p.) 30 min before i.p. injection of 70 mg/kg of LPS. Cytokine concentrations were determined from the plasma taken at 1.5, 2, 4, and 8 h after the LPS injection. Data are means ± SEM of $n = 8$ mice. * indicates $p < .05$. (Reproduced from Hasko, G. et al., Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice, *J. Immunol.*, 157, 4634, 1996. With permission of American Association of Immunologists.)

in mice. Furthermore, the same group of investigators confirmed that inosine also exerts local protective effects, as inosine downregulates the proinflammatory response and inhibits the resultant lung injury induced by tracheal instillation of endotoxin to mice.[21] As seen with systemic endotoxin administration, this local protective effect of inosine in the lung was secondary to a suppression of macrophage-mediated proinflammatory production of cytokines, including TNF-α and IL-1, as well as IL-6. In addition, inosine reduced both lung nitric oxide formation and nitrosative stress,[21] both contributors to acute lung injury after local instillation of endotoxin.

Initial pharmacological analysis using adenosine receptor antagonists revealed that the proinflammatory effects of inosine are mediated by adenosine receptors.[7] In a recent study using A_{2A} receptor and A_3 receptor knockout mice, it was demonstrated that the protective effect of inosine against the endotoxin-induced systemic inflammatory response requires both A_{2A} and A_3 receptors.[12] The suppressive effect of inosine on systemic endotoxin-induced TNF-α production was reversed in A_{2A}/A_3 receptor double knockout mice, but not in A_{2A} or A_3 receptor single knockout mice. Interestingly, inosine protected mice also from the concanavalin A-induced systemic inflammatory response, which is mediated by T lymphocytes and natural killer cells as well as macrophages and neutrophils. In this experimental system, however, the A_3 receptor was solely responsible for this therapeutic effect of inosine. Thus, although inosine has protective actions in various inflammatory states, the receptors mediating these salutary effects may well depend on the cell types and stimulus involved.

Although endotoxemic models provide information on the early inflammatory response encountered in some forms of sepsis, results in these models do not predict or correlate with the majority of cases of clinical sepsis and/or the development of multiple organ failure. The majority of patients survive this initial hyperinflammatory response, which is a direct consequence of the liberation of endotoxin from bacteria. In fact, it is the subsequent development of infection, secondary to an immunosuppressed state seen at the later stages of sepsis, that is the major cause of the development of multiple organ failure resulting in mortality in a high number of patients. A clinically more relevant model of sepsis-mediated multiple organ failure is the mouse cecal ligation and puncture model.[22] Liaudet and coworkers[23] reported that inosine was also protective in this model of sepsis. Inosine rescued mice from the lethal effects of cecal ligation and puncture and substantially ameliorated the course of multiple organ failure. In addition, a recent study in rats subjected to hemorrhagic shock demonstrated that inosine delays mortality in a severe lethal model of hemorrhagic shock, and these effects were associated with protection against the shock-induced hyperkalemic effect.[24]

15.6 INOSINE AND CHRONIC AUTOIMMUNE/INFLAMMATORY DISEASES

Macrophages play an important role in inducing tissue injury during autoimmune diseases. Presentation of autoantigens by macrophages as well as other antigen-presenting cells to $CD4^+$ T lymphocytes in association with major histocompatibility class II (MHC II) molecules is considered to be one of the first steps in the initiation of autoimmune diseases.[25] In addition, macrophages play an early role by producing chemoattractant agents, including the chemokine MIP-1α, which are instrumental in the recruitment of inflammatory cells to affected tissues. Macrophages are also terminal effectors of the inflammatory process, as they produce cytokines and free radicals that are capable of causing direct injury to parenchymal cells.

Because inosine exerts potent macrophage deactivating effects, it is not surprising that inosine affords protection from disease development in a number of autoimmune/inflammatory animal models. These include the multiple-low-dose

streptozotocin (MLDS) and non-obese diabetic (NOD) mouse models,[26] the dextran sulfate sodium model of colitis,[27] collagen-induced arthritis,[28] cerulein-induced pancreatitis,[29] and ligature-induced gingivitis.[30]

In the MLDS-induced diabetes model, inosine reduced diabetes incidence 21 d after disease induction from 80% when administered at 100 and 200 mg $kg^{-1}d^{-1}$ to 30 and 13%, respectively. Inosine reduced pancreatic infiltration by polymorphonuclear cells and monocytes resulting in decreased pancreatic lipid peroxidation and greater survival of β cells. Similarly in NOD mice, 200 mg $kg^{-1}d^{-1}$ inosine reduced diabetes incidence from 83 to 33% at 30 weeks of age and reduced islet leukocyte infiltration. In both type 1 diabetes models, the reduced diabetes incidence observed after inosine administration was associated with a shift from T-helper-1- to T-helper-2-type cytokine profile in the pancreas. Furthermore, inosine treatment prolonged graft survival after syngeneic islet transplantation to diabetic NOD mice, indicating that inosine is also able to block an established autoimmune process.[26]

The dextrane-sulfate-induced colitis is a widely used murine model that shows some similarity to the human inflammatory bowel diseases. Oral administration of dextrane sulfate sodium (DSS) induces epithelial cell damage in the colonic mucosa, and presentation of haptens produced from DSS-bound endogenous proteins to immune cells in the gut induces a strong inflammatory response in both the mucosa and submucosal layers of the colon. Continuous or repeated administration of DSS results in a sustained immune response and leads to chronic inflammation in the colon, similar to the human ulcerative colitis and Crohn's disease. Inosine treatment dose dependently attenuated the DSS-induced colitis when administered either orally or intraperitoneally. Inosine reversed the colon shortening and weight loss, and prevented rectal bleeding in the mice, all of which are the typical signs of the disease. Significantly reduced leukocyte infiltration and lipid peroxidation were detected after inosine treatment and, on day ten of the disease, only mild histological changes were seen in animals that also received inosine. In addition, inosine significantly improved survival when treatment was started after a 10-d disease induction period, indicating that it markedly attenuates established colitis as well. The underlying mechanism of the protective effect involves altered cytokine and chemokine production, because inosine significantly reduced the MIP-1 and MIP-2 (major intrinsic protein) expression, as well as IL-1, IL-6, IL12, and TNF-α production in the colon.[27]

In the collagen-induced arthritis model, inosine was similarly effective. It reduced both the incidence (from 90 to 25%) and the severity of the disease. A decreased level of leukocyte infiltration and oxidative stress was detected in the paws of the mice and reduced levels of MIP-1α and IL-12 were seen.[28]

A mild edematous pancreatitis can be induced in Wistar rats by the repeated injection of cerulein. Inosine administration prior to the first cerulein injection attenuated the increase in serum amylase level in this model, indicating lesser tissue damage. However, a similar degree of edema was seen in the inosine-treated group. Inosine reduced leukocytic infiltration and myeloperoxidase activity in the pancreas, but had little effect on other histological changes, such as cellular vacuolization, necrosis, and hemorrhage. It also failed to decrease pancreatic TNF-α production and showed an even milder efficacy when administered after the first cerulein

injection, which was only apparent 18 h after the disease induction. The mild protective effect of inosine seen in this model may be assigned to the reduced cellular infiltration as a result of decreased cytokine-induced neutrophil chemoattractant (CINC-1) expression in the pancreas.[29]

In a rodent periodontitis model induced by ligature placement around the tooth, inosine also proved to be protective. It decreased the number of infiltrating leukocytes, and thus significantly reduced the plasma extravasation and alveolar bone resorption of the ligated side, which is a later consequence of chronic inflammation.[30]

In line with the concept that inosine prevents disease development, at least in part, by suppressing macrophage activation, it is a common finding in all these disease states that the production of macrophage proinflammatory products, including TNF-α and MIP-1α, is substantially reduced following inosine administration. One possible exemption is experimental allergic encephalomyelitis, an animal model of multiple sclerosis, in which inosine treatment is protective. However, this protection does not appear to be associated with a direct macrophage deactivating effect.[31] Rather, inosine suppresses clinical signs of experimental allergic encephalomyelitis via its metabolism to uric acid. Because uric acid has potent antioxidant properties against peroxynitrite and other oxidant species,[32] all of which are important pathophysiological factors contributing to the clinical signs of experimental allergic encephalomyelitis, it is likely that the therapeutic effects of inosine are mediated by the antioxidant action of uric acid.

15.7 INOSINE AND ISCHEMIA-REPERFUSION INJURY

Although the immune response to tissue injury plays an essential role in preserving tissue homeostasis, uncontrolled inflammation or immune activation can inflict further damage on the affected tissues. This is especially true of ischemia-reperfusion injury, in which activation of neutrophils, macrophages, and other immune cell types is responsible for much of the tissue injury following the initiating insult. Inosine levels up to approximately 6 μM have been detected in human myocardial ischemia, and many times higher levels in various experimental models of ischemia-reperfusion.[33–36] It is unclear at present whether this endogenously produced inosine is sufficient to exert tissue-protective effects. Exogenous administration of larger doses of inosine has been reported to prevent ischemia-reperfusion injury in a number of tissues, including the heart and brain.[37–40] However, until recently its mode of action was unclear. Similar to the endotoxin-induced inflammatory response, inosine attenuates the production of macrophage-derived proinflammatory cytokines, including TNF-α, MIP-2, and IL-6, following both skeletal muscle[41] and gut ischemia-reperfusion.[42] This suppression of the inflammatory response to injury is a likely mode of inosine's protective action, because neutralizing antibodies to these cytokines provide similar protection against skeletal muscle and gut injury.[43,44]

A recent report by Shen and colleagues demonstrated the beneficial effects of inosine in a rat model of middle cerebral artery occlusion and reperfusion (stroke).[45] Stroke animals receiving inosine pretreatment demonstrated a higher level of locomotor activity and less cerebral infarction. Intracerebroventricular administration of the same dose of hypoxanthine, however, did not confer protection. Coadministration of a

selective A_3 receptor antagonist significantly attenuated the neuroprotective effects of inosine, suggesting that the protective effect against ischemia/reperfusion-related insults may involve activation of adenosine A_3 receptors.

15.8 INOSINE AND HYPEROXIC CELL INJURY

A recent study demonstrated a possible modulatory effect of inosine on the extent of hyperoxic damage to the pulmonary alveolar epithelium. Rats were treated with inosine, 200 mg/kg i.p., twice daily during a 48-h period of exposure to >90% oxygen. Alveolar epithelial type 2 cells (AEC2) were then isolated and cultured. Epithelial cells isolated from inosine-treated hyperoxic rats had less DNA damage and increased antioxidant status compared to cells from hyperoxic rats without inosine. Inosine treatment during hyperoxia also reduced the proportion of epithelial cells in S and G_2/M phases of the cell cycle and increased levels of the DNA repair enzyme 8-oxoguanine DNA glycosylase. Bronchoalveolar lavage (BAL) recovered from hyperoxic, inosine-treated rats contained threefold higher levels of active transforming growth factor-beta than BAL from rats exposed to hyperoxia alone, and Smad2 was activated in AEC2 isolated from these animals. As ERK1/2 was activated by *in vivo* inosine treatment and the blockade of the MAPK pathway *in vitro* reduced the protective effect of the *in vivo* inosine treatment, it is likely that inosine treatment during hyperoxic exposure results in protective signaling mediated through pathways downstream of MEK.[46]

15.9 INOSINE AND GLIAL AND NEURONAL CELL VIABILITY DURING HYPOXIA

It has long been known that adenosine has neuroprotective effects during cerebral ischemia.[47] However, under some conditions, the neuroprotective effects of adenosine cannot be explained as an action on adenosine receptors, raising the possibility of a mechanism related to the intracellular metabolism of adenosine. Haun and colleagues reported[48] that inosine mediates the protective effect of adenosine on cell viability in rat astrocyte cultures subjected to combined glucose–oxygen deprivation. The authors came to this conclusion following the observation that the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) reversed the protective effect of adenosine. Furthermore, adenosine was rapidly metabolized to inosine, and inosine mimicked the protective effect of adenosine. Subsequent studies provided further mechanistic insight, demonstrating that the function of purine nucleoside phosphorylase was necessary for the protective effect of inosine,[49] because a purine nucleoside phosphorylase inhibitor abolished this protective effect of inosine on glial cell viability. Because hypoxanthine did not reproduce the protective effect of inosine, the next possibility was that ribose 1-phosphate, the other degradation product of inosine, was responsible for the inosine prevention of glial cell death. In fact, when iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, was added to the cells, the protective effect of inosine was lost. Because glyceraldehyde-3-phosphate dehydrogenase is part of the glycolytic pathway downstream from the entry of

ribose, it was suggested that the ribose 1-phosphate moiety of inosine conferred protection against the consequences of glucose–oxygen deprivation by providing the ATP necessary for maintaining plasmalemmal integrity. In a separate line of investigation, Litsky and colleagues[50] demonstrated that inosine administration to neuronal cells yielded a protective effect against glucose–oxygen deprivation similar to that seen in glial cells.

15.10 INOSINE AND AXONAL REGROWTH AFTER INJURY

Under normal circumstances, neurons of the mature central nervous system are unable to regenerate injured axons. With alterations in the extracellular environment, however, many neurons that otherwise show no potential for growth extend injured axons over long distances. It is well known that a number of polypeptide factors secreted by glial cells, including nerve growth factor, ciliary neurotrophic factor, and axogenesis factor, can promote axon regeneration following injury. Studies by Benowitz and coworkers[51] have identified inosine as a small molecule factor that can foster axonal regrowth in the central nervous system. These investigators first demonstrated that inosine could trigger axonal outgrowth from goldfish retinal ganglion cells.[51] This axonal outgrowth stimulated by inosine was associated with a characteristic pattern of molecular changes, most notably, upregulation of GAP-43, a protein associated with the submembrane cytoskeleton and which is a crucial regulator of axonal regrowth.[52] The primary intracellular target responsible for these changes in gene expression after inosine treatment is a 47- to 50-kDa serine-threonine kinase (protein kinase N) that is normally activated within minutes of treating cells with nerve growth factor. These *in vitro* findings were later extended to demonstrate that inosine stimulates axonal regrowth after both corticospinal tract injury[53] and stroke[54] *in vivo*.

15.11 THE MULTIPLE MECHANISMS OF INOSINE'S ACTION

As overviewed in the previous sections and summarized in Table 15.1, inosine exerts protective effects in a wide variety of experimental models of disease. It is likely that a number of separate, but possibly interrelated actions (Figure 15.3) are responsible for the biological effects of inosine.

15.11.1 Interaction with Surface Receptors

Agonists of A_{2A} and A_3 adenosine receptors have been shown to downregulate inflammatory responses in various cell types *in vitro* and *in vivo*.[55–62] The suggestion that inosine may involve activation of these receptors comes from studies demonstrating that the inhibition by inosine of systemic endotoxin-induced TNF-α production is absent in A_{2A}/A_3 receptor double knockout mice, but not in A_{2A} or A_3 receptor single knockout mice. In the case of the inosine-mediated protection from the concanavalin-A-induced systemic inflammatory response, knockout studies in mice demonstrate that the A_3 receptor is solely responsible for this action. Other actions, for example, the effect of inosine on the ERK pathway (see the following text), may be related to binding to the A_1 adenosine receptors.

TABLE 15.1
The Effects of Inosine in Preclinical Disease Models

Disease Model	Main Results
	I. Systemic inflammation models
Endotoxemia	Inosine suppressed proinflammatory cytokine production and mortality in mice[7]
	Inosine exerted protective effects on LPS-induced intestinal hyperpermeability and ameliorated the increases in myeloperoxidase and malondialdehyde in the lung and gut, indicative of reduced tissue neutrophil infiltration and oxidative stress. It also improved vascular function[20]
	Inosine protected both A_{2A} receptor/and A_3 receptor/mice from inflammation, but not A_{2A}-A_3 receptor double-null mice[12]
Cecal ligation and puncture	Significant improvement in survival and reduced organ damage were detected after inosine treatment, which was associated with lower levels of circulating cytokines and lower tissue content of chemokines. An increased hepatic NAD^+/NADH ratio, decreased MPO activity in the lung, and reduced MDA formation in the gut and liver were also found[23]
	II. Organ-specific inflammation models
Lung injury after local LPS instillation	Total leukocyte counts, myeloperoxidase, and nitric oxide production were all significantly decreased by inosine and it also suppressed nitrosative stress in the lungs. Inosine induced a shift from T helper 1 to T helper 2 type cytokine production and reduced MIP-1 α and MIP-2 production[21]
Multiple low dose streptozotocin-induced diabetes, NOD diabetes, syngeneic islet transplantation into diabetic NOD mice	Inosine protected against both the development of diabetes and against the rejection of transplanted islets. It reduced the incidence of diabetes in both streptozotocin-induced diabetes and spontaneous diabetes in NOD mice. Inosine decreased pancreatic leukocyte infiltration and oxidative stress in addition to switching the cytokine profile from a Th1 to a Th2 profile. Inosine prolonged pancreatic islet graft survival, increased the number of surviving beta cells, and reduced the number of infiltrating leukocytes[26]
Experimental allergic encephalomyelitis (EAE)	Administration of inosine suppressed the appearance of clinical signs of EAE and promoted recovery from ongoing disease[31]
Dextran-sulfate-induced colitis	Inosine protected the colon from DSS-induced inflammatory cell infiltration and lipid peroxidation. Inosine also partially reduced these parameters in an experimental model of established colitis[27]
ConA-induced fulminant hepatitis	A_{2A}-receptor-expressing mice were protected by inosine whereas wild-type and A_3-receptor-deficient mice exhibited severe liver damage[12]
Collagen-induced arthritis	Inosine reduced both the incidence and severity of arthritis. Inosine decreased both the leukocytic infiltration and oxidative stress and the levels of the chemokine MIP-1α and the inflammatory cytokine IL-12 in the treated mouse paws[28]

(continued)

TABLE 15.1 (CONTINUED)
The Effects of Inosine in Preclinical Disease Models

Disease Model	Main Results
Cerulein-induced pancreatitis	Inosine significantly decreased the elevation of serum amylase, myeloperoxidase activity, and cytokine-induced neutrophil chemoattractant-1 concentrations in the pancreas and the lung, indicative of reduced inflammation and less pancreatic necrosis. Inosine improved the outcome even if it was administered 1 h after the first injection of cerulein[29]
Ligature-induced gingivitis	Inosine treatment significantly decreased the number of infiltrating leukocytes and also significantly reduced the plasma extravasation and the alveolar bone resorption[30]
	III. Hypoxic injury
Ischemia-reperfusion injury on left anterior descending coronary artery	Inosine significantly diminished the number of ischemic points and reduced an increase in R-wave voltage induced by coronary occlusion in the pig. Myocardial extraction of measured substrates was not significantly influenced by inosine administration[37]
Tourniquet-induced skeletal muscle reperfusion injury	Inosine pretreatment resulted in reduced neutrophil infiltration into muscle and lung. Similarly, muscle and lung edema was significantly reduced with inosine pretreatment. At the end of reperfusion, serum proinflammatory cytokine levels (TNF-α and MIP-2) were significantly reduced compared to preischemia levels following inosine pretreatment[41]
Ischemia-reperfusion-induced gut barrier dysfunction and associated lung injury	Rats treated with inosine exhibited significantly reduced degree of gut barrier dysfunction and lung injury. Serum cytokine levels were also significantly lower[42]
Stroke	In adult rats with unilateral cortical infarcts, inosine stimulated neurons on the undamaged side of the brain to extend new projections to denervated areas of the midbrain and spinal cord. This growth was paralleled by improved performance on several behavioral measures[54] Animals subjected to ischemic stroke receiving inosine pretreatment demonstrated a higher level of locomotor activity and less cerebral infarction. Inosine's protection was mediated by A_3 receptors[45]
Hemorrhagic shock	Inosine and other purine nucleosides stimulated Na^+/K^+ ATPase and prolonged survival in hemorrhagic shock in rats.[24]
	IV. Miscellaneous models
Hyperoxic lung injury	Type 2 alveolar epithelial cells (AEC2) isolated from inosine-treated hyperoxic rats had less DNA damage. Inosine treatment during hyperoxia also reduced the proportion of AEC2 in S and G_2/M phases of the cell cycle and increased levels of the DNA repair enzyme 8-oxoguanine DNA glycosylase. ERK1/2 was activated in AEC2 by *in vivo* inosine treatment[46]
Unilateral transsection of the corticospinal tract	Inosine stimulated intact pyramidal cells to undergo extensive sprouting of their axons into the denervated spinal cord white matter[53]

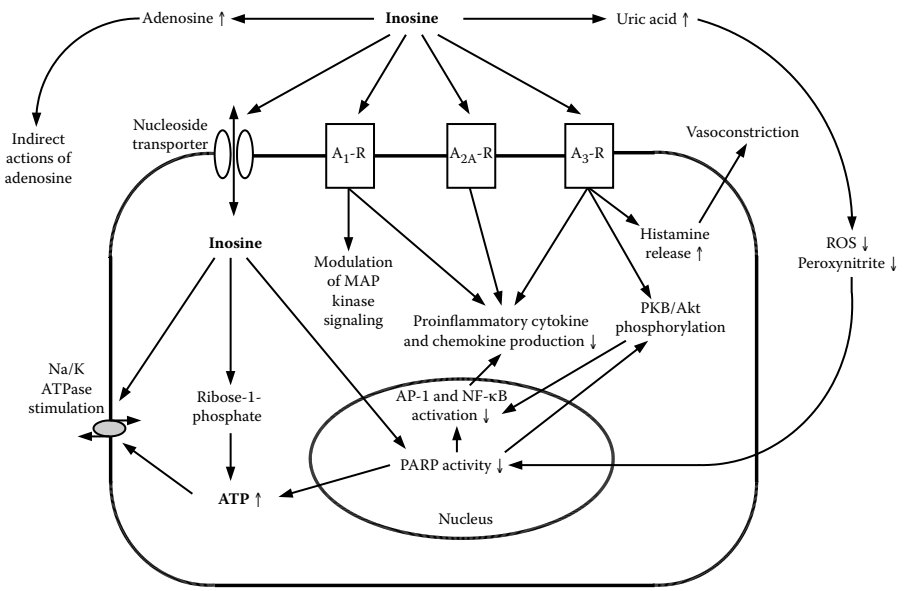

FIGURE 15.3 Multiple potential modes of the cellular action of inosine.

15.11.2 Indirect Enhancement of Adenosine's Actions

As mentioned earlier, extracellular inosine enters the intracellular space through both equilibrative and concentrative nucleoside transporters. Thus, extracellular inosine can augment extracellular adenosine levels by preventing adenosine uptake and thus generate indirect biological effects secondary to adenosine binding to its receptors.

15.11.3 Enhancement of Uric Acid Production

The breakdown of inosine leads to an increase in uric acid concentrations. Studies that began over two decades ago demonstrated that uric acid acts as a potent antioxidant against oxyradical-mediated processes.[63] More recent work also demonstrated that uric acid also acts as a neutralizer of the cytotoxic effects of peroxynitrite.[64,65] Thus, an additional mechanism of inosine's action, recently highlighted by studies in murine encephalomyelitis models,[32,66–68] may be related to direct antioxidant effects; inosine is broken down *in vivo* to produce uric acid, and uric acid has potent antioxidant properties against peroxynitrite and other reactive oxidant species. As many of the proinflammatory signal transduction pathways, including the activation of nuclear factor-κB, involve oxidant-mediated steps,[69,70] an antioxidant effect of uric acid may interfere with signal transduction pathways and consequently with the production of proinflammatory mediators.

15.11.4 Inhibition of Poly(ADP-Ribose) Polymerase (PARP)

Oxidative and nitrosative stress can trigger DNA strand breakage, which then activates the nuclear enzyme PARP. Rapid activation of the enzyme depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide, thus slowing the rate of glycolysis, electron transport, and, subsequently, ATP formation. This process

can result in cell dysfunction and cell death. PARP is also involved in the activation of proinflammatory signal transduction pathways as overviewed elsewhere.[71,72] *In vitro* studies demonstrated that various purines (hypoxanthine > inosine > adenosine) dose-dependently inhibit PARP activation in peroxynitrite-treated macrophages and also inhibit the activity of the purified PARP enzyme. Consistent with its PARP inhibitory effects, inosine also protected endotoxin-stimulated RAW macrophages from the inhibition of mitochondrial respiration.[73] Inhibition of PARP activity may be an additional cytoprotective/anti-inflammatory mechanism of inosine. It must be noted, nevertheless, that it has not yet been determined whether the concentration of inosine that is required to inhibit PARP activation in cultured cells can be reached *in vivo*.

15.11.5 Additional Cellular Mechanisms

In neuronal cell lines, several additional mechanisms of inosine's action have been identified, including protection by its breakdown product ribose 1-phosphate,[49] as well as upregulation by inosine of GAP-43,[52] a protein that is associated with the submembrane cytoskeleton and is a crucial regulator of axonal regrowth. In Sertoli cells, a recent report showed that extracellular inosine modulates ERK1/2 and p38 phosphorylation in cultured Sertoli cells, possibly through A_1 adenosine receptor activation. Interestingly, it was also demonstrated that inosine also participates in TNF-alpha modulation of ERK1/2[14] and ERK-related effects may be responsible for the protective effect of inosine on pulmonary epithelial cells exposed to hyperoxia *in vivo*.[46] In contrast, in immature human dendritic cells, the effects of inosine (increased chemotaxis, facilitation of Ca^{2+}-transients, and actin polymerization) appear to occur via a mechanism that is independent of adenosine receptors.[74] In erythrocytes, inosine (as well as other purine nucleosides, including guanosine and adenosine) stimulated Na^+/K^+ ATPase, via an effect that appeared to be independent of adenosine receptors, but was blocked by inhibitors of the equilibrative nucleoside transporter (dipyridamole or *S*-(4-nitrobenzyl)-6-thioinosine), suggesting that the mechanism of action is intracellular.[24]

In conclusion, multiple mechanisms may be responsible for the actions of inosine *in vitro* and *in vivo* (Figure 15.3). It is evident that marked cell-specific differences exist, and it is also clear that much additional work is needed to characterize the modes of inosine's cellular actions, as well as to understand the molecular basis underlying the marked cell-type-specific differences in the actions of inosine. Whatever the specific mechanisms of inosine's actions may be, these different actions may work in parallel or in synergy. Further work is needed to define the interactions between the multiple modes of inosine's action, and to determine the relative importance of these various pathways in various pathophysiological conditions.

15.12 FUTURE PROSPECTS

Inosine is a safe, naturally occurring purine, which appears to be nontoxic to humans, even when ingested at doses as high as 10 g/kg/d; in fact, inosine is widely available as a nutritional supplement in health food stores.[75–77] Inosine has been sporadically used in clinical practice for various forms of cardiovascular disorders, including certain ischemic events.[78–80] Furthermore, inosine has recently been used in small patient populations for the therapy of multiple sclerosis.[68] Table 15.2 overviews

TABLE 15.2
Some Effects of Inosine in Humans

Diseased Organ System or Functions Affected	Specific Disease or Condition	Major Findings
Cardiovascular diseases	Acute myocardial infarction and postinfarction period	Inosine improved recovery, increased cardiac output, and decreased peripheral resistance[79]
	Acute myocardial infarction	Inosine decreased free-radical lipid peroxidation and slightly increased cardiac contractility. It also stimulated the development of compensatory myocardial hypertrophy[80]
	Post-irradiation endomyocardial fibrosis	Inosine combined with potassium and magnesium aspartate caused no structural or functional improvement in the heart[87]
Neurological disorders	Gilles de la Tourette syndrome (tics)	Inosine treatment controlled the tic attacks in 75% of patients[88]
	Multiple sclerosis	Inosine increased serum uric acid level. 3 of 11 patients showed some clinical improvement and the others were protected from disease progression[68]
Recovery after surgical procedures	Renal ischemic surgery	Kidneys perfused at room temperature with fluid containing inosine showed comparable results to hypothermic protection. Only a moderate degree of transient depression in renal function was noted[89]
	Blood cardioplegia during coronary artery bypass surgery	High-dose adenosine increased serum levels of inosine and hypoxanthine, and was associated with higher ejection fraction and lower requirement of dopamine and nitroglycerine postoperatively[90]
Athletic performance	Three-mile treadmill run	Inosine supplementation had no significant effect on 3-mi run time in highly trained endurance runners. Moreover, time to exhaustion was significantly shorter after inosine treatment[76]
	Weight lifting	Inosine supplementation in top weightlifters elevated serum CPK and lipid levels and showed some effect on nerve conductivity[91]
	Cycling performance	Inosine had no ergogenic effect. Time to fatigue was shorter after inosine supplementation in a supramaximal cycling spring test, suggesting rather an ergolytic effect[77,92]
Neonatal nutrition	Milk formula supplementation with nucleotides in at-term neonates	Milk formula supplemented with cytidine, adenosine, guanosine, uridine, and inosine-5′-monophosphate increased the plasma level of polyunsaturated fatty acids[93]
	Nucleotide-supplemented milk formula in preterm neonates	Preterm formula supplementation with CMP, UMP, AMP, IMP, and GMP increased the triglyceride and cholesterol levels in the plasma[94]

some of the studies and results involving the administration of inosine to humans. Isoprinosine/inosine pranobex are immunomodulatory agents that have been used with some success in the therapy of some immune disorders.[81–84] Although the mechanism of action was never clearly understood, the possibility arises that some of the actions of these compounds may be related to the effects of inosine. As was shown by Barasoain et al., some of the effects of isoprinosine on T lymphocytes can be mimicked by inosine and adenosine *in vitro*, which suggests that these effects of isoprinosine are in fact mediated by inosine.[85] However, this is not the case with the inhibitory effect on mast cell degranulation, which is attributed to the acetamidobenzoic acid part of the complex.[86]

With an increasing body of preclinical evidence showing that inosine is efficacious in a wide variety of ischemic and inflammatory diseases, it may be worthwhile reevaluating the therapeutic potential of inosine (or prodrugs of inosine) in humans suffering from a variety of ischemic and autoimmune/inflammatory diseases.

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16 Adenosine and Infection

Christopher Moore, Edward Martin, Gail Sullivan, Joel Linden, and W. Michael Scheld

CONTENTS

16.1 INTRODUCTION

Adenosine, through its receptors, is an important mediator of inflammation reduction. In this chapter, we will discuss the role of the innate immune response to infection and its relationship to inflammation, which can often be deleterious to the host. The chapter will first describe the cellular response to adenosine and adenosine receptor agonists before discussing the role of adenosine and its analogs in the treatment of meningitis, peritonitis, septic arthritis, and sepsis.

16.2 BACKGROUND

The host response to infection is complicated and specialized. Although adaptive and cellular immunity are tailored to the specific host and pathogen, there are more conserved mechanisms that serve to protect against infection. Innate immunity utilizes specific receptors to recognize pathogen-associated molecular patterns (PAMPs), including bacterial cell wall components, to act as a first line of defense. The binding of PAMPs to toll-like receptors (TLRs) serves as the beginning of a cascade of intracellular signaling, including the translocation of nuclear transcription factor NF-κB, leading to production of several cytokines and chemokines. These cytokines and

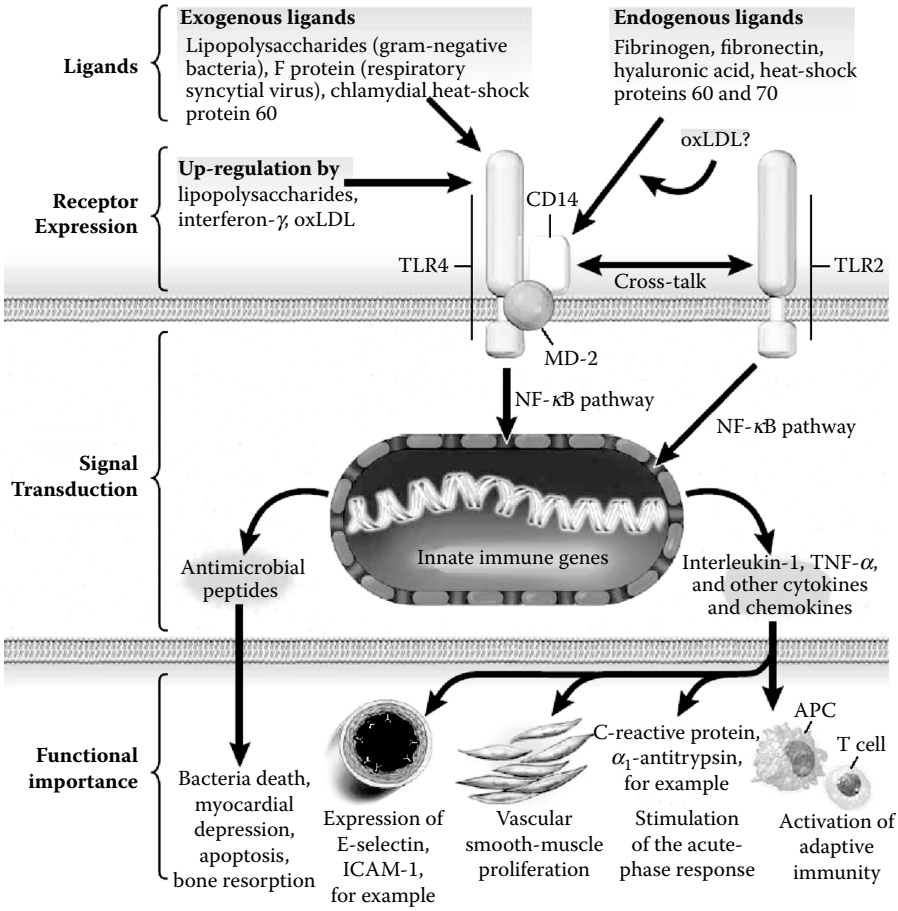


FIGURE 16.1 Pathophysiology of sepsis syndrome. (From Reference 1. With permission.)

chemokines are instrumental in recruiting inflammatory cells such as neutrophils and macrophages to sites of tissue damage (Figure 16.1).

Recruitment occurs through upregulation of endothelial adhesion molecules (e.g., selectins), which attach themselves to ligands expressed on the cellular surface of leukocytes. Ideally, this process of inflammation occurs as a local response to cellular injury and is marked by capillary dilatation and leukocytic infiltration, which serves as a mechanism to eliminate noxious agents, microbes, and damaged tissue. However, in many cases what should be a localized event becomes exaggerated and causes tissue damage beyond that caused by the original infection.[1]

Tissue damage due to infection leads to ischemia and the breakdown of adenosine triphosphate by intracellular and surface 5′-nucleotidases to adenosine. Intracellular adenosine is then transported out of the cell and may ligate to one of four

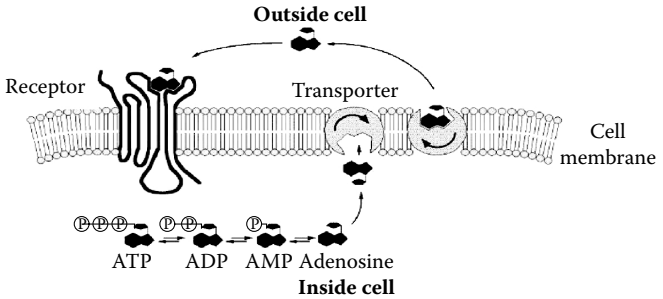


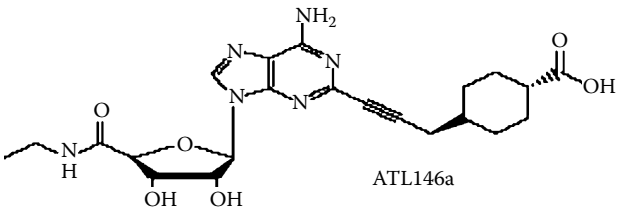
FIGURE 16.2 Tissue damage due to infection leads to ischemia and the breakdown of adenosine triphosphate by intracellular and surface 5′-nucleotidases to adenosine. Intracellular adenosine is then transported out of the cell and may then ligate to one of four adenosine receptors (ARs): A_1, A_{2A}, A_{2B}, or A_3.

adenosine receptors (ARs): A_1, A_{2A}, A_{2B}, and A_3[2] (Figure 16.2). These receptors serve different functions (Table 16.1), one of which is to decrease inflammation by way of the A_{2A} adenosine G-protein-coupled receptor. High-affinity and selective analogs that bind to the four subtypes of receptors have been synthesized (for examples, see Table 16.2). These are useful both as probes in experimental models and as potential therapeutic agents.[3]

TABLE 16.1
Adenosine Receptor Subtypes, Tissue Distribution, and Effect of Receptor Activation

Receptor Subtype	Tissue Distribution	Effect of Receptor Activation
A_1	Brain	Sedation
	Kidney	Antidiuresis
	Heart	Negative inotropy and preconditioning
A_{2A}	Striatum	Inhibition of locomotion and dilation
	Vascular smooth muscle	Anti-adhesion/anti-inflammatory
	Inflammatory cells (neutrophils, mast cells, macrophages, eosinophils, platelets, T cells)	
A_{2B}	Mast cells (dog, human)	Degranulation/proinflammatory
	Endothelium	Dilation
	Gut epithelium	Water secretion
A_3	Eosinophils	Activation

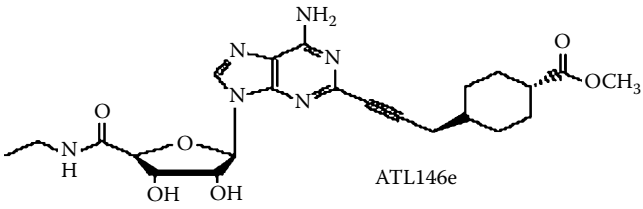
TABLE 16.2
Structure and Binding Affinities of ATL146e, ATL146a, and ATL313 for Recombinant Human Adenosine Receptor Subtypes



ATL146a

A_1 189 ± 19
A_{2A} 30 ± 2
A_{2B} > 1000
A_3 29 ± 10

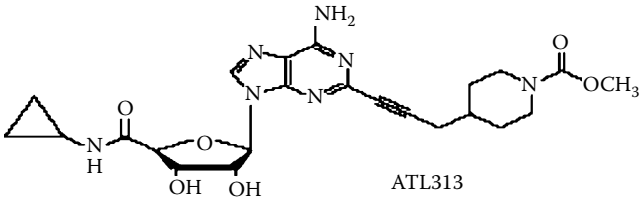
(a)



ATL146e

A_1 77 ± 12
A_{2A} 0.4 ± 0.1
A_{2B} > 1000
A_3 45 ± 15

(b)



ATL313

A_1 57 ± 3
A_{2A} 0.7 ± 0.3
A_{2B} > 1000
A_3 250 ± 70

(c)

Note: Data are the mean ± SEM for binding to the high-affinity conformational state of recombinant human adenosine receptors expressed in HEK-239 cells as determined by competition for radioligand binding.

Source: From Reference 3. With permission.

16.3 CELLULAR RESPONSE

16.3.1 Effects of Adenosine on Neutrophil Activation

The A_{2A}AR–adenylate cyclase–protein kinase A pathway appears to mediate the effects of adenosine on polymorphonuclear neutrophils (PMN; Figure 16.3; Reference 4–Reference 6). Studies of isolated human PMN show that adenosine analogs inhibit superoxide generation concentration-dependently in response to activation, but have no effect on unstimulated PMN. Potent, selective A_{2A}AR agonists, such as CGS 21680, WRC0470, and ATL146e, greatly inhibit the release of superoxide from activated PMN, strongly implying a modulatory role for that receptor.[4,5,7] Adenosine- and A_{2A}AR-selective agonists inhibit tumor necrosis factor (TNF)- or fMLP-stimulated

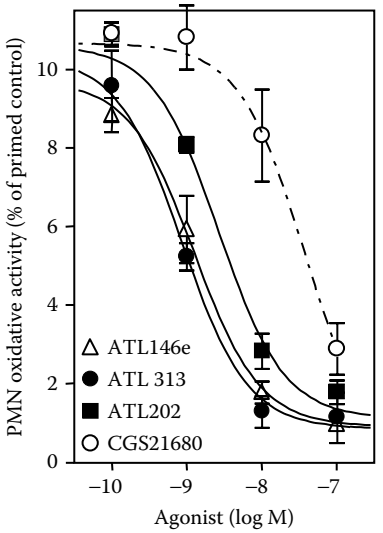


FIGURE 16.3 Inhibition of oxidative burst in human neutrophils by A_{2A}AR agonists. Neutrophils were activated with TNFα/fMLP.

oxidative activity of human PMN suspended from or adhering to a serum-coated surface.[5,8,9,10] In adherent PMN, TNF reduces cyclic adenosine monophosphate (cAMP) levels in PMN by half; conversely, pharmacologically augmenting cellular cAMP levels decreases TNF-induced PMN release of reactive oxygen species.[9] The combination of the A_{2A}AR agonist CGS 21680 and the type IV phosphodiesterase (PDE) inhibitor rolipram synergistically decreases superoxide release from adherent PMN stimulated by TNF,[11] suggesting that the adenosine effect is mediated by cAMP (Figure 16.4). The protein kinase A inhibitor H-89 abolished the ability of A_{2A}AR agonists to modulate the oxidative burst, further supporting a role for cAMP.[5]

16.3.2 Effect of Adenosine on Monocyte and Macrophage Activation

Monocytes and macrophages express the A_1AR, A_{2A}AR, and, possibly, the A_3AR.[12,13] The activation of A_1ARs increases indices of inflammation such as phagocytosis.[12,13] In contrast, activation of A_{2A}ARs decreases the release of inflammatory cytokines from endotoxin- and interleukin-1-stimulated human monocytes.[2,14,15] The activation of A_{2A}ARs alters lipopolysaccharide (LPS)-stimulated cytokine production by monocytes and macrophages, enhancing IL-10 production and altering the pattern from that of a Th1 to a Th2 response. Adenosine inhibits the release of TNF from mouse macrophages *in vitro*, and exogenous adenosine *in vivo* decreases serum concentrations of TNF in LPS-challenged mice.[16] TNF is proinflammatory, stimulating the expression of adherence factors on PMN and endothelial cells, priming PMN, and enhancing oxidative activity and degranulation of adherent PMN.[17] Endogenous adenosine and adenosine agonists reduce TNF release from human monocytes[18,19] and rat macrophages.[20]

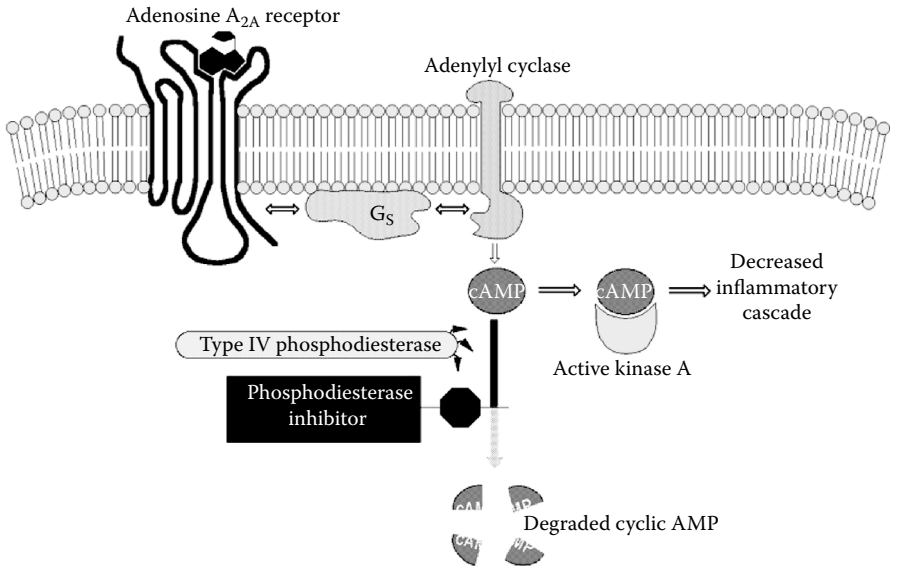


FIGURE 16.4 The A_{2A}AR–adenylate cyclase–protein kinase A pathway mediates the effects of adenosine on polymorphonuclear neutrophils. The anti-inflammatory effect of A_{2A}AR agonists can be increased with addition of a phosphodiesterase inhibitor.

The selective A_{2A}AR antagonists CSC[23] and ZM241395 block the activity of adenosine in human monocyte cultures. Thus, the activation of A_{2A}ARs suppresses TNF production *in vitro* and *in vivo* at both the transcriptional and posttranscriptional levels.

The binding of adenosine to A_{2A}ARs,[21] and perhaps A_3ARs,[22] decreases the production of interleukin-12 (IL-12) by human monocytes stimulated by LPS. Inhibition of IL-12 by passive immunization with anti-IL-12 antibody protects mice from LPS challenge, but decreases survival in a mouse Gram-negative sepsis (live *E. coli*) model.[23] In contrast to IL-12, interleukin-10 (IL-10) exerts anti-inflammatory activity in LPS-stimulated inflammation and promotes humoral immunity. It decreases LPS-stimulated release of TNF from monocytes, inhibits oxidative activity, and lowers the expression of leukocyte adhesion molecules. TNF and LPS stimulate the release of IL-10 from monocytes. Adenosine augments stimulated human monocyte production of IL-10.[24] The selective A_{2A}AR agonist CGS 21680 decreases LPS-stimulated TNF production and increases plasma IL-10 levels in mice.[25]

16.3.3 Regulation by the A_{2A}AR of T Cells

As with neutrophils, macrophages, and monocytes, T cells are also thought to play a role in the pathophysiology of inflammation and the immune dysregulation of sepsis. For example, there is large-scale T cell apoptosis during sepsis.[26] Additionally, a disruption of the balance of Th1 or Th2 response may occur during sepsis, causing an overly pro- or anti-inflammatory response that can be deleterious to the host.[27]

Similar to other hematopoeitic cells, adenosine has been shown to suppress lymphocyte activation. Adenosine, by way of A_{2A}ARs, decreases T cell production of IL-2, an important factor for the proliferation of T cells.[28,29] There is also evidence that adenosine can invoke apoptosis of T cells through DNA fragmentation, but this is thought to occur by a mechanism other than through known adenosine receptors.[30,31] Additionally, T cells are indirectly influenced by adenosine's direct effect on macrophages. IL-12 produced by macrophages is an important regulator of Th1 differentiation and lymphocyte activation. However, adenosine decreases IL-12 production by macrophages and therefore leads to a Th2-predominant T cell profile. This leads to a decrease in proinflammatory cytokine production and an increase in anti-inflammatory cytokine production.[27]

Furthermore, adenosine A_{2A}ARs are instrumental in decreasing IL-2 production by T cells, as evidenced in a study which revealed that CGS21680, an A_{2A}AR agonist, greatly reduced Tc1 and Tc2 cell interleukin-2 (IL-2) and TNF-α, with nominal effect on IFN-γ secretion. Notably, adenosine agonist concentrations that abrogated cytokine secretion did not inhibit Tc1 or Tc2 cell cytolytic function.[32] However, another recent study did document decreased IFN-γ production as a result of A_{2A}AR induction in $CD4^+$ T cells.[33]

16.4 MENINGITIS

Meningitis is characterized by central nervous system (CNS) inflammation, primarily derived from neutrophils, after bacterial entry into the cerebrospinal fluid (CSF). The bacteria release cell surface components, including LPS and peptidoglycan (PGN), that trigger resident CNS cells to produce inflammatory cytokines and chemokines, including TNF-α and IL-1β among others. These cytokines and chemokines attract and recruit PMN, which leads to adherence of neutrophils to cerebromicrovascular endothelium, and subsequent pleocytosis of the neutrophils into the CSF. Once in the CSF, neutrophils release reactive oxygen and nitrogen species and, with other resident CNS cells, cytokines and chemokines that contribute to the pathophysiology of bacterial meningitis.

A rat meningitis model has been devised to study the pathophysiology of bacterial meningitis.[4] Meningitis is induced via an intracisternal inoculation of LPS. The blood–brain barrier permeability (BBBP) is assessed by an intravenous injection of ^{125}I-labeled albumin concurrent with the intracisternal inoculation. Samples of CSF and blood are analyzed in a gamma counter, and after subtraction of background radioactivity, the percentage of BBBP is calculated by the following formula: %BBP = (cpm CSF/cpm blood) × 100.

Using this model, it has been shown that the A_{2A}AR agonist WRC-0470 (2-cyclohexylmethlidene-hydrazinoadenosine) caused a dose-dependent inhibition of pleocytosis into the subarachnoid space with 95% inhibition seen during WRC-0470 administration at 0.9 μg/kg/h ($p < .05$ vs. control). Additionally, BBBP was reduced in experimental animals with infusion of WRC-0470 at 0.6 to 0.9 μg/kg/h. A further finding revealed that the combination of the A_{2A}AR agonist WRC-0470 and the type IV phosphodiesterase inhibitor rolipram further reduced migration of leukocytes into the subarachnoid space (Figure 16.5). This same combination also decreased

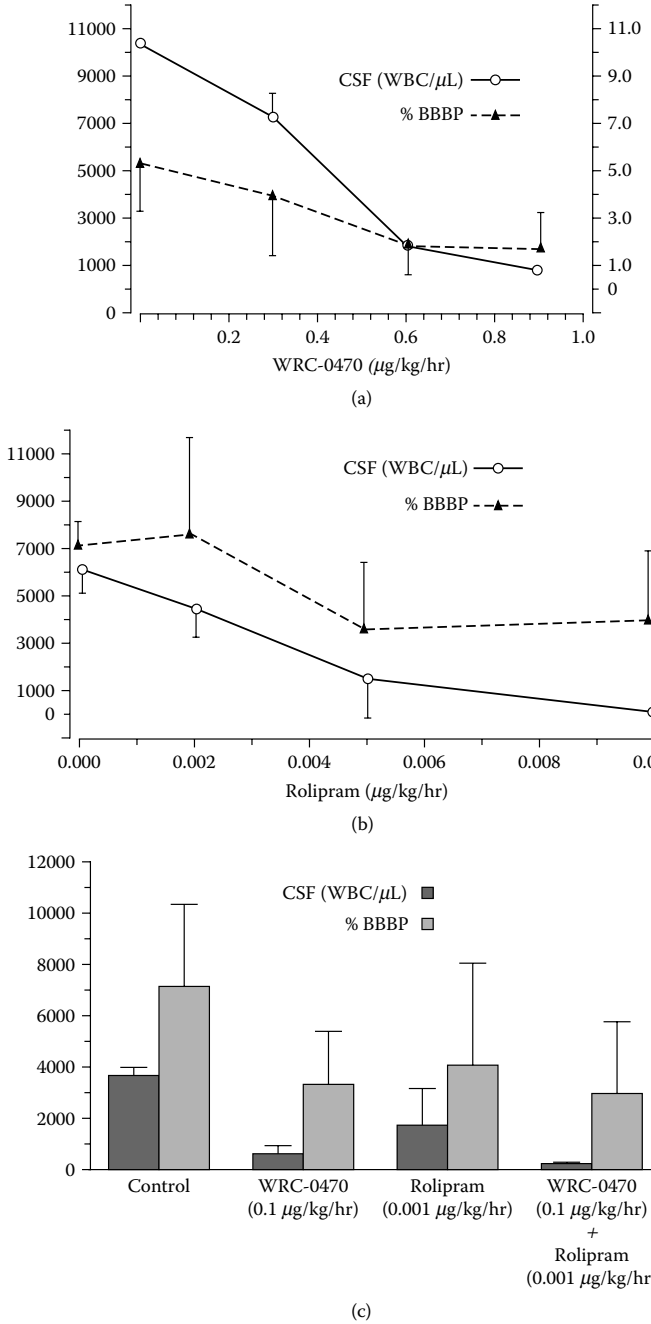


FIGURE 16.5 Effects of WRC-0470 and rolipram on lipopolysaccharide-stimulated white blood cell pleocytosis and blood–brain barrier permeability in rat bacterial meningitis model. A-C with and without WRC-0470 (0 to 0.9 μg/kg/h) (A), rolipram (0 to 0.01 μg/kg/h (B), and WRC-0470 (0.1 μg/kg/h) and rolipram (0.001 μg/kg/h) (C). Mean ± SD, n = 2 rats for each point. (From Reference 4. With permission.)

TABLE 16.3
WRC-0470 with Rolipram Synergistically Decreases Tumor Necrosis Factor (TNF)-α-Stimulated Adherent Polymorphonuclear Leukocyte (PMNL) Degranulation

PMNL Stimulated with the Following	Lysozyme (ng/ml) Released per 3×10^6 PMNL per ml per 120 min[a]
Unstimulated	15 ± 5
TNF-α	430 ± 100
TNF-α and WRC-0470 (300 n*M*)	360 ± 74
TNF-α and rolipram (300 n*M*)	400 ± 140
TNF-α and WRC-0470 (300 n*M*) + rolipram (300 n*M*)	140 ± 41

[a] Mean ± SE, 6 experiments.

Source: From Reference 4. With permission.

TNF-α stimulated adherent neutrophil oxidative activity and degranulation of activated neutrophils adhering to a biologic surface in an *in vitro* model (Table 16.3). This beneficial effect was counteracted when the A_{2A}AR antagonist ZM241385 was introduced into the model.

16.5 PERITONITIS

In a mouse sepsis model in which animals were injected ip with 20 million live *E. coli*, subsets of mice were treated with A_{2A}AR agonist ATL146e alone, or in combination with ceftriaxone.[34] Ceftriaxone was administered as a single dose of 25 mg/kg 8 h after the infection of mice with *E. coli*. ATL146e was dosed ip starting 8 h after *E. coli* injection, 8 times at 6 h intervals with 50 μg/kg.

In infected mice, treatment with ceftriaxone markedly reduced the live bacteria concentration in the peritoneal wash measured 120 h after the initial infection or within 30 min of the time of death ($p < .001$; Figure 16.6A). ATL146e did not significantly influence bacterial concentrations in the peritoneum, but treatment with ATL146e did reduce the number of live bacteria in blood, with or without concomitant treatment with ceftriaxone (Figure 16.6B). These data indicate that treatment with ATL146e does not interfere with bacterial killing by the immune system and does promote the containment of infection. In mice that received the combination of ceftriaxone and ATL146e, the number of white blood cells (WBCs; >95% neutrophils according to the results of hemocytometer examination) in the peritoneal fluid was significantly higher than that in untreated animals ($p < .01$; Figure 16.7).

Intraperitoneal injection of the nonspecific inflammatory stimulus zymosan into mice produces a brisk leukocytosis in the peritoneal cavity, which is inhibited in a dose-dependent manner by A_{2A}AR agonists (e.g., ATL146e; see Figure 16.8; Reference 35). This simple but elegant model is a useful screening tool to evaluate the

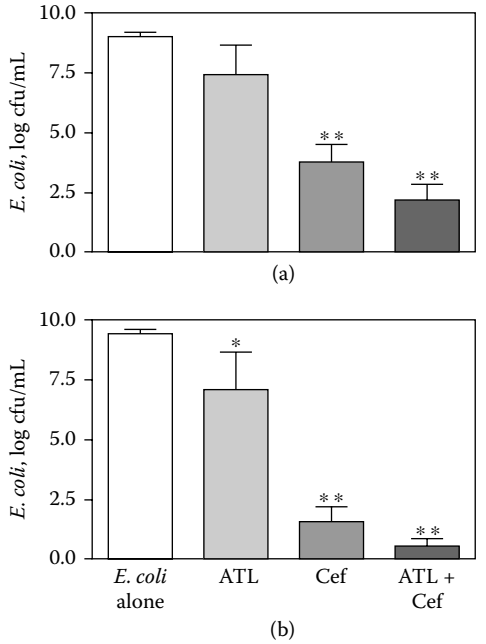


FIGURE 16.6 Treatment with ATL146e (ATL) and bacterial survival in the peritoneum vs. in the blood. Live *E. coli* organisms in a peritoneal wash (A) and blood (B) were done within 30 min of death or 120 h after infection and were counted by use of serial dilution and plate-count methods. ATL (50 μg/ml) decreased the number of live bacteria in blood (*p < .05). Treatment with ceftriaxone (cef) and treatment with cef and ATL decreased the number of live bacteria in the peritoneum and blood (**p < .001; n = 8 to 23 mice/treatment). (From Reference 34. With permission.)

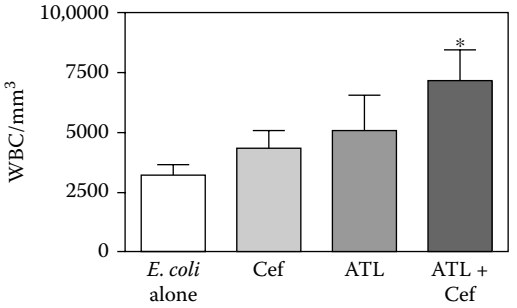


FIGURE 16.7 Treatment with ATL146e (ATL; 50 μg/kg) and ceftriaxone (cef; 25 mg/kg) increased white blood cell (WBC) migration into the peritoneum. Numbers of WBCs were determined by hemocytometer count in the peritoneal wash within 30 min of death or 120 h after infection. Treatment with ATL (50 μg/ml) and cef increased the number of WBCs in the peritoneum, compared to untreated *E-coli*-infected mice (* p < .05; n = 8 to 23 mice/treatment). (From Reference 34. With permission.)

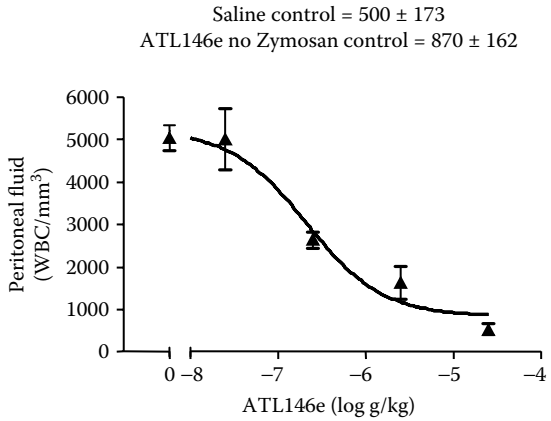


FIGURE 16.8 Increasing concentrations of ATL146e decrease peritoneal WBC concentrations.

in vivo potency of A_{2A}AR agonists prior to study in more expensive and labor-intensive discriminative models of infection in animals.

16.6 SEPTIC ARTHRITIS

Septic arthritis can result from direct inoculation of bacteria into the joint space, hematogenous seeding from another infectious source, or from direct extension from a soft-tissue infection. Morbid consequences of septic arthritis are common and include loss of range of motion, growth arrest of the involved limb, and degenerative arthritis. The pathophysiology that leads to joint destruction includes a combination of articular damage directly attributable to the bacteria themselves and the degenerative consequences of the immune response to the infectious stimulus. Proinflammatory cytokines including TNF-α and IL-1 act to recruit WBCs to the area of infection. Once in the joint space, inflammatory WBCs release reactive oxygen species along with metalloproteases, which degrade the type II collagen of the articular surface.

In general, treatment incorporates a combined medical and surgical approach. Arthroscopic irrigation and debridement, in addition to appropriate antibiotics, serve to decrease the bacterial burden and damage from proinflammatory leukocytes. There are currently no adjunctive therapies to decrease the inflammatory cytokine response to an ongoing infection. However, adenosine receptor agonists and phosphodiesterase inhibitors have been studied in a rabbit model of septic arthritis.[36] In this model, LPS was injected into the knee at varying doses followed by an injection of saline prior to needle aspiration for quantification of PMN concentrations.

Animals received systemic infusions of the A_{2A} adenosine receptor agonist WRC-0470, the type IV phosphodiesterase inhibitor rolipram, or both. Approximately 4000 WBCs were recovered from LPS-inoculated joints at an inoculum of 200 ng. With infusions of higher doses of WRC-0470 and rolipram, the WBC count dropped by

81 and 71%, respectively. The combination of WRC-0470 and rolipram at low doses showed increased activity, reducing the WBC concentration by 81% when similar doses of these compounds used alone had no effect.

These studies endorse prior *in vitro* studies[5] that implicated cAMP as part of the mechanism by which A_{2A}ARs exert their effects. Furthermore, these results suggest a potentially useful adjunctive therapy for septic arthritis. The use of A_{2A}AR agonists and type IV phosphodiesterase inhibitors would likely be most beneficial in the pre- and immediately postoperative periods when the WBC concentrations are highest and there is greater possibility of inflammatory damage.

A follow-up study[37] evaluated cartilage damage due to host inflammatory response following septic arthritis in the setting of treatment with an A_{2A}AR agonist, ATL 146e (0.1 mg/kg every 6 h). The right knees of rabbits were injected with 10^5 to 10^6 colony-forming units of *Staphylococcus aureus* (the most common cause of septic arthritis), while the left knees were injected with sterile saline. Animals were divided into one of five treatment groups: (1) untreated infected control, (2) antibiotic control, and ceftriaxone together with A_{2A}AR agonist for (3) 24, (4) 48, or (5) 72 h. Sixteen hours after joint inoculation, treatment was begun.

There was severe swelling and loss of motion of the joint at all time points in the infected-control knees. Mild joint swelling and loss of motion were observed in the antibiotic-control knees. No evidence of joint swelling or loss of motion was found in the infected knees after treatment with the A_{2A}AR agonist. Average synovial fluid aspirate WBC concentrations decreased 3.6-fold with administration of the A_{2A}AR agonist for 24 h in conjunction with antibiotic from days 1 to 7 when compared to synovial fluid aspirates of the infected-control knees. Average synovial WBC concentrations in the "antibiotic with 48 h of A_{2A}AR agonist administration" group were 2.8- to 4.8-fold lower than day 4 and day 7 concentrations in the infected-control group (p .05), and 1.2-fold lower than day 7 concentrations in the antibiotic-alone treatment group. Average WBC concentrations of the "antibiotic with A_{2A}AR agonist for 72 h" group at 7 days were 8.3-fold lower than the infected-control group ($p < .025$) and 2.2-fold lower than the antibiotics-alone group ($p = .40$).

Synovial fluid cultures were positive in three of six day-1 antibiotic-alone-treated rabbits and negative in all remaining day 4 and day 7 antibiotic-control knees. Synovial fluid cultures were positive at day 1 in all of the 24-h drug-treatment knees, but negative in the days 4 and 7 knees. All synovial fluid cultures of days 4 and 7 samples were negative in the groups following treatment with adenosine agonist for 48 and 72 h, indicating complete clearance of bacteria after a minimum of 48 h of drug treatment.

Analyses of histologic scores revealed that all treatment groups were found to have significantly lower (better) scores compared to the infected-control group ($p <$ .0002; *t*-test; $p = 2.26 \times 10^{-8}$; ANOVA). Rabbits treated with antibiotics plus A_{2A}AR agonist for 72 h had significantly improved scores compared to rabbits treated with antibiotics alone ($p < .05$; *t*-test; $p = .041$; ANOVA). Rabbits treated with antibiotics and A_{2A}AR agonist for 24 and 48 h had a trend of improved scores compared to those treated with antibiotics alone ($p > .05$; *t*-test; $p = .144$; ANOVA). Histologic qualitative examination of the joint surface stained with safranin O revealed a decrease in staining of the cartilage matrix, irregularity of the joint surface and tidemark, and

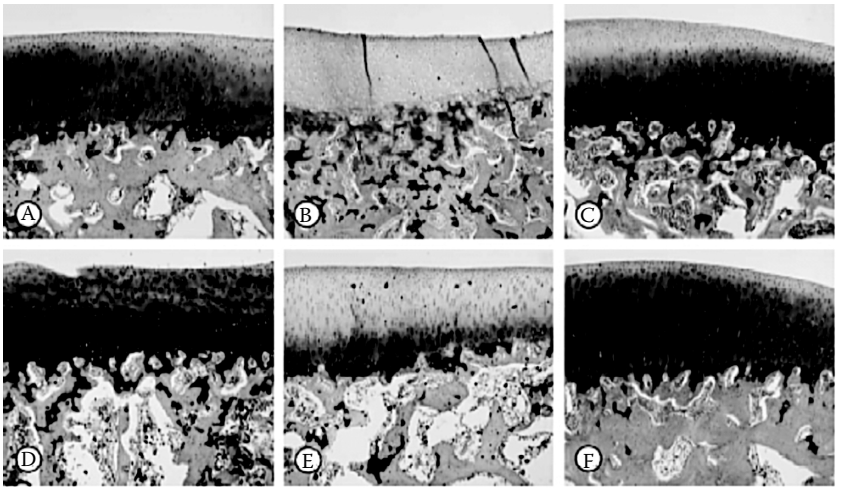


FIGURE 16.9 Histologic appearance of articular cartilage specimens at day 7 (safranin O stain, 10× magnification): (A) sterile control, (B) untreated infected control, (C) antibiotics control, (D) antibiotics + A_{2A}AR agonist × 24 h (E) antibiotics + A_{2A}AR agonist × 48 h, and (F) antibiotics + A_{2A}AR agonist × 72 h. Note the irregular joint surface, altered chondrocyte morphology and tidemark, and loss of safranin O staining in the infected control sample compared to the other groups. Note the near-normal appearance of the A_{2A}AR-agonist-treated samples, especially with 72 h of treatment. (Reprinted from Cohen, S.B. et al., Reducing joint destruction due to septic arthrosis using an adenosine 2A receptor agonist, *J. Orthopaed. Res*., 22, 427, 2004. With permission from Orthopaedic Research Society).

disorganization of articular chondrocytes in infected-control samples when compared to normal tissue (Figure 16.9). No eburnated areas of bone were found in any of the treatment groups.

Synovial tissue histology of infected-control knees showed a large, acute, inflammatory cell response when compared to normal tissue. The osteochondral and synovial histologic assessment of the antibiotic-control treatment group showed abnormal articular cartilage and moderate inflammatory changes when compared to normal, but were improved from untreated-control group specimens.

The addition of ATL146e to treatment groups resulted in diminished synovial inflammation and less articular cartilage degradation. Safranin-O-stained osteochondral tissue in the adenosine agonist 24-h treatment group revealed moderate cartilage matrix staining and a near-normal appearance of the articular surface and subchondral bone. Synovial tissue staining with H & E also demonstrated a minimal-to-moderate inflammatory response and cellular infiltrate. Histologic examination of the osteochondral sections and synovial tissue resembled nearly normal tissue after treatment with antibiotics and 48 h of A_{2A}AR agonist, with even further improvement noted in the 72-h treatment group. Despite only moderate differences in quantitative analysis of osteochondral sections treated with antibiotics alone and antibiotics with

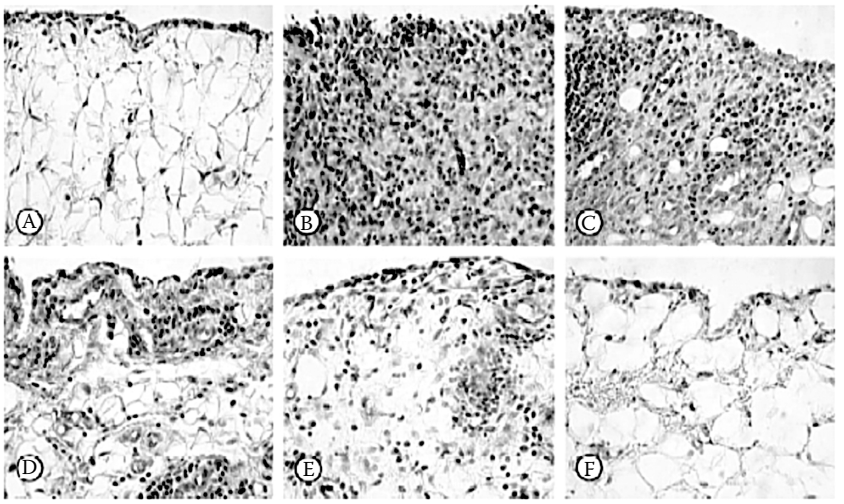


FIGURE 16.10 Histologic appearance of synovial tissue specimens at day 7 (H & E stain, 40× magnification): (A) sterile control, (B) untreated infected control, (C) antibiotics control, (D) antibiotics + A_{2A}AR agonist × 24 h, (E) antibiotics + A_{2A}AR agonist × 48 h, and (F) antibiotics + A_{2A}AR agonist × 72 h. Note the acute inflammatory response with abundant PMNLs in the infected-control and the antibiotics-only treatment groups, and the resolution of the acute inflammatory response and evidence of more chronic inflammation with occasional lymphocytes in the A_{2A}AR-agonist-treated groups. The predominance of lymphocytes appeared to diminish with increased A_{2A}AR agonist treatment. (Reprinted from Cohen, S.B. et al., Reducing joint destruction due to septic arthrosis using an adenosine 2A receptor agonist, *J. Orthopaed. Res*., 22, 427, 2004. With permission from Orthopaedic Research Society).

A_{2A}AR agonist, the histologic appearance of samples treated with A_{2A}AR agonist were noticeably more similar to normal articular cartilage than the antibiotic control (Figure 16.10).

16.7 SEPSIS

Approximately 900,000 cases of sepsis occur annually in the U.S., causing roughly 210,000 deaths and costing almost $17 billion.[38] The overwhelming inflammation that occurs along with infection during sepsis has been the target of several therapeutic interventions.[39] Unfortunately, other than the modest survival benefit offered by activated protein-C and low-dose corticosteroids,[40,41] there has been little progress in discovering an adjunctive therapy, used in parallel with antimicrobial agents, that improves survival by countering this inflammation.

Ideally, inflammation occurs as a local response to cellular injury and is marked by capillary dilatation and leukocytic infiltration, which serves as a mechanism to eliminate noxious agents, microbes, and damaged tissue. However, deleterious effects to the host manifest when this inflammatory response becomes systemic. The systemic inflammatory response syndrome (SIRS) is marked by an abnormality of

two or more of the following clinical findings: body temperature, heart rate, respiratory rate, or peripheral WBC concentration.[42] SIRS may occur in the setting of many unspecified insults, but is termed *sepsis* when it occurs in conjunction with an identifiable infection.

The clinical manifestations of septic shock are mimicked in both humans and laboratory animals after injection with endotoxin, a lipopolysaccharide generated from Gram-negative bacteria.[43] These manifestations include, but are not limited to, decreased blood pressure and increased heart rate; with higher doses, shock and death can ultimately occur.[44] Additionally, injected endotoxin induces high rates of cellular transcription and translation and increases circulating levels of inflammatory cytokines.[45]

Elevated plasma concentrations of adenosine are found in severely ill patients suffering from major trauma, cardiogenic shock, and septic shock.[44] Also, significantly higher levels of adenosine are found in nonsurvivors than in survivors of septic shock.[46] These findings are not wholly surprising when one considers that tissue hypoxia enhances breakdown of ATP, which leads to increased levels of ADP as well as ultimately to free AMP.[47] This AMP is then dephosphorylated by 5′-nucleotidases to adenosine. The release of adenosine may be further potentiated by prevention of its reutilization through the inhibition of salvage pathways.[48]

Currently, adenosine receptor agonists have not been used in clinical trials of sepsis or septic shock. However, there is now an accumulation of compelling data regarding the use of adenosine receptor agonists in animal studies. One early study evaluated adenosine and ATP in endotoxic rats. In this study, ATP-treated rats, but not adenosine-treated ones, had a statistically significant improval in survival when compared to controls (12/14 and 5/10 vs. 11/25). At that time it was suggested that ATP may be beneficial because the addition of this end product of metabolism might nonspecifically bypass mitochondrial deficits caused by endotoxemia.[49]

A later study in the early 1990s evaluated adenosine and a related synthetic adenosine analog MDL201112 (100 mg/kg).[15] This study revealed attenuation by these compounds of TNF-α, but not IL-1β, expression in activated peritoneal macrophages. A difference in mechanism between the two compounds was proposed as northern blot analysis revealed that the adenosine analog inhibited TNF-α RNA but adenosine did not, suggesting a posttranscriptional effect of adenosine. Additionally, it was noted that a survival benefit was conferred on LPS-challenged mice (90% vs. 18%) by the adenosine analog, but not adenosine. This finding supported the data presented in the prior study published approximately 20 years earlier. It was also shown that TNF-α expression in serum was attenuated in endotoxic animals treated with the analog. Lastly, this study suggested that the adenosine analog affected TNF-α production rather than abrogating TNF-α effect, given its failure to attenuate lethality caused by recombinant TNF-α.

There was concern that clinical application of adenosine and related compounds in the setting of sepsis would be limited by cardiovascular side effects, particularly hypotension. Therefore, one group took the novel approach of inhibiting adenosine kinase (AK) to increase local rather than systemic levels of adenosine. The AK inhibitor utilized in the study had been previously shown to increase endothelial cell adenosine production *in vitro*, as well as decreasing neutrophil adhesion.[50] In an LPS-induced endotoxic shock model, the investigators increased survival from 10

to 55% with a single dose of the AK inhibitor. This was a statistically significant increase in survival. A concomitant decrease in circulating TNF concentrations was also noted, as well as a decrease in neutrophil concentration in the lungs. Using a cecal ligation and puncture (CLP) model in rats, the same investigators showed a significant increase in survival with one dose of the AK inhibitor prior to CLP.

A further study in endotoxic animals attempted to elucidate a possible underlying mechanism for the enhanced survival produced by adenosine and its analogs. Hon et al.[51] studied the influence of adenosine receptors and their agonists on nitric oxide synthase and nitric oxide. Nitric oxide has been implicated as an effector of vasodilation and hypotension in the setting of sepsis. The investigators injected mice with adenosine agonists or vehicle 1 h prior to an injection of LPS. The adenosine agonists used included (1) NECA, which has equal A_1 and A_2 receptor affinity; (2) R-PIA and CHA, which have higher affinity for the A_1 receptor; and (3) CPCA, which is "specific" for the A_2 receptor. NO was maximally expressed at 8 h after the LPS inoculation (38 times control). Although each agonist was able to reduce NO production, it was found that NECA and R-PIA did so to the highest degree. NECA was studied further and found to decrease NO production early on after LPS injection. The authors hypothesized that this finding might be related to an effect on TNF production. The order of inhibitory potency for NO production was NECA ≥ R-PIA > CPCA > CHA, which was not specific for the A_1 or A_2 receptor. Therefore, it was proposed that because neither the A_1 nor A_2 receptor was specifically implicated, it was conceivable that adenosine's anti-inflammatory effect was mediated by an untested adenosine receptor.

Further work on the effect of adenosine receptor agonists and cytokine expression was undertaken by Hasko et al. in 1996.[52] This study evaluated plasma and macrophage cytokines in endotoxemic mice. Animals were exposed to 2-chloro-N^6-cyclopentyladenosine (CCPA; 0.5 mg/kg), an A_1 adenosine receptor agonist, CGS-21680 HCl (CGS; 0.2 to 2.0 mg/kg), an A_2 adenosine receptor agonist, 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-beta-D-ribofuranuronamide (IB-MECA; 0.5 mg/kg), a relatively nonselective A_3 adenosine receptor agonist,[53] and then inoculated with LPS. The findings of this study showed that LPS caused an elevation of plasma IL-10, which peaked at 90 min and returned to baseline at 6 h.

The selective A_1 agonist given 30 min prior to LPS resulted in augmented plasma IL-10 concentrations at 90 min, but only at the highest dose given (2 mg/kg). This pretreatment also decreased TNF-α concentrations in plasma at the same time point. The A_2 selective agonist also augmented IL-10 levels in plasma but delayed the peak to 180 min and the effect was dose dependent. There was also a dose-dependent decrease in TNF-α levels. Lastly, the A_3 agonist IB-MECA (0.2 and 0.5 mg/kg) also augmented IL-10 and decreased TNF-α in plasma at 90 min. However, in macrophages both the A_1 and A_2 receptor agonists decreased both IL-10 and TNF-α formation. Although LPS increased the nuclear translocation of the transcription factor NF-κB, neither the A_1 nor the A_2 receptor agonists affected translocation.

After this initial evaluation of cytokine expression, another group took on the challenge of investigating the effect of continuous intravenous adenosine on circulating neutrophils in endotoxemic pigs.[54] A total of 20 pigs received intravenous

endotoxin (5 μg/kg of body weight/hour over 330 min) with half receiving an additional continuous adenosine (150 μg/kg/min) infusion. Control animals received either adenosine or physiological saline.

The results of this study revealed the impact of LPS on circulating neutrophils including neutropenia, L-selectin shedding, upregulation of β2-integrins, increased binding of C3-coated zymosan particles, and subsequent phagocytosis by PMN. Extracellular release of superoxide anions was strongly enhanced, but phagocytosis-induced production of oxygen radicals was decreased. The addition of adenosine had no effect on neutropenia, expression of adhesion molecules, C3-induced adhesion, phagocytosis, or intracellular production of oxygen radicals. However, extracellular release of O^2 was strongly inhibited. The authors concluded that adenosine could have a positive effect on tissue damage related to oxygen radicals without impeding the bactericidal effect of PMN.

Rather than evaluate cytokine response in experimentally septic animals, Motew et al.[55] took a more macroscopic view of a rat model of sepsis and evaluated hepatosplanchnic blood flow in the presence of the nonspecific adenosine antagonist 8-phenyltheophylline (8-PTH). In this study, the investigators induced sepsis via ip inoculation of a cecal slurry (150 mg cecal material/kg). Septic animals were found to have elevated serum lactate levels compared to nonseptic controls. Hemodynamics were evaluated, and it was shown that septic animals were significantly tachycardic with a lower stroke volume. Upon administration of the adenosine antagonist 8-PTH, there was a significant reduction in blood flow to the small intestine, colon, and pancreas at 24 h. A change in mean arterial blood pressure or heart rate was not apparent. This suggests that during sepsis, adenosine-receptor-mediated vasodilation contributes to maintenance of blood flow in these regions.

A correlated finding to the hepatosplanchnic evaluation by Motew et al. was made by Thiel with a study of the effects of adenosine on cardiopulmonary functions and oxygen-derived variables during endotoxemia.[56] This study was carried out on pigs that were anesthetized and mechanically ventilated prior to the onset of endotoxemia. Endotoxemia was achieved with a 330-min infusion of *Salmonella abortus equi* endotoxin. The animals were divided into four groups. Group 1 received a continuous infusion of endotoxin. Group 2 animals received an additional intravenous infusion of adenosine, which started 30 min prior to endotoxin infusion. Groups 3 and 4 included control animals that received either saline or adenosine alone.

Intravascular catheters were deployed to measure hemodynamic parameters. Endotoxemia caused increased heart rate and decreased mean arterial pressure (MAP). Adenosine did not aggravate the arterial hypotension elicited by endotoxemia but significantly increased cardiac output by a comparably small decrease in systemic vascular resistance, prevention of pulmonary vasoconstriction, and improvement of left ventricular performance. Adenosine effectively inhibited the increase of pulmonary vascular resistance in the early phase of endotoxemia and, to a lesser degree, in the late phase of endotoxic shock. Adenosine infusion was associated with an improvement in the oxygenation index. In fact, as a result of the increase in cardiac output and the improvement of arterial oxygenation, systemic oxygen delivery increased and reached values that were almost twice as high as in

untreated endotoxic animals. The nucleoside did not significantly aggravate the endotoxin-induced decrease of the systemic vascular resistance. Further analysis included cytokine evaluation. The serum concentration of TNF-α increased to maximum values 60 min after the beginning of the infusion of endotoxin and decreased thereafter. This TNF-α expression was not altered by adenosine infusion.

Investigators then turned their attention to the effect of A_3 adenosine receptor agonists on murine cytokine production and survival during endotoxic shock.[57] Animals were injected intraperitoneally with a drug vehicle or an A_3 adenosine receptor agonist, IB-MECA (0.2 to 0.5 mg/kg), and then inoculated with LPS 30 min later. Cytokines were evaluated in treated and untreated animals, and it was shown that IL-12 and IFN-γ concentrations, which were elevated at 4 and 8 h, respectively, in untreated animals, could be reduced by pretreatment with IB-MECA. Less profound attenuation of MIP-1α and plasma nitrite and nitrate levels was noted. However, the A_3AR agonist failed to decrease the concentrations of IL-1β and IL-6, which were induced by LPS.

The anti-inflammatory cytokine IL-10 was augmented in endotoxic animals pretreated with IB-MECA (0.5 mg/kg). Therefore, the cytokine expression stimulated by LPS was studied in IL-10 null mice. In these animals, IB-MECA was still able to suppress expression of IL-12, IFN-γ, and nitrite/nitrate despite the lack of IL-10 expression. Survival studies showed a marked improvement in outcome for wild-type animals pretreated with IB-MECA (0.5 mg/kg) when compared to endotoxic controls.

Several studies have evaluated the role of adenosine in the pathogenesis of acute renal failure during endotoxic shock. For example, Churchill et al.[58] found that treatment with a nonselective antagonist, 1,3-dimethylxanthine ethylenediamine, prevented LPS-induced renal vasoconstriction. Later it was found that blocking A_1 adenosine receptors prevents LPS-induced reduction in renal blood flow and reduces the severity of acute renal failure during endotoxic shock.[59] With these findings, Nishiyama et al.[61] investigated the renal interstitial concentration of adenosine during endotoxic shock in dogs. These investigators found that LPS reduced renal blood flow and that renal interstitial adenosine concentrations were transiently increased approximately threefold. A further aim was to study the effects of an A_1 receptor antagonist (FK352) administered intravenously during LPS infusion. The A_1AR receptor antagonist reduced the renal interstitial concentration of adenosine and attenuated the reduced renal blood flow and mean arterial pressure caused by LPS. Given the rise in adenosine concentration during endotoxemia, it was suggested by the authors that adenosine is an important mediator of renal hemodynamic changes during endotoxic shock.

A study by Hasko et al.[61] evaluated the effect of inosine on inflammatory and immune processes. Inosine is another endogenous purine nucleoside, which is formed during the breakdown of adenosine by adenosine deaminase and also binds to adenosine receptors A_1, A_{2A}, and A_3. The investigators applied *in vitro* and *in vivo* techniques. Initially, peritoneal macrophages were procured from mice, inoculated with LPS after pretreatment with or without inosine, and then evaluated for cytokines. In the setting of inosine, TNF-α, IL-1, and IL-12 were suppressed in a dose-dependent manner. MIP-1α, but not MIP-2, production was also diminished under

similar conditions. Inosine did not significantly alter IL-10 or NO production in macrophages.

Use of dipyridamole, a selective blocker of nucleoside uptake, and inosine together suppressed the production of TNF-α. This finding suggests that the effect of inosine cannot be prevented by blockade of nucleoside uptake. Furthermore, the effect of inosine was similar in the absence and presence of pertussis toxin, a G protein inhibitor. Therefore, attention was focused on the adenosine receptor, and A_1 and A_2 antagonists (8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 3,7-dimethyl-1-propargylxanthine (DMPX), 3-isobutyl-1-methylxanthine) alone augmented TNF production, suggesting that endogenous adenosine inhibits cytokine production. Also, in the presence of both antagonists, the inhibition of TNF by inosine was significantly abrogated, although not completely, suggesting that the effect of inosine is at least partially mediated via adenosine receptors.

The investigators also studied the effect of inosine on signaling proteins MAPK, IκB, and JNK. The activation of these enzymes by LPS was not influenced by pretreatment with inosine. Although LPS induced the degradation of IκB at 15 min after stimulation, pretreatment with inosine did not change IκB degradation. Although LPS induced a strong increase of mRNA levels of TNF, MIP-1, MIP-2, RANTES, and TGF-β, inosine failed to suppress this response. Therefore, it was concluded that the effect of inosine on cytokine production is posttranscriptional. The same group also took an *in vivo* approach on the effect of inosine on cytokines and found that inosine decreased plasma levels of TNF, IL-1, IL-12, IFN-γ, and MIP-1, whereas it augmented the production of IL-10. It was also noted that inosine conferred significant survival protection on endotoxemic animals.

Another group tested the hypothesis that endogenous adenosine tempers proinflammatory cytokine responses and oxyradical-mediated tissue damage during endotoxemia and sepsis.[62] To do so, the investigators used a nonselective adenosine receptor antagonist, 8-sulfophenyltheophylline (8-SPT), and pentostatin, a potent inhibitor of adenosine deaminase. In rats administered endotoxin, pretreatment with pentostatin reduced TNF and IL-1β concentrations in a dose-dependent manner when compared to controls. Pentostatin had no effect on circulating IL-10 concentrations but did raise concentrations in liver and spleen when compared to controls.

In a second model of sepsis, in which rats were inoculated with cecal slurries, animals received either 8-SPT or pentostatin. Blood samples were obtained via a carotid catheter at 4 and 24 h after sepsis induction. Pentostatin reduced TNF levels in animals, whereas 8-SPT treatment resulted in significantly higher TNF concentrations in serum at 24 h. TNF was similarly reduced in concentration in the liver and spleen. The results indicate that preventing endogenous adenosine degradation with pentostatin diminishes the *in vivo* TNF response to sepsis, whereas blockade of adenosine receptors alone amplifies this response.

Oxyradical-mediated damage was also evaluated in the form of thiobarbituric acid-reactive substances (TBARS), which measure tissue products of lipid peroxidation. In septic rats treated with 8-SPT, the concentration of tissue TBARS was increased in the spleen relative to that in the water-treated septic rat group. Liver values were also consistently elevated, but not significantly. Pretreatment with pentostatin significantly reduced the tissue concentrations of TBARS relative to that of

control animals. These data indicate that endogenous adenosine is also an important modulator of oxyradical damage after a septic challenge. Additionally, pentostatin reduced mortality after septic challenge.

A subsequent link between adenosine and nitric oxide was found by Sam et al.[63] In an innovative study, these investigators utilized nonselective NOS inhibitors, as well as nonselective adenosine receptor blockade, to determine the interdependence of adenosine and nitric oxide mediation of regional hepatosplanchnic perfusion. Rats had sepsis induced via ip injection of a cecal slurry. Twenty-four hours after sepsis induction, animals were divided into two groups. One group received the adenosine antagonist 8-SPT (0.9 mg/kg × 10 min) followed by infusion with a combination of the adenosine and nitric oxide antagonists. The second group received the nitric oxide antagonist L-nitro-arginine-methyl ester (0.5 mg/kg × 10 min) followed by the antagonist combination. Each group had hemodynamic and regional hepatosplanchnic blood flow measurements recorded prior to infusions, 10 min after initiation of each single-agent infusion, and again after double-agent infusion.

Twenty-four hours after sepsis induction, rats exhibited evidence of systemic shock with elevated TNF concentrations. There were no significant changes in MAP or heart rate as a result of the infusion of either agent, given separately or combined, in nonseptic or septic animals. In septic rats, adenosine receptor blockade alone increased hepatosplanchnic and skeletal muscle vascular resistance, but no further increases were seen when the nitric oxide synthase inhibitor was added. Nitric oxide synthase inhibition alone increased hepatosplanchnic and skeletal muscle vascular resistances. When the adenosine receptor blocker was added to the infusion, skeletal muscle vascular resistance increased significantly more than with nitric oxide synthase inhibiton alone. However, there was no further increase in hepatosplanchnic resistance. Additionally, renal and adipose vascular resistance increased with nitric oxide synthase antagonism, but not with adenosine receptor blockade.

Therefore, in the hepatosplanchnic and skeletal muscle vasculature, all of the resting nitric-oxide-mediated vasodilation is secondary to endogenous adenosine action; but in adipose and renal vasculature, resting nitric-oxide-mediated vasodilation is independent of adenosine. Endogenous adenosine also appears to play a significant role in determining resting skeletal muscle resistance that is independent of nitric oxide synthase during sepsis.

Further cytokine analysis was done by Tofovic et al. in 2001.[64] In this study, the effects of endogenous adenosine on endotoxin-induced release of cytokines and changes in heart performance and neurohumoral status in early, profound endotoxemia in rats were investigated. Endogenous adenosine concentration was increased by the adenosine deaminase inhibitor erythro-9[2-hydroxyl-3-nonyl] adenine (EHNA), and the effects of endogenous adenosine were blocked by caffeine, a nonspecific adenosine receptor antagonist.

Time and pressure variables of heart performance and blood pressure were recorded continuously and plasma levels of TNF, IL-1β, plasma renin activity (PRA), and catecholamines were determined before and 90 min after administration of endotoxin (30 mg/kg). The adenosine deaminase inhibitor had no effects on measured time-pressure variables of heart performance under baseline conditions and during endotoxemia, yet significantly attenuated endotoxin-induced release of

cytokines and PRA. Pretreatment with caffeine not only prevented the effects of EHNA but also increased the basal release of cytokines and augmented PRA. Caffeine attenuated endotoxin-induced release of cytokines and augmented endotoxin-induced increases in plasma catecholamines and PRA. Pretreatment with propranolol abolished the effects of caffeine on heart performance and neurohormonal activation during the early phase of endotoxemia. The A_1 adenosine receptor agonist CPA induced bradycardia and negative inotropic effects, reduced workload, and inhibited endotoxin-induced tachycardia and renin release. However, the selective A_{2A} adenosine receptor agonist CGS 21680 decreased blood pressure under basal conditions but did not potentiate decreases in blood pressure during endotoxemia. This same agonist completely inhibited endotoxin-induced release of TNF, augmented sympathetic activity, and PRA. The authors concluded that inhibition of adenosine deaminase reduces cytokine release *in vivo* without producing significant hemodynamic and cardiac effects during the early phase of profound endotoxemia in rats.

Further investigation into the receptors responsible for the anti-inflammatory effect of inosine has also been made. Gomez et al.[65] investigated the effect of inosine on wild-type A_{2A} adenosine receptor knockout (KO) and A_3 adenosine receptor KO mice. Mice were pretreated with inosine followed by an injection of LPS. TNF in serum was measured 1 h later. LPS-stimulated TNF was inhibited in wild-type mice. This decrease in inflammation was manifested by an improvement in survival vs. control animals (15% mortality vs. 85%). TNF was also inhibited in the absence of A_{2A}ARs or A_3ARs in single-gene knockouts. However, combined A_{2A}AR and A_3AR knockout animals were unresponsive to the inhibitory effect of inosine and maintained high levels of LPS-induced TNF production *in vivo* compared to wild-type littermates. It was therefore thought that inosine utilized both A_{2A}AR and A_3 receptor subtypes to inhibit endotoxin-induced inflammation *in vivo* in this model of LPS-induced endotoxemia.

A second component of this study involved adenosine receptor agonists. Injection of an A_{2A}AR agonist, CGS21680 (0.5 mg/kg), or A_3AR agonist and A_1AR agonist (2-Cl-IB-MECA; 0.5 mg/kg) into wild-type mice resulted in decreased TNF levels in blood serum following lethal injection of LPS. Additionally, the A_{2A}AR agonist had no effect in A_{2A}AR KO mice and, likewise, the A_1AR and A_3AR agonists had no effect in A_3AR KO mice. These findings supported the notion that both A_{2A}AR and A_3 receptor subtypes play a role in the negative regulation of LPS-induced TNF and that inosine utilized both receptor subtypes to negatively regulate endotoxin-induced inflammation *in vivo*.

Finally, the authors of this chapter have shown that A_{2A}AR agonists reduce mortality in a murine model of septic shock.[34] In this study, repeated administration of the selective ATL146e, an A_{2A}AR agonist (8 bolus ip doses, beginning at the time of administration of the LPS challenge and repeated at 6-h intervals for 48 h), produced dose-dependent protection against LPS-induced mortality compared to the incidence of mortality in control animals. At the highest dose, ATL146e resulted in 100% survival on day 4 vs. 25% in untreated controls. Animals that survive four days continue to do well (no late deaths; Figure 16.11A). Importantly, it was shown that A_{2A}AR agonists reduce mortality in a murine model of endotoxin-induced septic shock even after a delay in the onset of therapy (Figure 16.11B). In separate studies,

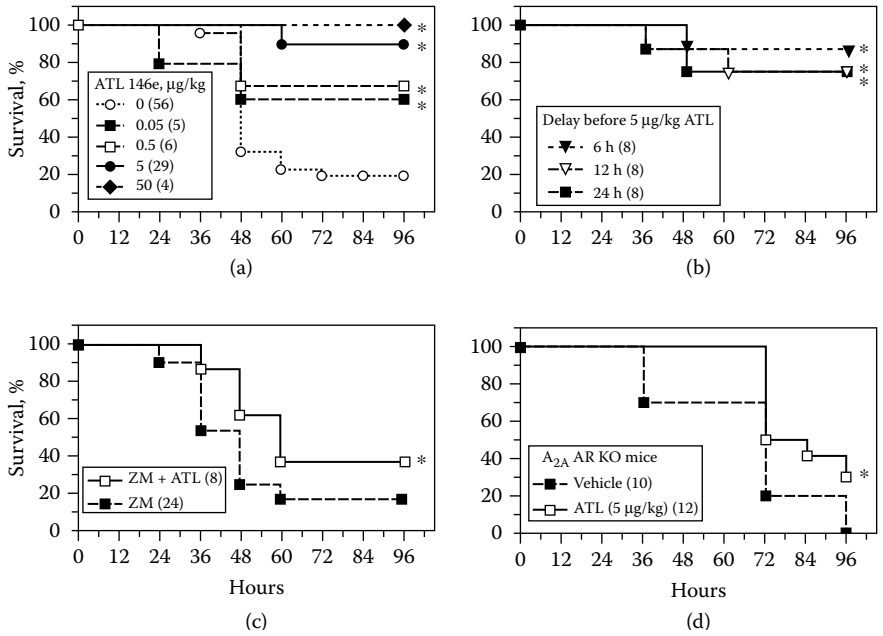


FIGURE 16.11 Treatment with ATL146e (ATL) and decreased lipopolysaccharide (LPS)-induced mouse mortality via A_{2A} adenosine receptor (AR)-mediated mechanisms. Female C57Bl/6 mice (~20 g) were injected intraperitoneally (ip) with LPS from *E. coli* (O26:B6; 12.5 mg/kg). (A) One hour before challenge with LPS, and at 6-h intervals, vehicle or ATL was injected (0.05 to 50 µg/kg) ip for a total of 8 doses/48 h. ATL protected the mice, compared to LPS-challenged mice in the absence of ATL (*$p < .05$). (B) Treatment with ATL (5 µg/kg) was delayed 6 to 24 h after challenge with LPS. ATL protected the mice, compared to LPS-challenged mice in the absence of ATL (*$p < .05$). (C) Selective A_{2A}AR antagonist ZM241385 (ZM) decreases ATL protection. Mice were treated with ATL (5 µg/kg) as in panel A and were also treated ip with ZM (3.4 µg/ml) on the same schedule as ATL dosing. ZM blocked ATL protection compared to treatment with ATL, in the absence of ZM (*$p < .05$). (D) ATL did not increase survival after challenge with LPS in mice that did not have the A_{2A}AR gene. The A_{2A}AR knockout (KO) mice were challenged with LPS and treated as described in panel A, with or without ATL (5 µg/kg). ATL did not provide as much protection in these mice as it did in wild-type mice (*$p < .05$). Numbers in parentheses are the numbers of mice per treatment. (From Reference 34. With permission.)

the effects of delayed administration of ATL146e on LPS-induced mortality in mice were examined. In these experiments ($n = 15–16$ per group; LPS 12.5 mg/kg), ATL146e was administered four times at a dose of 5 µg/kg ip at 6-h intervals, beginning 6, 12, or 24 h after challenge with LPS. ATL146e reduced mortality even when 24 h elapsed between LPS challenge and treatment. These findings suggest that A_{2A}AR agonists may be beneficial even in advanced, severe sepsis syndromes. These kinds of experiments are important because patients with sepsis syndrome and septic shock are often evaluated and treated many hours after the onset of symptoms.

These studies confirmed that the A_{2A}AR mediates the protective effect of ATL146e on mortality in the murine model of endotoxin-induced septic shock. Two other experiments were carried out to establish the relationship between the protective effects of ATL146e on mortality in the mouse model of sepsis and the activation of the A_{2A}AR. In the first experiment, ATL146e was administered alone or in combination with ZM 241385, a potent and highly selective antagonist of the A_{2A}AR; dosing with ATL146e alone or in combination with ZM 241385 began 12 h after LPS challenge followed by repeated administration of each regimen for 24 h. Figure 16.11C (left panel) shows the results from the first study; in this experiment, ZM 241385 given alone did not protect against LPS-induced mortality, whereas ATL146e alone protected against it. Administration of ZM241385 in combination with ATL146e nearly abolished the protective effect of ATL146e on LPS-induced mortality (Figure 16.11C).

It had previously been shown by Sitkovsky[66] that subthreshold doses of an inflammatory stimulus that caused minimal tissue damage in wild-type mice were sufficient to induce extensive tissue damage, more prolonged and higher levels of proinflammatory cytokines, and death of male animals deficient in the A_{2A}AR. Similar observations were made in studies of three different models of inflammation and liver damage, as well as during bacterial endotoxin-induced septic shock. These findings were corroborated by the Scheld group, which documented that the survival benefit of ATL146e was abolished in animals devoid of A_{2A}ARs.

PCR and immunohistochemical studies of A_{2A}ARs in wild-type (WT) mice and A_{2A}AR KO mouse basal ganglia using a selective A_{2A}AR monoclonal antibody confirmed the absence of A_{2A}ARs. Marker-assisted selection then transferred the mutant A_{2A}AR into C57BL/6J mice, permitting the use of congenic WT littermates as controls.

A_{2A}AR KO and congenic WT mice were inoculated with a 12.5 mg/kg dose of LPS, and the first dose of ATL146e (5 μg/kg) was administered 12 h later followed by three additional doses at 6-h intervals. Figure 16.11D shows that ATL146e protected against LPS-induced mortality in the WT but not the A_{2A}AR KO mice, evidence that the protection produced by administration of ATL146e is mediated through activation of the A_{2A}AR.

Further studies revealed that A_{2A}AR agonists (ATL146e) reduce mortality in a murine model of septic shock following challenge with live bacteria. These experiments compared mortality in animals receiving no treatment to those treated with ATL146e alone, ceftriaxone alone, or a combination of ceftriaxone and ATL146e. Ceftriaxone was administered at a single dose of 25 mg/kg 8 h following inoculation of *E. coli*. The dose of ATL146e was 50 μg/kg, commencing 8 h after the *E. coli* challenge and repeated at 6-h intervals for a total of eight doses. Survival was monitored for 10 d. All animals surviving after 5 d continued to do well. Figure 16.12 shows that the combination of a single dose of ceftriaxone with multiple doses of ATL146e was highly effective; 100% of the animals survived compared to the 92% death rate in the controls and a mortality of 62.5% in mice receiving ceftriaxone alone ($p < .001$ vs. controls and $p < .05$ vs. ceftriaxone alone). Mice receiving ceftriaxone had reduced bacterial concentrations in the peritoneal fluid (PF) and blood 2 d after inoculation, but bacterial concentrations were unaffected in mice

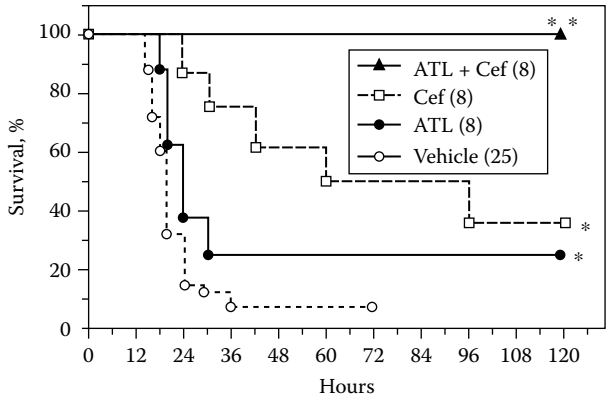


FIGURE 16.12 Treatment with ATL146e (ATL) in mice challenged with live *E. coli.* Mice were injected intraperitoneally (ip) with 20 million live *E. coli,* and were treated with ATL, the antibiotic ceftriaxone (cef), or a combination of cef and ATL. Cef was administered at a single dose of 25 mg/kg 8 h after the inoculation of mice with *E. coli.* ATL was dosed ip 8 h after the *E. coli* injection, 8 times at 6-h intervals with 50 µg/kg. ATL or cef alone protected the mice, compared with untreated mice (*$p < .05$). Treatment with ATL and cef protected mice better than treatment with cef alone (**$p < .05$). Numbers in parentheses are the number of mice per treatment. (From Reference 34. With permission.)

receiving ATL146e alone. Furthermore, bacterial concentrations in PF and blood were lower in mice receiving the combination regimen. The number of PMN in the peritoneal fluid was significantly higher in animals receiving combination therapy.

16.8 CONCLUSION

To date, a wealth of research has been performed in the area of adenosine, adenosine receptors, and infectious diseases. This work has demonstrated the powerful anti-inflammatory nature of adenosine and compounds that bind adenosine receptors, specifically the A_{2A}AR. Immunomodulatory therapies are urgently needed in many areas of infectious diseases and particularly in the setting of sepsis. Therefore, the results of studies that have documented the robust survival benefit of A_{2A}AR agonists in experimental sepsis are particularly encouraging given the need for adjunctive therapies for use in combination with targeted antibiotic therapy in the setting of clinical sepsis. Future research will be directed to the mechanism of the anti-inflammatory effect of adenosine and its receptors, including cellular and molecular mechanisms.

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17 Regulation of Peripheral Inflammation by Spinal Adenosine

Sanna Rosengren and Gary S. Firestein

CONTENTS

17.1 INTRODUCTION

Novel drug targets are being continually sought for the unmet medical needs associated with inflammatory diseases such as sepsis and rheumatoid arthritis. In the quest to identify naturally occurring substances that affect inflammation, the purine adenosine was discovered to have several properties that allow it to regulate inflammatory events, including inhibitory effects on neutrophil activation,[1–3] production of inflammatory mediators,[4,5] vascular permeability,[6,7] and pain pathways.[8–10] The therapeutic utility of adenosine itself is limited by cardiovascular side effects,[11] but compounds that enhance adenosine levels might offer a viable alternative. One such class of compounds is inhibitors of adenosine kinase, which exert their biological effect by increasing tissue levels of adenosine[12] and have demonstrated anti-inflammatory effects *in vivo*.

While examining several novel adenosine kinase inhibitors (AKI),[13,14] we found that highly lipophilic compounds that possessed analgesic and antiseizure characteristics, and were hence very likely to cross the blood–brain barrier in an

efficient manner, blocked peripheral inflammation much more effectively than water-soluble molecules (unpublished observations, 1995). In light of findings that describe dampening effects of intrathecally administered A_1 adenosine agonists[15,16] and systemically administered lipophilic AKI[17] on the development of hyperalgesia, which occurs following an inflammatory insult and is due to sensitization of neurons in the dorsal horn of the spinal cord,[18] we hypothesized that the enhanced anti-inflammatory effect of the analgesic AKI was due to increased adenosine levels, not only in the peripheral tissues but also in the spinal cord. Hence, we studied the effect of adenosine agonists administered intrathecally on acute and chronic peripheral inflammatory events. To our surprise, spinal adenosine receptor stimulation exhibits profound effects in both settings, which at least partly is due to inhibition of spinal pathways involving excitatory amino acids such as glutamate. These results allowed identification of a previously unknown pathway of interaction between the immune and nervous systems, in which adenosine appears to play a key role both centrally as well as peripherally, at the site of the inflammatory insult.

17.2 THE EFFECT OF INTRATHECAL ADENOSINE AGONISTS ON ACUTE INFLAMMATION

Using chronic catheters positioned in the intrathecal space (underneath the dura) of rats and ending in the lumbar area, compounds can be administered directly to the spinal area of interest,[19] permitting the study of the effects of several different adenosine agonists in inflammation models, such as the rat skin lesion model. In this model, carrageenan or other proinflammatory substances are injected intradermally, and the resulting neutrophil accumulation is monitored by measuring the skin content of the neutrophil-specific enzyme, myeloperoxidase. When the nonselective adenosine agonist *N*-ethyl-carboxyadenosine (NECA) was injected intrathecally, dose-dependent inhibition of neutrophil accumulation in response to carrageenan-activated complement, in the form of zymosan-activated plasma or immune complex, was observed.[20] The IC50 of intrathecal (IT) NECA was estimated at around 5 μg/kg, a dose too low to have any impact when injected systemically. In fact, a sixfold higher dose of NECA administered intraperitoneally had absolutely no effect. Thus, the action of NECA was due to a local, spinal action, and not simply a result of leakage from the dural space to the periphery. The A1-selective agonist cyclohexyl-adenosine (CHA) was also effective when injected intrathecally, whereas the A_2-selective agonist CGS-21680 had no effect (Figure 17.1a), lending support to the notion that activation of central A1 receptors is responsible for the inhibitory effect by IT NECA. The neutrophil-inhibitory effect of IT adenosine agonists was rapid; in fact, it was observed even when induction of dermal inflammation followed immediately after IT injection of compounds.[20]

These results suggest that local inflammation in the periphery induces a signal to the spinal cord, which in turn somehow helps sustain the inflammatory response. Activation of spinal A_1 adenosine receptors interferes with this process. The rapid nature of the effect suggests that the signal from or to the periphery is conducted along neural pathways, as opposed to being mediated by hormones. In support of this notion,

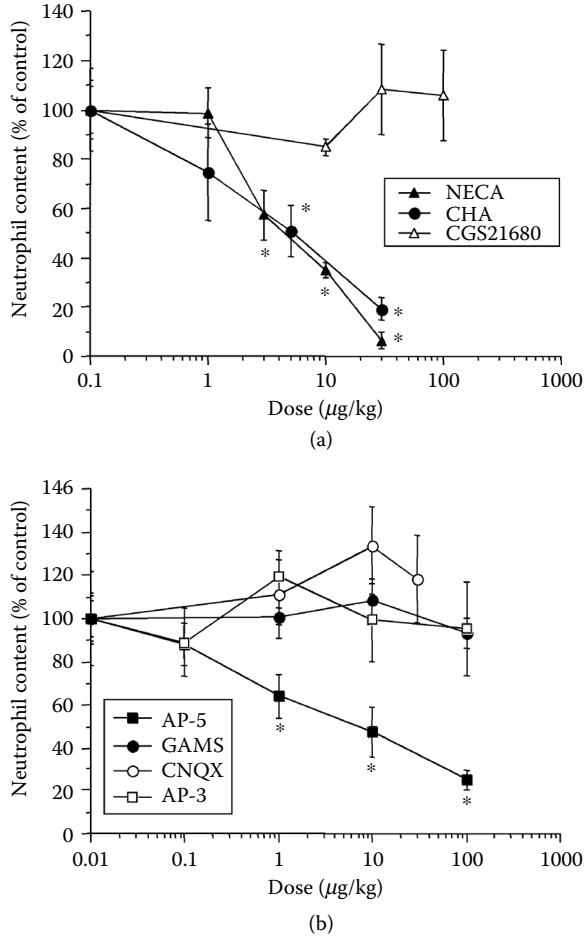

FIGURE 17.1 Inhibition of neutrophil accumulation in rat skin lesions by IT administration of (a) adenosine agonists and (b) an NMDA glutamate receptor antagonist. Adenosine receptor agonist specificities are NECA, nonselective; CHA, A1; and CGS21680, A2a. Glutamate receptor antagonist specificities are AP-5, NMDA; GAMS, AMPA; CNQX, AMPA/kainate; and AP-3, metabotropic. (From Bong, G.W., Rosengren, S., and Firestein, G.S., Spinal cord adenosine receptor stimulation in rats inhibits peripheral neutrophil accumulation: the role of *N*-methyl-D-aspartate receptors, *J. Clin. Invest.,* 98, 2779, 1996. With permission.)

experiments in rhizotomized rats, in which lumbar dorsal roots had been severed unilaterally, revealed that the effect of IT CHA on peripheral neutrophil accumulation was lost on the side with nerve injuries but not on the side with intact nerves.[21] This could be due to interruption of either the primary transmission from the periphery into the spinal cord along afferent sensory pathways, or alternatively of retrograde signals along sensory neurons back to inflammatory sites. In either case, this represents a potential pathway by which the spinal cord regulates peripheral inflammation.

17.3 AFFERENT INPUT: THE EFFECT ON PRIMARY SENSORY C FIBERS

Primary sensory C fibers are involved in sensing primary pain, and they are activated in carrageenan-induced peripheral inflammation.[22,23] These nerves terminate in the dorsal horn of the spinal cord, and upon activation the axon terminals release substance P and excitatory amino acids such as glutamate.[24–27] In order to investigate whether C fibers conduct the preceding inflammatory signal from the periphery, the involvement of spinal substance P and glutamate was studied in separate experiments. Substance P is known to be released peripherally in carrageenan-induced inflammation, and to play a role in the resulting edema;[28,29] however, its central involvement in conducting the inflammatory signal from the periphery was not known. Hence, we depleted nociceptive peptides such as substance P in the spinal cord by injecting capsaicin IT,[30] which substantially reduced levels of nociceptive peptides.[21] This treatment regimen had no effect on the peripheral inflammatory response; it also did not affect the ability of an adenosine agonist administered IT to inhibit carrageenan-induced PMN accumulation.[21]

On the other hand, the pharmacology of the inhibition of peripheral inflammation by IT adenosine agonists was reminiscent of that previously observed for the well-known inhibition of hyperalgesic responses by spinal adenosine receptor stimulation,[15,16] both in terms of the adenosine receptor subtype involved (A_1) as well as the time course of the inhibitory effect. In hyperalgesia, release of glutamate and its activation of NMDA subtype receptors constitute key events,[31] and indeed, adenosine is known to have both pre- and postsynaptic effects on glutamate receptor activity in neurons from various CNS locations, preventing glutamate release[32,33] as well as altering receptor thresholds.[34,35] Thus, we studied the effect of an IT coadministration of the glutamate agonist NMDA along with NECA in the rat skin lesion model. Whereas IT NMDA had no effect on peripheral neutrophil accumulation by itself, it completely reversed the inhibitory effect of IT NECA,[20] suggesting that the inflammatory signal from the periphery was mediated through release of spinal glutamate and subsequent activation of the NMDA receptor. In support of this hypothesis, IT injection of the NMDA glutamate receptor antagonist AP-5 inhibited carrageenan-induced neutrophil migration into skin lesions to the same extent as adenosine agonists (Figure 17.1b).[20] Antagonists of other glutamate receptor subclasses (metabotropic, AMPA, and kainate) were ineffective in this model. This suggested that the inflammatory signal from the periphery involved spinal glutamate release and activation of NMDA receptors, and that the anti-inflammatory effect of IT adenosine agonists was mediated through downregulation of this glutamate release. Other subclasses of glutamate receptors could also be involved in the regulation of inflammation, because earlier studies in an acute knee arthritis model in the rat found that an AMPA receptor antagonist, but not an NMDA antagonist, inhibited development of joint swelling.[36] Possibly, this discrepancy could be due to differential receptor involvement in the regulation of neutrophil infiltration and edema formation. In any event, based on the lack of involvement of substance P, this represents a selective effect of IT adenosine receptor stimulation on excitatory amino acids only, rather than a general C-fiber inhibitory effect. Although both

substance P and excitatory amino acids are released in the dorsal horn in response to peripheral acute inflammation,[24–27] only glutamate is apparently involved in facilitating peripheral inflammatory response, as indicated by our results.

17.4 PERIPHERAL ANTI-INFLAMMATORY EFFECT MEDIATED BY MAINTAINING ADENOSINE AT SITE OF INFLAMMATION

In order to elucidate downstream pathways, the mechanism in the periphery responsible for diminished neutrophil recruitment was studied further. To begin with, a change in gross hemodynamic parameters such as blood pressure and heart rate could certainly affect cutaneous perfusion and thus alter the influx of inflammatory cells. However, these parameters were not affected by IT injection of AP-5. We then hypothesized that the facilitation of peripheral inflammation by the spinal cord might involve the local release of some autacoid. To study this, an alternative model of acute inflammation, the rat air pouch model, was employed. A dorsal stable pouch with an epithelial lining can be formed by repeated subcutaneous injections of sterile air.[37] The resulting pouch is well suited for the instillation of inflammatory agents, and at the end of the experiment it can be lavaged with saline to yield an aspirate wherein cell populations and the concentrations of pro- and anti-inflammatory mediators can be studied. In such air pouches, carrageenan induced release of substance P that was undetectable in pouches injected with saline alone. Levels of TNF-α were also significantly elevated over fivefold compared to saline-injected pouches. However, IT AP-5 had no effect on the level of either mediator, although it greatly reduced carrageenan-induced infiltration of leukocytes as previously observed in the skin lesion model.[20] Unexpectedly, when concentrations of adenosine in air pouch lavages were determined after injection of carrageenan or saline, the purine normally present at around 500 to 700 n*M* decreased dramatically following carrageenan administration: mean adenosine levels at 4 h following carrageenan injection were only 107 ± 7 n*M*. Even more surprisingly, when rats were treated with an IT injection of AP-5, the carrageenan-induced drop in pouch adenosine levels was abolished and adenosine concentrations remained high (646 ± 127 n*M*).[20] Because adenosine can inhibit neutrophil activation and endothelial adhesion by activation of adenosine A2a receptors,[1,2] its disappearance following induction of inflammation would serve to facilitate accumulation of neutrophils, and this mechanism might underlie the effect of the spinal cord on acute inflammation described earlier. If this was true, then inhibitory effects by IT adenosine agonists and glutamate antagonists observed on neutrophil infiltration should be reversed by a peripherally administered A_{2A} antagonist. Indeed, this turned out to be the case: intraperitoneal injection of DMPX, an A_2 antagonist, reversed the inhibition of inflammatory cell accumulation by IT NECA, CHA, and AP-5 in both models of acute inflammation, i.e., the skin lesion and air pouch models[20] (Figure 17.2). This was not simply due to leakage from the periphery into the spinal cord, because IT injections of DMPX had no effect.

As a final test of our hypothesis that peripheral adenosine levels are maintained by IT injection of adenosine agonists or AP-5, the enzyme adenosine deaminase was injected into air pouches along with carrageenan to degrade any endogenous

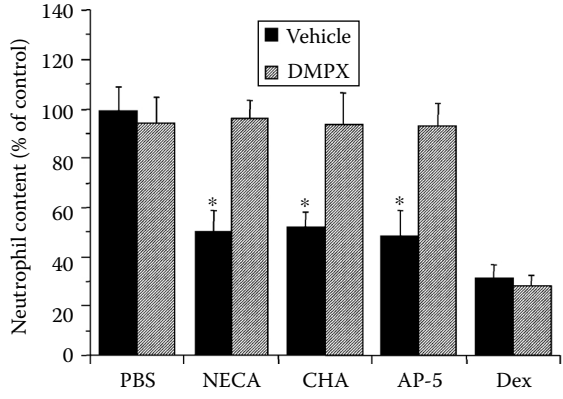

FIGURE 17.2 Inhibition of neutrophil accumulation in rat skin lesions by IT administration of adenosine agonists and an NMDA glutamate antagonist is reversed by systemic treatment with the adenosine A2 antagonist, DMPX. As a control, dexamethasone inhibited neutrophil accumulation, but DMPX had no effect. (Reproduced from Bong, G.W., Rosengren, S., and Firestein, G.S., Spinal cord adenosine receptor stimulation in rats inhibits peripheral neutrophil accumulation: the role of *N*-methyl-D-aspartate receptors, *J. Clin. Invest.,* 98, 2779, 1996. With permission.)

adenosine. This treatment reversed the inhibition of leukocyte accumulation caused by IT adenosinergic stimulation,[20] again supporting the theory that IT adenosine receptor stimulation inhibits peripheral neutrophil infiltration by preserving local adenosine concentrations. Hence, we identified a completely novel pathway in which spinal glutamate release is induced by peripheral acute inflammation and appears to facilitate the local inflammatory response. This series of events can be reversed by spinal adenosinergic stimulation, and is mediated through downregulation of peripheral adenosine, which normally acts to dampen local inflammatory responses. Thus, adenosine receptor stimulation is involved at two levels: centrally and peripherally.

The precise links that allow the spinal cord to regulate tissue levels of adenosine remain unknown. It is reasonable to assume that efferent neural pathways might be involved, and in fact both sympathetic and cholinergic mechanisms that downregulate inflammation have been identified previously.[38] For example, IT-administered adenosine blocks hyperalgesia partly through interaction with norepinephrinergic pathways,[39] and norepinephrine has long been known to downregulate production of inflammatory cytokines from tissue-residing macrophages.[40,41] Therefore, we conducted chemical sympathectomy experiments in which rats were pretreated with 6-hydroxydopamine, which resulted in significant norepinephrine depletion as evidenced by an 87% drop in concentrations in the spleen. In these rats, IT injections of CHA still led to inhibition of neutrophil accumulation in carrageenan-induced skin lesions,[21] suggesting that sympathetic pathways play no role. The possible involvement of cholinergic mechanisms similar to those observed following vagal nerve stimulation[42] has not yet been addressed.

17.5 THE EFFECT OF IT ADENOSINE AGONIST ON CHRONIC INFLAMMATION

After evaluating the effect of IT adenosine receptor stimulation in acute inflammation, we then determined whether chronic inflammation could also be influenced. For this purpose, the rat adjuvant arthritis model was used to study the effect of IT administration of the A_1 adenosine agonist CHA. In this model, a single injection of complete Freunds' adjuvant is used to induce an aggressive, destructive polyarthritis with clinical signs of joint swelling 7 to 12 d later.[43] Importantly, the adjuvant arthritis model can be inhibited by compounds that enhance tissue levels of adenosine, as demonstrated by us and others.[43,44] To evaluate the effects on inflammatory mechanisms without affecting the development of the initial immune response, IT treatment every other day with CHA was begun on day 8 following adjuvant injection. This significantly reduced hind paw edema throughout the experiment, which was terminated on day 20[45] (Figure 17.3a). A further delay of the initiation of IT

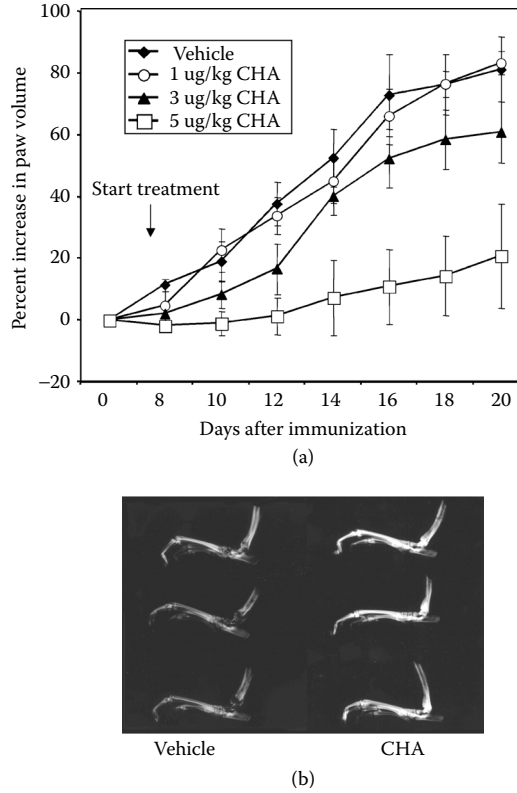

FIGURE 17.3 (a) Dose-dependent reduction of paw edema in the rat adjuvant arthritis model by IT CHA. (b) Preservation of hind paw bone and joint structures, as demonstrated by x-rays. (Reproduced from Boyle, D.L. et al., Spinal adenosine receptor activation inhibits inflammation and joint destruction in rat adjuvant-induced arthritis, *Arthritis Rheum.,* 46, 3076, 2002. With permission.)

treatment with CHA to day 14 led only to a minimal and not significant inhibition of paw swelling.

The effect of IT CHA in this model was partially reversed by daily systemic injections of a nonselective adenosine antagonist, theophylline.[45] This compound readily penetrates the blood–brain barrier in the rat[46] and therefore might act directly at the spinal level to block CHA-induced activation of A_1 receptors or, alternatively or additionally, at the peripheral site to reverse anti-inflammatory effects by elevated tissue adenosine levels as observed in the acute models (see earlier text). Further experimentation using more selective adenosine antagonists was hindered by the fact that the pharmacokinetic properties of many of these compounds remain unknown, complicating the design of a treatment regimen that would efficiently block adenosine receptor activation in a chronic manner.

Apart from the effect on development of paw edema, IT CHA also had a striking effect on the joint destruction normally observed in this model. On day 20 of the model, saline-treated animals displayed substantial demineralization, erosions, and loss of joint space in the hind paws, as determined by radiographic analysis. In contrast, IT treatment with CHA led to marked preservation of cartilage and bone[45] (Figure 17.3b). When joint extracts were examined by electrophoretic mobility shift assay, the oligonucleotide binding activity of the transcription factor AP-1 was also substantially decreased by IT treatment with CHA. Because AP-1 is a key regulator of matrix metalloproteinase expression[47] and MMPs are responsible for joint destruction in adjuvant arthritis,[48,49] the observed reduction in AP-1 activity might help explain the protective effects of adenosinergic stimulation in the spine.

17.6 POTENTIAL CENTRAL MECHANISMS IN THE INHIBITION OF CHRONIC INFLAMMATION BY SPINAL ADENOSINE RECEPTORS

The gene product c-Fos is known to be induced in postsynaptic neurons in the spinal cord dorsal horn after noxious cutaneous stimulation.[50] Relevant to our results, the induction of c-Fos following an inflammatory insult is reduced by peripheral treatment with an adenosine kinase inhibitor,[51] and that c-Fos expression in various pain models is also reduced by systemic[52] and central[53] administration of NMDA antagonists. In the adjuvant arthritis model, increased spinal c-Fos expression was also previously demonstrated[54] and was reduced by NMDA antagonist treatment.[55] When examining spinal dorsal horn sections from rats with adjuvant arthritis using immunohistochemistry, we, too, observed a large increase in the number of c-Fos-positive cells on day 20 of the model. A modest but significant reduction of c-Fos-positive cells was observed both in superficial and deeper laminae following chronic IT treatment with CHA.[45,56] Hence, inhibition of c-Fos expression might play a role in the reduction of chronic inflammmation following IT adenosine agonist administration in rat adjuvant arthritis.

Another potential central mechanism that might underlie the inhibition of chronic inflammation by IT adenosine agonists constitutes reversal of glial activation. Relatively recent data indicate that spinal glial cells (i.e., microglia and astrocytes) are activated in the course of acute[57] as well as chronic[58] peripheral inflammation.

These cells are increasingly recognized as powerful modulators of nociception and are thought to affect development of hyperalgesia following an inflammatory insult[59,60] through pathways that include production of proinflammatory cytokines.[61] For example, in the adjuvant arthritis model, the numbers of astrocytes and microglia that express the cytokines IL-1, IL-6, and TNF-α are significantly elevated over nonarthritic controls.[58] Activated by excitatory amino acids among other mediators, glial cells themselves release glutamate[62] and thus might be implicated in the anti-inflammatory effect of adenosinergic receptor stimulation in the spine.

Upon examination of the published literature on the consequences of adenosinergic stimulation in glial cells, it is clear that adenosine exhibits multiple and complex effects. For example, adenosine induces expression of IL-6, a pro- or anti-inflammatory cytokine depending on the context, in astrocytes through stimulation of A_{2B} receptors.[63,64] In astrocyte cultures, adenosine A_3 receptor stimulation leads to apoptosis or to cytoprotection, depending on the concentration of agonists.[65] Adenosine itself can stimulate[66] or inhibit[67] the proliferation of cultured microglia from newborn rats through mechanisms that at least partially implicate A_1-adenosine-receptor-mediated effects. Recent findings suggest that LPS induces TNF-α release from a glioma cell line, and this is inhibited by an adenosine kinase inhibitor.[68] Additionally, the xanthine derivative propentofylline, known to increase extracellular adenosine concentrations among other actions,[69] inhibits the LPS-activated release of TNF-α and IL-1 from cultured microglia.[70]

Activation of astrocytes causes an increase in production of glial fibrillary acidic protein (GFAP), which can be monitored by immunohistochemistry. When spinal cords from rats with adjuvant arthritis were examined for GFAP, elevated levels over those in nonarthritic rats as determined by image analysis were found on day 20 of the model,[56] as described earlier.[58] The difference was especially large in superficial laminae, in which approximately a threefold increase was observed. Notably, IT treatment with CHA every other day, beginning on day 8, significantly lowered expression of GFAP.[56] This might be due to a direct effect on A1 adenosine receptors, or alternatively could result from the earlier described inhibition of glutamate release, because GFAP expression in a neuropathic pain model is dependent on NMDA receptor activation.[71] It is currently unknown how, or if, glial activation and cytokine release participate in peripheral inflammatory processes; however, very recent results indicate that IT treatment with a TNF-α inhibitor reduces hyperalgesia induced by spinal nerve ligation.[72] If chronic inflammation is likewise affected, this would certainly present an intriguing therapeutic option.

17.7 POTENTIAL PERIPHERAL MECHANISMS IN THE INHIBITION OF CHRONIC INFLAMMATION BY SPINAL ADENOSINE RECEPTORS

The exact peripheral mechanism behind the effect of IT administration of CHA in the adjuvant arthritis model has not yet been fully elucidated. One might speculate that the events leading to inhibition of chronic inflammation involve peripheral adenosinergic pathways, as observed in the case of acute inflammation and neutrophil infiltration (see earlier text). In fact, neutrophils accumulate in large numbers in

arthritic joints from rats with adjuvant arthritis,[73] and depletion of neutrophils leads to significant reduction of arthritic symptoms in this model.[74] Thus, inhibition by IT CHA could potentially be due to reduction of neutrophil infiltration rather than constituting a separate, independent effect on chronic pathways. However, the significant suppressive effect of the IT A_1 agonist on AP-1 binding activity in the joint and the accompanying inhibition of joint destruction suggest that the mechanism is more complex. First, neutrophils have previously been reported to have a deficiency in their ability to activate AP-1 in response to a variety of stimuli,[75] although others have argued that there might be a difference in suspended and adherent neutrophils in this regard.[76] Second, although neutrophils express certain types of matrix metalloproteinases, especially MMP-8 and MMP-9, expression of these proteins might not be regulated through AP-1 activation. Rather, assuming adenosine levels are indeed maintained peripherally upon spinal A_1 adenosine receptor stimulation, as previously observed in acute inflammation models, an alternative potential mechanism involves effects on fibroblast-like synoviocytes. These cells constitute a crucial source of the matrix metalloproteinases that mediate joint destruction in arthritis, which are induced by cytokines such as IL-1. However, in the presence of NECA, collagenase production is significantly decreased by a mechanism that appears to involve A_{2B} receptors.[77] Other peripheral anti-inflammatory mechanisms by adenosine in chronic arthritis may include the inhibition of cytokine expression by macrophage-like synoviocytes or other infiltrating cells of monocytic or macrophage lineage.[4,5]

17.8 CONCLUSIONS AND IMPLICATIONS

The pharmacological effects of adenosine are many and varied, with new facets of its actions continuing to be elucidated. Its inhibitory effects on inflammatory cells have long been appreciated, but in addition, adenosine regulates inflammation by stimulation of spinal A_1 adenosine receptors. This leads to suppressed glutamate release and activation of NMDA receptors, earlier implicated in the development of inflammatory hyperalgesia and allodynia. In turn, this leads to significant downregulation of peripheral inflammatory events through the preservation of endogenous adenosine levels at the site of inflammation. Other potential central mechanisms include reduction in nuclear c-Fos expression as well as inhibition of glial activation, both of which are more likely to play significant roles in chronic settings.

These findings have implications for research in pain and inflammation. First, in response to pathological stimuli, adenosine itself is released in the spinal cord both from neurons and glial cells[78] and plays a protective role against development of hyperalgesia. Our findings extend the significance of this endogenous adenosine release to include modulation of peripheral inflammatory events, both acute and chronic. Second, this pathway presents a novel potential target for pharmaceutical intervention, both by drugs that enhance adenosine levels as well as compounds that interfere with the excitatory amino acid component. Finally, because some commonly used disease-modifying antirheumatic drugs have been proposed to act through the upregulation of tissue adenosine release,[79,80] the identification of these pathways might also further our understanding of the mechanisms underlying their anti-inflammatory effects.

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18 Adenosine, Tumors, and Immunity

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CONTENTS

18.1 INTRODUCTION

Considerable evidence has accumulated indicating that adenosine plays an important role in controlling tumorigenesis via its receptors. Adenosine, which is released to the microenvironment by metabolically active tumor cells, fulfills a multitude of functions in regulating tumor cell proliferation. At micromolar (μM) concentrations

it directly induces an antiproliferative effect toward various tumor cell types. Indirectly, it affects tumor development via its capability to affect cytokine release, cell migration, angiogenesis, and chemotaxis. Moreover, adenosine induces activation or suppression of T killer or natural killer cells that affect tumor cell development.

It is quite impossible to assess the effect of adenosine *in vivo* because of its rapid metabolization by adenosine deaminase (ADA). However, its effects are mediated via the four receptor subclasses: A_1, A_{2A}, A_{2B}, and A_3.

This chapter briefly reviews the role played by various adenosine receptors in mediating tumor cell response to adenosine, its agonists, and antagonists.

Interestingly, among the four receptor subtypes, the A_3AR was found to mediate a potent antitumor effect. The specificity of this target results from the finding that this receptor is highly expressed in tumor cells, whereas low receptor expression is reported in normal cells. In addition, the low affinity of adenosine to A_3AR and the finding that A_3AR knockout mice are considered normal indicate that the receptor does not mediate essential functions under normal conditions, and thus may be suggested as a specific target to combat cancerous diseases. The associated molecular mechanisms, including signal transduction pathways generated upon adenosine receptor activation in tumor cells, will be described in this chapter.

18.2 THE EFFECT OF ADENOSINE ON TUMOR CELL GROWTH

Rapidly growing solid tumor tissues are characterized by the high metabolic rate of the proliferating tumor cells and frequently insufficient microcirculation. As a result, local regions of ischemia and hypoxia are developed.[1] Under these conditions adenosine levels are elevated owing to three pathways that involve breakdown of ATP to AMP: via the 5'-nucleotidase, decreased AMP formation by the inhibition of adenosine kinase, and upregulation of *S*-adenosylhomocysteine hydrolase, which also participates in adenosine formation.[2–4] The concentration of adenosine in extracellular fluids of colorectal and lung carcinoma tumor tissues can reach up to 10^5 mol/l.[5]

The high adenosine level in the tumor microenvironment prompted the study of adenosine's effect on leukemia, lymphoma, and solid tumor cell types. The antitumor effect of adenosine, mediated via mechanisms dependent or independent of adenosine receptors, results in cell cycle arrest, proliferation inhibition, or the induction of apoptosis.[6–10]

In the human leukemia cell lines HL-60 and K-562 as well as in the Nb2-11C rat lymphoma cells, adenosine at μM concentrations causes inhibition of tumor cell growth. In some cell types the inhibitory effect was due to apoptosis induced via an active transport of adenosine into the cells.[8,11] In other cell types adenosine induced a cytostatic effect by arresting the cells in the G_0/G_1 phase of the cell cycle as a result of a decrease in the telomeric signal. Telomeres are repeated DNA sequences that guard the ends of chromosomes, serving as a checkpoint for cell-cycle progression, thus regulating cell senescence and apoptosis.[12]

Few studies have tested the response of solid tumors to adenosine. Low concentrations of adenosine (<10 μM) induced cell growth inhibition in A431 human epidermoid carcinoma cells, LNCaP human prostate adenocarcinoma, and murine B16-F10 melanoma. At higher concentrations, adenosine promoted cell proliferation of the

A431 human epidermoid carcinoma cells through activation of the A_2 adenosine receptors.[8,13]

Adenosine was shown to induce a differential effect on tumor and normal cell growth. The proliferation of lymphocytes derived from patients with chronic lymphocytic leukemia was suppressed by adenosine, whereas that of normal lymphocytes was inhibited to a lesser extent.[14] Moreover, stimulation of normal cell proliferation by adenosine has been demonstrated in Swiss mouse 3T3 and 3T6 fibroblasts, thymocytes, hemopoietic cells, endothelial cells, astrocytes, and myeloid bone marrow cells.[8,15–18]

18.2.1 The Antitumor Effect of Adenosine: Mediated via Adenosine Receptors

The effect of adenosine and its agonists/antagonists on tumor cells depends on their extracellular concentrations and on the expression of different adenosine receptor subtypes. Upon receptor activation, various signal transduction pathways are generated, resulting in a direct inhibitory effect on tumor growth. Other cell types, such as immunocytes or endothelial cells, may respond to receptor activation by the release of cytokines and mediators that indirectly affect tumor growth.

18.2.1.1 Tumor Cells and Expression of A_1 and A_3 Adenosine Receptors

Studies aimed at the analysis of adenosine receptor expression in tumor cells were conducted based on previous knowledge that high adenosine level in the tumor microenviroment may regulate receptor expression and that receptor density may affect cell response to adenosine or its synthetic agonist/antagonists. This led to studies exploring high receptor expression as a characteristic of tumor cells. There is evidence that the A_1 adenosine receptor is expressed in various tumor cell lines, including the A431 epidermal carcinoma, LOVO human colon carcinoma, A2058 melanoma, medullary thyroid carcinoma, and human hepatocellular carcinoma cells.[19–22] In human colorectal cancer tissue the mRNA expression level of A_1AR was found to be high compared to the peritumoral tissue, whereas A_{2A}A expression level was the same in both tissues.[23]

A_3AR expression was also found in tumor cell lines including astrocytoma, HL-60 leukemia, B16-F10 and A378 melanoma, human Jurkat T cell lymphoma, and murine pineal tumor cell.[24–30]

High A_3AR mRNA, protein expression level, and cell surface exhibition were reported in different tumor cell types. A retrospective study in pathological paraffin-embedded slides of breast, colon, pancreatic, small cell lung carcinoma, and melanoma showed high A_3AR expression in comparison to normal adjacent tissues. Protein A_3AR expression level was high in fresh tumors derived from colon and breast carcinoma when compared to normal adjacent tissue, as detected by Western blot (WB), immunohistochemistry, and binding assay analyses.[29,31]

Gradual increase in A_3AR density is seen during colorectal tumor progression, and is manifested by greater receptor density as the tumor develops from a small adenoma into a large adenoma and finally to colon carcinoma. Moreover, mRNA A_3AR expression level was higher in lymph node metastasis compared to primary

tumor and normal peritumoral tissue. Interestingly, in peripheral blood lymphocytes and neutrophils of colon carcinoma patients, high A_3AR density was detected and reflected A_3AR expression in the relevant tumor tissue.[29,31]

It thus seems that A_3AR levels are directly correlated to tumorigenicity and may be developed as an additional biomarker to monitor disease progression.

18.2.2 Direct Antitumor Effects Induced by A_3AR Agonists and Mechanisms Involved

Although various tumor cells highly express A_1 and A_3 adenosine receptors, the anticancer activity is attributed to the A_3AR. Growth inhibition of tumor cells upon A_3AR agonist treatment was reported to be mediated via cell cycle arrest, apoptosis, or necrosis, depending on cell type and agonist concentration. Moreover, inhibition of tumor growth by A_3AR synthetic agonists was found to be receptor dependent or receptor independent.

Tumor and normal cells respond differentially to the activation of A_3AR by natural or synthetic agonists. Utilizing nanomolar (n*M*) A_3AR agonist concentrations, inhibition of tumor cell growth *in vitro* was observed in melanoma, colon, breast, and prostate carcinoma.[32–36] On the other hand, the proliferation of normal cells such as murine bone marrow was stimulated.[37,38] At μM concentrations, the proliferation of both tumor and normal cells was inhibited. This differential effect may be explained by the high vs. low A_3AR expression level in tumor and normal cells, respectively.

18.2.2.1 Antitumor Effect of A_3AR Agonists at μM Concentrations

At μM concentrations, A_3AR agonists inhibited the growth of leukemia, lymphoma, and various solid tumor cell lines.

In HL-60 promyelocytic leukemia and U-937 hitiocytic lymphoma cells, A_3AR agonists, 1-deoxy-1-amino]-9H-purine-9-yl]-*N*-methyl-(-D-ribofuranuronamide (IB-MECA) and 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (Cl-IB-MECA), induced apoptosis via an elevation in intracellular calcium concentration and upregulation of the Bak gene. However, low concentrations of these agonists protected against apoptosis induced by A_3AR antagonists.[39,40] In an additional study that also included the HL-60 cells and MOLT-4 cells, apoptosis was induced by Cl-IB-MECA and was not mediated via the A_3AR. The mechanism proposed included upregulation of the death receptor, Fas, which was p53 independent.[41]

In solid tumors, the effect of IB-MECA at μM concentrations was tested in different breast cancer cell lines including MCF-7, ZR-75 and T47D (estrogen receptor α positive), MDA-MB468, and Hs578T (estrogen receptor α negative). Panjehpour et al.[42] reported that in the MCF-7 and the MDA-MB468 cell lines, IB-MECA treatment resulted in significant cell growth inhibition. The response was A_3AR mediated, as was demonstrated by pretreatment with the selective A_3AR antagonist MRS1220 and by the inhibition of forskolin-stimulated cAMP levels.[42] In another study Lu et al.[43] also demonstrated that IB-MECA induced growth inhibition of MCF-7 and ZR-75 and apoptosis in T47D and Hs578T cells.

The authors showed that the response to IB-MECA was not A_3AR mediated (because they did not detect A_3AR mRNA expression in these cells) and that the mechanism involved downregulation of the mRNA and protein expression level of the estrogen receptor.[43]

In melanoma tumor cells the A_3AR agonist Cl-IB-MECA inhibited the proliferation of the A375 melanoma cell line via an A_3AR dependent pathway. This was supported by studies showing that the response to A_3AR was neutralized by a specific antagonist and by A_3AR knock-down (using the siRNA technique). The mechanism of action included modulation of the phosphatidylinositide-3-OH kinase (PI3K)/Akt and the Raf/mitogen-activated protein kinase, MAPK/Erk kinase, and MEK/mitogen-activated protein kinase (MAPK) pathways. Stimulation of the PI3K-dependent phosphorylation of Akt leading to the reduction of basal levels of ERK1/2 phosphorylation levels took place.[44]

In an additional set of experiments IB-MECA and Cl-IB-MECA at a concentration of 10 μM inhibited the proliferation of murine melanoma (B16-F10), human colon (HCT-116), and prostate carcinoma (PC-3) cells. The inhibitory effect was only partially mediated via the A_3AR because the A_3AR antagonist MRS 1523 neutralized only 50% of the agonist inhibitory effect.[34,35]

18.2.2.2 Antitumor Effect of A_3AR Agonists at n*M* Concentrations

In contrast to adenosine, which binds to all the different receptor subclasses, A_3AR synthetic agonists, which have high affinity to the receptor, will activate A_3AR exclusively. This was the rationale behind the examination of the effect of IB-MECA and Cl-IB-MECA (having affinity values in the range of 0.5 to 1.5 n*M*) on the growth of different tumor cells at n*M* concentrations.

IB-MECA and Cl-IB-MECA inhibited the proliferation of murine melanoma (B16-F10), human colon (HCT-116), and prostate carcinoma (PC-3) in a dose-dependent manner. Cell proliferation was measured by [^{3}H]-thymidine incorporation at nanomolar concentrations (1 to 1000 n*M*). The response was A_3AR dependent, because the effect was fully neutralized by the antagonist MRS 1523, suggesting that at low n*M* concentrations, only A_3AR is activated, mediating the antitumor effect.[33–35]

Cross talk between the A_3AR and the Wnt signal transduction pathway was found to play a major role in mediating the inhibitory effect of IB-MECA (at 10 n*M* concentration) on the growth of melanoma, colon, and prostate carcinoma. Downregulation of receptor expression was noted shortly after A_3AR activation, in the three cell lines, demonstrating the rapid response of the tumor cells to agonist stimulation and the initiation of downstream responses. Indeed, the tumor cells responded to A_3AR activation by a decrease in protein kinase A (PKA) c level, an effector protein involved in the initiation, regulation, and cross talk between various signaling pathways. It phosphorylates and inactivates the enzyme glycogen synthase kinase 3 (GSK-3β), a key element in the Wnt signaling pathway.[45,46] GSK-3β suppresses mammalian cell proliferation and survival by phosphorylating the cytoplasmic protein β-catenin, leading to its ubiquitination. GSK-3β in its inactive form does not phosphorylate β-catenin, which accumulates in the cytoplasm and subsequently translocates to the nucleus, where it associates with Lef/Tcf to induce cyclin-D1 and c-myc transcription.[47] IB-MECA treatment decreased the PKA and protein

kinase B/Akt (PKB/Akt) levels. Subsequently, the phosphorylated form of GSK-3β was decreased and total GSK-3β levels were increased. This led to downregulation in the level of β-catenin, Lef/Tcf, and the β-catenin-responsive cell growth regulatory genes c-myc and cyclin D1. These observations, which link cAMP to the Wnt signaling pathway, provide mechanistic evidence for the involvement of the Wnt pathway, via its key elements GSK-3β and β-catenin, in the antitumor activity of A_3AR agonists (see Figure 18.1).

The expression level of NF-κB was downregulated in both *in vitro* and *in vivo* studies. NF-κB is also linked to the effector protein PKAc. The most abundant form of NF-κB is a heterodimer of p50 and p65 (Rel A) subunits in which the p65 contains the transcription activation domain. PKAc regulates the transcriptional activity of NF-κB by phosphorylating the p65 subunit of NF-κB, enabling its association with the coactivator CBP/p300 and efficient transcriptional activity.[48,49] Thus, IB-MECA's capability to suppress NF-κB expression may serve as part of the mechanism through which it exerts an inhibitory effect on tumor growth (see Figure 18.1).

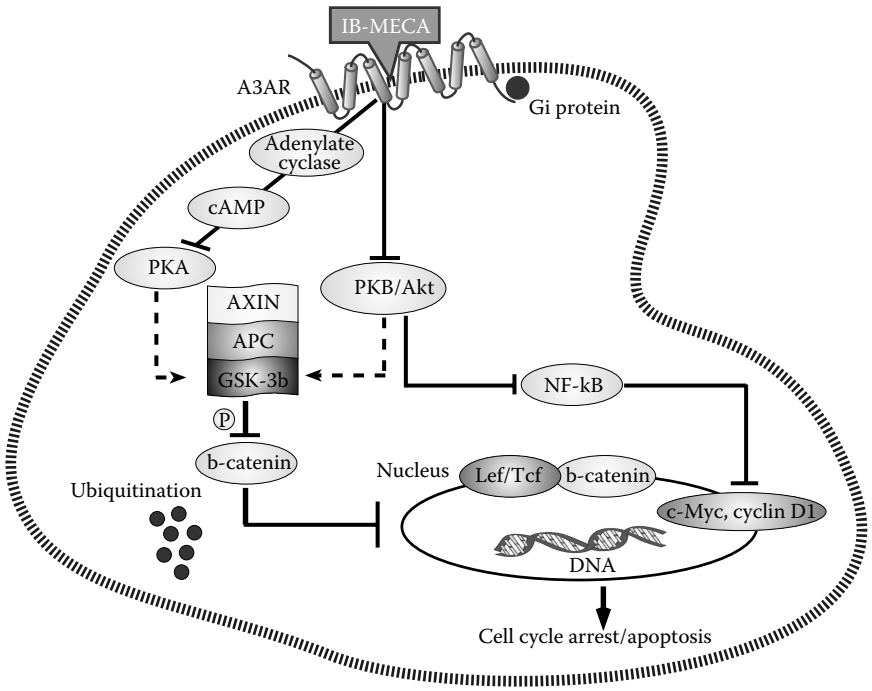

FIGURE 18.1 Activation of A_3AR by IB-MECA deregulates the Wnt and the NF-κB signaling pathways. Tumor cells responded to A_3AR activation by a decrease in the levels of PKAc and PKB/Akt. Both are known to control the phosphorylation and inactivation of GSK-3β. PKB/Akt also phosphorylates downstream proteins such as IKK and IκB. As a result the expression level and activity of β-catenin, Lef/TCF, and NF-κB is downregulated. This chain of events is followed by decreased transcription of cell growth regulatory genes, such as c-myc and cyclin D1, resulting in cell cycle arrest and apoptosis.

18.2.2.3 *In vivo* Effect of A_3AR Agonists

Oral administration of IB-MECA and Cl-IB-MECA was efficacious in inhibiting the development of primary tumors and metastasis in xenograft and syngeneic models. IB-MECA inhibited the development of primary colon carcinoma in syngeneic (murine CT-26 colon carcinoma and B16-F10 melanoma cells) and xenograft (HCT-116 colon and PC3 prostate human carcinoma cells) tumor models. Moreover, IB-MECA and Cl-IB-MECA suppressed the development of lung melanoma metastases and IB-MECA inhibited colon cancer liver metastases in syngeneic mice. In all *in vivo* experiments, mice were treated with a low dose of agonist (10 μg/kg) aimed at exclusively targeting the A_3AR.[32–38]

Exploration of the mechanism responsible for the *in vivo* antitumor effect was carried out in protein extracts derived from IB-MECA-treated melanoma prostate and colon–carcinoma-bearing animals. A similar protein profile to that described *in vitro* was found, suggesting that the Wnt and the NF-κB signal transduction pathways are involved in the *in vivo* antitumor effect.[32–38] Table 18.1 summarizes the different experimental animal models used and the percentage of tumor growth inhibition exerted by the two A_3AR agonists.

18.2.2.4 A_3AR Fate upon Chronic Activation

It is well established that upon activation of a G_i protein receptor, a chain of events takes place including phosphorylation, palmitoylation, desensitization, internalization, receptor downregulation, resynthesis, and recycling as a functional receptor to

TABLE 18.1
Effect of A_3AR Agonists on Tumor Growth

Percentage of Tumor Growth Inhibition (at Study Termination)	Tumor Type	Experimental Model
52 ± 2.8	B16-F10, murine melanoma	Primary
53 ± 4.7	CT-26, murine colon carcinoma	Primary
63 ± 8.2	HCT-116, human colon carcinoma	Primary
75 ± 9.7	PC3, human prostate carcinoma	Primary
59 ± 6.8	B16-F10, murine melanoma Lung metastases	Metastatic
52 ± 8.3	CT-26, murine colon carcinoma Liver metastases	Metastatic

Note: In vivo, oral administration of A_3AR agonists was efficacious in inhibiting the development of primary tumors and metastasis in xenograft and syngeneic models. IB-MECA inhibited the development of primary colon carcinoma in syngeneic (murine CT-26 colon carcinoma and B16-F10 melanoma cells) and xenograft tumor models (HCT-116 colon and PC3 prostate human carcinoma cells). Moreover, IB-MECA and Cl-IB-MECA suppressed the development of lung melanoma metastases, and IB-MECA inhibited colon cancer liver metastases in syngeneic mice.

the cell surface (see Figure 18.2). This process leads to the initiation of a downstream molecular mechanism that transmits the signal to the nucleus (Figure 18.2). As was specified earlier, the A_3AR expression level is highly expressed in various leukemias, lymphomas, and solid tumors. Given that A_3AR agonists inhibit the growth of cells, receptor fate upon chronic activation and the effects on the downstream molecular mechanisms leading to tumor growth inhibition, both *in vitro* and *in vivo*, are important. When the agonist is used as a drug, chronic targeting of the Gi protein receptor might lead to its desensitization and loss of function, resulting in a lack of response.

Only two studies were conducted that investigated receptor fate in tumor cells. In the ADF astrocytoma cell line, A_3AR fate was examined after short- and long-term agonist exposure. A_3AR desensitization, tested by the adenylyl cyclase activity assay, occurred shortly (15 min) after activation with the agonist Cl-IB-MECA (100 n*M*).

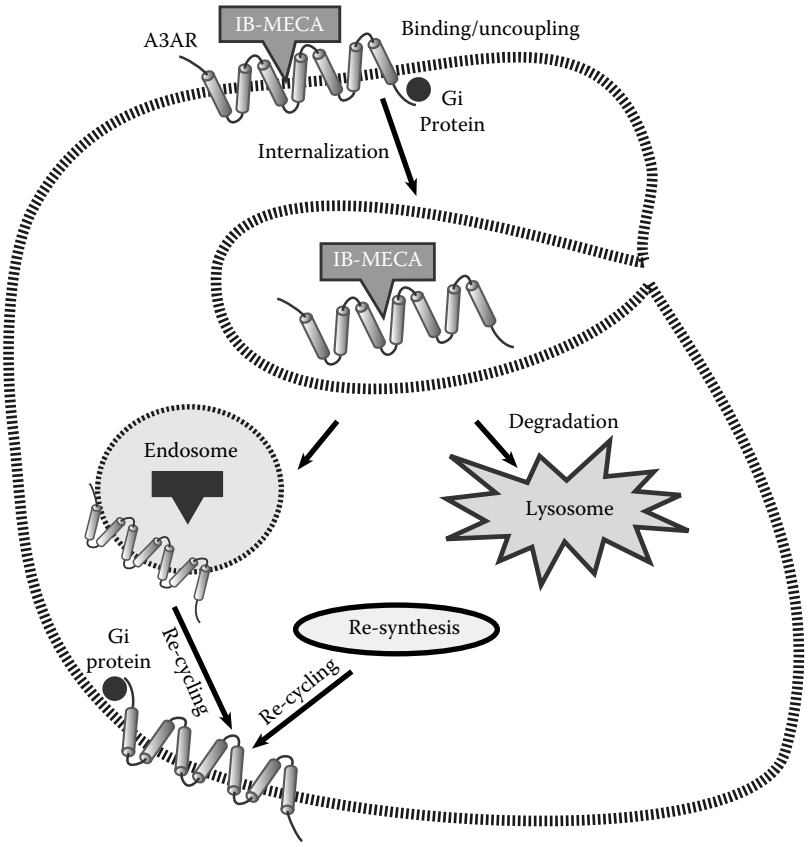

FIGURE 18.2 Upon activation of A_3AR by IB-MECA, desensitization and internalization of the receptor take place. Within the cells the receptor accumulates either in the endosome to be recycled to the cell surface or in the lysosome for degradation. In parallel, resynthesis of a functional receptor that will also be recycled to cell surface may occur.

Desensitization was accompanied by receptor internalization (30 min) and intracellular distribution to various compartments. Recycling and restoration of receptor function were observed upon agonist removal (120 min). The receptor was tracked by utilizing radioligand binding studies and by immunogold staining, followed by electron microscopy examination. Downregulation of the receptor, as analyzed by Western blotting analysis, took place after prolonged agonist exposure (1 to 24 h). Restoration of cell surface receptor to control values was followed by recovery of receptor functioning.[28]

A_3AR fate was also studied in B16-F10 melanoma cells, in which the receptor was highly expressed, and was tracked by confocal microscopy and radioligand binding. Upon chronic *in vitro* exposure to IB-MECA, gradual internalization occurred within a few minutes, whereas after 15 min receptor recycling to the cell surface was noted. Colocalization with FITC-dextran revealed that after internalization, the receptor was "sorted" to the early endosomes and recycled to the cell surface. In parallel, colocalization with FITC-transferrin showed that the receptor was targeted to lysosomes and then subjected to degradation. Receptor distribution in the lysosomes was consistent with the downregulation of receptor protein expression level. This was followed by mRNA and protein resynthesis and full receptor recovery (protein expression and cell surface exhibition) after 24 h. At each stage, receptor functionality was evidenced by the modulation in cAMP level and the downstream effectors PKA, GSK-3β, c-Myc, and cyclin D1.[36] Supporting the notion that the receptor is fully recovered upon chronic treatment are studies in experimental animal models of murine melanoma, as well as human prostate and colon carcinoma. Daily treatment with IB-MECA for one month resulted in receptor downregulation in tumor lesions shortly after treatment and full receptor recovery before the next treatment. The data showing receptor recovery after chronic activation *in vitro* and *in vivo* may suggest A_3AR as a stable target to combat tumor cell growth.[35–37]

18.2.3 Indirect Antitumor Effects Mediated via Adenosine and its Receptors

The high adenosine concentration in the tumor microenvironment raises the question of whether this natural ligand supports or inhibits tumor growth by activating or inhibiting immune cells and cytokines that play an important role in maintaining tumor development.

In a series of experiments, it was shown that adenosine prevented the induction of murine anti-CD-3-activated killer cells via A_3AR activation and suppressed β integrin-mediated adhesion of T lymphocytes to colon adenocarcinoma cells. In addition, high levels of adenosine or its agonists inhibited IL-12 and TNF-α production via activation of A_2 and A_3 adenosine receptors. IL-12 and TNF-α are known to act as anticancer cytokines and their inhibition supports tumor growth.[50–53]

On the other hand, the Cl-IB-MECA, at a low dose (10 μg/kg), potentiated the activity of NK cells in naïve and tumor-bearing mice. Cl-IB-MECA induced increased serum IL-12 levels followed by elevated NK cell activity and tumor growth inhibition.[54]

18.3 ENHANCEMENT OF CHEMOTHERAPEUTIC AND CHEMOPROTECTIVE EFFECTS VIA ADENOSINE RECEPTOR ACTIVATION

Adenosine's characteristic of inducing a differential effect on tumor and normal cells is mediated via the A_3AR and was the basis of examining the effect of A_3AR agonists as compounds that enhance the chemotherapeutic index and of testing their myeloprotective effects *in vivo*.

18.3.1 A_3AR Agonists and Antagonists and Chemotherapeutic Index Enhancement

NF-κB and the upstream kinase PKB/Akt are highly expressed in chemoresistant tumor cells and may hamper the apoptotic pathway. A_3AR agonists have been shown to downregulate PKB/Akt and NF-κB protein expression level, a result that prompted the evaluation of their effect on the response of tumor cells to cytotoxic drugs. Combined treatment of IB-MECA and 5-Flurouracil (5-FU) enhanced the cytotoxic effect of the latter on HCT-116 human colon carcinoma growth. Downregulation of PKB/Akt, NF-κB, and cyclin D1 and upregulation of caspase-3 protein expression level was observed in cells and tumor lesions upon treatment with a combination of IB-MECA and 5-FU.[55]

In three different experimental murine models, including syngeneic (B16-F10 melanoma in C57Bl/6J mice) and xenograft models (HCT-116 human colon carcinoma and PC-3 human prostate carcinoma in nude mice), IB-MECA or Cl-IB-MECA inhibited tumor growth when administered orally at low doses (5 to 100 mg/kg). The tumor inhibitory effect was of the same magnitude as that seen with a standard chemotherapy protocol. When combined with chemotherapy, a synergistic effect was seen, yielding an overall, greater response than treatment with the agonists or chemotherapy alone.[56] The protein profile in tumor lesions excised from colon-carcinoma-bearing mice treated with a combination of 5-FU and IB-MECA revealed downregulation of PKB/Akt, NF-κB, and cyclin D1 and upregulation of caspase-3 protein expression level.[55] These data suggest that IB-MECA opens the way for apoptosis to take place via the modulation of apoptotic proteins.

A_3AR antagonists, pyrazolotriazolopyrimidine derivatives (PTP-d), were also shown to sensitize the A375 human melanoma cells to the chemotherapeutic agents taxol and vindesine. PTP-d's reduced the EC50 doses of the G_2/M accumulation by an average of 1.7-fold for taxol and 9.5-fold for vindesine and increased the ability of the melanoma cells to undergo apoptosis. PTP-d's were found to interact with a binding site on multidrug-resistance-associated ATP binding cassette drug transporter and to control ATP hydrolysis produced during the drug transport, thus affecting the activity of the chemotherapeutic agent.[57]

18.3.2 The A_1 and the A_3 Adenosine Receptors: Mediating Chemoprotective Effects

Adenosine stimulates the proliferation of murine bone marrow cells *in vitro*. Pharmacological studies, using antagonists to the adenosine receptors, revealed that this activity was mediated through the binding of adenosine to its A_1 and A_3 receptors.

The two selective A_1 and A_3 receptor agonists, CPA and IB-MECA, induced bone marrow cell proliferation in a manner similar to adenosine.[58]

Adenosine's interaction with its A_1 and A_3 receptors induced G-CSF production *in vitro*, which led to its stimulatory effect on bone marrow cells. *In vivo* administration of low-dose adenosine (0.25 mg/kg) to mice pretreated with chemotherapy restored the number of leukocytes and neutrophils to normal levels, compared to the decline in these parameters after chemotherapy alone.[58]

Synthetic A_3AR agonists exhibited a myelostimulatory effect both *in vitro* and *in vivo*. IB-MECA and Cl-IB-MECA show an overall myeloprotective activity in mice pretreated with chemotherapy. Oral administration of IB-MECA to näive mice led to the elevation of serum G-CSF levels, an increase in absolute neutrophil counts (ANC), and bone marrow colony-forming cells. Splenocytes derived from these mice produced higher G-CSF levels than controls. The molecular mechanisms underlying the events prior to G-CSF production included the upregulation of NF-κB and the upstream PI3K, PKB/Akt, and IKK. Accelerated recovery of white blood cells and neutrophil counts were observed in cyclophosphamide-treated mice following IB-MECA administration. Thus, the NF-κB signaling pathway also plays a key role in mediating the myeloprotective effect of IB-MECA. In contrast to tumor cells, normal G-CSF-producing cells respond to IB-MECA by elevating NF-κB levels.[38]

In addition to the myeloprotective effect, A_3AR agonists were found to mediate a protective effect against doxorubicin-induced cardiotoxicity. Activation of A_3AR, but not A_1AR, attenuated doxorubicin cardiotoxicity in cultured cardiomyocytes. Protection by Cl-IB-MECA was manifested by a decrease in intracellular calcium, reduction of free-radical generation and lipid peroxidation, attenuation of mitochondrial damage, and attenuation of the decrease in ATP production. Cardioprotection caused by Cl-IB-MECA was antagonized considerably by the selective A_3AR antagonist, MRS1523, demonstrating the role of A_3AR in mediating the cardioprotective effect.[59]

18.4 CONCLUSIONS

The direct inhibitory effect of adenosine on tumor cell growth is mainly mediated via the A_3AR. Synthetic agonists to this receptor demonstrate an anticancer effect that is mediated via cell cycle arrest, apoptosis, or necrosis, dependent on agonist concentration and cell type. Molecular mechanisms involving modulation of the MAP kinase, Wnt, and the NF-κB signal transduction pathways are involved. The oral bioavailability of synthetic A_3AR agonists, and their induced systemic anticancer and myeloprotective effects, render them potentially useful in three different modes of treatment: as a stand-alone anticancer treatment, in combination with chemotherapy to enhance its therapeutic index, and for myeloprotection. A_3AR agonists are thus a promising new class of agents for cancer therapy.

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19 Adenosine in Atherosclerosis

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CONTENTS

19.1 INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of impairment and death in populations with Western lifestyles. The frequency and severity of this problem is well recognized, and advances are being made in our understanding of its pathobiology. Gradual progressive narrowing of the arterial lumen owing to evolution and expansion of a fibrous plaque impedes the flow of blood after more than 50 to 70% of the lumen diameter is obstructed. As fatty plaque is deposited in the arterial wall, the inward bulge of material reduces available space for blood flow, eventually resulting in symptoms of inadequate blood supply to the target organ in the event of increased metabolic activity and oxygen demand. In recent years, the role of inflammatory cells and signaling in the development and progression of atherosclerosis has been studied extensively. It is now well established that vascular inflammation plays a role in the pathogenesis of atherosclerosis. Atherosclerosis is now regarded as a systemic disease characterized by inflammatory changes with buildup of deposits of lipid-rich plaque within the walls of medium- and large-size muscular arteries.[1,2]

Clinical and statistical studies have identified several risk factors in the pathogenesis of ASCVD. Modifiable major risk factors include hyperlipidemia, hypertension, diabetes mellitus, physical inactivity, and cigarette smoking.[3] Therapies developed to target these factors are effective in reducing morbidity and mortality.[4] The advent

of the statin class of lipid-lowering agents represented a major advance in the treatment of lipid disorders.[5] Lipid-lowering therapy is also associated with reductions in inflammatory markers.[6,7] Despite the wide variety of treatment options, cardiovascular disease continues to pose a major public health problem. Many new molecular targets are being studied to improve atherosclerosis treatment and reduce the number of deaths. The action on these targets could lead to a less atherogenic lipid profile or produce a direct antiatherosclerotic or anti-inflammatory effect on the arterial wall.

The endogenous nucleoside adenosine exerts multiple biochemical effects that serve important roles in vascular biology.[8,9] The effects of adenosine are mediated by a family of four G-protein-coupled receptors, A_1, A_{2A}, A_{2B}, and A_3,[10] which are encoded by distinct genes and are differentiated based on their affinities for adenosine agonists and antagonists.[11–16] This chapter examines the role of adenosine in the molecular biological processes involved in the pathogenesis of atherosclerosis and explores potential approaches utilizing adenosine pathways to develop efficacious prevention and treatment strategies.

19.2 ADENOSINE IN THE VESSEL WALL

Adenosine is a critical mediator of blood flow changes that is released from cells in response to ischemia. Substantial amounts of adenosine are synthesized locally within the vessel wall, thus ensuring pharmacologically active concentrations of adenosine that can impact blood vessel function, most prominently under hypoxic conditions when adenosine increases from nanomolar to micromolar levels.[17] Cells of the vessel wall that have several metabolic pathways for generating large amounts of adenosine include smooth muscle cells[18] and endothelial cells.[19]

19.2.1 Vascular Smooth Muscle Cells

Adenosine is principally formed on degradation of its precursors ATP, ADP, and AMP when high-energy phosphate use exceeds its formation.[20] Nucleotides are rapidly converted to adenosine by a family of ectonucleotidases, including CD39 ectoapyrase (which converts ATP/ADP to AMP) and CD73 ecto-5′-nucleotidase (which converts AMP to adenosine). Vascular smooth muscle cells can also synthesize adenosine from exogenous cAMP.[12,21]

One of the key physiological actions of adenosine is to relax arterial smooth muscle, which results in decreased vascular resistance and augmented blood flow and oxygen delivery.[22] Exogenously administered adenosine is widely used clinically as a vasodilator for myocardial perfusion imaging, producing hyperemic coronary flow by stimulating A_{2A} adenosine receptors on arteriolar vascular smooth muscle cells.[23] In human coronary arteries, adenosine has been shown to induce near-maximal vasodilatation primarily via activation of specific adenosine A_{2A} receptors in vascular smooth muscle. Based on early studies, the vasodilator effect of adenosine was thought to be based solely on direct stimulation of A_2 receptors on vascular smooth muscle cells, which mediates an increase in the second messenger cAMP by stimulating adenylate cyclase.[24] However, several other mechanisms involving

endothelial-dependent vasodilator effects have also been implicated; these will be discussed in the next section. Adenosine may interact with both A_1 and A_2 receptor subtypes to activate ATP-sensitive K^+ channels, leading to relaxation of vascular smooth muscle.[25] Increased intracellular cAMP stimulates protein kinase A, which opens K^+ channels. K^+ channel activation hyperpolarizes smooth muscle and thereby weakens smooth muscle contraction via reduced Ca^{2+} entry through voltage-dependent Ca^{2+} channels.[26]

Sato[27] found that in patients with heart disease, adenosine-induced vasodilation in coronary arterioles was endothelium independent and potently induced by A_{2A} receptor activation. However, activation of A_1 receptors reduced the dilation due to adenosine, indicating opposing effects of A_2 and A_1 receptors.

The adenosine receptor subtype involved in causing relaxation of porcine coronary smooth muscle is mainly the A_{2A} subtype, although A_{2B} may also play a role, possibly through the p38 MAPK pathway.[28]

The excessive focal thickening of the intima during atherosclerosis is attributed to a large increase in smooth muscle cells, production of new connective tissue matrix by these smooth muscle cells, and intracellular and extracellular lipid deposits within the inner vessel wall. In addition to smooth muscle relaxation effects, adenosine inhibits vascular smooth muscle cell proliferation by A_2 receptor activation via the elevation of cAMP,[21,29] and a selective A_2 receptor agonist, 2-octynyladenosine, can reduce neointimal thickening in a rat femoral artery injury model.[30] Inhibitory effects of cAMP on smooth muscle cell proliferation are blocked by A_2 adenosine receptor antagonists, thus implying that the cyclic AMP-adenosine pathway regulates smooth muscle cell growth.[12,31]

19.2.2 Endothelial Cells

The endothelial lining cells of blood vessels form a metabolically active organ system that provides an interface for the exchange of nutrients, gases, wastes, hormones, and metabolites between blood and all tissues of the body. This endothelium rests on a basement membrane called the *intima* and is actively involved in maintaining vascular homeostasis. The endothelium is a focus for inflammatory processes. Endothelial cells receive signals from humoral factors, inflammatory mediators, and physical forces from both the circulation and the tissue.[32] Potential triggers capable of inducing proinflammatory responses include modified lipoproteins, proinflammatory cytokines, chemokines, vasoactive peptides, neuropeptides, hyperglycemia and advanced glycosylated end products, smoking, and oxidative stress. Adhesion of leukocytes to injured vascular endothelium is an early event in atherogenesis initiated by endothelial cell expression of cellular adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin. Adherent leukocytes can then undergo the process of transendothelial migration and subsequent activation.[33]

Endothelial cells have a very active adenosine and adenine nucleotide metabolism, characterized by a large capacity for uptake and release of the nucleoside,[34] and can be an important source of adenosine released during ischemia. ATP is released from endothelial cells during the shear stress produced by changes in blood flow, and there is an ectoenzymatic breakdown of ATP to adenosine.[35,36]

Functional A_{2A} and A_{2B} adenosine receptors have been identified on human aortic endothelial cells,[37] and A_1 and A_3 receptors are also present on vascular endothelium.[38] Adenosine has been shown to induce dose-dependent proliferation of endothelial cells obtained from the aorta.[39]

Both adenosine and homocysteine, the demethylated derivative of methionine, have been shown to enhance endothelial barrier function in cultured endothelial cells isolated from bovine main pulmonary artery and human lung microvasculature by inhibiting adherens junction disruption and intercellular gap formation.[40]

In guinea pig aortic rings, adenosine acts via its A_{2A} receptor to mediate vasodilation through both endothelial-dependent and endothelial-independent mechanisms,[41] not only via flow-mediated dilation[42] but also by directly stimulating A_1 receptors[43] and other purinergic receptors[44] on endothelial cells.

An important mechanism by which adenosine causes vasodilatation is its action on A_{2A} or A_1 receptors present on vascular endothelium to increase the synthesis of the vasodilator nitric oxide (NO).[45,46] NO derived from coronary endothelium causes dilation of the coronary vasculature. NO is generally regarded as an antiatherogenic compound not only because it is a vasodilator, but also owing to its antiproliferative[47,48] and antithrombogenic properties.[49] In addition, NO can interfere with monocyte and leukocyte adhesion to the endothelium.[50] NO is produced from the amino acid L-arginine by different NO synthase (NOS) isoforms.[51] NO is a highly reactive species that is released continuously by endothelial cells. Released NO is an important mediator for vasodilation in response to the lower concentrations of adenosine ($\leq 10^{-7}$ mol/l).[52] In isolated, cannulated porcine coronary arterioles, Hein and colleagues[53] found that microvascular dilation to adenosine was mediated by A_{2A} receptors found on both the microvascular endothelial cells and smooth muscle cells. A_{2A} receptor activation induced vasodilation by endothelial discharge of NO and by the smooth muscle opening of ATP-sensitive K^+ channels.

In guinea pig coronary endothelium, activation of adenosine receptors (order of potency $A_{2A} > A_1 >> A_3$) mediates a vasodilatory effect through NO release.[54] Rubio and Ceballos[54] estimate that in guinea pig coronary vessels 60 to 80% of the vasodilatory effect of adenosine is mediated through NO. In general, NO released from the endothelium subsequently activates soluble guanylyl cyclase in underlying vascular smooth muscle cells and thus produces vasodilation.[55] However, a direct activation of vascular smooth muscle ATP-sensitive K^+ channels and calcium-activated K^+ channels by NO was also reported.[56,57] Therefore, signal transduction mediating dilation of coronary arteries to adenosine downstream from NO remains unclear.

Extracellular ATP and adenosine cause endothelial apoptosis, as assessed by DNA ladder formation and ethidium bromide staining. The development of apoptosis is linked to intracellular metabolism of adenosine because the effect was inhibited by inhibitors of ectonucleotidases and adenosine transport.[58] Purine-induced endothelial cell apoptosis may be important in control of cell replication, limiting endothelial cell proliferation after vascular injury.

19.2.3 Platelets

The first event in hemostasis is the adhesion of platelets to exposed subendothelium. Increasing evidence shows the contribution of platelet–endothelial cell interactions in

the progression of atherosclerosis.[59] Platelets contribute to coronary events by promoting thrombosis, vasoconstriction, and fibroproliferation. When tissue or vascular injury occurs, endothelial cells are damaged and platelets encounter matrix proteins exposed by disrupted endothelium, causing them to adhere and become activated. Activation of platelets is essential to prevent excessive blood loss at the site of vascular injury, but also may lead to proatherogenic consequences such as synthesis of proinflammatory cytokines. Platelets participate in monocyte recruitment to early atherosclerotic and neointimal lesions by secretion and deposition of chemokines. Physiologic platelet stimuli include ADP, epinephrine, thrombin, and collagen. Activated platelets change shape from normal discoid to spiny sphere and spread over the vascular defect. They also undergo the release reaction, liberating the contents of alpha and dense granules during activation. Platelets stimulate, through a platelet-derived growth factor-dependent mechanism, the migration of fibroblasts from the media to the intima of the vessel wall and the proliferation of smooth muscle cells in the intima of arteries, both of which may contribute to intimal thickening in atherosclerosis.[60,61] Contact with activated platelets alters the chemotactic and adhesive properties of endothelial cells by inducing monocyte chemoattractant protein 1 (MCP-1) production and ICAM-1 surface expression by endothelial cells.[62] Both MCP-1 and ICAM-1 have been detected in high concentrations in atherosclerotic lesions.

Adenosine A_{2A} receptors are present in abundance in platelets, and their activation results in accumulation of cAMP and an anti-aggregatory effect.[63] Endogenous adenosine prevents platelet adhesion through A_2-receptor- and possibly also A_1-receptor-dependent mechanisms.[64,65] A_{2A} knockout mice exhibit increased platelet aggregation.[66] Under normal conditions, blood levels of adenosine exceed the threshold necessary to tonically activate platelet A_{2A} receptors, preventing excessive platelet responsiveness.[67]

Elevated circulating levels of cholesterol can influence platelet aggregation by decreasing responsiveness of platelets to adenosine. Platelets from patients with untreated heterozygous familial hypercholesterolemia had a reduced response to aggregation inhibition by adenosine, whereas platelets from patients treated with cholestyramine for their hypercholesterolemia responded normally to adenosine. Hypercholesterolemia may interfere with coupling of the platelet A_2 adenosine receptor to adenylyl cyclase.[68]

The importance of adenosine in platelet aggregation and thrombus formation is evidenced by the therapeutic efficacy of the drug dipyridamole in treating arterial vascular disease. Dipyridamole acts by inhibiting the uptake of adenosine into platelets, endothelial cells, and erythrocytes *in vitro* and *in vivo*. The inhibition occurs in a dose-dependent manner at therapeutic concentrations (0.5 to 1.9 μg/ml) and results in an increase in local concentrations of adenosine, which acts on the platelet adenosine A_2 receptor. Platelet aggregation is inhibited via this mechanism in response to various stimuli such as platelet activating factor (PAF), collagen, and ADP.[69]

19.3 ADENOSINE IN CHOLESTEROL HOMEOSTASIS

Autoimmune disorders such as rheumatoid arthritis accelerate progression of atherosclerosis.[70] Methotrexate (MTX) is a commonly used treatment for rheumatoid arthritis, whose antirheumatic, anti-inflammatory effects are mediated, in large part,

by adenosine.[71,72] MTX may provide a substantial survival benefit in rheumatoid arthritis, largely by reducing cardiovascular mortality.[73] Antiatherogenic effects of MTX may be mediated through adenosine as well. Because inflammatory processes are intricately involved in the pathogenesis of atheroma,[74] our laboratory investigated whether adenosine modulates cholesterol transport protein expression and thereby influences atherogenesis.

We found that selective A_{2A} receptor activation increased both 27-hydroxylase and ATP binding cassette transporter A1 (ABCA1) mRNA expression by about 80% in THP-1-differentiated macrophages derived from the THP-1 human monocytoid cell line.[75] The enzyme cholesterol 27-hydroxylase and the protein ABCA1 are central mediators of reverse cholesterol transport from peripheral tissues to liver. Cholesterol 27-hydroxylase both facilitates cholesterol transport to the liver and, by generating oxysterol signaling molecules, promotes expression of other cholesterol efflux molecules, specifically ABCA1.[76–78] ABCA1 mediates cholesterol secretion from cells and functions as a rate-controlling protein in the apolipoprotein A-I (apoA-I)-dependent active transport of cholesterol and phospholipids.[79] Mutations in the ABCA1 gene result in Tangier disease, a rare genetic disorder characterized by extremely low-plasma high-density lipoprotein and apoA-I levels, cholesteryl ester deposition in tissues, macrophage cholesterol ester accumulation, and a high prevalence of ASCVD.[80] We previously demonstrated that immune reactants, including immune complexes that have fixed complement (IC-C1q) and IFN-γ, affect cellular cholesterol flux by markedly decreasing expression of both cholesterol 27-hydroxylase and ABCA1 in human monocytes and macrophages.[81] Adenosine modulation of cholesterol 27-hydroxylase and ABCA1 gene expression implies a role for adenosine in cholesterol homeostasis and cardioprotection, which has been further validated in studies of foam cell formation. THP-1 macrophages can be overloaded with cholesterol ester to form typical foam cells, and foam cell formation increases in the presence of immune reactants such as IFN-γ and IC-C1q. In cholesterol-loaded THP-1 macrophages, occupancy of adenosine A_{2A} receptors not only counteracts immune reactant-induced suppression of cholesterol 27-hydroxylase and ABCA1 levels, but also diminishes immune reactant-induced foam cell transformation by between 25 and 40%.[75] Upregulation of cholesterol 27-hydroxylase and ABCA1 via A_{2A} receptor occupancy can restore a critical defense mechanism against atherosclerosis that is crippled by immune and inflammatory mediators.

19.4 CONCLUSIONS

Despite our knowledge of an array of ASCVD risk factors and despite aggressive medical intervention targeting elevated blood pressure and cholesterol, cardiovascular diseases still remain the most common cause of death in industrialized countries. Accumulating data have provided a new paradigm for understanding how inflammation, cholesterol metabolism, and cardiovascular disease are related to one another. This paradigm has opened up new areas of research and may lead to novel treatment approaches. New atheroprotective therapeutic strategies may include effective control of inflammation. An antiatherosclerotic effect on the vascular wall could

be achieved by using adenosine, which inhibits multiple steps in the inflammatory cascade.[82] It has been suggested that the establishment of adenosine therapy may be useful for the treatment of either ischemic heart diseases or chronic heart failure.[83] In a canine model of reperfused myocardial infarction, a novel highly selective adenosine A_{2A} receptor agonist, ATL-146e, reduced infarct size by 45% at a very low nonvasodilating dosage.[84]

Atheroprotection may be achieved by restoring or protecting endothelial functional integrity, preventing smooth muscle cell overgrowth, limiting thrombosis, and controlling inflammation. In murine $CD4^+$ T lymphocytes, A_{2A} receptor activation reduced T cell receptor-mediated production of the atherosclerosis-promoting cytokine IFN-γ by 98%.[85] Further, cellular release of adenosine is increased under pathophysiologic conditions of oxidative stress,[86] and adenosine receptor occupancy can suppress the generation of reactive oxygen species (ROS).[87] ROS, which mediate various signaling pathways that underlie vascular inflammation in atherogenesis, are generated under conditions of vascular injury secondary to processes such as ischemia/reperfusion, inflammation, and atherosclerosis.[88] Exposure of cells to oxidized low-density lipoprotein induces ROS formation.[89] Oxidized low-density lipoprotein accumulation in the arterial wall is a characteristic feature of atherosclerosis. Thus, adenosine may act as an endogenous protector against ROS, an underlying cause of atherosclerotic injury.

Insights into the multiple mechanisms by which adenosine may interfere with progression from normal arterial wall to atherosclerotic plaque may be of value in designing new treatment approaches for reducing ASCVD risk.

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20 Regulation of Macrophage-Dependent Angiogenesis by Adenosine and Toll-Like Receptors

Samuel Joseph Leibovich

CONTENTS

20.1 INTRODUCTION

Regulation of new blood vessel growth, the process of angiogenesis, is critical for inflammation, wound healing, granulation tissue formation, development of fibroproliferative diseases, and solid tumor growth. Numerous studies over the past three decades have indicated that angiogenesis is regulated by a series of growth regulatory factors affecting the principal cells that constitute the microvasculature, namely, endothelial cells, smooth muscle cells, and pericytes. The angiogenic and antiangiogenic growth regulatory factors are produced by these vascular cells themselves, as well as by nonvascular cells recruited to the vicinity of the microvasculature as a result of microenvironmental signals. These nonvascular cells include monocytes/macrophages, lymphocytes and platelets, recruited as part of inflammatory processes, and certain populations of tumor cells produced as a result of cellular transformation. Many transformed cells produce high levels of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and various isoforms of fibroblast growth factor (FGF), including oncogenic forms, in an unregulated,

constitutive fashion, due to genetic alterations in the transcription factors involved in the expression of these factors.[1,2] In contrast, in situations such as wound healing, in which granulation tissue forms and angiogenesis is prominent, these angiogenic factors are produced in a highly regulated and controlled fashion. Macrophages, which have been shown in numerous studies to be important mediators of angiogenesis, express angiogenic factors such as VEGF.[3–22] However, they do so in a manner that is exquisitely sensitive to microenvironmental cues. These cues include lipid-derived inflammatory molecules produced locally at the sites of injury, such as prostaglandins and leukotrienes derived from arachidonic acid, degradation products of extracellular matrix constituents, and chemokines and cytokines produced by cells of the microvasculature. In addition, local oxygen tension plays an important role in regulating the expression of angiogenic factors by numerous cell types.[23–27] We have found recently that production of inflammatory cytokines (TNFα, IL-12, and IL-10) and of the angiogenic growth factor VEGF by macrophages is regulated by adenosine, a metabolite produced by numerous cell types, and induced by conditions of ischemia and hypoxia.[28–32] In particular, we have found that adenosine synergizes with Toll-like receptor (TLR) agonists such as endotoxin (LPS) to strongly induce the expression of VEGF by macrophages and to strongly downregulate their expression of TNFα. We have proposed that this interaction between adenosine and TLR agonists constitutes an "angiogenic switch" that converts macrophages from an inflammatory phenotype engaged in combating infection to a wound-healing phenotype dedicated to inducing angiogenesis and fibrovascular proliferation.[7,33] In this chapter, we will consider the various roles of adenosine and TLRs in the regulation of inflammation and angiogenesis, with a special emphasis on the role of these agents in modulating the inflammatory vs. the angiogenic phenotype of macrophages.

20.2 PATHWAYS OF MACROPHAGE ACTIVATION

Macrophages derive from blood-borne monocytes that are recruited to sites of inflammation and injury in response to chemoattractants produced at the site of injury.[34,35] In addition to the monocytes recruited in response to injury, resident tissue macrophages are also present in most tissues, having taken up residence during fetal and embryonic development.[36–38] These resident macrophages have a very low rate of turnover in uninjured tissues, but following injury may proliferate to increase the local population of macrophages. Both recruited monocytes and local macrophages respond to local microenvironmental conditions by changing their phenotype and producing a series of factors that they do not produce in the resting state.[39–43] The nature of the factors induced depends critically on the nature of the microenvironment. Classical activation of macrophages induces an inflammatory (M1) phenotype, with the expression of inflammatory cytokines, including TNFα, IL-1, IL-6, and IL-12.[44–52] In contrast, activation of macrophages with glucocorticoid hormones, IL-4, IL-10, or IL-13, results in an alternatively activated phenotype characterized by the production of anti-inflammatory cytokines such as IL-10, IL1-Ra, and the type-II IL-1 decoy receptor.[42,44,53–55] Macrophages activated in this manner have been termed type II or M2-activated macrophages.[41,42] As discussed later, we have defined an

additional pathway for regulation of macrophage phenotype that depends upon a synergistic interaction between TLR agonists and adenosine receptor (AR) agonists. This alternative pathway results in a switch of macrophages from an inflammatory phenotype to an anti-inflammatory, angiogenic phenotype.[31,33] Pathways of classical vs. alternative activation of macrophages are shown in Figure 20.1.

Classical activation of macrophages (type I, M1) involves the response of macrophages to LPS and the T-cell-derived cytokine IFNγ.[41,56] This pathway induces an inflammatory phenotype characterized by the upregulation of expression of reactive oxygen radicals, the inducible nitric oxide synthase (iNOS) and nitric oxide (NO), matrix metalloproteinase-9 (MMP9), and inflammatory cytokines, including TNFα, IL-1, IL-6, and IL-12.[57,58] Classically activated macrophages also show upregulated expression of certain cell surface receptors, including MHC class II, Fcγ-RI, II, III (CD16, CD32, and CD64), CD80, and CD86.[41,42] These macrophages are thus primed for a role in the recognition, phagocytosis, and destruction of foreign organisms and tumor cells, and produce large amounts of proinflammatory cytokines. Factors such

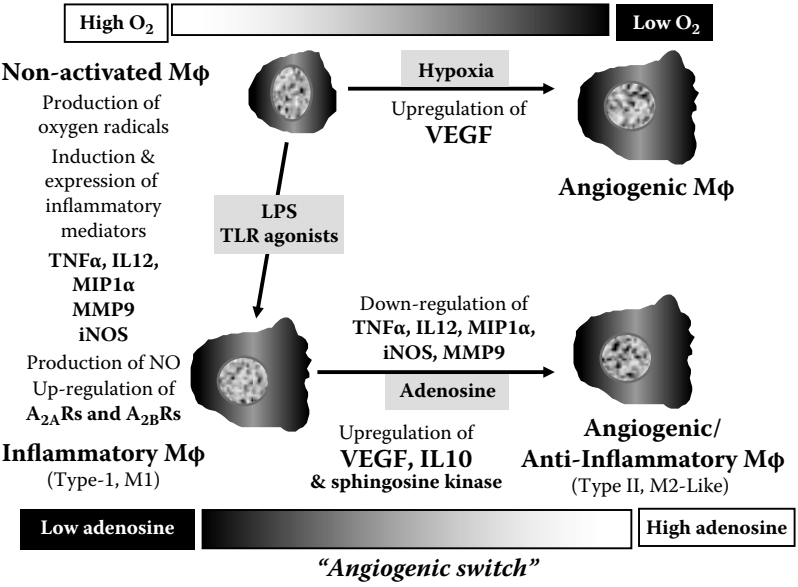

FIGURE 20.1 Alternative pathways of macrophage activation induced by TLR agonists and adenosine. LPS (TLR4 agonist) and TLR2, TLR4, TLR7, and TLR9 agonists activate macrophages to express an inflammatory (M1) phenotype, with upregulated expression of oxygen radicals, iNOS, inflammatory cytokines, MMP9, and adenosine receptors. Hypoxia induces the expression of VEGF, but does not affect the expression of inflammatory cytokines. Extracellular adenosine, produced as a result of ischemia, switches M1 macrophages to an M2-like, anti-inflammatory, angiogenic phenotype by downregulating the expression of inflammatory cytokines, iNOS and MMP9, and upregulating the expression of VEGF, SK1, and IL10.

as LPS from Gram-negative bacteria (TLR4 agonist) or lipotechoic acid from Gram-positive bacteria (TLR2 agonist) bind to TLRs on the cell surface, in conjunction with other cell surface receptors, including CD14, CD11b/CD18, and MD2 for TLR4, and CD36 for TLR2. Agonist binding activates intracellular signaling pathways that result in the activation of NF-κB and in the induction of expression of inflammatory cytokines.[59–63] Agonists of several TLRs have been shown to induce this pathway of activation. However, the pattern of genes induced by various TLR agonists is not identical. For example, TLR2 agonists such as *P. gingivalis* LPS induce a more limited repertoire of cytokines than does the TLR4 agonist *Escherichia coli* LPS.[64] Agonists of scavenger receptors also induce gene expression in macrophages, resulting in an inflammatory phenotype.[65–71]

Alternative pathways of macrophage activation that differ from the classical activation pathway have also been defined recently.[39,42,44,72–77] Exposure of macrophages to glucocorticoid hormones IL-4, IL-10, and IL-13 induces a phenotype characterized by induction of IL-10, IL1-Ra, Decoy IL1R type II, scavenger receptors A and B, FcεRII (CD23), CD163, and arginase, without induction of TNFα, IL-12, iNOS, or reactive oxygen species.[41,44] These macrophages have been termed type II or M2 macrophages (Figure 20.1). Hypoxia induces a phenotype in macrophages characterized by upregulation of VEGF expression and upregulation of iNOS, but no upregulation of inflammatory cytokines. Activation of macrophages by TLR agonists generally induces an inflammatory M1 phenotype, but the pattern of gene induction through specific TLRs differs. As discussed later, activation of macrophages by TLR2, 4, 7, and 9 agonists in the presence of adenosine (or adenosine A_2R agonists) changes macrophages from a classic M1 phenotype to a phenotype characterized by downregulated expression of inflammatory cytokines and upregulated expression of VEGF, IL-10, G-CSF, both adenosine A_{2A} and A_{2B} receptors, and sphingosine kinase-1 (SK-1), a phenotype that more closely resembles that of M2 macrophages.[31,33] However, whereas the original reports of the M2 phenotype require macrophage activation by glucocorticoid hormones or cytokines such as IL4, IL10, and IL13, the pathway we have described, mediated by TLR agonists together with adenosine, does not require adaptive immunity but functions as a basic part of the innate immune response to injury and infection, requiring only a TLR agonist and the extracellular accumulation of adenosine.

20.3 TOLL-LIKE RECEPTORS (TLRs)

TLRs are transmembrane receptors that play a critical role in the host cells' recognition of and response to foreign organisms. Toll was first described in *Drosophila* as a molecule involved in dorsal–ventral patterning.[78] Subsequently, Toll was shown to be essential for the resistance of *Drosophila* to fungi, with Toll mediating a signaling pathway that activates expression of antifungal peptides.[79] This pathway has many aspects in common with the NF-κB signaling pathway in mammalian cells.[62,63] Following the initial characterization of Toll as an innate immune signaling molecule in *Drosophila*, several mammalian homologs of Toll were described. The first of these was TLR4, which was shown to be the mammalian cell surface receptor for endotoxin of Gram-negative bacteria.[80,81] TLR4 was shown to be a transmembrane

molecule that transmits an LPS-mediated signal from the cell surface, inducing activation of intracellular signaling pathways.[62] These pathways result in the activation of NF-κB, and thus the induction of expression of numerous inflammatory cytokines, including IL-1, IL-6, IL-12, TNFα, and others.[61,62,82,83] TLR activation may also stimulate MAP-kinase-dependent pathways. To date, at least 23 TLRs have been described,[84] and agonists for several of these molecules have been defined. In general, TLRs act as recognition molecules for pathogen-associated molecular patterns (PAMPs). TLR4 is an essential receptor for endotoxin from Gram-negative bacteria.[80,83–86] TLR2 recognizes a variety of microbial components, including lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipotechoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, and LPS from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis,* and *Helicobacter pylori*.[85,87–90] TLR3 recognizes double-stranded RNAs, which are produced by most viruses during their replication.[91] TLR5 recognizes bacterial flagellins, a major protein constituent of bacterial flagellae.[92,93] TLR7 and TLR8 recognize synthetic imidazoquinolines, used for the treatment of genital warts associated with viral infections, and loxiribine, an antiviral and antitumor compound.[85,94–96] Both TLR7 and TLR8 recognize guanosine or uridine-rich single-stranded RNA from viruses such as HIV, vesicular stomatitis virus, and influenza virus.[97,98] TLR9 recognizes nonmethylated CpG DNA motifs, common in bacterial DNA but rare in mammalian DNA,[99,100] and TLR11 recognizes as yet unidentified epitopes on uropathogenic bacteria.[101]

TLRs are expressed on several cell types, but macrophages, in particular, express high levels of several TLRs, either constitutively or following induction with agents such as LPS and other activating agents. Homo- and heterodimerization of TLRs lead to their ability to recognize a huge range of pathogenic organisms, and TLRs clearly provide a critical first line of defense against invasion of the host by foreign organisms. Although TLRs are pattern recognition receptors for foreign organisms, it has been suggested that TLRs may also recognize certain endogenous ligands.[102] For example, TLR4 has been shown to be a receptor for Hsp60, an alternatively spliced form of fibronectin, certain forms of hyaluronic acid, and components of minimally modified LDL.[51,103–109] In addition to providing the basis for the innate immune response, TLRs are also required for the initiation of the adaptive immune response.[110–112]

TLR signaling pathways have been extensively studied recently. Following binding of agonists, the cytoplasmic adaptor protein MyD88 is recruited to the cytoplasmic domain of all TLRs with the exception of TLR3, and is critical for TLR-mediated signaling.[61–63] Signaling through TLR3 and TLR4 may also occur in an MyD88-independent manner, using adaptors such as Mal/TIRAP and TRIF/TRIM. However, MyD88 is clearly a critical signaling molecule for the activation of NF-κB and induction of inflammatory cytokines. TLR activation generally results in the activation of the NF-κB signaling pathway and in the upregulation of expression of inflammatory cytokines such as TNFα and IL-12. Activation of different TLRs, however, leads to varying patterns of gene expression.[64,113,114] Activation of TLR3 and TLR4 signaling pathways leads to the induction of type-1 interferons, whereas activation of TLR2- and TLR5-mediated pathways does not. TLR7, TLR8, and TLR9 signaling pathways may also lead to induction of type-1 interferons, through

mechanisms distinct from TLR3- and TLR4-mediated induction.[100,115] TLR-mediated signaling pathways have been reviewed in detail recently.[62,63]

20.4 ADENOSINE AND ADENOSINE RECEPTORS

Adenosine is a bioactive metabolite produced as a result of intracellular and extracellular breakdown of nucleotides and nucleosides.[116,117] Adenosine is actively released from cells in conditions of stress, including inflammation, ischemia, and hypoxia.[28,118–122] In addition, adenosine may be formed from ATP, which is actively released from viable cells under conditions of stress. Extracellular ATP can signal directly through purinergic type-2 (P2) receptors, or can be hydrolyzed by cell surface and extracellular nucleotidases (CD39 and CD73) to ADP, AMP, and adenosine.[123–126] Adenosine may then signal through a series of cell surface G-protein-coupled adenosine receptors (P1-type purinergic receptors).[118,127–132]

Although signaling through adenosine receptors may often involve regulation of adenylyl cyclase and cAMP levels, signaling through Gβ and Gγ proteins in a Gsα-independent and cAMP-independent manner has also been demonstrated. G-protein-independent signaling mediated through interactions with the β-arrestin family of signaling molecules may also occur.[133–135]

Numerous studies of the past two decades have shown that adenosine is a potent endogenous anti-inflammatory agent.[136–138] Adenosine, acting through A_2Rs, inhibits many functions of neutrophils, including phagocytosis, generation of toxic oxygen metabolites, and adhesion to endothelium.[136,139] Adenosine acts locally on the ARs of surrounding cells to protect tissues from injury through anti-inflammatory responses. For example, although activated platelets can release ADP, which binds to P2Y receptors and causes aggregation/adhesion of platelets,[117,140] ADP can also be dephosphorylated by the 5′-ectonucleotidase CD39 to form AMP, which can be further converted by the 5′-ectonucleotidase CD73 to form adenosine, which binds to ARs such as the A_{2A}R to inhibit platelet aggregation/adhesion, decrease expression of endothelial cell adhesion molecules, and decrease histamine and proinflammatory cytokine production by mast cells and macrophages. A_{2A}, A_{2B}, and A_3 receptors have been shown to be involved in the downregulation of cytokine expression by macrophages. Adenosine suppresses the expression of TNFα, IL-12, MIP-1α, and nitric oxide (NO) by LPS-activated macrophages.[28–30,141–143] We have found recently that adenosine also strongly suppresses the expression of MMP9.[144] Suppression of TLR-ligand-induced expression of these inflammatory mediators by adenosine is probably one of the key mechanisms by which adenosine receptor occupancy prevents inflammation-induced tissue injury. Adenosine suppresses induction of these factors not only by LPS, a TLR4 ligand, but also by TLR2-, TLR7-, and TLR9-activated macrophages,[31] indicating that this anti-inflammatory action of adenosine is a general effect, and not limited to antagonism of LPS. Although suppression of NF-κB has been suggested as the mechanism for downregulation of TNFα expression in macrophages by adenosine,[145] the mechanism by which adenosine suppresses TLR-agonist-induced TNFα expression is not yet entirely clear. Levels of LPS-induced TNFα mRNA are not reduced by adenosine, and activation of NF-κB, a critical factor involved in the transcriptional regulation of cytokines such as IL-12 and TNFα,

is not altered.[33,145,146] It would appear that the regulation by adenosine is posttranscriptional and may occur at the translational or posttranslational level. This aspect of adenosine signaling is discussed in more detail in Chapter 4.

Although adenosine strongly downregulates LPS-induced TNFα, IL-12, NO, and MMP9 expression by macrophages, this nucleoside also strongly upregulates expression of both IL-10 and VEGF. IL-10 expression is upregulated by LPS, but adenosine augments this expression at least two- to fourfold. This effect is mediated posttranscriptionally at the translational level.[147] In contrast, LPS alone has little effect on the expression of VEGF by murine macrophages, although it may cause a weak induction of VEGF expression by human monocytes.[33,148–150] In synergy with adenosine A_{2A}R agonists, however, LPS (and agonists of TLR2, TLR7, and TLR9) strongly upregulates the expression of VEGF by at least 10- to 12-fold. This upregulation of VEGF expression is as strong as, or stronger than, that induced by hypoxia alone and is independent of it. The mechanism by which VEGF expression is regulated by adenosine with TLR2, TLR4, TLR7, and TLR9 agonists will be discussed in more detail in the following text.

20.5 REGULATION OF MACROPHAGE ACTIVATION BY ADENOSINE

We have shown recently that adenosine and adenosine receptor analogs play a profound role in modulating the phenotype of macrophages.[31,33,146,151] Although macrophages activated by TLR2, 4, TLR7, and TLR9 agonists acquire a classically activated (M1) phenotype, activation of macrophages by these TLR agonists in the presence of adenosine (or adenosine receptor agonists) switches macrophages from the M1 phenotype to an alternatively activated state resembling an M2 phenotype, strongly downregulating the expression of TNFα, IL-12, and MMP9, and strongly upregulating the expression of the angiogenic growth factor VEGF, sphingosine kinase (SK1) and several other genes (see the following text). We have termed this phenotypic change induced in macrophages an "angiogenic switch."[31] We have been investigating the downstream pathways that mediate the synergistic interaction between TLR and adenosine receptor signaling. With regard to adenosine signaling in this synergistic pathway, upregulation of VEGF expression critically requires the adenosine A_{2A}R. This requirement was confirmed both pharmacologically and by the use of mice specifically lacking individual adenosine receptors. Mice lacking the A_{2A}R, but not those lacking the A_3R, do not show the synergistic upregulation of VEGF expression with TLR agonists.[33] The A_{2A}R-specific agonist 2-[p-(2-carboxyethyl)-phenylethyl amino]-5′-*N*-ethyl-carboxamido-adenosine (CGS21680) strongly synergizes with LPS to induce VEGF expression, whereas the A_1R agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) does not. 5′-*N*-ethylcarboxamidoadenosine (NECA), a nonspecific A_2R agonist, also strongly synergizes with TLR agonists to induce VEGF expression. The A_1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) did not antagonize the action of CGS21680 in stimulating VEGF production. Similarly, 3,7-dimethyl-1-propargyl xanthine (DMPX), a nonspecific A_2R antagonist, had little effect, but ZM241385, a selective A_{2A}R antagonist, strongly inhibited the effects of LPS with CGS21680 or NECA. These results clearly indicate

a critical requirement for A_{2A}Rs in this aspect of the angiogenic switch. Analysis of A_{2A}R expression in murine macrophages following LPS and/or CGS21680 or NECA treatment, using a specific anti-A_{2A}R antibody, did not indicate an increase in expression; however, a recent binding study using the specific A_{2A}R antagonist ZM241385 to determine specific A_{2A}R levels indicates that LPS induces a large increase in the expression of functional A_{2A}Rs on both murine macrophages and human monocytes.[152] This increased expression of A_{2A}Rs may play a key role in the synergistic upregulation of VEGF expression induced by LPS with A_{2A}R agonists.

The A_{2A}R is generally a G_s-coupled receptor that positively regulates adenylyl cyclase activity. However, pharmacological studies indicate that A_{2A}R-dependent signaling in this system upregulating VEGF expression is cAMP independent, and independent of protein kinase-A (PK-A). Inhibitors of cAMP formation or of PK-A activity do not block the synergistic upregulation of VEGF. Similarly, KT449, an inhibitor of G_{soc} has no effect on VEGF induction. It seems from preliminary experiments that the A_{2A}R signaling for this interaction may involve a pathway regulated by GRK-2 and the β-arrestins, but this remains to be rigorously confirmed.[144] We have also found that regulation of both phospholipase-Cβ2 (PLCβ2) and of its downstream target protein kinase-Cδ (PKCδ) is involved in the synergistic switch of macrophages from an inflammatory to an angiogenic phenotype, suggesting the involvement of Gq or Gβ/γ signaling. In contrast, no effect of PL-A_2, or of COX1 and COX2 inhibitors was found.

The upregulation of VEGF expression in this pathway is, at least in part, transcriptionally regulated. We have prepared a series of VEGF promoter constructs in the pGL3®-luciferase vector bearing specific deletions of various transcription factor binding elements.[153] Transfection of RAW264.7 cells with these constructs indicates that expression of luciferase under the control of the VEGF promoter is strongly induced by a combination of an adenosine A_{2A}R agonist (CGS21680) or NECA and a TLR agonist such as LPS (TLR4) or R848 (TLR7). CGS21680 alone, or LPS alone, had only minor effects on luciferase expression, indicating that the induction of VEGF expression is mediated, at least in part, at the transcriptional level. Deletion of the hypoxia-response element (HRE) from the promoter abrogates the upregulation of luciferase expression. This suggests that the HRE, which binds the HIF-1 transcription factor, is essential for this transcriptional regulation. These results indicate an important role for the HRE and the HIF-1 transcription factor in the nonhypoxic regulation of macrophage VEGF expression by adenosine and TLR agonists.

With regard to signaling through TLRs, we have found that MyD88, the immediate downstream molecule that interacts with the cytoplasmic TIR domain of most TLRs,[63] is critically required for the stimulation of VEGF expression by A_{2A}R agonists with TLR agonists. Macrophages from mice deficient in MyD88 (MyD88$^{-/-}$ mice) totally lack this pathway. Similarly, mice lacking the IRAK4 gene, which is recruited to the TLR-MyD88 cytoplasmic complex, are also deficient in this pathway. Downregulation of TRAF6 expression in macrophages using siRNA also abrogates this signaling pathway. TRAF6 is a downstream mediator of IRAK4 signaling.[154] TRAF6 signals through various pathways, of which the TAK/TAB pathway that activates IκB-kinase is a major branch. Activation of IκB-kinase results in the

phosphorylation of IκB, promoting the release of NF-κB from the inactive IκB-NF-κB complex. NF-κB isoforms (p50 and p65) then translocate to the nucleus, in which they form a complex on NF-κB binding sequences in the promoters of genes such as TNFα, which are involved in the regulation of inflammation. As discussed already, we have found that adenosine does not appear to alter the LPS-induced activation of NF-κB and that the downregulation of TNFα expression induced by adenosine in LPS-activated macrophages does not involve direct suppression of NF-κB activation.[33,146]

In regard to the induction of VEGF expression by adenosine with TLR agonists, the role of NF-κB is not entirely clear. The VEGF promoter contains putative NF-κB binding sequences.[153,155] Deletion of these sites from the VEGF promoter does not abrogate luciferase expression induced by LPS with adenosine A_{2A}R agonists in transfection experiments in RAW264.7 cells. In contrast, deletion of the HRE from the VEGF promoter causes a major reduction in luciferase expression in RAW264.7 cells treated with either hypoxia, or LPS with CGS21680 or NECA.[153,156] Treatment of hypoxic macrophages with NF-κB inhibitors such as BAY11-7082 or SN50 had little effect on VEGF expression; however, in LPS/NECA-treated cells, these NF-κB inhibitors induced a marked inhibition of VEGF expression,[144] suggesting the involvement of NF-κB in the regulation of VEGF expression by LPS/NECA. Our results suggest that this effect of NF-κB inhibitors in LPS-treated macrophages is indirect. We have found, using Q-RT-PCR, that BAY11 and SN50 reduce the expression of HIF1α at the mRNA level as well as at the protein level, suggesting that reduction in HIF1α levels may be involved in the reduction of VEGF expression by NF-κB inhibitors, and indicating a role for NF-κB in the regulation of HIF1α expression. In addition, it has been shown recently that BAY11-7082 reduces the LPS-induced expression of A_{2A}Rs,[152] providing an alternative explanation for the inhibitory effect of this NF-κB inhibitor.

Upregulation of VEGF expression by binding of the HIF1 complex to the HRE of the VEGF promoter requires stabilization of HIF1α, which is normally rapidly degraded by the proteosome under normoxic conditions.[157–164] HIF1α is a target for proline hydroxylases of the EGLN family. These enzymes use molecular oxygen, Fe^{++}, and α-keto-glutarate to hydroxylate specific proline residues to hydroxyproline. These hydroxyprolines then promote the binding of HIF1α to the Von-Hippel Landau tumor suppressor protein, pVHL.[158,165,166] This protein, a ubiquitin E3 ligase, targets HIF1α to the proteasome for degradation. Oxygen-dependent hydroxylation of an asparagine residue in the C-terminal domain of HIF1α by an asparagines-specific oxygen-dependent hydroxylase also regulates the transcriptional activity of HIF1α.[159] Under hypoxic conditions, the reduced availability of oxygen prevents these enzymes from hydroxylating HIF1α, thus preventing the binding of pVHL, ubiquitination, and subsequent targeting to the proteosome. Steady-state levels of HIF1α thus increase rapidly under hypoxia, and the stabilized HIF1α translocates to the nucleus, dimerizes with its constitutively expressed partner HIF1β (ARNT), and binds to the HREs in the promoters of genes such as VEGF that are transcriptionally regulated by HIF1. The mechanism of the HIF1 dependence of VEGF expression induced by the A_{2A}R agonists with TLR agonists is not yet fully understood. However, as discussed earlier, the effect appears to be independent of cAMP, PK-A, and Gsα, and to require signaling through PLCβ2 and PKCδ.

20.6 ROLE FOR THE ADENOSINE-TLR-MEDIATED ANGIOGENIC SWITCH *IN VIVO*

The roles of adenosine in mediating physiological and pathological angiogenesis *in vivo* have been elegantly reviewed recently.[128,129] Adenosine clearly plays an important role in the induction of angiogenesis, by inducing concentration-dependent proliferation and migration of endothelial cells; numerous studies have indicated that adenosine or inhibitors of adenosine uptake can stimulate angiogenesis *in vivo*.[117,129,167,168] The mechanism by which adenosine stimulates angiogenesis has been shown in many of these studies to involve regulation of VEGF production by a variety of cell types,[169–173] as well as the induction of VEGF receptors.[174,175] VEGF-independent effects of adenosine have also been demonstrated.

What is the significance of the TLR2-, TLR4-, TLR7-, and TLR9-mediated interaction with adenosine receptor signaling in the regulation of angiogenesis? As discussed earlier, macrophages are a primary mediator of angiogenesis in wound healing and fibroproliferative diseases in which angiogenesis is prominent, and regulation of macrophage activation is critical for inflammation, innate immunity, and wound healing. TLR agonists stimulate macrophages to produce inflammatory mediators such as TNFα, IL-12, iNOS, and MMP9, and prime macrophages toward a cytotoxic phenotype that exhibits increased antigen processing, thus providing an important line of defense against invasion of the host by foreign organisms.[42,176] TLR agonists also prime macrophages to increase their scavenger activity, thus aiding in debridement of sites of injury. In addition, this priming by TLRs sensitizes the macrophages to the effects of adenosine, in part by inducing the expression of transcription factors such as HIF1,[138] and in part by strongly upregulating the expression of both A_{2A}Rs and A_{2B}Rs.[146,152,177] If the TLR-primed macrophages then find themselves in a hypoxic or ischemic microenvironment in which adenosine accumulation is favored, the increased expression of adenosine receptors will result in a greatly amplified response by these cells to adenosine stimulation through these receptors. This will result in strong downregulation of the expression of inflammatory cytokines (e.g., TNFα and IL-12) and strong upregulation of VEGF expression — the angiogenic switch.[31] The roles of adenosine and TLRs in regulating the phenotype of macrophages are shown diagrammatically in Figure 20.2 and Figure 20.3.

The importance of A_{2A}R signaling *in vivo* for the regulation of angiogenesis is indicated by the observation that A_{2A}R knockout mice exhibit a markedly impaired wound healing phenotype characterized by a decreased formation of granulation tissue and decreased angiogenesis. Also, A_{2A}R agonists applied topically accelerate the healing rate of excisional skin wounds in normal mice by increasing both the rate of angiogenesis and the recruitment of endothelial progenitor cells from the bone marrow.[178–180] The role of adenosine in the regulation of wound healing is discussed in more detail in Chapter 11. In recent experiments, we have studied the healing of excisional skin wounds in mice lacking the MyD88 gene ($MyD88^{-/-}$ mice). As discussed earlier, MyD88 is the immediate downstream mediator of TLR signaling, interacting with the cytoplasmic TIR domain of most TLRs (with the exception of TLR3). Macrophages from MyD88 mice do not respond to stimulation by TLR2, TLR4, TLR7, or TLR9 agonists, and these mice are markedly

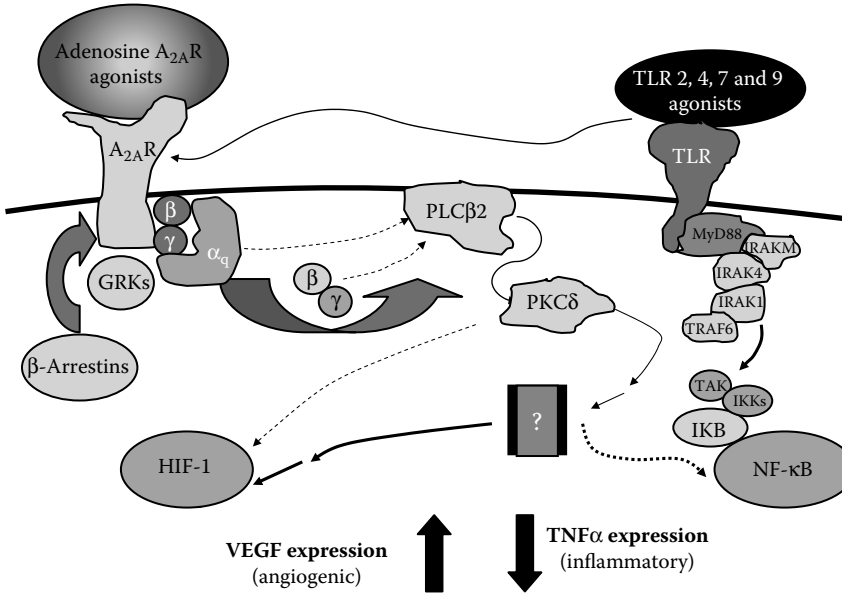

FIGURE 20.2 Signaling pathways involved in the regulation of the "angiogenic switch" in macrophages. Ligation of TLR2, TLR4, TLR7, or TLR9 activates the MyD88-dependent signal transduction pathway that induces activation of NF-κB, a transcription factor that is critical for the expression of inflammatory cytokines such as TNFα. This pathway also results in the upregulated expression of adenosine A_{2A} and A_{2B} receptors. In the presence of adenosine, a pathway shift occurs, which results in strong downregulation of TNFα expression and strong upregulation of VEGF expression. The pathway by which TNFα is downregulated is not yet fully elucidated, but does not involve inhibition of NF-κB activation. The upregulation of VEGF requires the expression of adenosine A_{2A}Rs and is dependent on the expression of HIF1α. The adenosine-dependent signaling is independent of Gsα and may require signaling through PLCβ2 and PKCδ. Signaling involving GRKs and β-arrestins may also be involved.

immunocompromised.[181–183] Although MyD88$^{-/-}$ mice develop normally with no apparent developmental defects, wounds in adult MyD88$^{-/-}$ mice heal at a much slower rate than do identical wounds in control MyD88$^{+/+}$ mice. This slow wound healing is characterized by delayed wound closure and re-epithelialization and delayed formation of granulation tissue.[184] Granulation tissue forming in these wounds also has a reduced neovascular density, as determined by anti-PECAM (CD31) immunohistochemical staining. Macrophage content of these wounds is relatively normal, as determined using staining with F4/80 antimacrophage antibody. Combined with our results indicating that macrophages from MyD88$^{-/-}$ mice do not respond to TLR agonists, these results suggest that TLR signaling through MyD88 plays an important role in normal wound healing. Interestingly, MyD88 signaling has been shown to be critical for the response of intestinal epithelium to injury, and macrophages may play an important role in this response.[185,186] To determine whether adenosine signaling plays any role in the wound-healing deficit

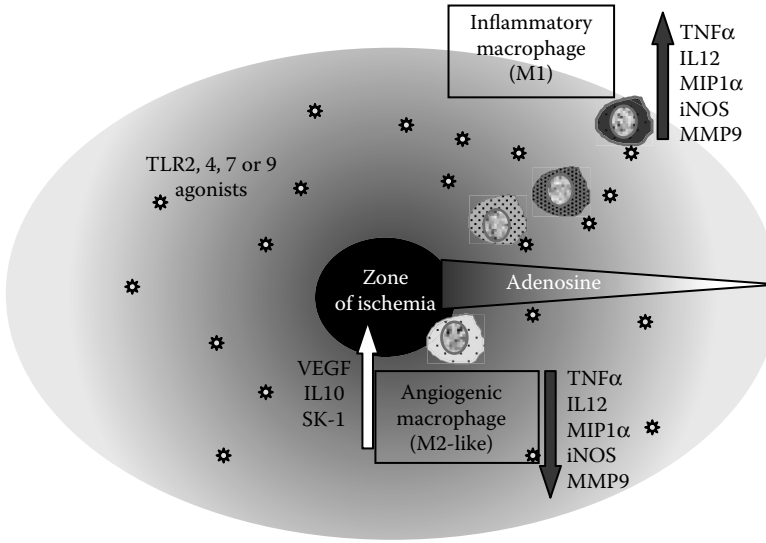

FIGURE 20.3 A model of the role of the TLR-adenosine-mediated "angiogenic switch" in regulating inflammation and angiogenesis in wound healing, fibroproliferative diseases, and cancer. Macrophages induced to migrate into an injured tissue site will be challenged locally by various TLR ligands. These may be exogenous ligands expressed by microorganisms or endogenous host tissue-derived agonists. Under nonischemic, nonhypoxic conditions, these agonists will induce macrophages to exhibit an inflammatory (M1) phenotype with increased ability to kill and remove offending stimuli and debride the site of injury. If the offending stimulus persists, however, and zones of ischemia or hypoxia are present, TLR-agonist-primed macrophages will respond to increased extracellular levels of adenosine by switching to an anti-inflammatory, angiogenic, M2-like phenotype with downregulation of inflammatory mediators and upregulation of VEGF. Thus, the microenvironmental conditions at particular sites of injury or infection, or in a developing tumor in which macrophages are located, will critically determine the particular phenotype — M1 vs. M2-like — that the macrophage will exhibit.

in MyD88$^{-/-}$ mice, we have studied the effects of CGS21680, an $A_{2A}R$ agonist, on wound healing in control and MyD88$^{-/-}$ mice. In control mice, CGS21680 induced an acceleration of wound healing, as reported previously, with increased angiogenesis and granulation tissue formation.[178–180] In MyD88$^{-/-}$ mice, however, CGS21680 had no effect on wound healing.[184] This suggests that the wound-healing effects of CGS21680 in normal mice may be in part dependent on MyD88 signaling. TLR agonists stimulate macrophages, first to an M1 phenotype that primes them for host defense, while strongly increasing their sensitivity to $A_{2A}R$ agonists, both by upregulating expression of A_{2A} and A_{2B} receptors and by inducing HIF1α expression. In the presence of adenosine produced as a result of inflammation, ischemia, or hypoxia, the macrophages will then switch to the M2-like alternatively activated phenotype, with downregulation of inflammatory cytokine expression and upregulation of VEGF expression. In the absence of MyD88 signaling, activation of macrophages to both the M1 and M2 phenotypes will be impaired, and their response to adenosine will

be weaker; thus, the signaling required for the induction of angiogenesis and wound healing is decreased.

This raises the interesting question of the potentially critical relationships between commensal organisms and the host response to injury, as well as the roles of endogenous TLR agonists in this response. Higher animals coexist normally with large numbers of commensal organisms, most of which have not yet been cultured or identified.[187–189] The roles of these potential TLR agonists in wound healing and angiogenesis — either those expressed by foreign organisms or those endogenous agonists exposed as a result of injury — are fruitful topics for research in the future. In addition there are numerous pathophysiological conditions in which TLR and AR signaling may interact. These include the following: (1) granulomatous skin diseases, in which infection results in fibroproliferation and skin lesion formation; (2) lung diseases such as tuberculosis, in which the persistence of a pathogenic organism results in granuloma formation with extensive angiogenesis; and (3) atherosclerosis, in which accumulation of macrophages in the aortic intima leads to foam cell formation, the development of necrotic lipid-rich zones, and the development of fibroproliferative vascular plaques. In all these situations, macrophages accumulate, are modified by local microenvironmental factors, including TLR agonists, and are prime sites for the accumulation of extracellular adenosine, due to development of local ischemia, or hypoxia, or both. Acquiring further insights into the interactions between TLR signaling and AR signaling should lead to the development of novel pharmacological interventions that will enable us to control the phenotype of macrophages and thus modulate developing disease processes.

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