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Adenosine: An Old Drug Newly Discovered

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Abstract

Over decades, anesthesiologists have used intravenous adenosine as mainstay therapy for diagnosing or treating supraventricular tachycardia in the perioperative setting. More recently, specific adenosine receptor therapeutics or gene-targeted mice deficient in extracellular adenosine production or individual adenosine receptors became available. These models enabled physicians and scientists to learn more about the biological functions of extracellular nucleotide metabolism and adenosine signaling. Such functions include specific signaling effects through adenosine receptors expressed by many mammalian tissues, for example vascular endothelia, myocytes, hepatocytes, intestinal epithelia or immune cells. At present, pharmacological approaches to modulate extracellular adenosine signaling are evaluated for their potential use in perioperative medicine, including attenuation of acute lung injury, renal, intestinal, hepatic and myocardial ischemia, or vascular leakage. If these laboratory studies can be translated into clinical practice, adenosine receptor based therapeutics may become an integral pharmacological component of daily anesthesiology practice.

Introduction

The observation that intravenous adenosine causes a temporary heart block dates back many years. In 1927, Drury and Szent-Gyorgyi from the University of Cambridge, United Kingdom, performed an experiment where they injected extracts from cardiac tissues intravenously into a whole animal. They were surprised to notice a transient disturbance of the cardiac rhythm and slowing of the heart rate.¹ Following several purification steps, the authors were able to identify the biologically active compound of the extract as an “adenine compound”.¹ Adenine is a purine-based nucleobase (similar to guanine) involved in many biological functions, including cellular respiration, or protein biosynthesis (as component of DNA and RNA). Looking back from today’s perspective, it seems likely that the induced slowing of the heart-rate was caused by the pharmacological activity of adenosine.¹ Adenosine belongs to the molecular group of nucleosides, composed of an adenine-group attached to a ribose sugar (fig. 1). It took almost 50 years from these early discoveries of the heart-rate-slowing effects of “adenine compounds”¹ to the clinical use of adenosine in treating patients with supraventricular tachycardia.² However, intravenous adenosine has remained a mainstay form of clinical therapy for diagnosing or treating patients with supraventricular arrhythmias since the 1980ies.^{3,4} In fact, intravenous adenosine is among the most frequently used anti-arrhythmic medications in the clinical practice of anesthesiology,⁴ including treatment of supraventricular tachycardia in many perioperative settings, such as cardiothoracic anesthesia,⁵ critical care

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medicine,⁶ or obstetric anesthesia.⁷ In addition, adenosine-induced induction of a transient cardiac arrest is frequently used for assisting accurate deployment of vascular stent grafts in the major blood vessels.^{8,9}

In addition to its clinical role as anti-arrhythmic agent, adenosine has been implicated in diverse areas of medicine. An important clinical application for extracellular adenosine signaling is its potent effect as arterial vasodilator. For example, the adenosine-uptake inhibitor dipyridamole is used during pharmacologically-induced stress-echocardiography to enhance vascular adenosine levels, causing coronary vasodilatation, and unmasking a clinically relevant coronary artery obstruction.¹⁰ In addition, adenosine functions as platelet aggregation inhibitor.¹¹ For example, a recent study investigated different platelet inhibitors in the prevention of recurrent stroke, and found that extended release dipyridamole in combination with aspirin is equally effective as the 5'-adenosine triphosphate (ATP)-receptor antagonist clopidogrel.¹² Moreover, the nonspecific adenosine receptor antagonist caffeine has been suggested for the prevention or treatment of postdural puncture headache.¹³ While this indication has been challenged,¹³ caffeine remains an important therapeutic agent in the treatment or prevention of caffeine withdrawal headache in perioperative patients.^{14,15} Similarly, the non-specific adenosine receptor antagonist theophylline has been used in the past for treating obstructive airway disease, but has been replaced by inhaled long-acting beta-agonist bronchodilators due to less drug-drug interactions and toxicity from drug overdosing.¹⁶

In addition to these well established clinical applications of adenosine, basic research has implicated extracellular adenosine as an endogenous distress molecule¹⁷ with profound impact on immune response,^{17,18} and adaptation to limited oxygen availability (hypoxia).^{19–23} In fact only recently, the research field of extracellular adenine nucleotide metabolism and adenosine signaling rapidly expanded to become an extremely dynamic and exciting field of investigation, as genetic models and specific pharmacological agents became available. For example, mice with genetic defects in the enzymes responsible for extracellular adenosine generation (*cd39*^{-/-} or *cd73*^{-/-} mice, see “Extracellular Adenosine Generation”), or for individual adenosine receptors allowed scientists to specifically target the contribution of extracellular adenosine generation or signaling through individual receptors in a wide range of disease models.

Biology of Extracellular Adenosine

Extracellular Adenosine Generation

Adenosine is implicated in a wide variety of basic biological functions, including nucleotide biosynthesis or cellular energy metabolism. On the outside of the cell, adenosine mainly serves as a signaling molecule and its biological functions occur through the activation of adenosine receptors (ARs) localized on the extracellular surface of the cell membranes. Particularly during conditions of cellular distress (inflammation, hypoxia, acute injury), extracellular adenosine stems from phospho-ester hydrolysis of its precursor molecules, ATP, 5'-adenosine diphosphate (ADP) or 5'-adenosine monophosphate (AMP).²⁴ These molecules (so called nucleotides) consist of the nucleoside adenosine, bound to a varying number of phospho-esters attached to the 5'-designated atom of its ribose sugar ring (fig. 1). In the absence of catalytic enzymes capable of hydrolyzing nucleotides, extracellular ATP, ADP and AMP would be relatively stable. However, most cell types express enzymes on their cell surface that catalyze nucleotide-phospho-hydrolyses. Typically, this process occurs in a three-step reaction. As first step, multiple cell types release intracellular stored nucleotides, particularly in the form of ATP and ADP.^{24–26} It is important to point out that intracellular ATP levels are very high (5–8 mmolar). Therefore, nucleotide release from intracellular sources can occur during cellular damage or death (lysis, necrosis, apoptosis, *etc.*), or through specific gradient driven channels.

^{27,28} Activated platelets that release ADP from stored intracellular vesicles *via* granular release provide an additional source of extracellular nucleotides.²⁹

As second step (Figure 1), extracellular ATP and ADP are rapidly converted to AMP by the ecto-apyrase (CD39). CD39 is a widely expressed surface-bound enzymes that is expressed on multiple cell types³⁰ and serves in a dual role. On the one hand, CD39 is responsible for extracellular adenosine production by generation of AMP. As such, pharmacological inhibition or genetic deletion of CD39 is associated with attenuated extracellular adenosine levels and defects in extracellular adenosine signaling.^{31,32} At the same time, CD39 catalyzes the key step for extracellular breakdown of ATP and ADP, which are both important signaling molecules, particularly during vascular thrombosis or inflammation.^{33,34} Thus, pharmacological inhibition or genetic deletion of CD39 is also associated with elevated ATP or ADP signaling effects.³⁵ Therefore, studies in *cd39*^{-/-} mice have to address the question if an observed phenotype is related to enhanced nucleotide (ATP/ADP), or attenuated adenosine signaling.^{31,32,36} While gene-targeted mice for CD39 are viable and do not exhibit obvious immunologic defects,³³ experimental approaches have identified several phenotypes, including a disordered homeostasis and thromboregulation,³³ and increased susceptibility to renal,^{31,37} myocardial,^{32,38,39} or central nervous^{34,40} system ischemia or acute lung injury.^{36,41} In addition, CD39 plays an important role in organ transplantation³⁸ or hepatic regeneration following partial hepatectomy⁴².

The third and final step in extracellular adenosine generation (fig. 1) is catalyzed by the 5'-ecto-nucleotidase (CD73), a membrane bound glycoprotein that rapidly converts extracellular AMP to adenosine. CD39 and CD73 belong to a family of ecto-nucleotidases that rapidly hydrolyze ATP/ADP to AMP (CD39), or AMP to adenosine (CD73), respectively.^{17,43} Genetic deletion or pharmacological inhibition of CD73 is associated with elevated AMP and attenuated adenosine levels within the extracellular fluids.⁴⁴⁻⁴⁸ Similar to gene-target mice for CD39, *cd73*^{-/-} mice appear to have a normal immune system and are healthy when housed in a specific pathogen-free animal facility.⁴⁹ Comparative measurements of CD73 activity in wild-type or *cd73*^{-/-} mice revealed that CD73 levels are particularly high in the intestine, brain, kidneys and lungs.⁴⁹ Studies of *cd73*^{-/-} mice indicate that these animals experience profound vascular leakage and pulmonary edema when exposed to ambient hypoxia (8% oxygen over 4 h) compared to littermate controls.⁴⁹ Consistent with the high enzymatic levels of CD73 in the lungs, kidneys and the intestine,⁴⁹ other studies also unveiled a critical role of CD73 in pulmonary⁴¹ or intestinal barrier function during ambient hypoxia,⁴⁸ colitis,⁵⁰ renal⁴⁵ or intestinal ischemia.^{46,51} *Taken together, extracellular adenosine mainly stems from phospho-hydrolysis of precursor nucleotides, a metabolic pathway that is highly regulated by transcriptionally controlled enzymes (CD39 and CD73, fig. 1).*

Extracellular Adenosine Signaling

Extracellular adenosine mainly serves signaling molecule that can activate any of four ARs.¹⁷ At present, four different receptors have been described (A1AR, A2AAR, A2BAR, and A3AR; fig. 2). Due to the fact that individual ARs result in different biologic functions, it is important to point out that extracellular adenosine signaling effects strongly depend on the relative expression pattern of ARs on the extracellular surface of an individual cell types or a specific tissue.^{18,24,41,44,51-55} ARs contain seven trans-membrane spanning domains and are coupled to intracellular GTP-binding proteins, utilizing intracellular cyclic AMP (cAMP) as second messenger.⁵⁶ Adenosine activates A1, A2A or A3ARS with EC₅₀ values between 10 nM to 1 μM.⁵⁶ In contrast, activation of the A2BAR generally requires adenosine levels that exceed 10 μM (EC₅₀ of 24 μM).^{56,57} Under physiological conditions, typical adenosine concentrations remain lower than 1 μM.⁵⁶ Therefore, activation of A1, A2A or A3ARs can occur during physiological conditions. While it remains difficult to estimate the adenosine

concentrations locally present during an intimate cell-cell contact, it appears that the higher adenosine concentrations required for activation of the A2BAR are mainly achieved during pathophysiological conditions (hypoxia, inflammation, ischemia).^{17,39,44,46,47,51,53–55,58} In addition, the A2BAR is dually coupled to cAMP and calcium signaling pathways, with activation of the A2BAR leading to a rise in intracellular calcium.⁵⁹ As biological functions elicited by adenosine signaling depend on the adenosine concentrations at the cell surface, several other factors including receptor density and the functionality of the intracellular signalling pathways coupled to adenosine receptors, are important determinants of signaling effects.⁵⁶ Moreover, specific transcriptional changes in the pattern of AR expression during pathophysiological conditions such as hypoxia, ischemia or inflammation have the potential to significantly alter AR signaling events.^{24,51,53,60,61}

AR signalling occurs through the changes in adenylyl cyclase activity, resulting in subsequent alteration of intracellular cAMP levels as second messenger.¹⁷ Based on their ability to elevate or to attenuate cAMP, ARs were initially classified as A1ARs (attenuation of cAMP) or A2ARs (elevation of cAMP), respectively.⁶² However, subsequent studies have refined the classification of A2ARs into two sub-groups, A2AARs with a high affinity, and A2BARs with a low affinity for adenosine.⁶³ More recently, the A3AR was discovered as fourth AR. Similar to the A1AR, the A3AR signaling is associated with attenuation of intracellular cAMP levels.⁵⁶ Examples for typical physiological responses associated with the activation of individual ARs include adenosine-mediated bradycardia *via* activation of the A1AR,⁶⁴ arterial vasodilatation or inhibition of platelet aggregation *via* activation of the A2AAR,^{11,65–67} ischemic preconditioning of different organs *via* activation of the A2BAR,^{44,54,55} or rodent mast-cell degranulation through A3AR-dependent attenuation of intracellular cAMP concentrations (fig. 2).⁶⁸ *Taken together, extracellular adenosine mainly exerts its biological actions through activation of four adenosine receptors (ARs). While activation of the A1AR or the A3AR leads to attenuation of intracellular cAMP levels, activation of the high affinity A2AAR or the low affinity A2BAR are associated with elevation of cAMP levels (fig. 2).*

Extracellular Adenosine Uptake

When using intravenous adenosine in the treatment of perioperative cardiac arrhythmias, anesthesiologists rely heavily on the short half-life of adenosine. In fact, the heart-block induced in patients treated with a rapid intravenous bolus of adenosine is terminated due to swift decline of plasma adenosine concentrations. Thus, the adenosine-induced heart block typically lasts for a period of only 5–10 seconds. The main mechanism responsible for the fast decline of vascular adenosine levels following intravenous injection is uptake of adenosine from the extracellular to the intracellular compartment,⁶⁹ followed by rapid intracellular metabolism *via* the adenosine deaminase (conversion to inosine) or adenosine kinase (conversion to AMP).^{70–72} Adenosine can traverse the cell membrane through concentrative or equilibrative nucleoside transporters. Equilibrative nucleoside transporters (ENT) 1 and ENT2 are functionally most relevant adenosine transporters (fig. 3).^{60,69} Equilibrative nucleoside transporters represent channels that allow adenosine to freely cross the cell membrane following a concentration gradient.⁶⁹ Under physiological conditions, differences between intra- or extracellular adenosine concentrations are very small. Therefore, net flow through ENTs is minimal under normal circumstances (fig. 4).⁶⁹ This is different following intravenous application of an adenosine bolus. In this setting, extracellular adenosine concentrations rise substantially, and flow through ENTs is directed from the extracellular compartment towards the intracellular space, resulting in swift uptake of adenosine. ENTs are widely expressed, including vascular endothelia, epithelia, erythrocytes or inflammatory cells. *Rapid adenosine transport as described above is the main mechanism for the prompt decline in adenosine plasma concentrations following an IV adenosine bolus, and is responsible for the swift termination of adenosine-induced heart block.*

Similar transport phenomena become important during conditions when extracellular adenosine levels are elevated as a response to hypoxia, ischemia, inflammation or other injurious conditions (“distress”, fig. 4). Again, these conditions cause adenosine transport through ENTs to be directed from the extracellular towards the intracellular space (fig. 4). *Taken together, extracellular adenosine signaling is terminated by rapid adenosine transport through diffusion limited channels across the cell membrane. These transporters (mainly ENT1 and ENT2) are placed in an anatomically ideal position to modulate extracellular adenosine levels and signaling events. Therefore, pharmacological inhibition of adenosine transporters (e.g., with dipyridamole) or transcriptional mechanisms to repress ENT-expression result in prolonged actions of extracellular adenosine.*⁷³ *As such, dipyridamole treatment will enhance extracellular adenosine signaling events, as long as the transport of adenosine is directed from the outside towards the inside of the cell (e.g., during hypoxia, ischemia, inflammation etc., fig. 4).*^{60,69,74}

Intracellular Adenosine Metabolism

Following uptake into the intracellular compartment, adenosine is rapidly metabolized. Two alternative metabolic pathways compete for the intracellular fate of adenosine. Adenosine can either be converted to inosine through enzymatic activity of the adenosine deaminase (fig. 5).^{71,72,75,76} Alternatively, adenosine can be converted by the adenosine kinase to AMP (fig. 5).^{17,70} *It is important to point out that intracellular adenosine metabolism represents an additional mechanism to modulate extracellular adenosine levels and signaling events. For example, genetic deletion of the adenosine deaminase in mice is associated with dramatic increases in extracellular adenosine levels, and a phenotype that is characterized by pulmonary adenosine toxicity.*^{76,77} *Similarly, inhibition of adenosine kinase is associated with increases in extracellular adenosine signaling, which can be beneficial to attenuate the detrimental effects of hypoxia or ischemia.*^{70,78}

Extracellular Adenosine and Hypoxia

Modulation of Extracellular Adenosine Signaling Events by Hypoxia

Extracellular adenosine plays a critical role in tissue adaptation to limited oxygen availability (hypoxia).^{20–22} Along these lines, several studies have found that extracellular adenosine levels are elevated during conditions of hypoxia. For example, one study exposed human volunteers to normoxia or moderate hypoxia (oxygen saturation of 80% over 20 min). Following hypoxia exposure, adenosine levels increased from approximately 20 nM to over 50 nM concentrations.⁷⁹ Moreover, adenosine tissue concentrations in hearts^{32,44} or kidneys^{31,45} exposed to ischemia increase approximately 5-fold. Similar studies with mice deficient in extracellular adenosine generation suggest that ischemia-induced increases of extracellular adenosine occur mainly in the extracellular space.^{31,32,44,45}

Over the past decade, convincing evidence has demonstrated a central role of hypoxia-inducible factor (HIF) in mammalian oxygen homeostasis.^{23,80} Therefore, it is not surprising that HIF is central in the transcriptional coordination of hypoxia-elicited adenosine responses.^{21,48,54,60,61,70,74} The transcription factor HIF-1 is composed of two subunits: constitutively expressed HIF-1 β and oxygen-regulated HIF-1 α .⁸⁰ Under normoxic conditions, HIF-1 α is subjected to hydroxylation on proline residues, resulting in proteasomal degradation.⁸¹ Under hypoxic conditions, hydroxylation is inhibited, allowing HIF to be active and to stimulate the transcriptional activation of HIF-dependent genes.⁵⁴ Transcriptional induction of HIF-target genes is tailored towards adapting the cells to limited oxygen availability (e.g., by inducing a switch from aerobic metabolism to anaerobic glycolysis)⁸² or towards restoring adequate tissue oxygen levels (e.g., induction of erythropoietin resulting in enhanced erythropoiesis).⁸¹

Acute hypoxia elicited changes of extracellular adenosine result in increased adenosine signaling events. Several steps of this transcriptionally controlled pathway are coordinated by HIF. As first step, hypoxia coordinates increases in extracellular adenosine production. This is achieved by a transcriptional induction of extracellular enzymes that produce extracellular adenosine. While CD39 transcription during hypoxia is driven by the transcription factor Sp1,³⁹ hypoxia-induced stabilization of HIF profoundly enhances CD73 transcription, translation and surface activity.⁴⁸ As such, studies of the CD73 promoter revealed a binding site for HIF-1, and additional studies with promoter constructs, including site-directed mutagenesis of the HIF-binding site or HIF-loss and gain of function studies confirmed HIF-1 in the transcriptional induction of CD73 during hypoxia.^{48,54}

Secondly, studies investigated the consequences of hypoxia or ischemia on extracellular adenosine signaling events. Such studies revealed a selective induction of the A2BAR with ambient hypoxia.^{24,61} Similarly to the studies performed with CD73, strong evidence supports a critical role for HIF-1 α in the transcriptional induction of the A2BAR with hypoxia or ischemia.^{54,61}

Thirdly, studies of extracellular adenosine uptake indicate that HIF-1 coordinates transcriptional repression of the adenosine transporters ENT1⁶⁰ and ENT2,⁷⁴ resulting in attenuated uptake of extracellular adenosine, and enhanced extracellular adenosine signaling events. Finally, HIF also coordinates transcriptional changes in intracellular adenosine metabolism.⁸³ As such, acute hypoxia results in HIF-dependent transcriptional repression of adenosine kinase, attenuated adenosine metabolism, and eventually in enhanced extracellular adenosine signaling events. *Taken together, extracellular adenosine signaling events are enhanced during hypoxia by a series of steps mainly coordinated by the transcription factor HIF-1. Hypoxia-induced increases in adenosine signaling are critical to counterbalance the deleterious effects of acute hypoxia, including attenuation of hypoxia-induced vascular leakage,^{49,58,70} ischemia-associated organ dysfunction^{46,51} or hypoxia-induced inflammation.^{18,23,24}*

Adenosine Signaling during Hypoxia-Induced Inflammation

Sites of acute inflammation are characterized by shifts in the supply and demand of metabolites that result in limited oxygen availability (inflammation-associated hypoxia).^{20,21,23} However, studies of ambient hypoxia provided strong evidence that hypoxia itself represents an inflammatory stimulus.^{84,85} For example, ambient hypoxia exposure causes activation of the transcription factor nuclear factor NF- κ B, which activates transcription of genes encoding pro-inflammatory molecules.^{84–86} Moreover, exposure of mice to ambient hypoxia (*e.g.*, 8% oxygen over 4–8 h) induces increased leakage through epithelial or endothelial barriers, and inflammatory cell accumulation in mucosal organs.^{18,24,48,58,60,70} Tissue inflammation caused by limited oxygen availability plays an important role in several human clinical conditions including solid organ transplantation (*e.g.*, lung or liver), when limited oxygen availability caused by graft ischemia is associated with increased inflammation, ischemia-reperfusion injury, and early organ failure.^{87,88} Similarly, hypoxia-associated inflammation strongly influences the clinical outcome of organ ischemia, and antiinflammatory therapeutic approaches have been proposed for myocardial,^{32,44,54,89} renal,⁵⁵ hepatic,⁴⁷ or intestinal ischemia.^{46,51} Due to their large surface areas, mucosal organs such as the lungs and the intestine are particularly prone to hypoxia-induced inflammation.^{23,46,51}

Whereas tissue hypoxia induces hypoxia-induced inflammation, hypoxia also drives hypoxia-associated antiinflammatory responses, particularly through changes in gene-expression coordinated by the transcription factor HIF-1.^{20,21} As outlined above, HIF-1 coordinates the metabolism and signaling properties of extracellular adenosine,^{21,54,60,70} as important antiinflammatory agent. HIF-1 also induces metabolic changes in immune cells by switching

from aerobic metabolism to glycolysis, and thereby markedly affects immune responses.²⁰ HIF-1 α is stabilized in inflamed^{90,91} or infected tissues⁹², and some data suggest an antiinflammatory and tissue protective role of HIF-1 α signaling during acute inflammation^{20,21,54,60,70,90–93} or bacterial infections.^{92,94} In this context, a recent study identified an important role of the neuronal guidance molecule netrin-1 in enhancing extracellular adenosine signaling pathways. Given that mucosal surfaces are particularly prone to hypoxia-elicited inflammation, this study sought to determine the role of netrin-1 in hypoxia-induced inflammation. The authors detected HIF-1 α -dependent induction of netrin-1 gene (*Ntn1*) expression in hypoxic epithelia. Neutrophil transepithelial migration studies showed that by engaging A2BARs on neutrophils, netrin-1 attenuates neutrophil transmigration. Exogenous netrin-1 suppressed hypoxia-elicited inflammation in wild-type, but not A2BAR-deficient mice, and inflammatory hypoxia was enhanced in *Ntn1*^{+/-} compared to *Ntn1*^{+/+} mice. *Taken together, these studies demonstrate that HIF-1 α -dependent induction of netrin-1 attenuates hypoxia-elicited inflammation at mucosal surfaces by enhancing extracellular adenosine signaling events (fig. 6).*

Utilizing Adenosine Signaling Pathways for Perioperative Medicine

The field of extracellular adenosine signaling has rapidly expanded over the past years. This goes hand in hand with the recent availability of gene-targeted mice that allow for studies of specific alterations in adenosine generation, signaling or metabolism in a large array of disease models. Therefore, the following examples for potential therapeutic applications of extracellular adenosine are not meant to be a complete list, but resemble therapeutic examples that could potentially be applied to the setting of perioperative medicine. Based on the biologic effects of acute hypoxia on enhancing extracellular adenosine effects, many of these examples include conditions of limited oxygen availability where adenosine signaling is pivotal to tissue adaptation and attenuation of the deleterious effects of hypoxia-associated inflammation.

Acute Lung Injury

Acute lung injury (ALI) is a syndrome consisting of acute hypoxemic respiratory failure with bilateral pulmonary infiltrates, not attributable to left heart failure.⁹⁵ Despite optimal management consisting of aggressive treatment of the initiating cause, vigilant supportive care, and the prevention of nosocomial infections, mortality ranges between 35 and 60%.⁹⁵ The pathogenesis of ALI is characterized by the influx of a protein-rich edema fluid into the interstitial and intraalveolar spaces as a consequence of increased permeability of the alveolar-capillary barrier. The importance of endothelial injury and increased vascular permeability to the formation of pulmonary edema in this disorder has been well established⁹⁵. Nevertheless, molecular details of how pulmonary capillary leakage is caused or maintained during ALI are largely unknown and studies linking its mechanisms with mechanical ventilation are currently areas of intense investigation.

Despite the large impact of ALI on morbidity and mortality in critically ill patients,⁹⁵ many episodes of ALI are self-limiting, and resolve spontaneously through unknown mechanisms. For example, patients undergoing major surgery requiring prolonged mechanical ventilation have an overall incidence of ALI between 0.2 and 5%, depending on the kind of surgery.^{96–98} Based on the rare occurrence of clinically relevant ALI in patients requiring mechanical ventilation, recent studies found that extracellular adenosine production *via* CD39 and CD73 are enhanced during cyclic mechanical stretch *in vitro*, or during ventilator-induced lung injury (VILI) *in vivo*. In fact, *cd39*^{-/-} or *cd73*^{-/-} mice experience enhanced lung inflammation and pulmonary edema in different models of ALI.^{36,41,99} Further studies examined the contribution of endogenous adenosine signaling to attenuation of VILI or endotoxin induced lung injury.⁵³ Initial profiling studies using gene-targeted mice for the *A1*, *A2A*, *A2B* or *A3AR* revealed that genetic deletion of the *A2BAR* was specifically associated with reduced survival-time and

increased pulmonary albumin leakage (5.3 ± 0.15 -fold) during VILI. Studies in wild-type mice showed that treatment with A2BAR-selective antagonist PSB1115 resulted in enhanced pulmonary inflammation, edema and attenuated gas-exchange, while treatment with the A2BAR-agonist BAY 60–6583 attenuated VILI. Studies in bone-marrow chimeric A2BAR mice demonstrated pulmonary A2BARs in VILI-induced albumin leakage and edema, while increases in pulmonary inflammation were, at least in part, bone-marrow mediated. Measurement of alveolar fluid clearance indicated that A2BAR signaling enhanced amiloride-sensitive fluid transport *via* elevation of pulmonary cAMP levels - similarly to beta-adrenergic agonist stimulation - suggesting that A2BAR-agonist treatment protects by drying out the lungs during VILI.⁵³ Taken together, such studies demonstrate that extracellular adenosine production *via* CD39 and CD73, in conjunction with A2BAR signaling represents in endogenous pathway to protect the lungs from pulmonary edema and excessive inflammation.^{41,53} Moreover, the A2BAR represents a potential therapeutic target for enhancing fluid transport and attenuating pulmonary edema and lung inflammation during ALI.⁵³ Other studies implicated A2BAR signaling in attenuating acute lung inflammation during hypoxia by dampening proinflammatory signaling pathways in the lungs, involving A2BAR-mediated cullin-1 deneddylation.¹⁰⁰ It is important to point out that while extracellular adenosine signaling appears to be protective during acute forms of lung injury, adenosine signaling may enhance aspects of chronic forms of lung injury, such as pulmonary fibrosis.⁷⁶ For example, adenosine-deaminase-deficient mice develop signs of chronic pulmonary injury in association with chronically elevated pulmonary adenosine levels. In fact, adenosine-deaminase-deficient mice die within weeks after birth from severe respiratory distress,⁷⁶ and recent studies suggest that attenuation of adenosine signaling may reverse the severe pulmonary phenotypes in adenosine-deaminase-deficient mice, suggesting that chronic adenosine elevation can affect signaling pathways that mediate aspects of chronic lung disease.^{76,101}

Iatrogenic Hyperoxia

Other studies have indicated a protective role of signaling through the A2AAR during inflammatory conditions, including different forms of ALI.^{19,22,102} As such, a recent study by Thiel *et al.*, in a team led by Michail Sitkovsky, tested the hypothesis that oxygenation weakens a tissue-protecting mechanism triggered by hypoxia. Similar to signaling through the A2BAR,⁵³ hypoxia also triggers a signaling pathway mediated by the A2AAR that attenuates lung inflammation and tissue damage.²² This hypoxia-driven pathway protects the lungs from the toxic effects of overactive immune cells such as neutrophils. Using a mouse model of acute lung injury induced by bacterial infection, Thiel *et al.* exposed one group of mice to 100% oxygen, mimicking therapeutic oxygenation, and left another group at normal ambient levels (21% oxygen).²² Five times more mice died after receiving 100% oxygen than died breathing normal oxygen levels. Mice given 60% oxygen—considered clinically safe—got worse, but didn't die. Hypoxia protects against lung damage, the authors conclude, by working through the A2AAR signaling pathway to control inflammation. Above-normal oxygen levels interrupt this anti-inflammatory pathway, paving the way for further lung injury.¹⁰³ *Taken together, such studies indicate that high levels of inspired oxygen - as maybe required to provide sufficient tissue oxygenation in patients suffering for ALI - may weaken the local tissue hypoxia-driven and AR-mediated antiinflammatory mechanism and thereby further exacerbate lung injury.*²²

Vascular Leakage during Hypoxia

Changes in vascular barrier function closely coincide with tissue injury of many etiologies, and result in fluid loss, edema, and organ dysfunction. Particularly during conditions of limited oxygen availability such as occurs during lung injury, sepsis, or a systemic inflammatory responses syndrome, the vascular barrier becomes leaky.⁷² The predominant barrier (~90%) to movement of macromolecules across a blood vessel wall is presented by the vascular

endothelium. As outlined above, extracellular nucleotide metabolites – particularly adenosine – may function as an endogenous protective mechanism during hypoxia and ischemia and could counterbalance hypoxia-induced increases in vascular leakage. As such, the vascular endothelium is the primary interface between a hypoxic insult and the surrounding tissues. This critical anatomic location places vascular endothelial cells in an ideal position to coordinate extracellular metabolic events important to endogenous responses to hypoxia. The pacemaker enzyme of extracellular adenosine generation is the ecto-5'-nucleotidase (CD73, extracellular conversion of AMP to adenosine). Endothelial cells of many origins express constitutive CD73. Thus, extracellular nucleotides are metabolized to adenosine by CD73 and subsequent activation of surface adenosine receptors have been shown to regulate endothelial barrier function. *In vivo* studies in models of murine whole-body hypoxia revealed that hypoxia-induced CD73 is critical for protecting the endothelial barrier, since *cd73*^{-/-} mice show increased vascular permeability and profound pulmonary edema upon hypoxia exposure (8% oxygen over 4 h).⁴⁹ *In vitro* studies of endothelial permeability suggested, that activation of endothelial adenosine receptors leads to a barrier resealing response following neutrophil transmigration.¹⁰⁴ In fact, all four ARs are expressed on vascular endothelia.²⁴ To identify the role of individual ARs in attenuating hypoxia-induced vascular leakage, a recent study found that small-interfering-RNA-mediated repression of the A2BAR selectively increased endothelial leak in response to hypoxia *in vitro*.⁵⁸ In parallel, vascular permeability was significantly increased in vascular organs of *A2BAR*^{-/-}-mice subjected to ambient hypoxia (8% oxygen, 4 h). By contrast, hypoxia-induced vascular leak was not accentuated in *A1AR*^{-/-}-, *A2AAR*^{-/-}-, or *A3AR*^{-/-}-deficient mice, suggesting a degree of specificity for the A2BAR. Further studies in wild type mice revealed that the selective A2BAR antagonist PSB1115 resulted in profound increases in hypoxia-associated vascular leakage while A2BAR agonist (BAY60–6583) treatment was associated with almost complete reversal of hypoxia-induced vascular leakage. *Taken together, these studies indicate extracellular adenosine production and signaling as central control point for hypoxia-associated vascular leak.*

Myocardial Ischemia

Myocardial ischemia is among the leading causes of morbidity and mortality in surgical patients.¹⁰⁵ Current therapeutic interventions for myocardial ischemia focus mainly on early and persistent coronary reperfusion. However, percutaneous coronary intervention in combination with anticoagulation and platelet inhibitors may not be suitable in the perioperative settings due to risk of bleeding from the surgical site.¹⁰⁵ Therefore, it is not surprising that the search for novel therapeutic approaches to prevent or treat perioperative myocardial ischemia is currently an area of intense investigation. A powerful strategy for cardioprotection would be to pharmacologically recapitulate the consequences of ischemic preconditioning (IP), where short and repeated episodes of ischemia and reperfusion prior to myocardial infarction result in attenuation of infarct sizes.^{73,106} Despite multiple attempts to identify the underlying molecular mechanisms, pharmacological strategies utilizing such pathways have yet to be further defined and introduced into clinical practice. Extracellular adenosine generation has been studied for its role in IP-responses and cardioprotection from ischemia for many years.¹⁰⁷ An important insight was gained from studies measuring cardiac adenosine levels following preconditioning. These studies revealed an about 5-fold increase of cardiac adenosine levels immediately following preconditioning. In contrast, adenosine levels derived from preconditioned myocardium in mice deficient of extracellular adenosine generation (*cd39*^{-/-} or *cd73*^{-/-} mice) was similar to un-preconditioned wild-type mice.^{32,44,54} *These studies indicate that extracellular adenosine levels are dramatically elevated with preconditioning.*

Functional studies revealed that pharmacological inhibition or genetic deletion of extracellular adenosine production is associated with abolished cardioprotection by IP-treatment.^{32,44} As

all four ARs have been associated with tissue protection in different settings, the question which AR mediates IP-dependent cardioprotection is controversial. While some studies found a critical role of the A1¹⁰⁸ or the A3AR,¹⁰⁹ a recent study compared IP responses in gene-targeted mice for all 4 ARs.⁴⁴ While IP-dependent cardioprotection was attenuated in different mice, including A1AR^{-/-} mice,¹⁰⁸ complete loss of IP-dependent cardioprotection was observed only in A2BAR^{-/-} mice.⁴⁴ Moreover, treatment with a specific A2BAR resulted in a robust reduction of infarct size in wild-type mice, but not in A2BAR^{-/-} mice.⁴⁴ Nevertheless, it is important to point out that also other AR signaling pathways have been associated with cardioprotection from ischemia, including the A1AR¹⁰⁸ or the A2AAR.⁸⁹ In addition, other studies have found critical roles for adenosine signaling in ischemic postconditioning¹¹⁰ - a recently described cardioprotective modality against reperfusion injury, through series of brief reflow interruptions applied at the very onset of reperfusion - including signaling events involving the A1AR,^{111,112} A2AAR¹¹³, A2BAR¹¹⁴ or A3AR.¹¹⁰ *Taken together, these studies indicate cardioprotection from myocardial ischemia through CD39- or CD73-dependent generation of extracellular adenosine and signaling via ARs.*^{32,44,54}

Attenuation of Ischemia Reperfusion Injury in other Organs

In addition to its role in cardioprotection,^{32,44,54} extracellular adenosine generation and signaling has also been implicated in protection from ischemia-reperfusion injury in other organ systems. For example, different studies pointed out a protective role of extracellular adenosine generation^{31,45} and signaling through the A2AAR¹¹⁵ or the A2BAR⁵⁵ in renoprotection from acute ischemic renal failure. Considerable progress has been made by studies in gene-targeted mice that only express a specific adenosine receptor on renal tissues or inflammatory cells. This can be achieved by irradiation of gene-targeted mice for a specific adenosine receptor, followed by bone marrow transplantation from wild-type animals, and *vice versa*. Studies in these “bone marrow chimeric mice” demonstrated that A2AARs expressed on inflammatory cells,^{115,116} and A2BARs expressed on renal tissues⁵⁵ attenuate renal ischemia reperfusion injury. Such studies indicate a potential cross-talk and/or functional compensation among the subtypes of adenosine receptors.

Other studies found protection from intestinal^{46,51} or hepatic ischemia reperfusion injury⁴⁷ by adenosine generation or signaling.⁴⁷ For example, a very elegant study demonstrated that adenosine-dependent attenuation of hepatic ischemia reperfusion injury involves activation of A2AARs localized on immune cells.¹¹⁷ Specifically, this study found a surprising role for adenosine-dependent inhibition of natural killer T-cells, a sub-population of lymphocytes representing about 0.2% of peripheral blood T-cells. Natural killer T-cells recognize the nonpolymorphic CD1d molecule, an antigen-presenting molecule that binds self- and foreign lipids and glycolipids. This study provides strong evidence that the activation of natural killer T-cells by a CD1d-dependent mechanism play a central role in initiating the inflammatory cascade responsible for reperfusion injury in the liver and that these cells are key targets of A2AAR agonist-protection in hepatic ischemia-reperfusion injury.¹¹⁷ Moreover, extracellular adenosine production appears to play a critical role in hepatic regeneration as observed following partial hepatectomy. As such recent studies demonstrate that regulated phosphohydrolysis of extracellular nucleotides by CD39 coordinates both hepatocyte and endothelial cell proliferation following partial hepatectomy. Lack of CD39 activity is associated with decreased hepatic regeneration and failure of vascular reconstitution.⁴²

Other Medical Applications for Adenosine Signaling Pathways

Many other treatment modalities have utilized adenosine-dependent signaling pathways. For example, experimental studies suggest a protective role of extracellular adenosine signaling in models of sepsis and acute inflammation, particularly through signaling events involving the A2AAR.¹⁹ Similarly, a protective and antiinflammatory role of A2BAR signaling has been

found during vascular inflammation¹¹⁸ or vascular injury.¹¹⁹ Other studies found a protective role of extracellular adenosine generation and signaling during murine colitis.^{50,120} As such, mice deficient in the A2BAR show increased sensitivity to chemically induced intestinal inflammation.¹²⁰ Other studies found a protective role of A2BAR agonist treatment in intestinal ischemia, highlighting a tissue protective and antiinflammatory role of intestinal A2BAR signaling.⁵¹ Similarly, gene-targeted mice for the A2BAR show dramatically increased responses to immunoglobulin E-elicited mast-cell activation, indicating that A2BAR functions as a critical regulator of signaling pathways within the mast cell, which act in concert to limit the magnitude of mast cell responsiveness when antigen is encountered.¹²¹ However, there may be different regulatory mechanisms involved in human mast cell degranulation (*e.g.*, the A2BAR).¹²² Other studies identified a critical role of adenosine in stimulating angiogenesis.¹²³ While this is not the focus of the present review, it is important to point out that adenosine signaling also plays a role as central nervous system signaling molecule. For example, signaling through the A1AR has been implicated in reducing hypersensitivity following peripheral nerve injury or following surgery.^{66,124,125}

Summary and Future Challenges

Adenosine has been used in the perioperative setting for the treatment of supraventricular tachycardia for many decades. More recently, research with specific AR agonists or antagonists in conjunction with studies in genetic models for adenosine generation or signaling have identified a rapidly expanding field of biomedical roles and potential therapeutic applications of extracellular adenosine signaling. Particularly during conditions of limited oxygen availability as occurs during acute inflammation, organ ischemia or acute lung injury, several pathways synergize to elevate extracellular adenosine levels and increase adenosine signaling effects. Such changes include increased extracellular adenosine production, increased expression patterns of specific adenosine receptors, and decreased adenosine uptake and intracellular metabolism (fig. 7). In this context, pharmacological strategies to enhance extracellular adenosine production (*e.g.*, treatment with apyrase or nucleotidase) or specific AR agonist appears to be particularly important to counterbalance the deleterious effects of hypoxia, such as for the treatment of hypoxia-induced vascular leakage, excessive inflammation, pulmonary edema or ischemia-reperfusion injury.

Most of the studies that were discussed in the present review were performed in murine models. While we are presently at a stage, where specific adenosine receptor agonists are explored in human volunteers or patients,¹²⁶ most of the studies discussed in the present review will require translation from murine models into a clinical setting. Moreover, specific side-effects and long-term safety of pharmacological agents utilizing adenosine signaling pathways have to be further defined. For example, it will be important to define the hemodynamic consequences of specific AR agonists. Also, it will be critical to address the question if long-term use of AR agonists may be associated with fibrotic changes of the liver or the lungs. Such observations may limit these drugs for a short-term use in an acute setting. Moreover, specific sub-sets of patients who may require exclusion from therapy will have to be defined.^{127,128}

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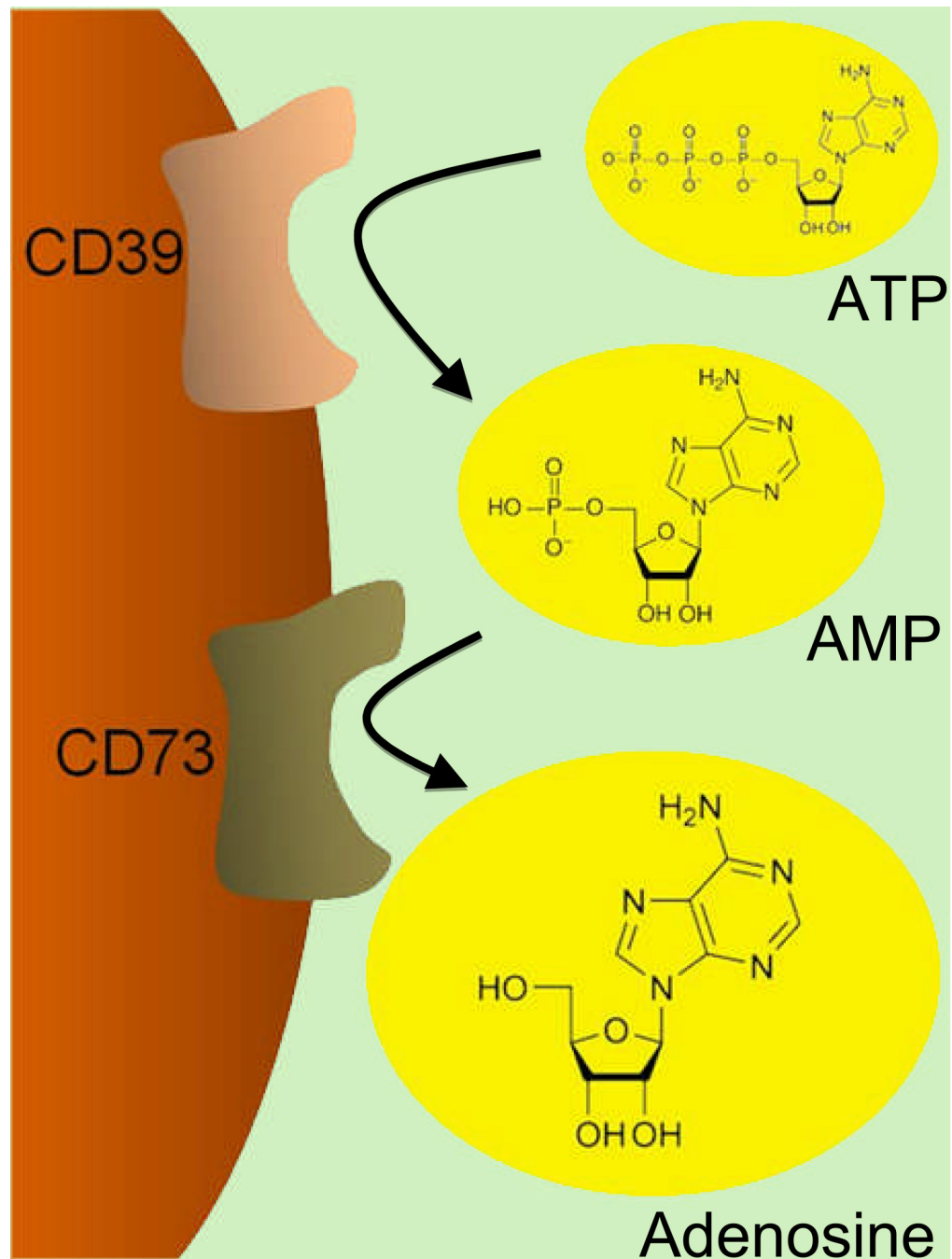


Figure 1. Extracellular Adenosine Generation

Adenosine is an extracellular signaling molecule that is generated from its precursor molecules 5'-adenosine triphosphate (ATP) and 5'-adenosine monophosphate (AMP). This process consists of a two-step enzymatic reaction. Extracellular ATP released by multiple cell types (e.g. platelets, endothelia, epithelia or inflammatory cells) is rapidly converted to AMP by the ecto-apyrase (CD39). As second step in extracellular adenosine generation, AMP is converted by the 5'-ecto-nucleotidase (CD73) to adenosine. Thus, extracellular adenosine is available on the cell surface to activate its receptors.

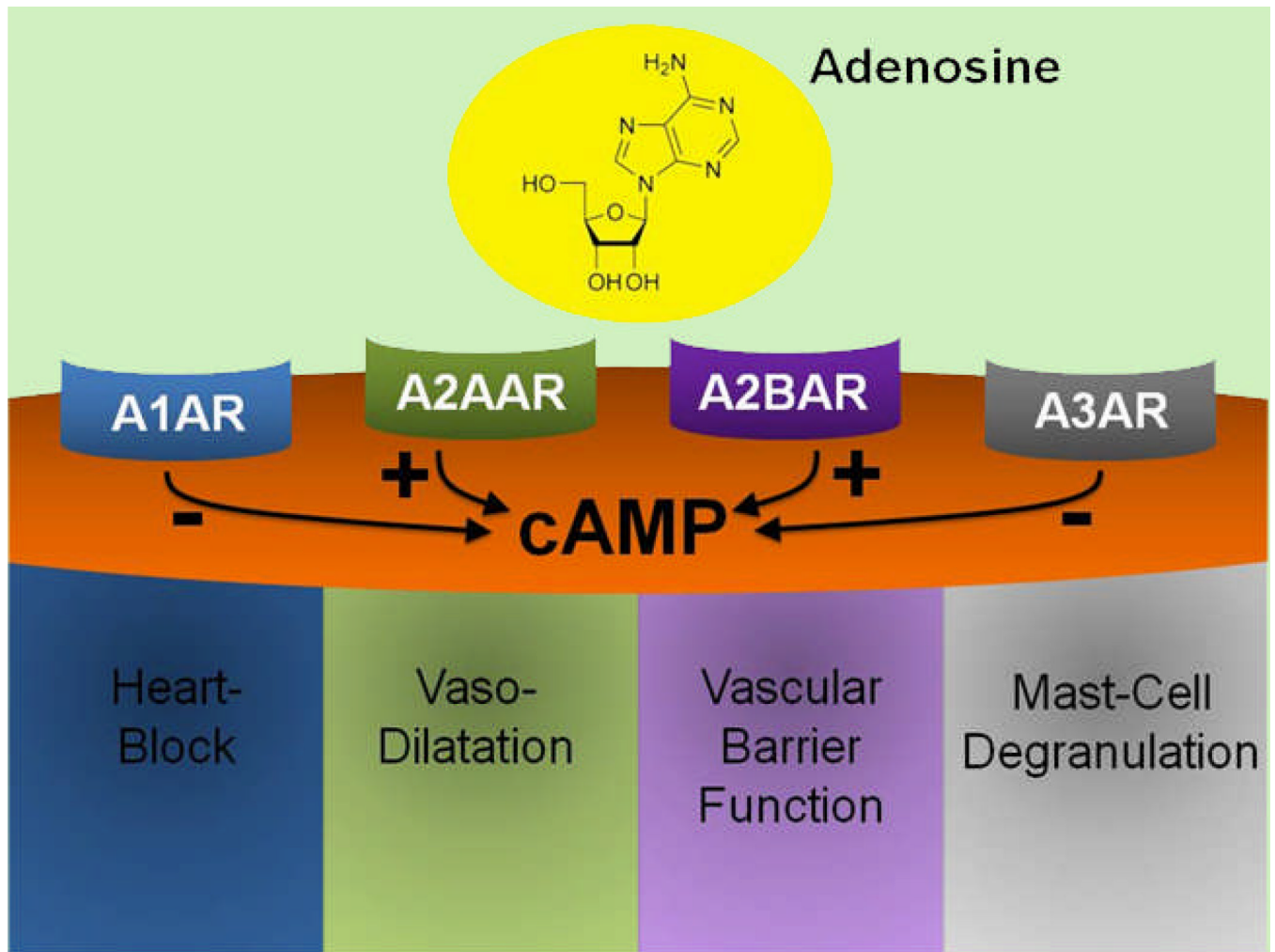


Figure 2. Extracellular Adenosine Signaling

Extracellular adenosine exerts its biological effects through activation of adenosine receptors (ARs). At present, four different ARs have been identified: A1AR, A2AAR, A2BAR and A3AR. ARs are G-protein coupled receptors that utilize cyclic adenosine monophosphate (cAMP) as a second messenger. While signaling events through the A1 or A3AR dampen intracellular cAMP levels, activation of the A2A or A2BAR elevates cAMP levels. Biological examples for AR signaling events include adenosine-elicited heart-block for activation of the A1, vaso-dilatation for the A2A, vascular barrier function for the A2B or murine mast-cell degranulation for the A3AR.

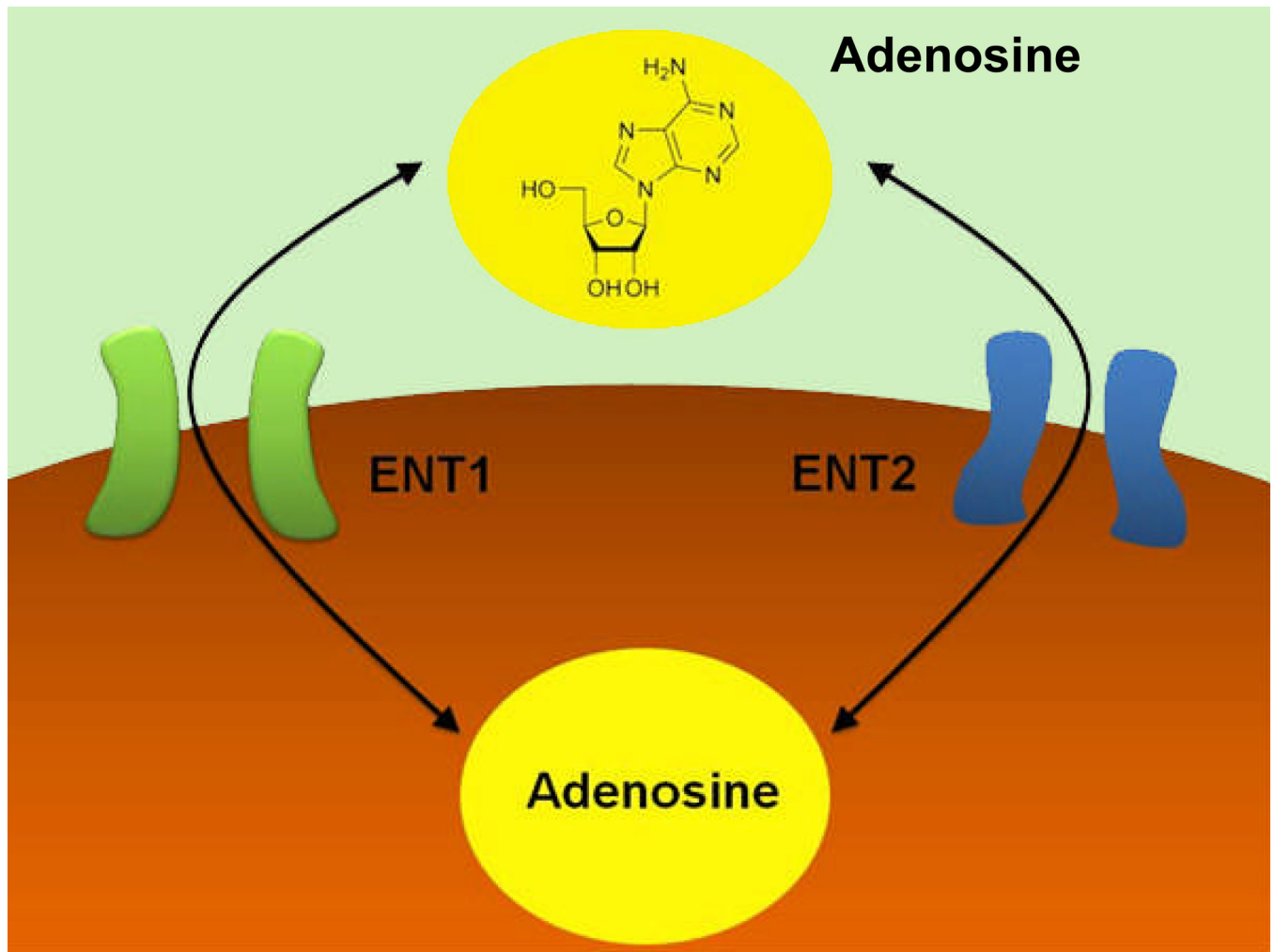


Figure 3. Extracellular Adenosine Uptake

Extracellular adenosine is taken up from the extracellular to the intracellular space via nucleoside transporters. Functionally, extracellular adenosine uptake is mainly achieved through “equilibrative nucleoside transporters”, ENT1 and ENT2. These transporters represent diffusion-limited channels that allow adenosine to freely cross the cell membrane following a concentration gradient.

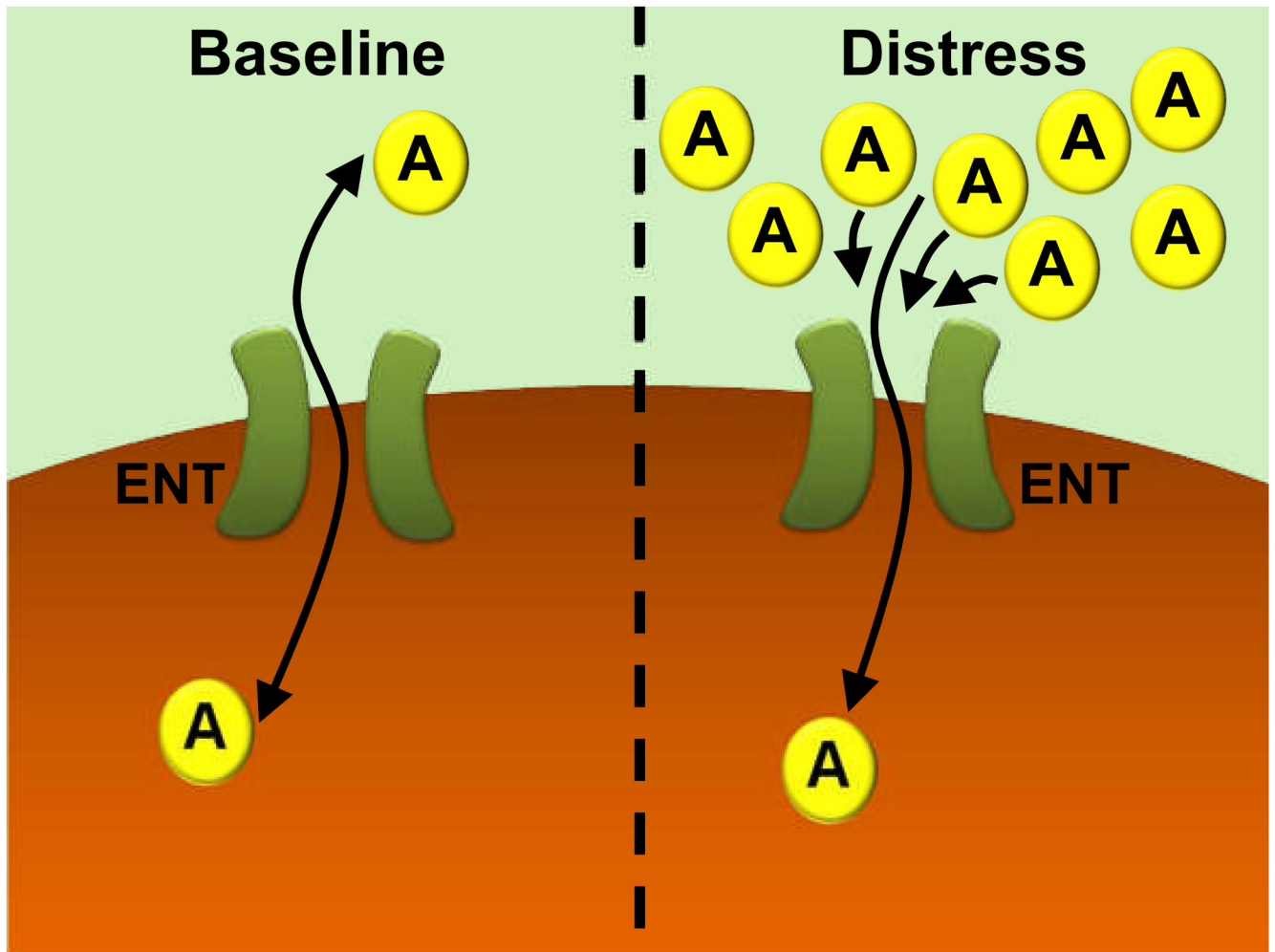


Figure 4. Extracellular Adenosine Uptake at “Baseline” or during “Distress.”

Adenosine (A) can freely cross the cell membrane via equilibrative nucleoside transporters (ENTs). ENTs represent diffusion limited channels that allow free passage of adenosine through the cell membrane. During baseline conditions, only a small concentration gradient for adenosine across the cell membrane is present. Therefore, flux through ENTs is minimal. In contrast, extracellular adenosine concentrations are elevated during conditions of hypoxia, ischemia, or inflammation (“distress”). Under these conditions, adenosine flux through ENTs is directed mainly from the extracellular space towards the intracellular compartment. As long as flux through ENTs is directed from the outside towards the inside of the cell, inhibitors of ENTs (such as dipyridamole) or transcriptional mechanisms that repress ENTs will attenuate adenosine transport, and result in increased extracellular adenosine concentrations and signaling effects.

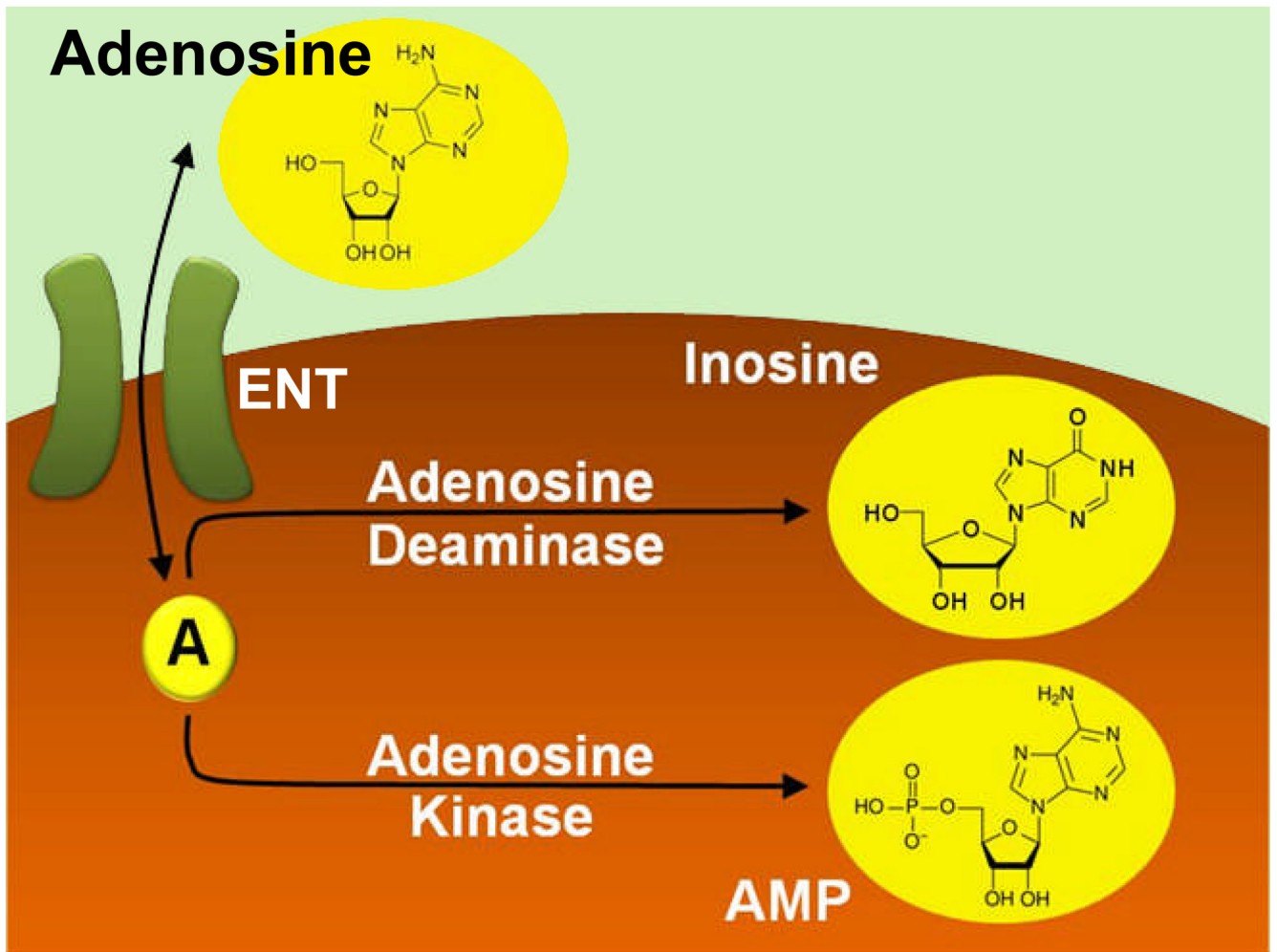


Figure 5. Intracellular Adenosine Metabolism

Following adenosine uptake via equilibrative nucleoside transporters (ENTs), intracellular adenosine is rapidly metabolized. Two competing pathways exist. Intracellular adenosine can be metabolized to inosine by the adenosine deaminase. Alternatively, adenosine is phosphorylated by the adenosine kinase to 5'-adenosine monophosphate (AMP).

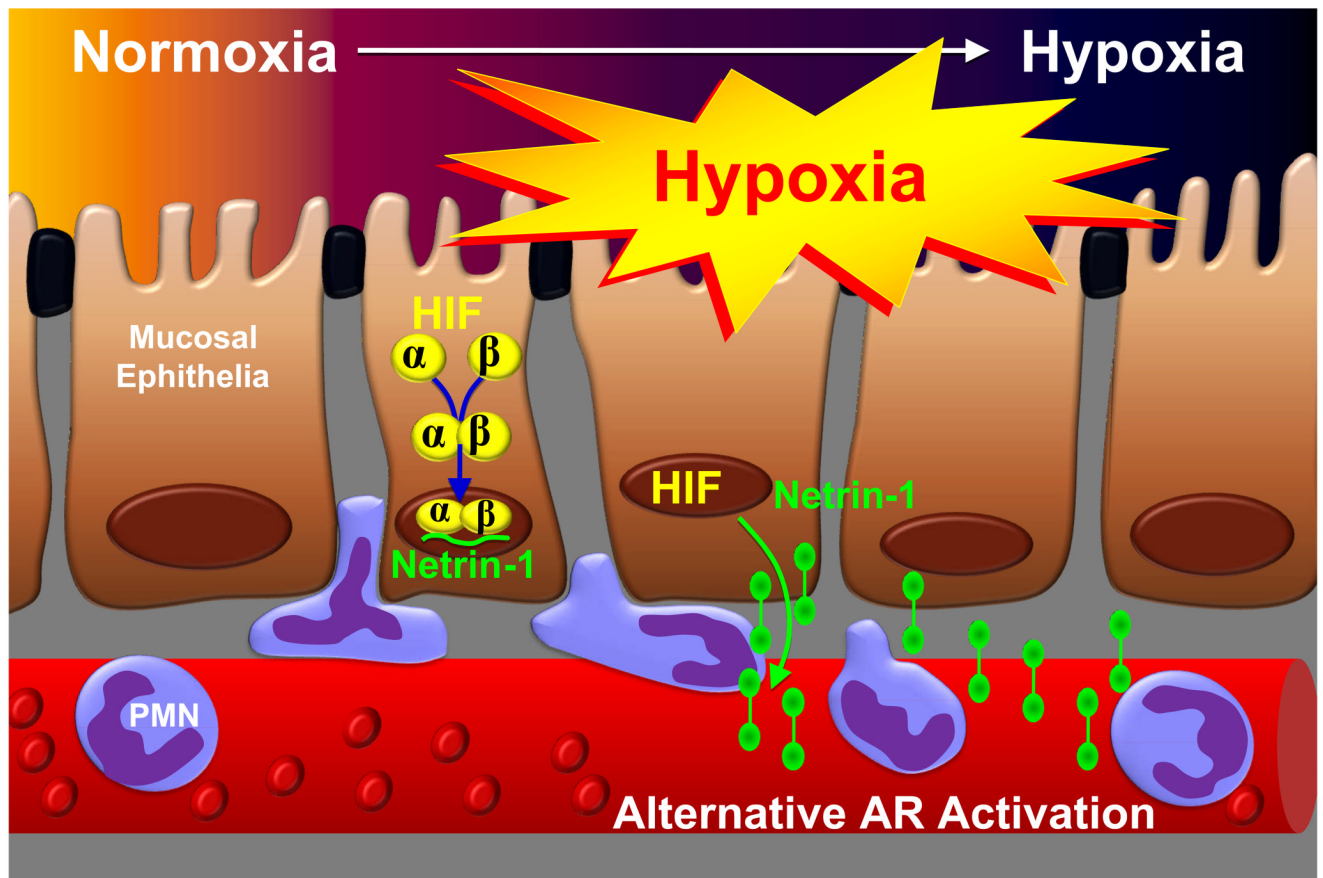


Figure 6. Netrin-1 Dampens Hypoxia-Induced Inflammation by Enhancing Extracellular Adenosine Signaling

During hypoxia-elicited inflammation of mucosal organs such as the lungs or the intestine, the transcription factor hypoxia-inducible factor (HIF) coordinates the induction of netrin-1. While originally described as neuronal guidance molecule, recent studies implicate netrin-1 in the regulation of inflammatory responses. Here, epithelial-released netrin-1 dampens neutrophil accumulation in the hypoxic mucosa. This process involves activation of A2B adenosine receptor (AR)-dependent signaling pathways of neutrophils (“alternative AR activation”).

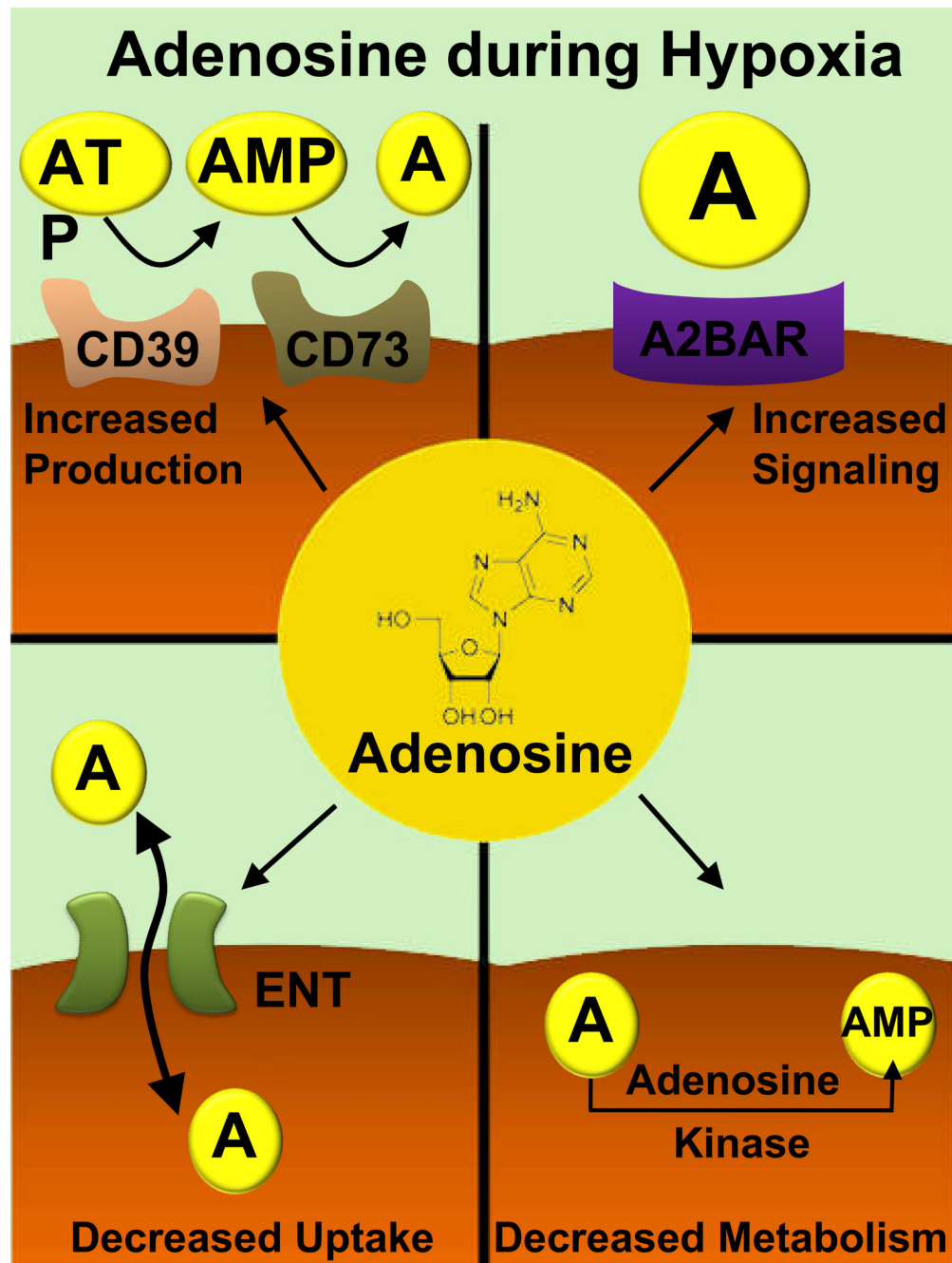


Figure 7. Consequences of Hypoxia on Adenosine Signaling Pathways

During situations of cellular distress (acute hypoxia, inflammation, ischemia reperfusion injury), hypoxia coordinates changes that lead to increases in extracellular signaling effects. These changes involve four mechanisms. First, extracellular adenosine production is enhanced through the transcriptional induction of the adenosine-producing enzymes ecto-apyrase (CD39; conversion of adenosine tri-phosphate, ATP, to adenosine mono-phosphate, AMP) and 5'-ecto-nucleotidase (CD73; conversion of AMP to adenosine). In addition, adenosine effects are also enhanced on the receptor level. As such, hypoxia coordinates the selective induction of the A2B adenosine receptor (A2BAR). Moreover, hypoxia leads to transcriptional repression of equilibrative nucleoside transporters (ENTs), resulting in attenuated adenosine

uptake, enhanced extracellular adenosine concentration and signaling. Finally, hypoxia also causes transcriptional repression of the adenosine kinase, the main enzyme for intracellular adenosine metabolism. Adenosine kinase catalyzes phosphorylation of adenosine to adenosine monophosphate (AMP). Hypoxia-dependent repression of adenosine kinase represents an additional hypoxia-elicited mechanism that enhances extracellular adenosine concentration and signaling during hypoxia.