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Targeting of Adenosine Receptors in Ischemia-Reperfusion Injury

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Abstract

Importance of the field—Ischemia-reperfusion (IR) injury is a common clinical problem after transplantation as well as myocardial infarction and stroke. IR initiates an inflammatory response leading to rapid tissue damage. Adenosine, produced in response to IR, is generally considered as a protective signaling molecule and elicits its physiological responses through four distinct adenosine receptors. The short half-life, lack of specificity, and rapid metabolism limits the use of adenosine as a therapeutic agent. Thus intense research efforts have focused on the synthesis and implementation of specific adenosine receptor agonists and antagonists as potential therapeutic agents for a variety of inflammatory conditions including IR injury.

Areas covered by this review—This review summarizes current knowledge on IR injury with a focus on lung, heart, and kidney, and examines studies that have advanced our understanding of the role of adenosine receptors and the therapeutic potential of adenosine receptor agonists and antagonists for the prevention of IR injury.

What the reader will gain—The reader will gain insight into the role of adenosine receptor signaling in IR injury.

Take home message—No clinical therapies are currently available that specifically target IR injury; however, targeting of specific adenosine receptors may offer therapeutic strategies in this regard.

Keywords

inflammation; innate immunity; therapeutic targets; transplantation; preconditioning

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Introduction

Ischemia is defined as a lack of blood supply to an organ or tissue, resulting in oxygen deprivation of cells. Without restoration of oxygenated blood, ischemic tissues soon undergo necrosis and die. Thus, restoration of blood flow, or reperfusion, is the definitive treatment for ischemia. Although ischemia itself is a serious condition, reperfusion of ischemic tissue paradoxically imparts further tissue injury, especially after prolonged ischemia. This injury, defined as reperfusion injury, has widespread clinical relevance and is encountered in a variety of surgical settings (e.g. transplantation, cardiopulmonary bypass and aneurysm repair) as well as non-surgical settings (e.g. myocardial infarction, stroke, hemorrhage, trauma and shock). The sum total of tissue injury imposed by ischemia and reperfusion is defined as ischemia-reperfusion (IR) injury. IR injury therefore involves a complex cascade of events including oxidative stress, inflammation, and interactions between many cell types. In the transplant setting, IR injury is unavoidable, and, in addition to short term effects on organ function, can have long-term effects on allograft survival [1]. If severe enough, IR injury can also lead to systemic inflammatory effects, multiorgan dysfunction and death [2].

Currently, no therapeutic agents are clinically available specifically for the prevention or treatment of IR injury. The single established strategy to limit IR injury is early reperfusion of the ischemic tissue. Despite a multitude of successful experimental interventions to reduce IR injury, very few have been successfully translated into clinical practice. Various pharmacological and non-pharmacological therapies may help reduce IR injury. For example, non-pharmacological, surgical strategies which may limit IR injury in patients include ischemic preconditioning, postconditioning, and remote preconditioning [3,4]. Improvements in hypothermic preservation solutions have been instrumental in reducing early graft failure. Several pharmacological preconditioning agents have been investigated such as nicorandil (K-ATP channel opener), adenosine, sodium-hydrogen exchange inhibitors, and statins. Adenosine is known to play an intimate role in preconditioning and postconditioning [5–7]. Other pharmacological strategies which have shown little to no success in clinical trials include antioxidants (e.g. allopurinol), anti-inflammatory drugs (e.g. methylprednisolone), and vasodilators (e.g. prostaglandins, dopamine) [3,8]. However, one example of a potentially promising agent has been the use of cyclosporine A to inhibit mitochondrial permeability transition pore (mPTP) opening [9].

In a variety of experimental settings and animal models, including models of IR, adenosine has been described as a retaliatory metabolite which serves as a protective agent with anti-inflammatory properties [10]. Adenosine is a purine nucleoside produced intracellularly and extracellularly during high stress conditions such as inflammation and IR [11]. Signaling through four known receptors, adenosine can have multiple effects depending on which receptors are activated and on which cells these receptors reside. Significant progress has been made in our understanding of the multifaceted role of adenosine signaling in tissue IR and inflammation, and new approaches and strategies are now being uncovered as a means to target adenosine receptor signaling as a therapeutic avenue to curtail tissue injury and inflammation. This review summarizes current knowledge on IR injury with a focus on the lung, heart, and kidney; and highlights studies that have advanced our understanding of the role of adenosine receptors and the therapeutic potential of adenosine receptor agonists and antagonists in the prevention of IR injury.

1.1 Ischemia-reperfusion injury

Within moments of reperfusion of ischemic tissue, reactive oxygen species (ROS) are generated (e.g. superoxide, peroxynitrite, hydrogen peroxide, and hydroxyl radical) [12,13]. ROS generation contributes to a rapid inflammatory response via direct oxidative injury to

cells and stimulation of proinflammatory mediators such as cytokines, chemokines and cell adhesion molecules. This inflammatory cascade is a key mediator of IR injury, and considerable studies have now established that an innate immune response is a significant component of this cascade. Reperfusion also entails complement activation and the release of cleavage fragments known as anaphylatoxins which are potent inflammatory mediators [14]. Microvascular dysfunction characterizes the “no-reflow” phenomenon, which refers to the failure of blood to reperfuse a previously ischemic tissue at the microvascular level even after blood flow has been restored at the macrovascular level. Mechanisms contributing to the no-reflow phenomenon include platelet-leukocyte aggregation, leukocyte-endothelial adhesion, reduced vasorelaxation capacity, and accumulation of interstitial fluid [15].

Circulating and resident leukocytes as well as tissue resident cells contribute to the immune response to IR injury. Orchestration of immune cell responses after IR has been made possible with the application of multi-color flow cytometry and the use of genetically modified mice, before which quantitative analysis relied primarily on immunohistochemical labeling of small samples of tissue. For example, a comprehensive leukocyte profile following kidney IR has been described using multi-color flow cytometry [16]. Tissue resident dendritic cells, neutrophils, macrophages, lymphocytes and platelets have been shown to contribute to IR injury [17]. Neutrophils, which are the major leukocyte population to rapidly infiltrate tissues following IR, are attracted by chemokines including MCP-1 (CCL2), KC (CXCL1), RANTES (CCL5), and IL-17. Infiltrating neutrophils can impose significant tissue injury through ROS generation and further release of cytokines and proteases. Damaged vascular endothelium provides an anchoring site for neutrophils via upregulation of adhesion molecules such as P-selectin, L-selectin and intercellular adhesion molecule (ICAM) [18–20]. Monocytes, macrophages and dendritic cells are heterogeneous and contribute to early and late phases of IR injury [21,22]. Recent studies have also documented that rapid activation and infiltration of T cells also mediate IR injury [23]. Depletion of neutrophils [24], macrophages [25], or T cells [26] have been shown to attenuate IR injury. Thus IR-induced inflammation entails a potent innate immune response involving the activation and infiltration of multiple leukocyte populations including neutrophils, macrophages, dendritic cells and T cells.

In addition to leukocytes, resident, tissue (non-leukocyte) cells are also involved in the pathogenesis of IR injury. For example, cardiac myocytes release IL-6 to contribute to myocardial dysfunction after IR [27], and alveolar epithelial cells contribute to pulmonary IR injury via release of chemokines such as KC and MIP-2 [28]. Thus, a variety of key cellular and molecular targets exist for which therapeutic interventions could be designed to attenuate the onset of inflammation initiated by IR.

1.2. Ischemic conditioning

Different organs have the ability to be preconditioned by a nonlethal period of ischemia, rendering the target organ refractory to further ischemia-induced dysfunction (ischemic preconditioning) such as the small intestine [29], liver [30], brain [31], lung [32], and kidney [33]. Mechanisms and pathways that are also involved in IR injury (described above in section 1.1), which are attenuated by activation of adenosine receptors, are important targets for ischemic preconditioning and postconditioning [34]. For example, many studies are now showing that preconditioning and postconditioning may involve similar signaling pathways upon reperfusion of the ischemic heart such as the reperfusion injury salvage kinase (RISK) pathway, redox signaling, and mPTP [35–37]. Adenosine is largely thought to serve as a mediator of ischemic preconditioning [38]. Similar to preconditioning, ischemic postconditioning was first described in 2003 and involves the interruption of reperfusion by intermittent, brief episodes of ischemia before extended reperfusion in order to provide protection [39]. Various studies suggest that the protection mediated by postconditioning is

also mediated in part by adenosine receptor signaling [40]. Finally, another concept, called remote conditioning, is defined as brief ischemia in an organ or tissue which provides a protective effect in a distant or remote organ which undergoes the primary ischemic insult. This brief ischemia can occur either before or after the primary ischemic event to be termed remote pre- or post-conditioning, and there is evidence that adenosine receptor signaling is also involved in remote conditioning [40–42]. Thus pre- and postconditioning as well as remote conditioning all appear to invoke similar protective pathways which may involve adenosine receptor signaling. It is important to note here that there is a distinct difference between the potential role of endogenous adenosine and the “preconditioning-like” protection afforded by the administration of adenosine either before or after ischemia.

1.3 Adenosine and adenosine receptors

Adenosine is a nucleoside locally released in response to cellular stress such as injury, IR, and inflammation [10]. Extracellular adenosine largely accumulates from the breakdown of adenine nucleotides that are liberated from damaged cells. These nucleotides are rapidly converted to adenosine by a family of nucleotidases including CD39 and CD73 [11]. In addition, two cytosolic enzymes important in the removal of adenosine from tissues, adenosine deaminase and adenosine kinase, will rapidly metabolize adenosine when extracellular adenosine traverses the cell membrane via the equilibrative nucleoside transporters (ENT) according to the concentration gradient of adenosine. Deficiency of adenosine deaminase results in adenosine levels which can increase dramatically in some tissues [43]. This mechanism is thought to contribute importantly to the pathophysiology underlying one form of severe combined immunodeficiency: adenosine deaminase (ADA) deficiency.

Adenosine exerts its effects through binding to any of four G protein-coupled adenosine receptors designated as A₁R, A_{2A}R, A_{2B}R and A₃R [44]. These receptors reside on a wide variety of cell types to control, either by inhibiting or stimulating, adenylyl cyclase and subsequently modulating intracellular levels of cyclic AMP (cAMP). A₁R and A₃R signaling is linked to inhibitory G proteins to down-regulate cAMP whereas A_{2A}R and A_{2B}R signaling is linked to stimulatory G proteins to up-regulate cAMP with subsequent transcriptional activation of cAMP-regulated genes (Figure 1). In addition to these classical pathways, adenosine receptor signaling can also activate other pathways involving phospholipase C, mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), or Akt pathways to influence gene expression [45] (summarized in Figure 1). The wide variety of physiological effects of adenosine, which may include not only protective, but also deleterious effects, depend upon many factors including: 1) differences in receptor affinities for adenosine, 2) extracellular concentration of adenosine, 3) expression of receptors among various cell types, 4) differences in receptor densities on cell membranes, and 5) duration of adenosine exposure.

Adenosine has the highest affinity for A₁R and A_{2A}R, an intermediate affinity for A₃R, and the lowest affinity for A_{2B}R [46,47]. Adenosine concentrations under basal conditions are sufficient to activate A₁R, A_{2A}R and A₃R. On the other hand, A_{2B}R requires higher adenosine concentrations to be significantly activated, concentrations typically attained after tissue injury. The expression of adenosine receptors are under dynamic regulation and can be up- or down-regulated depending on the cell type and nature of the physiological stress. For example, A_{2A}R expression can increase under inflammatory conditions [48], and the A_{2B}R expression can increase during hypoxic and ischemic conditions [49].

Research into the role of adenosine receptor signaling in organ injury and disease has undergone rapid and significant progress due to the development of several critical tools. First, potent and selective synthetic agonists and antagonists for each adenosine receptor

have been developed to enable researchers to specifically test the role of each receptor in almost any model [50]. Second, gene knockout ($-/-$) mice with targeted deletions of each adenosine receptor have now been generated and have been important in exploring the physiological and pathophysiological roles that these receptors play. The utilization of these agonists/antagonists and knockout mice in various studies of IR injury are discussed below.

2. Adenosine receptors in lung IR injury

All four adenosine receptors are expressed in lungs of mice [51] and humans [52]. Adenosine is well recognized as exhibiting protective effects in various models of IR injury [53], and a study by Hsu *et al.* was one of the first to demonstrate this for the lung [54]. Most studies into lung IR injury have focused on the A_2A R or A_3 R, where activation of either is protective. The roles of A_1 R and A_2B R remain poorly understood.

2.1 A_1 R in lung IR injury

Studies on the role of A_1 R in lung IR injury have been conflicting as the A_1 R has been shown to have both pro- and anti-inflammatory effects [55]. Activation of A_1 R in a variety of cell types can produce proinflammatory effects. For example, in neutrophils, A_1 R activation induces chemotaxis, adherence to endothelium, and generation of ROS [56]. Although A_1 R activation has been shown to be protective after IR in the heart [57] and kidney [58], an early study by Neely and Keith concluded that A_1 R antagonists (XAC and DPCPX) block IR injury in the lung [59]. More recent studies, however, have suggested that the A_1 R may be protective. Using a blood-perfused rabbit lung model, it was recently shown that activation of A_1 R (via CCPA) during reperfusion attenuates lung IR injury [60]. Yildiz *et al.* evaluated the role of adenosine in ischemic preconditioning using an *in vivo* rat lung IR model [61]. Here, preconditioning in the form of adenosine infusion or a brief ischemic exposures prior to sustained ischemia prevented injury, and, importantly, these protective effects were abolished by DPCPX. This suggests that preconditioning entails adenosine signaling via the A_1 R. Furthermore, studies with $A_1R^{-/-}$ mice suggest that A_1 R activation is protective in a model of sepsis [62], and it has recently been shown that the A_1 R on hematopoietic cells mediates (reduces) transendothelial and transepithelial migration in a model of LPS-induced lung injury [63]. In contrast, blockade of A_1 R was found to prevent injury in a feline model of LPS-induced lung injury [64]. Thus the role of A_1 R in lung IR injury and other inflammatory conditions remains controversial, which suggests that the A_1 R has a more complex role than previously realized. This complexity likely arises from a variety of differences among these studies including differences in organ and tissue models, A_1 R signaling pathways among cell/tissue types, and A_1 R expression among cell types within an organ or between species.

2.2 A_2A R in lung IR injury

A rich history of studies provides overwhelming evidence that A_2A R is the major adenosine receptor mediating anti-inflammatory properties of adenosine [55]. An early study by Khimenko *et al.* was the first to demonstrate that selective activation of A_2A R is protective in the setting of lung IR [65]. Here, treatment of isolated rat lungs with an A_2A R agonist, CGS-21680, reversed endothelial damage after IR. There have been a series of more recent supportive studies as well. Sharma *et al.* demonstrated that ATL-313, an A_2A R agonist [66], prevented lung IR injury in wild-type mice and that ATL-313 had no protective effect in A_2A R $^{-/-}$ mice [67]. In rabbit lungs, it was also shown that ATL-313 has an additive protective effect when administered both prior to ischemia as well as during reperfusion, an effect that was abolished by the A_2A R antagonist, ZM241385 [68]. There is now evidence that the protective effect of ATL-313 in lung IR injury is largely derived through the activation of A_2A Rs on CD4 $^{+}$ T lymphocytes and neutrophils [69]. In a more mechanistic

study, Rivo *et al.* concluded that A_{2A}R-mediated protection is associated with decreased apoptosis and involves ERK1/2 activation and modulation of Bcl-2 and Bax expression [70].

In an effort to move beyond small animal models toward more clinically relevant models, the protective effects of A_{2A}R activation was recently tested in a porcine lung transplantation model in which the lungs were ischemic for six hours prior to transplant [71]. In this study, recipient pigs were administered ATL146e, an A_{2A}R agonist, intravenously for three hours after lung transplantation. This study demonstrated that ATL146e attenuated lung injury and inflammation and preserved pulmonary function after transplantation. Collectively, these studies, and others, suggest that specific A_{2A}R activation may be a promising therapeutic target for the prevention of IR injury and primary graft function in lung transplant patients.

2.3 A_{2B}R in lung IR injury

The A_{2B}R is expressed on a wide variety of cells including most inflammatory cells and, in the lung, are highly expressed on alveolar epithelial cells [72]. Expression of A_{2B}R is induced under stresses such as injury, oxidative stress, or by TNF- α [73]. As with other adenosine receptors, both anti- and pro-inflammatory activities have been attributed to A_{2B}R activation. However, the fact that the A_{2B}R has relatively low affinity for adenosine suggests that it may serve a more prominent role in pathological conditions in which high levels of adenosine are achieved.

The role of A_{2B}R in lung IR injury has not been well studied. A recent study by Anvari *et al.* showed that lung IR injury is attenuated in A_{2B}R^{-/-} mice, suggesting that A_{2B}R is proinflammatory in this setting [74]. Also in this study, use of chimeric mice demonstrated that the proinflammatory effects of A_{2B}R were attributed to A_{2B}R signaling on resident pulmonary cells and not bone-marrow-derived cells. A proinflammatory role of A_{2B}R has been reflected in several other studies as well [75–77]. On the other hand, some studies suggest an anti-inflammatory role for A_{2B}R in different settings of acute lung injury. For example, Schingnitz *et al.* demonstrated a protective role of A_{2B}R signaling in endotoxin-mediated lung injury, and use of bone marrow chimeric mice demonstrated that this is largely mediated via A_{2B}R signaling in pulmonary cells, suggesting that A_{2B}R agonists may be potentially useful for the treatment of endotoxin-induced lung injury [78]. These seemingly contradictory studies of A_{2B}R may be due its various signaling partners in different tissues and whether the signal in the system used originates from bone marrow cells or from tissues. Continued efforts to better define the role of A_{2B}R in lung IR injury will be critical to the development of adenosine receptor agonist/antagonist-based therapeutics.

2.4 A₃R in lung IR injury

The A₃R is widely expressed with the highest levels found in the lung and liver. The role of A₃R in inflammatory conditions, including IR injury, is ambiguous. Although there is evidence for both protective and detrimental effects, most studies suggest that A₃R activation is protective in lung IR. Using a feline model, Rivo *et al.* found that IB-MECA, an A₃R agonist, administered before ischemia or during reperfusion, attenuated injury and apoptosis [79]. These protective effects of IB-MECA were blocked by MRS-1191, an A₃R antagonist. This same group has also provided evidence that the protective effects of A₃R activation may be mediated in part through upregulation of phosphorylated ERK [80] as well as inhibition of p38 MAPK [81]. In addition to IR injury, activation of A₃R was shown to serve an anti-inflammatory function in a bleomycin-induced model of pulmonary inflammation and fibrosis [82]. In LPS-induced pulmonary inflammation, a recent study by Wagner *et al.* demonstrated that administration of the A₃R agonist, CI-IB-MECA,

attenuated microvascular permeability and neutrophil migration in lungs after LPS exposure, suggesting that pharmacologic modulation of A₃R signaling may be a promising strategy for lung inflammation [83]. This study also found that CI-IB-MECA required A₃Rs on both hematopoietic and nonhematopoietic cells to reduce transmigration *in vivo*. Thus, overall, it appears that the most promising therapeutic strategies to prevent lung IR injury may involve the use of A_{2A}R and/or A₃R agonists in transplant recipients. It will likely be important for these agonists to be very specific so as to not engage potentially proinflammatory adenosine receptors such as A₁R or A_{2B}R.

3. Adenosine receptors in kidney IR injury

Extracellular adenosine has a critical role in mediating physiological properties in the kidney [84–86]. Adenosine constricts afferent arterioles, thereby reducing filtered sodium loading and controlling metabolic work of the kidney, vasodilation, and NaCl transport in the distal nephron. These vascular and epithelial effects control metabolic function in the kidney. In addition, adenosine has an important effect on the regulation of inflammatory processes in the kidney. Early studies using a nonselective adenosine receptor antagonist, theophylline, attenuated the reduction in renal blood flow and glomerular filtration rate observed after IR [87,88]. In contrast, intravenous infusion of adenosine protected kidneys from IR injury. Kidney IR in mice deficient in CD39 or CD73, enzymes necessary for adenosine production, resulted in enhanced injury, highlighting the protective effects of adenosine and a critical role for adenosine-generating enzymes [89,90]. These reports in which adenosine receptor antagonists and agonists reduced injury are likely explained by the nonselective effect of these compounds, and the use of more selective pharmacological reagents in recent studies have helped to shed more light onto the mechanism of action.

3.1 A₁R in kidney IR injury

In rodents, A₁R activation protected kidneys from IR injury [91,92]. In these studies, animals treated with an A₁R agonist before renal ischemia showed reduced IR injury. Further support of the role of A₁Rs in mediating protection comes from studies in which A₁R^{−/−} mice showed increased kidney IR injury [58]. Thus activation of A₁Rs appears to serve a protective role against renal IR injury. This protective effect could be due to: 1) over-expression of A₁Rs in proximal tubule cells leading to reduced renal proximal tubule cell necrosis and apoptosis via upregulation of heat shock protein 27 [93,94] and 2) enhanced A₁R-mediated vasoconstriction with subsequent induction of hypoxia-inducible factors (HIFs) and HIF-dependent, protective genes [93]. In other models of acute kidney injury (AKI); however, A₁R antagonism leads to renal protection. In cisplatin-induced AKI, tonapofylline, an A₁R antagonist, reduced injury [95]. In radiocontrast-induced AKI, A₁R^{−/−} mice or wild-type mice treated with a selective A₁R antagonist, DPCPX, were protected from kidney injury [96]. In both of these models, vasoconstriction contributed to injury, and A₁R blockade was thought to reduce vasoconstriction.

3.2 A_{2A}R in kidney IR injury

The use of A_{2A}R-specific agonists and antagonists has revealed in numerous studies that A_{2A}R signaling is protective in renal IR injury (reviewed in [97]). Since the initial observation that A_{2A}R activation ameliorates IR injury in rat kidneys [98], significant advances have been made in characterizing the mechanism and target of action for A_{2A}R. ATL146e, an A_{2A}R agonist, markedly reduced the damaging effects of IR when infusion was initiated prior to, or at the time of ischemia [99]. Dose-dependent studies in mice indicated that infusion of 1–10 ng/kg/min of ATL146e was the most effective dose [99] and that this protective dose was not due to changes in systemic blood pressure [98]. When mice were subjected to kidney IR and treated with rolipram, a PDE IV inhibitor, or ATL146e

during IR, delivery of either drug was associated with reduced renal injury in a dose-dependent manner, and maximal (additive) protection was observed when both compounds were administered together [99]. It was then demonstrated that the protective mechanism of ATL146e was, at least in part, mediated through neutrophils, where ATL146e significantly reduced neutrophil accumulation in renal tissues after IR [99]. Using high resolution microscopy combined with flow cytometry, it was also demonstrated that A_{2A}R agonists reduced transmigration of neutrophils from the intravascular space to the interstitium as well as decreased vascular permeability [100]. Additionally, the enhanced expression of adhesion molecules P-selectin and ICAM-1 following IR was reduced by A_{2A}R agonists [20]. These observations are consistent with the idea that A_{2A}R agonists limit IR injury due to an inhibitory effect on neutrophil adhesion.

Given the ubiquitous expression of A_{2A}Rs, the precise target of A_{2A}R agonist action in mediating tissue protection *in vivo* has only recently been described. Using bone marrow chimeric mice, it was determined that the protective effect of A_{2A}R agonists following IR was due to A_{2A}R signaling on bone marrow-derived cells [101]. Subsequent studies have now demonstrated that activation of A_{2A}Rs on CD4⁺ T cells, and not macrophages, mediates tissue protection [97].

3.3 A_{2B}R and A₃R in kidney IR injury

Few studies have examined the role of A_{2B}R or A₃R in kidney IR injury or AKI. In one recent study, renal protection by ischemic preconditioning was abolished in A_{2B}R^{-/-} mice and in wild-type mice administered PSB1115, an A_{2B}R antagonist [102]. In contrast, protection by ischemic preconditioning was observed in A₃R^{-/-} mice [102]. Using chimeric mice, this study also demonstrated a critical role of renal A_{2B}R (as opposed to A_{2B}R on hematopoietic cells) in mediating the protective effects of ischemic preconditioning.

Lee *et al.* demonstrated that an A₃R agonist worsened kidney injury while an antagonist improved renal function after IR in mice [103]. In accordance, A₃R^{-/-} mice displayed significant functional and morphological renal protection from IR injury [103]. A study by Lee *et al.* also demonstrated that A₃R activation prior to renal IR worsens injury whereas A₃R antagonism protects renal function [92]. On the other hand, it has been shown that A₃R activation confers significant protection from renal injury in murine septic peritonitis [104]. Thus further studies will be required to understand the roles of A_{2B}R and A₃R in kidney IR injury.

4. Adenosine receptors in cardiac IR injury

Gene expression of all four adenosine receptors have been detected in the rat heart, including cultured cardiomyocytes [105] and cardiac fibroblasts [106]. These results are consistent with the pharmacologic studies undertaken in many species over many years, which for the most part support a functionally significant role for each adenosine receptor in the heart [107]. Adenosine itself has long been known to exert cardioprotective effects in various models of IR injury [53,108], effects that are traditionally understood in terms of adenosine triggering a preconditioned state through A₁R stimulation [109]. However, cardioprotective roles for the A_{2A}R [110] and A₃R [111] were demonstrated not long thereafter. More recent studies have even suggested a cardioprotective role for the A_{2B}R [112]. Thus the cardioprotective effects of A₁R signaling are better understood and characterized than A_{2A}R and A₃R signaling, and until recently, little was known about the cardioprotective potential of the A_{2B}R.

4.1 A₁R in cardiac IR injury

The phenomenon of ischemic preconditioning was first discovered in the heart [113], and the role of adenosine in mediating the effects of preconditioning via A₁R activation was discovered soon after [6]. It is now well established that activation of not only the A₁R, but also other G-protein coupled receptors, can induce preconditioning. Indeed, opioids and bradykinin can stimulate signal transduction pathways which ultimately protect the mitochondria by inhibiting the opening of the mitochondrial permeability transition pore (mPTP), thereby protecting mitochondria from lysis due to calcium overload and salvaging cardiomyocytes from cell death. The downstream signal transduction mechanisms and end effectors mediating ischemic preconditioning have been the subject of intensive study for many years (reviewed in [114–116]). In addition to the many elegant pharmacologic studies conducted in a wide variety of animal models, the central importance of the A₁R in preconditioning has also been demonstrated in transgenic mouse lines that overexpress A₁R via a cardiac-specific promoter [57,117]. Nevertheless, it is important to note that classic ischemic preconditioning (and preconditioning mimetics such as adenosine, bradykinin and opioids) need to be applied in advance of the index event and are thought to protect cardiomyocytes against the effects of prolonged ischemia and/or calcium overload upon reperfusion. Thus while preconditioning induces a very effective and reproducible form of cardioprotection, the clinical utility is limited to applications in which the ischemic event can be predicted (such as cardiac transplantation). An alternative or supplemental strategy for cardioprotection is to limit myocellular death by minimizing the added damage imposed by calcium overload and inflammation during reperfusion. These approaches may be used in a wider range of clinical applications since they may be effective even when treatment is delayed until the time of reperfusion.

4.2 A_{2A}R in cardiac IR injury

Soon after the discovery of preconditioning, it was reported that the intracoronary administration of adenosine during reperfusion could also substantially reduce infarct size [118]. In contrast to the direct effects of preconditioning on cardiomyocytes, the cardioprotective effects of adenosine during reperfusion were primarily attributed to decreased neutrophil infiltration in the ischemic zone and preservation of endothelial structure in the subendocardium. Interestingly, the same group later reported that the salutary effect of adenosine on infarct size was lost if treatment was delayed by only three hours post-reperfusion [119]. Subsequent work using more selective adenosine receptor agonists established that this form of cardioprotection was mediated primarily by the A_{2A}R [120,121]. The ability of selective A_{2A}R agonists to reduce infarct size when administered at, shortly before, or during reperfusion has since been confirmed in studies of a wide variety of animal models including pigs [122], dogs [123,124], rabbits [125], rats [126] and mice. Of note, the cardioprotection afforded by selective A_{2A}R agonists is abrogated in A_{2A}R^{-/-} mice [127], as well as lymphocyte-deficient Rag-1^{-/-} mice reconstituted with CD4⁺ T cells from A_{2A}R^{-/-} mice, thus implicating CD4⁺ T cells in the pathophysiology of IR injury [128].

The timing of administration for A_{2A}R agonists during reperfusion makes them far more suitable than A₁R agonists for clinical applications such as direct percutaneous intervention for acute myocardial infarct. As such, the timing of reperfusion therapy shares much in common with the more recently discovered phenomenon of postconditioning [39]. Similar to preconditioning, postconditioning involves a series of coronary occlusions and reperfusions, but the series of ischemic episodes is imposed at the end (rather than the beginning) of the coronary occlusion. It remains to be definitively established whether postconditioning functions primarily by reducing ischemic injury to cardiomyocytes or by reducing the additional injury imposed by reperfusion. However, the efficacy of

postconditioning in reducing neutrophil infiltration and preserving endothelial function is well established [129], and the salutary effect of postconditioning on infarct size is retained in $A_1R^{-/-}$ mice whereas it is greatly attenuated in $A_{2A}R^{-/-}$ mice [130]. Thus, it would appear that preconditioning and A_1R agonists protect cardiomyocytes against ischemic injury, while postconditioning and $A_{2A}R$ agonists function primarily by inhibiting inflammation and thus attenuating reperfusion injury to the heart.

4.3 $A_{2B}R$ in cardiac IR injury

Similar to the $A_{2A}R$ and its agonists, the $A_{2B}R$ has also been implicated in ischemic postconditioning [112], and its agonists have been shown to reduce infarct size when administered during the first hour of reperfusion in rabbits [131]. Differences in animal models and experimental preparations (Langendorff vs. open-chest) led to healthy debate regarding the relative importance of the two receptors in reducing infarct size when their agonists were administered during reperfusion. However, a recent study found that both $A_{2A}R$ and $A_{2B}R$ activation were required to achieve maximal protection against IR injury in rats [132], and this observation was supported by an independent laboratory using an open-chest murine model of IR injury [133]. The mechanisms underlying the additive effect of combined $A_{2A}R$ and $A_{2B}R$ stimulation have yet to be fully defined, but the signal transduction pathways implicated in $A_{2B}R$ -mediated cardioprotection involve cardiomyocytes, whereas the inflammatory system has been more strongly implicated in $A_{2A}R$ -mediated cardioprotection, suggesting that the two-pronged strategy targets both ischemic and reperfusion injury.

4.4 A_3R in cardiac IR injury

The $A_{2A}R$ and $A_{2B}R$ are primarily coupled through the G_s G-protein coupled receptors, whereas the A_1R and A_3R are G_i -coupled [53]. Thus one might expect A_3R activation to share aspects with A_1R activation, which exerts only modest cardioprotection and then principally during ischemia [134]. However, unlike selective A_1R agonists [135], selective A_3R agonists have been shown to trigger cardioprotection not only when applied prior to ischemia, but also when administered at reperfusion [136]. When studied in isolated rat cardiomyocytes subjected to simulated ischemia/reoxygenation injury, A_3R activation exerted anti-apoptotic and anti-necrotic effects when administered at reoxygenation [137]. Another proposed mechanism by which A_3R reduces infarct size is by attenuating neutrophil activation and infiltration. For example, Jordan *et al.* have shown that A_3R activation at reperfusion attenuates neutrophil-mediated reperfusion injury [111]. Thus, therapeutic strategies targeting A_3R activation may also be promising for the reduction of cardiac reperfusion injury. It remains to be seen whether the combination of $A_{2A}R$, $A_{2B}R$ and A_3R stimulation during reperfusion offers any additional cardioprotection over the combination of $A_{2A}R$ and $A_{2B}R$ in pre-clinical models of myocardial infarction. Even so, the relative tissue abundance (and perhaps even the primary function) of the four adenosine receptors may vary from species to species, and more work will be needed to identify the most potent combination of adenosine receptor agonists (or mixed agonists) for reducing infarct size in humans.

5. Adenosine receptors in liver IR injury

All four adenosine receptors are expressed in hepatocytes, where they stimulate the rates of glycogenolysis, gluconeogenesis, and ureagenesis [138]. One of the first reports of the protective effects of adenosine in the liver after IR was a study by Satoru *et al.* which showed that augmentation of endogenous adenosine by inhibition of nucleoside transport attenuated liver IR injury in dogs [139].

5.1 A₁R in liver IR injury

Most studies, but not all, suggest that A₁R activation is protective in the setting of liver IR. The protective effects of ozone oxidative preconditioning have been attributed to A₁R activation [140], and, in another study, A₁R activation was found to induce hepatic delayed preconditioning while A₁R antagonist blocked preconditioning [141]. Although A₁R^{-/-} mice and wild-type mice treated with an A₁R antagonist developed worse liver IR injury, the A₁R agonist, CCPA, failed to protect against injury [142], suggesting that the endogenous A₁R is protective. On the other hand, administration of A₁R antagonists have been shown to elicit beneficial effects against hepatic IR injury in a canine model [143] and a pig model of non-heart beating donor liver transplantation [144]. Thus, as with other organs, the role of A₁R in liver IR injury remains unclear.

5.2 A_{2A}R in liver IR injury

As with the lung, liver, and heart, ample evidence exists which demonstrates that A_{2A}R activation confers protective and anti-inflammatory effects against liver IR injury. An early study by Harada *et al.* demonstrated that selective A_{2A}R agonists, YT-146 and CGS-21680C, reduce liver IR injury in rats by inhibiting leukocyte activation [145]. Several more recent studies have now determined that the protective effects of A_{2A}R against liver IR injury are due to the inhibition of NKT cell activation and chemokine production [146–148].

5.3 A_{2B}R and A₃R in liver IR injury

The roles for A_{2B}R and A₃R in liver IR injury remain obscure. To date, no studies have examined the A_{2B}R and very few have evaluated the A₃R. Guinzberg *et al.* [149] have provided evidence for a protective role for A₃R in the liver by evaluating the role of A₃R in the responses of hepatocytes to inosine, a purine nucleoside formed by adenosine deamination which has been shown to bind to A₃R to promote its effects [150]. Guinzberg *et al.* also showed that, in perfused rat liver as well as isolated hepatocytes, hypoxia-reoxygenation stimulated the release of inosine, adenosine, and glucose; effects which were attenuated by an A₃R antagonist [149]. They concluded that after ischemia, inosine contributes to the maintenance of homeostasis by releasing glucose from the liver through A₃R activation. Further studies will be required to better define the roles for A_{2B}R and A₃R in liver IR injury.

6. Adenosine receptors in IR injury of other tissues

Literature on the role of adenosine receptors in IR injury of tissues other than lung, kidney, heart and liver is much less extensive. An A_{2A}R agonist was shown to improve islet cell transplant outcome by attenuating innate immune responses [151]. Most studies of ischemic brain injury conclude that A_{2A}R antagonists and adenosine appear to be neuroprotective, depending on the nature of the brain injury and associated pathology [152,153]. The mechanisms by which ischemic brain injury is modulated by A_{2A}R are poorly understood. Several studies have demonstrated that A_{2A}R agonists are protective in the setting of spinal cord IR [154,155]. In addition, Miyazaki *et al.* demonstrated the involvement of A₁R activation in the neuroprotective effects of adenosine in spinal cord ventral horn [156]. In models of intestinal IR injury, agonists for both A₁R [157] and A_{2B}R [158] have been shown to provide protection.

7. Conclusion

The most extensively studied adenosine receptors in the setting of IR injury have been the A₁R and A_{2A}R. Nearly all studies indicate that A_{2A}R activation results in potent, protective, anti-inflammatory effects. This is likely the primary mechanism responsible for adenosine-

mediated protection, at least in acute inflammatory conditions. However, controversy remains regarding the role of other adenosine receptors, where both pro- and anti-inflammatory effects have been observed. Potent and selective adenosine receptor agonists and antagonists have been, and will remain, important tools to help define these roles. While the anti-inflammatory properties of A_{2A}R activation are well characterized in multiple tissue systems, it appears that the roles of other adenosine receptors in IR injury may be more complex and tissue-dependent, which is likely due to multifaceted patterns of expression and signaling pathways for these receptors. However, there is now building and significant evidence which support protective effects of A₁R and A₃R in IR injury. Further studies will be necessary to decipher the roles for these receptors in various settings of IR injury.

8. Expert Opinion

A general conceptual framework for understanding IR injury is presented in Figure 2, which employs data derived from a murine model of myocardial infarction as an example [159]. Figure 2 illustrates the concept that the experimentally-defined relationship between infarct size and duration of ischemia (the sigmoidal curve) is actually the sum total of two separate components: ischemic injury (gray region) and reperfusion injury (white area between the sigmoidal curve and the gray region). In this scheme, the gray area representing ischemic injury is hypothetical and could differ in shape and/or size. Nevertheless, ischemic injury clearly dominates in the setting of extended or long-term occlusion and certainly disappears if the period of occlusion is extremely brief. A major component of reperfusion injury is inflammatory injury, although other mechanisms are also known to contribute to reperfusion injury. Figure 2 illustrates the point that the efficacy of agents that focus on reperfusion injury, such as A_{2A}R agonists, is predicted to wane with increasing durations of ischemia. Conversely, therapies that minimize both ischemic injury and reperfusion injury will be needed to achieve maximal tissue protection over the widest range of ischemic events. It should be noted that the slope of the line defining ischemic injury almost certainly varies depending on the tissue undergoing ischemia. Thus heart and brain would likely exhibit steeper slopes than other tissues (e.g., liver, lung and kidney) where metabolic demand is far lower and reperfusion injury may become the predominant mode of injury.

Currently, no therapeutic strategies are available that specifically target IR injury. Perhaps strategies aimed toward augmenting adenosine-mediated protective effects could provide such a therapy. The development and use of selective adenosine receptor agonists and antagonists have been instrumental in understanding the role of adenosine receptors in IR injury. More importantly, these agents, and newer generation agents with greater selectivity and potency, will be key to the development of future adenosine receptor-targeted therapies to prevent IR injury. In light of current research, the most promising therapeutic strategies will likely involve the administration of selective A₁R, A_{2A}R, or A₃R agonists to patients immediately upon organ transplantation, or as quickly as possible after episodes of IR such as myocardial infarct or trauma to minimize injury imposed by inflammation.

As pointed out in the introduction, despite many experimental studies which suggest a beneficial effect of anti-inflammatory agents in preventing IR injury, most anti-inflammatory therapies, at least in the heart [8] and kidney [160], which have been tested in the clinical setting have failed. Possible reasons for these negative results are nicely discussed in a recent review by Dirksen *et al.* [8] and may involve differences in the methodology and biology between experimental and clinical studies. This could reflect an incomplete understanding of the intricacies of IR injury. Indeed, IR injury is a very complex process, involving more than just inflammation, and it is quite possible that a single intervention cannot be expected to completely prevent IR injury. However, experimental anti-inflammatory studies are progressing, and novel anti-inflammatory strategies continue

to be explored such as neutralization of proinflammatory cytokines, prevention of cytokine secretion, administration of anti-inflammatory cytokines, and cell-based therapies [161].

The application of A_{2A}R agonists toward treatments for inflammation has been discussed in detail by Lappas and colleagues [162]. One advantage of using an A_{2A}R agonist is that minimal side effects have been reported in animals. For example, there are no adverse side effects of ATL146e treatment, due to its short half life (minutes), with the exception of transient hypotension following administration at high doses. However, at much lower doses, which effectively block inflammation in IR studies (1–50 ng/kg/min), there are no hemodynamic effects [71,99]. Thus A_{2A}R agonists show promise in future clinical studies directed towards the treatment and prevention of IR injury.

The role of adenosine receptors other than A_{2A}R in IR injury and whether agonists or antagonists for these receptors can be used to attenuate injury remains unclear and will likely vary depending on the organ/species affected. Overall, studies suggest a protective role for A₁R and A₃R agonists in IR injury, although A₃R antagonists appear to attenuate kidney injury. The role of A_{2B}R in modulating inflammatory responses to injury is confounding, complex, and likely tissue-specific. Animal studies of the A_{2B}R have been hindered by the limited availability of well-characterized, selective agonists. In addition, A_{2B}R^{-/-} mice have only recently been generated [163]. Further investigations will be required to better understand the diverse A_{2B}R-mediated signaling pathways in various cell types after IR.

An advantage to utilizing adenosine receptor agonists to attenuate IR injury is that an acute treatment would likely be sufficient and efficacious; possibly in the range of hours rather than days. This therapeutic window is based upon many studies in both animals and humans that have documented acute inflammation that begins immediately upon reperfusion that is initiated by rapid activation and infiltration of leukocytes. Blocking this inflammatory cascade appears to be sufficient to reduce acute graft dysfunction after transplant or cardiac dysfunction after infarct. Because IR injury to single organs leads to systemic inflammation and multiorgan dysfunction, compounds such as adenosine receptor agonists may not only benefit the ischemic organ but may also attenuate dysfunction of distal organs.

A continued push to test the efficacy of adenosine receptor agonists in humans to ameliorate IR injury may be a worthwhile pursuit. One caveat to keep in mind for this strategy is that different adenosine receptors can result in different effects, and in fact, activation of some receptors, especially under high adenosine levels, may even be detrimental under certain conditions. Thus, the specificity of adenosine receptor agonists will be paramount so as to not engage other, potentially detrimental receptors. In conclusion, the development of small molecule therapies to prevent IR injury is sorely needed, and may now be getting nearer to reality due to the many pre-clinical studies that continue to advance our understanding of the actions of adenosine receptor agonists in various models. Such a therapy will not only help the patient acutely after IR, but will also have a significant impact on organ survival in the long-term.

Article highlights

- Ischemia-reperfusion injury involves a complex cascade of events including oxidative stress, inflammation, and interactions between many cell types.
- Adenosine is a retaliatory, protective metabolite locally released in response to cellular stress such as ischemia-reperfusion and inflammation.
- Adenosine exerts its effects through binding to any of four G protein-coupled adenosine receptors designated as A₁R, A_{2A}R, A_{2B}R and A₃R.

- Adenosine receptor signaling has a wide variety of physiological effects, often protective, depending on receptor type activation, cell/tissue type, and physiologic conditions.
- In the lung, A₁R and A_{2A}R have been shown to be protective, A_{2B}R may be detrimental, and the role of A₁R remains controversial.
- In the kidney, A₁R and A_{2A}R have been shown to be protective, whereas the roles of A_{2B}R and A₃R are less understood.
- In the heart, A₁R is cardioprotective via preconditioning effects, A_{2A}R, and A₃R are also protective, and there is building evidence that A_{2B}R may be protective.
- In the liver, A₁R and A_{2A}R have been shown to be protective, whereas the roles of A_{2B}R and A₃R remain obscure.
- Less extensive studies suggest that adenosine receptor signaling, especially A_{2A}R, is protective in other tissues such as islet cell transplant, brain, spinal cord, and intestine.
- Adenosine receptor agonists may be a promising therapeutic strategy against ischemia-reperfusion injury, and continued push to test the efficacy of adenosine receptor agonists in humans to treat ischemia-reperfusion injury is likely a worthwhile pursuit.

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Abbreviations

–/–	gene knockout
ADA	adenosine deaminase
AKI	acute kidney injury
cAMP	cyclic AMP
ENT	equilibrative nucleoside transporter
GPCR	G-protein coupled receptor
HIF	hypoxia-inducible factor
ICAM	intercellular adhesion molecule
IR	ischemia reperfusion
MAPK	mitogen activated protein kinase
MODS	multiple organ dysfunction syndrome
mPTP	mitochondrial permeability transition pore
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome

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Figure 1. Adenosine receptor signaling pathways

A_{2A}R and A_{2B}R activation increase cyclic AMP (cAMP) levels with subsequent activation of the PKA → CREB pathway to increase transcription of cAMP-regulated genes, while A₁R and A₃R activation decrease cAMP levels. In addition to mediating gene expression via cAMP, adenosine receptor activation can also influence gene expression via the PI3K → Akt/PKB or PLC → MAPK pathways, among others not shown here. CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PK, protein kinase.

Figure 2. Conceptual framework for separating reperfusion injury from ischemic injury
 As shown, the final infarct size indicated by the empirically-determined sigmoidal line represents the sum total of the ischemic damage to the tissue and the additional injury imposed by reperfusion, a major component of which is inflammation. The white area directly underneath the sigmoidal curve represents reperfusion injury, while the shape and size of the gray area is hypothetical due to difficulties in determining it experimentally. Adopted with permission from Xu *et al.* [159] and used with permission from the American Physiological Society.