Protection from ischemic liver injury by activation of A2A adenosine receptors during reperfusion: inhibition of chemokine induction

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Submitted 11 August 2003; accepted in final form 9 September 2003

Am J Physiol Gastrointest Liver Physiol 286: G285–G293, 2004; 10.1152/ajpgi.00348.2003.—Ischemia-reperfusion (I/R) injury occurs as a result of restoring blood flow to previously hypoperfused vessels or after tissue transplantation and is characterized by inflammation and microvascular occlusion. We report here that 4-[3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-prop-2-ynyl]-cyclohexanecarboxylic acid methyl ester (ATL146e), a selective agonist of the A2A adenosine receptor (A2A AR), profoundly protects mouse liver from I/R injury when administered at the time of reperfusion, and protection is blocked by the antagonist ZM241385. ATL146e lowers liver damage by 90% as assessed by serum glutamyl pyruvic transaminase and reduces hepatic edema and MPO. Most protection remains if ATL146e treatment is delayed for 1 h but disappears when delayed for 4 h after the start of reperfusion. In mice lacking the A2A AR gene, protection by ATL146e is lost and ischemic injury of short duration is exacerbated compared with wild-type mice, suggesting a protective role for endogenous adenosine. I/R injury causes induction of hepatic transcripts for IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-18, INF-β, INF-γ, regulated on activation, normal T cell expressed, and presumably secreted (RANTES), major intrinsic protein (MIP)-1α, MIP-2, IFN-γ-inducible protein (IP)-10, and monocyte chemotactic protein (MCP)-1 that are suppressed by administering ATL146e to wild-type but not to A2A AR knockout mice. RANTES, MCP-1, and IP-10 are notable as induced chemokines that are chemotactic to T lymphocytes. The induction of cytokines may contribute to transient lymphopenia and neutrophilia that occur after liver I/R injury. We conclude that most damage after hepatic ischemia occurs during reperfusion and can be blocked by A2A AR activation. We speculate that inhibition of chemokine and cytokine production limits inflammation and contributes to tissue protection by the A2A AR agonist ATL146e.

Adenine receptor; A2A adenosine receptor knockout mouse; PI purinergic; receptors; regulated on activation, normal T cell expressed, and presumably excreted; monocyte chemotactic protein-1; interferon-γ-inducible protein-10

ISCHEMIA-REPERFUSION (I/R) injury is characterized by inflammation and microvascular occlusion during the reperfusion period (51). I/R injury of liver is a clinically significant manifestation of several surgical procedures, such as liver transplantation, partial hepatic resection, hepatic tumor, or trauma repair (18). The degree of liver cell damage that occurs as a consequence of these procedures depends in part on primary injury that occurs during ischemia and in part on secondary damage that occurs during reperfusion. Severe hepatic I/R injury causes not only liver failure but damage to other organs (14). Inflammatory events that occur during reperfusion lead to disruption of the integrity of the vascular endothelium and sinusoids, platelet aggregation, immunocyte activation (monocytes/macrophages, Kupffer cells, neutrophils), chemokine and cytokine secretion, and complement activation (32, 45).

Induction of chemokines has been suggested as a possible contributory factor in I/R injury-induced inflammation (28). Certain chemokines act as activators of neutrophil and monocyte diapedesis in the early stages of reperfusion injury (9) and may function as chemotactic molecules. A2A adenosine receptors (A2A AR) have been shown to be anti-inflammatory and to reduce I/R injury in liver (2, 17) as well as in spinal cord (12), heart (11), kidney (38), and lung (44). Pharmacological data suggest that A2A AR activation also causes liver protection from I/R injury, but these studies have been somewhat inconclusive due to the limited selectivity of the compounds used. In this study, we sought to better define the effects of A2A AR activation on protection of the liver from I/R injury and to determine whether suppression of hepatic inflammatory chemokine production plays a role in this protection. We show that the potent and highly selective agonist of the A2A AR, 4-[[3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-prop-2-ynyl]-cyclohexanecarboxylic acid methyl ester (ATL146e), produces a profound protection of wild-type C57BL/6 mice from liver I/R injury that is absent in congenic animals lacking the A2A AR gene. We also show for the first time that both CC and CXC chemokines, such as major intrinsic protein (MIP)-1α, MIP-1β, regulated on activation, normal T cell expressed, and presumably secreted (RANTES), and IFN-γ-inducible protein (IP)-10 are all induced after liver I/R injury and that this induction is suppressed by ATL146e.

MATERIALS AND METHODS

ATL146e was a gift from Jayson Rieger of Adenosine Therapeutics (Charlottesville, VA), and ZM241385 was a gift from Simon Poucher of AstraZeneca Pharmaceuticals (Cheshire, UK). Model 1003D minipumps were from Alza (Palo Alto, CA); glutamyl pyruvic transaminase (GPT) kit 505 was from Sigma (St. Louis, MO); blood collection tubes (cat. no. S22E-06) were from StatSpin (Norwood, MA); RNAzol B was from Leedo Medical Laboratories (Houston, TX); RiboQuant multiprobe RNAase protection systems were from TX); RiboQuant multiprobe RNAase protection systems were from

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BD Pharmingen; rat anti-mouse anti-neutrophil antibodies (MCA771G) were from Serotec; biotinylated rabbit anti-rat secondary antibody was from Vector Labs (Burlingame, CA); and peroxidase ABC elite kit was from Vectastain.

Marker-assisted genetic selection. Mice with the disrupted A2A AR gene (13) B6;129P-adora2a^{tm1jfc} were moved onto a C57BL/6 background by using 96 microsatellites for flanking the adora2a locus on chromosome 1. Male mice 10–12 wk of age and sex- and age-matched C57BL/6 mice (Hilltop) were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care according to National Institutes of Health guidelines. The University of Virginia Animal Care and Use Committee approved experimental procedures.

Surgical protocol and drug treatment. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Robinul-V (0.05 mg/kg), was delivered subcutaneously before the operation. Ambient temperature was controlled in the range of 24–26°C. Mice were placed on a 37°C heating pad. Core body temperature of some animals was monitored by using a TH-8 ThermaTel monitoring thermometer (Physitemp) and maintained at ±0.5°C of the widely used A2A-selective agonist CGS-21680 is 4.9 nM, or 24.5-fold higher than ATL146e. Figure 1A shows that a lower concentration of ATL146e attenuates liver I/R injury in radioligand binding assays. ATL146e is a highly selective agonist of the A2A AR (43). Inhibitor constant (K_i) values for the high-affinity concentration forms of human adenosine receptor subtypes are (in nM) 77 A1, 0.2 A2A, >100 A2B, and 45 A3. By comparison, the A2A K_i of the widely used A2A-selective agonist CGS-21680 is 4.9 nM, or 24.5-fold higher than ATL146e. Figure 1A shows the effect of ATL146e to inhibit liver I/R injury when administered for 24 h beginning at the time of reperfusion. Treatment consisting of an intraperitoneal loading dose of 1 µg/kg and a subcutaneously Alzet minipump-infused dose of 10 ng·kg^{-1}·min^{-1} for 24 h results in a 90% reduction in the liver injury marker serum GPT measured at 24 h and was used for subsequent experiments. This dose was chosen because infusion of ATL146e at 10 ng·kg^{-1}·min^{-1} has been shown to produce maximal protection of the kidney from I/R injury and to be devoid of any effects on heart rate or blood pressure (38). No additional liver protection was noted at higher doses (data not shown). Figure 1A shows that a lower concentration of ATL146e consisting of a loading dose of 1 µg/kg and an infused dose of 0.1 ng·kg^{-1}·min^{-1} also produced significant but submaximal protection. Figure 1B shows the time course of the effect of ATL146e to decrease serum GPT levels measured at various reperfusion times after 1 h of ischemia. Liver protection by ATL146e is evident within 3 h after reperfusion.
ZM241385 or deletion of the A2A AR gene prevents ATL146e-mediated liver protection. To confirm that the A2A AR mediates tissue protection by ATL146e, our first approach was to add an equimolar concentration of the A2A AR antagonist ZM241385. This compound is highly selective for the A2A AR compared with the A1 and A3 receptors and moderately selective (30-fold) compared with the A2B receptor (34). Figure 2A shows that ZM241385 effectively abolishes tissue protection by ATL146e. As further evidence that ATL146e acts through A2A ARs, we showed that ATL146e does not protect livers from I/R injury in A2A AR knockout mice (Fig. 2B). The C57BL/6 wild-type mice match the strain of mice lacking the A2A AR used in this study. The magnitude of I/R injury after 60 min of ischemia in vehicle-treated wild-type animals is not statistically greater than in knockout animals (Fig. 2B). This result is somewhat surprising, because we anticipated that endogenous adenosine produced in ischemic liver would exert some protective effect. To examine the possible protective role of endogenous adenosine further, we investigated the time course of injury in wild-type and A2A AR knockout animals. Figure 3 shows that wild-type animals are less injured by I/R injury than knockout animals after ischemia times of 30 or 40 min, but this difference dissipates and is not statistically significant by 50 or 60 min. We conclude that endogenous adenosine imparts some degree of protection from I/R injury in wild-type animals, but compared with the synthetic agonist ATL146e, endogenous adenosine produces a smaller and more transient protection.

Serum levels of GPT provide a relative measure of liver damage but do not provide a good sense of the absolute magnitude of injury. A more quantitative assessment of liver injury was provided by hematoxylin and eosin staining of liver 24 h after 1 h of liver ischemia. Necrotic tissue has a smooth pink appearance, whereas living tissue has a blue granular appearance. Figure 4 shows that 1 h of ischemia followed by 24 h of reperfusion causes severe liver necrosis (A and C) and confirms that ATL146e produces substantial protection from I/R injury in wild-type (B) but not in A2A AR knockout animals (D).

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1Although equimolar ZM241385 and ATL146e were placed in Alzet minipumps, it is likely that plasma levels of ZM241385 reach higher levels due to its slower metabolism. The use of equimolar doses of the two compounds in minipumps was based on the preliminary finding that this dosing regimen was sufficient to fully block A2A AR-mediated responses.
Effects of ATL146e on liver edema and MPO activity. Liver I/R injury is associated with tissue edema. As shown in Fig. 5A, I/R injury increases liver water content in vehicle-treated animals, and ATL146e significantly attenuates this edema ($P < 0.05$). We also examined MPO as a biochemical marker of neutrophils and macrophages. As shown in Fig. 5B, animals treated with ATL146e have much less MPO activity than vehicle-treated animals. We noted above that liver protection by ATL146e assessed by serum GPT levels is lost in A2A AR knockout animals. Figure 5 shows the same pattern of loss of protection in knockout animals based on the other parameters of liver injury, i.e., tissue edema and MPO activity. We also examined the time course of MPO accumulation during liver reperfusion after 1 h of ischemia (Fig. 6).
pared with the extent of MPO activity after 24 h of reperfusion, very little MPO is detected at 2 h, but even at this early time point, MPO is significantly elevated by 2.5-fold over the time 0 control (Fig. 6, inset). This could represent early margination of neutrophils to the walls of blood vessels during the first few hours of reperfusion injury. The pattern of neutrophil infiltration at 24 h after reperfusion is shown in Fig. 7. Neutrophil accumulation is more extensive in necrotic than in living tissue (Fig. 7A), and treatment with ATL146e reduces neutrophil accumulation particularly in living tissue (Fig. 7B). In liver of animals lacking the A2A AR, ATL146e has no effect on the extensive neutrophil accumulation seen even in living tissue (Fig. 7, C and D). Many neutrophils are located within small blood vessels as shown in Fig. 7D; these neutrophils may play a role in exacerbating reperfusion injury by inhibiting microvascular blood flow.

Effect of delaying treatment after reperfusion on liver protection by ATL146e. ATL146e gradually loses its ability to protect the liver from I/R injury (measured as GPT release at 24 h) when treatment is delayed after reperfusion (Fig. 8A). The effect of ATL146e to reduce neutrophil accumulation in the liver after I/R injury is lost over a similar time frame, as shown in Fig. 8B. Most liver protection is lost if treatment is delayed beyond 2 h. However, it is notable that if treatment is delayed for as much as 1 h, ATAL146e is still very effective at reducing I/R injury. These data indicate that protection by the A2A agonist is not limited to the first few minutes after reperfusion when there is a burst of oxygen radical production.

Effect of I/R injury on circulating leukocytes. Lymphopenia develops immediately following reperfusion after liver ischemia, and the blood lymphocyte count reaches its lowest level at 4 h (Fig. 9). Neutrophils accumulate in the blood within 2 h of I/R injury, and their numbers continue to increase for 8 h before declining. Accumulation of neutrophils may be due to release from the reperfused liver of cytokines that mobilize neutrophils from other tissues, such as bone marrow.

ATL146e suppresses chemokine and cytokine transcript induction in wild-type but not in A2A AR knockout mice. We next examined the expression (at 24 h after reperfusion) of different cytokine and chemokine transcripts in hepatic tissue by ribonuclease protection assays. As shown in Fig. 10, A–C, liver I/R injury causes induction of transcripts for the cytokines IL-10, IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, INF-γ, MIF, IL-6, INF-β, TGF-β, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1 and TCA-3. With the exception of MIF and possibly TGF-β1, the induction of all of these cytokine and chemokine transcripts is attenuated by ATL146e treatment. Little or no transcripts for

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**Fig. 6.** Time course of neutrophil infiltration of liver after I/R injury. Mice were subjected to liver ischemia for 1 h and reperfused for various times. Livers were removed and assayed for MPO activity. Each point is the mean ± SD of 6 animals. Inset, MPO activity at 0 and 2 h on an expanded scale; *P < 0.05 compared with time 0.

**Fig. 7.** Effects of liver I/R injury and ATL146e on histological evidence of liver neutrophil infiltration. Wild-type mice (A and B) or A2A AR knockout mice (C and D) were subjected to liver ischemia for 1 h and treated with vehicle (A and C) or ATL146e (B and D) for 24 h during reperfusion. Liver sections were immunostained for neutrophils, which appear dark brown. The sections are representative of 6 similar experiments.
IL-12p35, IL-12p40, LT-/H9251, LT-/H9252, TNF-/H9251, TGF-/H9252, TGF-/H9252 or eotaxin were detected 24 h after I/R injury. Certain of these transcripts, such as TNF-/H9251, may have peaked and returned toward baseline expression within 24 h. Interestingly, the RNase protection assay data show that in normoxic liver, levels of MCP-1 transcript are somewhat higher in A2A AR knockout mice than in wild-type mice (Fig. 10C). This expression of MCP-1 in A2A AR knockout animals could result in some constitutive chemoattraction into liver of monocytes, T cells, and natural killer (NK) cells.

DISCUSSION

Previous studies (2, 17) using the A2A AR agonist CGS-21680 suggest that activation of A2A AR protects rat liver from I/R injury. These conclusions are tempered by the fact that CGS-21680 has only limited adenosine receptor subtype selectivity, particularly over A3 AR, and the concentration of the compound in various tissue compartments was not determined. Here, we utilize a more selective agonist, ATL146e, as well as a selective A2A AR antagonist, ZM241385, and deletion of the A2A AR gene to show convincingly that A2A AR activation during reperfusion reduces by as much as 90% murine liver damage from I/R injury. ATL146e attenuates liver damage and inflammation as assessed by serum GPT, edema, MPO, histology, immunohistochemistry, and reduced induction of proinflammatory cytokine and chemokine transcripts. A2A agonist treatment during reperfusion can be delayed for up to 1 h with little attenuation of protection. This suggests that A2A agonist-mediated protection occurs downstream of oxygen radical production that occurs early after reperfusion (Fig. 11).

It has been shown recently that endogenous adenosine, by activating A2A AR, can reduce injury in response to hepatic toxins (36). Here we show that endogenous adenosine also produces some protection from liver I/R injury. This observation is consistent with the idea that endogenous adenosine is part of an innate mechanism to minimize tissue inflammation and injury. Compared with endogenous adenosine, ATL146e was found to produce much greater protection. This may be due in part to the greater stability of ATL146e than adenosine in blood, resulting in its greater accessibility to receptors on blood cells and vascular endothelial cells. Also, endogenous adenosine produced in the ischemic liver may rapidly dissipate during reperfusion and may have proinflammatory effects by activation of A1 receptors on neutrophils (15) and A3 receptors on mast cells (20).

ATL146e reduces I/R injury in tissues other than liver, including kidney, skin (40), and spinal cord. Although high concentrations of A2A agonists produce vasodilation by acting on receptors on vascular smooth muscle, the doses of ATL146e

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**Fig. 8.** Effect of delaying ATL146e administration on liver I/R injury. Wild-type mice were subjected to liver ischemia for 1 h and treated after various times of delay after the start of 24 h of reperfusion with ATL146e. Serum GPT (A) and tissue MPO activity (B) were measured. Each bar is the mean ± SD of 6 animals; *P < 0.05 compared with vehicle.

**Fig. 9.** Time course of changes in peripheral blood cells during reperfusion after liver ischemia. C57BL/6 mice were subjected to liver ischemia for 1 h. Animals were killed at various times after the start of reperfusion to collect peripheral blood and measure lymphocyte number (A) and neutrophil number (B). Each point is the mean ± SD of 9 animals.
found to produce tissue protection in previous studies are well below those required to change blood pressure. In the present study, we confirm that A2A AR-mediated protection from I/R injury is conferred during reperfusion rather than during ischemia. These findings are consistent with the hypothesis that A2A AR activation protects tissues from I/R injury resulting from inflammation initiated by a burst of oxygen free radicals that occurs at the time of reperfusion.

Figure 11 shows a hypothetical scheme of the sequence of events leading from reperfusion to tissue necrosis that is supported by results of this and previous studies. A2A AR activation may inhibit several steps in this scheme. Chemokines are induced during reperfusion by reactive oxygen species. In a murine myocardial I/R injury model, free radical scavengers have been demonstrated to preserve myocardial function only when administered before or immediately after reperfusion. This is probably because a free radical burst occurs during the first few minutes after reperfusion (6, 10, 49). In transgenic mice overexpressing free radical scavenging glutathione peroxidases, there is a reduction of chemokine expression during renal I/R injury (19). Moreover, oxygen free radicals directly elicit chemokine production during the first two hours of liver reperfusion in a cytokine-independent manner (3, 7, 26). These data suggest that there is a link between I/R injury and induction of inflammatory chemokines.

Most investigators agree that neutrophils contribute to I/R injury. If the primary target of A2A AR activation is the neutrophil per se, then ATL146e would be expected to produce protection when it is delivered 4–8 h after reperfusion, at a time before a large increase in MPO activity in the reperfused tissue (Fig. 6). In fact, ATL146e exerts little or no protection if treatment is delayed for 4 h after the start of reperfusion. Hence, we conclude that ATL146e probably suppresses early inflammatory events that precede the recruitment of large numbers of cells that contain MPO.
Our RNase protection assay data show that MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and IP-10 are all upregulated 24 h after reperfusion injury. Most of the upregulated chemokines are chemotactic to neutrophils and monocytes (25, 39, 46). Possible cellular sources of chemokines are platelets, vascular endothelium, dendritic cells, tissue resident mast cells, macrophages, neutrophils, T and B lymphocytes, and/or hepatocytes (1, 35). Chemokines may trigger the expression of adhesion molecules on vascular endothelium and circulating platelets and leukocytes. For example, P-selectin and ICAM-1 are expressed on the microvascular endothelium after renal I/R injury and the expression of these adhesion molecules is inhibited by ATL146c (37). A2AR agonists also may inhibit adhesion of inflammatory cells to the endothelium via receptors that have been demonstrated on platelets, T cells, monocytes/macrophages, and neutrophils (27). Other mechanism(s) by which A2AR activation may reduce I/R injury are by direct effects on vascular smooth muscle cells or hepatocytes.

Inhibition of CXC chemokine production from liver macrophages (Kupffer cells) decreases the degree of tissue damage from liver I/R injury (33, 50). Neutralization of MIP-2 was found to protect brain and kidney from I/R injury (31, 48), and neutralization of MCP-1 protects heart and brain (8, 22). Upregulation of some of these chemokine transcripts may occur in response to free radicals produced during reperfusion or secondary to induction or proinflammatory cytokines. For example, TNF-α (29), IL-1β (30), and IFN-γ (23) have been reported to elicit release of chemokines from many cell types. It may be informative in future experiments to carefully explore the kinetics of chemokine and cytokine gene induction and to investigate the consequences of genetic deletion of certain of these inflammatory mediators.

It has long been thought that T cells are recruited in late-stage (48–72 h) but not early-stage (24 h) reperfusion injury. However, recent studies (21, 41, 47) demonstrate recruitment of small numbers of T lymphocytes that participate in early I/R injury. RANTES has been proposed as a major mediator of antigen-independent T lymphocyte activation. RANTES can directly initiate T lymphocyte signaling, initially via a G protein-coupled pathway and later via activation of a tyrosine kinase pathway (4, 5). Another chemokine transcript we found to be elevated after liver I/R injury, IP-10, is induced by INF-γ and attracts activated T lymphocytes and NK cells by binding to CXCR3 receptors (16). Hydrogen peroxide generated during reperfusion activates macrophages and T lymphocytes by inhibiting tyrosine phosphatases (42). On the basis of our results, it is reasonable to hypothesize that A2A receptor stimulation inhibits the activation of T lymphocytes and/or macrophages that participate in I/R injury-induced chemokine induction during reperfusion before the recruitment of large numbers of monocytes and neutrophils (Fig. 11).

ACKNOWLEDGMENTS

We thank Jiang-Fan Chen of Boston University for mice lacking adora2a and Rob Pursley for his help with the preparation of this manuscript.

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