

Additive protection against lung ischemia-reperfusion injury by adenosine A_{2A} receptor activation before procurement and during reperfusion

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Objective: Adenosine A_{2A} receptor activation during reperfusion improves lung ischemia-reperfusion injury. In this study we sought to determine whether pretreatment of rabbits with a potent and selective adenosine A_{2A} receptor agonist, ATL-313, before transplantation or whether adding ATL-313 to the preservation solution results in equivalent or additional protection compared with ATL-313 added during reperfusion.

Methods: An isolated, ventilated, ex vivo blood-perfused rabbit lung model was used. All groups underwent 2 hours of reperfusion after 18 hours of cold ischemia (4°C). ATL-313 was administered 1 hour before ischemia intravenously, with the preservation solution, and/or during reperfusion.

Results: Both pretreatment of donor animals with ATL-313 or adding ATL-313 just during reperfusion improved pulmonary function, but significantly greater improvement was observed when pretreatment and treatment during reperfusion were combined (all $P < .05$). Myeloperoxidase levels, bronchoalveolar lavage tumor necrosis factor α levels, and pulmonary edema were all maximally decreased in the combined treatment group. The administration of an equimolar amount of the potent and highly selective adenosine 2A receptor antagonist, ZM 241385, along with ATL-313, resulted in the loss of protection conferred by ATL-313.

Conclusions: Adenosine A_{2A} receptor activation with ATL-313 results in the greatest protection against lung ischemia-reperfusion injury when given before ischemia and during reperfusion. Improved pulmonary function observed with adenosine A_{2A} receptor activation was correlated with decreased bronchoalveolar lavage tumor necrosis factor α and decreased lung myeloperoxidase. The loss of protection observed with the concurrent administration of the adenosine A_{2A} receptor antagonist, ZM 241385, supports that the mechanism of ATL-313 protection is specifically mediated via adenosine A_{2A} receptor activation.

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Although the yearly number of lung transplants worldwide has increased 130-fold over the past 20 years, critical shortages in the donor pool exist, and survivals remain low.¹ In most series, 30-day mortality rates are close to 15%, and approximately 33% of patients who receive a lung transplant die within 3 years.^{2,3} Lung ischemia-reperfusion injury (LIRI) after lung transplantation continues to be one of the most common and significant causes of morbidity and mortality and has been found to increase the risk of bronchiolitis obliterans.⁴ The 30-day mortality of recipients who have reperfusion injury is as high as 40% compared with 7% in patients without graft failure.^{2,4} Improvements in the care of patients with end-stage lung disease hinge on the ability to attenuate the robust inflammatory response characteristic of LIRI.

Abbreviations and Acronyms

A _{2A} R	= A _{2A} receptor
ANOVA	= analysis of variance
BAL	= bronchoalveolar lavage
ELISA	= enzyme-linked immunosorbent assay
IR	= ischemia-reperfusion
LIRI	= lung ischemia-reperfusion injury
MPO	= myeloperoxidase
PA	= pulmonary artery
TNF- α	= tumor necrosis factor α

Adenosine potentially plays a critical role in minimizing ischemia-reperfusion (IR) injury inasmuch as it is known to confer protection against IR in the lungs, heart, liver, and kidney secondary to its widely described anti-inflammatory effects.^{5,6} The adenosine A_{2A} receptor (A_{2A}R), one of four subtypes of the G protein-coupled adenosine receptor family that includes A₁, A_{2A}, A_{2B}, and A₃, has been associated with many anti-inflammatory properties. Adenosine receptor subclassification has shown specifically that activation of the A_{2A}R and resultant increases in cyclic adenosine monophosphate prevent leukocyte adhesion to endothelial cells as well as inhibiting the release of toxic oxygen products and inflammatory cytokines.^{5,6} The A_{2A}R is predominantly expressed on inflammatory cells including neutrophils, mast cells, macrophages, monocytes, and platelets.⁷ Current evidence supports diverse mechanisms of IR injury and complex interactions between the aforementioned inflammatory cells.

Whereas the anti-inflammatory and tissue-protective effects of A_{2A}R activation during reperfusion are well documented, protective effects of activating the A_{2A}R in donor animals before transplantation have not been reported. Our group has previously shown that A_{2A}R activation during rabbit lung reperfusion reduces IR injury after lung transplantation.^{8,9} Pretreatment of donor lung before ischemia, however, may confer better protection. Brief periods of ischemic insult before prolonged ischemia, commonly referred to as ischemic preconditioning, protect against IR injury and can be pharmacologically manipulated through activation of its key mediators.¹⁰ Growing evidence supports the emerging role of adenosine as one of these key mediators as the accumulation of adenosine from the breakdown of adenosine triphosphate is a natural method of protection against ischemia and inflammation.^{11,12} In this study we examined the effect of pretreatment of donor animals with the highly selective A_{2A}R agonist, ATL-313, on the development of LIRI. We hypothesized that activation of A_{2A}R in the lung before ischemia might lead to enhanced protection compared with A_{2A}R activation during reperfusion alone.

Materials and Methods**Animal Care**

New Zealand White rabbits (Burlson Enterprises, Inc, Unionville, Va) of both sexes (3.0–3.5 kg) were used for all studies and received humane care in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication No.85-23, revised 1995). The Animal Care and Use Committee at the University of Virginia reviewed and approved the protocol for this study before experimentation.

Experimental Protocol

Seven experimental groups (n = 6/group, based on our most recent study¹³) were compared using an isolated, whole blood-perfused, ventilated rabbit lung model (model TIS3862; Kent Scientific, Litchfield, Conn). All groups were reperfused for 120 minutes after 18 hours of cold ischemia at 4°C (see Table 1 for the experimental protocol for each group). We used 4-(3-[6-amino-9-(5-cyclopropylcarbonyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl)-piperidine-1-carboxylic acid methyl ester, ATL-313, a gift from Adenosine Therapeutics, LLC (Charlottesville, Va), as a potent, selective activator of the A_{2A}R, and 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol, ZM 241385 (Tocris, Ellisville, Mo), as a selective A_{2A}R antagonist. ATL-313 and ZM 241385 were dissolved in saline. Lung donors undergoing pretreatment received an intravenous injection of the drug (ATL-313 [100 nmol/L] or ATL-313 [100 nmol/L] + ZM 241385 [100 nmol/L] [*note: drug concentrations are final concentrations in blood determined by calculations based on blood volume/kilogram*]) 1 hour before harvest. When given during reperfusion (ATL-313 [100 nmol/L] or ATL-313 [100 nmol/L] + ZM 241385 [100 nmol/L]), the respective drugs were administered to whole blood at the beginning of reperfusion. In the group receiving ATL-313 in the lung preservation solution, the ATL-313 was added to the preservation solution at 100 nmol/L. The chosen dose of A_{2A}R agonists and antagonists was based on previous experiments.¹⁴⁻¹⁷

Harvest procedure. New Zealand White rabbits of both sexes (3.0–3.5 kg) were randomly assigned to 7 experimental groups. Intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg) was used to anesthetize each animal. Tracheal intubation was performed via a tracheostomy and mechanical ventilation (model RSP1002; Kent Scientific, Litchfield, Conn) was instituted with room air at a respiratory rate of 30 breaths/min. A median sternotomy and thymectomy were performed. After the pericardium was opened, the pulmonary artery (PA) and the aorta were dissected free and encircled. A purse-string suture was then placed in the right ventricular outflow tract and intravenous heparin was administered (1000 U/kg). The PA was then injected with 30 μ g of prostaglandin E₁, and the venae cavae were ligated to initiate ischemia 5 minutes after heparin administration. The PA was then cannulated through a right ventriculotomy. After the left ventricle was vented through a left ventriculotomy and the aorta was ligated, 100 mL/kg of Perfadex (Vitrolife, Kungsbacka, Sweden) preservation solution was infused into the PA at 30 cm H₂O pressure at 4°C. Topical cooling was achieved with cold saline solution slush. The left atrium was cannulated through the left ventriculotomy

TABLE 1. Summary of study protocol (n = 6 per group)

Group	Treatment	Method of treatment			End points studied
		Pretreat donor animal	Pretreat preserved lung (flush)	During reperfusion	
1	None				PA pressure, pulmonary compliance, lung histology, MPO activity, TNF- α , wet/dry weight
2	ATL313	✓			
3	ATL313		✓		
4	ATL313			✓	
5	ATL313	✓		✓	
6	ZM 241385 + ATL313	✓			
7	ZM 241385 + ATL-313	✓		✓	

PA, Pulmonary artery; MPO, myeloperoxidase; TNF- α , tumor necrosis factor α .

with an outflow catheter. The lung–heart block was excised. The lungs were then inflated and stored at 4°C for 18 hours.

Reperfusion procedure. After harvest, the lung–heart block was suspended from a force transducer and ventilation was initiated with a gas mixture of 95% oxygen and 5% carbon dioxide (model RSP1002, Kent Scientific). All groups underwent 120 minutes of whole-blood perfusion. Atelectasis was grossly eliminated by administering one breath of approximately 30 cm H₂O positive end-expiratory pressure once per minute in the first 5 minutes of the stabilization period. Lungs were ventilated at a constant tidal volume of 10 mL/kg with 3 cm H₂O of positive end-expiratory pressure at a rate of 30 breaths/min. The PA and the outflow catheters connected the lung–heart block to a venous blood reperfusion circuit. New Zealand White rabbits served as fresh venous blood donors. Blood was circulated through a pediatric oxygenator set to deoxygenate the blood and add carbon dioxide to simulate venous blood (Po₂ = 60 mm Hg/PCO₂ = 60 mm Hg). The lungs were subsequently perfused via the PA cannula at 60 mL/min with “venous” blood at 37°C.

Physiologic Parameters

Recordings of PA pressure and compliance were collected by a dynamic data acquisition program (DASYLab, DASYTEC, USA, Bedford, NH). Pulmonary venous blood samples were collected for blood gas analysis (Bayer 348 pH/Blood Gas Analyzer; Bayer Corp, E Walpole, Mass) at 15, 30, 60, 90, and 120 minutes after initiation of reperfusion.

Lung Wet/Dry Weight Ratio

Lung wet/dry weight ratios were used as a measure of pulmonary edema. Samples of right lower lobe lung tissue were blotted and weighed immediately after 120 minutes of reperfusion. These samples were desiccated under vacuum at 55°C until a stable dry weight was achieved.

Lung Tissue Myeloperoxidase

Myeloperoxidase (MPO) assay was performed on lung tissue to quantify neutrophil sequestration as previously described.¹⁷

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed on all lungs after the reperfusion period ended. The right upper and middle lobes were isolated and lavaged with 10 mL of normal saline. The BAL fluid was then centrifuged at 1500g for 5 minutes at 4°C. The supernatant was snap-frozen for subsequent cytokine analysis.

Enzyme-linked Immunosorbent Assay

The protein levels of tumor necrosis factor α (TNF- α) in BAL fluid were examined with a TNF- α enzyme-linked immunosorbent assay (ELISA) purchased from BD Biosciences (San Diego, Calif) and performed according to manufacturer directions. Samples were run in triplicate.

Lung Injury Score

A blinded pathologist graded each lung sample after appropriate tissue processing and staining (hematoxylin and eosin). Each sample was graded on the basis of the number of macrophages, amount of interstitial infiltrate, and percentage of alveolae affected by fibrin deposition. Each of these three categories was then given a score of 0 to 3, resulting in a possible score ranging from 0 for uninjured lungs to 9 for the most severely injured lungs.

Statistics

Values were expressed as the mean \pm standard deviation. Analysis of variance (ANOVA) was used to determine whether significant differences existed between groups. The Tukey honest significant difference multiple-comparison test was used to determine which groups were significantly different when the ANOVA results were significant. Repeated-measures analysis of variance was performed and ultimately allowed us to conclude that PA pressure, lung compliance, and oxygenation change over time and depend on group. The test for between-subject effects was significant, which implies significance in the aforementioned variables between groups.

Results

Physiologic Measurements

Pretreatment of donor lungs with ATL-313 and the administration of ATL-313 during reperfusion (group 5) resulted in

TABLE 2. Lung function

Group	PA pressure	15 min	30 min	60 min	90 min	120 min
1	Control	28.3 ± 1.0†‡§	25.7 ± 1.4†‡§	25.0 ± 0.6†‡§	26.7 ± 1.2†‡§	32.8 ± 1.2†‡§
2	Pretreatment (ATL 313)	19.0 ± 1.8*	16.3 ± 1.0*	19.0 ± 1.0*§	19.5 ± 1.0*	22.2 ± 1.2*†
3	Flush (ATL 313)	27.3 ± 1.0†‡§	25.8 ± 1.0†‡§	24.8 ± 0.9†‡§	27.3 ± 0.8†‡§	31.2 ± 1.4†‡§
4	Reperfusion (ATL 313)	21.2 ± 1.2*§	16.0 ± 1.4*	16.8 ± 1.7*	17.5 ± 1.2*	18.5 ± 1.5*†
5	Pretreatment and reperfusion (ATL 313)	16.7 ± 1.2*†	14.2 ± 1.7*	16.5 ± 1.0*†	17.7 ± 0.8*	19.8 ± 1.9*
6	Pretreatment (ATL 313 + ZM 243185)	27.2 ± 2.8†‡§	26.5 ± 2.6†‡§	26.8 ± 2.3†‡§	29.3 ± 2.3†‡§	31.6 ± 2.1†‡§
7	Pretreatment and reperfusion (ATL 313 + ZM 243185)	26.8 ± 1.5†‡§	25.0 ± 0.9†‡§	24.2 ± 1.2†‡§	25.2 ± 1.2*†‡§	29.3 ± 1.9†‡§
Group	Pulmonary compliance	15 min	30 min	60 min	90 min	120 min
1	Control	6.42 ± 0.09†‡§	5.93 ± 0.10†‡§	5.58 ± 0.12†‡§	5.30 ± 0.14†‡§	4.93 ± 0.08†‡§
2	Pretreatment (ATL 313)	6.90 ± 0.14*§	6.62 ± 0.09*§	6.42 ± 0.13*§	6.18 ± 0.17*§	5.46 ± 0.16*§
3	Flush (ATL 313)	6.43 ± 0.08†‡	5.88 ± 0.08†‡	5.70 ± 0.11†‡	5.43 ± 0.10†‡	5.07 ± 0.08†‡
4	Reperfusion (ATL 313)	6.82 ± 0.12*	6.63 ± 0.10*	6.38 ± 0.13*§	6.18 ± 0.18*§	5.87 ± 0.20*§
5	Pretreatment and reperfusion (ATL 313)	6.95 ± 0.19*	6.85 ± 0.10*†	6.70 ± 0.09*†	6.58 ± 0.13*†‡	6.25 ± 0.15*†‡
6	Pretreatment (ATL 313 + ZM 243185)	6.20 ± 0.24†‡§	5.85 ± 0.22†‡§	5.55 ± 0.32†‡§	5.28 ± 0.35†‡§	5.08 ± 0.33†‡§
7	Pretreatment and reperfusion (ATL 313 + ZM 243185)	6.40 ± 0.14†‡§	5.82 ± 0.12†‡§	5.68 ± 0.13†‡§	5.45 ± 0.11†‡§	5.12 ± 0.12†‡§
Group	Pao ₂	15 min	30 min	60 min	90 min	120 min
1	Control	524.3 ± 9.1†§	550.8 ± 11.1†‡§	552.5 ± 7.9†‡§	539.5 ± 6.7†‡§	529.7 ± 2.7†‡§
2	Pretreatment (ATL 313)	578.7 ± 10.8*†‡§	600.5 ± 16.9*§	583.8 ± 11.9*§	580.2 ± 8.0*§	563.8 ± 7.7*§
3	Flush (ATL 313)	525.5 ± 8.5†‡§	547.2 ± 9.3†‡§	553.5 ± 7.4†‡	537.7 ± 9.9†‡§	527.0 ± 5.1†‡§
4	Reperfusion (ATL 313)	550.5 ± 7.7†§	584.2 ± 6.7*§	581.0 ± 5.8*§	575.2 ± 5.4*§	569.2 ± 2.8*§
5	Pretreatment and reperfusion (ATL 313)	669.7 ± 20.3*†‡	695.8 ± 15.7*†‡	686.3 ± 12.9*†‡	663.7 ± 20.0*†‡	655.7 ± 13.7*†‡
6	Pretreatment (ATL 313 + ZM 243185)	532.7 ± 29.2†§	545.7 ± 31.7†‡§	547.3 ± 32.9†‡§	534.0 ± 35.1†‡§	521.8 ± 38.2†‡§
7	Pretreatment and reperfusion (ATL 313 + ZM 243185)	525.7 ± 8.0†§	550.3 ± 10.6†‡§	553.7 ± 10.6†‡§	541.7 ± 6.3†‡§ ^b	525.3 ± 7.1†‡§

PA, Pulmonary artery; Pao₂, arterial oxygen saturation. Data are reported at each time point as mean ± standard deviation. All ANOVA *P* values = .000. Pairwise comparisons between specific groups (Tukey honest significant difference test): **P* < .05 versus control; †*P* < .05 versus pretreatment (ATL-313); ‡*P* < .05 versus reperfusion (ATL-313); §*P* < .05 versus pretreatment and reperfusion (ATL-313).

significant decreases in PA pressure compared with all groups (*P* < .05) except group 4 (ATL-313, reperfusion; *P* = .32). No significant differences in PA pressure were observed between the administration of ATL-313 during reperfusion alone

(group 4) versus ATL-313 pretreatment alone (group 2), although these interventions did result in significant decreases in PA pressure versus control (*P* < .01). Significant increases in lung compliance and oxygenation were observed with pretreat-

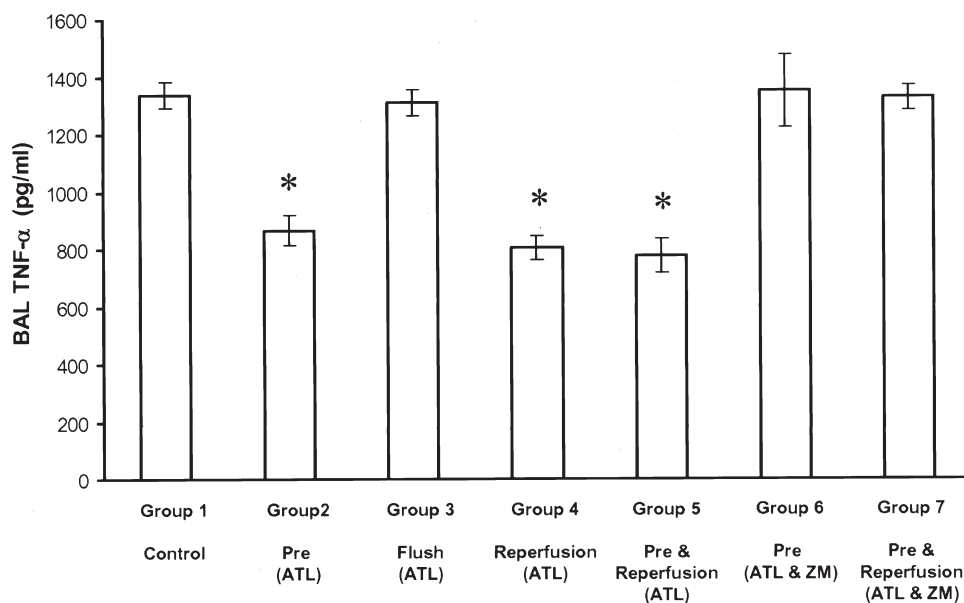


Figure 1. BAL TNF- α levels. All groups were compared with control. Lowest TNF- α levels were observed in group 5. * $P < .01$.

ment of donor lungs with ATL-313 and the concurrent administration of ATL-313 during reperfusion (group 5) versus all other groups (all $P < .05$). The concurrent administration of ATL-313 with its antagonist, ZM 241385, nullified the physiologic effects of ATL-313 pretreatment and its administration with reperfusion. Pairwise comparisons between groups at specific time points (ie, at 15, 30, 60, 90, and 120 minutes) are found in Table 2.

ELISA for TNF- α

ELISA was used to evaluate the effect of the timing of ATL-313 administration on the quantity TNF- α in BAL fluid (Figure 1). ATL-313 pretreatment and its concurrent administration during reperfusion (group 5) resulted in the greatest decrease in TNF- α levels (42%) versus control ($P < .01$). This decrease in TNF- α was abolished by the simultaneous administration of ZM 241385 (group 7). Pretreatment with ATL-313 alone (group 2) and ATL-313 treatment during reperfusion alone (group 4) resulted in a 35% and 40% decrease in TNF- α levels, respectively, compared with control (both $P < .01$). The administration of ATL-313 in the preservation solution (group 3) did not affect TNF- α levels versus control ($P = .99$).

MPO Activity

MPO activity was used as an indicator of lung tissue neutrophil sequestration (Figure 2). ATL-313 pretreatment and concomitant ATL-313 treatment during reperfusion (group 5) led to a 48% reduction in MPO activity compared with control ($P < .01$). ATL-313 pretreatment alone (group 2) or

administration during reperfusion alone (group 4) reduced MPO levels (39% and 44%, respectively) versus control ($P < .01$), although no significant differences were seen when these groups were compared with group 5 (concurrent administration of ATL-313 during pretreatment and reperfusion). The decrease in MPO activity observed with ATL-313 pretreatment and with its concurrent administration during reperfusion was blocked by the administration of the A_{2A}R antagonist, ZM 241385.

Wet/Dry Analysis

Wet/dry ratios were examined as an indicator of pulmonary edema (Figure 3). Pretreatment of donor lungs with ATL-313 (group 2) resulted in a 28% reduction in the wet/dry ratio versus control ($P < .01$). Compared with control, ATL-313 treatment during reperfusion alone (group 4) reduced the wet/dry ratio by 32% ($P < .01$). ATL-313 administration during reperfusion along with pretreatment (group 5) led to the greatest reduction in wet/dry ratio versus control (37%; $P < .01$). ATL-313 was protective against the development of pulmonary edema when given before ischemia and/or with reperfusion. This protective effect was lost with the concurrent administration of the A_{2A}R antagonist, ZM 241385. The addition of ATL-313 to the preservation solution (group 3) failed to significantly reduce edema.

Lung Histologic Condition

Overall lung histologic condition (Figure 4) was most improved and lung injury severity score (Table 3) most de-

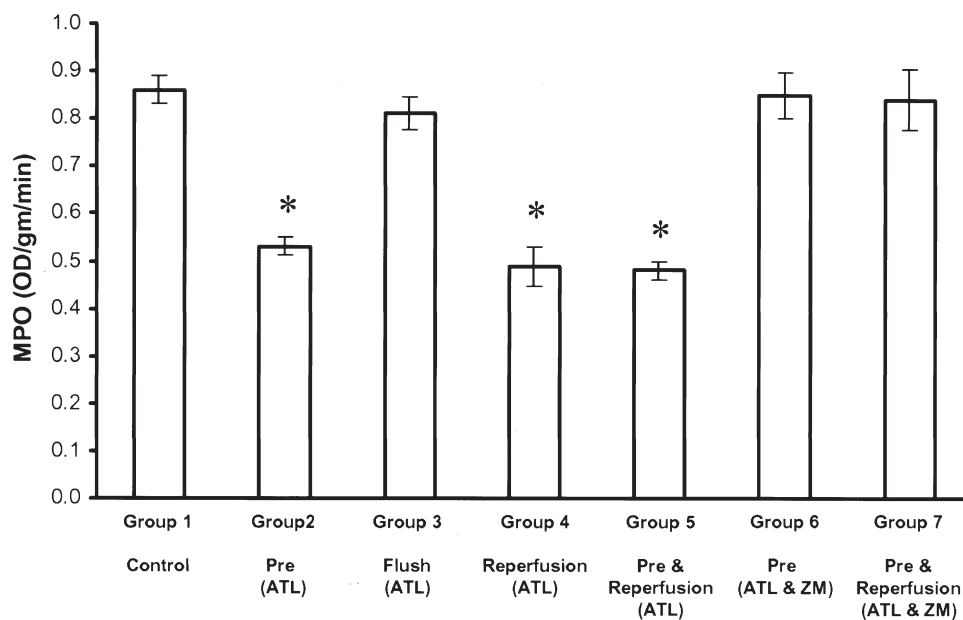


Figure 2. Lung tissue MPO. All groups were compared with control. Lowest MPO activity was observed in group 5. * $P < .01$. OD, Optimal dose.

creased in group 5 (lungs pretreated with ATL-313 and also treated during reperfusion) compared with all other groups including group 2 (ATL-313 pretreatment) and group 4 (ATL-313 treatment during reperfusion) (all $P < .05$). Al-

though the total lung injury severity score was not different between group 2 (ATL-313 pretreatment) and group 4 (ATL-313 treatment during reperfusion), pretreatment (group 2) significantly decreased the number of macro-

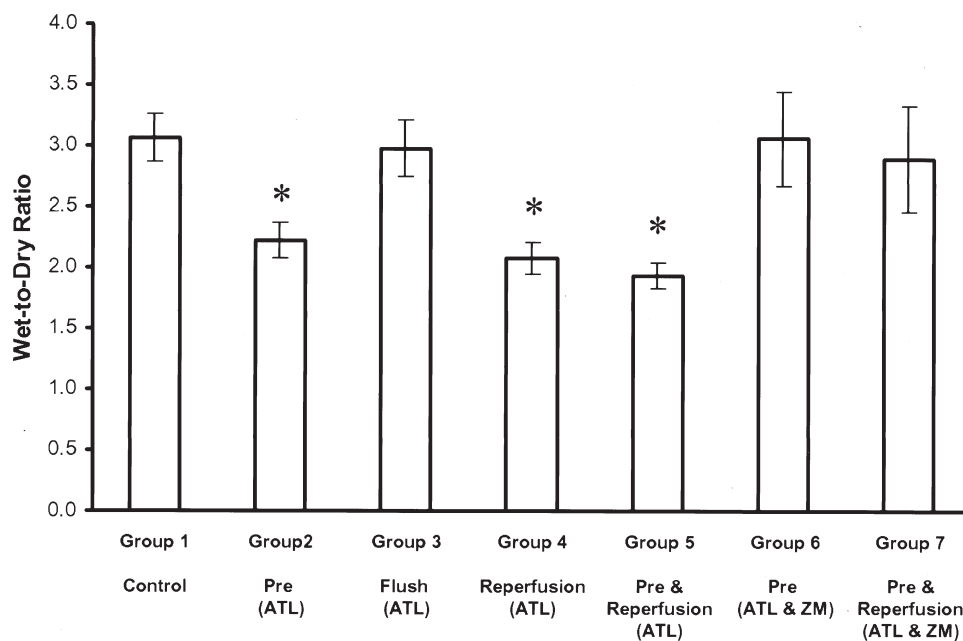


Figure 3. Wet/dry analysis. All groups were compared with control. Lowest wet/dry ratio was observed in group 5. * $P < .01$.

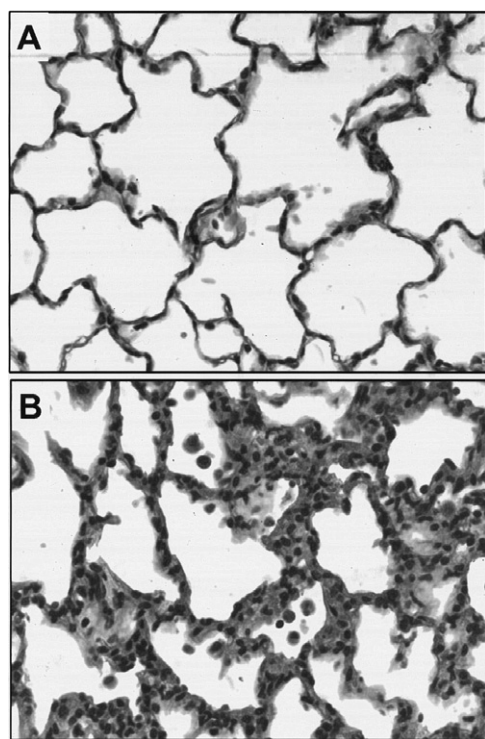


Figure 4. Representative hematoxylin–eosin sections of lung tissue. A, Group 5 (ATL-313 pretreatment and administration during reperfusion). B, Group 1 (control).

phages in the alveolar space compared with treatment during reperfusion alone (group 4). The severity of interstitial infiltrate, however, was similar between these two groups. The administration of the A_{2A}R antagonist, ZM 241385, simultaneously with ATL-313 during pretreatment (group 6) and during pretreatment and reperfusion (group 7) resulted in similar scores compared with control (all *P* > .05) as did the administration of ATL-313 with the preservation solution (data not shown).

Discussion

Adenosine, widely known for its anti-inflammatory properties, exerts a protective role against the development of ischemia-induced cell injury.^{5,6} Activation of the A_{2A}R subtype has been specifically associated with protection against the development of LIRI.^{8,18} Studies to date have revealed the benefits of pharmacologic A_{2A}R activation during lung reperfusion associated with inhibition of inflammation, but have not previously examined the effect of treating the host animal with an A_{2A} agonist before tissue transplantation. Growing evidence supports the concept that ischemic preconditioning, brief periods of ischemic insult before prolonged ischemia, protects against IR injury in various organ systems. The protection resulting from ischemic preconditioning is associated with increased levels of extracellular adenosine and thus provides the concept that the pharmacologic activation of adenosine receptors before ischemia may confer protection against LIRI.¹¹ It is notable, however, that ischemic preconditioning has been reported to be due to activation of A₁, A_{2B}, or A₃ receptors, and not A_{2A} receptors. This is the first study to our knowledge that specifically investigates the effect of A_{2A}R activation before lung ischemia.

Our study demonstrates that A_{2A}R activation before lung ischemia confers significant protection against the deterioration of lung function seen during reperfusion. Improved lung physiology seen with ATL-313 pretreatment correlated with significantly decreased levels of the potent proinflammatory cytokine, TNF-α, pulmonary edema, neutrophil sequestration (decreased MPO), and preserved lung histology. Not surprisingly, A_{2A}R activation during reperfusion also provided significant protection against LIRI.^{8,19} More important, clinically relevant and additive improvements in oxygenation and lung compliance were seen with the concomitant administration of ATL-313 before lung harvest and with reperfusion versus ATL-313 pretreatment alone or administration during reperfusion alone. The loss of protection observed with the administration of the A_{2A}R antagonist, ZM 241385, with ATL-313 indicates that the mecha-

TABLE 3. Lung severity score

Group		Interstitial infiltrate	Macrophages	Fibrin	Total score
1	Control	2.17 ± 0.52*	2.67 ± 0.51*	1.58 ± 0.66*	6.42 ± 1.24*
2	Pretreatment (ATL 313)	1.33 ± 0.26*	0.92 ± 0.21*†	0.08 ± 0.21	2.5 ± 0.45*
4	Reperfusion (ATL 313)	1.08 ± 0.21	1.67 ± 0.51*†	0.17 ± 0.40	2.92 ± 0.92*
5	Pretreatment and reperfusion (ATL 313)	0.92 ± 0.20	0.08 ± 0.21	0.0 ± 0.0	1.0 ± 0.44

P < .05 in all pairwise comparisons versus control; **P* < .05 in pairwise comparison versus group 5; †*P* < .05 in pairwise comparison of group 2 versus group 4.

nism of ATL-313 protection is specifically mediated via $A_{2A}R$ activation. Since $A_{2A}R$ activation is known to inhibit the activation of leukocytes, the protection afforded by pretreating rabbits with ATL-313 may be due to inhibition of resident leukocytes in the transplanted lung, whereas the protection observed during reperfusion is likely due to reduced adhesion and extravasation of leukocytes in blood or reperfused lung. Of note, the temperature (4°C) of the preservation solution may account for the lack of effect of ATL-313 in this solution.

This study also highlights the emerging therapeutic role of simulating preconditioning pharmacologically by exploiting different mediators of protection. The use of adenosine and $A_{2A}R$ agonists, such as ATL-313, is a logical extension of many intrinsic defense mechanisms inasmuch as adenosine is known to accumulate in response to ischemia and hypoxia.²⁰ Whereas the exact mechanisms of protection are complex and poorly understood, increased levels of adenosine before a sustained ischemic event have been shown to improve end-organ function in the liver,²¹ kidney,²² and heart,²³ although some of these effects are not mediated by $A_{2A}R$ activation. Treatment with $A_{2A}R$ agonists has also been associated with inhibition of inflammatory cytokine release, reduction of IR-induced apoptotic injury, and diminution of free radical production.^{18,24} In an isolated, buffer-perfused rat lung model, Yildiz and colleagues²⁵ demonstrated that adenosine “preconditioning” conferred similar protection against IR-induced pulmonary vasoconstrictor dysfunction, edema, and lipid peroxidation as did their ischemic preconditioning protocol. Moreover, the administration of the nonselective adenosine receptor antagonist, theophylline, abolished the protective effects of ischemic preconditioning, thereby implying adenosine’s critical role as a mediator of preconditioning. It has not yet been determined whether a selective $A_{2A}R$ antagonist such as ZM 241385 will block all of the preconditioning effects of adenosine. Nitric oxide has also been described as a protective mediator in preconditioning, decreasing LIRI with 10 minutes of nitric oxide pretreatment at 15 ppm. Limitations of the use of adenosine and nitric oxide per se as preconditioning agents are their cardiovascular side effects. Interestingly, $A_{2A}R$ activation has also been associated with enhanced production of nitric oxide in cultured coronary artery cells.²⁶

The role of inflammatory cells in the pathogenesis of IR injury is unquestioned. A greater body of evidence further supports the emerging role of donor alveolar macrophages in early LIRI and its subsequent priming of other inflammatory cells such as neutrophils.²⁷ Adenosine has also been shown to decrease not only the release of TNF- α and other proinflammatory cytokines from macrophages but also the activation of neutrophils from TNF- α released from macrophages.²⁸ Moreover, $A_{2A}R$ activation also directly inter-

feres with neutrophil-induced IR injury. Treatment of rats with the $A_{2A}R$ agonist, ATL-146e, resulted in decreased expression of the adhesion molecules P-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 in renal²⁹ and myocardial³⁰ IR models. $A_{2A}R$ activation was shown to decrease the release of neutrophil elastase and cytokine-induced neutrophil chemoattractant, an effect that was blocked with the administration of the $A_{2A}R$ antagonist, ZM 241385. Again, the administration of ATL-313 in the current study did significantly attenuate TNF- α levels in BAL fluid, although the specific sources of TNF- α release were not examined. Interestingly, ATL-313 pretreatment resulted in a significant decrease in the number of macrophages seen on histologic examination compared not only with control but also with the administration of ATL-313 during reperfusion alone and may account, in part, for the decreased levels of TNF- α seen with pretreatment. Minimizing the quantity of macrophages may have also improved lung function by minimizing the respiratory burst activity of macrophages. Although there is indirect evidence of diminished neutrophil mediated injury with ATL-313, significant decreases in neutrophil sequestration (decreased MPO) and interstitial infiltrate seen by lung histologic studies support the anti-inflammatory properties of $A_{2A}R$ activation in our model of LIRI.

Aspects of $A_{2A}R$ agonism still require further elucidation. The effect of $A_{2A}R$ agonism on T cells and natural killer cells, known contributors to lung IR injury, were not evaluated in this study. $A_{2A}R$ agonism has, however, been observed to mitigate T cell-related interferon γ production and secondary macrophage activation in inflamed tissue.³¹ The immunologic effect of $A_{2A}R$ agonism, especially in terms of the immunosuppressed transplant patient, also requires further inquiry. $A_{2A}R$ agonists may also cause systemic hypotension and thus potentially limit their clinical applicability.

The concept that IR injury is attenuated by $A_{2A}R$ pretreatment and that concurrent $A_{2A}R$ activation during reperfusion further improves lung function is poorly described yet clinically relevant, despite the aforementioned uncertainties. The elimination of the improvements observed with $A_{2A}R$ activation with the addition of a highly selective $A_{2A}R$ antagonist provides strong evidence that the mechanism of protection is specifically mediated via $A_{2A}R$ activation. The specific downstream mediators of protection conferred by pharmacologic adenosine preconditioning remain elusive. Early evidence supports the activation of mitogen-activated protein kinase subtypes including extracellular signal-related kinase 1/2, jun N terminal kinase 1/2, and p38 and the attenuation of apoptosis as a few of the possible mechanisms of protection conferred by adenosine receptor activation before major ischemic insult.^{19,32} The pharmacologic activation of the $A_{2A}R$ along with optimized

timing of activation may prove to be an important therapeutic intervention in attenuating the robust inflammatory response after lung transplantation.

In conclusion, this report demonstrates that pretreatment with an A_{2A}R agonist protects the lung against IR injury and that further improvements in oxygenation and lung compliance occur with concurrent A_{2A}R activation during reperfusion. The ability to exploit and further characterize innate physiologic mechanisms of protection that minimize the contribution of inflammatory cells will likely prove to be critical in reducing the destructive consequences of LIRI and ultimately improve the care of patients with end-stage lung disease.

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Discussion

Dr Mark J. Krasna (Towson, Md). What is the anti-inflammatory effect that you think ATL-313 has? Tell us what your view is.

Dr Gazoni. A_{2A}R agonists significantly decrease the amount of leukocyte activation and decrease the interaction of neutrophils, for example, with endothelial cells. One of the mechanisms behind that is decreasing the expression of adhesion molecules such as intercellular adhesion molecule and P-selectin. These are some of the few widely documented anti-inflammatory effects of the A_{2A}R agonists.

Dr Frank W. Sellke (*Boston, Mass*). I have a technical question. You subjected the lungs to 18 hours of ischemia and then reperfusion. Did you reperfuse with blood or with a crystalloid solution?

Dr Gazoni. We reperfused with whole blood. After working on this model for 2 years, I do not think I can handle seeing another rabbit. We actually have to exsanguinate 2 rabbits to reperfuse the harvested lung. So for every lung that we get data from there are actually 3 rabbits that are put to death total.

Dr Sellke. That was my next question. Did you kill the rabbits right before you were going to reperfuse the lungs or did you kill them at the same time and freeze the blood?

Dr Gazoni. As I am priming the reperfusion apparatus, I actually administer anesthesia to the blood donors with a combination of ketamine, xylazine, and vecuronium. As I open up the chest, the heart is still beating and I then cannulate the right atrium/ventricle. The blood then goes directly to the reperfusion apparatus.

Dr Sellke. One other question has to do with statistics. Did you do a multiple comparison test, or how did you compare the different groups? If you have 6 groups you have to have tremendous differences to reach statistical significance.

Dr Gazoni. We performed an ANOVA initially to see that there are actual differences between all groups throughout all time periods. All ANOVA tests were significant. The Tukey honest significant difference multiple-comparison test was then used to determine which groups were significantly different.

Dr David H. Harpole (*Durham, NC*). This is a very elegant technique. The only question I have is very simple. I read the paper and read this, and I could not exactly see. You had standard deviations. How many animals were in each group?

Dr Gazoni. Six. This is the third experiment I have performed with this model. I have probably harvested and reperfused about 400 lungs. By the time I did this experiment, which is the last experiment I have done with this model, I have gotten the technique down fairly well.

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