

Adenosine A_{2A} receptor activation reduces inflammation and preserves pulmonary function in an in vivo model of lung transplantation

T. Brett Reece, MD,^a Peter I. Ellman, MD,^a Thomas S. Maxey, MD,^a Ivan K. Crosby, MBBS,^a Patrick S. Warren, BS,^a Tae W. Chong, MD,^a Robin D. LeGallo, MD,^b Joel Linden, PhD,^c John A. Kern, MD,^a Curtis G. Tribble, MD,^a and Irving L. Kron, MD^a

Background: Reperfusion injury continues to significantly affect patients undergoing lung transplantation. Isolated lung models have demonstrated that adenosine A_{2A} receptor activation preserves function while decreasing inflammation. We hypothesized that adenosine A_{2A} receptor activation by ATL-146e during the initial reperfusion period preserves pulmonary function and attenuates inflammation in a porcine model of lung transplantation.

Methods: Mature pig lungs preserved with Viaspan (Barr Laboratories, Pomona, NY) underwent 6 hours of cold ischemia before transplantation and 4 hours of reperfusion. Animals were treated with (ATL group, n = 7) and without (IR group, n = 7) ATL-146e (0.05 $\mu\text{g kg}^{-1} \cdot \text{min}^{-1}$ ATL-146e administered intravenously for 3 hours). With occlusion of the opposite pulmonary artery, the animal was maintained for the final 30 minutes on the allograft alone. Recipient lung physiology was monitored before tissue evaluation of pulmonary edema (wet-to-dry weight ratio), myeloperoxidase assay, and tissue tumor necrosis factor α by means of enzyme-linked immunosorbent assay.

Results: When the ATL group was compared with the IR group, the ATL group had better partial pressure of carbon dioxide (43.8 ± 4.1 vs 68.9 ± 6.3 mm Hg, $P < .01$) and partial pressure of oxygen (272.3 ± 132.7 vs 100.1 ± 21.4 mm Hg, $P < .01$). ATL-146e-treated animals exhibited lower pulmonary artery pressures (33.6 ± 2.1 vs 47.9 ± 3.5 mm Hg, $P < .01$) and mean airway pressures (16.25 ± 0.08 vs 16.64 ± 0.15 mm Hg, $P = .04$). ATL-146e-treated lungs had lower wet-to-dry ratios (5.9 ± 0.39 vs 7.3 ± 0.38 , $P < .02$), lower myeloperoxidase levels ($2.9 \times 10^{-5} \pm 1.2 \times 10^{-5}$ vs $1.3 \times 10^{-4} \pm 4.0 \times 10^{-5}$ $\Delta\text{OD mg}^{-1} \cdot \text{min}^{-1}$, $P = .03$), and a trend toward decreased lung tumor necrosis factor α levels (57 ± 12 vs 96 ± 15 pg/mL, $P = .06$). The ATL group demonstrated significantly less inflammation on histology.

Conclusion: Adenosine A_{2A} activation during early reperfusion attenuated lung inflammation and preserved pulmonary function in this model of lung transplantation. ATL-146e and similar compounds could play a significant role in improving outcomes of pulmonary transplantation.

Despite improvements in lung preservation techniques and organ-specific preservation solutions, ischemia-reperfusion injury after lung transplantation continues to occur in 10% to 20% of recipients.^{1,2} Ischemia-reperfusion injury has been shown to be clinically relevant because of increased in-hospital mortality and

From the Departments of Surgery^a and Pathology^b and the Cardiovascular Research Center,^c University of Virginia Health System, Charlottesville, Va.

Supported by American Heart Association grant 0250222N and The Dr Frank and Marion Falk Research Trust Fund.

Read at the Thirtieth Annual Meeting of The Western Thoracic Surgical Association, Maui, Hawaii, June 23-26, 2004.

Received for publication June 21, 2004; revisions received Oct 15, 2004; accepted for publication Nov 17, 2004.

Address for reprints: T. Brett Reece, MD, University of Virginia Health System, Department of Surgery, PO Box 801359, Charlottesville, VA 22908 (E-mail: tbr5q@virginia.edu).

J Thorac Cardiovasc Surg 2005;129:1137-43

0022-5223/\$30.00

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doi:10.1016/j.jtcvs.2004.11.042

morbidity, resulting in prolonged ventilation, protracted intensive care unit stay, and increased cost of hospitalization.³ Furthermore, the prospect of ischemia-reperfusion injury limits the number of acceptable organs for transplantation to less than a third of organ donors.⁴ Therefore the prevention or attenuation of ischemia-reperfusion injury in transplanted lungs could result in profound effects in both those patients receiving organs in our current system and could improve the usefulness of marginal lung donors, thus possibly expanding the number of suitable lungs for transplantation.

Over the past several years, lung transplantation research has focused on prevention of lung injury by improving preservation solutions to attenuate injury from storage and transport of the donor organ.⁵ However, significant inflammatory reaction still occurs with reperfusion. Recent studies have demonstrated that pharmacologic intervention at reperfusion rather than during the preservation period could preserve function in spinal cord, renal, and cardiac models of ischemia-reperfusion injury.⁶⁻⁹ Most important to this study, adenosine A_{2A} receptor activation during reperfusion in isolated lung models improved graft function while decreasing cellular and molecular markers of inflammation.^{10,11} On the basis of these studies, activation of this receptor is thought to have great potential in modulation of reperfusion injury in lung transplantations. The adenosine A_{2A} receptor is predominantly expressed on inflammatory cells, including neutrophils, mast cells, macrophages, monocytes, and platelets.¹² The mechanism of injury attenuation in ischemia and reperfusion of various organs by activation of this receptor appears to involve attenuation of the secondary inflammatory reaction through a purinergic regulatory process.¹³ The adenosine A_{2A} receptor is coupled to a stimulatory G protein, which leads to an increase in intracellular cyclic adenosine monophosphate. Increased cyclic adenosine monophosphate through this pathway leads to an inactivation of inflammatory cells, resulting in reduced cytokine release, accumulation of other inflammatory cells, and possibly even the modulation of cellular immunity through impaired T-cell activity.¹⁴ Although several inflammatory cells certainly play a role in ischemia-reperfusion injury, this study focused on the neutrophil and tumor necrosis factor α (TNF- α) as representatives of inflammation.

The aim of this study was to demonstrate that the inflammatory response in a whole animal lung transplantation model could be lessened and that pulmonary function would parallel this inflammatory reduction. We hypothesized that ATL-146e, a specific adenosine A_{2A} receptor agonist, given at reperfusion would both impair the inflammatory response to lung transplantation, as demonstrated by neutrophil sequestration and TNF- α production, and preserve pulmonary

functional physiology.

Methods

Animal Care

All animals received humane care in accordance with the "Guide for care and use of Laboratory animals" published by the National Institute of Health (National Institutes of Health publication no. 85-23, revised 1985). The Animal Care and Use Committee at the University of Virginia reviewed and approved the protocol for this study before experimentation.

Study Groups

Domestic swine of both sexes (18-30 kg) were randomly separated into 2 groups (both n = 7). The first group was an ischemia-reperfusion control group (the IR group) that underwent 6 hours of ischemia, followed by 4 hours of reperfusion. The second group (the ATL group) underwent the same procedure but also received ATL-146e (ATL) for the first 3 hours of reperfusion. The dosing of ATL was based on previous experience with this drug in the isolated lung model of ischemia-reperfusion injury, as well as experience with this drug in the prevention of ischemic spinal cord injury.¹¹ A dose of 0.05 $\mu\text{g kg}^{-1} \cdot \text{min}^{-1}$ for 3 hours was chosen for this study because this was the optimal *in vivo* dose, as determined in earlier studies on spinal cord injury.

Donor Procedure

The donor lung harvest procedure was identical for both groups. The pigs were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). After endotracheal intubation, they were ventilated with a volume-cycled ventilator with a 15 mL/kg tidal volume at a rate of 10 to 16 breaths/min. The fraction of inspired oxygen was 100% for the procedure. Pigs were anesthetized with halothane and anticoagulated with 200 U/kg heparin sodium. A left thoracotomy was performed to expose the heart and left lung. The inferior pulmonary ligament was taken down before the pulmonary artery was dissected. Prostaglandin E₁ (PGE₁; 10 mg/kg) was injected directly into the main pulmonary artery. After the main pulmonary artery was clamped with a large vascular clamp, the lung was submerged in saline slush, and 1 L of Viaspan (Barr Laboratories, Pomona, NY), the commercially available version of the University of Wisconsin solution that was being used clinically at the time of this study, was infused from a height of 30 cm. The heart and both lungs were excised en bloc. The left pulmonary artery and bronchus were isolated, as well as the part of the left atrium that received the left pulmonary veins. The rest of the heart and the right lung were discarded. The donor lung was doubly wrapped in a sterile bag. It was then stored in the preservation solution at 4°C for 6 hours before implantation. This ischemic time was chosen because previous studies had shown adequate injury for study at this time point, and the ischemic time correlated with the clinical scenario.¹⁵

Recipient Procedure

Recipient swine were size matched to the donor before induction with ketamine (50 mg/kg) and xylazine (5 mg/kg). The recipient was intubated before placement on a volume ventilator. After neck dissection with carotid arterial line placement and internal jugular line placement, a pulmonary artery catheter

was floated until it wedged. Then a left thoracotomy was performed, with exposure of both the right and left main pulmonary arteries. A vessel loop was placed around the right pulmonary artery to facilitate subsequent occlusion. Next a left pneumonectomy was completed by ligating the pulmonary veins individually before clamping the left pulmonary artery and bronchus proximally and cutting them distally. The donor lung was attached to the left main stem bronchus by using 4-0 Prolene sutures (Ethicon, Inc, Somerville, NJ). After confirming an airtight bronchial anastomosis, the left pulmonary artery was anastomosed with 5-0 Prolene sutures. Finally, the atrial cuff of the donor was sewn to a section of the recipient left atrium isolated with a side-biting vascular clamp by using a 4-0 Prolene suture. After adequate hemostasis was achieved, the skin was reapproximated with towel clamps for the reperfusion period.

IR group animals were reperfused for 3.5 hours before the right pulmonary artery was clamped, maintaining the recipient animal on the donor lung for the final 30 minutes of reperfusion. The ATL group underwent an identical procedure with the addition of receiving intravenous ATL-146e ($0.05 \mu\text{g kg}^{-1} \cdot \text{min}^{-1}$) for the first 3 hours of reperfusion beginning 10 minutes before reperfusion.

After 4 hours of reperfusion, an arterial blood gas measurement was taken. By using a pressure ventilator, ventilation parameters were taken, including mean airway pressure, tidal volume, peak inspiratory pressure, minute volume, and compliance. With a pulmonary artery catheter, the cardiac output, cardiac index, pulmonary artery pressure, wedge pressure, systemic vascular resistance, and pulmonary vascular resistance were determined. The mean arterial pressure and heart rate were also monitored throughout the procedure. Ventilator settings were used to maintain normal pH, PCO_2 , and PO_2 during reperfusion in both lungs. No changes were made once single-lung reperfusion began.

Samples were taken from the donor and recipient lung, as well as from the left ventricle, which were both frozen in liquid nitrogen and fixed in 10% formalin. Samples were also taken from both lungs for the wet-to-dry weight ratios, the myeloperoxidase (MPO) assay, and the TNF- α assay. Serum samples were taken at 0, 1, 2, 3, and 4 hours of reperfusion before being frozen for the TNF- α assay.

Wet-to-Dry Weights

For wet-to-dry weight ratios, parenchymal samples of whole lungs were taken after reperfusion. The tissue was blotted to remove excess blood in all samples. The samples were weighed before placement in a vacuum oven for 48 to 72 hours. The samples were weighed daily until the weight was stable. These data were used as an indicator of the amount of pulmonary edema. Lungs with higher wet-to-dry ratios would be expected to have had more pulmonary edema.

MPO Activity

Neutrophil sequestration was quantified by using a MPO activity assay. Lung and ventricular tissue was frozen in liquid nitrogen after the reperfusion period. The tissue was resuspended in 50 mmol/L KPO_4 (pH 7.4) and then homogenized for 30 seconds at 4°C. The solution was centrifuged for 15 minutes at 15,000g (Sorvall RC-5b Refrigerated Superspeed Centrifuge; Kendro Lab-

oratory Products, Newton, Conn). The supernatant was discarded before the pellet was resuspended in 0.5% hexadecyltrimethylammoniumbromide (HTAB) in 50 mmol/L KPO_4 (pH 6.4). The solution was then homogenized a second time for 90 seconds at 4°C. The solution underwent sonication and 3 freeze-thaw cycles (alternating liquid nitrogen for 1 minute and 37°C circulating bath for 5 minutes). The solution was centrifuged for a second time at 15,000g for 15 minutes. The supernatant was divided into 1-mL aliquots. The assay buffer was made from 10 mg of *o*-diananosine in 1 mL of deionized water, 100 mL of H_2O_2 , and 8.9 mL of 50 mmol/L potassium phosphate (pH 6.0). The 15 mL of supernatant was combined with 135 mL of assay buffer and incubated at room temperature in a 96-well flat-bottom plate. Absorbance at 460 nm was measured over 2 minutes by means of spectrophotometry (MRX Revelation Plate Reader; Dynex Technologies, Inc, Chantilly Va). MPO activity was expressed as change in absorbance per milligram per minute.

TNF- α Assay

Lung tissue lysate was generated from a minimum of 5 mg of tissue sample (lung wedge) in Triton X-100 lysis buffer by using a tissue homogenizer (Fisher Scientific International, Pittsburgh, Pa). The lysis buffer consisted of 1% Triton X-100 (Sigma-Aldrich Corp, St Louis, Mo), 20 mmol/L Tris pH 7.4 (Sigma-Aldrich Corp, St Louis, Mo), 150 mmol/L NaCl (Sigma-Aldrich Corp), 2 mmol/L ethylenediamine tetraacetic acid (Sigma-Aldrich Corp), 2 $\mu\text{g/mL}$ Leupeptin (Roche Diagnostics Corp, Indianapolis, Ind), 0.7 $\mu\text{g/mL}$ Pepstatin (Roche Diagnostics Corp), 100 $\mu\text{mol/L}$ Chymostatin (Roche Diagnostics Corp), and 100 $\mu\text{mol/L}$ Antipain (Roche Diagnostics Corp). The samples were centrifuged at 10,000 rpm and 0°C for 10 minutes to remove debris. The supernatant was then aspirated and used for enzyme-linked immunosorbent assay (ELISA). TNF- α levels of lung tissue lysates and serum were determined by using commercially available ELISA kit (Pierce Biotechnology, Inc, Rockford, Ill), according to the manufacturer's protocols. All samples were tested in duplicate and averaged. The ELISA kit sensitivity for TNF- α was 20 pg/mL. All readings were performed on an MRX II Absorbance Reader (DYNEX Technologies Inc). TNF- α concentration was expressed in picograms per milliliter.

Lung Injury Score

After tissue processing and staining for hematoxylin and eosin, lung samples were graded by a blinded pathologist. Each sample was graded with a lung injury score on the basis of the amount of neutrophils, interstitial infiltrate, and alveolar edema. A modified version of this scoring system has been used previously in an isolated mouse model of lung ischemia and reperfusion.¹⁶ Each of these 3 categories was given a score of 0 to 3, resulting in a possible range of 0 for normal lung to 9 for most injured lung. Details of the lung injury scoring are depicted in Table 1.

Statistical Analysis

All statistics were performed by an independent statistician. For this study, a Student *t* test for independent samples was conducted to compare the IR group with the ATL group.

TABLE 1. Lung injury score description

Category	0	1	2	3
Neutrophils per high-powered field	<5	6-10	11-20	>20
Alveolar edema: edema per alveolar area	<5%	6%-25%	25%-50%	>50%
Interstitial infiltrate	None	Minimal	Moderate	Severe

This is the scoring system used to evaluate lung injury in the current study on the basis of neutrophil infiltration, alveolar edema, and interstitial edema.

Results

Physiologic

Arterial blood gases were significantly different between the ATL and IR groups in terms of pH, Pco₂, and Po₂. The ATL group's mean pH was 7.39 ± 0.04 compared with a more acidotic mean pH of 7.27 ± 0.03 in the IR group (*P* < .05). ATL treatment preserved Pco₂ levels compared with those seen in the IR group (43.8 ± 4.1 vs 68.9 ± 6.3 mm Hg, respectively; *P* < .01). Oxygenation was significantly improved among animals receiving ATL (272.3 ± 50.2 mm Hg) compared with that of animals in the IR group (100.1 ± 21.4 mm Hg, *P* < .01).

This study identified no statistically significant difference between groups in terms of mean arterial pressure, cardiac output or index, systemic vascular resistance, or pulmonary capillary wedge pressure. In addition, minute volume and pulmonary compliance were similar. However, ATL-treated animals demonstrated significantly lower mean pulmonary artery pressures (33.6 ± 2.14 vs 47.9 ± 3.5 mm Hg, *P* < .01) and pulmonary vascular resistance (933.1 ± 109.3 vs 1772.3 ± 313.9, *P* < .03) than animals in the IR group. Finally, mean airway pressures were significantly lower in the ATL group compared with those in the IR group (16.3 ± 0.08 vs 16.6 ± 0.15 mm Hg, *P* = .04).

Pulmonary Edema

Wet-to-dry weight ratios were determined to quantify the amount of lung edema in the transplanted lungs. ATL-treated donor lungs had a significantly lower mean ratio than those of the control animals (5.88 ± 0.31 vs 7.25 ± 0.38, *P* < .02). Both of these groups had higher values than the mean wet-to-dry weight ratio of lungs from pigs undergoing anesthesia alone (4.70 ± 0.09). There was also no discernible difference between recipient (nonischemic) lungs of the ATL and IR groups (5.62 ± 0.27 vs 5.68 ± 0.15, *P* = .85).

Neutrophil Sequestration

The MPO assay demonstrated less neutrophil sequestration in ATL animals compared with that in animals in the IR group (2.9 × 10⁻⁵ ± 1.2 × 10⁻⁵ vs 1.3 × 10⁻⁴ ± 4.0 × 10⁻⁵ ΔOD mg⁻¹ · min⁻¹, *P* = .03).

TABLE 2. Lung injury score for ATL-treated and IR animals

	ATL	IR	<i>P</i> value
Neutrophils	0.86 ± 0.26	2.00 ± 0.31	.016*
Alveolar edema	0.29 ± 0.18	1.14 ± 0.51	.12
Interstitial infiltrate	0.86 ± 0.14	1.03 ± 0.30	.15
Total	2.00 ± 0.44	4.57 ± 0.87	.027*

*Statistical significance.

TNF-α

TNF-α concentrations were less than the detectable levels, as determined by means of ELISA, in serum at 0, 1, 2, 3, and 4 hours of reperfusion. TNF-α concentrations were also not detectable in animals that did not undergo pulmonary ischemia. However, after 4 hours of reperfusion, the mean TNF-α concentration trended lower in ATL-treated animals than in the IR group (57 ± 12 vs 96 ± 15 pg/mL, *P* < .06).

Lung Injury Score

The lung injury score was significantly lower in ATL-treated animals compared with that in the IR group (2.00 ± 0.44 vs 4.57 ± 0.87, *P* < .03). When broken down further, the majority of the difference between the 2 groups stemmed from less pulmonary neutrophil infiltration in the ATL group compared with that in control animals (2.0 ± 0.3 vs 0.9 ± 0.3, *P* < .02). These scores are summarized in Table 2.

Discussion

The mechanism of lung ischemia and reperfusion is sufficiently complex that precise processes of injury progression and manipulation are poorly understood. Ischemia-reperfusion injury stems from the interplay between ischemic injury to the donor graft and the secondary injury from reperfusion, including the recipient inflammatory response.¹⁷ Clinical manipulation of the ischemic insult has not yet reached the point of providing benefit to the 20% of lung transplant recipients who experience reperfusion injury.³

Our recent research has focused on attenuation of ischemia-reperfusion injury by means of pharmacologic intervention at the time of reperfusion. The use of adenosine A_{2A} agonists has been shown to augment several specific cellular and molecular targets that play considerable roles in the progression of this injury. Adenosine A_{2A} receptors are present on almost all inflammatory cells (including neutrophils, mast cells, macrophages, eosinophils, platelets, and T-cells).¹² In these cells activation of this receptor is almost universally inhibitory.¹⁸ Most important to this study is the inhibition of the macrophage and the neutrophil. Adenosine A_{2A} receptors are also present on vascular smooth muscle. Activation of these vascular receptors leads to muscular relaxation and vascular dilatation. Because both inflammation and vasoconstriction play major roles in ischemia-reperfusion injury, this receptor would appear to have great potential for ameliorating this process.

TX

As previously stated, attenuation of the inflammatory response can be targeted at numerous sites. First, resident pulmonary macrophages play a role in early injury.^{19,20} Macrophage activation instigates a local inflammatory cascade through the release of TNF- α and proinflammatory cytokines. At reperfusion, cellular injury leads to resident macrophage activation, which in turn releases more inflammatory mediators. Ultimately, the cytokines stimulate T-cell and neutrophil activation. The inflammatory cascade amplifies into a systemic response, which overflows into further graft injury. In fact, this inflammatory response is sufficiently exaggerated that the inflammation leads to injury to other organs, including the heart and the contralateral lung.^{21,22} The overview of this process provides multiple cellular and molecular targets for manipulation.

Recent literature on pulmonary ischemia-reperfusion injury on one of these cellular targets has demonstrated the significance of macrophage function and the reduction of injury from macrophage impairment.^{19,20,23} The resident macrophage appears to be particularly important in the early phases of reperfusion injury. Early in reperfusion, the resident macrophage releases TNF- α , leading to upregulation of the inflammatory cascade. Reduced levels of TNF- α in tissue can demonstrate a blunted inflammatory response and an inhibition of resident macrophage function. In an isolated mouse model focusing on the resident pulmonary mechanism of reperfusion injury, Maxey and colleagues¹⁶ demonstrated that TNF- α knockout mice had preserved pulmonary function, impaired inflammatory response, and preserved cytoarchitecture compared with wild-type animals. This study concluded that TNF- α played a major role in the early progression of pulmonary ischemia-reperfusion injury. The current study demonstrated a nearly significant trend in the reduction of TNF- α levels after lung transplantation. This reduction is most likely from the inactivation of macrophages by adenosine A_{2A} activation, but this is far from proved here. No matter the source of TNF- α affected, there is a reduction with use of ATL-146e. This might lead to further downregulation of the inflammatory cascade.

Although their role is limited in the first several hours after reperfusion, neutrophils play a significant role in ischemia-reperfusion injury.²³ Numerous studies have shown that adenosine A_{2A} activation impairs neutrophil function and activation in response to various modes of injury.²⁴⁻²⁶ Isolated lung studies have demonstrated decreased neutrophil sequestration in lungs receiving ATL-146e. In an isolated rabbit lung model, Ross and coworkers¹¹ demonstrated that perfusion of the ischemic lung with an adenosine A_{2A} receptor agonist limited the MPO activity in the lungs within 30 minutes of reperfusion. In a similar model with high-flow reperfusion in isolated rabbit lungs, Fiser and associates¹⁰ showed a trend toward decreased MPO activity in animals receiving ATL-146e compared

with ischemic control animals. The reduction in neutrophil sequestration is echoed in this study by a significant decrease in the MPO activity in ATL-treated animals compared with activity in animals in the IR group. Furthermore, this evidence of impaired neutrophil activation or recruitment is confirmed by the histologic findings of fewer neutrophils in the lung tissue. The combination of molecular and histologic data strengthens the argument of neutrophil impairment with adenosine A_{2A} receptor activation. This study does not show directly that adenosine A_{2A} receptor activation inactivates neutrophils, but a growing body of evidence suggests neutrophil inactivation by ATL-146e.

This study does have its limitations. With respect to inflammation, the markers used are crude. MPO assays alone do not prove increased neutrophil infiltration into tissue. Moreover, increased infiltration does not prove increased neutrophil activity but rather only suggests compartmental recruitment. However, the histologic scoring by our pathologist tends to support the MPO findings. The neutrophil infiltration scoring alone showed significant differences between the groups. We believe that these 2 markers of impaired neutrophil recruitment imply that the use of ATL in this injury does attenuate the neutrophil response to this injury. The reperfusion period chosen for this study is probably not optimal for identifying the peak TNF- α concentrations in serum or tissue. Although low levels of mRNA of TNF- α and TNF- α have been demonstrated very early in the reperfusion period, the peak concentration might occur later in the process.^{27,28} The TNF- α data were also limited to a trend toward lower concentrations in ATL-treated transplant lungs compared with in that seen in lungs of the IR group. But the trend approaches significance even at this early time point. Despite the lack of statistical difference, we believe that ATL will indeed modulate TNF- α production in this injury, given a longer reperfusion period and larger sample size, which we hope to show in future studies. The short reperfusion period remains valid because we are trying to demonstrate that early intervention during reperfusion can preserve lung allograft function from the deleterious effects of ischemia-reperfusion injury. Finally, this study does not exclude other mechanisms of injury attenuation. This study did not demonstrate any appreciable changes in systemic hemodynamics, but the microvasculature response to adenosine A_{2A} activation has not been studied. Even ATL-146e can reduce systemic blood pressure through vasodilatation at a much higher dose than those used in this study.²⁹

Additionally, the idea of direct cellular protection by this compound remains theoretical but possible. Studies in spinal cord ischemia and reperfusion have found that the functional preservation is demonstrable before any appreciable increase in markers of inflammation.⁷ Although the study has limitations, we believe that the conclusions re-

main valid and that, more importantly, this therapy will prove to benefit lung transplant recipients in the future.

The ultimate goal of modulation of ischemia-reperfusion injury remains the preservation of lung transplant function. On the basis of our limited understanding of reperfusion injury, inflammatory reduction should lead to improved lung function. This study demonstrated preserved lung function as defined by blood gases in terms of pH, oxygenation, and ventilation. Pulmonary mechanics were also preserved, with improved pulmonary vascular resistance and lower pulmonary artery pressures. ATL-treated animals also had lower mean airway pressures, which were probably exacerbated by less pulmonary edema, as indicated by wet-to-dry weight ratios. Functionally, ATL-treated animals experienced far superior physiologic performance than animals in the IR group. These results are consistent with previous studies in isolated lung models.^{10,11} Adenosine A_{2A} activation in the early reperfusion period improves physiology in a fashion that parallels inflammatory inhibition.

This study shows that in a large animal model of transplantation, activation of the adenosine A_{2A} receptor at reperfusion can impair the inflammatory response and preserve pulmonary function during the first 4 hours after transplantation. In conclusion, the use of ATL-146e or a similar compound can improve outcomes by reducing the detrimental effects of ischemia-reperfusion injury on lung transplant function.

We thank Tony Herring, Cindy Dodson, and Sheila Hammond for their invaluable technical assistance during this study. Also, we would like to thank Jayson Reiger of Adenosine Therapeutics, LLC, for providing us with the ATL-146e used in this study. Finally, we disclose that Dr Kron and Dr Linden have a proprietary relationship with Adenosine Therapeutics, LLC.

References

- Burdine J, Hertz MI, Snover DC, Bolman RM 3rd. Heart-lung and lung transplantation: perioperative pulmonary dysfunction. *Transplant Proc.* 1991;23:1176-7.
- Fiser SM, Kron IL, Long SM, Kaza AK, Kern JA, Tribble CG. Early intervention after severe oxygenation index elevation improves survival following lung transplantation. *J Heart Lung Transplant.* 2001;20:631-6.
- King RC, Binns OA, Rodriguez F, Kanithanon RC, Daniel TM, Spotnitz WD, et al. Reperfusion injury significantly impacts clinical outcome after pulmonary transplantation. *Ann Thorac Surg.* 2000;69:1681-5.
- Ware LB, Wang Y, Fang X, Warnock M, Sakuma T, Hall TS, et al. Assessment of lungs rejected for transplantation and implications for donor selection. *Lancet.* 2002;360:619-20.
- de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med.* 2003;167:490-511.
- Okusa MD, Linden J, Macdonald T, Huang L. Selective A_{2A} adenosine receptor activation reduces ischemia-reperfusion injury in rat kidney. *Am J Physiol.* 1999;277:F404-12.
- Reece TB, Okonkwo DO, Ellman PI, Warren PS, Smith RL, Hawkins SL, et al. The evolution of ischemic spinal cord injury in function, cytoarchitecture, and inflammation, and the effects of adenosine A_{2A} receptor activation. *J Thorac Cardiovasc Surg.* 2004;128:925-32.
- Cargnoni A, Ceconi C, Boraso A, Bernocchi P, Monopoli A. Role of A_{2A} receptor activation in the modulation of myocardial reperfusion damage. *J Cardiovasc Pharmacol.* 1999;33:883-93.
- Pearl J, Flood A, Linden J, Matherne GP, Headrick JP. Adenosine-mediated cardioprotection in ischemic-reperfused mouse heart. *J Cardiovasc Pharmacol.* 2002;39:117-29.
- Fiser SM, Tribble CG, Kaza AK, Long SM, Kern JA, Cassada DC, et al. Adenosine A_{2A} receptor activation decreases reperfusion injury associated with high-flow reperfusion. *J Thorac Cardiovasc Surg.* 2002;124:973-8.
- Ross SD, Tribble CG, Linden J, Gangemi JJ, Lanpher BC, Wang AY, et al. Selective adenosine-A_{2A} activation reduces lung reperfusion injury following transplantation. *J Heart Lung Transplant.* 1999;18:994-1002.
- Sullivan GW, Linden J, Buster BL, Scheld WM. Neutrophil A_{2A} adenosine receptor inhibits inflammation in a rat model of meningitis: synergy with the type IV phosphodiesterase inhibitor, rolipram. *J Infect Dis.* 1999;180:1550-60.
- Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Ann Rev Pharmacol Toxicol.* 2001;41:775-87.
- Link AA, Kino T, Worth JA, McGuire JL, Crane ML. Ligand-activation of the adenosine A_{2A} receptors inhibits IL-12 production by human monocytes. *J Immunol.* 2000;164:436-42.
- Ross SD, Kron IL, Gangemi JJ, Shockey KS, Stoler M, Kern JA, et al. Attenuation of lung reperfusion injury after transplantation using an inhibitor of nuclear factor-kappaB. *Am J Physiol Lung Cell Mol Physiol.* 2000;279:L528-36.
- Maxey TS, Enelow RI, Gaston B, Kron IL, Laubach VE, Doctor A. Tumor necrosis factor-alpha from resident lung cells is a key initiating factor in pulmonary ischemia-reperfusion injury. *J Thorac Cardiovasc Surg.* 2004;127:541-7.
- de Perrot M, Keshavjee S. Lung preservation. *Ann Thorac Surg.* 2002;74:629-31.
- Sullivan GW, Rieger JM, Scheld WM, Macdonald TL, Linden J. Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A_{2A} receptor agonists. *Br J Pharmacol.* 2001;132:1017-26.
- Eppinger MJ, Deeb GM, Bolling SF, Ward PA. Mediators of ischemia-reperfusion injury of rat lung. *Am J Pathol.* 1997;150:1773-84.
- Fiser SM, Tribble CG, Long SM, Kaza AK, Kern JA, Kron IL. Pulmonary macrophages are involved in reperfusion injury after lung transplantation. *Ann Thorac Surg.* 2001;71:1134-8.
- Reece TB, Laubach VE, Tribble CG, Maxey TS, Ellman PI, Warren PS, et al. Adenosine A_{2A} receptor agonist improves cardiac dysfunction from pulmonary ischemia-reperfusion injury. *Ann Thorac Surg.* In press.
- Palazzo R, Hamvas A, Shuman T, Kaiser L, Cooper J, Schuster DP. Injury in nonischemic lung after unilateral pulmonary ischemia with reperfusion. *J Appl Physiol.* 1992;72:612-20.
- Fiser SM, Tribble CG, Long SM, Kaza AK, Cope JT, Laubach VE, et al. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. *J Thorac Cardiovasc Surg.* 2001;121:1069-75.
- Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M. The adenosine/neutrophil paradox resolved: human neutrophils possess both A₁ and A₂ receptors that promote chemotaxis and inhibit O₂ generation, respectively. *J Clin Invest.* 1990;85:1150-7.
- Cronstein BN, Levin RI, Phillips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A₁ receptors and inhibited via adenosine A₂ receptors. *J Immunol.* 1992;148:2201-6.
- Mullane K, Bullough D. Harnessing an endogenous cardioprotective mechanism: cellular sources and sites of action of adenosine. *J Mol Cell Cardiol.* 1995;27:1041-54.
- Krishnadasan B, Naidu BV, Byrne K, Fraga C, Verrier ED, Mulligan MS. The role of proinflammatory cytokines in lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg.* 2003;125:261-72.
- de Perrot M, Fischer S, Liu M, Jin R, Bai XH, Waddell TK, et al. Prostaglandin E₁ protects lung transplants from ischemia-reperfusion

injury: a shift from pro- to anti-inflammatory cytokines. *Transplantation*. 2001;72:1505-12.

29. Linden J. Autoregulation of cyclic AMP in vascular smooth muscle: a role for adenosine receptors. *Mol Pharmacol*. 2002;62:969-70.

Discussion

Dr David Fullerton (*Denver, Colo*). I enjoyed your talk very much, and I have enjoyed following the progress of your laboratory at the University of Virginia as you have pursued the application of A_{2A} receptor stimulation in a variety of models and now into lung transplantation. I had 3 questions I wondered if you might expand a little bit on.

First, as you pointed out in your slide, ultimately the intracellular mechanism that is activated by A_{2A} receptor stimulation is through cyclic adenosine monophosphate (cAMP) and through phosphokinase A. I am curious whether you might have any thoughts on whether this is an A_{2A} receptor-specific phenomenon or whether it might be possible to activate phosphokinase A through any other membrane-based receptors.

Dr Reece. There are certainly other possibilities to explore in the manipulation of intracellular cAMP in response to injury. We are exploring pathways to synergize the response to ATL-146e with the addition of a phosphodiesterase inhibitor to further accentuate cAMP and the resulting functional preservation. We would never entertain the idea that we have the only substance that can affect this mechanism, but we believe that ATL-146e effectively attenuates ischemia-reperfusion injury.

Dr Fullerton. You did a nice job in your study of associating your findings with neutrophil-mediated injury, particularly with your MPO assays, and your work that has led up to this has demonstrated an antineutrophil effect, if you will, of adenosine. Could you speculate on whether there might be other mechanisms of injury, such as an oxidative stress injury, that the adenosine might also affect?

Dr Reece. I think that direct cellular protection with adenosine receptor activation is definitely a possibility. The more we study

activation of this receptor, the more we observe that the level of protection might extend beyond what can be accounted for by the reduction of anti-inflammation. We have seen this phenomenon in the spinal cord studies, in which we believe we are limiting the inflammation, but the animals are improving much beyond the relative inflammatory attenuation. There could be both direct effects on the tissue because some adenosine compounds have a preconditioning effect, and then also there is a potential for vasodilatation in at least the smaller vessels, although we do not see it in terms of our hemodynamics systemically.

Dr Fullerton. Finally, TNF is an appropriate suspect for any sort of injury like this, and you did a nice job of demonstrating increased tissue levels of TNF in your control group. I was curious whether you might have looked at any other cytokines, either proinflammatory cytokines or potentially anti-inflammatory cytokines, like IL-10 for instance, which is recognized to diminish the production of TNF.

Dr Reece. We have not looked at other inflammatory cytokines using these models yet. We are trying to move in that direction and are looking at mRNA signaling and upregulation of other inflammatory cytokines to identify where in the process of injury to inflammation these substances are attenuating ischemia and reperfusion. We have no results to report at this time, but this is the direction in which we are heading.

Dr John Chen (*Honolulu, Hawaii*). You studied this in a model of lung transplantation. Can you speculate as to whether this compound would be useful in cardiac surgery, for instance, in reducing inflammation in cardiopulmonary bypass?

Dr Reece. There are published studies with ATL-146e in cardiac infarction models that have shown improvement in the reversibility of ischemic injury and the resultant function of hearts in mice. As we have demonstrated with the spinal cord ischemia-reperfusion attenuation, adenosine A_{2A} receptor activation might have a role in a wide array of organ preservation, including cardiac transplantation, in the future.