

Adenosine A_{2A} Activation Attenuates Nontransplantation Lung Reperfusion Injury

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Background. Lung reperfusion injury is a significant problem in cardiothoracic surgery. Previous studies have demonstrated that an adenosine A_{2A} agonist can attenuate lung reperfusion injury in a lung transplantation model. There has been little work, however, examining its effects in the setting of nontransplant ischemia reperfusion. Our hypothesis was that an A_{2A} agonist would attenuate lung reperfusion injury in a warm ischemia hilar clamping model.

Study design. Sprague Dawley rats underwent 90 min of left hilar clamping followed by 4 h of reperfusion. Group 1 ($n = 13$) received an intravenous infusion of 0.06 ug/kg/min of ATL-146e, which was started 10 min before reperfusion. Group 2 ($n = 16$) received an equivalent saline infusion. A third sham group ($n = 14$) received the same protocol as Group 2 but no lung ischemia.

Results. Animals receiving ATL-146e showed significant improvements in oxygenation (Group 1: 447 ± 26.02 mmHg versus Group 2: 223 ± 24.46 mmHg ($P < 0.001$)) as well as ventilation (pCO₂ Group 1: 48.78 ± 3.88 versus Group 2: 63.56 ± 4.80 ($P = 0.009$)). Total protein in the bronchoalveolar lavage was significantly higher in the saline group compared with the adenosine as well as a higher proportion of neutrophils. Histological analysis demonstrated a significantly higher number of neutrophils in the IR group compared with the adenosine group.

Conclusions. ATL-146e, an adenosine analogue that is a specific agonist for the A_{2A} receptor, attenuates reperfusion injury in an *in vivo* rat lung model. Arterial blood gas measurements demonstrate a statistically significant increase in oxygenation and improved ventilation. © 2008 Elsevier Inc. All rights reserved.

Key Words: adenosine; adenosine A_{2A}; lung reperfusion injury.

INTRODUCTION

Lung ischemia-reperfusion injury (IR) is a significant clinical problem faced by cardiothoracic surgeons, cardiologists, and pulmonologists, and is most commonly associated with lung transplantation [1, 2]. Although it is most commonly associated with transplantation, IR injury causes significant morbidity and mortality in other areas of thoracic surgery including cardiopulmonary bypass, trauma, pulmonary thromboendarterectomy, and pulmonary resection with major vascular reconstruction [2–8]. Thus, given the widespread preponderance of this phenomenon, it is of interest to the practicing cardiothoracic surgeon to better understand the process and look for potential therapies.

IR injury in the lung is thought to be due in large part to collateral damage caused by an inflammatory response initiated by a number of possible etiologies. Thus, many investigators have looked at drugs and compounds that could potentially attenuate this inflammation. Adenosine has been described as a “retaliatory metabolite” to inflammation [9]. However, given the fact that it has a short half life and causes significant vasodilation, adenosine is not a viable option as a therapeutic drug. In recent years, specific adenosine receptors have been isolated and their functions delineated. With these discoveries has also come the production of receptor specific analogues that can agonize or block these receptors. Specifically, it has been well demonstrated that a drug that agonizes the adenosine A_{2A} receptor can cause significant attenuation in the inflammatory response; in other words, activation of the A_{2A} receptor works as a breaking mechanism in inflammation. Use of an A_{2A} agonist has demonstrated attenuation of IR injury in spinal cord, renal, and cardiac models [10–13].

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Adenosine A_{2A} agonists have also demonstrated significant attenuation of IR injury in lung transplantation models [14, 15]. While one could theoretically apply these data to nontransplantation IR injury, one must consider the fact that IR injury in transplantation introduces a number of variables, each of which may increase or decrease the amount of injury. The cumulative effect of these variables is difficult to quantify. For example, it is known that hypothermia can lead to the production of reactive oxygen species [16]. This may amplify the effect of ischemia. Also, the immunological milieu is different in nontransplantation IR injury given the fact that the lung is reperfused by autologous blood rather than allogenic blood. The effect on reperfusion injury of this mixing of resident donor leukocytes with recipient circulating white cells is not well understood [17]. In the case of reperfusion injury in the nontransplantation setting, these variables are not at play and, thus, the actual disease process may be different. Thus, given the number of differences one can describe when comparing transplantation *versus* nontransplantation lung reperfusion injury, it is also unclear whether or not a drug will have the same effect. Our hypothesis was that an A_{2A} agonist would attenuate reperfusion injury in a hilar clamping model of lung reperfusion injury.

MATERIALS AND METHODS

Surgery

Adult male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) weighing between 325 and 375 g were anesthetized with 100 mg/kg of ketamine and 10 mg/kg xylazine, shaved, and prepared for surgery (Fig. 1). A tapered 16 gauge catheter was inserted into the trachea at the second tracheal ring via midline neck incision and secured with a 3-0 silk suture passed around the trachea with a small right angle. The animals were ventilated using a Harvard rodent ventilator (Harvard Apparatus Inc, Holliston MA) with a FIO₂ of 100%, PEEP of 3 cm H₂O, and 0.5% to 1% halothane for anesthesia.

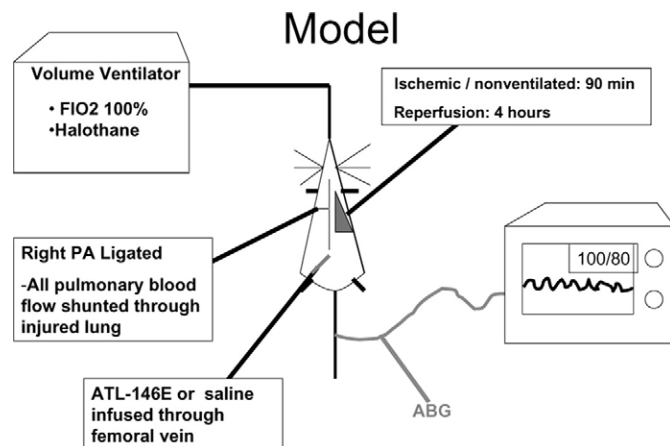


FIG. 1. Diagram of experimental protocol.

Via tail artery cut down, a 24 gauge angiocath was placed in the tail artery, allowing continuous blood pressure monitoring of the rat, as well as a port for arterial blood gases. Blood pressure was maintained in a physiological range for all animals throughout the experiment. Through a left anterolateral thoracotomy through the fifth interspace, dissection was carried out to create a window through the inferior pulmonary ligament. The hilum was bluntly stripped, and a silicon vessel loop was doubly passed around the left hilum. One hundred units/kg of heparin was flushed antegrade through the tail artery catheter, and approximately 5 min later the hilum was clamped using a Rammel tourniquet technique. The left lung was rendered hypoxic and nonventilated for 90 min, followed by 4 h of reperfusion. A 24 gauge catheter was also placed in the femoral vein for delivery of drug or saline infusion, which was delivered over a 3 h period starting 15 min before reperfusion and ending 2 h and 45 min after reperfusion.

Three hours post-reperfusion, a right sided anterolateral thoracotomy was performed. After dissection and skeletonization, the right pulmonary artery was clipped with a titanium clip (Horizon ligating clips; Weck Closure Systems, Research Triangle Park, NC), shunting all pulmonary flow to the left lung and allowing for physiological measurements of the left lung, which we drew at 4 h post-reperfusion. Sham animals received the same procedure as the control group but did not have their lung clamped.

Drug Administration

ATL-146e (4-[3-[6-Amino-9-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl]-9H-purin-2-yl]-prop-2-ynyl)-cyclohexanecarboxylic acid methyl ester) was synthesized and chemically characterized within the chemistry department of our institution [17a]. This compound is a selective agonist of A_{2A} adenosine receptors that has ubiquitous anti-inflammatory effects on the major inflammatory cells involved in the early inflammatory cascade, namely macrophages, neutrophils and endothelial cells. One group of animals ($N = 13$) received 0.06 ug/kg/min of ATL-146e, whereas the control ischemia reperfusion group ($N = 16$) received only saline vehicle. A sham group ($N = 14$) had the same entire procedure as the IR group but did not get hilar clamping. Drug or saline was administered in saline solution over a 3 h period at a rate of 1 cc/h using a calibrated syringe pump (Harvard Apparatus Inc., Holliston, MA).

Functional Lung Measurements

With all pulmonary arterial blood flow shunted through the left lung, we were able to assess the function of the lung. Arterial blood gasses were obtained at 4 h post-reperfusion in all animals. With this information we were able to assess oxygenation by pO₂ as well as ventilation by pCO₂.

Histology

After tissue processing and staining for hematoxylin and eosin, lung samples were graded by a blinded pathologist in a manner previously described [15]. Each sample was graded with a lung injury score on the basis of the amount of neutrophils, interstitial infiltrate, and alveolar edema. Each of these three 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.

Myeloperoxidase (MPO) Assay

A MPO assay was performed to quantify neutrophils sequestration. Lung tissue was placed in 5 mL of 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate solution (pH7.4) and disrupted by homogenizing at 4 C. The solution was centrifuged at 15,000 g for 15 min at 4°C and the supernatant was discarded. The pellet was resuspended in 2 mL of 0.5% hexadecyl-

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

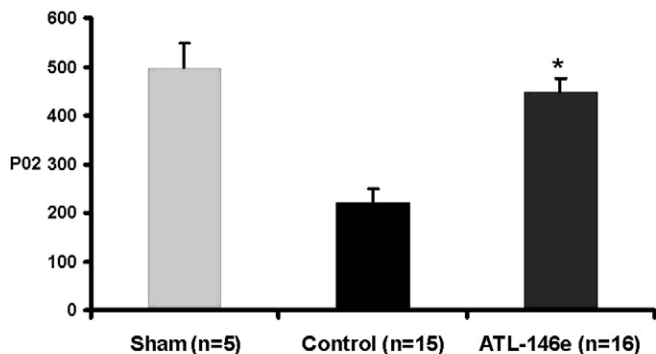
Note. Scoring system used to evaluate lung injury in the current study on the basis of neutrophil infiltration, alveolar edema, and interstitial edema.

rimethylammonium in 50 mmol/L potassium phosphate solution (pH 6.0) and homogenized. Tissue was disrupted further by sonication and three freeze-thaw cycles (liquid nitrogen bath/37°C water bath). The solution was again centrifuged at 15,000 g for 15 min at 4°C. Aliquots of (0.1 mL) of supernatant were added to the assay buffer of O-dianisidine dihydrochloride, H₂O₂, and 50 mmol/L potassium phosphate (pH 6.0). Absorbance at 460 nm was measured during a period of 2 min by spectrophotometry (LKB model 4050, Cambridge, United Kingdom). Protein concentration for each of the lung samples was measured by using a BCA protein assay kit from Pierce (Rockford, IL). Protein concentrations were calculated by comparing the absorbance at 595 nm of the experimental samples with that of known bovine serum albumin standard concentrations in the same assay. Lung tissue MPO activity was expressed as change in absorbance per gram of protein per min.

Bronchoalveolar Lavage (BAL)

BAL was performed at the end of the experiment using 3 cc of normal saline injected into the left lung while clamping the right hilum. The recovered fluid was then centrifuged (model 5804R; Eppendorf North America, Westbury, NY) for 10 min at 4°C. Protein concentration in the supernatant was measured by using the BCA protein assay (Pierce Biotechnology, Inc, Rockford, IL) and using a spectrophotometer (BioMate 3; Thermo Electron Corp, Waltham, MA). Cytospin (Thermo Shandon, Ltd, Astmoor, United Kingdom) was used to plate the cells and using the Diff-Quick staining process

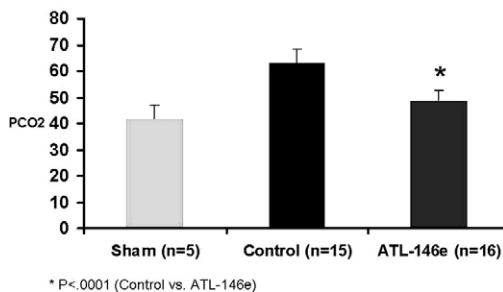
OXYGENATION: 4 HOURS POST REPERFUSION



* P<.0001 vs. Control

FIG. 2. PO₂ obtained via the tail artery arterial blood gas obtained at 4 h post-reperfusion. Right pulmonary artery had been clamped, thus arterial blood gas is measurement of left lung function.

VENTILATION: 4 HOURS REPERFUSION



* P<.0001 (Control vs. ATL-146e)

FIG. 3. PCO₂ obtained via the tail artery arterial blood gas obtained at 4 h post-reperfusion.

(Dade Behring AG, Düringen, Switzerland), the percentages of lymphocytes, neutrophils, and macrophages were assessed by blinded microscopic evaluation.

RESULTS

Lung Function

Based on blood gases obtained after 4 h of reperfusion, the group that received the drug ATL-146e had significantly better lung function as measured by oxygenation compared with the IR group (Fig. 2). The sham group had a mean PO₂ of 532.39, and the ATL and IR groups had PO₂s of 447.92 and 233.26, respectively (P < 0.001 ATL versus IR). Ventilation as measured by PCO₂ at 4 h of reperfusion was also significantly better in the ATL group compared with the IR group (48.78 versus 63.56 (P = 0.009 ATL versus IR) (Fig. 3).

BAL

BAL demonstrated significantly lower levels of total protein in the adenosine group when compared with the IR group (Fig. 4). Again, this represents decreased lung inflammation and decreased capillary leak. A dif-

BAL Protein Concentration

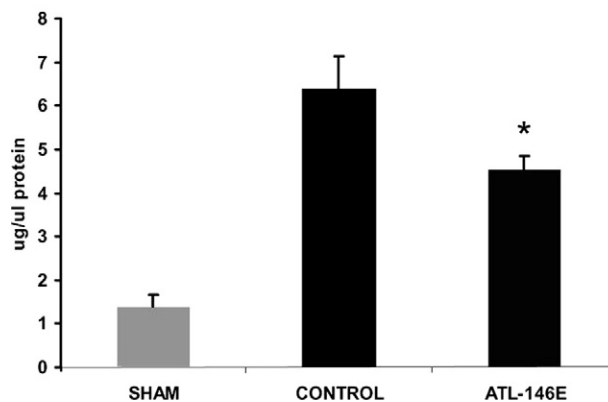


FIG. 4. Protein concentration of BAL obtained after 4 h of reperfusion.

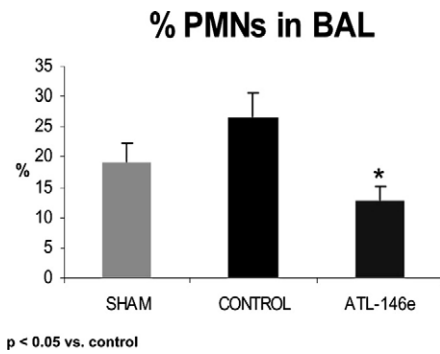


FIG. 5. Percentage of polymorphonuclear lymphocytes in BAL obtained after 4 h of reperfusion.

ferential count of the BAL cell count was performed. The count demonstrated half as many neutrophils in the drug group *versus* controls, which does correlate with the drug's known specific effect on decreasing neutrophil activation and margination (Fig. 5). Macrophage and lymphocyte concentrations were not significantly different between groups.

MPO activity, which is an indicator of tissue neutrophils, demonstrated a trend toward decreased activity in the adenosine group, again supporting evidence for the drug's inhibitory effect on neutrophil margination and activation (Fig. 6).

Histology

Histologically, the adenosine group appeared to have less interstitial edema and inflammation with preservation of alveoli and thinner septae compared with the IR group, and appeared more like the sham group (Fig. 7). Blinded histological analysis also demonstrated findings consistent with the trends toward higher neutrophil content seen in the MPO analysis (Fig. 8). There were significantly fewer neutrophils seen on histological analysis compared with the sham and IR groups. Interstitial infiltrate and alveolar edema were not significantly different.

MYELOPEROXIDASE ACTIVITY

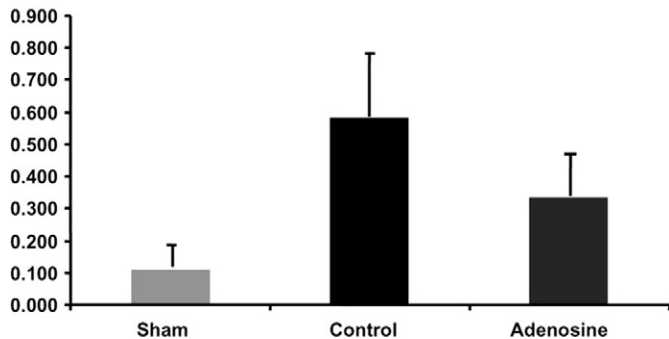


FIG. 6. MPO activity in lung tissue obtained after 4 h of reperfusion. This is an indirect measurement of neutrophil activity.

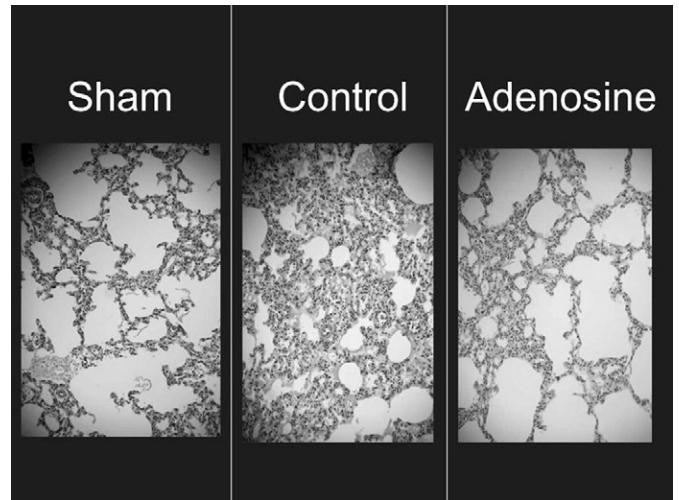


FIG. 7. Micrographs of lung tissue stained with hematoxylin and eosin. Note the increased interstitial edema of the control group compared with the adenosine group.

DISCUSSION

Lung reperfusion injury continues to be a problem in lung transplantation [2, 18]. A number of strategies are currently used to decrease the chances of having lung reperfusion injury, though the multitude of initiating factors that lead to IR injury are difficult to control. Therefore, it would be ideal to find a drug that can be used at the time of transplantation that could theoretically attenuate the lung reperfusion injury, no matter what the cause.

The pathogenesis of ischemia-reperfusion injury in clinical lung transplantation is complex and multifaceted [1]. Although the cause of IR injury is still not well known, once initiated, IR injury follows a relatively consistent pathophysiologic pattern. At the time of reperfusion there is a generation of free radicals partially due to the buildup of xanthine and the conversion of xanthine reductase to xanthine oxidase. This production of toxic metabolites, potentially exacerbated by

Histology

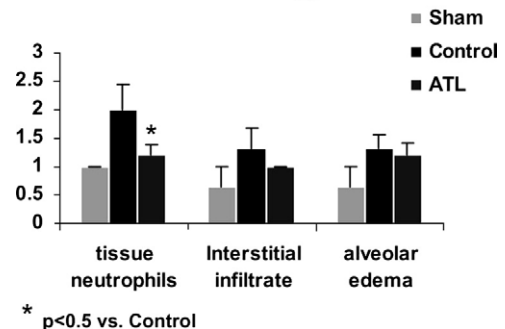


FIG. 8. Histologic grading performed by a blinded pathologist. A significantly lower number of neutrophils was noted in the adenosine group.

hyperoxic oxygen delivery, leads to lung inflammation and IR injury [19]. IR injury is then thought to occur in a two hit process, with donor macrophages initiating the early injury and initiating the early inflammatory cascade, followed by the recruitment of recipient neutrophils and lymphocytes [20, 21]. Given the fact that IR injury is thought to be caused largely by what is considered collateral damage caused by the host's immune system, drugs that could potentially attenuate this response have long been the holy grail of IR research.

For a number of years, it has been known that adenosine has anti-inflammatory effects at the tissue level and acts as a breaking mechanism in inflammation [9]. It has now become more evident that the anti-inflammatory effects are the result of A_{2A} receptor agonists. There are a number of mechanisms by which adenosine is thought to attenuate lung reperfusion injury. One mechanism that has been proposed is adenosine's ability to inhibit the leukocyte-endothelial interaction normally needed for margination, which then leads to the migration of leukocytes into the interstitium where they promote further inflammation [22]. It has also been shown that adenosine decreases superoxide production by vascular endothelial cells [23]. Cronstein *et al.* have demonstrated adenosine's ability to directly down-regulate neutrophil activation [24].

Use of an adenosine A_{2A} receptor agonist has led to attenuation of reperfusion injury in heart, kidney, spinal cord, and in the isolated as well as *in vivo* lung transplantation models [12, 14, 15, 25]. This attenuation is manifested by decreased neutrophil priming and superoxide production, decreased neutrophil adherence to the vascular endothelium, a marked decrease in systemic tumor necrosis factor-alpha levels, and in each organ examined, marked functional improvements compared with controls [25–29].

We have previously demonstrated that an A_{2A} agonist can decrease lung reperfusion injury in transplantation models of lung reperfusion injury. Using an isolated rabbit lung preparation perfused with allogeneic blood, Ross demonstrated improved oxygenation, decreased pulmonary vascular resistance, improved airway compliance, decreased microvascular permeability, and lower MPO in the group receiving ATL-146e, an adenosine A_{2A} agonist [14]. More recently, Reece demonstrated similar results using ATL-146e in an *in vivo* pig lung transplantation. Compared with the control group, the adenosine group demonstrated improved ventilation, oxygenation, mean airway pressures, lower MPO levels, lower wet to dry ratios, and a trend toward decreased tumor necrosis factor-alpha levels [15].

While these results are promising for the use of an adenosine agonist in the prevention of reperfusion injury, they are specific to lung transplantation. There

are a number of reasons why it is important to examine the effects of A_{2A} agonism in a nontransplant model of lung reperfusion injury. First, reperfusion also occurs in an injury not specific to lung transplantation, as it is seen in cardiopulmonary bypass, pulmonary resection with vascular reconstruction, and pulmonary thromboendarterectomy [4, 5, 7]. It is also well known that allogeneic blood transfusion can have significant immunomodulatory effects [30]. Furthermore, allogeneic transfusion can cause acute lung injury that manifests itself in a clinically similar way to lung reperfusion injury [31]. It is also known that using a leukocyte filter can decrease lung reperfusion injury, giving further credence to the hypothesis that the circulating blood has a significant contribution to reperfusion injury [32]. And finally, the effect of resident donor leukocytes in the transplanted lung is unknown [17]. Therefore, it was important to take the potential immunomodulatory or potentially harmful effects of allogeneic blood out of the equation and examine the effects of an A_{2A} agonist on an IR model that used the animal's own blood for reperfusion.

Recently, Rivo *et al.* published a paper examining the effects on an *in vivo* model of lung reperfusion injury in the cat using an A_{2A} agonist, ATL-313. Rivo *et al.* used a different model than ours, as they use an endovascular technique to block the pulmonary artery to the left lower lobe, as well as a bronchial blocker [33]. Although their model is not designed to assess the physiological derangements caused by ischemia reperfusion injury like ours, they also demonstrated attenuation of lung reperfusion injury as evidenced by increased wet to dry ratios, increased MPO, and increased alveolar infiltration with neutrophils, macrophages, and red blood cells. They also demonstrated decreased apoptosis using a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay in the groups that received adenosine compared with those that did not.

Hasko *et al.* demonstrated that an A_{2A} agonist cgs-21680, reduced lung injury in a hemorrhagic shock model [34]. In concordance with our study, they showed that treatment with the adenosine agonist decreased lung injury in the hemorrhagic shock model, which was manifested by decreased lung permeability and MPO levels. Interestingly, they looked at both pretreatment and post-treatment and had similar results with both groups. The half-life of our compound in theory would not make it effective in a pretreatment setting; however compounds with longer half-lives are now becoming more available.

Using a novel modification of the hilar clamping model, we were able to demonstrate attenuation of lung reperfusion injury using an adenosine A_{2A} agonist. We demonstrated significant improvements in oxygenation and ventilation. We also demonstrated sig-

nificantly less edema, lower protein and neutrophils in the BAL, and fewer tissue neutrophils on histological examination. These data seem to demonstrate that the effects of A_{2A} agonism are indeed specific to attenuating the reperfusion injury itself regardless of how the lungs are reperfused. This is the first such study that we are aware of that has demonstrated attenuation of lung reperfusion injury with an adenosine A_{2A} agonist using a hilar clamping model.

In conclusion, we have demonstrated conclusively that A_{2A} activation leads to attenuation of lung reperfusion injury. This attenuation occurs in the presence or absence of allogeneic blood. It likely causes its effects in a number of ways, immunomodulating both tissue and hematological immune factors. Further study will be needed to elucidate precisely where this immunomodulation is occurring.

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