

## Adenosine A<sub>2A</sub> receptor activation decreases reperfusion injury associated with high-flow reperfusion

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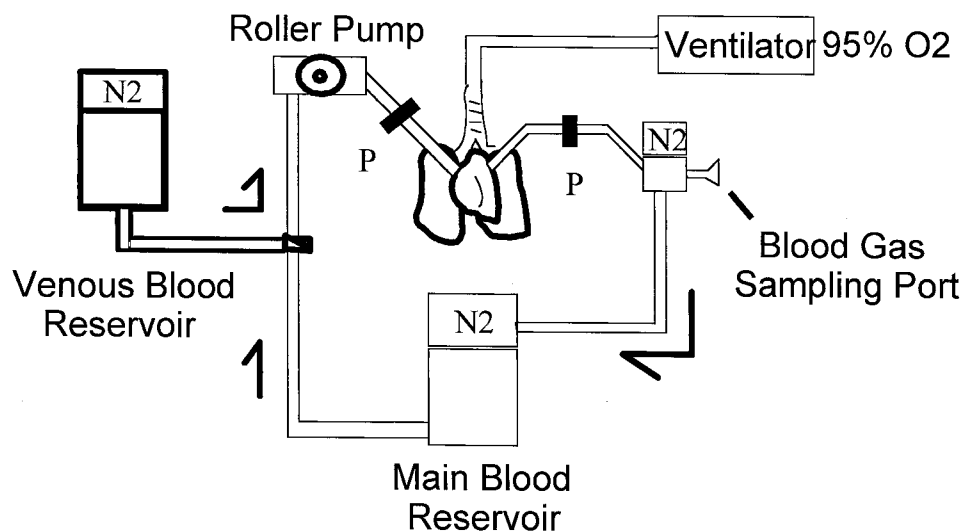
**Introduction:** High pulmonary artery flow rates can result in severe reperfusion injury after lung transplantation. Our hypothesis was that selective activation of the adenosine A<sub>2A</sub> receptor with a highly specific analog (ATL-146e) would inhibit leukocyte activation and decrease reperfusion injury after high-flow reperfusion.

**Methods:** Using our isolated, ventilated, blood-perfused rabbit lung model, all groups (n = 8 per group) underwent lung harvest, 4 hours of cold storage, and blood reperfusion for 30 minutes. Measurements of pulmonary artery pressure (in millimeters of mercury), arterial oxygenation (in millimeters of mercury), myeloperoxidase, peak inspiratory pressure, and wet/dry weight ratio were obtained. Groups 1 (high flow) and 2 (high flow ATL-146e) underwent reperfusion at 120 mL/min for 30 minutes. Groups 3 (controlled high flow) and 4 (controlled high flow ATL-146e) underwent controlled reperfusion with an initial reperfusion of 60 mL/min for the first 5 minutes, followed by a rate of 120 mL/min for 25 minutes. During reperfusion, groups 2 and 4 received ATL-146e at 4  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ .

**Results:** ATL-146e significantly improved lung physiologic measurements under both high-flow (group 1 vs group 2) and controlled high-flow (group 3 vs group 4) conditions after 30 minutes.

**Conclusions:** The adenosine A<sub>2A</sub> receptor analogue ATL-146e significantly decreases the severity of reperfusion injury in the setting of both high-flow and controlled high-flow reperfusion.

**D**espite improvements in lung transplantation, transplanted lungs remain vulnerable to ischemia-reperfusion injury, with severe graft dysfunction occurring in 20% of lung transplant recipients.<sup>1,2</sup> Although severe graft dysfunction can be reversible, it is often associated with the need for prolonged intensive care and increased mortality.<sup>3,4</sup> It has become increasingly evident that reperfusion is responsible for the majority of tissue injury after lung transplanta-



**Figure 1. Isolated, blood-perfused rabbit lung model: diagram of the isolated lung model. P, Pressure transducer; N<sub>2</sub>, nitrogen gas; O<sub>2</sub>, oxygen gas.**

tion.<sup>5</sup> Additionally, some investigators have suggested that high pulmonary artery flow rates increase the risk of severe reperfusion injury after lung transplantation. In our clinical practice, patients with preexisting pulmonary hypertension are at increased risk for reperfusion injury.<sup>6</sup> Other authors have also shown that a more severe reperfusion injury seems to occur in these patients as well.<sup>7-9</sup> The increased risk for reperfusion injury in these patients might be the result of activation of the inflammatory cascade against endothelium that is damaged as a result of the high-flow state. Our hypothesis was that selective activation of the adenosine A<sub>2A</sub> receptor with a highly specific analogue (ATL-146e) would inhibit leukocyte activation and decrease reperfusion injury after high-flow reperfusion.

## Materials and Methods

### Experimental Protocol

Using our isolated, ventilated, blood-perfused rabbit lung model, all groups (n = 8 per group) underwent lung harvest, 4 hours of cold storage, and blood reperfusion for 30 minutes. Groups 1 (high flow) and 2 (high flow ATL) underwent reperfusion at 120 mL/min for 30 minutes. Groups 3 (controlled high flow) and 4 (controlled high flow ATL) underwent controlled reperfusion with an initial reperfusion rate of 60 mL/min for the first 5 minutes, followed by a rate of 120 mL/min for 25 minutes. During reperfusion, groups 2 and 4 received ATL-146e at  $4 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ . This dose was selected after a preliminary experiment with doses of ATL-146e at 0.01, 0.1, 1.0, and  $4.0 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ .

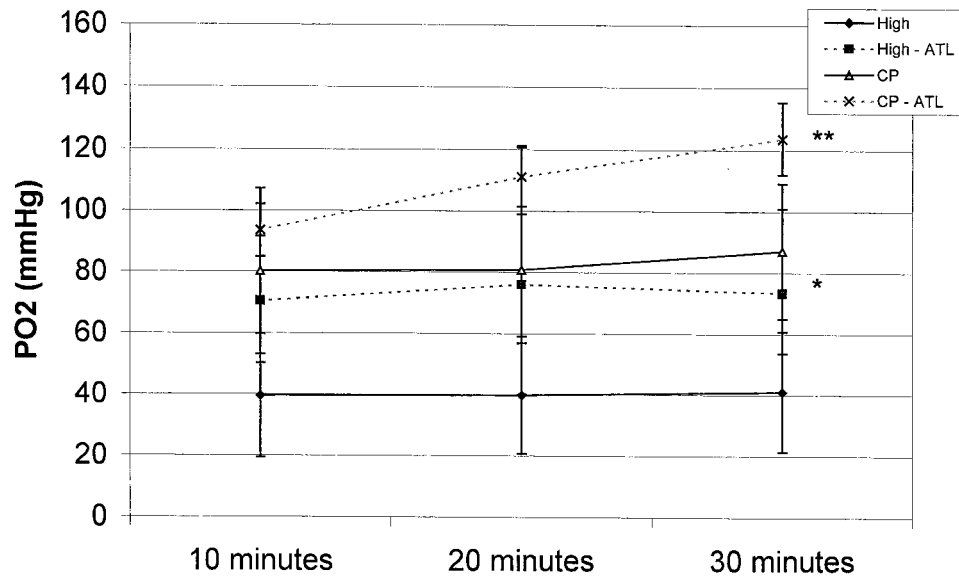
### Harvest Procedure

Adult New Zealand White rabbits of both sexes weighing 3.0 to 3.5 kg were randomly assigned to the 3 experimental groups. Animals were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg). Tracheal intubation was performed

through a tracheostomy, and mechanical ventilation was instituted with a constant pressure ventilator (#RSP1002; Kent Scientific Corp, Litchfield, Conn) with room air at a rate of 18 breaths/min. A median sternotomy and thymectomy were then performed. The 2 superior and 1 inferior venae cavae were loosely encircled with ligatures, and the pericardium was opened. Both the pulmonary artery (PA) and aorta were dissected free and similarly encircled. A purse-string suture was placed in the free wall of the right ventricle, and intravenous heparin was administered (500 U/kg). After injection of  $30 \mu\text{g}$  of alprostadil (prostaglandin E<sub>1</sub>; Upjohn Company, Kalamazoo, Mich) into the PA, the cavae were interrupted, and onset of ischemia was noted. The PA was then cannulated through a right ventriculotomy placed in the center of the purse-string suture. Both the right ventricle and PA ligatures were tied to secure the cannula. After venting the left ventricle with a left ventriculotomy and ligating the aorta, 50 mL/kg Euro-Collins (Hamburg, Germany) preservation solution was infused at 4°C into the PA from a height of 30 cm. Topical cooling was achieved with cold saline solution slush. During the PA flush, the left atrium was cannulated through the left ventriculotomy with an outflow catheter and a catheter to directly transduce left atrial pressures. A purse-string suture was placed to secure these cannulas. After completion of the PA flush, the inflow and outflow cannulas were clamped. The heart-lung block was then excised, and the tracheostomy tube was clamped at end inspiration. The inflated lungs were stored immersed in saline solution at 4°C for 4 hours. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (National Institutes of Health publication No. 85-23, revised 1996).

### Reperfusion Procedure

After organ harvest and ischemic storage, the heart-lung block was suspended in a warm, humidified tissue chamber, and ventilation was reestablished with a 95% oxygen and 5% carbon dioxide gas



**Figure 2. Arterial oxygenation. The high-flow ATL group (*High-ATL*) had significantly improved arterial oxygenation after 30 minutes of reperfusion compared with that of the high-flow (*High*) group. Similarly, the controlled high-flow ATL (*CP-ATL*) group had significantly improved arterial oxygenation compared with that of the controlled high-flow (*CP*) group. \* $P = .002$ , high-flow ATL group versus high-flow group; \*\* $P = .004$ , controlled high-flow group ATL versus controlled high-flow group.**

mixture at a respiratory rate of 18 breaths/min by using the constant pressure ventilator (Figure 1). The inflow and outflow cannulas were then connected to a venous blood reperfusion circuit. New Zealand White rabbits weighing 3.5 to 5.0 kg served as fresh venous blood donors. The lungs were reperfused with venous blood from a main reservoir. A second nonrecirculated venous blood reservoir was used to challenge the lungs and to determine the single-pass oxygenation values during reperfusion. The circuit (Kent Scientific Corp) was designed to recirculate 150 mL of warmed blood with a roller pump (#7521-40; Cole Palmer Instrument Co, Chicago, Ill).

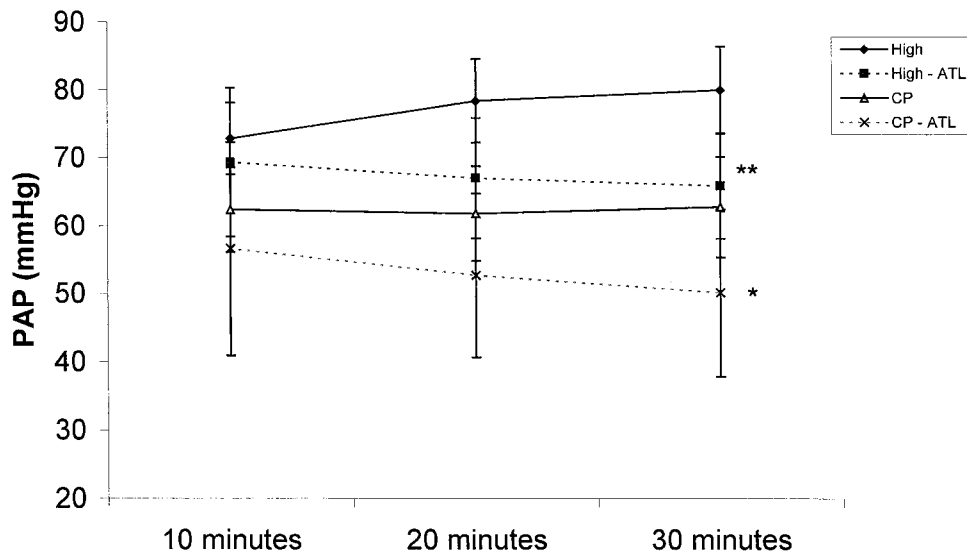
Continuous recordings of pulmonary artery pressure (PAP), left atrial pressure, and air flow were performed with a dynamic data-acquisition program (Workbench PC; Strawberry Tree, Inc, Sunnydale, Calif) on a personal computer (#470A; Compaq Prolinea, Houston, Tex). This program automatically calculated and displayed tidal volume, pulmonary vascular resistance, and dynamic airway compliance. Pulmonary venous blood samples were collected for blood gas analysis (Corning 178pH/Blood Gas Analyzer; Corning Inc, Corning, NY) after 10, 20, and 30 minutes of reperfusion. At each sampling interval, inflow from the main reservoir was temporarily interrupted, and the circuit was filled with nonrecirculated blood from the second inflow reservoir. A 30-mL sample of venous blood was passed through the pulmonary vasculature at each interval to obtain accurate measurements of pulmonary venous oxygen content. Oxygen contact with exposed blood surfaces was minimized by means of continuous passive infusion of 100% nitrogen.

### Lung Wet/Dry Weight Ratios

Lung wet/dry weight ratios were used as a measurement of pulmonary edema. Samples of lung tissue were weighed immediately after reperfusion. These samples then underwent passive desiccation at room temperature until a stable dry weight was achieved. The weight immediately after reperfusion and the stable dry weight were then used to calculate the lung wet/dry weight ratios.

### Lung Tissue Myeloperoxidase

A myeloperoxidase assay was performed to quantify neutrophil sequestration. Lung tissue was placed in 5 mL of 0.5% hexadecyltrimethyl-ammonium bromide in 50 mmol/L potassium phosphate solution (pH 7.4) and disrupted by means of homogenizing at 4°C. The solution was centrifuged at 15,000g for 15 minutes at 4°C, and the supernatant was discarded. The pellet was resuspended in 2 mL of 0.5% hexadecyltrimethyl-ammonium bromide in 50 mmol/L potassium phosphate solution (pH 6.0) and homogenized. Tissue was disrupted further by means of ultrasonication and 3 freeze-thaw cycles (liquid nitrogen bath/37°C water bath). The solution was again centrifuged at 15,000g for 15 minutes at 4°C. Aliquots (0.1 mL) of supernatant were added to the assay buffer of *o*-dianisidine dihydrochloride, H<sub>2</sub>O<sub>2</sub>, and 50 mmol/L potassium phosphate (pH 6.0). Absorbance at 460 nm was measured over 2 minutes by means of spectrophotometry (LKB Model 4050, Cambridge, England). Lung tissue myeloperoxidase activity was expressed as the change in absorbance per milligram of dry weight per minute.



**Figure 3. Pulmonary artery pressure. The high-flow ATL group (*High-ATL*) had a significantly lower PAP compared with that of the high-flow (*High*) group. Similarly, the controlled high-flow ATL (*CP-ATL*) group had significantly improved PAP measurements compared with those of the controlled high-flow (*CP*) group. \* $P < .015$ , high-flow ATL group versus high-flow group; \*\* $P < .003$ , controlled high-flow ATL group versus controlled high-flow group.**

**Statistical Analysis**

Statistical analysis was performed by using analysis of variance on SPSS software (SPSS Inc, Chicago, Ill). Significant differences were determined by using the Tukey significant difference test. Data are expressed as means  $\pm$  SD.

**Results**

**Physiologic Measurements**

The high-flow ATL group ( $73.4 \pm 22.0$  mm Hg,  $P = .002$ , Figure 2) had significantly improved arterial oxygenation measurements compared with those of the high-flow group ( $41.0 \pm 11.7$  mm Hg) after 30 minutes of reperfusion. Similarly, the controlled high-flow ATL group ( $123.6 \pm 19.7$  mm Hg,  $P = .004$ ) had significantly improved arterial oxygenation measurements compared with those of the high-flow group ( $87.0 \pm 27.5$  mm Hg) after 30 minutes of reperfusion.

PAP was significantly lower in the high-flow ATL group ( $66.0 \pm 7.4$  mm Hg,  $P = .015$ , Figure 3) compared with that of the high-flow group ( $80.1 \pm 12.3$  mm Hg). Similarly, the controlled high-flow ATL ( $50.3 \pm 6.4$  mm Hg,  $P = .003$ ) group had significantly lower PAP measurements compared with those of the controlled high-flow group ( $62.9 \pm 7.8$  mm Hg) after 30 minutes of reperfusion.

There was a trend toward decreased peak airway pressure in the high-flow ATL group ( $11.8 \pm 0.45$  mm Hg,  $P = .086$ ) compared with that in the high-flow group ( $13.7 \pm 0.57$  mm Hg). There were no significant differences in peak airway pressure in the controlled high-flow ATL group

( $18.5 \pm 0.64$  mm Hg,  $P = .29$ ) compared with values in the controlled high-flow group ( $19.5 \pm 0.68$  mm Hg).

**Lung Wet/Dry Weight Ratio**

The wet/dry weight ratio was significantly lower in the high-flow ATL group ( $11.8 \pm 1.28$ ,  $P = .018$ ) compared with that in the high-flow group ( $13.7 \pm 1.62$ ). Wet/dry weight ratio was not significantly different between the controlled high-flow ATL group ( $6.0 \pm 1.49$ ,  $P = .184$ ) and the controlled high-flow group ( $6.9 \pm 1.01$ ).

**Tissue Myeloperoxidase Assay**

Myeloperoxidase activity was not significantly different between the high-flow ( $0.31 \pm 0.09$   $\Delta$ absorbance/mg dry weight/min) and high-flow ATL groups ( $0.29 \pm 0.09$   $\Delta$ absorbance/mg dry weight/min). Similarly, myeloperoxidase activity was not significantly different between the controlled high-flow ( $0.22 \pm 0.08$   $\Delta$ absorbance/mg dry weight/min) and controlled high-flow ATL ( $0.17 \pm 0.06$   $\Delta$ absorbance/mg dry weight/min) groups.

**Discussion**

Approximately 20% to 30% of all lung transplant recipients experience reperfusion injury, which has an associated mortality of 40% in some series.<sup>1,2</sup> Although the exact mechanism of this process has not been fully discerned, clinical studies have suggested that certain patient populations, such as patients with preexisting pulmonary hypertension, might be at increased risk for reperfusion injury after lung trans-

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plantation.<sup>7-9</sup> In our own clinical practice, patients with preexisting pulmonary hypertension are at increased risk of reperfusion injury.<sup>6</sup> A more severe reperfusion injury seems to occur in patients with preexisting pulmonary hypertension as well.<sup>7-9</sup> This patient population would include patients with primary pulmonary hypertension in addition to patients with secondary pulmonary hypertension as a result of congenital heart defects. Additionally, however, patients undergoing lung transplantation for restrictive lung diseases often have severe pulmonary hypertension as a result of pulmonary fibrosis. Other authors, in addition to the present ones, have shown an increased risk of reperfusion injury in patients with restrictive lung disease.<sup>6,10</sup>

One possible mechanism by which those patients have reperfusion injury is through mechanical disruption of the pulmonary vascular endothelium. This would result in increased pulmonary edema and lung dysfunction as a result of the disrupted endothelium. Another possible mechanism for the increased reperfusion injury seen in those patients is through increased hydrostatic gradient created by increased PAP, which might serve to drive fluid across endothelial membranes. Additionally, endothelial disruption results in collagen exposure, which might be serving to activate circulating leukocytes against the transplanted lung. Many studies have shown that circulating white blood cells are one of the major mediators of reperfusion injury after lung transplantation.<sup>11-15</sup>

One of the major anti-inflammatory mechanisms used by endothelial cells is mediated through the release of adenosine.<sup>2</sup> Adenosine receptors are ubiquitous membrane receptors found on many inflammatory cell types, including macrophages and neutrophils.<sup>16-20</sup> Activation of these receptors results in suppression of the inflammatory function of these cells. Adenosine has been shown to be a protective agent in models of warm lung ischemia-reperfusion injury,<sup>21</sup> cardiac ischemia-reperfusion injury,<sup>22</sup> intestinal reperfusion injury,<sup>23</sup> and hepatic ischemia-reperfusion injury.<sup>24</sup> Different types of adenosine receptors exist ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ ) and have different functions. For example, the adenosine  $A_1$  receptor can have proinflammatory effects, including neutrophil chemotaxis and neutrophil adherence to endothelial cells.<sup>22</sup> To avoid these proinflammatory effects, we chose a selective  $A_{2A}$  receptor analogue. Adenosine receptor subclassification has shown specifically that activation of the adenosine  $A_{2A}$  receptor prevents leukocyte adhesion to endothelial cells, as well as inhibiting the release of toxic oxygen products.<sup>17</sup>

The present study demonstrates that leukocyte inhibition with ATL-146, which is a highly potent and specific activator of the adenosine  $A_{2A}$  receptor,<sup>25</sup> results in improved arterial oxygenation and PAP under both high-flow and controlled high-flow reperfusion conditions. Additionally, pulmonary edema, as assessed on the basis of wet/dry

weight ratio, was significantly lower in the high-flow ATL group compared with in the high-flow group. These data would suggest that the inflammatory cascade has a role in reperfusion injury after high-flow reperfusion.

Interestingly, although myeloperoxidase activity was lower in the high-flow ATL group compared with that in the high-flow group and in the controlled perfusion ATL group compared with that in the controlled perfusion group, these comparisons did not reach statistical significance. Had the experiment been carried out for a longer time period, the gradual buildup of sequestered leukocytes might have brought out more of a difference between the various groups. Another possibility, however, is that the adenosine analogue is acting on cells other than circulating leukocytes. Previously, we have shown that circulating leukocytes are not significantly involved in reperfusion injury until after 2 hours of reperfusion.<sup>15</sup> Furthermore, our studies have also suggested that intrinsic pulmonary macrophages might be involved in the earliest phase of reperfusion injury after lung transplantation.<sup>26</sup> Alveolar macrophages, in addition to circulating neutrophils, are known to carry the  $A_{2A}$  receptor and thus might be the initial key target for the  $A_{2A}$  analogue. Another possibility is that the  $A_{2A}$  analogue was acting directly on the pulmonary vasculature, leading to vasodilation, although many studies have shown the action of this drug is on white blood cells.<sup>16-20</sup>

One further observation in this study was the effect of controlled perfusion on reperfusion injury. Controlled perfusion seemed to improve physiologic measurements compared with high-flow reperfusion. Our group has previously published on this topic.<sup>27</sup>

In summary, high-flow reperfusion can cause significant lung dysfunction after lung transplantation. Adenosine  $A_{2A}$  receptor activation with ATL-146 significantly reduces reperfusion injury associated with high-flow and controlled high-flow conditions, possibly by inhibiting white blood cells. Thus adenosine  $A_{2A}$  agonists might have a future clinical role in lung transplantation.

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## Discussion

**Dr Ralph A. Schmid** (Berne, Switzerland). Why did you use the Euro-Collins solution in these experiments? I think the era of Euro-Collins is over.

**Dr Fiser.** We still use Euro-Collins at our institution.

**Dr Schmid.** Did you look at reperfusion for longer times than 30 minutes? This is a very short observation time, and therefore the differences might not be significant.

**Dr Fiser.** No, we only looked at 30 minutes. I think our future studies involving high-flow reperfusion should be carried out for more extended time periods.