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Adenosine A_{2A} Agonist Improves Lung Function During Ex-vivo Lung Perfusion

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Abstract

Background—Ex-vivo lung perfusion (EVLP) is a novel technique to assess, and potentially repair marginal lungs that may otherwise be rejected for transplantation. Adenosine has been shown to protect against lung ischemia-reperfusion injury through its A_{2A} receptor. We hypothesized that combining EVLP with adenosine A_{2A} receptor agonist treatment would enhance lung functional quality and increase donor lung usage.

Methods—Eight bilateral pig lungs were harvested and flushed with cold Perfadex. After 14 hours storage at 4°C, EVLP was performed for 5 hours on two explanted lung groups: 1) Control group lungs (n=4), were perfused with Steen Solution and Dimethyl sulfoxide (DMSO), and 2) treated group lungs (n=4) received 10μM CGS21680, a selective A_{2A} receptor agonist, in a Steen Solution-primed circuit. Lung histology, tissue cytokines, gas analysis and pulmonary function were compared between groups.

Results—Treated lungs demonstrated significantly less edema as reflected by wet-dry weight ratio (6.6 vs. 5.2, p<0.03) and confirmed by histology. In addition, treated lung demonstrated significantly lower levels of interferon gamma (45.1 vs. 88.5, p<0.05). Other measured tissue cytokines (interleukin (IL) 1 beta, IL-6, and IL-8) were lower in treatment group, but values failed to reach statistical significance. Oxygenation index was improved in the treated group (1.5 vs. 2.3, p<0.01) as well as mean airway pressure (10.3 vs. 13 p<0.009).

Conclusions—EVLP is a novel and efficient way to assess and optimize lung function and oxygen exchange within donor lungs, and the use of adenosine A_{2A} agonist potentiates its potential. EVLP with the concomitant administration of A_{2A} agonist may enhance donor lung quality and could increase the donor lung pool for transplantation.

Keywords

Lung transplantation; Lung preservation; Ex vivo lung perfusion

Introduction

Lung transplantation remains a widely accepted treatment for the majority of end stage lung diseases. However, there are still major obstacles in the way of successful lung transplantation that include: 1) shortage of suitable donors; 2) ischemia-reperfusion (IR)

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injury; 3) allograft rejection and 4) the development of bronchiolitis obliterans (BO) and chronic graft rejection (1).

Despite breakthroughs in donor management and organ preservation, fewer than 30% of lungs are considered transplantable by currently accepted criteria (2), and the majority of lungs are removed from the donor pool because of their “marginal” status. Donor lung injury may be a result of mechanisms of death, excessive fluid resuscitation, aspiration, or neurogenic edema (3, 4). Shortages in suitable lungs for transplantation contribute to delays in lung transplantation and increase patient morbidity and mortality. Many lung transplant centers utilize “extended criteria” or “marginal” lungs with acceptable short and long-term results. Utilizing organs from non-heart beating donors (NHBD) provides an additional alternative to increase the number of available donor lungs. Several groups have successfully transplanted lungs from NHBD (5–7). However, proper assessment of such organs prior to transplantation is crucial. Steen et al (8) addressed this problem by developing an ex vivo perfusion circuit, in which lungs are perfused and ventilated in physiologic condition. While the primary use of ex vivo perfusion is to assess lung quality, recent studies have demonstrated its use in the preservation and salvage of previously unacceptable organs (9).

Adenosine is an endogenous compound released in response to inflammation, exerting both pro- and anti-inflammatory effects through binding to four adenosine receptors (A_1 , A_{2A} , A_{2B} , and A_3). Based on our prior experience examining acute lung injury and the effects of adenosine, we hypothesized that ex-vivo perfusion of lungs with adenosine A_{2A} agonist after significant ischemic injury would attenuate the acute inflammatory response to IR injury and prove beneficial in maintaining pulmonary function and mitigating edema formation.

Materials and Methods

Animals 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. 86-23, revised 1996). The study protocol was approved by the University of Virginia Animal Care and Use Committee before experimentation.

Study design

Domestic pigs ($n=8$) with a weight range of 30–40 kg were randomly assigned into 2 groups. The control group underwent 14 hours of ischemia followed by 5 hours of EVLP with Steen Solution (Vitrolife, Denver, CO) and Dimethyl sulfoxide (DMSO). The treatment group underwent the same procedure but received CGS-21680 (Sigma-Aldrich, St. Louis, MO), a selective A_{2A} receptor agonist. A_{2A} receptor agonist dosing was determined based upon our previous experiments in cell culture and murine model of IR injury. A dose of 10 μ M of the compound was added to the prime at the beginning of perfusion and at 3 hours.

Donor lung retrieval and preservation

The donor lung harvest procedure was identical for both groups and is described in detail elsewhere (10). Briefly, pigs were anesthetized with intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Endotracheal intubation was performed and lungs were ventilated with an inspired oxygen fraction (FiO_2) of 1.0, tidal volume of 8.0 ml/kg, respiratory rate of 18–20 and positive end expiratory pressure (PEEP) of 5.0 cm H_2O . Anesthesia was maintained by 2.0% isoflurane throughout the procedure. Following administration of 200 U/kg heparin sodium, a median sternotomy was performed, and the

pericardium and bilateral pleura were opened. Following pulmonary artery (PA) cannulation with a cardioplegia cannula (Sarns, Ann Arbor, MI,) prostaglandin E1 (PGE1; 10 mg/kg) was injected directly into the main pulmonary artery. Lungs were then flushed with 1 liter of cold Perfadex (Vitrolife, Denver, CO) through the PA cannula while both lungs were cooled using saline slush. The heart and bilateral lungs were resected en bloc. Lungs were inflated with 100% oxygen to tidal volume, and the trachea was clamped. The heart and lung bloc was dissected ex-vivo, and the heart was excised leaving a generous left atrial (LA) cuff in place. A funnel shape plastic cannula (Vitrolife, Denver, CO) was then sewn to the atrial cuff using prolene suture. An additional 1 liter retrograde flush of cold Perfadex was then performed through the newly sewn cannula. Lungs were immersed in cold Perfadex and kept at 4°C for 14 hours prior to ex-vivo perfusion. This ischemic time was chosen based upon the results of previous studies that had shown adequate lung injury for a similar period of time (11).

Priming the ex-vivo circuit and preparation of lungs for perfusion

The ex-vivo perfusion circuit consisted of a bypass centrifugal pump (Medicus, Minneapolis, MN), cardiopulmonary bypass membrane oxygenator and venous reservoir (Dideco, Arvada, CO), a heat exchanger (Dideco, Arvada, CO), and polyethylene tubes. The circuit was primed with 1 liter of Steen Solution (Vitrolife, Denver, CO), sodium heparin 10,000 U (APP Pharmaceutical, Schaumburg, IL), cefazolin 500 mg (HikmaFarmaceutica, Portugal) and methylprednisolone 500 mg (Pfizer, New York, NY).

After 14 hours of cold ischemia, the trachea and pulmonary artery were cannulated. Specially designed cannulae with built-in pressure catheters were used for PA and LA cannulations (Vitrolife, Denver, CO). In addition, 0.5 ml/min of mixed gases (nitrogen 86%, carbon dioxide 8%, and oxygen 6%) were supplied to the oxygenator to deoxygenate the perfusate returning from the lungs to a PCO₂ of 35–45 mm Hg.

EVLP

Experimental lungs were transferred to an XVIVO chamber (Vitrolife, Denver, CO). The LA cannula was connected first and retrograde flow was initiated to de-air the pulmonary vasculature and cannulae. Once the flow ascended the PA cannula, the outflow cannula was connected to the PA cannula, and antegrade flow was initiated. Perfusion began with low flow (0.1 L/min) at 25°C for 20 minutes. When the perfusate temperature reached 32°C, ventilation was initiated (Harvard Apparatus, Holliston, MA) with FiO₂ of 0.2, ventilatory rate of 8 breaths/min, tidal volume of 8.0 ml/kg, and a PEEP of 5.0 cm H₂O. Within the first hour, antegrade PA flow was gradually increased to a maximum flow of 40% of cardiac output (calculated as 100 ml/kg of animal weight), while PA pressure was continuously maintained between 10–15 mm Hg. Similarly, LA pressure was maintained in a range of 0 to 5 mm Hg by adjusting the height of the venous reservoir.

Assessment

Pulmonary artery flow, pulmonary artery pressure (PAP), and left atrial pressure (LAP) were recorded continuously during EVLP. Inflow and outflow perfusate samples were collected hourly to analyze oxygenation. A recruitment maneuver was performed 10 minutes prior to each gas analysis to a maximum pulmonary artery wedge pressure of 20 Cm H₂O. A small tissue sample was obtained from the left lower lobe at 3 hours for wet/dry weight ratio assessment and histological examination. Oxygenation index was calculated as $[\text{FiO}_2 (\%) \times \text{mean airway pressure (mm Hg)}] / \text{PO}_2 (\text{mm Hg})$ and pulmonary vascular resistance (PVR) = $\text{PAP} - \text{LAP} / \text{pulmonary artery flow (Wood Units)}$. At the end of EVLP, pieces of tissue from all lobes were collected, weighed and desiccated under vacuum at 55°C until a stable dry weight was achieved. The lung wet/dry weight ratio was used to assess the degree of

pulmonary edema following EVLP. A tissue sample from the left upper lobe was excised, fixed in 4% formalin, and stained with hematoxylin and eosin for histologic examination. In addition, 100 ml of Steen Solution was replaced at 2 and 4 hour time points, to ensure freshness of perfusate components, and to maintain consistent glucose and bicarbonate levels.

Lung tissue collection and protein extraction

Lung tissues from left and right lower lobes were collected after EVLP was finished (5 hours). Tissues were weighed, minced, and homogenized in 10X PBS (g:ml=1:10) with FastPrep FP120 (Thermo Scientific). Samples were then centrifuged at 8,000 rpm for 10 minutes at 4°C and were stored at -80 °C for future ELISA analysis.

ELISA Analysis

Porcine enzyme linked immunosorbant assay (ELISA) kits for interleukin (IL)-1 beta, IL-6, IL-8, and interferon gamma (IFN- γ) were purchased from RayBiotech, Inc. (NorcrossGA). Cytokines were measured in porcine lung extraction according to the manufacture's instruction. Briefly, the lung extraction samples (100 μ l/well) were added to 96 well plates, which were pre-coated with specific antibodies separately and incubated over night at 4°C. One hundred micro liters of biotin antibodies were added to each well and incubated 1 hour at room temperature followed by adding 100 μ l Streptavidin solution and incubation of 45 minutes at room temperature. A 100 μ l TMB One-Step Substrate Reagent was then added to each well and incubated 30 minutes at room temperature. Finally, 50 μ l Stop Solution was added to each well and read at 450 nm immediately. Each sample was analyzed in triplicate.

Statistical analysis

Values are expressed as mean $\pm$ standard error of the mean. For comparison of variables between the 2 groups, two-tailed Student t-test was used. A *p*-value of less than 0.05 was considered statistically significant.

Results

Swine weight and perioperative physiologic parameters (cardiac output, arterial PaO₂, time from median sternotomy to cold flush, and cannulae sewing time) were not significantly different between the experimental groups. All lungs underwent 5 hours of continuous EVLP.

Oxygenation index was significantly improved in the treated group compared to control lungs (1.5 ± 0.17 vs. 2.3 ± 0.07 , $P < 0.01$) (Figure 1). Although the lungs in treated group had better oxygenation reflected as higher pulmonary vein PaO₂, this improvement failed to reach statistical significance ($P = 0.3$).

Significant functional differences existed between experimental lung groups. Following treatment with A_{2A} agonist, mean airway pressures were significantly decreased compared to control lungs (10.3 ± 0.6 vs. 13 ± 0.06 , $P < 0.009$) (Figure 2). Similarly, expiratory minute volume was improved within the treated group compared to control lungs (7.7 ± 0.5 ml vs. 7.0 ± 0.8 ml, $P < 0.04$). A_{2A} treatment also improved both inspiratory (403 ± 61.5 vs. 332 ± 52.7 ml, $P = 0.1$) and expiratory (428 ± 7.7 vs. 319 ± 62.3 ml, $P = 0.07$) tidal volumes compared to controls, however, these improvements failed to reach statistical significance. Pulmonary vascular resistance (PVR), although lower in the treated group was not significantly different compared to the controls ($P = 0.4$).

Significant differences in lung edema and proinflammatory cytokine expression were observed among experimental lungs following EVLP. W/D weight ratio was significantly lower for the treated group compared to control lungs (5.2 ± 0.1 vs. 6.6 ± 0.3 , $P < 0.03$) (Figure 3). Moreover, decreased interstitial edema was noted on histologic examination of treatment lungs compared to control lungs at 3 hours and 5 hours of EVLP (Figure 4). In addition to more pronounced intra-alveolar fluid accumulation, control lung samples showed thicker inter alveolar septa with more inflammatory cell infiltration into alveolar walls by 5 hours compared to compound exposed lungs. With respect to cytokine expression, lung tissue IFN- γ level was significantly lower in treatment group compared to the control group (45.1 ± 10.0 vs. 88.5 ± 12.2 pg/ml, $P < 0.05$) (Figure 5). Similarly, tissue levels of IL-1 beta, IL-6, and IL-8 were also lower among A_{2A} agonist treated lungs; however, these trends failed to reach statistical significance.

Discussion

Herein, we have reported the novel use of ex-vivo lung perfusion with the concomitant administration of an adenosine A_{2A} agonist to attenuate acute ischemia-reperfusion injury in porcine lungs. Specifically, we have demonstrated that treatment of experimental lungs with A_{2A} agonist during EVLP results in an improved oxygenation index, decreased mean airway pressure, and improved expiratory minute volume compared to control lungs. In addition, A_{2A} agonist perfused lungs incurred significantly decreased lung edema and tissue IFN- γ , and displayed histologic evidence of reduced acute lung injury. These results corroborate the previously established benefits of A_{2A} receptor agonism on the attenuation of acute IR lung injury and further extend the application of ex-vivo lung perfusion to the successful reconditioning of explanted lungs in preparation for transplantation.

Although lung transplantation is the accepted definitive treatment for many end-stage lung conditions, availability of acceptable donor organs remains a major obstacle. In 2007, only 30% of available lungs from deceased donors were used for transplantation (2). EVLP is a technique that allows visual evaluation of donor lungs, monitoring of ventilator and perfusion capacities of the explanted organ, and gas analysis (11). In addition, EVLP provides an environment to restore and re-expand atelectatic lungs more efficiently, decrease the presence of hypoxic vasoconstriction, and remove small clots from the pulmonary microvasculature via retrograde flush. Thus, use of EVLP could translate into fewer postoperative pulmonary complications in lung transplant patients. In fact, EVLP has been utilized for prolonged periods to assess, preserve, and treat organs (8, 9, 11). Steen et al (12) has demonstrated that lung ischemic time following explantation may be extended up to 20 hours.

The use of treatment modalities in concert with EVLP has been a recent focus of investigation. Although solitary perfusion of lungs with Steen Solution has been shown to be superior to cold preservation of the organ (13), the use of various treatment strategies during EVLP has recently been explored for both NHBD and brain dead donors with marginal lungs. Inci et al (14) demonstrated that addition of a fibrinolytic agent, urokinase, to the perfusate in a pig EVLP model of NHBD improved graft function by reporting a reduction in pulmonary vascular resistance and an increase in oxygenation at the end of perfusion. Cypel and colleagues (13) used an adenoviral vector to transfer interleukin 10 (IL-10), an anti-inflammatory cytokine, gene into airways and alveoli at the beginning of EVLP. Kakishita et al (15) showed no significant improvement in pulmonary function following neutralization of pro-inflammatory cytokines, tumor necrosis factor (TNF) α and IL-8, from the perfusate using an adsorbent membrane.

In this study, we tested the impact of an adenosine A_{2A} agonist on the pulmonary function of acutely injured experimental lungs during normothermic EVLP. Adenosine is a purine nucleoside produced in response to ischemia that acts through its interaction with four different adenosine receptors (A₁, A_{2A}, A_{2B} and A₃) (16). Adenosine A_{2A} receptors are highly expressed on bone marrow-derived cells, including T lymphocytes, neutrophils, monocytes, macrophages, platelets, and mast cells (1). Activation of the A_{2A}R on bone marrow-derived cells is the primary mechanism for the protective effects of A_{2A} receptor agonists in acute lung injury (17). The cellular responses appear to be mostly mediated through cAMP (17) and result in inhibition of oxidative burst in neutrophils (18), reduced cytokine release (19), and, inhibition of leukocyte activation (20). We have previously reported the inhibitory effects of A_{2A} receptor agonism on acute lung IR injury within several animal models (21–23). A_{2A} agonists have been shown to attenuate pulmonary dysfunction, edema, neutrophil infiltration, proinflammatory cytokine production, and microvascular leak after lung transplantation (23). As a result, we sought to further investigate the potential protective effect of an adenosine A_{2A} agonist during EVLP to salvage acutely injured lungs. While the anti-inflammatory role of A_{2A} agonists following reperfusion has been extensively studied in past, the reconditioning of injured lungs, as we have described prior to transplantation, has not yet been investigated. In our EVLP model, the addition of a selective adenosine A_{2A} agonist to the perfusate resulted in significant reduction of edema and better oxygenation index. Functionally, treated lungs showed improved mean airway pressure and expiratory minute volume. Interestingly, we found that the most optimal recorded gases occurred during the first and second hours of EVLP, an observation that may be secondary to the compound's short half-life (25 to 30 minutes) (24).

Acute lung ischemia reperfusion injury is modulated by specific bone marrow derived cells and proinflammatory cytokines. IFN- γ has been shown to be involved in migration and cytokine production of macrophages (25–27). It has also been demonstrated as an important mediator in development of inflammatory response in lung injury (28). Eppinger et al (29), showed that vascular leakage of serum albumin was increased in a rat model of lung ischemia-reperfusion injury. They noticed an increased level of cytokines released from macrophages, including IFN- γ , shortly after reperfusion started. Our group and others have previously shown that alveolar macrophages have a pivotal role in lung ischemia-reperfusion injury by releasing proinflammatory cytokines and chemokines, such as IFN- γ , in response to oxidative stress (30–32). As the lung possesses resident macrophages as a defense against environmental pathogens (29), it seems likely that these cells were targets of anti-inflammatory effect of adenosine A_{2A} agonist in our study. One of the pulmonary protective mechanisms of adenosine A_{2A} agonist is through activation of receptors on CD4⁺ T lymphocytes and neutrophils. These cells would then attenuate downstream intercellular signaling cascade events involving other cell types such as alveolar macrophages and epithelial and endothelial cells (33).

We have previously shown that adenosine A_{2A} receptor activation results in the greatest protection against lung IR injury when given before ischemia and during reperfusion (22). With the experience gained through this experiment, it can be proposed that treating lungs with adenosine A_{2A} agonist *ex vivo*, in addition to recipient treatment, would result in even better outcomes. Future experiments combining the superior impact of adenosine A_{2A} on donor lung and recipient are warranted. We are currently planning a series of experiments using treated EVLP lungs for subsequent lung transplantation to continue our assessment of the impact of A_{2A} administration and the benefits of EVLP. These studies will help to further determine the potential clinical use of A_{2A} receptor agonists.

Limitations

Select limitations deserve discussion in this study. The admittedly small sample size in each experimental group limited the ability to detect statistically significant differences between observed trends in various measures of lung injury between control and treatment groups. In addition, the most significant differences between the control and treatment groups occurred during the first 2 hours of EVLP. While not surprising as adenosine A_{2A} receptor treatment typically acts very early in the inflammatory process following reperfusion, it is unclear whether prolonged treatment will derive any additional benefits during the EVLP process. Regarding cytokine expression, one potential reason inflammatory tissue cytokines were not significantly decreased after 5 hours could be a function of the short half-life of the A_{2A} agonist itself. Future studies are planned with slow infusion of the agent throughout the EVLP to further assess this possibility. Finally, the acellular nature of perfusate in this experiment likely excluded important sources of inflammatory cytokine production.

Conclusions

We conclude that the combined use of adenosine A_{2A} agonist and EVLP significantly attenuates the inflammatory response in acutely injured lungs following ischemia-reperfusion to enhance pulmonary function. This combined treatment modality offers a novel and clinically translatable approach to enhance donor lung quality and a potential to increase the donor lung pool for transplantation.

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Figure 1.

Oxygenation index was significantly improved after 5 hours of ex vivo lung perfusion with Steen Solution and A_{2A} agonist compared to lungs perfused with Steen Solution only. * $P < 0.01$

EVLP, Ex vivo lung perfusion; A_{2A}R, A_{2A} receptor

Figure 2.

Lungs after five hours of ex vivo lung perfusion with A_{2A} agonist revealed significantly lower mean airway pressure compared to control lungs. * $P < 0.009$

EVLP, Ex vivo lung perfusion; A_{2A}AR, A_{2A} receptor

Figure 3.

Comparison of wet to dry (W/D) weight ratio between the treatment and control group. Low edema formation rates were observed in lungs exposed to the A_{2A} agonist. $*P < 0.03$

EVLP, Ex vivo lung perfusion; A2AR, A_{2A} receptor

Figure 4. Histological appearance of the lung after 5 hours ex vivo lung perfusion (hematoxylin and eosin stain). The magnification of the pictures is x100. (A) Control group, (B) Treatment group. There is marked alveolar wall thickening with inflammatory cell infiltration in control lungs as compared to compound exposed lung.

Figure 5. Tissue level of Interferon gamma (IFN- γ) was significantly lower in treatment lungs after 5 hours of ex-vivo lung perfusion (EVL) and exposure to adenosine A2A agonist. * $P < 0.05$