Adenosine A_1 receptor activation attenuates lung ischemia–reperfusion injury

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Objectives: Ischemia–reperfusion injury contributes significantly to morbidity and mortality in lung transplant patients. Currently, no therapeutic agents are clinically available to prevent ischemia–reperfusion injury, and treatment strategies are limited to maintaining oxygenation and lung function. Adenosine can modulate inflammatory activity and injury by binding to various adenosine receptors; however, the role of the adenosine A_1 receptor in ischemia–reperfusion injury and inflammation is not well understood. The present study tested the hypothesis that selective, exogenous activation of the A_1 receptor would be anti-inflammatory and attenuate lung ischemia–reperfusion injury.

Methods: Wild-type and A_1 receptor knockout mice underwent 1 hour of left lung ischemia and 2 hours of reperfusion using an in vivo hilar clamp model. An A_1 receptor agonist, 2-chloro-N6-cyclopentyladenosine, was administered 5 minutes before ischemia. After reperfusion, lung function was evaluated by measuring airway resistance, pulmonary compliance, and pulmonary artery pressure. The wet/dry weight ratio was used to assess edema. The myeloperoxidase and cytokine levels in bronchoalveolar lavage fluid were measured to determine the presence of neutrophil infiltration and inflammation.

Results: In the wild-type mice, 2-chloro-N6-cyclopentyladenosine significantly improved lung function and attenuated edema, cytokine expression, and myeloperoxidase levels compared with the vehicle-treated mice after ischemia–reperfusion. The incidence of lung ischemia–reperfusion injury was similar in the A_1 receptor knockout and wild-type mice; and 2-chloro-N6-cyclopentyladenosine had no effects in the A_1 receptor knockout mice. In vitro treatment of neutrophils with 2-chloro-N6-cyclopentyladenosine significantly reduced chemotaxis.

Conclusions: Exogenous A_1 receptor activation improves lung function and decreases inflammation, edema, and neutrophil chemotaxis after ischemia and reperfusion. These results suggest a potential therapeutic application for A_1 receptor agonists for the prevention of lung ischemia–reperfusion injury after transplantation. (J Thorac Cardiovasc Surg 2013;145:1654-9)

Ischemia–reperfusion (IR) injury and its more severe form, primary graft dysfunction, lead to significant morbidity and mortality after lung transplantation, with mortality rates approaching 40%. Ischemia is unavoidable during transplantation, and the subsequent effect of reperfusion results in significant cellular damage, oxidative stress, innate immune responses, and lung inflammation.[1,2] During organ inflammation and IR injury, such as after transplantation, adenosine is a retaliatory metabolite released from many cell sources and serves largely as a protective agent with anti-inflammatory effects. Adenosine mediates its effects through binding of 4 different G protein-coupled receptors: A_1 receptor (A_1R), A_{2A}R, A_{2B}R, and A_3R, and adenosine receptor signaling typically entails second messenger pathways such as the cyclic adenosine monophosphate-dependent protein kinase A pathway or the phospholipase C pathway.

All 4 adenosine receptors are expressed in the lungs of mice[3] and humans.[4] Studies from our laboratory have suggested that activation of A_1R or A_3R, by selective agonists, offers protection from lung IR injury in an isolated rabbit lung model.[5] We have also demonstrated potent anti-inflammatory effects of A_{2A}R activation in both a mouse lung IR model[6] and a porcine lung transplant model.[7] However, our studies suggested a proinflammatory role for the A_{2B}R in the setting of lung IR.[8] The role of the A_1R in lung IR injury remains controversial and not well understood. The A_1R is expressed largely on endothelial, epithelial, and inflammatory cells and signals through Gi or G0 proteins. Its effects include inhibition of adenylyl cyclase, activation of K^+ channels, inhibition of N-, P-, and Q-type Ca^+ channels, and activation of phospholipase Cβ.[9] Earlier studies reported that A_1R antagonism attenuates lung inflammation after IR.[10] However, more recent studies,

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Abbreviations and Acronyms

$A_1R-/-$ = congenic A_1 receptor knockout
BAL = bronchoalveolar lavage
CCPA = 2-chloro-N6-cyclopentyladenosine
FBS = fetal bovine serum
IR = ischemia–reperfusion
MPO = myeloperoxidase
TNF-α = tumor necrosis factor-α
WT = wild-type

including our own, have suggested that A_1R activation has protective effects in several models of inflammatory lung injury.[5,11,12] Using A_1R knockout mice and a specific pharmacologic A_1R agonist, the present study tested the hypothesis that specific A_1R activation would be anti-inflammatory and provide significant protection against lung IR injury.

METHODS

Animals and Study Design

Adult male C57BL/6 wild-type (WT) mice (Jackson Laboratories, Bar Harbor, Me) and congenic A_1R knockout ($A_1R-/-$) mice 8 to 12 weeks old were used. The $A_1R-/-$ mice[13] were a gift of Dr Jurgen Schnermann (Institute of Pharmacology and Toxicology, University of Tübingen, Tübingen, Germany). The mice underwent either sham or IR surgery (n = 6 mice/group). The mice were randomly allocated to the various control and experimental groups. They were treated with either vehicle (0.1% dimethyl sulfoxide in saline) or 2-chloro-N6-cyclopentyladenosine (CCPA; Sigma Aldrich, St Louis, Mo) by intravenous injection 5 minutes before ischemia. CCPA is a highly selective and potent A_1R agonist (affinity Ki value, 0.4 and 3900 nM for rat A_1R and $A_{2A}R$, respectively), and CCPA is nearly 10,000-fold more selective for A_1R than for $A_{2A}R$.[14] The Institutional Animal Care and Use Committee at the University of Virginia approved all animal procedures and protocols used in the present study, which conformed to the National Institute of Health guidelines *(Guide for the Care and Use of Laboratory Animals).*

Lung IR Model

The IR group underwent 1 hour of left lung ischemia and 2 hours of reperfusion (IR) using an established in vivo hilar clamp model.[6,8] The sham group underwent anesthesia, left thoracotomy (without the hilar clamp), and 2 hours of reperfusion. Analgesia was administered to all the mice after surgery.

Lung Function Measurement

After reperfusion, pulmonary function was evaluated using an isolated, buffer-perfused lung system (Hugo Sachs Electronik, March-Huggstetten, Germany), as previously described by our laboratory.[8] After a 5-minute equilibration period, data regarding the pulmonary arterial pressure, pulmonary compliance, and airway resistance were recorded for an additional 5 minutes using the Pulmodyn data acquisition system (Hugo Sachs Elektronik).

Bronchoalveolar Lavage

After lung function was measured, left lungs were lavaged with 0.4 mL saline. The bronchoalveolar lavage (BAL) fluid was then centrifuged (1500 rpm for 8 minutes) and stored at −80°C.

Lung Weight/Dry Weight Ratio

Using separate groups of mice (n = 6/group), the left lung was excised after the reperfusion period, blotted dry, and immediately weighed and desiccated until a stable dry weight was reached. The lung wet/dry weight ratio was calculated as a measure of lung edema.

Analysis of Cytokines and Myeloperoxidase

Cytokines were measured in BAL fluid using a mouse Bio-plex cytokine assay (Bio-Rad Laboratories, Hercules, Calif), as previously reported.[6,8] The myeloperoxidase (MPO) levels were measured in BAL fluid using a mouse MPO enzyme-linked immunosorbent assay kit (Hycult Biotech, Uden, The Netherlands). MPO is abundant in the azurophilic granules of polymorphonuclear neutrophils and was used as an indicator of neutrophil activation and infiltration into alveolar airspaces.

Chemotaxis Assay

In vitro migration was assessed in bone marrow–derived murine neutrophils using a commercially available kit (QCM 5-μm Chemotaxis Cell Migration Assay; Millipore, Billerica, Mass). In brief, bone marrow cells were harvested from mouse femurs and tibias by flushing with 10 mL of phosphate-buffered saline containing 10% fetal bovine serum (FBS). The suspended cells were centrifuged at 500*g* for 10 minutes. After red blood cell lysis for 2 minutes at room temperature, the cells were washed, counted, and resuspended in Roswell Park Memorial Institute buffer. Neutrophil isolation was then performed using a commercially available anti-Ly-6G mouse microbead kit (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The neutrophils were resuspended at 2×10^6 cells/mL in Roswell Park Memorial Institute buffer with 0.5% FBS, and 250 μL of the cell suspension was incubated in a 5-μm insert, with or without A_1R agonist CCPA (10 ng/mL), for 30 minutes at 37°C. Next, 500 μL of serum-free medium was then added to the lower chamber, with or without chemoattractant (10% FBS), and the plate was incubated for 4 hours at 37°C in a carbon dioxide incubator. After the incubation period, the upper chamber was removed, and the insert was incubated in 400 μL of cell stain for 20 minutes at room temperature. Nonmigratory cells were removed from the interior of the insert with a cotton swab, and the insert was transferred to a new well containing 200 μL of extraction buffer for 15 minutes. Colorimetric measurement of the reaction was performed at 570 nm in a plate reader (Quant; Bio-Tek Instruments, Winooski, Vt). Two independent in vitro experiments were performed.

Statistical Analysis

The results were analyzed using 2-way analysis of variance to determine whether significant differences existed between the 2 groups. Comparisons between the 2 groups were analyzed using unpaired Student *t* test. Data are expressed as the mean ± standard deviation.

RESULTS

A_1R Activation Improves Lung Function After IR

To measure the effects of exogenous A_1R activation on pulmonary function after IR, WT and $A_1R-/-$ mice underwent lung IR after pretreatment with vehicle (0.1% dimethyl sulfoxide) or CCPA (A_1R agonist, 0.1, 1, and 2 mg/kg). Lung function was assessed after 2 hours of reperfusion. IR in the vehicle-treated mice significantly increased the pulmonary artery pressure and airway resistance and significantly decreased pulmonary compliance (Figure 1). Treatment of WT mice with 1 or 2 mg/kg CCPA resulted in significantly decreased pulmonary artery

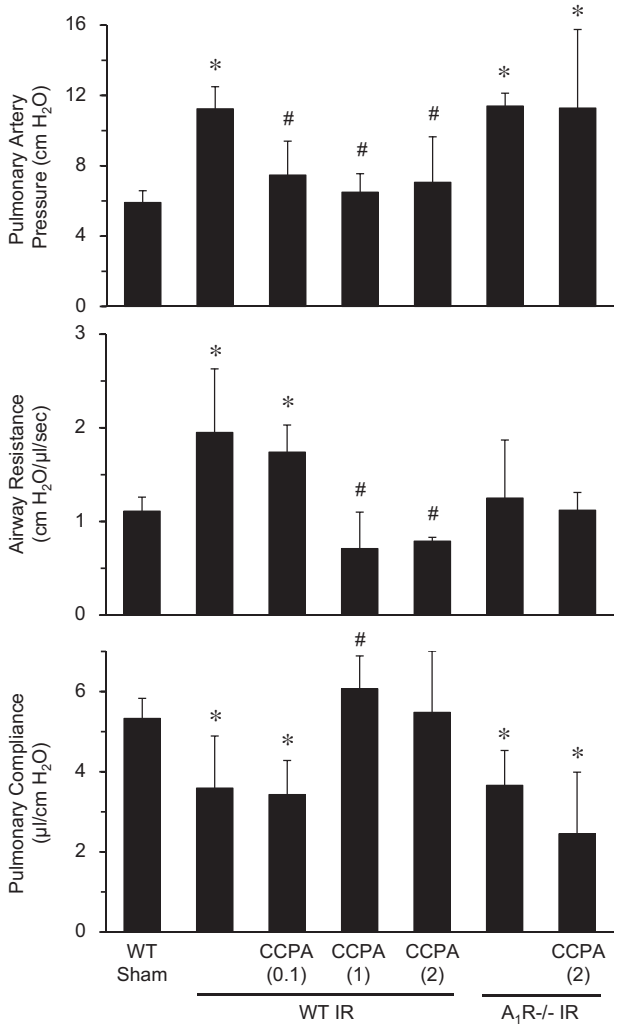

FIGURE 1. Lung function after ischemia–reperfusion *(IR)* is significantly improved by 2-chloro-N6-cyclopentyladenosine *(CCPA)*. Wild-type *(WT)* and congenic A_1 receptor knockout *($A_1R-/-$)* mice were treated with vehicle or CCPA (doses in mg/kg shown in *parentheses*) before ischemia. In WT mice after IR, CCPA significantly decreased pulmonary artery pressure and airway resistance and increased pulmonary compliance. Lung function in $A_1R-/-$ mice after IR was not significantly different from that of WT mice after IR, and CCPA did not significantly affect the lung function in $A_1R-/-$ mice. The lung function was similar between WT and $A_1R-/-$ mice after sham surgery (data not shown). *$P < .03$ versus WT sham, #$P < .03$ versus WT IR. Data presented as mean ± standard deviation.

pressure and airway resistance and significantly increased pulmonary compliance after IR. The lung function in the $A_1R-/-$ mice after IR was impaired similarly to that in the WT mice after IR; however, CCPA had no protective effects in the $A_1R-/-$ mice after IR (Figure 1). This demonstrates the specificity of the agonist for A_1R. No significant differences in lung function were found between the WT and $A_1R-/-$ sham mice (data not shown); thus, only data from WT sham mice have been reported for the subsequent experiments.

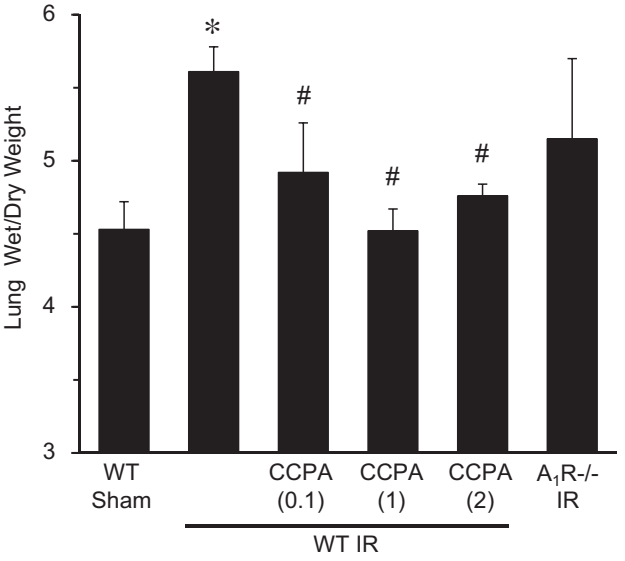

FIGURE 2. Pulmonary edema after ischemia–reperfusion *(IR)* is significantly reduced by 2-chloro-N6-cyclopentyladenosine *(CCPA)*. Lung wet/dry weight ratio was measured after IR or sham surgery in congenic A_1 receptor knockout *($A_1R-/-$)* mice and wild-type *(WT)* mice treated with vehicle or CCPA (doses in mg/kg shown in *parentheses*) before ischemia. Lung wet/dry weight ratio was significantly increased after IR in WT mice, which was significantly attenuated by CCPA treatment. Lung wet/dry weight ratio after IR was also elevated in $A_1R-/-$ mice after IR similar to that in WT mice after IR. Lung wet/dry weight ratio was similar between WT and $A_1R-/-$ mice after sham surgery (data not shown). *$P < .05$ versus WT sham, #$P < .05$ versus WT IR. Data presented as mean ± standard deviation shown.

Activation of A_1R Reduced Lung Edema After IR

To evaluate the effects of exogenous A_1R activation on lung edema after IR, the lung wet/dry weight ratio was measured in mice pretreated with vehicle or CCPA (0.1, 1, and 2 mg/kg). The WT mice had a significantly greater wet/dry weight ratio after IR compared with the sham group (Figure 2), which was significantly decreased by CCPA (all doses). The lung wet/dry weight ratio after IR was also elevated in the $A_1R-/-$ mice, similar to that of the WT mice after IR (Figure 2).

A_1R Activation Attenuated Proinflammatory Cytokine Expression After IR

To measure the lung inflammatory response after IR, the levels of proinflammatory cytokines and chemokines were measured in the BAL fluid of mice pretreated with vehicle or CCPA (0.1, 1, and 2 mg/kg). IR in the vehicle-treated WT mice resulted in significantly increased levels of interleukin-6, CXCL1, CCL2, and tumor necrosis factor (TNF)-α compared with the sham mice (Figure 3). Treatment with 2 mg/kg CCPA significantly reduced the levels of interleukin-6, CXCL1, and CCL2 compared with WT mice after IR. TNF-α was also reduced by CCPA, but this did not reach statistical

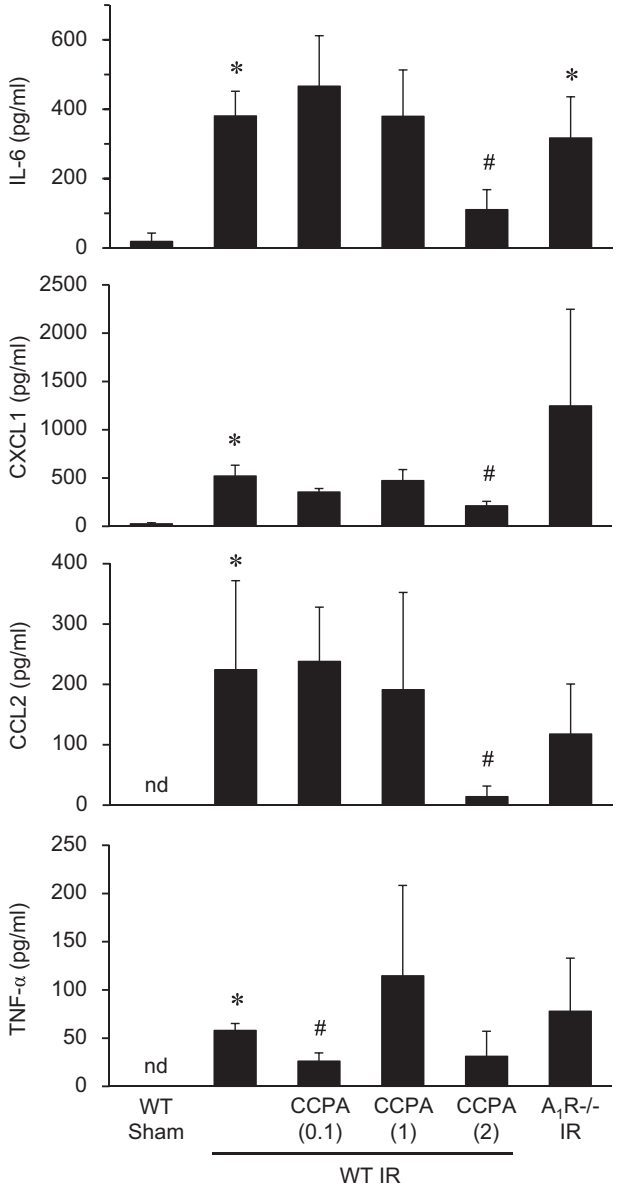

FIGURE 3. Expression of proinflammatory cytokines after ischemia–reperfusion *(IR)* is attenuated by 2-chloro-N6-cyclopentyladenosine *(CCPA)*. Cytokine levels in bronchoalveolar lavage fluid after IR or sham surgery was measured in congenic A_1 receptor knockout *($A_1R-/-$)* mice and wild-type *(WT)* mice treated with vehicle or CCPA (doses in mg/kg are shown in *parentheses*) before ischemia. Expression of interleukin-6 *(IL-6),* CXCL1, CCL2, and tumor necrosis factor-α *(TNF-α)* were all significantly increased after IR in WT mice; CCPA treatment significantly attenuated cytokine levels after IR. Cytokine levels after IR were elevated in $A_1R-/-$ mice after IR similar to those in WT mice after IR. *$P < .05$ versus WT sham, #$P < .05$ versus WT IR. Data presented as mean ± standard deviation. *nd,* Not detectable.

significance ($P = .069$). Similar to lung function and the wet/dry weight ratio, the $A_1R-/-$ mice after IR had elevated levels of cytokines comparable to that of the WT mice after IR (Figure 3).

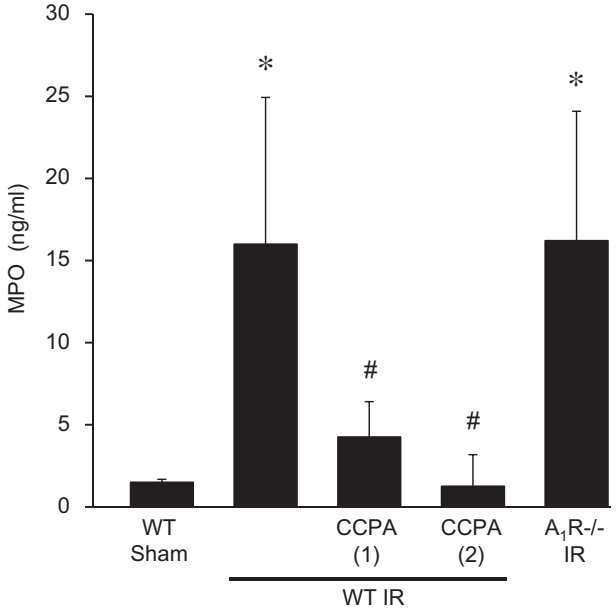

FIGURE 4. Myeloperoxidase *(MPO)* levels after ischemia–reperfusion *(IR)* are significantly reduced by 2-chloro-N6-cyclopentyladenosine *(CCPA)*. MPO levels in bronchoalveolar lavage fluid, as an estimate of neutrophil infiltration into alveolar airspaces, after IR or sham surgery were measured in congenic A_1 receptor knockout *($A_1R-/-$)* mice and in wild-type *(WT)* mice treated with vehicle or CCPA (doses in mg/kg are shown in *parentheses*) before ischemia. Elevated MPO in WT mice after IR was significantly attenuated by CCPA. MPO levels were also elevated in $A_1R-/-$ mice after IR similar to those in WT mice after IR. *$P < .05$ versus WT sham, #$P < .05$ versus WT IR. Data presented as mean ± standard deviation.

Activation of A_1R Reduces MPO Levels After IR

MPO was measured in BAL fluid as an indication of neutrophil activation and infiltration into the alveolar airspaces. Vehicle-treated WT mice after IR had significantly elevated MPO levels compared with the sham mice (Figure 4). Treatment with 1 or 2 mg/kg CCPA significantly reduced the MPO levels after IR to levels similar to those in the sham group. The $A_1R-/-$ mice had elevated MPO levels after IR comparable to those of the WT mice (Figure 4).

A_1R Agonist Impairs Neutrophil Chemotaxis

To evaluate a possible direct effect of A_1R activation on neutrophil migration, in vitro neutrophil chemotaxis was evaluated using the Boyden chamber method, as described in the ''Methods'' section. Bone marrow–derived neutrophils exposed to 10% FBS as a chemoattractant demonstrated significant chemotaxis (Figure 5). Incubation of neutrophils with CCPA (10 ng/mL, 30 minutes before stimulation) significantly attenuated the chemotaxis (Figure 5).

DISCUSSION

Studies on the effects of A_1R activation in IR have been controversial. Evidence has been shown for an anti-inflammatory role for A_1R in the lung,[5] liver,[15] kidney,[16]

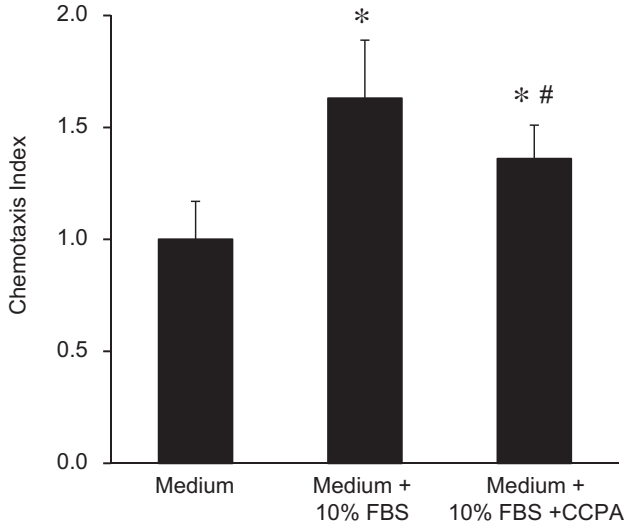

FIGURE 5. Neutrophil chemotaxis was significantly reduced by 2-chloro-N6-cyclopentyladenosine *(CCPA)*. Chemotaxis was measured in murine bone marrow–derived neutrophils, as described in the "Methods" section. Chemotaxis was significantly increased in medium containing 10% fetal bovine serum *(FBS)* and significantly reduced by 10 ng/mL CCPA. *$P < .001$ versus medium, #$P = .03$ versus medium plus 10% FBS. Data presented as mean ± standard deviation.

heart,[17] intestine,[18] and skeletal muscle.[19] However, other studies have suggested that A_1R antagonism has beneficial effects in lung and heart IR models.[10,20] In the present study, we used an in vivo hilar clamp model in WT and $A_1R-/-$ mice to clarify a protective role of exogenous activation of A_1R in lung IR injury. Our results have demonstrated the anti-inflammatory effects of CCPA, a potent and selective A_1R agonist, against lung IR injury. The parameters of lung injury, dysfunction, and inflammation showed a significant dose-dependent improvement after exogenous A_1R activation. These results are in agreement with other reports of improved lung function after A_1R activation in acute lung injury models.[5,21]

In addition to defining a protective role of exogenous A_1R activation in lung IR injury, our results have provided insight into the potential mechanisms for A_1R-mediated anti-inflammatory effects. One mechanism of lung protection by A_1R agonist is the modulation of cytokines and chemokines, which are important for the recruitment and activation of effector cells such as neutrophils. In the present study, CCPA attenuated the levels of several proinflammatory cytokines/chemokines after IR, including interleukin-6, CXCL1 (KC), CCL2 (monocyte chemotactic protein-1), and TNF-α. This suggests that activation of A_1Rs on cells such as the alveolar epithelial cells or macrophages could directly affect the secretion of CCL2 and TNF-α, respectively, by these cells and that an A_1R agonist, such as CCPA, can exogenously activate A_1R-mediated anti-inflammatory effects. The elevation in CXCL1, CCL2, and TNF-α in $A_1R-/-$ mice after IR has also been described in a model of kidney IR injury, in which reconstitution of A_1R by lentivirus injection encoding for the human A_1R gene decreased CCL2 and TNF-α expression to sham levels.[22]

Another potential mechanism of A_1R agonist-mediated protection is the modulation of inflammatory cell recruitment. Studies have shown that activation and migration of neutrophils are crucial events that modulate the inflammation process after IR, and the important role of neutrophil recruitment in lung IR injury has been demonstrated in our laboratory.[23] The results of the present study showed that WT mice had significantly elevated MPO levels in the BAL fluid after IR and that A_1R activation by CCPA reduced the MPO levels, indicating a reduction in alveolar neutrophil activation and recruitment. These results are in agreement with previous studies of lung[5] and kidney[16] IR models.

In vitro studies have shown that the A_1R agonist decreases albumin permeability across confluent bovine pulmonary artery endothelial cell monolayers.[24] Our present study evaluated the direct effect of selective A_1R activation on neutrophil chemotaxis in vitro. Bone marrow–derived neutrophils incubated with 10% FBS demonstrated increased chemotaxis compared with cells incubated with medium alone, and CCPA treatment significantly attenuated neutrophil chemotaxis. These results have demonstrated that A_1R agonist can act directly on neutrophils to impair migration.

Although we anticipated that the $A_1R-/-$ mice would have worse lung IR injury than the WT mice, these mice displayed a level of injury similar to that of the WT mice. These results could have several explanations. First, one explanation might be because endogenous adenosine production during IR can act on any or all of 4 adenosine receptors to initiate the signaling pathways that can have synergistic or antagonistic effects. The pharmacology of adenosine receptor signaling is complex, and the role of these individual receptors can be proinflammatory or anti-inflammatory during lung IR injury, as noted. Hence, in $A_1R-/-$ mice, endogenous adenosine could still exert an anti-inflammatory effect by other adenosine receptors such as A_3R or $A_{2A}R$, thereby preventing worse lung injury after IR. Second, adenosine receptor signaling cascades that occur in $A_1R-/-$ mice remain unknown and could be disrupted, and it is possible that potential crosstalk between A_1R and other adenosine receptors could be altered in $A_1R-/-$ mice. Several studies have now pointed to synergistic mechanisms between various adenosine receptors, especially A_1R, $A_{2A}R$, and $A_{2B}R$. For example, Urmaliya and colleagues[25,26] found that endogenous adenosine contributes to A_1R-mediated cardioprotection by cooperative interactions with both $A_{2A}R$ and $A_{2B}R$. The complex and cooperative adenosine receptor signaling mechanisms involved in lung IR injury remain unknown, and it is likely that similar cooperative pathways are disrupted in

the $A_1R-/-$ mice. Importantly, however, the exogenous activation of A_1R by CCPA in WT mice in the present study clearly resulted in significant protection from lung injury and dysfunction after IR, supporting the therapeutic use of A_1R agonists to prevent lung IR injury.

CONCLUSIONS

We have demonstrated the protective, anti-inflammatory effects of an A_1R agonist in the setting of lung IR injury. Exogenous A_1R activation by CCPA improved lung function and decreased the injury and inflammation after IR. These protective effects involved the attenuation of edema, proinflammatory cytokines, and the infiltration and chemotaxis of neutrophils. These results suggest that the pharmacologic activation of A_1R by selective agonists could be a promising strategy to prevent and/or treat lung IR injury and primary graft dysfunction after transplantation.

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