Protective effects of adenosine A_{2A} receptor agonist in ventilator-induced lung injury in rats

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Abstract

Objectives—Mechanical ventilation is associated with overwhelming inflammatory responses that are associated with ventilator-induced lung injury (VILI) in patients with acute respiratory distress syndrome. The activation of adenosine A_{2A} receptors has been reported to attenuate inflammatory cascades.

Hypothesis—The administration of A_{2A} receptors agonist ameliorates VILI.

Methods—Rats were subjected to hemorrhagic shock and resuscitation as a first hit to induce systemic inflammation. The animals randomly received the selective A_{2A} receptor agonist CGS-21680 or a vehicle control in a blinded fashion at the onset of resuscitation phase. They were then randomized to receive mechanical ventilation as a second hit with a high tidal volume of 20 mL/kg and zero positive end-expiratory pressure, or a low tidal volume of 6 mL/kg with positive end-expiratory pressure of 5 cm H₂O.

Results—The administration of CGS-21680 attenuated lung injury as evidenced by a decrease in respiratory elastance, lung edema, lung injury scores, neutrophil recruitment in the lung, and production of inflammatory cytokines, compared with the vehicle treated animals.

Conclusions—The selective A_{2A} receptor agonist may have a place as a novel therapeutic approach in reducing VILI.

Keywords

acute respiratory distress syndrome; inflammation; neutrophils

Introduction

Hemorrhagic shock and resuscitation (HSR) triggers inflammatory responses as reflected by an increased infiltration of polymorphonuclear neutrophils into the lung and an enhanced expression of inflammatory cytokines including interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α . This inflammatory response has been associated with acute lung injury and its worst form the acute respiratory distress syndrome (ARDS) (1). It has been shown that HSR can prime the immune system to enhance excessive host reaction in response to a secondary stimulus such as endotoxin (2, 3) and mechanical ventilation (4). We have recently shown in a rat model that HSR renders the lung more susceptible to mechanical ventilation resulting in ventilator-induced lung injury (VILI) and kidney dysfunction (4, 5).

Adenosine—a purine nucleoside released during hypoxia, inflammation, and ischemia/reperfusion—is a potent agent to protect organ systems from injury by its anti-inflammatory properties (6–10) to decrease macrophage activation, block endothelial polymorphonuclear neutrophils interactions, and reduce polymorphonuclear neutrophils-mediated injury and vascular derangement (11, 12). A couple of recent studies have demonstrated that mechanical ventilation in rats and mice increases the level of pulmonary adenosine (13, 14), and that mice deficient for extracellular adenosine generation show increased pulmonary edema and inflammation after VILI (14). Once in the extracellular milieu, adenosine exerts its biological effects through binding to the G-protein coupled adenosine receptors including A₁R, A_{2A}R, A_{2B}R, and A₃R (8, 15, 16). The A_{2A}R expressed on leukocytes and lung displays high affinity for adenosine (8). Several studies have shown that activation of A_{2A} receptor is protective against ischemia/reperfusion injury by decreasing the expression of endothelial adhesion molecules and the production of cytokines and reactive oxygen species (17–21). We thus hypothesized that a pharmacologic intervention with an A_{2A}R agonist protects the lung from injury in a two-hit model of HSR followed by mechanical ventilation.

Methods

Surgical Preparation

The study protocol was approved by the Institutional Animal Care Committee at St. Michael's Hospital. Male Sprague-Dawley rats weighing 280–360 g (Charles River Labs, St. Constant, Quebec, Canada) were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) intraperitoneally. Tracheostomy was performed for endotracheal intubation (14-gauge Angiocath Catheter, Becton Dickinson Infusion Therapy Systems, Sandy, UT). The animals were then ventilated (Servo 300 ventilator, Siemens, Sweden) using a tidal volume (V_t) of 6 mL/kg, positive end-expiratory pressure of 5 cm H₂O, and a respiratory rate of 50 breaths/min under 40% FIO₂. Anesthesia and muscle paralysis were maintained by a continuous infusion of ketamine (15 mg/kg/h), xylazine (3 mg/kg/h), and pancuronium (0.35mg/kg/h) through a tail vein. The right carotid artery was catheterized for monitoring mean arterial pressure (MAP), blood gas analysis (Ciba-Corning Model 248 blood gas analyzer; Corning Medical, Melfield, MA), and conduction of controlled hemorrhagic shock.

Hemorrhagic Shock/Resuscitation and A_{2A}R Agonist Administration

Hemorrhagic shock was induced by blood withdrawal over 15 minutes to reach an MAP of 40 ± 5 mmHg. The target MAP was achieved by further blood withdrawal if the MAP was > 45 mm Hg, or by injection of shed blood collected in a 0.1% citrate solution if the MAP was < 35 mm Hg. The hypotension status was maintained for 60 minutes, followed by resuscitation by transfusion of the shed blood and Lactated Ringer's (1:1 volume) to achieve an MAP of 70–80 mm Hg within 15 minutes.

The A_{2A}R agonist CGS-21680 (Sigma, St. Louis, MO) dissolved in 45% (w/v) aqueous 2-hydroxypropyl- β -cyclodextrin, or an identical volume of placebo-control solution was administered at 0.3 mg/kg intravenously over 30 seconds at the onset of resuscitation phase. The administration of CGS-21680 and placebo control solution was chosen in a random and blinded fashion to the investigators who conducted the experiments.

Randomization of Mechanical Ventilation

After HSR and CGS-21680 administration, the animals were randomly assigned to receive mechanical ventilation for 3 hours at two different ventilator settings. In the high Vt group, the animals were ventilated with a Vt of 20 mL/kg and zero positive end expiratory pressure and in the low Vt group, the animals were ventilated with a Vt of 6 mL/kg at a positive end-expiratory pressure level of 5 cm H₂O. Thus, there were a total of four groups: group 1 (high Vt + placebo, n=10); group 2 (high Vt + CGS-21680, n = 10); group 3 (low Vt + placebo, n = 10); and group 4 (low Vt +CGS-21680, n = 10).

Monitoring and Fluid Maintenance

In addition to MAP, heart rate and oxygen saturation were monitored in a real time fashion by placing a veterinary pulse coximeter (SpO₂) (Nonin 8600, Plymouth, MN). Airway plateau pressure was measured by holding breath for 4 seconds at the end inspiratory phase. Lung elastance was calculated by using the formula: Lung elastance = (plateau pressure – positive end-expiratory pressure)/Vt. Lactated Ringer's solution was infused (3 mL/hr) to maintain MAP, and a bolus of 1 mL shed blood was given hourly to replace blood loss due to sampling. Rectal temperature was maintained at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ by using a heating pad.

Measurements

On completion of the experiments, animals were sacrificed by injecting overdose of sodium pentobarbital followed by lung pressure-volume curve construction. The left lung was then lavaged for cell differentiation assay (n =10 per group). The right lung was used for estimation of wet-to-dry (W/D) weight ratio (n = 5 per group) or histology analysis by hematoxylin and eosin staining (n = 5 per group). The lung injury score (22) was graded by a pathologist (C.-F.L.) who was blinded to the experimental groups.

Cytokine profiles (multiplex including rat IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, granulocytemacrophage colony stimulating factor [GM-CSF], interferon- γ and TNF- α ; Bio-Rad, Hercules, CA) were analyzed in plasma and lung-lavage fluids by a technician in a blinded manner.

Statistical Analysis

Results are expressed as mean $\pm$ SE. Comparison between the groups was performed using a two-way analysis of variance for repeated measurement. *Post hoc* analysis was conducted using the Bonferroni method. One-way analysis of variance followed by a Student's *t* test was used where appropriate. A *p* value <0.05 was considered as significant.

Results

Model

The amount of blood withdrawn was similar to reach target MAP during hemorrhagic shock (13.5 ± 0.5 mL in HV + placebo vs. 13.2 ± 0.7 mL in HV + CGS-21680, and 13.3 ± 0.6 mL in LV + placebo vs. 13.6 ± 0.6 mL in LV + CGS-21680 group, *p* = not significant for all). The volume of fluid infused was identical in all animals in the resuscitation phase and thereafter during the mechanical ventilation period.

The MAP was similar at baseline and during HSR in all animals. For a given amount of fluid infused in all animals, the two groups of animals receiving CGS-21680 had greater MAP than the two placebo groups between 30 and 90 minutes after randomization to the two ventilatory strategies (Fig. 1).

Blood Gas Analysis and Respiratory Mechanics

The mean values of PaO₂ at baseline were similar in all animals and were lower in the HV + placebo group than in the LV groups 60 minutes after randomization into the two mechanical ventilatory strategies. The administration of CGS-21680 reserved the PaO₂ in the HV group (Fig. 2A). The SpO₂ value was significantly lower in the HV + placebo group than other groups, respectively, at the end of the experiments (Fig. 2B). The CGS-21680-treated groups showed a higher level of pH and HCO₃⁻ compared with the placebo group (Table 1) with no differences in PaCO₂ values (Fig. 2C).

The levels of lung elastance at baseline were similar in all groups, but increased in the HV + placebo group than the other groups (Fig. 3A). Similarly, the static pressure–volume curve showed that the HV + placebo group had a lower compliance that was reversed by the treatment with CGS-21680 (Fig. 3B).

PMN Lung Infiltration and Lung Injury

The total cell counts in lung lavage (bronchoalveolar lavage) fluid were significantly higher in the HV + placebo group ($1.67 \pm 0.21 \times 10^6/\text{mL}$) than in the other groups ($0.99 \pm 0.11 \times 10^6/\text{mL}$ in HV + CGS-21680, $0.80 \pm 0.08 \times 10^6/\text{mL}$ in LV + CGS-21680, and $1.01 \pm 0.10 \times 10^6/\text{mL}$ in LV + placebo, respectively, *p* < 0.05 for all; Fig. 4A). Similarly, the HV + placebo group had a higher percentage of neutrophils than the other groups ($27\% \pm 2\%$ vs. $12\% \pm 2\%$ in HV + CGS-21680, $9\% \pm 1\%$ in LV + CGS-21680, and $14\% \pm 3\%$ in LV + placebo, respectively, *p* < 0.05 for all; Fig. 4B).

The lung wet-to-dry ratio was 7.03 ± 0.71 in the HV + placebo group, which was significantly higher than the other groups (5.28 ± 0.13 in HV + CGS-21680, 5.05 ± 0.12 in

LV + CGS-21680, and 5.42 ± 0.02 in LV + placebo, respectively, $p < 0.05$ for all; Fig. 4C). The lung injury score was higher in the HV + placebo group (15.8 ± 0.9) than in the other groups (2.5 ± 0.6 in HV + CGS-21680, 7.1 ± 1.2 in LV + CGS-21680 [both $p < 0.05$], and 13.5 ± 1.3 in LV + placebo, respectively; Fig. 4D).

Lung histology shows that the administration of CGS-21680 attenuated alveolar collapse, perivascular and peribronchial edema, and polymorphonuclear neutrophils infiltration seen in the placebo group (Fig. 5).

Cytokine Profiles

Among the ten cytokines assayed, the plasma level of IL-1 β was significant and TNF- α tended to be higher in the HV + placebo group than in the other groups ($p < 0.05$, Figs. 6, 7, and 8). The lung lavage levels of IL-2, -6, -10, and TNF- α were higher, and GM-CSF tended to be higher in the HV + placebo group than in the other groups ($p < 0.05$, Figs. 6–8).

Discussion

The main finding of the present study is that the administration of a specific A_{2A} receptor agonist dramatically attenuated inflammatory responses thus VILI in the two-hit model of ischemia and reperfusion followed by mechanical ventilation in rats. It is noteworthy that these beneficial effects of CGS-21680 were observed where investigators were blinded to the compound administration during the studies.

Over seven decades ago, Drury and Szent-Györgyi (23) discovered the role of adenosine as an extracellular signaling molecule, which later was shown to protect organ systems from injury in response to stress conditions including ischemia and reperfusion (22). The administration of adenosine has displayed a beneficial effect in protecting tissue from ischemia and reperfusion. However, an extremely short half-life (1–2 seconds) of injected adenosine in plasma had limited its role for clinical application (23–25).

The cloning of cell surface ligands to recognize adenosine provides one with a power approach to modulate the various effects of adenosine (8, 15). There are four G-protein coupled receptors A₁, A_{2A}, A_{2B}, and A₃, and each of them mediates specific effects of adenosine (8, 15).

The anti-inflammatory effects of adenosine are generally attributed to the occupancy of A_{2A} receptor that is widely distributed in the human body with high expression in leukocytes and platelets, while expressed at intermediate levels in the lung (26). In ischemia reperfusion models, the use of A_{2A} receptor agonists resulted in a decrease in macrophage activation and adhesion molecular expression on endothelial cells, blockage of endothelial-neutrophil interactions, and attenuation of vascular derangement (11, 12, 17–21). The administration of the A_{2A} receptors agonist CGS-21680 has been shown to ameliorate lung injury in animal models of ischemia and reperfusion (12, 17). Furthermore, a reduced inflammatory response and preserved pulmonary function was achieved by using an A_{2A} receptor agonist in a rabbit model of lung transplantation (18). We thus speculated that the activation of A_{2A} receptor may have a place in the attenuation of VILI by its anti-inflammatory properties.

It is noteworthy that a recent study demonstrated in a model of LPS induced acute lung injury in mice that deletion of the A_{2B} receptor gene was associated with reduced survival time and increased lung permeability (27). Furthermore, treatment with A_{2B} receptor-selective antagonist resulted in enhanced pulmonary inflammation and lung permeability while an A_{2B} receptor agonist attenuated VILI in wild-type mice. Taken together, our current and the recent study (27) suggest that both A_{2A} and A_{2B} receptor agonists may become a therapeutic approach in VILI.

Our HSR model is different from those ischemia reperfusion models previously described (12, 17). We used a shorter period of reperfusion (30 minutes) than the previous models in which a longer time of reperfusion (3 hours) was used (12, 17, 18). Thus, an inflammatory response was induced by HSR to prime the lung without causing lung injury in our rat model (18). Our model of HSR followed by mechanical ventilation is clinically relevant, since approximately 20% of patients who have hemorrhagic shock require mechanical ventilation. We have previously reported that the HSR renders the lung more susceptible to VILI (4). It has been well established that mechanical ventilation can cause biotrauma by releasing inflammatory cytokines in experimental and clinical settings (28–30). Several clinical trials including the ARDS Network study demonstrated that patients with ARDS ventilated with low tidal volume had an improved survival rate associated with a decrease in circulating cytokine levels compared with those ventilated with high tidal volume (31, 32). However, the low tidal volume strategy may not apply to all patients with ARDS (33), thus an effective pharmacologic intervention is required to treat these patients. In the present study, we demonstrated that the administration of the A_{2A} receptors agonist CGS-21680 protected the lung from injury associated with a decreased cytokine response.

We observed that the plasma levels of IL-1 β and TNF- α decreased in the CGS-21680-treated animals ventilated with high tidal volume. Because IL-1 β and TNF- α are early and central proinflammatory cytokines in response to stresses, the attenuation of these cytokines may have contributed to beneficial effects in the CGS-21680-treated animals.

Interestingly, the levels of IL-2, GM-CSF, IL-10, and TNF- α in lung-lavage fluid decreased in the CGS-21680-treated animals than those in the placebo group under HV mechanical ventilation. IL-2 is produced by helper T (Th1) cells. The intrinsic death-sensitizing activity of IL-2 was believed to be a key mediator for apoptosis of peripheral autoreactive T cells. However, recent studies redefine the pivotal role for IL-2 as the major inducer for the developmental production of suppressive T regulatory cells (34). GM-CSF is a glycoprotein cytokine secreted by monocytes that regulate the proliferation and differentiation of myeloid cells and the activation of monocytes (32). GM-CSF has a stimulatory effect on a number of neutrophil functions (35). The IL-10 secretion was significantly increased by stimulation with GM-CSF in spleen cells compared with similarly cultured saline spleen cells (36). IL-10 was originally described as a cytokine synthesis inhibitory factor produced by Th2 cells that inhibit Th1 function. Much of the early studies with IL-10 focused on its ability to inhibit production of proinflammatory cytokines, including IL-1 β and TNF- α . In fact, a major stimulus for the production of IL-10 is inflammation itself, because IL-1 β , TNF- α , and GM-CSF can stimulate IL-10 production directly, suggesting the existence of a negative feedback loop, whereby inflammatory processes are self limited by the endogenous

production of IL-10 (37). Taken together, these findings suggest that the magnitude of the endogenous IL-10 response correlates with both the severity of the inflammatory insult and the concentrations of proinflammatory cytokines such as TNF- α (38).

The cytokine profiles observed in the present study provide one with novel information suggesting that T-cell activation may have well taken place in response to a short term (i.e., 3.5 hours) of mechanical ventilation in an ARDS lung. This was evidenced by the release of the Th cytokines including IL-2 and IL-10. Clinical studies have suggested that the ratio of T cells expressing CD4⁺ over CD8⁺ could help diagnose ARDS and predict its outcome. Although the direct consequences of the cytokine responses in relation to VILI were not addressed in the present study, previous studies have demonstrated the importance of cytokines in the context of ARDS by showing that increased blood levels of cytokines were correlated with mortality rate in patients with ARDS (39).

Neutrophils are considered to be central to the pathogenesis of ARDS (40). The decreased neutrophil migration in the lung is believed to be related to the anti-inflammatory properties of CGS-21680 in the treated groups (41, 42). Furthermore, the transient higher MAP values observed in the CGS-21680-treated animals may be due to preserved vascular integrity for a given amount of fluids infused in all animals.

We did not measure the level of adenosine in the present study. However, previous *in vitro* studies have revealed that supernatants from hypoxia- or stretch induced injury contained adenosine that diminished endothelial leakage (14, 43). Additional studies *in vivo* demonstrated prominent increases in pulmonary adenosine levels with mechanical ventilation (13, 14). The increased adenosine level was associated with the enhanced expression of ectoaprase (CD39) and ecto-5'-nucleotidase (CD73) that hydrolyze ATP to adenosine at the cell surface of neutrophils and endothelial cells (43). The CD39 deficient mice have been shown to have pressure- and time-dependent increases in pulmonary edema and inflammation following mechanical ventilation, and administration of CD39 deficient or CD73 deficient mice with soluble apyrase or 5' nucleotidase, respectively, abolished such increases (14). An alternate source for adenosine formation is cyclic adenosine monophosphate. A previous study has reported an elevation of pulmonary cyclic adenosine monophosphate levels following VILI (27). In the present study, we noted that the CGS-21680-treated animals in the high volume group had the lowest lung injury score. This observation is in agreement with those reported by Eckle et al who have recently demonstrated a 1.5-fold increase in the expression of A_{2A} receptor associated with a significant increase in A_{2A} receptor mRNA level in a mouse model of VILI, and the A_{2A} receptor signaling pathway further increased pulmonary levels of cyclic adenosine monophosphate (27).

Taken together, the previous work suggests that a more severe VILI resulted in a higher expression of A_{2A} receptor and adenosine in the given lung conditions (27). Because both cyclic adenosine monophosphate and adenosine regulate the activities of the epithelial sodium channel and the chloride channel cystic fibrosis transmembrane conductance regulator to balance between fluid secretion and absorption in the alveolar space, the adenosine pathways to dry out the lungs during VILI may have contributed to the better

preserved lung histology by surpassing the effects of leukocyte migration seen in the HV + CGS21680 group.

In summary, the present study suggests that a selective activation of adenosine A_{2A} receptors by using CGS-21680 is protective in the two-hit model of VILI in rats. Further studies are warranted to confirm the observation in other models of VILI.

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Figure 1.

Time course of mean arterial pressure (*MAP*) during hemorrhagic shock (*HS*) and resuscitation (*Resc.*) and after randomization to mechanical ventilation (*MV*) of low volume (*LV*) with placebo, low volume with drug (CGS21680), high volume (*HV*) with placebo, and high tidal volume with drug, $n=10$ each. * $p < 0.05$ CGS21680 vs. placebo in identical conditions, respectively.

Figure 2.

PaO₂, SpO₂, and PaCO₂ during the 3-hr period of mechanical ventilation after randomization (n=10 each). *p<0.05 LV groups vs. HV groups (except LV + GCS21680 vs. HV + GCS21680 at 60 min); †p<0.05 HV + placebo vs. other groups. LV, low volume; HV, high volume.

Figure 3.

Elastance changes during mechanical ventilation after randomization. * $p < 0.05$ HV groups vs. LV groups. B, Static compliance curves of total respiratory system at the end of the experimental protocol. * $p < 0.05$ HV + placebo vs. other group. *LV*, low volume; *HV*, high volume.

Figure 4.

A, The total cell counts in lung lavage fluids (n=10 each). B, The percentage of neutrophil counts in lung lavage fluids. C, Lung wet-dry ratio (n=5 each) and D, lung injury scores.

*p < 0.05 HV + placebo vs. other groups, respectively. †p < 0.05 LV + GCS21680. LV, low volume; HV, high volume; BAL, bronchoalveolar lavage.

Figure 5. Representative lung histology slides (original magnification X 400). A, high volume + CGS21680, (B) high volume + placebo, (C), low volume + CGS21680 and (D) low volume + placebo.

Figure 6.

Levels of interleukin (IL)-1α, -1β, and -2 in plasma (n= 5 each) and lung lavage fluid (n=10) at the end of the experiments. *p<vs. other groups, respectively. *LV*, low volume; *HV*, high volume.

Figure 7.

Levels of interleukin (IL)-4, IL-6 and IL-10 in plasma (n=5 each) and lung lavage fluid (n=10) at the end of the experiments. *p < 0.05 vs. other groups, respectively. LV, low volume; HV, high volume.

Figure 8.

Levels of granulocyte-macrophage colony stimulation factor (GM-CSF), interferon (IFN)- γ , and tumor necrosis factor (TNF)- α in plasma (n=5 each) and lung lavage fluid (n=10) at the end of the experiments. *p< 0.05 vs. other groups, respectively. LV, low volume; HV, high volume.

Table 1

Time course for pH and HCO_3^- at baseline and after randomization for mechanical ventilation.

	Baseline	5 Mins	60 Mins	120 Mins	180 Mins
pH					
LV + placebo	7.390 ± 0.010	7.297 ± 0.013	7.340 ± 0.016	7.310 ± 0.024	7.313 ± 0.025
LV + CGS21680	7.374 ± 0.010	7.289 ± 0.015	7.357 ± 0.012	7.368 ± 0.017	7.344 ± 0.022
HV + placebo	7.384 ± 0.009	7.273 ± 0.015	7.295 ± 0.015 ^a	7.294 ± 0.013 ^a	7.242 ± 0.014 ^a
HV + CGS21680	7.381 ± 0.009	7.269 ± 0.013	7.332 ± 0.012	7.365 ± 0.015	7.363 ± 0.018
HCO_3^-					
LV + placebo	22.5 ± 0.6	18.2 ± 0.5	19.3 ± 0.7	18.5 ± 1.0	18.2 ± 0.9
LV + CGS21680	21.6 ± 0.5	17.6 ± 0.5	20.3 ± 0.7	20.7 ± 0.8	19.8 ± 0.9
HV + placebo	23.4 ± 0.6	17.3 ± 0.8	17.9 ± 0.6	17.5 ± 0.4 ^a	16.1 ± 0.7 ^a
HV + CGS21680	22.0 ± 0.5	16.9 ± 0.7	20.1 ± 0.8	20.8 ± 0.9	20.4 ± 1.0