Protective effect of adenosine receptors against lipopolysaccharide-induced acute lung injury

Joyce N. Gonzales,1,2 Boris Gorchkov,2 Matthew N. Varn,2 Marina A. Zemskova,2 Evgeny A. Zemskov,2 Supriya Sridhar,2 Rudolf Lucas,2,3 and Alexander D. Verin1,2
1Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, 2Vascular Biology Center, and 3Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, Georgia

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Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) affect 200,000 people a year in the USA. Pulmonary vascular and specifically endothelial cell (EC) barrier compromise is a hallmark of these diseases. We have recently shown that extracellular adenosine enhances human pulmonary (EC) barrier via activation of adenosine receptors (ARs) in cell cultures. On the basis of these data, we hypothesized that activation of ARs might exert barrier-protective effects in a model of ALI/ARDS in mice. To test this hypothesis, we examined the effects of pre- and posttreatment of adenosine and 5′-N-ethylcarboxamidoadenosine (NECA), a nonselective stable AR agonist, on LPS-induced lung injury. Mice were given vehicle or LPS intratracheally followed by adenosine, NECA, or vehicle instilled via the internal jugular vein. Postexperiment cell counts, Evans Blue Dye albumin (EBDA) extravasation, levels of proteins, and inflammatory cytokines were analyzed. Harvested lungs were used for histology and myeloperoxidase studies. Mice challenged with LPS alone demonstrated an inflammatory response typical of ALI. Cell counts, EBDA extravasation, as well as levels of proteins and inflammatory cytokines were decreased in adenosine-treated mice. Histology displayed reduced infiltration of neutrophils. NECA had a similar effect on LPS-induced vascular barrier compromise. Importantly, posttreatment with adenosine or NECA recovers lung vascular barrier and reduces inflammation induced by LPS challenge. Furthermore, adenosine significantly attenuated protein degradation of A2A and A3 receptors induced by LPS. Collectively, our results demonstrate that activation of ARs protects and restores vascular barrier functions and reduces inflammation in LPS-induced ALI.

adenosine; lipopolysaccharide; pulmonary edema; receptors

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are commonly seen in Intensive Care Units and carry a high mortality. Breakdown of the pulmonary endothelial cell (EC) barrier is one of the hallmarks of these lung diseases. The results can be noncardiogenic pulmonary edema and hypoxemia (51).

As a breakdown product of ATP, adenosine is an endogenous purine nucleoside that modulates many physiological processes in all cells of the body. Four adenosine receptors (ARs) (A1, A2A, A2B, and A3) have been identified. Activation of ARs leads to inhibition of inflammatory pathways in many cell types (18, 25). The A2AR is expressed in lung tissues, where it is activated by endogenous/exogenous adenosine or A2AR agonists and where it exerts anti-inflammatory effects (24, 39, 45). Hasko et al. (19) showed that activation of the A2AR ameliorated lung permeability and protein levels in bronchoalveolar lavage fluid (BALF) in traumatic hemorrhagic shock. It has been shown that an A2AR agonist protects against ischemia-reperfusion injury in porcine allogeneic lung transplantation (23). The A2BR has been evaluated in lung injury and is known to be protective against ALI (4, 22, 44). Several studies have also suggested that the beneficial effects of adenosine occur through the effects of the A2AR and A2BR (7, 22, 27, 43). Limited information is available regarding involvement of other ARs in ALI/ARDS. The A1R has been associated with reduction in lung edema (20).

Recently, our research team showed that the A1R is responsible primarily for enhancement of the EC barrier in the vasa vasorum of bovine EC in vitro (48), suggesting that the A1R can also be involved in protection of vascular barriers against ALI. A3R activation provides lung protection against ischemia-reperfusion injury as well as in LPS-induced ALI (41). Collectively, these data reflect the complexity of AR-mediated barrier protection.

Our research team has shown recently that adenosine enhances the human pulmonary artery endothelial (HPAEC) barrier in vitro via activation of A2 receptors that couple with G-stimulatory (Gs) trimeric G proteins, thereby stimulating adenylyl cyclase and producing an increase in cAMP levels. The subsequent intracellular molecular mechanisms were found to decrease the formation of F-actin stress fibers and increase cortical actin ring, thus increasing cell junctions and decreasing contractility (46). On the basis of these findings, we hypothesized that adenosine stimulates AR-mediated signaling cascades, leading to protection and restoration of vascular barriers in a murine model of ALI induced by intratracheal instillation of LPS.

To test the effect of adenosine and 5′-N-ethylcarboxamidoadenosine (NECA) over time, we used pretreatment and posttreatment protocols. We found that adenosine exerted barrier-protective effects with respect to lung permeability and injury. Treatment with NECA was found to exert similar effects. These findings suggested that the protective effects of adenosine against LPS-induced ALI in a murine model were dependent on activation of ARs, but not products of adenosine degradation. Furthermore, adenosine prevents the degradation of A2A and A3 receptors on the protein level induced by LPS. Collectively, our data suggest that adenosine stabilized and restored vascular barriers via multiple mechanisms, which

Address for reprint requests and other correspondence: J. N. Gonzales, Assistant Prof. of Medicine, Div. of Pulmonary and Critical Care Medicine, Georgia Regents Univ., Rm. BB-R-5513, 1120 15th St., Augusta, GA 30912 (e-mail: JGONZALES@gru.edu).

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included activation of AR-mediated signaling as well as preservation of ARs against LPS-induced degradation.

**MATERIALS AND METHODS**

**Ethical approval of the study protocol.** The study protocol was approved by the Animal Care and Use Committee of Georgia Regents University (Augusta, GA). The care and treatment of animals was undertaken according to guidelines set by the National Institutes of Health (Bethesda, MD).

**Chemicals and reagents.** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless indicated otherwise. LPS is Escherichia coli serotype 0111:B5. PBS and Hanks’ balanced salt solution (HBSS) were obtained from Invitrogen (Carlsbad, CA). NECA was purchased from Tocris Bioscience (Ellisville, MO). A bicinechonic acid (BCA) protein assay kit (Pierce, Rockford, IL) was used to measure total protein.

**Animals.** CD-1 mice or C57BL/6 mice in some experiments weighing 20–25 g were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in cages until the time of experimentation. They had free access to food and water in a temperature- and light-controlled room with a 12-h:12-h dark/light cycle.

**Animal procedures.** Mice were anesthetized with ketamine (150 mg/kg ip) and acetylpromazine (15 mg/kg ip) before exposure of the trachea and right internal jugular vein (IJV) via a neck incision. Escherichia coli LPS solution (serotype 0111:B5, 1.58 mg/kg) or sterile normal saline (NS) 0.9% was instilled via the intratracheal route using a 20-G catheter. Fifteen minutes before or 3 h after instillation mice received adenosine [final calculated plasma concentration 100 μM (4.05 mg/kg), NECA (3.5 μM), or NS] in the control groups via the right IJV. Mice were allowed to recover overnight for 18–24 h. Two hours before termination of the experiment, Evans Blue Dye albumin (EBDA, 30 mg/kg) was instilled via the intravenous route. Mice were weighed at the start of the experiment and at 24 h. Mice were euthanized 18–24 h after LPS challenge. BAL was conducted by instillation of 1 ml of 10% HBSS, and samples were collected for cell counts and measurement of protein levels. Lungs were collected and stored at −80°C for the evaluation of permeability. The posttreatment time was based on our previous studies with pulmonary ECs. Those studies demonstrated that the effective decrease in transendothelial resistance (an inverse index of permeability) elicited by LPS begins to reach a nadir after 3 h (47).

**Quantification of total protein and white blood cells in BALF.** Collected BALF was centrifuged (500 g, 15 min, 4°C). The supernatant was centrifuged again (16,500 g, 10 min, 4°C). Total protein in pure BALF was measured using a BCA protein assay kit (Thermo Scientific). Cell pellets were suspended in ACK Lysing Buffer (Invitrogen, Grand Island, NY) for red blood cell lysis, centrifuged (500 g, 15 min, 4°C), and resuspended in 3.7% formalin. The total number of white blood cells (WBCs) was counted using a hemocytometer.

**Measurement of the EBDA concentration in lungs.** Measurement of the EBDA concentration in the lungs was undertaken by injection of EBDA (30 mg/kg) into the right IJV 2 h before termination of the experiment to assess vascular leakage. Lungs free of blood were weighed and snap-frozen in liquid nitrogen. The left lung was homogenized, incubated with two volumes of formamide (18 h, 60°C), and centrifuged (5,000 g, 30 min, 20°C). The optical density of the supernatant was determined by spectrophotometric means at 620-nm and 750-nm band pass filters. The extravasated EBDA concentration in lung homogenates was calculated against a standard curve (micrograms of EBDA per gram of lung).

**Lung histology.** Lungs perfused free of blood with EDTA were immersed in 4% buffered paraformaldehyde for 18 h before histological evaluation using hematoxylin & eosin (H&E) staining. Right lung lobes were used for histological evaluation. H&E staining was done by deparaffinizing and hydrating the slides to water. Slides were stained in Harris hematoxylin for 15 min and eosin for 30 s. Slides were dehydrated, cleared, and mounted with Cytoseal. A designated scoring system to quantify the extent of histological lung injury in animals has been published by the American Thoracic Society (ATS) and was used to assess lung injury (30). Lung fields were evaluated after H&E staining as described above. Each of the five independent variables set by the ATS Workshop Report on the features and measurements of ALI in experimental animals was evaluated in 20 random high-power fields (×400 magnification). The independent variables were 1) neutrophils in the alveolar space, 2) neutrophils in the interstitial space, 3) hyaline membranes, 4) proteinaceous debris filling the airspaces, and 5) sepal thickening. To generate a lung injury score, the sum of each of the five independent variables was weighted and relevance ascribed to each feature and then normalized to the number of fields evaluated.

**Immunohistochemical analyses of myeloperoxidase.** Sections (4-μm thickness) were cut from paraffin blocks and mounted on treated slides (Superfrost Plus; VWR Scientific Products, Suwanee, GA). Slides were air-dried overnight and then placed in a 60°C oven for 30 min. Slides were then deparaffinized in two changes of xylene for 7 min. They were then passed through a graded series of alcohol solutions. That is two changes of absolute ethanol for 2 min each, two changes of 95% ethanol for 2 min, 80% ethanol for 2 min, and 70% ethanol for 2 min to distilled water. Slides were pretreated with Target Retrieval Solution (Dako, Carpentry, CA) using a Rice Steamer (Black and Decker, Towson, MD), and slides were rinsed in distilled water. Endogenous peroxidase was quenched with 0.3% H2O2 in distilled water for 5 min followed by distilled water for 2 min and then placed in 1× PBS for 5 min. Slides were then incubated with myeloperoxidase (MPO) primary antibody (Dako) for 30 min at room temperature followed by two changes of 1× PBS. Slides were then incubated with secondary antibody peroxidase-conjugated AffiniPure F(ab)2 Fragment donkey anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA) for one hour rinse in two changes of 1× PBS. Bound antibody was detected with a 3,3’-diaminobenzidine substrate kit for horseradish peroxidase (Dako). Slides were then counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI).

**Isolation of total RNA real-time quantitative PCR.** Total RNA was isolated from mouse lung tissue using Trizol reagent (Invitrogen) according to manufacturer instructions. The concentration and purity of RNA was determined by measurement of absorbance at 260 nm and 280 nm (Ultraspec 3000; Pharmacia Biotech, Uppsala, Sweden). Then ∼4 μg of cDNA was synthesized by reverse transcription with Maloney murine leukemia virus reverse transcriptase (Invitrogen) using oligo(dT) primers (Bio-Rad, Hercules, CA). Next, real-time PCR was conducted on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) using iTag Universal SYBR Green Supermix (Bio-Rad), 100 ng of cDNA, and a primer concentration of 1 μM (Integrated DNA Technologies, Coralville, IA). Real-time PCR primers (detailed in Table 1) were designed to target sequences of mRNA of mouse origin using NCBI Primer-Blast, and their quality was confirmed on NetPrimer (Premier Biosoft, Palo Alto, CA). Analyses of qPCR data were completed using the comparative Ct method (also known as the 2−ΔΔCT method), in which the mRNA levels of interest were normalized to the levels of the control (18S rRNA).

**Preparation of total lung lysates.** Frozen lung lysates were weighed. A total of 20–50 mg of tissue was collected and placed on dry ice for processing. Tissue was pulverized cryogenically using a liquid nitrogen-cooled mini mortar and pestle (Thomas Scientific, Swedesboro, NJ). The resulting powder was suspended in sample lysis buffer, supplemented with a 1× Halt Protease and Phosphatase Inhibitor Cocktail, sonicated on ice twice for 10 s at 20%, and centrifuged (14,000 g, 15 min, 4°C). The supernatant was saved and protein concentration determined using the BCA Protein Assay Kit (Pierce). The homogenate was then stored at −80°C.

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Gel electrophoresis and Western blotting. Total lung lysates (40 μg) and cell lysates (15 μg) were combined with 2× Laemmli sample buffer, containing 5% β-mercaptoethanol, and boiled for 8 min at 100°C. Equal volumes were loaded in each lane of a 4–20% precast polyacrylamide gel (Bio-Rad) and resolved by SDS-PAGE at 80 V for 1.5 h. They were then transferred to 0.2-μm PVDF membranes (Immun-blot PVDF; Bio-Rad) at 40 V for 4 h at 4°C. Membranes were blocked for 1 h in blocking buffer at room temperature and immunoblotted overnight at 4°C with the primary antibody of interest in blocking buffer. The next day, the membrane was washed with Tris-buffered saline and Tween-20 (4 × 5 min), and horseradish peroxidase-conjugated secondary antibody was added for 1 h. After being washed, the immune complexes were visualized by enhanced chemiluminescent detection (Pierce) using a Kodak 440CF Image Station (Carestream, Rochester, NY). Equal loading of protein was confirmed by β-actin or GAPDH, (Cell Signaling, Danvers, MA). The intensities of protein signals were quantified based on the optical densities of immunoblots using ImageJ software (National Institutes of Health).

Quantification of proinflammatory mediators in BALF. Collected BALF was centrifuged (500 g for 15 min at 4°C). Supernatants were centrifuged again (500 g for 15 min at 4°C). The concentrations of cytokines, chemokines, and growth factors in pure BALF were quantified using the multiplex MCYTOMAG-70K assay (EMD Millipore, Darmstadt, Germany), according to manufacturer instructions. In other experiments, levels of the inflammatory cytokines IL-6 and TNF-α were measured in lung tissue lysates using real-time PCR at 6 h. The results for IL-6 and TNF-α are expressed as 2−ΔΔct (26).

Statistical analyses. Values are the means ± SE of 3–5 independent experiments. For multiple comparisons, ANOVA and post hoc multiple comparison tests were applied. The Student’s t-test was used for comparisons of the mean values of two samples. P ≤ 0.05 was considered significant.

RESULTS

LPS increased vascular permeability in a time-dependent manner in murine model of ALI. Endotoxin challenge by intratracheal instillation of LPS results in injury to lung tissue that can be evident within 1 h, and maximal injury is observed at 24 h (31). To optimize conditions in an in vivo model, the effect of LPS on pulmonary vascular permeability in mice was tested. LPS significantly (7 h, P ≤ 0.01; 24 h, P ≤ 0.0008) increased EBDA leakage into lung tissue lysates (Fig. 1A) and protein levels in BALF (Fig. 1B) in a time-dependent manner, with a significant (P ≤ 0.001) increase at 7 h and a greater increase at 24 h (P ≤ 0.0005).

Pre- and posttreatment with adenosine or NECA significantly attenuates LPS-induced vascular leakage in a murine model of ALI. Increased microvascular lung permeability is a cardinal feature of ALI/ARDS. Initially, we compared the efficacy of pretreatment (15 min) and posttreatment (3 h) with adenosine in preventing vascular leakage in an LPS-induced model of ALI in mice. Mice were intubated and given vehicle or LPS intratracheally (for 24 h, maximal LPS response, see Fig. 1). Vehicle or adenosine was instilled via the IJV 2 h before the end of the experiment. EBDA was injected into the IJV (33). In the pretreatment group, adenosine was injected into the right IJV 15 min before intratracheal LPS installation. At the end of the experiment, BALF was collected and tested for protein content. Pretreatment with adenosine significantly reduced the vascular permeability of EBDA (Fig. 2A) and protein content in BALF (Fig. 2B). Posttreatment with adenosine (intravenous into the IJV 3 h after intratracheal LPS)
provided similar results as those seen with pretreatment. A significantly decreased permeability of EBDA (Fig. 2D) and protein content in BALF was noted (Fig. 2E). Consistent with these results, microscopic assessment demonstrated significantly reduced WBC counts in the BALF of the LPS/adenosine-treated mice compared with those treated with LPS alone (Fig. 2, C and F).

It is well known that adenosine activates cell signaling primarily through ARs (13, 44). However, adenosine is rapidly degraded in the blood stream (16a), and products of adenosine degradation can affect EC permeability (17). To further define the role of ARs in adenosine-induced pulmonary vascular barrier protection, we conducted similar pre and posttreatment studies with stable AR agonist, NECA. NECA significantly reduced vascular permeability of EBDA (Fig. 3, A and C) and BALF protein content (Fig. 3, B and D) in LPS-treated mice. These data confirmed that adenosine exerted its barrier-protective effects via activation of ARs but not via signaling pathways initiated by adenosine degradation products.

Histological evaluation of adenosine effect on LPS-induced lung inflammation. Histological assessment of the effect of adenosine on LPS-challenged lung tissue showed decreased numbers of neutrophils and injury (Fig. 4A). Histology specimens showed increased sequestration of neutrophils, as well as increased edema and injury in the LPS-treated group compared with the LPS/adenosine group, vehicle, or adenosine-alone group. Injury was assessed using the lung injury score set by the ATS (32). Total lung injury scores for LPS/adenosine-treated mice were significantly (P ≤ 0.04) decreased compared with the LPS group (Fig. 4B).

**Immunohistocytostaining for MPO.** MPO is an enzyme expressed abundantly in neutrophil granulocytes. An increased concentration of MPO in BALF is suggestive of neutrophil activation. MPO was evaluated upon immunohistocytotoxic chemical criteria. MPO levels were increased in all samples and abundant in LPS-treated lung samples. MPO levels were decreased in LPS/adenosine-treated mice (Fig. 5).

**Adenosine attenuates LPS-induced loss of body weight.** The proinflammatory state is associated with cachexia, dehydration, and weight loss. Attenuated weight loss was observed in our models upon treatment with adenosine (Fig. 6).

**Effect of LPS and adenosine on expression of ARs.** Adenosine-dependent cell signaling is mediated via the activation of four ARs, A1, A2A, A2B, and A3. Therefore, the levels of

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Fig. 2. Adenosine (ADO) pre- and posttreatment significantly attenuates an LPS-induced increase in vascular leakage in a murine model of ALI. Adenosine (intravenous, i.v.) was instilled 15 min before or 3 h after LPS (intratracheally, i.t. for 24 h) challenge. A: EBDA was injected (i.v.) 2 h before termination of the experiment. LPS challenge increased EBDA leakage from the vascular space into surrounding lung tissue in the LPS group, and adenosine (100 µM, i.v.) instilled 15 min before LPS significantly attenuated LPS-induced vascular leakage in mice. B: BALF was collected 24 h after treatment and centrifuged. Adenosine pretreatment reduced total protein accumulation in the BALF of mice with LPS-induced lung injury. C: adenosine pretreatment reduces white blood cell (WBC) accumulation in the BALF of LPS-treated mice compared with untreated mice. BALF was collected at the end of the experiment and centrifuged. Cells were counted using a hemocytometer. Adenosine reduced the total number of WBCs in BALF. D: EBDA was injected into the internal jugular vein 2 h before termination of the experiment. LPS challenge increased EBDA leakage from the vascular space into surrounding lung tissue in the LPS group, and adenosine (100 µM, i.v.) instilled 3 h after LPS significantly attenuated LPS (2.0 mg/kg, i.t.)-induced vascular leakage in mice. E: BALF was collected 24 h after treatment and centrifuged. Protein content was estimated in the clear supernatant using a bicinchoninic acid (BCA) protein assay kit. Adenosine treatment reduced total protein accumulation in the BALF of mice with LPS-induced lung injury. F: adenosine reduced WBC accumulation in the BALF of LPS-treated mice compared with untreated mice. *value differed significantly (P < 0.05) from that of the vehicle-treated group; #value differed significantly (P < 0.05) from the LPS group (n = 4–8). The error bars represent ± SE.
their expressions can be critical for adenosine-dependent protection of LPS-challenged tissues. To evaluate the relative expression of ARs in murine lung, we undertook qPCR analyses (Fig. 7). Our results indicated that, under normal physiological conditions (Fig. 7, left), all four ARs were expressed in murine lung, with predominant expression of A2BR and relatively low expression of A3R. To study the effects of LPS and adenosine on the expression of ARs in the lung, mice were treated with vehicle, adenosine, LPS, or LPS/adenosine, and lung tissue was harvested after 6 h. Then, respective mRNA expressions were evaluated by qPCR. Interestingly, LPS treatment significantly decreased transcription of A1 and A2BR receptors but not A2A and A3 receptors (Fig. 7). Adenosine alone had no significant effect on the transcription levels of any of the receptors (Fig. 7) and on the LPS-induced decrease in the transcription of A1 and A2BR receptors (Fig. 7, A and C). Next, we evaluated the effects of LPS and adenosine on AR expressions at the protein level using immunoblotting with receptor-specific antibodies. We conducted these analyses only for the A2AR and A3R due to the lack of satisfactory commercially available antibodies against A1R and A2BR. LPS treatment significantly decreased protein expression of the A2AR and A3R (Fig. 8, A and B). Surprisingly, adenosine protected these receptors from LPS-induced protein degradation (Fig. 8, A and B). These data demonstrated that LPS can specifically downregulate expression of ARs at the different levels. A1 and A2B, but not A2A and A3, are downregulated by LPS at the mRNA level, and this downregulation was not affected by adenosine treatment. In contrast, A2A and A3 receptors are downregulated at the protein level, and this effect is attenuated by adenosine. To the best of our knowledge, this is the first evidence of differential regulation of ARs by LPS/adenosine. The mechanisms involved in the protective effect of adenosine are unknown. Recent studies demonstrate that adenosine can affect ATP-mediated regulation of proteasome activity (29). Further studies are needed to evaluate the mechanisms of adenosine-induced receptor protection and the role of this pathway in adenosine signaling.

Effect of LPS and adenosine on the levels of proinflammatory cytokines. LPS is known to elicit a robust inflammatory response from the immune system and produces various effects linked to lung injury (32). To ascertain whether the protective effect of adenosine on barrier dysfunction occurs due to an inhibitory effect on inflammatory mediators affecting barrier integrity, we examined its effect on LPS-induced production of inflammatory cytokines and growth factors. First, we evaluated the effect of adenosine on LPS-induced gene expression of the well-known markers of inflammation IL-6 (Fig. 9A) and
TNF-α (Fig. 9B) in the lung lysates of LPS- and LPS/adenosine-treated mice by qPCR. Adenosine significantly attenuated LPS-induced increase in mRNA level of these cytokines \((P \leq 0.05)\). Consistent with these results, adenosine significantly attenuated LPS-induced upregulation of the other proinflammatory cytokines/chemokines and growth factors reported to directly affect pulmonary barrier function (Fig. 10, A–K) as quantified by the multiplex MCYTOMAG-70K assay in BALF. These findings suggest that adenosine can modulate broad immune responses in mice, and, importantly, this modulation can lead to a downregulation of the expression and/or secretion of a number of clinically relevant proinflammatory factors.

**DISCUSSION**

Pulmonary EC dysfunction, as characterized by parenchymal edema caused by neutrophil extravasation, is a major feature of ALI/ARDS (14, 51). Pulmonary injury at the
capillary EC barrier is observed with increased gaps between cells and increased permeability (5). Injury induced by LPS in mice has been shown to be consistent with sepsis-induced ALI (1, 32).

LPS is a component of the outer membrane of Gram-negative bacteria. LPS is an endotoxin and induces strong responses from the immune systems of healthy animals (32). The effects of LPS are exerted through the generation and secretion of proinflammatory cytokines. These substances cause lung inflammation, as well as the subsequent edema and injury in lungs that occur in ALI/ARDS (2, 28, 39). We evaluated the effects of LPS-induced inflammation and vascular leakage over time. We observed significant increases in lung permeability in our LPS-generated model of lung injury. Extravasation of EBDA into lung tissue and protein into BALF was significantly increased in a time-dependent manner at 7 h and 24 h (Fig. 1, A and B).

Adenosine is a potent mediator of stress that is present in an inflammatory environment (22, 50). Studies with adenosine have shown its barrier-protective benefits in various ALI models (7, 10, 42). However, it was also shown that effects of adenosine on the vascular barrier depends on adenosine concentration (10). Specifically, intratracheal instillation of low adenosine concentration leads to increased vascular leak. In contrast, physiological adenosine concentration reduced alveolar clearance (10). Importantly, intratracheal instillation of adenosine described by Factor et al. (10) initially affected alveolar epithelium but not endothelium. In our study, we instilled adenosine via an intravenous route, thus primarily affecting EC barrier. Recent studies have described the bene-

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)

Fig. 7. LPS treatment decreased gene expression of the A1R and A2BR but not the A2AR and A3R. qPCR analyses of adenosine receptor (A1, A2A, A2B, A3) mRNA expression from murine lung tissues at 6 h. A and C: LPS and LPS/adenosine (adenosine 3 h after LPS treatment) decreased A1R and A2BR mRNA expression compared with vehicle and adenosine. B and D: LPS did not affect the mRNA expression of the A2AR and A3R compared with vehicle and adenosine alone. The bar represents the mean fold change compared with vehicle. Expression levels were normalized to the value of 18S rRNA mRNA and expressed relative to the A3R. Data shown are ± SE, and all results are representative of 3 separate experiments. *Value differed significantly (P < 0.05) from the vehicle-treated group.

![Graph F](image6.png)

Fig. 8. Effects of LPS on the expression of A2AR and A3R proteins on the protein level in murine lung. LPS decreased expression of A2AR and A3R proteins. A: LPS decreased expression of the A2AR compared with vehicle-treated control 6 h after LPS treatment. The decrease was attenuated by adenosine treatment 3 h after LPS treatment. B: LPS decreased expression of the A3R compared with the vehicle-treated control 6 h after LPS treatment. The decrease was attenuated by adenosine treatment 3 h after LPS treatment. Data are expressed as the relative fold changes compared with controls (controls are vehicle-treated lysates, value = 1) 6 h after LPS treatment. *Value differed significantly (P < 0.05) from that of the vehicle group; #value differed significantly (P < 0.05) from that of the LPS group (n = 3 for each group). The error bars represent ± SE, and all results are representative of 3 separate experiments.

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ficial effects of adenosine on LPS-disrupted EC barrier in vitro. Many beneficial effects are thought to be through the A2AR or A2BR (3, 4, 6, 8, 20, 40). In vitro studies from our research team have demonstrated that addition of adenosine significantly increases transendothelial electrical resistance of cultured HPAEC. Furthermore, a siRNA mechanistic strategy confirmed that the A2AR and a cAMP-dependent signaling pathway elicit changes in actomyosin cytoskeleton via activation of myosin light chain phosphatase (46). We confirmed the barrier-protective properties of adenosine in an in vivo murine model of ALI with adenosine (Fig. 2). Furthermore, experiments with the stable AR agonist, NECA (Fig. 3), indicate that the effect of adenosine is attributed to the activation of ARs but not to the signaling induced by adenosine degradation. These data can explain the long-lasting effects of adenosine on the vascular barrier despite the fact that adenosine is degraded rapidly in the vasculature (16a). AR activation transfers signals across the membrane into the cytoplasm, thereby eliciting cellular changes (53). We speculate that AR activation rapidly stimulates downstream signaling cascades, leading to vascular cytoskeletal remodeling, which can last for hours. More importantly, our data demonstrated that activation of ARs is able to restore vascular barrier after LPS-induced injury. We believe that it was not described and is important in the clinical setting (Figs. 2 and 3).

ALI is characterized by neutrophil infiltration into the lung with an associated increase in the levels of inflammatory mediators and interstitial edema, which contribute to the early death of the host. Histological evaluations with H&E staining confirmed increased numbers of neutrophils and interstitial edema in LPS-induced ALI (Fig. 4). MPO stored in the azurophilic granules of neutrophils is released in response to leukocyte activation by adhesion and extravasation (15). Released MPO in the granules is consistent with neutrophil-dependent increased lung permeability, and MPO has a broad spectrum of antimicrobial activity (mainly against Gram-negative bacteria). MPO is a multifunctional inflammatory mediator with cell-contracting effects (52). Our results are consistent with reduced infiltration of neutrophils into the lung tissue in adenosine-treated mice, suggesting a positive role of adenosine in the resolution of lung injury (Fig. 5).

Influx of inflammatory cells into alveolar air spaces is another important feature of ALI (11). Proinflammatory cytokines induce fever and multi-organ dysfunction. They also induce lung injury associated with remodeling of the EC cytoskeleton (resulting in a contractile phenotype), thereby leading to the neutrophil extravasation and noncardiogenic pulmonary edema prevalent in ALI/ARDS. We also showed an increase in the levels of proinflammatory cytokines in the BALF of mice after endotoxin-induced ALI. Posttreatment with adenosine downregulated the mRNA and polypeptide levels of multiple proinflammatory cytokines and chemokines (Figs. 9 and 10). These results are consistent with very recent data indicating that activation of the A2AR leads to attenuation of levels of TNF-α and IL-6 that are increased after LPS inhalation in a murine model of ALI (21).

Previously, we showed that HPAECs express A2AR and A2BR (46) but not the A1R or A3R. In contrast, we found that all four ARs were expressed in lung tissue. qPCR analysis demonstrated that intratracheal LPS instillation reduced gene expression of the A1R and A2BR (Fig. 7, A and C) but not the A2AR and A3R (Fig. 7, B and D). Our research team has recently shown that LPS effect on gene expression can be attributed to decreased mRNA stability of specific genes (37). However, it has been demonstrated that administration of LPS and/or inflammatory cytokines resulted in increased A2AR expression in various cells. Examples include macrophages, polymorphonucleocytes (PMNs) and dermal human microvas-
cular endothelial cells (dHMVEC) (12, 34, 36). In contrast, allergen challenges in asthmatic patients lead to downregulation of the A1R and A2BR (49). Furthermore, at the conditions of experimentally induced endotoxemia in humans, LPS induced changes in the gene expression of ARs in isolated lymphocytes in a complementary manner. Expression of the A1R and A3R were decreased by endotoxin, and, as the A3R mRNA expression levels recovered from the initial insult, A2BR levels decreased and returned to the level shown by the control (38). Studies on dHMVEC demonstrated a time-dependent effect of proinflammatory cytokines on A2BR mRNA expression (36).

Interestingly, our results demonstrated that LPS induced protein degradation of A2A and A3R without affecting gene expression (Figs. 7 and 8). Furthermore, we believe that we are the first who demonstrated that exogenous adenosine can protect ARs against LPS-induced degradation on the protein level (Fig. 8, A and B). The mechanism of such protection is unknown; however, recently published data demonstrated that adenosine can affect ATP-mediated regulation of proteasome activity. The effect is complex and depends on intracellular ATP concentration and may involve AR-independent pathways (29). Further studies are required to define mechanisms of AR protection induced by adenosine.

The A2AR increases cAMP levels via G-stimulating (Gs) coupling mechanisms. In contrast activation of the A3R induces G-inhibitory (Gi) activation, leading to decreasing cAMP production. The A2AR is known to have an anti-inflammatory role in many cells (34). Reports describing expression of the A2AR in cells that do not express the A3R (or with decreased transcriptions of the A3R) have shown that LPS or cytokines are associated with greatly increased mRNA expression for the

**Fig. 10.** A–K: adenosine (posttreatment) significantly attenuates LPS-induced production of proinflammatory cytokines/chemokines. The levels of proinflammatory cytokines/chemokines in mice BALF at 24 h with LPS compared with LPS/adenosine. *†Value differed significantly ($P < 0.05$) from that of the vehicle-treated group; #value differed significantly ($P < 0.05$) from that of the LPS and LPS/adenosine group ($n = 4–10$). MIP, macrophage inflammatory protein; INF, interferon; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; RANTES, regulated and normal T cell expressed and secreted.
A2AR (34, 36). The inhibitory effect of the A3R may attenuate the greatly increased expression of the A2AR reported in other cell models (34). The A3R has been reported to decrease LPS-stimulated expression of TNF-α and inducible nitric oxide synthase genes in macrophages via inhibition of a critical step for Ca²⁺ release (30). Perhaps our A3R transcriptional results with LPS/adenosine (Fig. 7D) reflect mediation of the A3R in a similar fashion. The apparent relationship between the A2AR and A3R in the present study is one illustration of the delicate balance between the effects of adenosine-activated Gi and Gs trimeric G proteins in the setting of an inflammatory milieu, but further investigation is needed to clarify these effects.

The A2BR has been shown to be lung protective (9, 22, 44). We found decreased expression of A2BR mRNA in lung tissue, but another group has noted an increase in A2BR transcription (44). Expression of the A2BR is known to be increased by hypoxia and subsequent induction of hypoxia-inducible factor-1α (HIF-1α) (6). Our mouse models showed increased permeability and induction of cytokines, but they may not have been hypoxic and did not induce HIF-1α to increase A2BR transcription. Notably, even with significant depletion of A2BR induced by LPS in our model (Fig. 7), adenosine and the nonselective AR agonist, NECA, are able to attenuate the edemagenic effects of endotoxin (Figs. 2 and 3). These data suggest that the A2BR is not involved in lung protection under normoxic conditions, which is consistent with our previous observations indicating that A2AR, but not A2BR, is involved in adenosine-induced EC barrier enhancement in HPAEC (46). However, further studies are needed to define the role of A2ARs in barrier protection against lung injury in the murine LPS model.

A recent literature report indicates that A1R expression/activation decreases LPS-induced microvascular permeability and reduces migration of PMNs into the lungs (35). Our findings showed that, despite LPS-induced decreases in A1R mRNA transcription (Fig. 7), adenosine and NECA still attenuate the effects of LPS on permeability and cytokine production (Figs. 2, 3, 9, and 10), suggesting that other ARs can also be involved in adenosine-induced protective effects.

LPS is known to induce cytokine production and expression of the proinflammatory cytokines IL-6 and TNF-α. Adenosine downregulates Toll-like receptor 4 expression at the surface of human macrophages, and this is thought to be protective (16). The levels of the proinflammatory cytokines, IL-6 and TNF-α, in lung lysates were decreased 6 h after adenosine treatment (Fig. 9, A and B). We also tested murine BALF for multiple cytokines (Fig. 10, A–K). Overall, we found significantly decreased levels of cytokines/chemokines/growth factors, suggesting that adenosine decreased the inflammatory burden and that this decrease was significant 24 h after injury.

Collectively, the present study demonstrates that adenosine acting via its receptors attenuated LPS-induced lung permeability and inflammation and restores the vascular barrier after LPS-induced lung injury in vivo. Future studies are needed to define the role of different receptors as well as downstream molecular mechanisms involved in adenosine effect.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

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PROTECTIVE ADENOSINE SIGNALING IN LPS-INDUCED ALI


