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Identification of Ectonucleotidases CD39 and CD73 in Innate Protection during Acute Lung Injury

Tobias Eckle,* Lars Füllbier,† Manfred Wehrmann,‡ Joseph Khoury,‡ Michel Mittelbronn,§ Juan Ibla,‡ Peter Rosenberger,* and Holger K. Eltzschig2*

Acute lung injury (ALI), such as that which occurs with mechanical ventilation, contributes to morbidity and mortality of critical illness. Nonetheless, in many instances, ALI resolves spontaneously through unknown mechanisms. Therefore, we hypothesized the presence of innate adaptive pathways to protect the lungs during mechanical ventilation. In this study, we used ventilator-induced lung injury as a model to identify endogenous mechanisms of lung protection. Initial in vitro studies revealed that supernatants from stretch-induced injury contained a stable factor which diminished endothelial leakage. This factor was subsequently identified as adenosine. Additional studies in vivo revealed prominent increases in pulmonary adenosine levels with mechanical ventilation. Because ectoapryrase (CD39) and ecto-5′-nucleotidase (CD73) are rate limiting for extracellular adenosine generation, we examined their contribution to ALI. In fact, both pulmonary CD39 and CD73 are induced by mechanical ventilation. Moreover, we observed pressure- and time-dependent increases in pulmonary edema and inflammation in ventilated cd39−/− mice. Similarly, pharmacological inhibition or targeted gene deletion of cd73 was associated with increased symptom severity of ventilator-induced ALI. Reconstitution of cd39−/− or cd73−/− mice with soluble apyrase or 5′-nucleotidase, respectively, reversed such increases. In addition, ALI was significantly attenuated and survival improved after i.p. treatment of wild-type mice with soluble apyrase or 5′-nucleotidase. Taken together, these data reveal a previously unrecognized role for CD39 and CD73 in lung protection and suggest treatment with their soluble compounds as a therapeutic strategy for noninfectious ALI. The Journal of Immunology, 2007, 178: 8127–8137.

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cute lung injury (ALI)1 is a syndrome consisting of acute hypoxemic respiratory failure with bilateral pulmonary infiltrates, not attributable to left heart failure (1). Despite optimal management consisting of aggressive treatment of the initiating cause, vigilant supportive care, and the prevention of nosocomial infections, mortality ranges between 35 and 60% (1). In fact, ~200,000 patients develop ALI annually in the United States, leading to 75,000 deaths and accounting for up to 3.6 million hospital days (2). The pathogenesis of ALI is characterized by the influx of a protein-rich edema fluid into the interstitial and intraalveolar spaces as a consequence of increased permeability of the alveolar-capillary barrier. The importance of endothelial injury and increased vascular permeability to the formation of pulmonary edema in this disorder has been well-established (1). Nevertheless, molecular details of how pulmonary capillary leakage is caused and maintained during ALI are largely unknown and studies linking its mechanisms with mechanical ventilation are currently areas of intense investigation (3, 4).

Despite the large impact of ALI on morbidity and mortality in critically ill patients (1), many episodes of ALI are self-limiting, and resolve spontaneously through unknown mechanisms. For example, patients undergoing major surgery requiring prolonged mechanical ventilation have an overall incidence of ALI between 0.2 and 5%, depending on the kind of surgery (5–7). Based on the rare occurrence of clinically relevant ALI in patients requiring mechanical ventilation, we hypothesized the existence of innate adaptive pathways to dampen acute increases in the capillary-alveolar permeability elicited by mechanical stretch or ventilation. In fact, the present study revealed a protective role of extracellular adenosine generation via the ectoapryrase (CD39, ATP/ADP conversion to AMP) and the ecto-5′-nucleotidase (CD73, AMP conversion to adenosine) during ALI induced by mechanical ventilation.

Materials and Methods

Cell culture

Human microvascular endothelial cells (HMEC-1) and human bronchial epithelial cells (Calu-3) were cultured as described previously (8, 9).

In vitro stretch model

To study the consequences of cyclic mechanical stretch on the release of barrier protective mediators, we adopted a previously described in vitro model resembling mechanical ventilation by applying cyclic mechanical stretch (10). In short, Calu-3 human bronchial epithelial cells were plated on BioFlex culture plates-collagen type I (BF-3001C; FlexCell International) and allowed to attach and grow to ~80% confluence. The medium was changed to MEM plus 10% FBS. Plates were then placed on a FlexCell
FX-4000T Tension Plus System and stretched at 20% stretch maximum, 0.7% stretch minimum, sine wave 5 s, on 5 s off. Supernatants were collected at indicated time points, flash-frozen, and stored at −80°C for further analysis. In controls, supernatants from Calu-3 human epithelia cultured under similar conditions at rest (without application of cyclic stretch) were used. In duplicate wells, cells adherent to the plates were used for transcriptional analysis (see below).

**HPLC analysis**

Supernatants were resolved by HPLC with a pump P6800 and a UVD 170 detector on a reverse-phase column (Grom-Sil 120-ODS-ST-5µ; 150 × 3 mm; Grom) using a mobile-phase gradient from 0 to 25% acetonitril/0.3 mM KH₂PO₄ (pH 5) in 5 min as described previously (9, 11, 12). UV absorption spectra were measured at 260 nm. In subsets of experiments, fractions were collected, evaporated to dryness by speed-vac, reconstituted in HBSS (20-fold concentrated), and bioactivity was determined by permeability assay (see below). In additional experiments, lungs from chronically ventilated animals (pressure controlled ventilation, inspiratory pressure 15 millibar (mbar), 100% inspired oxygen concentration) were harvested at indicated time points and flash-frozen in liquid nitrogen. Adenosine nucleosides were extracted from frozen lungs using 0.4 N perchloric acid and adenosine levels were determined as described previously (13). In subsets of experiments, adenosine concentrations were determined in the bronchoalveolar lavage (BAL) fluid derived from mice exposed to mechanical ventilation over indicated time periods (inspiratory pressure of 15 mbar, 100% inspired oxygen concentration). To obtain BAL fluid, the tracheal tube was disconnected from the mechanical ventilator and the lungs were lavaged three times with 0.5 ml of PBS containing 200 μM dipiridamole and 1 μg/ml deoxycoformycin. Removed fluid was centrifuged immediately, shock-frozen with liquid nitrogen for HPLC analysis.

**Macromolecule paracellular permeability assay**

Using a modification of methods previously described (14), HMEC-1 were grown on polycarbonate permeable supports (0.4-μm pore, 6.5-mm diameter; Costar) and studied 7–10 days after seeding (2–5 days after confluency). Paracellular flux rates were determined as described previously. In a subset of experiments, the nonspecific adenosine receptor antagonist 8-phenyl-theophylline (8-PT, 10 μM; Sigma-Aldrich) was added to the incoming buffer, before the start of the experiments and as described previously, supernatants were treated with bovine adenosine deaminase at a concentration that was previously shown effective in abolishing adenosine-mediated barrier responses (0.1 nM; Sigma-Aldrich) (11).

**Murine mechanical ventilation**

All animal protocols were in accordance with the German guidelines for use of living animals and were approved by the Institution Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. Mice deficient in the A2A adenosine receptor (AR) antagonist ZM241385 (Tocris Cookson; 1 mg/kg i.p.), or the A₂₅AR antagonist MRS1754 (Tocris Cookson; 1 mg/kg i.p.) 30 min before induction of anesthesia.

**Transcriptional analysis**

To examine the influence of mechanical ventilation on CD39 and CD73 transcript levels, C57BL/6d mice (Charles River Laboratories) were ventilated in a pressure-controlled fashion with indicated settings. Mice were euthanized at indicated time points and the remaining blood was removed from the pulmonary circulation by injection of 1 ml of PBS into the right heart. Lungs were excised and immediately frozen at ~80°C until transcriptional profiling. For this purpose, total RNA was isolated using the total RNA isolation NucleoSpin RNA II kit as described previously (Mauchery & Nagel) (17). RNA was washed and the concentration was quantified. cDNA synthesis was performed using reverse transcription according to the manufacturer’s instructions (i-script kit; Bio-Rad). The primer sets for the RT-PCR contained 1 μM sense and 1 μM antisense with SYBR Green 1 (Molecular Probes). Primer sequences for murine CD39/CD73 were 5′-TACACCCCCATCGTGCTT-3′ and 5′-GGAGCTTGTGTTT-3′ (sense/antisense) and 5′-CAGATCCACACAGACACACG-3′ and 5′-TGCCTACTGTTGCAACAGGC-3′, respectively. Primer sequences for human CD39/CD73 were 5′-ACG AGC TTA ATG CTG GC-3′, 5′-GAG ACA GTA TCT GCA GAA C-3′ and 5′-ATT GCA AAG TGG TTC AAA GTC A-3′ (sense/antisense), respectively. The primer set was amplified using increasing numbers of cycles of 94°C for 1 min, 58°C for 0.5 min, 72°C for 1 min. Murine or human β-actin (sense primer, 5′-ACAT TGGCATGGCTTTGT-3′; antisense primer, 5′-GGTGGCTCAAC CAACATGTGCT-3′ (murine); sense primer 5′-GGT GCC TTC TAG TAG G-3′ antisense primer 5′-ACT GGA ACG CGT AGG GTG ACA G-3′ (human)) in identical reactions were used to control for the starting template. Levels and fold change in mRNA were determined as described previously (18).

**Immunoblotting experiments**

In subsets of experiments, we determined CD39 and CD73 protein content from whole tissue. For this purpose, C57BL/6d mice (Charles River Laboratories) were ventilated with indicated ventilator settings and euthanized. Remaining blood was removed from the pulmonary circulation by injection of 1 ml of PBS into the right heart. The lungs were excised and immediately frozen at ~80°C until immunoblotting. For this purpose, tissues were homogenized and lysed for 10 min in ice-cold lysis buffer (10° polymerphoronal neutrophils (PMN)/500 μl; 150 mM NaCl, 25 mM Tris (pH 8.0), 5 mM EDTA, 2% Triton X-100, and 10% mammalian tissue protease inhibitor mixture; Sigma-Aldrich), and collected into microfuge tubes. After spinning at 14,000 × g to remove cell debris, the pellet was discarded. Proteins were solubilized in reducing Laemmli sample buffer and heated to 90°C for 5 min. Samples were resolved on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in PBS supplemented with 0.2% Tween 20 (PBST) and 4% BSA. The membranes were incubated in 1 μg/ml CD39 goat polyclonal Ab raised against the C terminus (Santa Cruz Biotechnology) or CD73 rabbit polyclonal Ab raised against the amino acids 275–574 (Santa Cruz Biotechnology) for 1 h at room temperature, followed by 10-min washes in PBS. The membranes were incubated in 1:3,000 donkey anti-goat HRP for CD39 (Santa Biotechnology), or goat anti-rabbit HRP for CD73 (Perbio Science). The wash was repeated and proteins were detected by ECL.

**Immunohistochemistry**

To examine the influence of mechanical ventilation on pulmonary CD39 and CD73 expression, mice were ventilated in a pressure-controlled fashion over indicated time periods. Mice were euthanized and the lungs were perfused via the right ventricle with 5 ml of PBS. Lungs were subsequently removed and stained with CD39 goat polyclonal Ab raised against the C terminus (Santa Cruz Biotechnology) or CD73 rabbit polyclonal Ab raised against the amino acids 257–574 (Santa Cruz Biotechnology) as described previously (17). In controls, normal goat or rabbit IgG was used at identical concentrations and staining conditions as the target primary Abs.

**Bronchoalveolar lavage**

To obtain BAL fluid, the tracheal tube was disconnected from the mechanical ventilator and the lungs were lavaged three times with 0.5 ml of PBS. All removed fluid was centrifuged immediately and the supernatant was aliquoted for albumin or MIP-2 determination.
Measurement of albumin and MIP-2 in BAL fluid

BAL fluid samples were thawed to 4°C and albumin or MIP-2 concentrations were measured using murine quantitative ELISA systems (albumin: Bethyl; MIP-2: R&D Systems), according to the instructions given by the manufacturer. All analyses were made in triplicate and mean values were used for statistical analysis.

Myeloperoxidase assay (MPO)

Pulmonary neutrophil sequestration was quantified using a myeloperoxidase (MPO) assay as described previously (19–21). In short, animals were euthanized and lungs were perfused with 5 ml of PBS through the right ventricle. Lungs were excised, frozen in liquid nitrogen, lyophilized, mechanically homogenized, and washed in a 50 mM potassium...
phosphate buffer solution (pH 6.0) to remove all hemoglobin. The resulting pellet was resuspended in 1.5 ml of a solution containing hexadecyltrimethylammonium bromide (5 g/L). The solution was subjected to three cycles of freezing (on dry ice) and thawing (at room temperature), sonicated for 40 s, and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was then assayed for MPO activity using a spectrophotometric reaction with o-dianisidine hydrochloride (Sigma-Aldrich) at 450 nm. Absorbance at 450 nm was measured and reported as difference in OD (ΔOD) over 5 min.

**Wet-to-dry ratios**

Wet-to-dry ratios were measured as previously described (12, 15). In short, following ventilation with indicated settings, lungs were excised en bloc. The weight was obtained immediately to prevent evaporative fluid loss of the tissues. Lungs were than lyophilized for 48 h and the dry weight was measured. Wet-to-dry ratios were then calculated as milligrams of water per milligram of dry tissue.

**Blood gas analysis**

To assess pulmonary gas exchange, blood gas analyses were performed in subsets of experiments by obtaining arterial blood via cardiac puncture. In short, a lateral thoracotomy was performed to access the left heart and blood was obtained via cardiac puncture. Analysis was performed immediately after collection with the I-STAT Analyzer (Abbott) and the arterial partial pressure of oxygen (PaO₂) was measured, in addition to arterial partial carbon dioxide pressure and pH values.

**Histopathological evaluation of ALI**

Following ventilation at indicated settings, the mice were euthanized and lungs were fixed by instillation of 10% formaldehyde solution via the tracheal cannula at a pressure of 20 mbar. Lungs were then embedded in paraffin and stained with H&E. Two random tissue sections from four different lungs in each group were examined by a pathologist who was blinded to the genetic background/treatment of the mice. ALI was scored as described previously, (22) according to the following criteria: 1) alveolar congestion, 2) hemorrhage, 3) infiltration or aggregation of neutrophils in airspace or vessel wall, and 4) thickness of the alveolar wall/hyaline membrane formation. For each subject, a 5-point scale was applied: 0, minimal (little) damage; 1+, mild damage; 2+, moderate damage; 3+, severe damage; and 4+, maximal damage. Points were added up and are expressed as median ± range (n = 4).

**Data analysis**

Lung injury score and survival data are given as median (range), all other data are presented as mean ± SD from four to six animals per condition. We performed statistical analysis using the Student t test (two sided, α < 0.05) or ANOVA to determine group differences. Lung injury score was analyzed with the Kruskal-Wallis rank test. Kaplan Maier curves were plotted using the log-rank test (Mantel-Haenszel) test. A value of p < 0.05 was considered statistically significant.

**Results**

**Supernatants from stretched pulmonary epithelia decrease endothelial paracellular permeability**

Many episodes of ALI are self-limiting and resolve spontaneously through unknown mechanisms. Therefore, we hypothesized the existence of metabolic pathways responsible for the innate protection of the capillary-alveolar barrier function during conditions of stretch or mechanical ventilation. To pursue this hypothesis, we adopted an in vitro model of cyclic mechanical stretch that attenuates endothelial leakage. As shown in Fig. 1, B and C, only fraction 5 improved endothelial barrier function (82 ± 6.2% decrease in

**Identification of epithelial-derived adenosine as mediator of endothelial permeability attenuation**

We next sought to determine the existence of soluble mediators released from Calu-3 cells undergoing cyclic mechanical stretch into their supernatant—responsible for endothelial barrier protection. To do this, we used HPLC to fractionate supernatants derived from Calu-3 exposed for 48 h to cyclic mechanical stretch and tested bioactivity of individual fractions on endothelial barrier effects. As shown in Fig. 1, B and C, only fraction 5 improved endothelial barrier function (82 ± 6.2% decrease in...
FITC-flux rate, $p < 0.001$). Based on further analysis of size, stability, UV spectroscopy, and HPLC retention time (see red dotted line, Fig. 1B), the bioactive content of fraction 5 was subsequently identified as adenosine. Moreover, treatment of fraction 5 with adenosine deaminase (ADA) (0.1 nM) (15) completely abolished its barrier protective effects (2 \% decrease in FITC-flux rate, data not shown). Consistently, endothelial barrier effects of supernatants from Calu-3 were neutralized using the nonspecific adenosine receptor antagonist 8-PT (10 $\mu$M) or following treatment with ADA (0.1 nM, Fig. 1D). Quantification of the adenosine concentration within the supernatant by HPLC measurements confirmed increased adenosine concentrations with exposure to cyclic mechanical stretch (Fig. 1E). To confirm that the observed increases in adenosine within the supernatants are not limited to the above in vitro model of cyclic stretch, we performed mechanical ventilation in mice for 0–3 h (pressure-controlled ventilation, 100% inspired oxygen) for 0 or 90 min at an inspiratory pressure of 45 mbar. Albumin concentration in the BAL was determined by ELISA. B, Lung water content. C, Neutrophil accumulation (myeloperoxidase assay). D, Arterial partial pressure of oxygen (PaO$_2$). E, Pulmonary adenosine levels (determined via HPLC). Results are presented as mean $\pm$ SD and derived from six animals in each condition.

**FIGURE 3.** ALI is enhanced in cd39$^{+/+}$ mice. A, Cd39$^{+/+}$ mice or littermate controls (cd39$^{-/-}$) were mechanically ventilated (pressure controlled ventilation, 100% inspired oxygen) for 0 or 90 min at an inspiratory pressure of 45 mbar. Albumin concentration in the BAL was determined by ELISA. B, Lung water content. C, Neutrophil accumulation (myeloperoxidase assay). D, Arterial partial pressure of oxygen (PaO$_2$). E, Pulmonary adenosine levels (determined via HPLC). Results are presented as mean $\pm$ SD and derived from six animals in each condition.

CD39 and CD73 are induced by cyclic stretch or mechanical ventilation

As previous studies had shown that CD39 and CD73 are rate limiting for extracellular adenosine generation (9, 15), we next investigated transcriptional consequences of cyclic stretch on CD39 and CD73 expression patterns in vitro and in vivo. As shown in Fig. 1G, CD39 and CD73 mRNA levels are induced in Calu-3 epithelia following exposure to cyclic mechanical stretch in vitro. As next step, we performed murine mechanical ventilation to investigate transcriptional consequences on ectonucleotidase expression. For this purpose, we ventilated mice for 0–150 min using pressure-controlled ventilation (100% inspired oxygen concentration, inspiratory pressure 15 mbar). After sacrificing the animals at indicated time points, harvesting
the lungs, and isolation of RNA, we assessed transcript levels of nucleotidases with real-time RT-PCR. These experiments revealed a prompt and robust induction of CD39 and CD73 transcript levels with increasing time periods of ventilation (Fig. 2A). As a previous study had shown oxygen-dependent modulation of adenosine signaling effects (3), we measured relative transcriptional levels of CD39/73 following 150 min of ventilation at different inspired oxygen concentrations (Fig. 2B). We observed no transcriptional effects of the inspired oxygen concentration on CD39/73 expression patterns. Therefore, all additional experiments were performed at 100% inspired oxygen concentration. As next step, we measured CD39/73 protein by Western blot analysis from whole lungs of ventilated mice. We found ventilation time-dependent increases in CD39/73 protein (Fig. 2C). Similarly, immunohistochemical staining confirmed CD39/CD73 induction with mechanical ventilation on both pulmonary epithelia and endothelia (Fig. 2, D and E), while IgG controls were negative. Taken together, these results reveal oxygen-independent increases of pulmonary CD39 and CD73 with mechanical ventilation.

**ALI is enhanced in cd39<sup>−/−</sup> mice**

After having shown increased pulmonary adenosine concentrations and induction of ectoapyrase and ecto-5′-nucleotidase with mechanical ventilation, we next pursued their functional contribution to ALI induced by mechanical ventilation. For this purpose, we used previously characterized cd39<sup>−/−</sup> mice (16) or age-, gender-, and weight-matched littermate controls and induced ALI via high-pressure ventilation (90 min, 45 mbar). As shown in Fig. 3A, cd39<sup>−/−</sup> mice showed a profound increases of albumin leakage into their BAL compared with littermate controls. In addition, increases in lung water (Fig. 3B) and PMN infiltration (MPO tissue activity, Fig. 3C) were significantly enhanced in cd39<sup>−/−</sup> mice. The fact that cd39<sup>−/−</sup> mice were more prone to ALI induced by mechanical ventilation was also reflected in functional studies: cd39<sup>−/−</sup> mice had significantly lower arterial oxygen partial pressure levels than control animals (Fig. 3D). Moreover, increases of pulmonary adenosine with ALI were attenuated in cd39<sup>−/−</sup> mice (Fig. 3E).

Taken together, these data reveal a functional role of CD39 in ALI induced by mechanical ventilation.

**Inhibition of CD73 enhances ALI**

After having shown a protective role for CD39 in ALI, we next pursued the functional contributions of CD73 which catalyzes the final conversion of AMP to adenosine and is considered the pacemaker for extracellular adenosine generation (9, 15, 23). For this purpose, we treated BL6 mice with the CD73 inhibitor
APCP (100 mg/kg i.p.). In fact, BAL-albumin leakage (1.6 ± 0.1-fold, p < 0.001), lung water content (7.3 ± 0.5 vs 8.0 ± 0.1 mg/dry tissue, p < 0.05), MPO (1.8- ± 0.06-fold, p < 0.0001) were increased after APCP treatment in comparison to vehicle control (90 min ventilation time, 45 mbar inspiratory pressure, data not shown). Consistent with our findings in cd39<−/−> mice, the PaO2 was decreased from 39 ± 3.6 to 30 ± 2.8 mmHg following APCP treatment (90 min ventilation at 45 mbar inspiratory pressure, p < 0.01, data not shown). Taken together, these studies provide pharmacological evidence that inhibition of CD73 function results in increased susceptibility to ALI from mechanical ventilation.

**ALI is increased in cd73<−/−> mice**

Based on these pharmacological studies showing increased severity of ALI with CD73 inhibition, we next pursued the effects of mechanical ventilation on ALI in previously characterized cd73<−/−> mice (15). Similar to cd39<−/−> mice, these studies revealed significant increases of albumin leakage, pulmonary edema, and tissue-MPO activity, as well as decreased PaO2, values in cd73<−/−> mice compared with their littermate controls (Fig. 4, A–D). Similar to cd39<−/−> mice, increases of pulmonary adenosine with ALI were attenuated in cd73<−/−> mice (Fig. 4E). Taken together, these studies provide genetic and pharmacological evidence for a protective role of extracellular adenosine generation via CD73 during ALI induced by mechanical ventilation.

**Macroscopic and histological characteristics of ALI in cd39<−/−> and cd73<−/−> mice**

To confirm increased susceptibility of cd39<−/−> and cd73<−/−> mice to lung injury induced by mechanical ventilation, we examined lungs from cd39<−/−> or cd73<−/−> mice after 90 min of ventilation at 15 or 45 mbar in comparison to littermate controls. As shown in the macroscopic and histological images of whole lungs from ventilated littermates, edema, alveolar congestion, neutrophil infiltration, and hemorrhage are increased with high-pressure ventilation (Fig. 5, A and B). However, these findings were dramatically increased in cd39<−/−> or cd73<−/−> mice. This could also be confirmed by quantitative analysis of pulmonary histology (Fig. 5, C and D). Taken together, these results confirm increased susceptibility of cd39<−/−> and cd73<−/−> mice to ventilator-induced ALI.

**Reconstitution of cd39<−/−> and cd73<−/−> mice with soluble apyrase or 5’-nucleotidase, respectively**

As proof of principle, we reconstituted cd39<−/−> and cd73<−/−> mice via i.p. injection with soluble apyrase or 5’-nucleotidase, respectively (10 U i.p.). In fact, increased albumin leakage, inflammation, and attenuated gas exchange of cd39<−/−> (Fig. 6A) or cd73<−/−> mice (Fig. 6B) was reversed with apyrase or 5’-nucleotidase treatment. Similar treatment of littermate controls was therapeutic, as it attenuated ALI-induced albumin leakage and inflammation while simultaneously improving gas exchange. Taken together, these studies provide strong evidence that CD39 and CD73 are critical control points for maintaining the capillary-alveolar barrier function and attenuating inflammation during episodes of noninfectious ALI.

**Treatment with soluble nucleotidases attenuates lung injury and improves survival**

To further pursue the therapeutic effects of soluble apyrase or 5’-nucleotidase, we treated C57BL/6/129 mice with soluble apyrase or BL6 mice with soluble 5’-nucleotidases (10 U i.p.). As shown in Fig. 7, A–D, such treatment was associated with improved survival and attenuated histological signs of ventilator induced tissue damage. Moreover, increases of pulmonary adenosine levels with mechanical ventilation were “hyper”-elevated by treatment with soluble apyrase or 5’-nucleotidase (Fig. 7, E and F). Taken together, these data suggest treatment with soluble nucleotidases as therapeutic strategy for noninfectious ALI.

**Protective effects of adenosine during ALI are mediated through A2<sub>2A</sub>AR signaling**

After having shown strong protective effects of CD39- and CD73-dependent adenosine production during ALI induced by mechanical ventilation, we were interested through which individual adenosine receptor this protective effect is mediated. Based on previous studies showing attenuation of acute injury or vascular barrier dysfunction by A2<sub>2A</sub>AR (24) or A2<sub>B</sub>AR signaling (9, 14, 17, 25), we used specific antagonists of these receptors. In this study, we treated BL6 mice with the A2<sub>2A</sub>AR antagonist.
antagonist ZM241385 (Fig. 8, A and B) or the A2AR antagonist MRS 1754 (Fig. 8, C and D). Although A2AAR antagonist treatment did not alter survival or albumin leakage during ALI, treatment with the A2BAR antagonist was associated with profound increases in albumin leakage into the BAL fluid and decreased survival. These pharmacological studies suggest a role of the A2BAR in adenosine-dependent protection of the alveolar-capillary barrier function during ALI induced by mechanical ventilation.

Discussion

ALI significantly contributes to critical illness, as it occurs frequently (2) and carries a high mortality rate (1). Moreover, the only therapeutic interventions currently available are elimination of the causative agents and supportive therapy (1). Nevertheless, in many instances, ALI resolves spontaneously through unknown mechanisms. In this study, we used ventilator-induced lung injury as model to identify novel endogenous mechanisms of lung protection. Initial in vitro studies revealed that supernatants from stretch-induced injury contained a stable factor, which diminished endothelial leakage. This factor was subsequently identified as adenosine. Further studies in vivo revealed prominent increases in pulmonary adenosine levels with ALI. Because ectoapyrase (CD39) and ecto-5’-nucleotidase (CD73) are rate limiting for extracellular adenosine generation, we examined their contribution to ALI. In fact, both CD39 and CD73 are induced by mechanical ventilation. Moreover, capillary-alveolar leakage, inflammatory responses, and symptom severity were increased following targeted gene deletion of cd39 or cd73 during ALI. Treatment with soluble apyrase or 5’-nucleotidase resulted in reconstitution of the phenotype of cd39−/− or cd73−/− mice and was therapeutic in wild-type animals. Additional studies with individual adenosine receptor antagonists suggest a role of signaling through the A2BAR in lung protection. Taken together, these studies identify CD39- and CD73-dependent adenosine generation as innate metabolic pathway elicited by mechanical ventilation. This transcriptionally regulated adaptation is important for the protection of the capillary-alveolar barrier during mechanical ventilation.
The observation of increased capillary-alveolar leakage with targeting CD39 or CD73 during mechanical ventilation suggests a protective role of extracellular adenosine signaling for maintaining the pulmonary barrier function. This is consistent with previous studies showing barrier protective effects of extracellular adenosine signaling during acute inflammation or hypoxia (26). Previous studies found different adenosine receptors responsible for tissue protection under such conditions (9, 17, 25, 27). For example, a study in mice deficient in the A2AAR found increased inflammation-associated tissue damage (27, 28) providing evidence for A2AAR signaling as a mechanism for regulating acute inflammatory responses in vivo. As previous studies have found an important contribution of leukocyte recruitment and neutrophil chemoattractants (such as MIP-2) in ventilator-induced lung injury (22), adenosine-dependent attenuation of neutrophil responses via A2AAR may be important for lung protection during mechanical ventilation. However, based on pharmacological approaches, the present studies suggest a protective role of the A2BAR during ALI induced by mechanical ventilation. This is consistent with studies on endothelial permeability (9, 14, 29), attenuated neutrophil responses (25), and myocardial protection from ischemia and reperfusion injury via A2BAR signaling.

At present, the exact source from which extracellular adenosine is generated during mechanical ventilation remains unclear. Some very carefully executed studies have shown that pulmonary epithelia and endothelia release ATP in different models of injury.
In contrast to the present study of acute injury, studies investigating chronic pulmonary disease have identified a detrimental role of elevated adenosine levels. For example, levels of adenosine are chronically increased in the lungs of asthmatics (39), and correlate with the degree of inflammatory insult (40), suggesting a provocative role of adenosine in asthma or chronic obstructive pulmonary disease (41). In addition, ADA-deficient mice develop signs of chronic pulmonary injury in association with chronically elevated pulmonary adenosine levels. In fact, ADA-deficient mice die within weeks after birth from severe respiratory distress (42) and recent studies suggest that attenuation of adenosine signaling may reverse the severe pulmonary phenotypes in ADA-deficient mice, suggesting that chronic adenosine elevation can affect signaling pathways that mediate aspects of chronic lung disease (36, 37).

In summary, the present study identifies endogenous adenosine production and signaling through the A2B AR as part of innate metabolic adaptation during ALI. In fact, genetically targeted mice with defects in the major extracellular pathway of adenosine generation (CD39 or CD73) show increased susceptibility to ALI during mechanical ventilation. In addition, treatment with their soluble compounds improves symptoms and survival during high-pressure ventilation.

Acknowledgments

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Disclosures

Treatment of ALI with apyrase or 5′-nucleotidase is currently under consideration for a patent by the Tufts University Hospital.

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