Adenosine protected against pulmonary edema through transporter- and receptor A2-mediated endothelial barrier enhancement

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Submitted 16 September 2009; accepted in final form 9 March 2010

Lu Q, Harrington EO, Newton J, Casserly B, Radin G, Warburton R, Zhou Y, Blackburn MR, Rounds S. Adenosine protected against pulmonary edema through transporter- and receptor A2-mediated endothelial barrier enhancement. Am J Physiol Lung Cell Mol Physiol 298: L755–L767, 2010. First published March 12, 2010; doi:10.1152/ajplung.00330.2009.—We have previously demonstrated that adenosine plus homocysteine enhanced endothelial basal barrier function and protected against agonist-induced barrier dysfunction in vitro through attenuation of RhoA activation by inhibition of isoprenylcysteine-O-carboxyl methyltransferase. In the current study, we tested the effect of elevated adenosine on pulmonary endothelial barrier function in vitro and in vivo. We noted that adenosine alone dose dependently enhanced endothelial barrier function. While adenosine receptor A1 or A3 antagonists were ineffective, an adenosine transporter inhibitor, NBTL, or a combination of DPMX and MRS1754, antagonists for adenosine receptors A2A and A2B, respectively, partially attenuated the barrier-enhancing effect of adenosine. Similarly, inhibition of both A2A and A2B receptors with siRNA also blunted the effect of adenosine on barrier function. Interestingly, inhibition of both transporters and A2A/A2B receptors completely abolished adenosine-induced endothelial barrier enhancement. The adenosine receptor A2A and A2B agonist, NECA, also significantly enhanced endothelial barrier function. These data suggest that both adenosine transporters and A2A and A2B receptors are necessary for exerting maximal effect of adenosine on barrier enhancement. We also found that adenosine enhanced Rac1 GTPase activity and overexpression of dominant negative Rac1 attenuated adenosine-induced increases in focal adhesion complexes. We further demonstrated that elevation of cellular adenosine by inhibition of adenosine deaminase with Pentostatin significantly enhanced endothelial basal barrier function, an effect that was also associated with enhanced Rac1 GTPase activity and with increased focal adhesion complexes and adherens junctions. Finally, using a non-inflammatory acute lung injury (ALI) model induced by α-naphthylthiourea, we found that administration of Pentostatin, which elevated lung adenosine level by 10-fold, not only attenuated the development of edema before ALI but also partially reversed edema after ALI. The data suggest that adenosine deaminase inhibition may be useful in treatment of pulmonary edema in settings of ALI.

Lung vascular endothelium; adenosine receptors; adenosine transporters

Adenosine is a purine nucleoside with potent signaling capabilities. Normally found in low concentrations within the cellular milieu, extracellular levels of adenosine can be rapidly increased in response to inflammation and ALI. Adenosine has been noted to be elevated during sepsis (38), tissue ischemia (49), and in the bronchoalveolar lavage (BAL) fluid and exhaled breath condensate in patients with asthma (15, 32). These increases in adenosine have been implicated in pathological progression of lung diseases, as well as in protection against ALI. For example, in animals deficient in adenosine deaminase, increased levels of adenosine have been associated with pulmonary fibrosis, inflammation, and alveolar air space enlargement through proinflammatory and profibrotic signaling in the lung (8, 12, 52). The effects have been suggested to be involved in the pathogenesis of asthma and chronic obstructive pulmonary disease. Conversely, adenosine signaling has been shown to be anti-inflammatory and to attenuate the degree of ALI in settings of hyperoxia and transplantation-induced lung ischemia-reperfusion (46, 53). Adenosine has also been shown to enhance endothelial barrier function and attenuate oxidant-induced barrier dysfunction in vitro (28, 42, 45). In addition, elevated adenosine has been shown to attenuate lung edema in several models of inflammation-related ALI (2, 3, 9, 13, 20, 22, 35, 41). The divergent inflammatory, fibrotic, and barrier-enhancing responses of cells to adenosine are thought to occur via signaling through distinct adenosine receptors and/or transporters.

Extracellular adenosine is produced through actions of CD39 and CD73 (21). Increased extracellular adenosine will interact with receptors, or be taken up into cells, or metabolized by extracellular adenosine deaminase. The adenosine receptors, including A1, A2A, A2B, and A3, are classic seven-transmembrane receptors. Each signal through heterotrimeric G proteins and with distinct cellular expression patterns, ligand binding affinities, and functional responses (reviewed in Ref. 23). As depicted in Fig. 1, upon activation, adenosine receptors A1 and A3 signal through Gi1/2/3 causing inhibition of adenylyl cyclase and various calcium channels, as well as activation of several potassium channels. Activated adenosine receptors A2A and A2B couple with Gs and promote cAMP production through adenylyl cyclase and calcium mobilization. Additionally, adenosine may elicit its effects intracellularly via uptake space. Identification of factors that maintain or enhance the integrity of the pulmonary vascular endothelial barrier, as well as the elucidation of signaling mechanisms by which barrier enhancement occurs, may be useful in development of new therapeutic strategies to limit lung injury and speed recovery to normal lung function.

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LUNG VASCULAR ENDOTHELIUM is a selective barrier for the pulmonary circulation. Acute lung injury (ALI) is characterized by damage to the pulmonary microvascular endothelium resulting in barrier dysfunction and infiltration of inflammatory cells, proteins, and water into the lung interstitium and alveolar

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through facilitated diffusion or active transporters (54). Once within the cell, intracellular adenosine is metabolized through three pathways (Fig. 1): 1) adenosine deaminase irreversibly deaminates adenosine, converting it to the related nucleoside inosine by the removal of an amino group; 2) adenosine kinase phosphorylates adenosine to form adenosine monophosphate (AMP), which can be subsequently converted to cAMP; 3) S-adenosylhomocysteine (SAH) hydrolase catalyses the reversible formation of SAH from adenosine plus homocysteine. Accumulation of SAH subsequently inhibits methyltransferase activity. Thus, the biological function of adenosine may depend highly on receptors or transporters that are activated.

Previously, we have demonstrated that increased levels of adenosine, in combination with homocysteine, enhanced endothelial basal barrier function and protected against agonist-induced barrier dysfunction in vitro through isoprenylcysteine-O-carboxyl methyltransferase (ICMT) inhibition-mediated attenuation of RhoA activation and subsequent increases in adherens junctions (27, 37). In the current study, we tested the effects of elevated adenosine alone on pulmonary endothelial barrier function. We noted that adenosine alone enhanced endothelial basal barrier function in vitro through adenosine receptor A2A and A2B activation, as well as via uptake by adenosine transporters. Adenosine enhanced Rac1 GTPase activity and overexpression of dominant negative Rac1 attenuated adenosine-induced increases in focal adhesion complexes. We further demonstrated that elevation of adenosine by inhibition of adenosine deaminase with Pentostatin significantly enhanced endothelial basal barrier function, an effect that was also associated with elevated Rac1 activation and with increased focal adhesion complexes and adherens junctions. Finally, using a non-inflammatory ALI model induced by α-naphthylthiourea (ANTU) (30, 31), we found that administration of Pentostatin, which elevated lung adenosine level by 10-fold, not only attenuated the development of edema before ALI, but also partially reversed edema after ALI. Our data suggest that both adenosine transporters and A2A and A2B receptors are necessary for exerting maximal effect of adenosine on barrier enhancement. We speculate that strategies to increase both intracellular and extracellular levels of adenosine may be effective treatment for ALI.

MATERIALS AND METHODS

Cell lines and reagents. Bovine pulmonary artery endothelial cells (PAEC) were purchased from Vec Technologies (Rensselaer, NY) and used between passages 3 and 9. The PAEC were propagated in MEM containing 10% FBS and sodium pyruvate.

The chemical inhibitors directed against the adenosine receptors A1, A2A, A2B, or A3 (8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 1,3-dipropyl-7-methylxanthine (DPMX), 8-[(4-cyanophenylcarbamoyl)oxy][phenyl]-1,3-di-(n-propyl)xanthine (MRS1754), and 3-ethyl 5 benzyl 2-methyl-6-phenylethynyl-1,4-(±)-dihydro-pyridine-3,5-dicarboxylate (MRS1191), respectively) or adenosine transporter nitrobenzylthioinosine (NBTI) were purchased from Sigma Chemical (St. Louis, MO). Similarly, adenosine and the adenosine receptor A2A and A2B agonist, N-ethylcaboxygenadenosine (NECA), were purchased from Sigma Chemical. Deoxycoformycin, also referred to as Pentostatin (Nipent), was a gift from SuperGen (Pleasanton, CA) and Hospira (Lake Forest, IL). ANTU was obtained from Avocado Research Chemicals (Heysham, UK). Antibodies directed against β-catenin and vinculin were purchased from BD Biosciences and Sigma Chemical, respectively. Antibodies directed against Rac1 were purchased from Millipore (Billerica, MA). pGST-PBD construct was a gift from Dr. Richard A. Cerione (Cornell Univ., Ithaca, NY). Bovine adenosine receptor A2A and A2B siRNAs were purchased from Integrated DNA Technologies. The sequence of siRNA directed against bovine adenosine receptor A2A was 5'-ACA AGA CTT TGG CCA GGT GTC TCA-3'. The sequence of siRNA directed against bovine adenosine receptor A2B was 5'-ACA CCA TCA ACT GTG CCT CAC TCT-3'. Control (non-silencing, scrambled) siRNA was purchased from Qiagen (Valencia, CA). The primers used to amplify bovine adenosine receptor A2A and A2B were also purchased from Integrated DNA Technologies. The sequence of primers for bovine adenosine receptor A2A; forward primer: 5'-ATT GAC CGC TAC ATT GCC ATC CGA-3'; reverse primer: 5'-TCT CCT GCT GAC TGC AGT TGT T-3'. The sequence of primers for bovine adenosine
Transfection of siRNA. PAEC were grown to 50% confluence and transfected with 50 nM control siRNA, or 50 nM A2A siRNA plus 50 nM A2B siRNA, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. At 72 h posttransfection, cells were used for further analysis (permeability assay and real-time PCR).

Real-time RT-PCR. Total RNA was purified from lysates using TRIzol reagent (Invitrogen) and reversely transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad). Adenosine receptor A2A and A2B cDNAs or GAPDH cDNA were then amplified using respective primers and SYBR GreenER qPCR SuperMixes (Invitrogen). The levels of adenosine receptor A2A, and A2B mRNAs were detected and quantified by 7300 Real-Time PCR System (Applied Biosystems).

Rac1 GTPase activity assay. Rac1-GTP was precipitated by GST-PBD beads, which pull down both Rac1-GTP and Cdc42-GTP. The precipitated proteins were then resolved by SDS-PAGE, and Rac1-GTP was detected by immunoblot analysis.

Transfection of cDNA. PAEC were grown to 80% confluence and transfected with cDNAs coded for GFP (pEGFP-C1) or dominant negative Rac1 (pcDNA3-EGFP-Rac1-T17N), using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. At 24 h posttransfection, cells were treated with vehicle or adenosine and then subjected to immunofluorescence microscopy.

Immunofluorescence microscopy. PAEC on coverslips were treated as described, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100, as we previously described (37). Cells were stained with primary antibodies directed against receptor A2B: forward primer: 5'-GCA CAG AGC TCA TGG ATC ACT CAA-3'; reverse primer: 5'-GAT GCC TGT GTF TAT CCG CTA CTT-3'; pEGFP-C1 construct was purchased from Clontech (Palo Alto, CA). pcDNA3-EGFP-Rac1-T17N construct was obtained from Dr. Gary Bokoch (Scripps Research Institute, Scripps, CA) through Addgene.

Endothelial monolayer permeability assay. Changes in endothelial monolayer permeability were assessed using the electrical cell impedance sensor (ECIS) technique (Applied Biophysics, Troy, NY). Equivalent numbers of endothelial cells were seeded on collagen-coated gold electrode (8W10E) arrays to confluence and permitted to adhere overnight. Cells were serum starved 24 h before performing experiments. Experiments were performed, and changes in electrical resistance were measured over time, as previously described (25, 26, 36).

Transfection of siRNA. PAEC were grown to 50% confluence and transfected with 50 nM control siRNA, or 50 nM A2A siRNA plus 50 nM A2B siRNA, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. At 72 h posttransfection, cells were used for further analysis (permeability assay and real-time PCR).

Real-time RT-PCR. Total RNA was purified from lysates using TRIzol reagent (Invitrogen) and reversely transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad). Adenosine receptor A2A and A2B cDNAs or GAPDH cDNA were then amplified using respective primers and SYBR GreenER qPCR SuperMixes (Invitrogen). The levels of adenosine receptor A2A, and A2B mRNAs were detected and quantified by 7300 Real-Time PCR System (Applied Biosystems).

Rac1 GTPase activity assay. Rac1-GTP was precipitated by GST-PBD beads, which pull down both Rac1-GTP and Cdc42-GTP. The precipitated proteins were then resolved by SDS-PAGE, and Rac1-GTP was detected by immunoblot analysis.

Quantification of cellular and lung adenosine levels. Rats were anesthetized, and the lungs were removed rapidly and frozen in liquid nitrogen. Cultured PAEC were rapidly collected, and the cell pellets were frozen in liquid nitrogen. Adenine nucleosides were extracted from frozen lungs or cell pellets using 0.4 N perchloric acid as previously described (7), and adenosine was separated and quantified using reverse-phase HPLC.

Assessment of lung edema. For measuring wet and dry lung weights, adult male Sprague-Dawley rats (~200 g) were killed, and the lungs were removed. Lungs were blotted on gauze to remove excess fluid, and wet weights were recorded immediately. The lungs were then dried for 72 h at 90°C, and weights of the dried lungs were recorded. Data are presented as the ratio of wet lung weight relative to dry lung weight, as we previously described (33, 47).

For the ex vivo lung edema studies, lungs were isolated from anesthetized adult male Sprague-Dawley rats and perfused, as previously described (33). Briefly, following a tracheotomy, the animals were ventilated at 6 ml/kg with 5% CO2 balanced air at 60 breaths/min−1; peak airway pressure was recorded at 12 cmH2O with PEEP at 2 cmH2O. The heart and lungs were exposed via a subdiaphragmatic incision, and the pulmonary artery (PA) and left atrium were cannulated and perfused with modified Earle’s buffer containing 4% albumin maintained at 37°C, pH 7.4. The pH of the perfusate was monitored before and after each experiment. The heart and lungs were then removed en bloc and suspended on a force transducer (Grass FT03C). Arterial (P), venous (Pv), and airway pressures were monitored using a Grass model 78D polygraph (Grass Instrument, Quincy, MA). Perfusate flow was adjusted to 0.04 ml/g body wt. Capillary filtration coefficient (Ki) was measured, following lung recruitment to reach zone III conditions and after achieving isogravimetric state. Ki was determined by the rate of weight gain during the final 2 min following an increase in P0.1 pressure by ~8 cmH2O (high hydrostatic challenge) for 15 min divided by the change in capillary pressures (Pc) taken by the double occlusion technique.

For histological assessment of pulmonary edema, the lungs were inflation fixed under constant pressure of 20 cmH2O with 4% paraformaldehyde and immersed in paraformaldehyde for 24 h, as we previously described (30). The lungs were subsequently fixed, and 15-μm sagittal sections were stained with a 1% hematoxylin and eosin solution. Representative images are presented.

All animal protocols were approved by the Providence Veterans Affairs Medical Center and Brown University Institutional Animal Care and Use Committee and comply with the Health Research Extension Act and the PHS policy.

Statistical analyses. For three or more groups, differences among the means were tested for significance in all experiments, using ANOVA with the Fisher least significance difference test. For two groups, differences among the means were tested for significance using Student’s unpaired or paired t-test. Significance was reached when P < 0.05. All data are presented as means ± SE; n is indicated for each set of data.

RESULTS

Adenosine enhanced endothelial basal barrier function partially through transporters. Adenosine has been demonstrated to decrease endothelial monolayer permeability, an effect that was not attenuated by the adenosine transporter inhibitor, dipyridamole, suggesting that adenosine transporter may not be important in mediating the barrier-enhancing effect of adenosine (28, 42). Conversely, we have previously shown that adenosine plus homocysteine (Ado/HC) enhanced endothelial barrier function in pulmonary endothelial monolayers, suggesting that elevated intracellular adenosine due to uptake via adenosine transporters may play a role in mediating the barrier enhancement (27, 37). Thus, in the current study, we first examined the effect of adenosine alone on endothelial monolayer permeability, as assessed by electrical resistance across monolayers using ECIS, in primary cultured bovine PAEC. As often noted, addition of media to endothelial monolayers causes a transient decrease in electrical resistance across monolayers, as seen in Fig. 2A. Adenosine attenuated this effect in a dose-dependent manner, with a maximal effect occurring at 50 μM (Fig. 2A), suggesting an enhancement of endothelial basal barrier function.

To determine whether adenosine transporters play a role in mediating the adenosine barrier enhancement, we next assessed the effect of adenosine transporter inhibitors on adenosine-induced barrier enhancement. PAEC were treated with vehicle or adenosine in the presence or absence of the adenosine transport inhibitor dipyridamole (10 μM) for 1 h, and electrical resistance across monolayers was recorded continuously. Similar to previous reports (28, 42), dipyridamole did not attenuate the barrier-enhancing effect of adenosine (data not shown). Since dipyridamole has been

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shown to decrease the loss of intracellular adenosine by altering adenosine metabolism, in addition to inhibiting adenosine transporters (50), we then tested the effect of another, more specific adenosine transporter inhibitor, NBTI. We noted that NBTI did not alter endothelial basal barrier function (data not shown), but significantly, although not completely, decreased the barrier-enhancing effects of adenosine (Fig. 2B). This result suggests that adenosine enhanced endothelial basal barrier function partially through transport into the intracellular space. The data also suggest that in addition to transporters, adenosine receptors may also be involved in adenosine-induced barrier enhancement.

Adenosine receptors A₁ and A₃ were not involved in adenosine enhancement of endothelial barrier function. The endothelial barrier-enhancing effect of adenosine has been mimicked by an adenosine A₁, but not A₂, receptor agonist, suggesting that A₁, but not A₂, may be important (42). Thus we further examined the effect of A₁ receptor blockade on adenosine-induced barrier enhancement. PAEC were treated with vehicle or adenosine in the presence or absence of an adenosine A₁ receptor antagonist, DPCPX, for 1 h, and electrical resistance across monolayers was recorded continuously. Unlike the previous report (42), adenosine A₁ receptor antagonist DPCPX did not show any significant effect on the adenosine barrier-enhancing effect (Fig. 3A). DPCPX alone did not alter endothelial basal barrier function compared with vehicle (data not shown). Similarly, inhibition of the adenosine receptor A₃ with MRS1191 also did not significantly alter barrier-enhancing effect of adenosine (Fig. 3B). Similar to DPCPX, MRS1191 alone also did not alter endothelial basal barrier function compared with vehicle (data not shown). These results led us to hypothesize that adenosine A₂ receptors play a role in mediating the effect of adenosine on barrier enhancement.

Fig. 2. Effects of adenosine and its transporters on pulmonary artery endothelial monolayer barrier function. Pulmonary artery endothelial cells (PAEC) were incubated with indicated concentrations of adenosine (A) or incubated with vehicle (0.02% DMSO in medium) or 50 μM adenosine (in 0.02% DMSO-containing medium) in the presence or absence of 10 μM NBTI for 1 h (B). Changes in endothelial monolayer permeability were assessed by assaying changes in electrical resistance across the monolayers using the electrical cell impedance system (ECIS). A representative tracing from 6 independent experiments is presented in A. The means ± SE of the normalized resistance are presented in B, n = 6, *P < 0.05 vs. vehicle; ‡P < 0.05 vs. adenosine or vehicle. Arrows indicate the time of addition of treatments.

Fig. 3. Effects of A₁ and A₃ receptors on the barrier-enhancing effect of adenosine. PAEC were incubated with vehicle (0.4% DMSO in medium) or 50 μM adenosine (in 0.4% DMSO-containing medium) in the presence or absence of 50 μM DPCPX (A) or 10 μM MRS1191 (B) for 1 h. Changes in endothelial monolayer permeability were assessed by assaying changes in electrical resistance across the monolayers using ECIS. The means ± SE of the normalized resistance are presented. A: n = 3–4; B: n = 5–6, *P < 0.05 vs. vehicle. Arrows indicate the time of addition of treatments.
Fig. 4. Effect of A2A and A2B receptors on the barrier-enhancing effect of adenosine. PAEC were incubated with vehicle (0.1% DMSO in medium) or 50 μM NECA for 1 h (A) or incubated with vehicle (0.5% DMSO) or 50 μM adenosine (in 0.5% DMSO-containing medium) in the presence or absence of 50 μM DPMX (B) or 10 μM MRS1754 (C) or 50 μM DPMX + 10 μM MRS1754 (D) for 1 h. E and F: PAEC were transfected with control (scrambled) siRNA or A2A siRNA or A2B siRNA for 72 h. Knockdown of A2A and A2B mRNA was assessed by real-time RT-PCR. The transfected cells were then treated with vehicle or 50 μM adenosine for 1 h. Changes in endothelial monolayer permeability were assessed by assaying changes in electrical resistance across the monolayers using ECIS. The means ± SE of the normalized resistance are presented. A: n = 4–5; B: n = 3–4; C: n = 5–6; D: n = 5–6; E is a representative tracing from 3 independent experiments; F represents the means ± SE of the normalized resistance at 15-min posttreatments. *P < 0.05 vs. vehicle; ξP < 0.05 vs. adenosine or vehicle. Arrows indicate the time of addition of treatments.
Adenosine receptors A_{2A} and A_{2B} partially mediated adenosine enhancement of endothelial barrier function. We found that an A_{2A} and A_{2B} agonist NECA significantly enhanced endothelial barrier function compared with vehicle-treated cells (Fig. 4A), suggesting that A_{2A} and A_{2B} receptors are important in mediating the effect of adenosine on barrier enhancement.

To identify which A2 receptors were responsible for adenosine-induced barrier protection, we next used A_{2A} and A_{2B} receptor antagonists and assessed their effects on adenosine-induced endothelial barrier enhancement. We noted that neither an adenosine receptor A_{2A} antagonist DPMX nor an adenosine receptor A_{2B} inhibitor MRS1754 blunted the effect of adenosine on barrier enhancement (Fig. 4, B and C). Although neither DPMX nor MRS1754 alone alters the basal barrier function (data not shown), a combination of DPMX and MRS1754 partially, but significantly, attenuated the effect of adenosine on barrier enhancement (Fig. 4D), suggesting that both A_{2A} and A_{2B} receptors play a role in mediating the effect of adenosine on barrier enhancement.

Since chemical inhibitors may have some nonspecific effects, we further addressed the role of adenosine A_{2} receptors in mediating adenosine barrier enhancement by using RNAi approach. By relative qPCR, we noted approximately fourfold decreases in A_{2A} and A_{2B} mRNA levels by respective siRNA (data not shown). Similar to the results seen by using A_{2A} and A_{2B} chemical inhibitors, a combination of A_{2A} siRNA and A_{2B} siRNA significantly, but not completely, attenuated the effect of adenosine on barrier enhancement (Fig. 4, E and F).

Both adenosine transporters and A_{2A}/A_{2B} receptors were necessary for mediating the effect of adenosine on barrier enhancement. Since either blockade of adenosine transporters or inhibition of both A_{2A} and A_{2B} receptors partially blunted the effect of adenosine on barrier enhancement, we next tested the effect of a combination of inhibition of adenosine transporters with inhibition of A_{2A} and A_{2B} receptors on the adenosine barrier enhancement. As for comparing with cells treated with vehicle (0.3% DMSO), cells treated with adenosine (+ 0.3% DMSO) displayed a much higher electrical resistance (Fig. 5), suggesting a barrier enhancement of adenosine. A combination of inhibitors (DPMX + MRS1754 + NBTI) alone did not alter barrier function compared with vehicle (Fig. 5). Cells treated with a combination of adenosine, DPMX, MRS1754, and NBTI demonstrated a complete attenuation of the barrier-protective effect of adenosine in endothelial cells (Fig. 5). As expected, this complete attenuation effect was not seen when adenosine receptor A_{1} or A_{3} concomitant with adenosine transporters were inhibited (data not shown). Together, the data suggest that the barrier-enhancing effect of adenosine on pulmonary endothelial cells is mediated through both the adenosine transporters and the adenosine A_{2A} and A_{2B} receptors.

Adenosine enhanced focal adhesion complex formation through Rac1 GTPase. Adenosine has been demonstrated to cause cAMP production through A_{2A} and A_{2B} receptors. cAMP-induced endothelial barrier enhancement is thought to act through Epac/Rap-mediated Rac1 activation (4–6, 14, 24). Thus we tested the effect of adenosine on Rac1 GTPase activity and noted that adenosine dramatically increased Rac1 GTPase activity (Fig. 6A).

We next evaluated the effects of adenosine on formation of stress fibers, adherens junctions, and focal adhesion complexes. While stress fiber formation was not significantly altered (data not shown), adenosine increased focal adhesion complexes, as indicated by extensive staining of vinculin, a component of focal adhesion complexes, and also increased adherens junctions, as indicated by enhanced peripheral staining of β-catenin (Fig. 6B), a component of adherens junctions. Interestingly, overexpression of GFP-conjugated dominant negative Rac1 attenuated the effect of adenosine on focal adhesion complex formation, not on adherens junction formation (Fig. 6B). Adenosine decreased intercellular gap formation, an effect prevented by overexpression of dominant negative Rac1 (Fig. 6B). We also noted that overexpression of GFP alone did not alter basal or adenosine-induced changes in focal adhesion complexes and adherens junctions (data not shown). These results suggest that adenosine enhances endothelial barrier function partially through adenosine receptor A_{2}-mediated Rac1 activation and subsequent focal adhesion complex formation.

Adenosine deaminase inhibitor increased cellular adenosine level and enhanced endothelial basal barrier function. We next tested if increasing adenosine by inhibiting adenosine deaminase activity with deoxycoformycin (also referred to as Pentostatin) would enhance endothelial barrier function in vitro and attenuate lung edema in vivo. To test the effect of Pentostatin on cellular adenosine level and endothelial barrier function, PAEC were treated with vehicle or varying doses of Pentostatin for 1 h. Cells were collected for measuring cellular adenosine level using reverse-phase HPLC. In parallel, endothelial monolayer permeability were assessed by assaying changes in electrical resistance across the monolayers using ECIS. The means ± SE of the normalized resistance are presented. *P < 0.05 compared with all other treatments. Arrows indicate the time of addition of treatments.
Adenosine deaminase inhibitor enhanced endothelial adherens junction and focal adhesion complex formation, as well as Rac1 GTPase activity. To elucidate the cellular mechanisms underlying Pentostatin enhancement of endothelial barrier function, we assessed the effects of Pentostatin on organization of adherens junctions and focal adhesion complex and noted that Pentostatin significantly increased adherens junction formation, as indicated by enhanced periphery staining of β-catenin (Fig. 7D). We also observed a dramatic increase in focal adhesion complexes, as indicated by intense vinculin staining (Fig. 7D). The data suggest that adenosine deaminase inhibition enhanced endothelial basal barrier function by increasing adherens junction and focal adhesion complex formation likely through elevation of cellular adenosine levels.

To determine if Rac1 is involved in Pentostatin-induced endothelial barrier enhancement, we tested the effect of Pentostatin on Rac1 GTPase activity and found that Pentostatin enhanced Rac1 GTPase activity at 30-min exposure (Fig. 7E).

Adenosine deaminase inhibitor attenuated lung edema in vivo. Since Pentostatin enhanced pulmonary endothelial basal barrier function in vitro, we next tested its effects on lung edema using a rodent model of ALI. We have previously shown that ANTU causes pulmonary edema and ALI without the accumulation of inflammatory cells in lung (30, 31, 47). Adenosine has been reported to be protective against lung injury through A1 and A2A receptor-mediated anti-inflammatory actions (39, 52, 56). Thus, we used the ANTU-induced non-inflammatory ALI model to test whether Pentostatin-induced adenosine elevation attenuated pulmonary edema through modulation of vascular endothelial permeability, rather than through effects on the inflammatory response. In the first series of experiments, rats were subcutaneously administered two doses of saline (vehicle) or Pentostatin (40 mg/kg) at 0 and 15 h. The rats were then intraperitoneally given vehicle (Tween 80) or 10 mg/kg ANTU at 16.5 h. Animals were then killed at 20.5 h, lungs were isolated and perfused at a constant flow, and filtration coefficients were determined, as previously described (33, 47). Figure 8A shows that ANTU caused a fivefold increase in the filtration coefficient in the isolated perfused lungs. Inhibition of adenosine deaminase with Pentostatin, which is presumed to elevate lung adenosine level, significantly attenuated the degree of lung edema caused by ANTU (Fig. 8A). Experiments assessing the dose of Pentostatin (0–40 mg/kg) needed to elicit this protective response yielded the 40 mg/kg as optimal.
L762 ADENOSINE AND LUNG EDEMA

A

Adenosine levels in cultured EC (mole/mg protein)

Vehicle

Pentostatin (1mg/ml)

B

Normalized Resistance

Time (minutes)

2mg/ml PS

1mg/ml PS

Vehicle

C

Normalized Resistance

Time (minutes)

2mg/ml PS

1mg/ml PS

Vehicle

D

Vehicle

Pentostatin

Vinculin

β-Catenin

E

Vehicle

PS

GTP-Rac1

Total Rac1

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Next, to test if Pentostatin could elicit the same protective effects if given after ALI, rats were intraperitoneally administered vehicle or 10 mg/kg-1 ANTU and then given vehicle or Pentostatin (40 mg/kg-1) subcutaneously at 1 h following the initial ANTU injection. Lungs were isolated at 3 h after Pentostatin injection. The filtration coefficient and the lung wet-to-dry weight ratio were determined. To confirm elevation of adenosine in lung after the administration of Pentostatin, parallel experiments were performed, and adenosine levels were measured in the isolated lung. We noted a 10-fold increase in lung adenosine level in animals receiving Pentostatin compared with that of animals not exposed to Pentostatin (Fig. 8B). We further demonstrated that elevated lung adenosine, by administration of Pentostatin after ALI, also significantly diminished the degree of lung edema caused by ANTU (Fig. 8, C and D). Furthermore, histological assessment of lung sections also demonstrated an attenuation of perivascular cuffing around arteries of animals receiving Pentostatin post-ANTU compared with animals not exposed to Pentostatin (Fig. 8E). Together, the data suggest that increased lung adenosine level, via Pentostatin administration, and subsequent enhancement of vascular endothelial barrier function not only prevented the development of pulmonary edema, but also partially attenuated lung edema after ALI.

**DISCUSSION**

In the current study, we show that adenosine enhanced endothelial basal barrier function, an effect that was dependent on the adenosine A2A and A2B receptors as well as the adenosine transporters. Adenosine also activated Rac1 and overexpression of dominant negative Rac1 attenuated adenosine-induced increases in focal adhesion complexes. We further noted that inhibition of adenosine deaminase with Pentostatin significantly elevated cellular adenosine level in pulmonary artery endothelial cells, an effect that was associated with enhanced endothelial barrier function, as well as with increased adherens junctions and focal adhesion complexes. Finally, we demonstrated that administration of Pentostatin, either before or after ALI, dramatically elevated lung adenosine levels and significantly attenuated the degree of lung edema in animals exposed to ANTU.

Adenosine is known to exert its effects through either activation of cell surface G protein-coupled receptors (A1, A2A, A2B, and A3) or through intracellular uptake and subsequent metabolism (Fig. 1). Each of the four adenosine receptors is expressed by endothelial cells (38). However, which receptor is actually activated and mediates the barrier-enhancing effect of adenosine is less clear. For example, Paty and colleagues (42) have shown that the barrier-enhancing effect of adenosine was mimicked by an adenosine A1, but not A2, receptor agonist. Conversely, Haselton and coworkers (28) have reported that the effect of adenosine on endothelial barrier enhancement was mimicked by an A2, but not A1, receptor agonist. They also showed that an A2 receptor antagonist attenuated the barrier-enhancing effect of adenosine, suggesting that A2 receptors play a role in mediating the barrier-enhancing effect of adenosine (28). Recent studies have demonstrated that adenosine A2B receptor stimulation attenuated hypoxia- and ventilator-induced vascular leak (16, 17). Consistently, our data indicate that both A2A and A2B receptors, but not A1 or A3 receptor, are important in mediating the barrier-enhancing effect of adenosine in pulmonary artery endothelial cells. The different results from our studies and studies by Paty et al., whereas both pharmacological inhibitors/agonists and molecular approaches were used in our study. In addition, in our in vitro model, we examined the effect of adenosine on basal endothelial barrier function rather than effects on agonist-induced barrier dysfunction.

Adenosine uptake via active transporters in the epithelium has been demonstrated to regulate alveolar fluid clearance (22). However, little data exist describing a role for adenosine uptake via transporters in attenuation of endothelial monolayer permeability. The adenosine transporter inhibitor dipyridamole did not attenuate adenosine-induced endothelial barrier enhancement, suggesting an independence of adenosine transporters (28, 42). Similarly, we also noted that dipyridamole was incapable of attenuating the barrier-enhancing effect of adenosine (data not shown). Since dipyridamole can elevate intracellular adenosine levels by inhibiting adenosine metabolism (50), we used a more specific adenosine transporter antagonist, NBPTI, and found that NBPTI partially attenuated the barrier-enhancing effect of adenosine, suggesting that adenosine uptake via transporters is also involved in mediating adenosine barrier enhancement.

cAMP has been shown to serve as a barrier-enhancing agent in the lung in vivo (1) and in pulmonary endothelium in vitro (11, 48, 51, 57). cAMP-induced endothelial barrier enhancement is thought to act through Epac/Rap-mediated Rac1 activation (4–6, 14, 24). Extracellular adenosine can induce cAMP production via activation of adenosine A2A and/or A2B receptors. In addition, adenosine can be taken up into cells through transporters and converted to cAMP through adenosine kinase-mediated metabolism pathway (Fig. 1). Both extra- and intracellular adenosine is also metabolized by adenosine deaminase. The adenosine deaminase inhibitor, Pentostatin, is cell permeable, thus likely increasing both extra- and intracel-
lular adenosine levels. We have shown that Pentostatin significantly increased adenosine levels in both lung homogenates and cell lysates, indicating an increase of intracellular levels of adenosine. Because adenosine levels in plasma or cell culture media were not assessed, our data can’t rule out the possibility that Pentostatin may also increase extracellular adenosine levels. We have shown that both adenosine and Pentostatin increased Rac1 GTPase activity, an effect that was associated

Fig. 8. The effect of Pentostatin on α-naphthylthioureia (ANTU)-induced lung edema. A: Sprague-Dawley rats were subcutaneously administered 2 doses of saline (vehicle) or Pentostatin (40 mg/kg⁻¹) at 0 and 15 h. The rats were then intraperitoneally given vehicle (Tween 80) or 10 mg/kg⁻¹ ANTU at 16.5 h. Animals were then killed at 20.5 h, lungs were isolated and perfused at a constant flow, and filtration coefficients were determined. B–E: rats were intraperitoneally administered vehicle or 10 mg/kg⁻¹ ANTU and then subcutaneously given vehicle or Pentostatin (40 mg/kg⁻¹) at 1 h following the initial ANTU injections. Lungs were isolated at 3 h after Pentostatin injection. Lung adenosine level (B), filtration coefficient (C), lung wet-to-dry weight ratio (D), and lung histology (E) were determined. A: n = 3–7, *P < 0.001 vs. vehicle or Pentostatin, §P < 0.05 vs. ANTU. B: n = 3, *P < 0.001 vs. vehicle or ANTU. C: n = 3–8, *P < 0.001 vs. vehicle or Pentostatin, §P < 0.05 vs. ANTU. D: n = 3–8, *P < 0.0001 vs. vehicle or Pentostatin, §P < 0.05 vs. ANTU. E: n = 2, representative images were obtained at ×200 magnification and are presented. Arrows indicate perivascular cuffing. Scale bar is 50 μm.
with enhanced focal adhesion complexes, adherens junctions, and endothelial barrier function. Overexpression of dominant negative Rac1 attenuated adenosine-induced increases in focal adhesion complexes. Therefore, we speculate that Pentostatin may activate Rac1 GTPase through extracellular adenosine-induced A2A and A2B activation and subsequent cAMP production. In addition, Pentostatin may directly increase intracellular adenosine levels by intracellular adenosine deaminase inhibition or through uptake of increased extracellular adenosine. Increased intracellular adenosine may activate cAMP-Rac1 pathway through adenosine kinase-mediated metabolism pathway. Thus, there are multiple mechanisms by which adenosine deaminase inhibition can increase intracellular cAMP-Rac1 pathway (Fig. 1).

Various studies have suggested that Rac1 activation also plays a role in enhancement of adherens junctions (55). To our surprise, overexpression of dominant negative Rac1 did not attenuate adenosine-induced increases in β-catenin staining. Thus we speculate that other mechanisms may also be involved in adenosine-induced enhancement of adherens junctions. Another critical pathway for intracellular adenosine metabolism is that SAH hydrolase catalyses the reversible formation of SAH from adenosine plus homocysteine. As a product of S-adenosylmethionine methylation reaction, accumulation of SAH subsequently can inhibit methyltransferase activity (Fig. 1). We have previously shown that adenosine plus homocysteine enhanced endothelial barrier function through elevation of SAH and subsequent inhibition of ICMT, attenuation of RhoA activity, and enhancement of adherens junctions (37). In this study, we demonstrated that adenosine transporters are necessary for mediating the barrier-enhancing effect of adenosine. Thus, it is possible that adenosine may decrease RhoA GTPase activity through intracellular metabolism pathway and that RhoA inactivation plays a role in mediating adenosine-induced enhancement of adherens junctions.

Transcriptional induction of adenosine A2B receptor has been implicated in vascular barrier protection against hypoxia-induced lung injury (34). Hypoxia also upregulates CD39 and CD73, leading to elevation of extracellular adenosine and enhanced protection against vascular leak (21). In addition, hypoxia transcriptionally represses equilibrative nucleoside transporter (ENT)1 and adenosine kinase, which are proposed to promote vascular barrier function and dampen inflammation through accumulation of extracellular adenosine (19, 40). Together, these studies suggest that extracellular adenosine through A2B receptor action plays an essential role in barrier protection against hypoxia-induced vascular leak. Our data suggest that both adenosine transporters and A2 receptors are necessary to mediate the barrier-enhancing effect of adenosine on baseline barrier function of pulmonary artery endothelial cells. Endothelial cells express multiple nucleoside transporters. Identification of adenosine transporters important in mediating barrier enhancement of adenosine is needed. Recent studies have also shown that induction of netrin-1 attenuated hypoxia-induced inflammation in epithelial cells by enhancing extracellular adenosine signaling pathways (18). Whether pulmonary endothelial netrin interacts with adenosine or its receptors to mediate the barrier-enhancing effect of adenosine remains to be elucidated.

Intravenous administration of adenosine attenuated lung edema during ALI induced by endotoxin in vivo (35, 41). Infusion of adenosine before injury also attenuated phorbol ester-induced lung edema ex vivo in isolated, perfused lung models (2, 3, 9). Similar to in vitro studies, the role of adenosine receptors or transporters in mediating adenosine protective effect against lung edema is controversial. Adenosine signaling through A1 receptor was suggested to attenuate endotoxin-induced lung edema (41), whereas activation of A2 receptor by adenosine was responsible for the mitigation of phorbol ester-induced increase in lung permeability (2). Likewise, adenosine receptor A1 or A2 agonists completely ablated lung edema induced by LPS in rats (29), suggesting an important role of both A1 and A2 receptors in protection against lung edema. Stimulation of adenosine A2B receptor has been shown to attenuate hypoxia- and ventilator-induced vascular leak (16, 17). The role of increased adenosine in protection against vascular permeability has also been suggested by demonstrations that adenosine deaminase inhibitor, deoxycoformycin (Pentostatin), which is presumed to elevate adenosine levels, attenuated microvascular dysfunction and improved survival in sepsis (13). In the current study, we demonstrated that administration of Pentostatin, either before or after ALI, significantly attenuated the degree of edema in lungs of animals exposed to ANTU, which causes a non-inflammatory ALI (30, 31). In previous studies of deoxycoformycin attenuation of hypoxia-induced lung edema (13, 20), lung adenosine levels were not measured. We found a 10-fold increase in lung adenosine levels in animals receiving Pentostatin. Second, in each of the previous studies, the protective effects of adenosine were noted only in settings whereby adenosine was elevated before or at the time of the induction of ALI. Indeed, Adkins and colleagues (2) have shown that infusion of adenosine after administration of phorbol ester in isolated, perfused lungs was ineffective in attenuating lung edema, suggesting inability of adenosine to reverse lung edema. We provide novel findings demonstrating that Pentostatin, given either before or after ALI, significantly attenuated lung edema induced by ANTU.

The differences in the ability of increased adenosine to mitigate the edematous effects after injury may be due to the differences between injury models. Endotoxin, LPS, phorbol esters, or hypoxia-induced lung injuries are characterized by massive inflammation (43, 44), which may contribute to edema formation. Elevated adenosine has been reported to protect against lung injury through anti-inflammatory effects (39, 52, 56). Thus, it is possible that increased adenosine prevents inflammation and thus edema formation, but is ineffective in reversing inflammation and subsequent edema formation. ANTU causes pulmonary edema and ALI without accumulation of inflammatory cells in lung (30, 31). Thus, the ANTU-induced non-inflammatory model of ALI allowed us to demonstrate that adenosine attenuates pulmonary edema through modulation of vascular endothelial permeability, rather than via effects on the inflammatory response. Thus, our data suggest that Pentostatin prevents lung edema directly through actions on vascular barrier function.

It is likely that other cell types within the lung are involved in the adenosine-protective effect against lung edema. In fact, adenosine A2A and A2B receptor agonist NECA has been shown to increase alveolar fluid clearance through actions on both sodium and chloride ion channels of the alveolar epithelium, suggesting an important effect of adenosine on alveolar epithelial cells in protection of lung edema (22). Adenosine...
A2B receptor is highly expressed on type II alveolar epithelial cells (10). We noted that Pentostatin completely prevented ANTU-induced increase in wet-to-dry weight ratio, but partially attenuated ANTU-induced increase in Ki. Thus our data suggest that adenosine mitigates the injurious effect of ANTU through adenosine-mediated barrier enhancement of the pulmonary endothelium, as well as through increase in alveolar epithelial water clearance.

In summary, we have demonstrated that adenosine enhanced endothelial basal barrier function through signaling mediated by A2A and A2B receptors, as well as by adenosine transporters. Adenosine enhanced Rac1 GTPase activity, which played an important role in adenosine-induced focal adhesion complex formation. We also noted that inhibition of adenosine deam- inase with Pentostatin significantly elevated cellular adenosine level and enhanced endothelial barrier function. Furthermore, administration of Pentostatin, either before or after ALI, dramatically elevated lung adenosine and significantly attenuated lung edema in the ANTU-induced ALI animal model. These results suggest that adenosine deaminase inhibition by Pentostatin may be useful in treatment of pulmonary edema due to ALI.

ACKNOWLEDGMENTS

Some of these results were presented at the American Thoracic Society 2008 International Conference, May 16–21, Toronto, Canada, and published in abstract form in American Journal of Respiratory and Critical Care Medicine. Other results were presented at the American Thoracic Society 2009 Interna- tional Conference, May 16–20, San Diego, CA, and published in abstract form in American Journal of Respiratory and Critical Care Medicine. We thank SuperGen, Incorporated (Pleasanton, CA) and Hospira, Incorporated (Lake Providence Veterans Affairs Medical Center and supported with VA Merit Review and National Heart, Lung, and Blood Institute Grants HL-64936 (S. Forest, IL) for providing Pentostatin (Nipent) for this study.

GRANTS

This study was supported with resources from and the use of facilities at the Providence Veterans Affairs Medical Center and supported with VA Merit Review and National Heart, Lung, and Blood Institute Grants HL-64936 (S. Rounds) and HL-67795 (E. O. Harrington), and a Parker B. Francs Fellowship, an American Lung Association research award, and an American Tho- racic Society/Pulmonary Hypertension Association research award (Q. Lu). We thank SuperGen, Incorporated (Pleasanton, CA) and Hospira, Incorporated (Lake Forest, IL) for providing Pentostatin (Nipent) for this study.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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