Protective effect of purinergic agonist ATPγS against acute lung injury

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Kolosova IA, Mirzapoiuzova T, Moreno-Vinasco L, Sammani S, Garcia JG, Verin AD. Protective effect of purinergic agonist ATPγS against acute lung injury. Am J Physiol Lung Cell Mol Physiol 294: L319–L324, 2008. First published November 9, 2007; doi:10.1152/ajplung.00283.2007.—Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are major causes of acute respiratory failure associated with high morbidity and mortality. Although ALI/ARDS pathogenesis is only partly understood, pulmonary endothelium plays a major role by regulating lung fluid balance and pulmonary edema formation. Consequently, endothelium-targeted therapies may have beneficial effects in ALI/ARDS. Recently, attention has been given to the therapeutic potential of purinergic agonists and antagonists for the treatment of cardiovascular and pulmonary diseases. Extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) are important signaling molecules that mediate diverse biological effects via cell-surface P2Y receptors. We previously described ATP-induced endothelial cell (EC) barrier enhancement via a complex cell signaling and hypothesized endothelial purinoreceptors activation to exert anti-inflammatory barrier-protective effects. To test this hypothesis, we used a murine model of ALI induced by intratracheal administration of endotoxin/lipopolysaccharide (LPS) and cultured pulmonary EC. The nonhydrolyzed ATP analog ATPγS (50–100 μM final blood concentration) attenuated inflammatory response with decreased accumulation of cells (48%, P < 0.01) and proteins (57%, P < 0.01) in bronchoalveolar lavage and reduced neutrophil infiltration and extravasation of Evans blue albumin dye into lung tissue. In cell culture model, ATPγS inhibited junctional permeability induced by LPS. These findings suggest that purinergic receptor stimulation exerts a protective role against ALI by preserving integrity of endothelial cell-cell junctions.

endotoxin/lipopolysaccharide; mice; inflammation; endothelial barrier

THE ENDOTHELIAL LINING of blood vessels forms a selective barrier between plasma and interstitial spaces. In the course of acute lung injury (ALI), the endothelial barrier of the pulmonary microvasculature becomes compromised, leading to pulmonary edema, a characteristic feature of ALI. It is widely accepted that endothelial cell (EC) barrier dysfunction is initiated by agonist-induced cytoskeletal remodeling, which leads to disruption of cell-cell contacts and formation of paracellular gaps, allowing penetration of protein-rich fluid and inflammatory cells. Little is known about processes that determine barrier enhancement or protection. Recently, it has been shown that extracellular ATP stabilizes barrier function of endothelial monolayers (15, 18, 21) and attenuates barrier disruption caused by thrombin (12) and reperfusion (13). Extracellular purines (adenosine, ADP, and ATP) are important signaling molecules that mediate diverse biological effects via cell-surface receptors. Purinergic receptors are divided into two classes: P1 or adenosine receptors, and P2, which recognize primarily extracellular ATP, ADP, UTP, and UDP (26). The P2 receptors are further subdivided into two subclasses. P2X receptors are ATP-gated calcium-permeable nonselective cation channels. The P2Y receptors are coupled to G proteins. Both P2Y and P2X are capable of triggering calcium-dependent signal transduction cascades. Purinergic receptors are expressed in the respiratory system by diverse cell types where they are possible targets for therapeutic strategies in pulmonary diseases. Purinoreceptors may regulate several physiological responses in lungs, such as alteration of vascular tone, immune cell activation, ciliary beat frequency and mucus secretion, pulmonary surfactant release from alveolar type II epithelial cells, and bronchoconstriction (22). Although in some systems, purinergic receptor activation triggers inflammatory cascades (9), several studies suggest that purinergic stimulation may produce beneficial effects on pulmonary and vascular physiology. P2Y2 receptor agonists are being evaluated as a treatment for the pulmonary manifestations of cystic fibrosis (17). Extracellular ATP is critical for survival of pulmonary endothelial and epithelial cells in high oxygen and ozone concentrations (3). A recent study on transgenic mice showed that P2Y1 and P2Y2 receptors exert a protective role against infection of the lungs by Pseudomonas aeruginosa by decreasing protein leak (11). ATP, either released from cells or exogenously applied, protects against reperfusion-induced failure of the coronary endothelial barrier (13). At the cellular level, extracellular ATP and other purinergic agonists enhance barrier properties of different types of EC in culture (18, 21). Furthermore, ATP is able to rescue endothelial barrier function disturbed by the inflammatory agonist thrombin (12, 29). It is possible that ATP is an autocrine regulator of EC. Although ATP is constantly released from EC (30), inflammatory stimuli, such as thrombin (23) and LPS (4), enhance ATP release from EC.

We sought to extend our previous findings (15, 18) and tested whether purinergic stimulation of P2 receptors can protect against ALI induced by bacterial LPS in vivo. Since ATP is rapidly degraded in the cardiovascular system by ectonucleotidases (27), we used the nonhydrolyzed analog ATPγS. Previous studies show that ATPγS exerts a barrier-protective effect on endothelium, similar to that of ATP (18, 21). The ability of ATPγS to protect the endothelial barrier against LPS-induced injury was tested in cultured human lung microvascular endothelial monolayers.

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METHODS

Animals. All animal care and treatment procedures were approved by University of Chicago Institutional Animal Care and Use Committee and were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6 (20–25 g) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed until the time of experiments in the cages, with access to food and water in a temperature-controlled room with a 12-h dark/light cycle.

Animal procedures. Mice (8–10 wk) were anesthetized with intra-peritoneal ketamine (150 mg/kg) and acetylprocainamide (15 mg/kg) before the exposure of the trachea and the right internal jugular vein via neck incision. *Escherichia coli* LPS solution, serotype O55:B5 (Sigma, St. Louis, MO), or sterile saline was instilled intratracheally via a 20-gauge catheter. Simultaneously, mice received either ATPS (final calculated plasma concentration 50–100 μM) or saline in the control group intravenously through the internal jugular vein. The animals were allowed to recover for 18 h. Bronchoalveolar lavage (BAL) and lungs were collected and stored at −70°C for evaluation of lung injury.

Histopathology. Lungs were perfused free of blood with PBS and inflated with 0.5% low melting agarose for histological evaluation by hematoxylin and eosin staining as described previously (16).

Quantification of total protein and white blood cells in BAL. Collected BAL was centrifuged (500 g, 20 min, 4°C), supernatant was centrifuged again (16,500 g, 10 min, 4°C), and pure BAL fluid was used to measure total protein (BCA Protein Assay kit; Pierce Chemical, Rockford, IL). Cell pellets were suspended in Hanks’ solution, and red blood cells were lysed by hypotonic shock (0.2% NaCl) for 5 min. Cell suspensions were centrifuged (500 g, 10 min, 4°C), resuspended in Hanks’ solution, and examined for total number of white blood cells using a hemocytometer. In addition, cytospin slides were prepared from cell suspensions. After Diff-Quik staining, differential cell counts of neutrophils and macrophages were determined by counting 300–400 cells under a microscope.

Determination of MPO activity. MPO was isolated from snap-frozen right lungs as previously described (19, 24). Enzymatic detection was performed according to Daemen et al. (7).

Measurement of Evans blue dye albumin concentration in lungs. Evans blue dye albumin (EBA; 20 mg/kg) was injected into the jugular vein 120 min before the termination of the experiment to assess vascular leak (24). Lungs free of blood were weighed and snap-frozen in liquid nitrogen. The right lung was homogenized, incubated with two volumes of formamide (18 h, 60°C), and centrifuged (10,000 g, 30 min). The optical density of the supernatant was determined spectrophotometrically at 620 nm. The extravasated EBA concentration in lung homogenate was calculated against standard curve (micrograms of Evans blue dye per gram lung).

Accumulation of biomediators in lungs. Mouse TNF-α and IL-6 in BAL were measured using ELISA-based kits according to the manufacturer’s protocols (R&D Systems, Chantilly, VA).

Cell culture. The experiments were carried out using human lung microvascular endothelial cells available from Clonetics (San Diego, CA). The cells were cultured in complete media available from Clonetics and used at passages 3–7.

Transendothelial resistance measurement. Decrease in cell monolayer resistance to electrical current flow correlates with paracellular gap formation and can be measured in real time using the electric cell-substrate impedance sensor system (Applied BioPhysics, Troy, NY) described previously (10, 31). Accordingly, tightening of cell-cell contacts correlates with increased resistance. For the resistance measurement, endothelial cells were plated onto a sterile, gelatin-coated, small, gold-plated electrode and then grown to confluence. The resistance was recorded continuously while cells were challenged with reagents according to experimental protocol.

Statistical analysis. Two-way ANOVA was used to compare the means of data from two or more different experimental groups. If a significant difference was present by ANOVA (P<0.05), a least significant differences test was performed post hoc. Subsequently, differences between groups were considered statistically significant when P<0.05. Results are expressed as means±SE.

RESULTS

ATPγS decreases LPS-induced lung inflammation. Mice challenged with LPS for 18 h demonstrated inflammatory response typical for ALI compared with saline-treated control (Fig. 1). Histological examination of lung tissue showed alveolar wall thickening and infiltration of white blood cells into the lung interstitium and alveolar space. Microscopic examination of BAL showed that control lungs contained mostly macrophages and appeared as giant cells with nonsgmented nuclei. LPS treatment led to an increased number of polymorphonuclear leukocytes (neutrophils) with segmented nuclei. ATPS treatment led to an increased number of white blood cells in BAL (Fig. 2A). Quantitatively, this effect was confirmed by increased total number of white blood cells in BAL (Fig. 2B).

MPO activity, an index of neutrophil sequestration in the lungs, was also increased (Fig. 3). All these LPS-induced changes were diminished in ATPγS-treated mice (Figs. 1–3). ATPγS...
alone had no effect on lung histology or quantitative parameters of inflammation (Figs. 1–3).

ATPγS reduced LPS-induced pulmonary vascular leak. LPS challenge induced pulmonary microvascular leakage as evidenced by increased BAL protein concentration (Fig. 4A) and the extravasation of EBA into lung parenchyma (Fig. 4B). The intravenous administration of ATPγS alone did not alter basal levels of BAL protein (Fig. 4A) or EBA extravasation in control animals (Fig. 4B) but attenuated parameters of LPS-induced microvascular leakage (Fig. 4). Administration of ATPγS resulted in a dose-dependent effect on EBA extravasation in LPS-treated mice. A trend towards reduction in EBA extravasation was suggested after treatment with 50 μM ATPγS, whereas a significant reduction was achieved by treatment with 100 μM ATPγS (Fig. 4).

ATPγS attenuates LPS-induced loss of body weight. Bacterial endotoxin-induced cachexia is characterized by weight

Fig. 2. Effect of ATPγS on LPS-induced accumulation of white blood cells (WBC) in lungs. Mice were treated intratracheally either with LPS (2.5 mg/kg) or with vehicle (PBS). Simultaneously, mice received intravenously either ATPγS (50 μM final, intravenous) or vehicle. Bronchoalveolar lavage (BAL) or lungs were collected 18 h after the treatments. A: BAL from vehicle or ATPγS-treated animals contained only macrophages (M). They appeared as giant cells with nonségmented nuclei. BAL of LPS-treated animals contained mainly neutrophils (N) with segmented nuclei, and they were smaller than macrophages. ATPγS decreased number of neutrophils in BAL of LPS-treated mice. B: ATPγS reduced total cell count in BAL of LPS-treated mice. Data are expressed as means ± SE (n = 3–5 in each group, *P < 0.01 vs. LPS).

Fig. 3. MPO activity in lungs. MPO activity (quantification of pulmonary neutrophil infiltration) was elevated in lung tissue of LPS group (*P < 0.001 vs. vehicle group); ATPγS significantly decreased LPS-induced MPO activity (**P < 0.001 vs. LPS group). Data are expressed as means ± SE (n = 3–6 in each group).

Fig. 4. Effect of LPS and ATPγS on pulmonary vascular leak. A: total protein accumulation in BAL. Data are expressed as means ± SE (*significantly different vs. LPS group, n = 5). B: Evans blue albumin dye extravasation into lungs. Evans blue dye albumin (EBA) was injected into the jugular vein 120 min before the termination of the experiment. LPS induced EBA leakage from the vascular space into surrounding lung tissue (LPS group). This leakage was noticeably reduced in ATPγS-treated mice (LPS/ATPγS group). Whereas a trend was suggested at lower concentrations of ATPγS (50 μM final, intravenous), a significant effect was evident at a higher ATPγS concentration (100 μM final, intravenous).
loss, anorexia, and a disturbance in lipid metabolism (25). LPS-challenged mice exhibited considerable decline in body weight 18 h after the treatment. ATP$_7$/H$_9253$S administration significantly attenuated loss of body weight in LPS-treated mice (Fig. 5).

**Effect of ATP$_γ$S on LPS-induced pulmonary endothelium barrier disruption.** Since vascular leak due to disruption of endothelial barrier significantly contributes to edema formation, we tested the potential protective effects of ATP$_γ$S on endothelial barrier in vitro using cultured endothelial cells. Transendothelial resistance (TER), an indicator of endothelial monolayer integrity, was measured in human pulmonary endothelial cell cultures challenged with LPS and ATP$_γ$S. As shown in Fig. 6A, LPS (100 ng/ml) stimulation caused significant and sustained decrease in TER, which reflects dramatic endothelial cell barrier dysfunction induced by LPS treatment. The effect reached maximum by 10 h of LPS stimulation. In accordance with our previous results (18), treatment of human pulmonary endothelial cell cultures with ATP$_γ$S alone caused significant increase in TER, reflecting ATP$_γ$S barrier-protective effects. ATP$_γ$S (100 μM) introduced simultaneously with LPS did not affect the drop of TER induced by LPS. However, ATP$_γ$S promoted partial restoration of endothelial cell barrier properties at later time points (10–15 h poststimulation) in contrast to cells incubated with LPS alone (Fig. 6B).

**DISCUSSION**

ALI is characterized by a significant pulmonary inflammatory response resulting in microvascular endothelial barrier failure and protein-rich pulmonary edema. Purine receptors are expressed in different types of cells in the cardiovascular system and are possible targets for therapeutic strategies in cardiovascular and pulmonary diseases (1). Our data show that purinergic agonist ATP$_γ$S applied intravenously produces a beneficial effect in an LPS-induced model of ALI, reducing accumulation of inflammatory cells and total protein in lungs. As a recent study showed, P2Y$_1$/P2Y$_2$ mice infected with *P. aeruginosa* accumulated more water and total protein in lungs than did wild-type mice, suggesting impaired epithelial/endothelial barrier in purinergic receptor-deficient mice (11). Our in vitro data shows that ATP$_γ$S promotes the restoration of endothelial barrier after LPS-induced injury. These data are in agreement with the previous studies showing that ATP prevents endothelial leakage induced by thrombin (12) and reperfusion (13). Several studies demonstrated barrier-protective properties of ATP in EC in the absence of inflammatory stimuli as well (12, 18, 21, 29). The exact mechanism of ATP$_γ$S-induced barrier augmentation is not well defined. We have recently shown that ATP promotes endothelial cell barrier enhancement via a complex cell signaling including G proteins,
protein kinase A, myosin phosphatase, small GTPase Rac, and cortactin (15, 18). An additional mechanism of “anti-inflammatory” effect of ATPγS may involve immune cells participating in inflammatory response, such as neutrophils and macrophages. There are data indicating that extracellular ATP and its analogs have an anti-inflammatory effect in LPS-treated murine macrophages as it decreased both IL-12 and TNF-α production, whereas it enhanced the release of the anti-inflammatory cytokine IL-10 (14). Generally, however, purinergic nucleotides are considered as proinflammatory stimuli for immune cells (5). Extracellular nucleotides are released from damaged cells at the site of injury; thus, neutrophils and macrophages migrate toward the injured tissue, where they become activated. In our experimental system, intravenous ATPγS inhibited accumulation of neutrophils in LPS-challenged lung tissue, indicating that purinergic stimulation of neutrophils in circulating blood may affect their ability to migrate toward the site of injury. The role of purines in immunity and inflammation is extremely complex and depends on expression patterns of purinergic receptors on particular immune cells. Since ATPγS is a nonhydrolized ATP analog, it activates a less broad spectrum of purinoreceptors (most likely P2Y2 and P2Y11) than ATP, which, when hydrolyzed, produces other signaling molecules ADP and adenosine. The effect of ATPγS on specific receptors in immune cells has not been investigated.

Our data also demonstrated that ATPγS prevented loss of body weight in LPS-challenged mice. Interestingly, a randomized clinical trial showed that intravenous ATP infusions had a favorable effect on fatigue, appetite, body weight, muscle strength, functional status, and quality of life in patients with lung cancer (2).

Pulmonary epithelium plays a leading role in edema clearance. Considering accumulated data, it is unlikely that the protective effect of ATPγS is related to pulmonary epithelial function. Resolution of alveolar edema or alveolar fluid clearance is driven by active sodium transport across the alveolar epithelium. Water moves passively primarily via transcellular water channels, aquaporins (28). Extracellular luminal nucleotides have been shown to be prominent regulators of ion transport (20). Studies on different epithelia suggest that purinergic stimulation of epithelium causes water secretion rather than absorption (6). The predominant P2 receptor in respiratory epithelium is the P2Y2 receptor, which is activated by ATP or UTP (6). The net effect of P2Y2 activation is to increase Cl− secretion and K+ secretion and inhibit electrogenic Na+ absorption, all of which lead to water secretion (6). It has been shown recently that instillation of UTP into mouse lung in vivo inhibited alveolar fluid clearance after respiratory syncytial virus infection (8).

It should be noted that in our model, pulmonary epithelium was not directly stimulated with ATPγS, since it was administered intravenously. In epithelia, P2Y receptors are found in both the basolateral and the luminal membranes. Extracellular luminal nucleotides have been shown to be prominent regulators of ion transport (20), whereas the role of basolateral stimulation of P2Y receptors is not clear.

In conclusion, we have shown that purinergic agonist ATPγS given intravenously attenuates lung injury by reducing edema via enhancement of the vascular endothelial barrier. Subsequent systematic investigation is required to clarify the mechanisms of ATPγS-induced vascular barrier restoration.

GRANTS

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REFERENCES


