Nucleotides induce IL-6 release from human airway epithelia via P2Y2 and p38 MAPK-dependent pathways


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Nucleotides induce IL-6 release from human airway epithelia via P2Y2 and p38 MAPK-dependent pathways. Am J Physiol Lung Cell Mol Physiol 291: L734–L746, 2006. First published April 21, 2006; doi:10.1152/ajplung.00389.2005.—Extracellular nucleotides can mediate a variety of cellular functions via interactions with purinergic receptors. We previously showed that mechanical ventilation (MV) induces airway IL-6 and ATP release, modifies luminal nucleotide composition, and alters lung purinoceptor expression. Here we hypothesize that extracellular nucleotides induce secretion of IL-6 by small airway epithelial cells (SAEC). Human SAEC were stimulated with nucleotides in the presence or absence of inhibitors. Supernatants were analyzed for IL-6 and lysates for p38 MAPK activity by ELISA. RNA was analyzed by real-time RT-PCR. Rats (n = 51) were randomized to groups as follows: control, small-volume MV, large-volume MV, large-volume MV-intratracheal apyrase, or small-volume MV-intratracheal adenosine 5′-O-(3-thiotriphosphate) (ATPγS). After 1 h of MV, bronchoalveolar lavage fluid was analyzed for ATP and IL-6 by luminometry and ELISA. ATP and ATPγS increased SAEC IL-6 secretion in a time- and dose-dependent manner, an effect inhibited by apyrase. Agonists were ranked in the following order: ATPγS > ATP = UTP > ADP = adenosine > 2-methylthio-ADP = control. SB-203580, but not U-0126 or JNK1 inhibitor, decreased nucleotide effects. Additionally, nucleotides induced p38 MAPK phosphorylation. Inhibitors of Ca2+ signaling, phospholipase C, transcription, and translation decreased IL-6 release. Furthermore, nucleotides increased IL-6 expression. In vivo, large-volume MV increased airway ATP and IL-6 concentrations. IL-6 release was decreased by apyrase and increased by ATPγS. Extracellular nucleotides induce P2Y2-mediated secretion of IL-6 by SAEC via Ca2+, phospholipase C, and p38 MAPK-dependent pathways. This effect is dependent on transcription and translation. Our findings were confirmed in an in vivo model, thus demonstrating a novel mechanism of nucleotide-induced IL-6 secretion by airway epithelia.

cytokine; inflammation; purine; purinergic; ventilator-associated lung injury; interleukin-6; mitogen-activated protein kinase

AIRWAY INFLAMMATION IS A COMMON process observed in pathologic conditions such as asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), and ventilator-induced lung injury (VILI) (29, 44, 64, 66, 67). Because the airway epithelium is the primary site at which lung tissue is exposed to various physiochemical stresses, it serves as a target and as a mediator of inflammation via production of cytokines and chemokines (12, 38, 65).

Locoregional cytokine release can, via autocrine and paracrine mechanisms, directly augment epithelial inflammation or amplify airway stimuli by activating resident immune cells (65). Interleukin-6 (IL-6) is a pleiotropic cytokine that is generally considered proinflammatory. It exerts numerous functions, including the induction of pulmonary neutrophil infiltration, airway mucus secretion, lung fibrosis, and hyperplasia and hypertrophy of airway smooth muscle cells (16, 19, 28, 42). Increased production of IL-6 has been observed in the airways of patients with asthma, COPD, ARDS, and VILI (5, 11, 40, 44, 54).

Extracellular nucleotides are ubiquitous molecules that have been shown to modulate numerous physiological functions, including vasomotor tone, apoptosis, ionic conductance, and transalveolar fluid regulation, via binding to specific membrane-bound purinoceptors (2, 8, 33, 60). Nucleotides are released in a stress-responsive fashion, as has been demonstrated by their release into the extracellular milieu by pulmonary epithelial cells in response to physical stimuli such as shear stress (30). In numerous cell or tissue culture systems (e.g., splenic tissue, macrophages, fibroblasts, thyrocytes, microglial cells, dendritic cells, and smooth muscle cells), it has been shown that extracellular nucleotides induce the synthesis and release of cytokines (1, 9, 21, 22, 61–63).

We previously demonstrated that mechanical ventilation (MV) induces cytokine and ATP release into the airways and that extracellular ATP is involved in the regulation of alveolar fluid dynamics (56, 57). We also showed that MV alters the concentration and relative proportions of adenyl-based nucleotides in the airway and also modifies purinoceptor expression patterns in the lung and extrapulmonary tissues (18). Thus we hypothesized that extracellular nucleotides could stimulate the synthesis and release of IL-6 directly from airway epithelial cells. We sought to examine the effect of extracellular nucleotides on airway IL-6 release in vivo and in vitro and to characterize potential signaling pathways involved in these effects.

MATERIALS AND METHODS

Cell Culture

Human normal small airway epithelial cells (SAEC) were obtained from Clonetics (Cambrex, Walkersville, MD). The cells were cultured on 12-well plates in serum-free medium (SAGM, a proprietary formula, Cambrex). The cells were used between passages 2 and 4 when grown to 70–80% confluence. To minimize the effect of

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cellular ATP release during media changes, we introduced fluid to the wells by slowly pipetting along the walls of each well to prevent significant perturbation of the cells. The plates were then allowed to stabilize for 15 min before stimulation with ATP, UTP, 2-methylthio-ADP (2-MeS-ADP), ADP, and adenosine (all from Sigma) and adenosine 5'-O-(3-thiotriphosphate) (ATPγS; Roche Diagnostics, Mannheim, Germany). For each plate, unstimulated control cells were treated with drug vehicle only. In some experiments, the cells were pretreated with apyrase (Sigma), U-0126 (Biosource, Camarillo, CA), SB-203580 (Sigma), JNK inhibitor I, U-73122, U-73343, ET18-OCH3, adenylyl cyclase toxin inhibitor, and actinomycin D (all from Calbiochem, La Jolla, CA) and thapsigargin, dantrolene, BAFTA-AM, and cycloheximide (all from Sigma). Conditioned media were collected 2, 6, 24, or 48 h and centrifuged at 3,000 g for 10 min at 4°C. The supernatants were frozen until IL-6 analysis by ELISA.

IL-6 Analysis

Cell culture supernatants and bronchoalveolar lavage (BAL) samples were analyzed by ELISA for human and rat IL-6, respectively (R&D Systems, Minneapolis, MN) according to the manufacturer’s directions. Results are expressed as a percentage of IL-6 constitutive release from unstimulated control cells.

Assay of p38 MAPK Phosphorylation

In some experiments, the cells were stimulated as described above for 1 min–2 h and then washed twice with cold PBS and lysed with cell extraction buffer [100 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na2HPO4, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, and 1 mM PMSE (all from Sigma) and protease inhibitor cocktail] according to the manufacturer’s guidelines (Sigma). The cell lysates were centrifuged at 13,000 g for 10 min at 4°C. The supernatants were frozen at −80°C until p38 MAPK analysis. Phosphorylated p38 MAPK and total pMAPK were analyzed on the same day by ELISA (Biosource). The ratio of phosphorylated to total p38 MAPK was then calculated, and results are expressed as a percentage of the ratio calculated for unstimulated control cells.

RNA Expression

Some cells were plated in six-well plates, stimulated as described above, and then subjected to RNA extraction using RNasea-free DNase (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. Treatment with RNase-free DNase (Qiagen) was performed on a column according to the manufacturer’s protocol. RNA quality was assessed spectrophotometrically and by denaturing agarose gel electrophoresis. cDNA was prepared using random primers and Moloney’s murine leukemia virus RT (SuperScript II RNase H− RT, Invitrogen, Carlsbad, CA) as directed by the manufacturer. For conventional and real-time PCR, primer sets described in Table 1 were designed to be intron spanning when possible (IL-6 and GAPDH). The National Center for Biotechnology Information website and Primer3 (Whitehead Institute, Cambridge, MA) and Genosys (Sigma) software applications were used to design the primers, which were synthesized by MWG Biotech (Charlotte, NC). Conventional PCR was performed using MyCycler (Bio-Rad, Hercules, CA), and PCR products were run on agarose gel electrophoresis and visualized by Multi-Image Light Cabinet (Alpha Innotech, San Leandro, CA). Real-time PCR was performed on a LightCycler (Roche Diagnostics, Indianapolis, IN) using LightCycler FastStart DNA Master SYBR Green I (Roche) according to the manufacturer’s instructions. The crossing point (CP) for each sample was determined by LightCycler LCS4 4.00.23 software (Roche). Each sample was amplified at least in duplicate, for which the CP standard deviation was <0.5. For each run, a negative control was performed; i.e., the cDNA template was replaced by water. Melting curves facilitated discrimination between potential primer dimer formation and specific amplified products and controlled for the homogeneity of a single amplified sequence. Serial dilutions of a control template permitted the establishment of a standard curve. The slope of the linear regression of CP vs. the logarithm of cDNA concentration was used to calculate amplification efficiency as follows: E = 10(−1/slope). The relative quantification of gene expression was calculated as a ratio (R) compared with a reference gene, i.e., GAPDH. The equation for R, as described by Pfaffl (52), was as follows

\[ R = \frac{E_{\text{target}}}{E_{\text{ref}}} \]

where \( E_{\text{target}} \) and \( E_{\text{ref}} \) represent efficiencies of the target gene and the reference gene (GAPDH), respectively. \( CP_{\text{target control}} \) is the average of CP of control cell cDNA for the target gene, \( CP_{\text{target sample}} \) is the CP of the sample for the target gene, \( CP_{\text{ref control}} \) is the average of CP of control cell cDNA for the reference gene, and \( CP_{\text{ref sample}} \) is the CP of the sample for the reference gene.

Animals

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Male Sprague-Dawley rats (n = 51, 410 ± 47 g body wt) were obtained from Harlan (Indianapolis, IN). The animals were acclimatized for 72 h and then anesthetized with USP grade pentobarbital sodium (50 mg/kg ip; Abbott Laboratories, North Chicago, IL). After a surgical plane of anesthesia was verified, the animals were chemically paralyzed with pancuronium bromide (0.4 mg/kg iv; Abbott Laboratories), and the cervical trachea was cannulated via a midline cervical incision. Control animals were subjected to immediate sampling of BAL fluid (BALF; see below). Experimental animals were randomized to an MV group and immediately connected to a mechanical ventilator (Inspira ASV, Harvard Instruments, Holliston, MA), which defined time 0. The animals were randomized as follows: 1) control (sham surgery, no ventilation, n = 6), 2) small-volume MV [tidal volume (VT) = 7 ml/kg, n = 13], 3) large-volume MV (VT = 40 ml/kg, n = 13), 4) large-volume MV with 2.5 U (n = 3) or 5 U (n = 3) of intratracheal apyrase (n = 6), 5) small-volume MV with intratracheal saline (n = 6), and 6) SV with 5 nmol of intratracheal ATPγS (n = 7). All MV animals were ventilated in a volume-controlled mode with room air, a respiratory rate of 40 breaths/min, zero end-expiratory pressure, and a 1:1 inspiratory-to-expiratory ratio.

BAL Sample Collection

Immediately after tracheal cannulation (control animals) or after 60 min of MV, BAL was performed by slow intratracheal instillation of 2 ml of sterile solution (0.9% NaCl containing 0.1 mM EDTA) followed by 2 ml of room air to promote distal fluid dispersion. Lung fluid was drained by gravity and gentle abdominal massage, and specimens were collected on ice. BAL was immediately centrifuged (1,100 g for 5 min), and the supernatant was stored at −80°C until analysis.

ATP Analysis

Immediately after cold collection, BAL samples were centrifuged to remove potential cell contaminants, and the supernatants were
boiled for 2 min to inactivate any nucleotide-active enzymes. No loss of ATP was observed during boiling. A luciferin-luciferase mixture [300 μM luciferin, 5 μg/ml luciferase, 25 mM HEPES (pH 7.8), 6.25 mM MgCl₂, 0.63 mM EDTA, 75 μM dithiothreitol, and 1 mg/ml bovine serum albumin (BD Pharmingen, San Diego, CA)] was added to the samples. A luminometer (LB953 Auto Lumat, Berthold) was used to compare luminescence of the sample with that of an ATP standard curve performed for each experiment. The threshold value for ATP detection was 100 pM; luminescence was a linear function of ATP concentration up to 1 μM (37).

Statistical Analysis

Values are means ± SE. Data were analyzed by ANOVA and Fisher’s protected least significance test where appropriate. Significance was defined as \( P < 0.05 \).

RESULTS

In Vitro Study

Nucleotides induce IL-6 release from SAEC. SAEC were stimulated with increasing doses of ATP or its stable agonist ATPγS (10⁻⁸–10⁻⁴ M) for 2 h (Fig. 1). Although the smallest dose had no significant effect, ATP and ATPγS increased IL-6 release from SAEC in a dose-dependent manner. The IL-6 release induced by ATP and ATPγS was time dependent, with a maximal release 6 h after stimulation with ATP or 24 h after stimulation with ATPγS (Fig. 2).

Apyrase inhibits ATP-induced IL-6 release. It is known that ATP and its metabolites can stimulate purinoceptors. To determine whether ATP itself is responsible for the release of IL-6, we stimulated SAEC for 2 h with nucleotides (ATP or ATPγS, both at 10⁻⁴ M) in the presence or absence of apyrase (0.66 U/ml), an enzyme that degrades ATP (Fig. 3). Although apyrase alone resulted in IL-6 release comparable to that of control, it significantly decreased the effect of ATP. In contrast, apyrase did not alter the release induced by the stable ATP agonist ATPγS.

Agonist potency profile on IL-6 release. To determine which purinergic receptor(s) is involved in IL-6 release, we stimulated SAEC with several purinoceptor agonists (adenosine, ADP, 2-MeS-ADP, ATP, ATPγS, and UTP, all at 10⁻⁵ M) for 6 h and compared their potency (Fig. 4). The potency ranked in order as follows: ATPγS > UTP = ATP > adenosine > ADP > 2-MeS-ADP = control. To confirm that SAEC express P2Y₂ receptors, we performed RT-PCR for P2Y₂ and GAPDH genes (Fig. 5). We observed that P2Y₂ was amplified in SAEC after RT of extracted RNA but not in extracted RNA without

Fig. 1. ATP and its stable agonist adenosine 5'-O-(3-thiotriphosphate) (ATPγS) stimulate IL-6 release from small airway epithelial cells (SAEC) in a dose-dependent manner. IL-6 was measured in conditioned medium 2 h after stimulation with indicated doses. Values are means ± SE (n = 5). *P < 0.05 vs. control.

Fig. 2. ATP and ATPγS stimulate IL-6 release from SAEC in a time-dependent manner. SAEC were stimulated with 10⁻⁴ M ATP or ATPγS, and IL-6 was measured in conditioned medium at 2 h (n = 7), 6 h (n = 5), 24 h (n = 4), and 48 h (n = 5) after stimulation. Values are means ± SE. *P < 0.05 vs. control (i.e., constitutive release after the same incubation times).
RT. This indicates that amplified sequences did not originate from genomic DNA but, rather, from mRNA.

**Intracellular signaling of IL-6 release.** To determine the intracellular signaling pathway involved in nucleotide-induced IL-6 release, we pretreated SAEC with inhibitors of phospholipase C (PLC). We observed that the inhibition of ATP-induced IL-6 release by U-73122 was dose dependent within the range of concentrations tested (200 nM–5 μM; Fig. 6). In the subsequent experiments, we used the dose of U-73122 that inhibited the effect of ATP by 50% (1.5 μM) or the same concentration of U-73343, a negative control of U-73122. At 20 min after these treatments, the cells were stimulated for 2 h with ATP, ATPγS, or UTP (all at 10^{-4} M; Fig. 7). The IL-6 release induced by ATP, ATPγS, or UTP was significantly inhibited by the PLC inhibitor. On the contrary, treatment with U-73343, the negative control, did not alter the nucleotide-induced IL-6 release.

To confirm the involvement of PLC, we tested the effects of ET18-OCH3, a phosphatidylcholine-specific PLC inhibitor. Inhibition of the ATP response was dependent on the dose of ET18-OCH3 (Fig. 8). The basal and the purine-induced IL-6 release was significantly reduced by pretreatment of the cells (20 min before) with 12.5 μM ET18-OCH3, which suggests that IL-6 secretion, either constitutive or stimulated, is dependent on phosphatidylcholine-specific PLC (Fig. 9).

To examine whether nucleotide-induced IL-6 release is dependent on adenylate cyclase signaling, we pretreated SAEC with adenylyl cyclase toxin inhibitor (90 μM) 20 min before stimulation with ATP, ATPγS, or UTP (all at 10^{-4} M) for 2 h (Fig. 10). ATP, ATPγS, and UTP significantly increased IL-6

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**Fig. 3.** ATP-induced IL-6 release is abolished in the presence of apyrase, an ATP-degrading enzyme. IL-6 was measured 2 h after SAEC were stimulated with 10^{-4} M ATP or ATPγS in the presence or absence of apyrase (0.66 U/ml). Values are means ± SE (n = 5). *P < 0.05 vs. control. †P < 0.05 vs. ATP.

**Fig. 4.** Agonist potency profile for IL-6 release suggests involvement of P2Y2 receptor. IL-6 was measured in conditioned media 6 h after stimulation with 10^{-5} M ATPγS, ATP, UTP, ADP, 2-methylthio-ADP (2-MeS-ADP), or adenosine (Ado). Values are means ± SE (n = 6). *P < 0.05 vs. control. †P < 0.05 vs. ATP, UTP, ADP, 2-MeS-ADP. ‡P < 0.05 vs. adenosine, ADP, or 2-MeS-ADP.

**Fig. 5.** SAEC express P2Y2 receptor. RNA was extracted from 13 SAEC preparations, and P2Y2 expression was assessed by conventional RT-PCR and compared with expression of GAPDH. In some samples, PCR followed a negative control RT.

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**Fig. 6.** U-73122 (200 nM–5 μM) inhibits ATP-induced release of IL-6 in a dose-dependent manner. Values are means ± SE (n = 4).

**Fig. 7.** SAEC express P2Y2 receptor. RNA was extracted from 13 SAEC preparations, and P2Y2 expression was assessed by conventional RT-PCR and compared with expression of GAPDH. In some samples, PCR followed a negative control RT.

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**Fig. 8.** U-73343 (200 nM–5 μM) inhibits ATP-induced release of IL-6 in a dose-dependent manner. Values are means ± SE (n = 4).

**Fig. 9.** SAEC express P2Y2 receptor. RNA was extracted from 13 SAEC preparations, and P2Y2 expression was assessed by conventional RT-PCR and compared with expression of GAPDH. In some samples, PCR followed a negative control RT.

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**Fig. 10.** U-73343 (200 nM–5 μM) inhibits ATP-induced release of IL-6 in a dose-dependent manner. Values are means ± SE (n = 4).
secretion, as observed previously; however, treatment with adenylate cyclase toxin inhibitor resulted in a very small decrease of IL-6 release, which failed to reach significance.

To test whether purine-induced IL-6 release involves Ca\(^{2+}\) signaling, we treated SAEC with 13 nM thapsigargin, an inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPase, 20 min before stimulation with ATP, ATP\(\beta\)S, or UTP (all at 10\(^{-4}\) M) for 2 h (Fig. 11). Although thapsigargin alone did not alter constitutive IL-6 release, it significantly inhibited nucleotide-induced IL-6 release.

To confirm the role of Ca\(^{2+}\) signaling, we also pretreated the cells with dantrolene, an inhibitor of Ca\(^{2+}\) release from sarcoplasmic reticulum, or with BAPTA-AM, a cell-permeable Ca\(^{2+}\) chelator. Each drug inhibited ATP-induced IL-6 release in a dose-dependent manner, which allowed determination of IC\(_{50}\) (Figs. 12 and 13). Pretreatment of the cells with 25 \(\mu\)M dantrolene (30 min before) or 1.2 \(\mu\)M BAPTA-AM (1 h before) significantly reduced the responses to ATP, ATP\(\beta\)S, or UTP.
and UTP but did not affect the unstimulated IL-6 release (Figs. 14 and 15).

We also examined whether MAPKs are involved in nucleotide-induced IL-6 release by treating SAEC with 12 μM SB-203580 (a p38 MAPK inhibitor), 1.44 μM U-0126 (an extracellular mitogen-regulated kinase ERK1/ERK2 inhibitor), or 20 μM JNK inhibitor 1 (a peptide inhibiting specifically c-Jun NH₂-terminal kinase). At 20 min after inhibitor treatment, the cells were stimulated with ATP, ATPγS, or UTP (all at 10⁻⁴ M) for 2 h (Fig. 16). Although MAPK inhibitors had no significant effect on constitutive IL-6 release, SB-203580 significantly decreased the release of IL-6 induced by ATP, ATPγS, and UTP, implicating the p38 MAPK pathway. U-0126 and JNK inhibitor 1 had no significant effect on nucleotide-induced IL-6 release, with the exception of an effect of U-0126 on ATPγS-induced release.

Nucleotides induce p38 MAPK phosphorylation. To verify that p38 MAPK is implicated in nucleotide-induced IL-6 release, we stimulated SAEC for 1–120 min with ATP, ATPγS, and UTP (all at 10⁻⁴ M) and measured p38 MAPK activation by determining the ratio of phosphorylated to total p38 MAPK.
The three nucleotides activated p38 MAPK in a time-dependent manner, with a maximum activation after stimulation for 5 min with ATP, 15 min with ATPγS, and 10 min with UTP. In a separate experiment, we verified that nucleotides activate p38 MAPK by stimulating the cells for 10 min with ATP, ATPγS, or UTP (all at 10⁻⁴ M; Fig. 18). SB-203580 significantly reduced nucleotide-induced p38 MAPK activity to a level similar to that of control.

Nucleotide-induced IL-6 release is dependent on mRNA expression and protein translation. To determine whether nucleotide-induced IL-6 release is dependent on mRNA expression and protein translation, we pretreated SAEC with cycloheximide (10 μg/ml) or actinomycin D (1 μg/ml) 45 min before stimulation with ATP, ATPγS, or UTP (all at 10⁻⁴ M; Fig. 19). IL-6 release was analyzed after 2, 6, and 24 h of stimulation. Because similar results were obtained at 2 and 6 h of stimulation, Fig. 19 represents IL-6 released after 6 h of stimulation. Cycloheximide and actinomycin D had no effect on constitutive IL-6 release. Both drugs significantly and markedly reduced nucleotide-induced release of IL-6, involving expression and translation. The effects of cycloheximide and actinomycin D were not significant 24 h after stimulation with the natural agonists ATP and ATPγS (data not shown).

Nucleotides increase IL-6 expression. We examined SAEC IL-6 expression by RT of extracted RNA followed by real-time PCR for IL-6 and GAPDH (Fig. 20). At 2 h after stimulation with ATP, ATPγS, and UTP, we observed a marked increase in IL-6 expression. This nucleotide-induced IL-6 expression was sustained for 6 h and decreased after 24 h in the case of the natural agonists. To verify that nucleotides induced the synthesis of IL-6 mRNA, some cells were stimulated in the presence of actinomycin D. Incubation with this inhibitor of DNA-primed RNA polymerase abolished the nucleotide-induced IL-6 expression to a level similar to that of controls.

In Vivo Study

Large-volume MV induces ATP release. To verify our hypothesis that stress during MV results in ATP release, we used a rat model where animals received small-Vt (7 ml/kg) or large-Vt (40 ml/kg) ventilation for 1 h. Experimental animals...
were compared with nonventilated controls. We performed BAL and measured ATP concentrations in BALF (Fig. 21). ATP in BALF of animals ventilated with small VT remained comparable to that of nonventilated controls. In contrast, ventilation with large VT resulted in a significant increase in ATP concentration in BALF.

**IL-6 release induced by MV is nucleotide dependent.** IL-6 elevation was not detected in BALF of control animals (Fig. 22). However, a significant elevation of IL-6 concentration was measured in the LV group. Pretreating animals that received large-VT ventilation with 2.5 U of apyrase resulted in a decrease in IL-6 in BALF, despite VT remaining constant at 40 ml/kg. When a larger amount of apyrase (5 U) was injected, the IL-6 concentration decreased further. Small-VT ventilation resulted in IL-6 concentrations that remained low and comparable to that of controls. However, when animals receiving small-VT ventilation were pre-treated with 5 nmol of ATP, a significant increase in IL-6 in BALF was observed.

**Fig. 17.** P2Y<sub>2</sub> receptor agonists induce p38 MAPK phosphorylation in a time-dependent manner. Phosphorylated and total p38 MAPK was analyzed after stimulation with 10<sup>-6</sup> M ATP (n = 5), ATPγS (n = 6), or UTP (n = 4) at 0–120 min. Values are means ± SE. *P < 0.05 vs. control.

**Fig. 18.** Phosphorylation of p38 MAPK induced by P2Y<sub>2</sub> receptor agonists is inhibited by SB-203580. p38 MAPK activity, as defined by the ratio of phosphorylated to total p38 MAPK, was measured 10 min after SAEC were stimulated with 10<sup>-4</sup> M ATP, ATPγS, or UTP in the presence or absence of 12 μM SB-203580. Values are means ± SE (n = 3). *P < 0.05 vs. control. †P < 0.05 vs. ATP, ATPγS, or UTP.

**Fig. 19.** IL-6 release induced by P2Y<sub>2</sub> receptor agonists is inhibited by cycloheximide and actinomycin D. IL-6 was measured in conditioned media 6 h after SAEC were stimulated with 10<sup>-4</sup> M ATP, ATPγS, or UTP in the presence or absence of cycloheximide (10 μg/ml, Cyclohex), a protein translation inhibitor, or actinomycin D (1 μg/ml, Actinom), an mRNA synthesis inhibitor. Values are means ± SE (n = 3). *P < 0.05 vs. control. †P < 0.05 vs. ATP, ATPγS, or UTP.
DISCUSSION

In this study, we have demonstrated that the extracellular nucleotides ATP and UTP induce IL-6 secretion from SAEC. We have shown that nucleotide-stimulated IL-6 release is dependent on Ca\textsuperscript{2+}-sensitive intracellular signaling pathways that involve activation of PLC and p38 MAPK but are not dependent on adenylate cyclase. In addition, nucleotide-mediated IL-6 release is dependent on mRNA expression, including that of IL-6, and protein translation. Furthermore, this effect is likely associated with activity of the P2Y\textsubscript{2} purinergic receptor. Studies in mechanically ventilated rats suggest that stress-mediated release of airway ATP induces pulmonary IL-6 release in vivo.

Extracellular nucleotides are ubiquitous molecules that regulate a multitude of cellular functions in various cell types and tissues. Well-known nucleotide-mediated processes include vascular and bronchial smooth muscle contraction/relaxation,
cellular proliferation, cell migration, nitric oxide synthase regulation, apoptosis, airway mucociliary clearance activities, and transmembrane ion transport and fluid regulation (2, 8, 15, 23, 26, 36, 39, 53). In recent studies, ATP has been shown to stimulate IL-6 expression and release in a murine microglial cell line model, and P2Y receptor activation has been shown to stimulate cytokine release in CD11c⁺ murine dendritic cells (41, 61). Furthermore, P2Y-mediated IL-8 and IL-1β activation has been demonstrated in human monocytes and astrocytes, respectively (34, 71). Recently, P2Y₁₄, a receptor stimulated by UDP-glucose, has been shown to induce IL-8 secretion in lung epithelial cell lines and P2Y₅ to mediate the secretion of IL-8 induced by human neutrophil peptides in airway cells (35, 46). In this study, we observed that ATP induces the release of IL-6 from human primary SAEC. In addition, the ATP-induced IL-6 release is dose and time dependent. Several studies have shown that airway epithelial cells are able to produce cytokines such as IL-6 when stimulated, for example, by cigarette smoke, rhinovirus infection, and prostaglandin E₂ (10, 24, 65). To our knowledge, this is the first study that has reported that ATP, as a sole stimulus, induces IL-6 release from human SAEC.

Extracellular nucleotides undergo rapid enzymatic degradation via cell surface ectonucleotidases (17). The effect in cell culture models after the addition of ATP to the extracellular milieu can therefore be due to ATP itself or one of its active metabolites, which also have potent effects on certain purinoceptors. Indeed, Scholz-Pedretti et al. (59) showed that, in rat mesangial cells, ATP has a proinflammatory effect via its P2Y₁ receptor. In our experiments, the effect of SAEC stimulation with a stable agonist of ATP, ATP₂S, was also dependent on that of ATP stimulation. To confirm that IL-6 release is not due to an ATP metabolite, we also stimulated the cells in the presence of apyrase, an enzyme that degrades ATP. Apyrase treatment inhibited ATP-induced IL-6 release, confirming the role of ATP receptor activation. Furthermore, apyrase treatment does not modify the effect of the stable agonist ATP₂S, thus leading to our conclusion that IL-6 release occurs in response to an ATP-induced interaction with the purinoceptor.

A rapidly growing family of purinergic receptors consists of two main classes. P1 receptors, which include the A₁, A₂a, A₂b, and A₃ subtypes, bind adenosine and have been implicated in bronchoconstriction as well as cytokine release from fibroblasts and bronchial smooth muscle cells (39, 76, 77). P2 receptors are further divided into two subclasses. P2X purinoceptors, which comprise seven different receptors (P2X₁–P2X₇), are ligand-gated cation channels with ionotropic or apoptotic effects (the latter in the case of P2X₇). P2Y purinoceptors, of which ≥15 subtypes have been described, are said to be metabotropic, signaling via G protein-coupled cAMP, inositol trisphosphate, or Ca²⁺ pathways (6, 14, 69, 73).

Purinoceptors are characterized by the order in which the potency of natural and synthetic agonists is ranked (7, 73). Immunohistological and mRNA expression studies have shown that A₂b, P₂Y₁, P₂Y₂ (which was also called P₂U), P₂Y₄, and P₂Y₅ are localized in airway epithelium. To determine which of these receptors is responsible for ATP-induced IL-6 release, we stimulated the cells with several different agonists to compare their relative potencies. The potency ranks in order as follows: ATP₂S > ATP = ATP > adenosine > ADP > 2-MeS-ADP = control. Although agonist profiles vary slightly between different species and tissues, the potency for P₂Y₁ agonism ranks as ADP > ATP >> UTP, that for P₂Y₅ as ATP = UTP > ADP, that for P₂Y₄ as UTP >> ATP > ADP, and that for P₂Y₆ as UDP > UTP > ATP (4, 73). Hence, the potency profile we observed suggests involvement of the P₂Y₂ receptor. We have shown by RT-PCR that SAEC express P₂Y₂ receptor, confirming that P₂Y₂ could indeed be responsible for the effects we observed.

In addition, we noted that adenosine had a small but significant effect on IL-6 release compared with control. Adenosine is known to be involved in anti-inflammatory mechanisms, as in the case of A₂a receptor-mediated inhibition of rolling, adhesion, and oxygen free radical production in neutrophils, as well as the reduction of macrophage TNF-α production via A₂a, A₂b, and A₃ activity (73). Furthermore, A₁ receptor activation has been shown to inhibit pleural and peritoneal inflammatory mechanisms, as well as IL-6 production in splenic tissue (63, 73). However, adenosine has also been described as a proinflammatory molecule (3, 13). In our study, adenosine increases SAEC IL-6 release. Because of the known presence of A₂b receptors in SAEC, this effect could be mediated via A₂b. The adenosine-induced IL-6 release in our experiments supports recently reported data describing A₂b-associated IL-6 release in fibroblasts and bronchial smooth muscle cells (76, 77).

Because specific P2 receptor antagonists are scarce and P₂Y₂ antagonists are commercially unavailable, study of specific P₂Y₂ receptor inhibition is difficult. However, several of the intracellular signaling pathways associated with P₂Y receptors have been described. We therefore turned our attention to characterizing the downstream signaling pathways implicated in the nucleotide-induced IL-6 release described above. It is known that P₂Y₂, via its G₄ protein coupling, activates PLC, which in turn produces inositol trisphosphate and induces Ca²⁺ release from the endoplasmic reticulum. Using U-73122, ET18-OCH₃, thapsigargin, dantrolene, and BAPTA-AM, we observed a decrease of nucleotide-induced IL-6 release, indicating the involvement of PLC and Ca²⁺ in the process. These results are consistent with activation of the P₂Y₂ receptor (14, 73). Nucleotide-induced cytokine release, including that of IL-6 via P₂Y receptors, has been described previously; however, the specific P₂Y receptor involved is not always clear (61, 63). To our knowledge, we are the first to report that nucleotides, likely through activation of the P₂Y₂ receptor, increase IL-6 release in SAEC.

The involvement of p38 MAPK activity in the production of IL-8 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells has been reported previously (24, 27, 43). In addition, it has been reported that nucleotides can activate MAPK (31, 70), including p38 MAPK (20, 32, 45, 76). Our experiments demonstrate that ATP, ATP₂S, and UTP-induced IL-6 release is dependent on p38 MAPK phosphorylation. Although the effect of ATP₂S was also dependent on the p42/44 MAPK pathway, this was not observed with the naturally occurring agonists ATP and UTP. We observed a trend for a decreased UTP response in the presence of U-0126 and JNK inhibitor, which could suggest involvement of JNK and p42/44 MAPK in UTP-induced release. However, these reductions were not statistically significant, indicating similar ATP and UTP pathway profiles. Our observations that nucleo-
otide-induced IL-6 secretion is p38 MAPK dependent are comparable to those of Shigemoto-Mogami et al. (61), who reported that ATP induces IL-6 release via an unspecified P2Y receptor in a PLC-, Ca<sup>2+</sup>-dependent manner, and p38 MAPK-dependent fashion. In addition, we have shown that ATP, ATP<sub>γ</sub>S, and UTP activate p38 MAPK phosphorylation, with a maximal activation after 5–15 min. These findings were confirmed by stimulating the cells with nucleotides in the presence of SB-203580, a specific p38 MAPK inhibitor. It is known that p38 MAPK can phosphorylate numerous downstream substrates, including heat shock protein (HSP27), transcription factors ATF2/6, CHOP, Max, MEF2C, nuclear histone H3, or other kinases, such as PRAK (p38-regulated/activated protein kinase), MNK1/2 (MAPK-interacting kinase 1/2), MSK1 (mitogen- and stress-activated kinase 1), and MAPK-activated protein kinase 2 (MAPKAP-K2) (51). It has also been reported that p38 MAPK-dependent synthesis of cytokines, including IL-6, involves MAPKAP-K2. Additionally, cross talk between P2Y receptors and MAPKAP-K2 has been observed (47, 49, 74). On the basis of these data and our results, we can speculate that similar downstream processes may also occur in the system we describe.

The nucleotide-induced IL-6 release we observed was detected 2 h after stimulation. This relatively short period could indicate that nucleotides induce the release of some intracellularly presynthesized protein. However, our experiments with cycloheximide and actinomycin D show that purine-induced IL-6 release is dependent on mRNA transcription and protein translation. This could involve the expression and translation of IL-6 and/or other factors involved in IL-6 secretion. Although we cannot exclude the involvement of other factors, we found that IL-6 expression is strongly induced by ATP, ATP<sub>γ</sub>S, and UTP, as analyzed by quantitative real-time RT-PCR. This IL-6 expression is, however, inhibited in the presence of actinomycin D, indicating that the nucleotides induce the synthesis of new IL-6 mRNA. We also observed that this synthesis is a rapid event that we can detect only 2 h after stimulation. IL-6 expression is sustained for several hours and then decreases after 24 h in the case of the natural agonists ATP and UTP. This confirms a similar time effect we observed in IL-6 release, as analyzed by ELISA in our experiments with cycloheximide and actinomycin D.

The results we obtained in our cell culture model add to the understanding of the complex lung pathophysiology involved in asthma, ARDS, COPD, and VILI. To further validate our findings, we proceeded with in vivo experiments using a well-studied model of rat VILI (55, 67, 68). Using this model, we previously showed that 1) during high-pressure MV, ATP is released in the airway space; 2) ATP is implicated in the lung fluid regulation and the edema observed during MV; 3) MV alters the concentration and composition of the different adenyl-based molecules (ATP, ADP, AMP, and adenosine) in the lung air space; and 4) large-volume MV also alters the expression of purinoceptors in the lung and extrapulmonary tissues (18, 57).

In the present study, we confirmed that large-volume MV resulted in a marked increase in the amount of ATP measured in the BALF and, therefore, the airway lumen. Mechanical stress-induced ATP release has been previously demonstrated in numerous models and various cell types, including fibroblasts, osteoblasts, renal cells, ascites cells, endothelium, and human nasal epithelium (25, 48, 50, 58, 72, 75). The increased ATP in BALF could potentially originate from the airway epithelium and/or subepithelial structures such as bronchial smooth muscle, fibroblasts, and blood vessels. Regardless of its source, increased concentrations of ATP in the lung lumen activate airway epithelial purinoceptors. To assess whether the nucleotide-induced IL-6 release we observed in vitro occurs also in vivo, we measured IL-6 in BALF in unventilated control animals, animals with large-volume ventilation, and animals with large-volume ventilation associated with intratracheal apyrase treatment. Large-volume ventilation resulted in a large release of IL-6 into the airway lumen, which could be overcome by apyrase treatment in a dose-dependent manner. This suggests that ATP is involved in ventilation-induced IL-6 release. To confirm these findings, we also measured IL-6 in BALF in animals ventilated with small Vr with and without intratracheal ATP<sub>γ</sub>S treatment. The small-volume ventilation itself did not appear injurious, inasmuch as IL-6 from those animals was similar to that of controls. However, ATP<sub>γ</sub>S resulted in an increase in IL-6 release, suggesting that ATP itself is implicated in ventilation-induced IL-6 release. Together, these findings suggest that large-volume MV releases ATP into the airways, which in turn stimulates IL-6 secretion, validating the effects observed in vitro.

Disease states and conditions that produce mechanical stress, such as the arduous coughing associated with asthma, COPD, and other mechanical stimulation, such as orotracheal intubation, might result in ATP release and, consequently, IL-6 release. IL-6, by virtue of its proinflammatory properties on airway epithelium and immune cells, can then amplify lung inflammation. Potential implications of these findings include the development of novel therapeutic agents that target purinoceptors as a means of potentially modulating airway inflammation.

In summary, we report a novel mechanism of airway inflammation observed in vitro and in vivo: ATP or UTP, released in response to mechanical stress, stimulates P2Y<sub>γ</sub> receptor-mediated IL-6 release from airway epithelial cells via a pathway involving PLC, Ca<sup>2+</sup>, and p38 MAPK activation, as well as mRNA transcription and protein translation.

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