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Adenosine Promotes IL-6 Release in Airway Epithelia[1]

Ying Sun,[2] Fan Wu,[2] Fengqiang Sun, and Pingbo Huang[3]

In the airway epithelia, extracellular adenosine modulates a number of biological processes. However, little is known about adenosine's role in the inflammatory responses of airway epithelial cells. Recent studies suggest that the chronic elevation of extracellular adenosine in mice leads to pulmonary inflammation and fibrosis. Yet, the underlying molecular mechanism has not been well understood and little attention has been paid to the role of airway epithelia in adenosine-triggered inflammation. In the present work, we examined the role of adenosine in releasing IL-6 from airway epithelia. In Calu-3 human airway epithelial cells, apical but not basolateral adenosine elicited robust, apically polarized release of IL-6, along with proinflammatory IL-8. Both protein kinase A and protein kinase C mediated the adenosine-induced IL-6 release, at least partly via phosphorylation of CREB. Protein kinase C appeared to phosphorylate CREB through activating ERK. In addition, A2A but not A2B adenosine receptors were specifically required for the adenosine-induced IL-6 release. Furthermore, in rat bronchoalveolar lavage fluid, adenosine triggered the release of IL-6 as well as proinflammatory IL-1β. Adenosine also mediated the release of a considerable portion of the LPS-induced IL-6 in rat bronchoalveolar lavage fluid. Our findings provide a possible molecular link between extracellular adenosine elevation and lung inflammation and fibrosis. *The Journal of Immunology,* **2008, 180: 4173–4181.**

Extracellular adenosine, released from the cell or converted from released adenine nucleotides, acts as a signaling molecule via cell surface adenosine receptors in various biological processes. Adenosine modulates inflammation responses in a wide array of tissues through its potent and selective regulation of the production of proinflammatory or anti-inflammatory cytokines. For instance, adenosine stimulates the release of proinflammatory IL-6 and IL-8 (1, 2) and anti-inflammatory IL-10 (3) and suppresses the production of proinflammatory IL-12 (3, 4).

In healthy airway epithelia, extracellular adenosine primarily comes from ATP release and metabolism (5, 6). Adenosine signaling regulates distinct Cl^- channels, mucin secretion, and ciliary mobility (7–11) and is believed to play a key role in mucociliary clearance (8, 9). Despite the rather rich information about its involvement in other physiological processes, little is known about the role of adenosine in the inflammatory responses of airway epithelial cells. Adenosine elevation in the lung, including bronchoalveolar lavage (BAL),[4] accompanies several types of pulmonary inflammation and fibrosis (12–14) and is merely considered as a consequence or byproduct of tissue inflammation and injury. Most recently, pulmonary inflammation and fibrosis have been found in adenosine deaminase-deficient mice that have a chronic elevation of extracellular adenosine (15, 16), suggesting that extracellular adenosine, in addition to being an inflammation byproduct, promotes pulmonary inflammation and tissue damage. Yet, the underlying molecular mechanism has not been well understood and little attention has been paid to the role of the airway epithelia in adenosine-triggered inflammation (15–18).

Increased neutrophil infiltration in the airway lumen is not only associated with acute lung inflammation, it is also the hallmark of many types of chronic inflammation including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (19–21). Because neutrophil degranulation releases a variety of inflammatory mediators, proteases, and oxygen radicals, the excessive and prolonged activation of neutrophils has been suggested to contribute to tissue damage and fibrosis in the airway. Extracellular adenosine has been implicated in neutrophil infiltration and activation in a number of tissues and organs, as it induces the release of IL-6, a potent mediator for neutrophil recruitment (22–24) and activation (2, 25, 26). Furthermore, adenosine-induced IL-6 from these tissues and organs has been observed in epithelial cells (2, 27), which have been increasingly appreciated as active participants in immune responses (28, 29). For example, in inflamed intestines, neutrophils in the lumen release 5′-AMP and the ectonucleotidase on the apical surface of the intestinal epithelial cells then converts the 5′-AMP into adenosine (2). Subsequently, adenosine induces apical IL-6 release from the intestinal epithelial cells and IL-6 in turn activates neutrophil degranulation in the lumen. This local interplay between adenosine from neutrophils and IL-6 from intestinal epithelial cells forms a positive feedback loop and could enhance microbicidal activity of the neutrophils or, if excessive, cause tissue damage (2). Conceivably, adenosine released from damaged tissues could further augment the interplay of adenosine and IL-6.

Little is known regarding whether or not adenosine induces IL-6 release from the airway epithelia. In this study, we examined whether and how adenosine induces the release of IL-6 from the airway epithelia in vitro and in vivo to understand the role that adenosine plays in airway inflammation and fibrosis.

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[4] Abbreviations used in this paper: BAL, bronchoalveolar lavage; BIM, bisindolylmaleimide; BALF, BAL fluid; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; siRNA, small interfering RNA; 8-SPT, 8-(*p*-sulfophenyl)theophylline.

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Materials and Methods

Reagents

All tissue-culture supplies were obtained from Invitrogen Life Technologies. PD98059, U0126, bisindolylmaleimide (BIM) I, BIM II, PMA, and U73122 were purchased from Calbiochem; anti-ERK, anti-phospho-ERK, anti-CREB, and anti-phospho-CREB Abs were purchased from Cell Signaling; the reagents for SDS-PAGE and the polyvinylidene difluoride membranes came from Bio-Rad; and the oligonucleotides were from Proligo. All other reagents were acquired from Sigma-Aldrich.

Cell culture

Human Calu-3 cells (American Type Culture Collection HTB-55) were grown in Eagle's MEM supplemented with 10% FBS, 1.0 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged by 1/3 dilution every 5–7 days. To measure the bilateral IL-6 release, cells were grown on clear Transwell plates (Costar) to confluence with a resistance of 900–1,200 ohm · cm^2. Cells were first grown submerged in medium for 3–4 days and then switched to an air-interface culture. For all other experiments the cells were grown on plastic cell culture plates. All reagents and media used in the cell cultures were free of endotoxin (<0.06 endotoxin units/ml) as measured by the *Limulus* endotoxin detection assay kit (E-TOXATE kit) from Sigma-Aldrich.

RT-PCR

Total RNA was isolated from Calu-3 cells with TRIzol reagents (Invitrogen Life Technologies) according to the manufacturer's instructions. RT-PCR was performed with OneStep RT-PCR kits (Qiagen). The primer pairs used were as follows: for IL-6, 5′-TGTAGCCGCCCCACACAGACAGCC-3′ (sense)/5′-GGCAAGTCTCCTCATTGAATCCAGATTG-3′ (antisense); for A2A adenosine receptors, 5′-GCCATCGCCATTGACCGCTAC-3′ (sense)/5′-GCAGTCGGGGCAGAAGAAAGT-3′ (antisense); and for A2B adenosine receptors, 5′-CTCTTCCTCGCCTGCTTCGT-3′ (sense) and 5′-GGGCA GAACACACCCAAAGAA-3′ (antisense). β-Actin or GAPDH was used as a loading control. The identities of the PCR products were confirmed by DNA sequencing.

Cytokine release experiments in Calu-3 cells

The confluent Calu-3 monolayer grown on plastic 12-well plates (surface area, 4 cm^2) or Transwell plates (surface area, 1.12 cm^2) was prewashed in HBSS and equilibrated in 500 μl of FBS-free MEM in the apical compartment (and the basolateral compartment for the bilateral assays) at 37°C for 30 min. Cells were exposed to various challenges. The apical (and basolateral) medium was then collected and centrifuged for 10 min at 1,000 × *g* to remove debris for the cytokine assays.

Western blot analysis

Calu-3 cells were washed with PBS containing 1 mM Na_3VO_4, followed by the addition of lysis buffer containing 10 mM HEPES, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, and 5 mM PMSF (pH 7.5). Following 5 min of incubation on ice, the cells were scraped off the plate and transferred to microcentrifuge tubes. Cell debris was removed from the whole cell lysates by 10 min of centrifugation at 12,000 × *g* at 4°C and the total cell lysates were subjected to SDS-PAGE. Subsequently, proteins were electrotransferred to polyvinylidene difluoride membranes and probed with 1/1,000 diluted primary Abs. The membranes were washed three times with TBST (0.1% Tween 20, 20 mM Tris-Cl, and 500 mM NaCl (pH 7.4)) and incubated for 1 h with a 1/10,000 diluted HRP-conjugated secondary Ab. After several washes with TBST, bound Abs were detected by ECL kits (Pierce).

RNA interference

RNA oligonucleotides were synthesized by Dharmacon. The small interfering RNA (siRNA) sequences targeting A2A and A2B adenosine receptors were 5′-ACAACUGCGGUCAGCCAAA-3′ and 5′-AACCGAGAC UUCCGCUACA-3′, respectively. The nontargeting siRNA of Dharmacon (catalog no. D-001210-01-05) was used as a negative control. Cells grown in DMEM on 12-well plates with 70–80% confluency were incubated for 6 h with 100 nM siRNA and Lipofectamine 2000 (Invitrogen Life Technologies) in FBS-free DMEM. The cells were then incubated in MEM. After 24 h, the mRNA levels of A2A and A2B receptors were analyzed by RT-PCR. IL-6 was assayed after 48 h.

Bronchoaveolar lavage

All experimental procedures and protocols were approved by the University Committee on Research Practices at Hong Kong University of Science and Technology (Hong Kong, People's Republic of China). The procedures were largely the same as those published by Nonas et al. (30) with modifications. Adult male Sprague-Dawley rats weighing 250–350 g were randomly assigned to control and treatment groups. After being anesthetized with an i.p. injection of ketamine (3 ml/kg) and xylazine (1.5 ml/kg), the rats were intratracheally intubated with a microsprayer (model IA-1B; Penn-Century) and given an aerosolized spray of either 150 μl of sterile water alone or various test compounds dissolved in water. After 18 h, the rats were killed by exsanguination under anesthesia. Following tracheotomies on the sacrificed rats, their tracheae were cannulated with a polyethylene tube. Their lungs were lavaged with 3 ml of sterile PBS by slow instilling and withdrawing the instillation. The collected BAL fluid (BALF) was centrifuged for 5 min at 1,100 × *g*, and the supernatant was stored at −80°C until analysis.

Cytokine assays

Human and rat IL-6 were measured using ELISA kits from Diaclone Research and R&D Systems, respectively. The other human and rat cytokines were analyzed using a Bio-Plex human cytokine four-plex panel for IL-1β, IL-4,IL-8, and IL-10 and a rat cytokine three-plex panel for IL-1β, IL-4, and IL-10 (Bio-Rad Laboratories) with a Bio-Plex suspension array system at the Genome Research Center at Hong Kong University as a paid service.

Statistics

All data are expressed as means ± SE. Unless indicated otherwise, ANOVA with Tukey's post hoc test was used for statistical analysis. $p < 0.05$ was considered as statistically significant.

Results

Apical adenosine induced polarized IL-6 release from Calu-3 human airway epithelial cells

The Calu-3 cell line is a well-accepted cellular model of serous cells in submucosal glands in the airway. Serous cells are thought to play a critical role in the innate immunity of the lung because of their secretion of liquid and antibacterial, antiprotease, antioxidant, and anti-inflammatory proteins (31–34). Hence, Calu-3 cells offer a good model system for studying cytokine release in human airway epithelial cells. A series of concentrations of adenosine was added on the apical side or basolateral side, and IL-6 release in both apical and basolateral compartments was then analyzed after 6 h. In the control groups with no adenosine addition, a relatively high basal IL-6 release was observed in the apical but not in the basolateral compartment (Fig. 1*A*). Apical application of adenosine induced a highly polarized IL-6 release to the apical compartment. One hundred μM adenosine induced a submaximal response with ~92% increase. In marked contrast, basolateral application of adenosine, even up to 500 μM, failed to evoke any significant IL-6 release (Fig. 1*A*).

A very recent study suggests that nucleotides, mainly in the form of ATP, promote IL-6 release from small airway epithelial cells via $P2Y_2$ receptors (35). Adenosine, however, was found to have a much smaller effect on IL-6 release (35). We therefore assessed the relative contribution of P2 receptors to IL-6 release in Calu-3 cells. Apical application of 100 μM ATPγS induced an apically polarized IL-6 release with a 32% increase (Fig. 1*B*), which was considerably smaller compared with the adenosine-induced IL-6 release. These observations indicate that adenosine receptors play a more important role than do P2 receptors in IL-6 release in Calu-3 cells.

In normal airway epithelia, adenosine seems to be preferentially released in the apical compartment (36). In inflamed airways, neutrophils in the lumen could be an additional source of extracellular adenosine (2). Thus, the function of apical adenosine is more physiologically and pathologically relevant than is that of basolateral adenosine. Furthermore, the data in Fig. 1 show that apical adenosine was much more potent than was basolateral adenosine. Therefore, our subsequent studies focused on apical adenosine.

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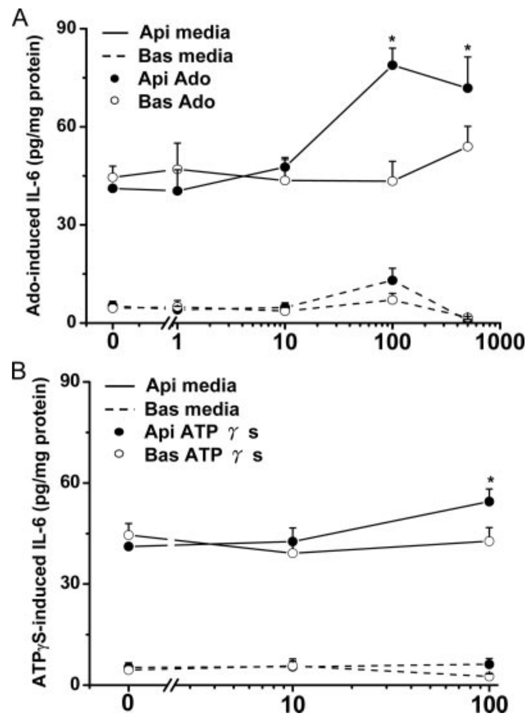

FIGURE 1. Polarized release of IL-6 induced by apical adenosine. *A*, Adenosine (Ado; 0–500 μM) was added apically or basolaterally to polarized Calu-3 cells grown on permeable Transwell membranes. IL-6 levels in the culture media from apical (Api) and basolateral (Bas) compartments were analyzed at 6 h. Released IL-6 was normalized to the protein content of the cells. *B*, Similar experiments were conducted on ATPγS (0–100 μM). Water was added as vehicle controls. All data in Fig. 1 were obtained from 5–10 independent experiments with each performed in duplicate. *, $p < 0.05$, different from the control (0 μM adenosine or ATPγS).

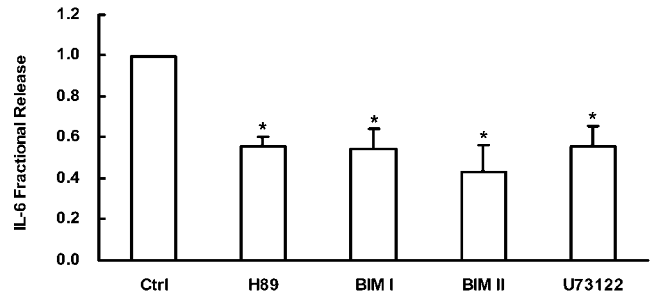

FIGURE 3. Effects of PKA and PKC/PLC inhibitors on adenosine-induced IL-6 release. Net IL-6 release induced by 6 h treatment with 100 μM adenosine with or without PKA or PKC/PLC inhibitors, including 20 μM H89, 1 μM BIM I, 1 μM BIM II, and 20 μM U73122. Data were normalized to the control group (Ctrl; adenosine alone). *, $p < 0.05$, compared with control; n = 5–7 independent experiments, each performed in duplicate. Net IL-6 release is defined as the difference between the adenosine/test compound group and the test compound alone group in this and all other figures.

The effect of apical adenosine on IL-6 release was further investigated over a time course of 9 h. One hundred μM adenosine was used in these experiments, as it elicited a submaximal response (Fig. 1*A*). The IL-6 release reached a maximal level in 6–8 h (*inset* in Fig. 2*A*), similarly as in previous studies of other cell types (2, 37, 38). The level of IL-6 production, normalized to the cell surface area (maximal ~70 pg IL-6/cm^2), is double that induced by adenosine in intestinal epithelial cells (2) and is comparable to that induced by TNF-α or *Pseudomonas aeruginosa* in Calu-3 cells (39). These results demonstrate that adenosine is a potent inducer of IL-6 release in Calu-3 cells. Because the basolateral IL-6 release was insignificant (*inset* in Fig. 2*A*), we subsequently examined only the apical IL-6 release; IL-6 release hereafter means apical IL-6 release unless indicated otherwise.

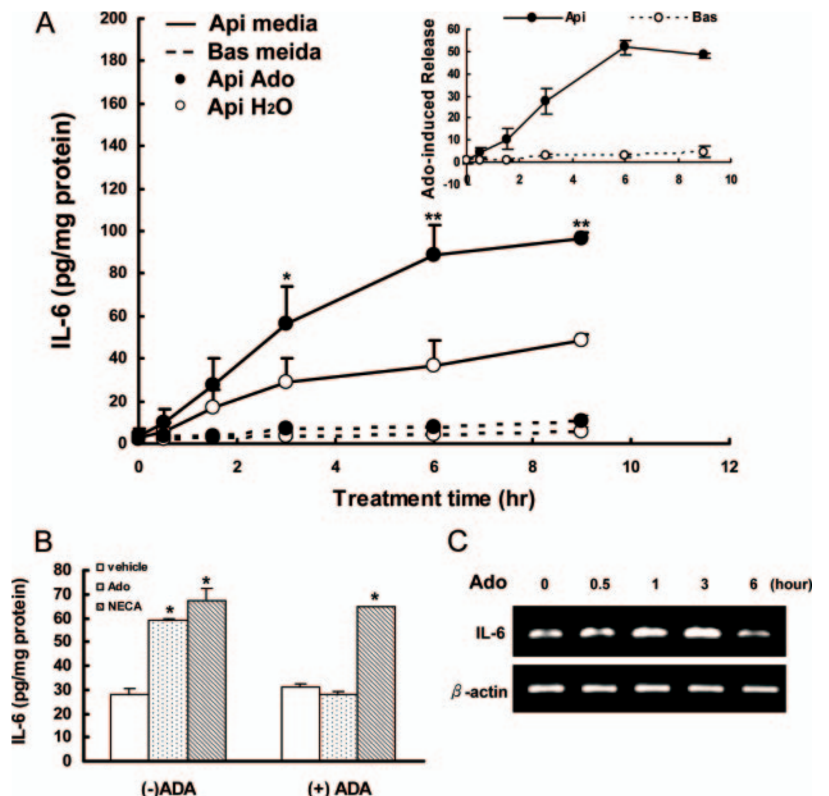

FIGURE 2. Time course of adenosine-induced IL-6 release. *A*, Adenosine (Ado; 100 μM) or water (H_2O; vehicle for the adenosine stock solution) was added apically to polarized Calu-3 cells grown on permeable Transwell membranes. The amount of IL-6 at different time points in the apical (Api) and basolateral (Bas) compartments was analyzed. The difference between the adenosine- and water-treated groups was taken as the net cytokine release induced by adenosine ("Ado-induced Release" in the *inset*). *, $p < 0.05$, different from the control (H_2O), Student's unpaired t test; **, $p < 0.01$. *B*, Four U/ml adenosine deaminase (ADA), adenosine (100 μM), or ADA-resistant NECA (10 μM) or vehicle were added to confluent Calu-3 grown on plastic culture plates. IL-6 released after 6 h was measured. Adenosine and NECA were used as positive and negative controls, respectively. HCl (0.1 N) was used as the vehicle for the NECA stock solution and was not significantly different from water on the basal release. *, $p < 0.05$, different from the vehicle. *C*, Adenosine-induced up-regulation of IL-6 mRNA expression. Calu-3 cells were incubated with 100 μM adenosine and mRNA expression of IL-6 at various time points was determined by RT-PCR. β-Actin was used as a control for cDNA input. DNA sequencing confirmed the identities of the PCR products. All data in Fig. 2, *A*–*C*, were obtained with three or five independent experiments, with each performed in duplicate for *A* and *B*.

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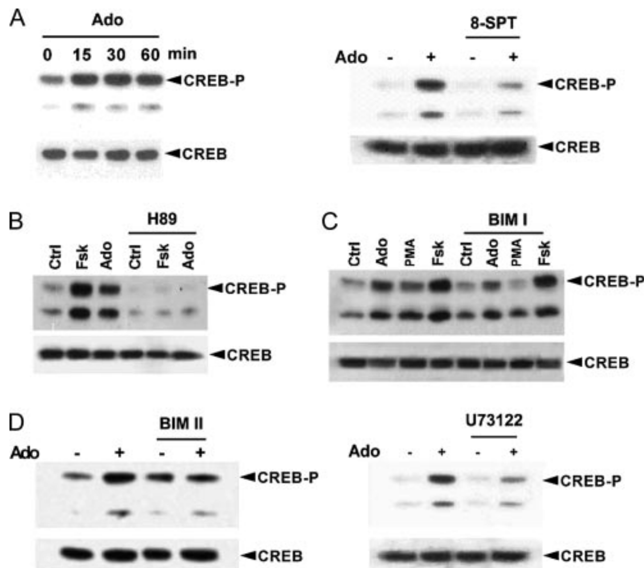

FIGURE 4. Adenosine induced PKA- and PKC-dependent CREB phosphorylation in Calu-3 cells. Shown are Western blots of phosphorylated (CREB-P) and total CREB in one of a total at least three similar experiments. *A*, *Left panel*, treatment with 100 μM adenosine (Ado) for the various times indicated; *right panel*, treatment for 30 min with 100 μM adenosine with or without 200 μM 8-SPT. *B*, Treatment for 30 min with 100 μM adenosine and 2 μM forskolin (Fsk) with or without 20 μM H89. *C*, Treatment for 30 min with 100 μM adenosine, 10 nM PMA, and 2 μM forskolin with or without 1 μM BIM I. Control (Ctrl) in *B* and *C* indicates no treatment. *D*, Treatment for 30 min with 100 μM adenosine with or without 1 μM BIM II (*left panel*) or 20 μM U73122 (*right panel*). The band below the phosphorylated CREB in *A–D* is activating transcription factor 1 (ATF-1), a transcription factor of the CREB/ATF family.

As shown in Fig. 2*A*, the basal IL-6 release was rather high, which may be a result from endogenous adenosine produced by ATP release and metabolism. Adenosine deaminase treatment that removes adenosine had virtually no effect on the basal release of IL-6 (Fig. 2*B*) while it eliminated the effect of adenosine. In addition, the inclusion of 200 μM adenosine 5′-[α,β-methylene]diphosphate (AMPCP), an ectonucleotidase inhibitor that blocks the conversion of ATP to adenosine, failed to alter the basal IL-6 release (not shown). These data suggest that the high basal IL-6 release is independent of the endogenous basal adenosine level under our experimental conditions. In addition, the high basal release did not seem to result from endotoxin contamination, because no endotoxin (<0.06 endotoxin units/ml) was found in the vehicle control groups in Fig. 2*B*.

Next, the effect of 8-(*p*-sulfophenyl)theophylline (8-SPT), a nonselective blocker of adenosine receptors, was assessed. The adenosine-induced IL-6 release was substantially reduced by 66.5 ± 8.1% in the presence of 200 μM 8-SPT ($n = 5$), confirming that the effect of adenosine is mediated by adenosine receptors rather than the product of adenosine metabolism. This is reinforced by the observation that 5′-*N*-ethylcarboxamidoadenosine (NECA), a metabolically stable analog of adenosine, also induced IL-6 release (Fig. 2*B*).

In addition, adenosine increased IL-6 mRNA synthesis in Calu-3 cells (Fig. 2*C*), further supporting the notion that adenosine induces IL-6 release and also indicating that adenosine-induced secretion of IL-6 involves de novo synthesis.

Adenosine-induced IL-6 release was protein kinase A (PKA)- and protein kinase C (PKC)-dependent

Both PKA and PKC signaling pathways couple with adenosine receptors and mediate adenosine-induced IL-6 production in a cell-type-specific fashion in other cell types (2, 27, 40). Next, the role of PKA and PKC in adenosine-induced IL-6 release was examined in Calu-3 cells. PKA seems to mediate the IL-6 release, because

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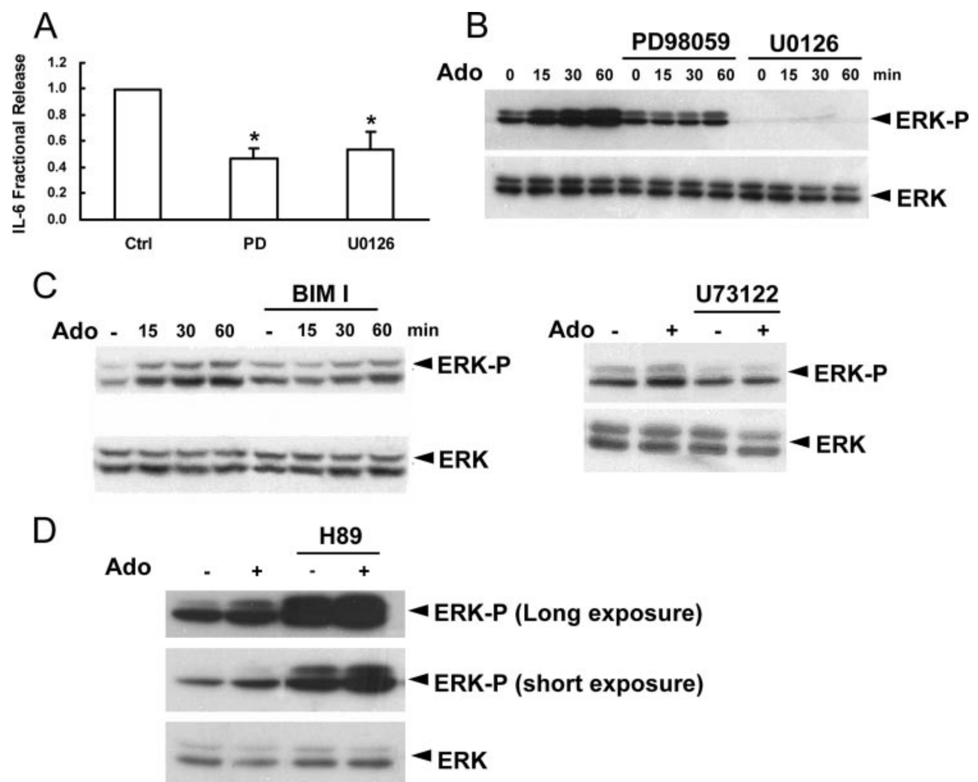

FIGURE 5. Involvement of ERK in adenosine-induced IL-6 release. *A*, Net IL-6 release induced by a 6-h treatment of 100 μM adenosine with or without 10 μM PD98059 (PD) or 10 μM U0126. Data are normalized to the control (Ctrl) group (adenosine alone). *, $p < 0.05$, compared with control; $n = 5–7$ independent experiments with each performed in duplicate. *B–D*, Western blots of phosphorylated ERK (ERK-P) and total ERK in one of total at least three similar experiments with treatment of 100 μM adenosine (Ado) with or without ERK inhibitors PD98059 (10 μM) or U0126 (10 μM) for the various times indicated (*B*); with or without BIM I (1 μM) for various times and U73122 (20 μM) for 30 min (*C*); or with or without H89 (20 μM) for 30 min (*D*). In *D*, two autoradiographs with different exposure times for ERK-P are shown. The two bands in *B–D* are ERK1 (*top band*) and ERK2 (*lower band*), respectively.

H89, a specific PKA inhibitor, blocked the IL-6 release (Fig. 3). In addition, BIM I and BIM II, two specific PKC inhibitors, suppressed the IL-6 release (Fig. 3), implicating the involvement of the PKC signaling pathway. Because PKC activation by adenosine receptors is often a downstream event of phospholipase C (PLC) activation, the effect of disrupting the PLC function was tested. U73122, a PLC-specific inhibitor, decreased IL-6 release (Fig. 3), reinforcing the idea that the PLC/PKC signaling pathway is required for IL-6 release.

Adenosine induced PKA- and PKC-dependent CREB phosphorylation

The promoter of the IL-6 gene contains a cAMP response element (CRE) (2) that is stimulated by the CRE binding protein (CREB). CREB is activated upon phosphorylation by various kinases such as PKA, PKC, MAPK (ERK and P38), and Ca^{2+}/calmodulin-dependent kinase (41). Because adenosine up-regulated IL-6 transcripts (Fig. 2*C*), we reasoned that adenosine elicits CREB phosphorylation in Calu-3 cells. Apical application of adenosine led to a robust, 8-SPT-sensitive CREB phosphorylation that peaked in 15~30 min after adenosine addition (Fig. 4*A*). Because PKA and PKC are required for adenosine-induced IL-6 release (Fig. 3), their roles in adenosine-induced CREB phosphorylation were evaluated. Forskolin, an adenylyl cyclase agonist, markedly elevated CREB phosphorylation (Fig. 4*B*), consistent with the general involvement of PKA in CREB phosphorylation. More importantly, adenosine-induced CREB phosphorylation was blocked by the PKA blocker H89 (Fig. 4*B*), pointing to a specific role of PKA signaling in adenosine-induced CREB phosphorylation. PMA, a PKC activator, also robustly increased CREB phosphorylation (Fig. 4*C*). In addition, adenosine-induced CREB phosphorylation was suppressed by the PKC inhibitor BIM I, and this effect was not due to the nonspecific action of BIM I on PKA because BIM I had no effect on forskolin-stimulated CREB phosphorylation (Fig. 4*C*). Moreover, adenosine-induced CREB phosphorylation was suppressed by BIM II and U73122 (Fig. 4*D*). Evidently, the PLC/PKC signaling pathway is also required for adenosine-induced CREB phosphorylation.

ERK links PKC with adenosine-induced IL-6 release by phosphorylating CREB

ERK acts as the downstream target of PKC in adenosine-induced cytokine release in some cell types (37), but the coupling of adenosine receptors and ERK in airway epithelial cells is poorly understood. U0126 and PD98059 are two structurally unrelated, highly selective inhibitors of ERK kinase (MEK) 1/2 and MEK 1, respectively. Because ERK activation is dependent on MEK activity, U0126 and PD98059 are widely used to block MEK and subsequent ERK activation. Adenosine-induced IL-6 release was substantially reduced by PD98059 and U0126 (Fig. 5*A*), implying that ERK plays an important role. To test more rigorously the coupling of adenosine receptors and ERK activation, we determined whether adenosine induces tyrosine phosphorylation of ERK because the phosphorylation of Tyr^{185} residue at the TEY motif is one of readouts of ERK1/ERK2 activation. Apical addition of adenosine significantly enhanced ERK phosphorylation without any alteration of total ERK (Fig. 5*B*). Both U0126 and PD98059 blocked adenosine-induced ERK phosphorylation (Fig. 5*B*), verifying that extracellular adenosine activates ERK in Calu-3 cells.

As shown in Fig. 5*C*, adenosine-induced ERK phosphorylation was suppressed by the PKC inhibitor, BIM I, and the PLC inhibitor, U73122. Stimulation of PKC by PMA also phosphorylated ERK (not shown). On the contrary, H89, the PKA blocker, seemed

FIGURE 6. The effect of ERK inhibitors on CREB phosphorylation. Western blots of phosphorylated CREB (CREB-P) and total CREB in one of total at least three similar experiments. Treatment for 30 min with 100 μM adenosine (Ado) in the presence or absence of 10 μM PD98059 (*left panel*) or 10 μM U0126 (*right panel*).

to have no or little effect on adenosine-induced ERK phosphorylation despite its stimulatory effect on basal ERK phosphorylation (Fig. 5*D*). Thus, ERK is a specific downstream target of PKC in adenosine-induced signaling.

Next, we examined whether ERK signaling is involved in CREB phosphorylation. As shown in Fig. 6, both ERK inhibitors

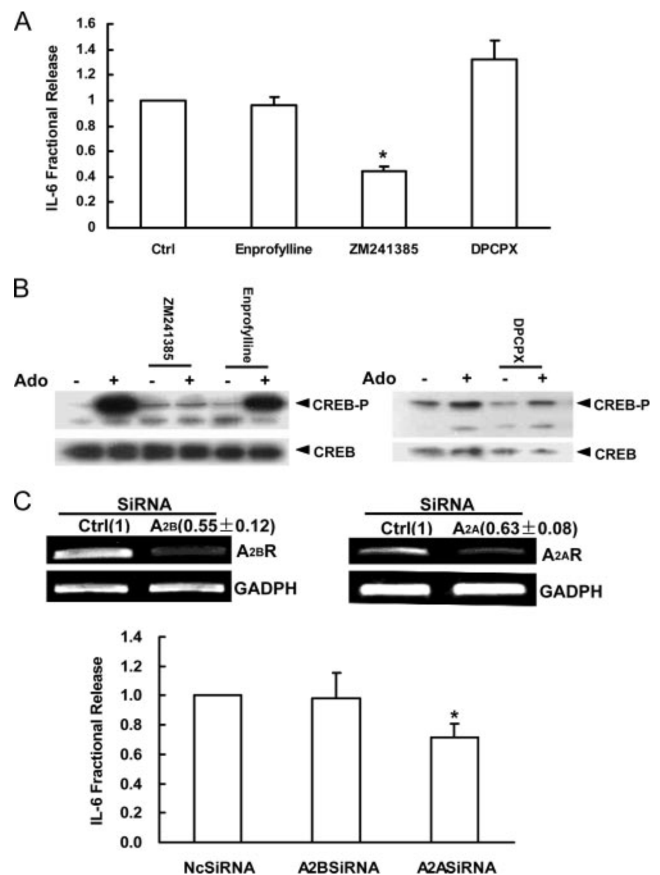

FIGURE 7. A2A adenosine receptors are specifically responsible for IL-6 release. *A*, Net IL-6 release induced by a 6-h treatment with 100 μM adenosine with or without 10 μM enprofylline, 1 μM ZM241385, or 0.3 μM DPCPX. Data were normalized to the control group (adenosine alone). *, $p < 0.05$, compared with control; $n = 3$–9 independent experiments with each performed in duplicate. *B*, Western blots of phosphorylated CREB (CREB-P) and total CREB in one of total at least three similar experiments. Treatment for 30 min with 100 μM adenosine (Ado) with or without 1 μM ZM241385 or 10 μM enprofylline (*left panel*) or 0.3 μM DPCPX (*right panel*). *C*, *Upper panel*, mRNA expression of A2A and A2B adenosine receptors after treatment with the control and their respective specific siRNA. The numbers in parentheses represent the normalized intensities of adenosine receptor mRNA ($n = 3$). GADPH was used as a control. *Lower panel*, Net IL-6 release induced by a 6-h exposure of 100 μM adenosine after treatment with control (NcSiRNA), A2A- (A2ASiRNA), or A2B-specific (A2BSiRNA) siRNA. *, $p < 0.05$, different from control.

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Table I. *The level of cytokines in Calu-3 (pg/ml)*[a]

	IL-1β	IL-4	IL-8	IL-10
H_2O	<1.95	<7.81	951.0 ± 149.3	<1.95
Adenosine (100 μM)	<1.95	<7.81	1474.5 ± 49.2*	<1.95
ATPγs (100 μM)	<1.95	<7.81	1072.2 ± 175.9	<1.95

[a] $n = 5–6$.
*, $p < 0.05$, significantly different from control.

Table II. *The level of cytokines in rat BAL (pg/ml)*[a]

	IL-1β	IL-4	IL-10
H_2O	62.0 ± 12.7	<1.95	<7.81
Adenosine (50 nmol)	196.4 ± 30.9*	<1.95	<7.81
NECA (50 nmol)	180.5 ± 26.5*	<1.95	<7.81
ATPγs (5 nmol)	270.4 ± 45.4*	<1.95	<7.81

[a] $n = 7–8$.
*, $p < 0.05$, significantly different from control.

suppressed adenosine-induced CREB phosphorylation. These data suggest that ERK, as a downstream target of PKC (Fig. 5*C*), bridges PKC signaling and CREB phosphorylation in adenosine-induced IL-6 release (Fig. 4).

Adenosine receptor subtypes in IL-6 release

We further sought to identify the adenosine receptor subtype(s) in the IL-6 release. Early studies suggested the presence of A2B and A2A but not A1 and A3 adenosine receptors in Calu-3 cells (11, 42). ZM241385, an A2A receptor-selective blocker, attenuated adenosine-induced IL-6 release (Fig. 7*A*). Consistent with this observation, ZM241385 virtually eliminated adenosine-induced CREB phosphorylation (Fig. 7*B*). Enprofylline and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), selective blockers for the A2B and A1 receptors, respectively, had no effect on either adenosine-induced IL-6 release (Fig. 7*A*) or CREB phosphorylation (Fig. 7*B*). In addition, knocking down A2A but not A2B receptors by RNA interference techniques resulted in a significant reduction in adenosine-induced IL-6 release (Fig. 7*C*). These results demonstrate that A2A adenosine receptors specifically mediate IL-6 release in Calu-3 cells.

Adenosine also induced proinflammatory IL-8 release in Calu-3 cells

Additional cytokines, including proinflammatory IL-1β and IL-8 and anti-inflammatory IL-4 and IL-10, were examined in parallel in the samples collected in the apical compartment with 100 μM apical adenosine or ATPγS treatment as described in Fig. 1. Although both adenosine and ATPγS failed to cause any change in IL-1β, IL-4 and IL-10, adenosine, but not ATPγS, triggered a significant elevation in IL-8 release (Table I). These results imply that adenosine-induced IL-6, together with proinflammatory IL-8, likely prompts inflammation, although IL-6 alone could be either proinflammatory or anti-inflammatory.

Adenosine-induced IL-6 release in rat BALF

A rat model was used to test whether or not adenosine induces IL-6 release in airway epithelia in vivo. Treatment with 5 nmol of adenosine induced some increase in IL-6 in BALF in rats; 50 nmol of adenosine further increased the IL-6 release by ~6-fold (Fig. 8). Similar results were observed with NECA, a nonhydrolyzable analog of adenosine. The effect of ATPγS was tested for comparison. Five nmol of ATPγS resulted in a 7-fold increase in IL-6 release (Fig. 8), similar to the previously reported observation (35). However, 50 nmol of ATPγS seemed to have some inhibitory effects on IL-6 release (Fig. 8).

Several other cytokines were also examined in the BALF of three experimental groups selected from Fig. 8 because of their relatively high IL-6 release, i.e., the groups treated with 50 nmol of adenosine, 50 nmol of NECA, and 5 nmol of ATPγS. Adenosine, NECA, or ATPγS failed to induce any changes in IL-4 and IL-10, the two anti-inflammatory cytokines, while they all evoked a robust 3- to 4-fold increase in proinflammatory IL-1β (Table II).

Adenosine and ATP mediated LPS-induced IL-6 release in rat BALF

Adenosine elevation in the lung, including BAL, has been observed in several types of pulmonary inflammation and fibrosis (12–14) and has been merely considered as a consequence or by-product of tissue inflammation and injury. We hypothesize that adenosine released during the inflammation process induces IL-6 release from airway epithelia. To test this hypothesis, LPS was used to induce lung inflammation in rats. LPS treatment elicited a robust increase in IL-6 release as expected (Fig. 9). Strikingly, adenosine seemed to contribute considerably to LPS-induced IL-6 release, because treatment with 150 nmol of 8-SPT reduced the LPS-induced IL-6 release by 42%. Thirty or 150 nmol suramin, a P2 receptor antagonist, also greatly attenuated the LPS-induced IL-6 release, indicating a role of ATP in IL-6 release during inflammation as well. A combination of 150 nmol of 8-SPT and 30 nmol of suramin did not lead to a significant additive effect (Fig. 9), implying that adenosine and P2 receptors may largely share common downstream signaling pathways in inducing IL-6 release.

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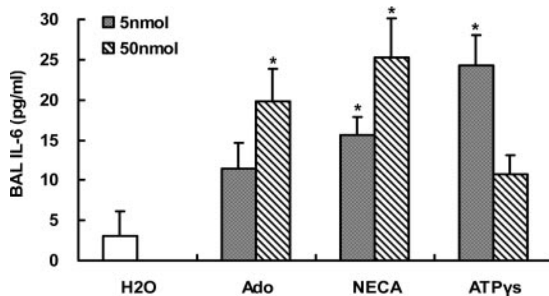

FIGURE 8. Adenosine induced IL-6 release in BALF. Rats were treated with different doses of adenosine (Ado), NECA, or ATPγS. IL-6 levels in BALF were analyzed at 18 h. *, $p < 0.05$, different from control (H_2O); $n = 6–10$.

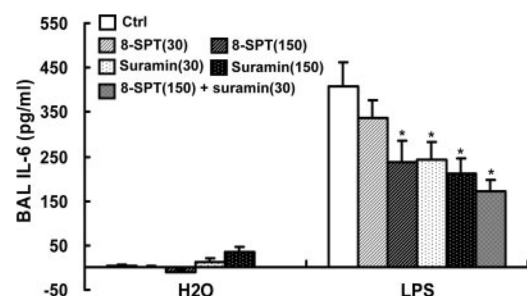

FIGURE 9. LPS-induced IL-6 release is dependent on adenosine and ATP. Rats were treated with LPS (1 mg/kg) in the presence or absence of 8-SPT and suramin. IL-6 levels in BALF were analyzed at 18 h. *, $p < 0.05$, different from LPS alone; $n = 6–10$.

Discussion

Biological implications of adenosine-induced IL-6 release

A recent study suggests that nucleotides, mainly in the form of ATP, promote IL-6 release from small airway epithelial cells via $P2Y_2$ receptors. Adenosine was also found to have a small effect on IL-6 release (35). However, adenosine-promoted IL-6 release from the airway epithelia remains largely unexplored.

Using Calu-3 human airway epithelial cells as an in vitro model, we demonstrated that extracellular adenosine induced a robust, apically polarized release of IL-6. In addition, adenosine also induced the release of IL-8 into the apical compartment (Table I) but not into the basolateral compartment (not shown). The preferential apical secretion of IL-6/IL-8 from airway epithelial cells, to the best of our knowledge, has not been reported before. IL-8 is a key chemokine of neutrophil infiltration, and IL-6 is able to induce neutrophil infiltration as well (22–24). Thus, apically polarized release of IL-6 and IL-8 presumably provides chemical gradients for neutrophils and other leukocytes to move from the subepithelium to the mucosal surface and remain there.

In an in vivo model, adenosine or NECA also induces a several fold increase in IL-6 release in rat BALF (Fig. 8), further strengthening the notion that adenosine induces IL-6 release in airway epithelia. The single application of exogenous adenosine or even NECA tested in this study, however, may not be a good representation of endogenous adenosine levels in inflammation, in which the continuous release of adenosine from airway epithelial cells and infiltrated leukocytes offsets the reduction of adenosine as a result of constant breakdown, cellular transport, and mucociliary clearance. To assess the contribution of adenosine to IL-6 production in inflammation properly, LPS was used to induce inflammation. Remarkably, blocking adenosine receptors led to a significant reduction in LPS-induced IL-6 release (Fig. 9). These results suggest that adenosine plays a pivotal role in IL-6 production in airway inflammation.

In partially ADA-deficient mice, several leukocytes, including neutrophils, were markedly increased in airway lumen, although the underlying molecular mechanism was not examined (17). Our findings provide a possible molecular link between adenosine elevation and neutrophil infiltration in airway lumen. Furthermore, because IL-6 and IL-8 could synergistically recruit and activate neutrophils in airway lumen, the release of adenosine (converted from 5′-AMP instead of ATP) from neutrophils (2, 43) and stressed/damaged tissues and the release of IL-6/8 from epithelial cells form a positive feedback loop. This positive feedback loop may exaggerate the inflammation and potentially contribute to tissue injury/fibrosis of the lungs in asthma, cystic fibrosis, and other types of airway inflammation disorders. Consistent with this notion, neutrophil infiltration is accompanied by the elevation of IL-6, IL-8, and adenosine in asthma and cystic fibrosis (20, 21). Further studies in animal models and clinical investigations are needed to establish the causal relation between adenosine elevation and the recruitment and activation of neutrophils in airway lumen.

In addition to inducing IL-6 release from airway epithelia, the presence of adenosine in airway lumen has other proinflammatory and tissue-destructive effects. In this study, adenosine was found to promote the release of IL-8 from human Calu-3 cells and IL-1β from rat lungs (Tables I and II). Moreover, adenosine was found to induce the synthesis of TNF-α and matrix metalloproteinase and directly promote neutrophil chemotaxis (12, 44). In contrast, IL-6 has target cells other than neutrophils. IL-6 can directly activate fibroblasts and thus is involved in the downstream events in fibrosis progression as well (45). IL-6 has been implicated not only in acute inflammation but also in chronic inflammation and is thought to play an essential role in the transition from acute to chronic inflammation (46). All these actions of adenosine and IL-6 could synergistically contribute to inflammation and tissue damage and further augment the positive feedback loop between adenosine and IL-6.

The link between adenosine and IL-6/IL-8 release in airway epithelia may also account for mechanical ventilator-induced lung injury (VILI). Overventilation may mechanically stimulate excessive ATP/adenosine release (47–49), which would increase IL-6/IL-8 release and lead to inflammation and tissue injury (35). Indeed, ventilation or stretch up-regulates IL-6/IL-8 and other proinflammatory cytokines in several types of lung cells, including epithelial cells (26, 49). Thus, adenosine receptor blockers might potentially be developed as treatment for ventilator-induced lung injury.

Adenosine vs ATP

Douillet and colleagues found that ATP is more potent than adenosine in promoting IL-6 release in cultured human small airway epithelial cells (35). In contrast, we found that adenosine is more potent than ATP in inducing IL-6 release in cultured Calu-3 human airway epithelial cells (Fig. 1). This is not too surprising considering that two different cell lines were used.

Although Douillet et al. did not directly test the effect of adenosine in the rat animal model they used, they found that apyrase, which catalyzes the hydrolysis of ATP to yield AMP and inorganic phosphate, attenuated ~85% of the IL-6 release induced by mechanical ventilation (35). Their data imply that adenosine does not play a major role in mechanical ventilation-induced IL-6 release, because AMP is converted to adenosine by ectonucleotidases as well (2). On the contrary, we found that adenosine was almost equally as potent as ATP in promoting IL-6 release when adenosine and ATP were applied exogenously (Fig. 8). Furthermore, adenosine seemed to contribute to LPS-induced IL-6 release as effectively as ATP does (Fig. 9). One major difference is that Douillet et al. measured IL-6 release after 1 h of mechanical ventilation while we challenged rats with different stimuli for 18 h. Our in vitro study suggests that adenosine did not take effect for 3 h (Fig. 2). If the action of adenosine has a similar latency in vivo, its effect could not have been observed in the study of Douillet et al. Nevertheless, the exact reasons for the discrepancy in these two studies remain to be determined.

Signaling pathways in adenosine-induced IL-6 release

Adenosine induces IL-6 release in a variety of cells and tissues with complex signaling mechanisms. PKA and PKC signaling have been previously implicated in adenosine-induced IL-6 secretion, although the involvement of these signaling pathways appears to be cell type specific (1, 2, 27, 40). Therefore, we examined the role of the two signaling pathways in adenosine-induced IL-6 release in Calu-3 cells. We found that both PKA and PKC mediate IL-6 release. Because the promoter of the IL-6 gene is regulated by CREB, we subsequently assessed the role of PKA and PKC in adenosine-induced CREB phosphorylation. Our results revealed that both PKA and PKC signaling control adenosine-induced CREB phosphorylation. Moreover, we found that ERK is also involved in adenosine-induced IL-6 release. Because ERK acts as a downstream target of PKC in adenosine signaling and phosphorylates CREB (Figs. 4 and 5), we propose that ERK bridges PKC signaling and CREB phosphorylation in adenosine-induced IL-6 release.

Although the PKA blocker H89 failed to block adenosine-induced ERK phosphorylation, it robustly stimulated basal ERK

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phosphorylation (Fig. 5*D*). These data suggest that basal PKA activity tonically suppresses ERK phosphorylation, probably through inhibiting Raf-1 kinase and thus the Raf-1/MEK/ERK signaling cascade (50, 51). It is not clear why adenosine-induced ERK phosphorylation was not enhanced in the presence of H89, which presumably blocked the inhibitory effect of adenosine receptor-coupled PKA on ERK phosphorylation. One explanation is that the PKA pool coupled with adenosine receptors does not target Raf-1. Alternatively, basal ERK phosphorylation in the presence of H89 (Fig. 5*D*) was too high for further increase.

A2A and A2B adenosine-receptor subtypes have been found in Calu-3 cells (11, 42). Previous studies suggest that both apical and basolateral A2B receptors are coupled with cystic fibrosis transmembrane conductance regulator (CFTR) activation and transepithelial anion secretion (8, 11, 42). However, the role of apical A2A adenosine receptors has not been described, although the basolateral A2A receptors appear to mediate transepithelial anion secretion (11). Using adenosine receptor subtype-specific blockers and siRNA, we found that apical adenosine induced IL-6 release specifically via A2A but not A2B receptors. Both A2A and A2B receptors are positively coupled with AC/PKA signaling, but A2A not A2B receptors are more often associated with PLC/PKC signaling (40, 52, 53). Indeed, adenosine-induced IL-6 release requires both the adenylate cyclase/PKA and PLC/PKC signaling pathways, consistent with the observation that A2A receptors play a role.

High basal cytokine release in Calu-3 cells

High basal IL-6 and IL-8 release was observed in Calu-3 cells (Fig. 2 and Table I). High basal release of IL-8 and other chemokines has been seen in human airway epithelial A549 cell lines (54). The high basal cytokine release is a result of neither basal endogenous adenosine release (Fig. 2*B*) nor endotoxin contamination in collected samples or the culture media (see *Results* and *Materials and Methods*, respectively). The use of FBS in culture media is believed to stimulate cytokine release, although removing FBS from the culture medium or using serum-free AIM V culture medium (Invitrogen Life Technologies) did not affect the basal cytokine release in our experiments (not shown). The basal ERK phosphorylation in Calu-3 cells, relatively high compared with other cell types (37), seems to contribute to the high basal release because ERK inhibitors attenuated not only basal ERK phosphorylation (Fig. 5*B*) but also 30–50% of basal IL-6 and IL-8 release (not shown). Constitutive ERK activity has been previously observed in many other cell types as well (55–58).

Summary

We found that apical adenosine induced robust, apically polarized release of IL-6, along with proinflammatory IL-8, in human airway epithelia. In addition, we showed that adenosine triggered the release of IL-6, together with proinflammatory IL-1β, in rat BALF. Furthermore, we demonstrated that adenosine mediated LPS-induced IL-6 release in rats. These observations may partly provide a molecular link between adenosine elevation and lung inflammation and fibrosis. We have also explored the signaling mechanisms for adenosine-induced IL-6 release in Calu-3 cells. Further clinical investigation is needed to establish the full physiological implications of these findings.

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Disclosures

The authors have no financial conflict of interest.

References

1. Feoktistov, I., and I. Biaggioni. 1995. Adenosine A2b receptors evoke interleukin-8 secretion in human mast cells: an enprofylline-sensitive mechanism with implications for asthma. *J. Clin. Invest.* 96: 1979–1986.
2. Sitaraman, S. V., D. Merlin, L. Wang, M. Wong, A. T. Gewirtz, M. Si-Tahar, and J. L. Madara. 2001. Neutrophil-epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of adenosine and IL-6. *J. Clin. Invest.* 107: 861–869.
3. Link, A. A., T. Kino, J. A. Worth, J. L. McGuire, M. L. Crane, G. P. Chrousos, R. L. Wilder, and I. J. Elenkov. 2000. Ligand-activation of the adenosine A2a receptors inhibits IL-12 production by human monocytes. *J. Immunol.* 164: 436–442.
4. Hasko, G., D. G. Kuhel, J. F. Chen, M. A. Schwarzschild, E. A. Deitch, J. G. Mabley, A. Marton, and C. Szabo. 2000. Adenosine inhibits IL-12 and TNF-α production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J.* 14: 2065–2074.
5. Picher, M., L. H. Burch, and R. C. Boucher. 2004. Metabolism of P2 receptor agonists in human airways: implications for mucociliary clearance and cystic fibrosis. *J. Biol. Chem.* 279: 20234–20241.
6. Picher, M., L. H. Burch, A. J. Hirsh, J. Spychala, and R. C. Boucher. 2003. Ecto 5′-nucleotidase and nonspecific alkaline phosphatase: two AMP-hydrolyzing ectoenzymes with distinct roles in human airways. *J. Biol. Chem.* 278: 13468–13479.
7. Cobb, B. R., L. Fan, T. E. Kovacs, E. J. Sorscher, and J. P. Clancy. 2003. Adenosine receptors and phosphodiesterase inhibitors stimulate Cl- secretion in Calu-3 cells. *Am. J. Respir. Cell Mol. Biol.* 29: 410–418.
8. Huang, P., E. R. Lazarowski, R. Tarran, S. L. Milgram, R. C. Boucher, and M. J. Stutts. 2001. Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells. *Proc. Natl. Acad. Sci. USA* 98: 14120–14125.
9. Morse, D. M., J. L. Smullen, and C. W. Davis. 2001. Differential effects of UTP, ATP, and adenosine on ciliary activity of human nasal epithelial cells. *Am. J. Physiol.* 280: C1485–C1497.
10. Pereira, M. M., C. Lloyd Mills, R. L. Dormer, and M. A. McPherson. 1998. Actions of adenosine A1 and A2 receptor antagonists on CFTR antibody-inhibited β-adrenergic mucin secretion response. *Br. J. Pharmacol.* 125: 697–704.
11. Szkotak, A. J., A. M. Ng, S. F. Man, S. A. Baldwin, C. E. Cass, J. D. Young, and M. Duszyk. 2003. Coupling of CFTR-mediated anion secretion to nucleoside transporters and adenosine homeostasis in Calu-3 cells. *J. Membr. Biol.* 192: 169–179.
12. Spicuzza, L., G. Di Maria, and R. Polosa. 2006. Adenosine in the airways: implications and applications. *Eur. J. Pharmacol.* 533: 77–88.
13. Driver, A. G., C. A. Kukoly, S. Ali, and S. J. Mustafa. 1993. Adenosine in bronchoalveolar lavage fluid in asthma. *Am. Rev. Respir. Dis.* 148: 91–97.
14. Li, Y., W. Wang, W. Parker, and J. P. Clancy. 2006. Adenosine regulation of cystic fibrosis transmembrane conductance regulator through prostenoids in airway epithelia. *Am. J. Respir. Cell Mol. Biol.* 34: 600–608.
15. Chunn, J. L., J. G. Molina, T. Mi, Y. Xia, R. E. Kellems, and M. R. Blackburn. 2005. Adenosine-dependent pulmonary fibrosis in adenosine deaminase-deficient mice. *J. Immunol.* 175: 1937–1946.
16. Chunn, J. L., H. W. Young, S. K. Banerjee, G. N. Colasurdo, and M. R. Blackburn. 2001. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J. Immunol.* 167: 4676–4685.
17. Chunn, J. L., A. Mohsenin, H. W. Young, C. G. Lee, J. A. Elias, R. E. Kellems, and M. R. Blackburn. 2006. Partially adenosine deaminase-deficient mice develop pulmonary fibrosis in association with adenosine elevations. *Am. J. Physiol.* 290: L579–L587.
18. Sun, C. X., H. Zhong, A. Mohsenin, E. Morschl, J. L. Chunn, J. G. Molina, L. Belardinelli, D. Zeng, and M. R. Blackburn. 2006. Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *J. Clin. Invest.* 116: 2173–2182.
19. Louis, R., and R. Djukanovic. 2006. Is the neutrophil a worthy target in severe asthma and chronic obstructive pulmonary disease? *Clin. Exp. Allergy* 36: 563–567.
20. Chmiel, J. F., and P. B. Davis. 2003. State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? *Respir. Res.* 4: 8.
21. Conese, M., E. Copreni, S. Di Gioia, P. De Rinaldis, and R. Fumarulo. 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *J. Cyst. Fibros.* 2: 129–135.
22. Romano, M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, et al. 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* 6: 315–325.
23. Hierholzer, C., J. C. Kalff, L. Omert, K. Tsukada, J. E. Loeffert, S. C. Watkins, T. R. Billiar, and D. J. Tweardy. 1998. Interleukin-6 production in hemorrhagic shock is accompanied by neutrophil recruitment and lung injury. *Am. J. Physiol.* 275: L611–L621.
24. McLoughlin, R. M., J. Witowski, R. L. Robson, T. S. Wilkinson, S. M. Hurst, A. S. Williams, J. D. Williams, S. Rose-John, S. A. Jones, and N. Topley. 2003. Interplay between IFN-γ and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J. Clin. Invest.* 112: 598–607.

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25. Pardo, A., R. Barrios, M. Gaxiola, L. Segura-Valdez, G. Carrillo, A. Estrada, M. Mejia, and M. Selman. 2000. Increase of lung neutrophils in hypersensitivity pneumonitis is associated with lung fibrosis. *Am. J. Respir. Crit. Care Med.* 161: 1698–1704.
26. Goodman, R. B., J. Pugin, J. S. Lee, and M. A. Matthay. 2003. Cytokine-mediated inflammation in acute lung injury. *Cytokine Growth Factor Rev.* 14: 523–535.
27. Hershko, D. D., B. W. Robb, G. Luo, and P. O. Hasselgren. 2002. Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells. *Am. J. Physiol.* 283: R1140–R1148.
28. Diamond, G., D. Legarda, and L. K. Ryan. 2000. The innate immune response of the respiratory epithelium. *Immunol. Rev.* 173: 27–38.
29. Stick, S. M., and P. G. Holt. 2003. The airway epithelium as immune modulator: the LARC ascending. *Am. J. Respir. Cell Mol. Biol.* 28: 641–644.
30. Nonas, S., I. Miller, K. Kawkitinarong, S. Chatchavalvanich, I. Gorshkova, V. N. Bochkov, N. Leitinger, V. Natarajan, J. G. Garcia, and K. G. Birukov. 2006. Oxidized phospholipids reduce vascular leak and inflammation in rat model of acute lung injury. *Am. J. Respir. Crit. Care Med.* 173: 1130–1138.
31. Jacquot, J., E. Puchelle, J. Hinnrasky, C. Fuchey, C. Bettinger, C. Spilmont, N. Bonnet, A. Dieterle, D. Dreyer, A. Pavirani, et al. 1993. Localization of the cystic fibrosis transmembrane conductance regulator in airway secretory glands. *Eur. Respir. J.* 6: 169–176.
32. Engelhardt, J. F., J. R. Yankaskas, S. A. Ernst, Y. Yang, C. R. Marino, R. C. Boucher, J. A. Cohn, and J. M. Wilson. 1992. Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nat. Genet.* 2: 240–248.
33. Zhang, Y., W. W. Reenstra, and A. Chidekel. 2001. Antibacterial activity of apical surface fluid from the human airway cell line Calu-3: pharmacologic alteration by corticosteroids and β_2-agonists. *Am. J. Respir. Cell Mol. Biol.* 25: 196–202.
34. Joo, N. S., D. J. Lee, K. M. Winges, A. Rustagi, and J. J. Wine. 2004. Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. *J. Biol. Chem.* 279: 38854–38860.
35. Douillet, C. D., W. P. Robinson, III, P. M. Milano, R. C. Boucher, and P. B. Rich. 2006. Nucleotides induce IL-6 release from human airway epithelia via P2Y2 and p38 MAPK-dependent pathways. *Am. J. Physiol.* 291: L734–L746.
36. Lazarowski, E. R., R. Tarran, B. R. Grubb, C. A. van Heusden, S. Okada, and R. C. Boucher. 2004. Nucleotide release provides a mechanism for airway surface liquid homeostasis. *J. Biol. Chem.* 279: 36855–36864.
37. Feoktistov, I., A. E. Goldstein, and I. Biaggioni. 1999. Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol. Pharmacol.* 55: 726–734.
38. Kiriyama, Y., H. Tsuchiya, T. Murakami, K. Satoh, and Y. Tokumitsu. 2001. Calcitonin induces IL-6 production via both PKA and PKC pathways in the pituitary folliculo-stellate cell line. *Endocrinology* 142: 3563–3569.
39. Cobb, L. M., J. C. Mychaleckyj, D. J. Wozniak, and Y. S. Lopez-Boado. 2004. *Pseudomonas aeruginosa* flagellin and alginate elicit very distinct gene expression patterns in airway epithelial cells: implications for cystic fibrosis disease. *J. Immunol.* 173: 5659–5670.
40. Rees, D. A., B. M. Lewis, M. D. Lewis, K. Francis, M. F. Scanlon, and J. Ham. 2003. Adenosine-induced IL-6 expression in pituitary folliculostellate cells is mediated via A2b adenosine receptors coupled to PKC and p38 MAPK. *Br. J. Pharmacol.* 140: 764–772.
41. Johannessen, M., M. P. Delghandi, and U. Moens. 2004. What turns CREB on? *Cell. Signal.* 16: 1211–1227.
42. Cobb, B. R., F. Ruiz, C. M. King, J. Fortenberry, H. Greer, T. Kovacs, E. J. Sorscher, and J. P. Clancy. 2002. A_2 adenosine receptors regulate CFTR through PKA and PLA_2. *Am. J. Physiol.* 282: L12–L25.
43. Madara, J. L., T. W. Patapoff, B. Gillece-Castro, S. P. Colgan, C. A. Parkos, C. Delp, and R. J. Mrsny. 1993. 5′-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J. Clin. Invest.* 91: 2320–2325.
44. Chen, Y., R. Corriden, Y. Inoue, L. Yip, N. Hashiguchi, A. Zinkernagel, V. Nizet, P. A. Insel, and W. G. Junger. 2006. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 314: 1792–1795.
45. Knight, D. A., M. Ernst, G. P. Anderson, Y. P. Moodley, and S. E. Mutsaers. 2003. The role of gp130/IL-6 cytokines in the development of pulmonary fibrosis: critical determinants of disease susceptibility and progression? *Pharmacol. Ther.* 99: 327–338.
46. Gabay, C. 2006. Interleukin-6 and chronic inflammation. *Arthritis Res. Ther.* 8 (Suppl. 2): S3.
47. Grygorczyk, R., and J. W. Hanrahan. 1997. CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. *Am. J. Physiol.* 272: C1058–C1066.
48. Watt, W. C., E. R. Lazarowski, and R. C. Boucher. 1998. Cystic fibrosis transmembrane regulator-independent release of ATP: its implications for the regulation of P2Y2 receptors in airway epithelia. *J. Biol. Chem.* 273: 14053–14058.
49. Rich, P. B., C. D. Douillet, H. Hurd, and R. C. Boucher. 2003. Effect of ventilatory rate on airway cytokine levels and lung injury. *J. Surg. Res.* 113: 139–145.
50. Hafner, S., H. S. Adler, H. Mischak, P. Janosch, G. Heidecker, A. Wolfman, S. Pippig, M. Lohse, M. Ueffing, and W. Kolch. 1994. Mechanism of inhibition of Raf-1 by protein kinase A. *Mol. Cell. Biol.* 14: 6696–6703.
51. Dumaz, N., Y. Light, and R. Marais. 2002. Cyclic AMP blocks cell growth through Raf-1-dependent and Raf-1-independent mechanisms. *Mol. Cell. Biol.* 22: 3717–3728.
52. Gardner, A. M., and M. E. Olah. 2003. Distinct protein kinase C isoforms mediate regulation of vascular endothelial growth factor expression by A2A adenosine receptor activation and phorbol esters in pheochromocytoma PC12 cells. *J. Biol. Chem.* 278: 15421–15428.
53. Ralevic, V., and G. Burnstock. 1998. Receptors for purines and pyrimidines. *Pharmacol. Rev.* 50: 413–492.
54. Lin, Y., M. Zhang, and P. F. Barnes. 1998. Chemokine production by a human alveolar epithelial cell line in response to *Mycobacterium tuberculosis*. *Infect. Immun.* 66: 1121–1126.
55. Chadee, D. N., D. Xu, G. Hung, A. Andalibi, D. J. Lim, Z. Luo, D. H. Gutmann, and J. M. Kyriakis. 2006. Mixed-lineage kinase 3 regulates B-Raf through maintenance of the B-Raf/Raf-1 complex and inhibition by the NF2 tumor suppressor protein. *Proc. Natl. Acad. Sci. USA* 103: 4463–4468.
56. Satyamoorthy, K., G. Li, M. R. Gerrero, M. S. Brose, P. Volpe, B. L. Weber, P. Van Belle, D. E. Elder, and M. Herlyn. 2003. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res.* 63: 756–759.
57. Aguirre-Ghiso, J. A., Y. Estrada, D. Liu, and L. Ossowski. 2003. ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). *Cancer Res.* 63: 1684–1695.
58. Conner, S. R., G. Scott, and A. E. Aplin. 2003. Adhesion-dependent activation of the ERK1/2 cascade is by-passed in melanoma cells. *J. Biol. Chem.* 278: 34548–34554.

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