Neutrophil elastase stimulates human airway epithelial cells to produce PGE$_2$ through activation of p44/42 MAPK and upregulation of cyclooxygenase-2

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Perng, Diahn-Warng, Yu-Chung Wu, Mei-Chuan Tsai, Ching-Ping Lin, Wen-Hu Hsu, Reury-Perng Perng, and Yu-Chin Lee. Neutrophil elastase stimulates human airway epithelial cells to produce PGE$_2$ through activation of p44/42 MAPK and upregulation of cyclooxygenase-2. Am J Physiol Lung Cell Mol Physiol 285: L925–L930, 2003. First published June 27, 2003; 10.1152/ajplung.00182.2002.—The responses of airway epithelium following exposure to neutrophil elastase (NE) were investigated. Human bronchial epithelial cells were explanted on insert surfaces of a modified air-liquid interface culture system to which NE was added to stimulate epithelial cells. PGE$_2$ release significantly increased within 10 min of incubation with NE and peaked 3 h after NE (20 μg/ml) stimulation. This action required proteolytic activity as α1-antitrypsin blocked NE-induced PGE$_2$ release. The production of PGE$_2$ was also inhibited by indomethacin; a selective cyclooxygenase (COX)-2 inhibitor, celecoxib; and dexamethasone. Moreover, the mRNA expression for COX-2 relative to that for a housekeeping gene was approximately eightfold that of the unstimulated cells. Dexamethasone inhibited COX-2 gene transcription. We further observed that NE-induced PGE$_2$ release involved activation of p44/42, but not p38, MAP kinases. Such p44/42 MAP kinases were rapidly phosphorylated, with the concentration of phosphorylated p44/42 MAP kinases peaking at 10 min after stimulation and declining in culture at 90 min. The specific p44/42 MAP kinase inhibitor U0126 completely blocked p44/42 phosphorylation and, subsequently, PGE$_2$ production. The airway epithelium may play important bronchoprotective and immunomodulatory roles in chronic neutrophil inflammation.

human bronchial epithelial cell; prostaglandin E$_2$; chronic neutrophil inflammation

rather than functioning as a passive barrier, the human airway epithelium can respond to injury, infection, or inflammation by producing various cytokines and mediators to modulate airway inflammation. Certain exogenous or endogenous stimuli can directly affect epithelial cells to increase the generation of IL-8; granulocyte-macrophage colony-stimulating factor; regulated on activation, normal T cell expressed, and presumably secreted; TNF-α; or PGE$_2$ (5, 16, 21, 30). PGE$_2$, a metabolite of cyclooxygenase (COX), has been shown to exert a variety of anti-inflammatory and bronchoprotective effects both in vitro and in vivo. PGE$_2$ may prevent allergen-induced bronchoconstriction (9), relax airway smooth muscle (17), inhibit cholinergic neurotransmission (12), and modulate fibroblast proliferation (20).

Neutrophil elastase (NE), stored in the azurophilic granules, has been reported to play an important role in stimulating mucus secretion (7), decreasing ciliary function (3), and increasing epithelial permeability (27) and tissue destruction (15). NE may contribute to several inflammatory disorders, including emphysema (29), chronic bronchitis (32), bronchiectasis (31), cystic fibrosis (13), and adult respiratory distress syndrome (18).

Mitogen-activated protein kinases (MAP kinases), a widely studied family of serine/threonine protein kinases, have been reported to participate in multiple directions of cellular programs (28). These MAP kinases are activated by dual phosphorylation on tyrosine/threonine residues by distinct MAP kinase kinases. c-Jun NH2-terminal kinase and p38 MAP kinase can be activated by a variety of extracellular stimuli and may play critical roles in regulating cytokine production. ERK1 (p44) and -2 (p42) are considered to be involved mostly in cell growth, differentiation, and development.

Airway epithelium is likely to be exposed to high levels of NE in chronic neutrophilic inflammation. This investigation attempts to determine the effects of NE on airway epithelium and its signaling pathways. Human airway epithelial cells (HAEC) were grown on modified air-liquid interface culture inserts. NE was employed to stimulate epithelial cells in both the apical and basal directions. We report that NE activates MAP kinases p44/42 and upregulates COX-2 gene expression, which subsequently enhances PGE$_2$ production from HAEC. These results demonstrate that human airway epithelium may play an important bronchopro-

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tective and immunomodulatory roles in chronic neutrophilic inflammation.

MATERIALS AND METHODS

Reagents. RPMI 1640, Medium 199, Leibovitz's L-15 medium, fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, insulin, recombinant human epidermal growth factor, COX-2 oligonucleotides, and superscript preamplification systems for cDNA synthesis were obtained from GIBCO-BRL Life Technologies (Gaithersburg, MD); type IV collagen, all-trans retinoic acid, transferrin, hydrocortisone, dexamethasone, and indomethacin were from Sigma (St. Louis, MO); NE (16,000 units per mg of protein on the substrate Meo-Suc-Al-Al-Pro-Val-pNA; 1 unit hydrolyzes 1 μmol of substrate per min at pH 7.5 and 25°C) from Owensville Elastin Products (St. Louis, MO); cell culture/H9262 lyzes 1 cortisone, dexamethasone, and indomethacin were from

Modified air-liquid interface culture for HAEC. This cell culture procedure was modified from methods described previously (33, 34). Human bronchus, obtained from surgical lobectomy for lung cancer, was rinsed several times with Leibovitz's L-15 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The tissue was cut into 1- to 2-mm² pieces, and 3–4 pieces of tissue were planted with the epithelium side facing down onto six-well culture inserts (growth area of membrane 4.2 cm², pore size 0.4 μm) coated with type IV collagen (50 μg/cm²). Two milliliters of medium containing antibiotics/antimycotic, human epidermoid growth factor (1 ng/ml), insulin (2.5 μg/ml), transferrin (2.5 μg/ml), hydrocortisone (1 μg/ml), 2 mM glutamine, and 0.1% FBS in RPMI 1640 and Medium 199 (vol/vol 1:1) were added to the basal chamber, and 100 μl were added to the insert. Culture medium in the basal chamber was changed every 48–72 h, and no medium was added to the insert. The aerial epithelial cells were grown on a porous membrane, on which they formed a continuous epithelial sheet with the basal aspect exposed to medium and the apical surface exposed to air.

Cells grown on the inserts were confluent after 7–10 days of incubation. The tissue fragments were then transferred to fresh inserts to obtain new growth of epithelial cells. Cells were then dissociated using 0.02% trypsin-EDTA solution and seeded in 24-well culture inserts (growth area of membrane 0.3 cm², pore size 0.4 μm) coated with collagen to determine the extent of mediator release following NE stimulation.

Mediator release. Cells (100 μl) were seeded in 24-well culture inserts at a density of 1 × 10⁶ cells/ml and grown in culture medium (500 μl per basal chamber). The purity of epithelial cells appeared to be >98%, as determined by morphology and by immunocytochemistry with antibodies against cytokeratin for epithelial cells, vimentin for fibroblasts, and myosin for smooth muscle cells. At confluence, NE was added to either apical or basal compartment at a concentration of 5–20 μg/ml. To suppress the effect of mediator release induced by NE, we treated cells with α2-antitrypsin (200 μg/ml), dexamethasone (1–100 μM), indomethacin (0.1–1 μM), or celecoxib (0.5–10 μM suspended in dimethyl sulfoxide). Supernatants were collected at each time point and stored at −80°C until assayed for mediators. Cell viability was determined by light microscopy and dye exclusion with trypan blue. Levels of PGE₂ were assayed by ELISA according to the manufacturer's instructions.

Analysis of COX-2 mRNA expression. After the removal of supernatants for mediator measurement, total cellular RNA was isolated from cell monolayers using a high pure RNA isolation kit. The RNA (1 μg) was reverse transcribed into cDNA using Superscript II RNase H⁻ reverse transcriptase. An aliquot of cDNA was then subjected to 35 cycles of PCR using a standard procedure denaturing at 94°C for 1 min, annealing at 52°C for COX-2 for 30 s, and elongating at 72°C for 1.2 min. The COX-2-specific primer pair amplified a 769-bp PCR product composed of 5’ primer GTGTCGGTG CCTGGTCTGATGATG and 3’ primer CCAATAGGAGGAAACATATTAAA. The constitutively expressed gene adenine phosphoribosyltransferase (APRT) was used as an internal control. The primers for APRT were 5’ primer GTGGGATCCCATCGGAAA and 3’ primer CTCTAGACGAGGTCAGCTCC, generating a 246-bp PCR product. The respective amplified products were subjected to electrophoresis in a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under a UV illuminator. The image was photographed, stored, and analyzed by a photo documentation system with Photo-Capt software (ETS Vilber-Lourmat, Marne VuVallee Cedex, France). Each band was quantified by calculating the ratio of target cDNA signal to the APRT control, and the mRNA expression was presented as a percentage of the APRT signal.

Western blot analysis of MAP kinases. The primary epithelial cells were exposed to NE in the presence or absence of inhibitors for various time intervals. At the end of treatment, the cells were lysed on ice in lysis buffer containing 50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.2 U/ml), leupeptin (0.5 μg/ml), and 1 mM Na3VO4. The protein concentration was determined by using a bicinchoninic acid protein assay (Pierce Chemicals, Rockford, IL) with bovine serum albumin as the standard. Equal amounts of total cell lysates (15 μg) were solubilized in a sample buffer by boiling for 10 min, fractionated on a 14% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was washed with 0.1% Tween 20 supplemented with Tris-buffered saline (TBS) and incubated in a blocking buffer (TBS containing 5% nonfat dry milk and 0.1% Tween 20). Anti-phospho-p44/42 (Th200/Tyr202) antibody or anti-phospho-p38 (Th118/Tyr115) antibody (Cell Signaling Technology, Beverly, MA) in a 1/1,000 dilution was then applied at 4°C overnight with gentle shaking. After being washed with TBS three times, blots were incubated with a 1/2,000 dilution of a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h. The protein bands were visualized using enhanced chemiluminescence (Amersham Pharma Biotech, Sunnyvale, CA) and autoradiography with Kodak X-ray film.

Statistics. Data were expressed as means ± SE. Statistical analysis for multiple comparisons was performed by ANOVA. Student’s t-test (for cytokine assay data) or the paired Student’s t-test (for the mRNA expression data) was employed. Differences at P < 0.05 were considered significant.

RESULTS

Effects of NE on PGE₂ release. The effect of NE upon the generation of PGE₂ from HAEC is shown in Fig. 1. In our preliminary experiments, subsequent to the addition of NE (0.1–80 μg/ml, either to the luminal or
submucosal side of the epithelial layers), most of the stimulated release of PGE₂ appeared to be in the direction of the submucosa (data not shown). No significant increase in PGE₂ production was observed when NE was <5 µg/ml. Cells incubated with NE (40 µg/ml) began to detach within 6 h of its addition. An increase in PGE₂ release was observed after NE (20 µg/ml) had been added for a period of 10 min (417 ± 49 vs. 164 ± 25 pg/ml; *P < 0.05, n = 4). The maximal concentration of PGE₂ (1,387 ± 130 vs. 418 ± 34 pg/ml, stimulated vs. control) was detected in the presence of NE (20 µg/ml) at 3 h of incubation. Under the light microscope, cell monolayers appeared to be intact following incubation with NE at the concentration ~20 µg/ml. The dye exclusion test using trypan blue indicated that cell viability was >98% at the end of each experiment.

Inhibition of NE-induced PGE₂ release. To suppress the NE-induced PGE₂ release, we treated HAEC with dexamethasone (1–100 µM), indomethacin (0.1–10 µM), or celecoxib (0.1–10 µM). NE-induced PGE₂ generation was abolished by coincubation of cells with dexamethasone (10 µM), indomethacin (1 µM), or celecoxib (1 µM) (Fig. 2). Dexamethasone, indomethacin, or celecoxib alone had no effect on PGE₂ release (data not shown). The induction of PGE₂ release required proteolytic activity. Preincubation of NE (20 µg/ml) with α₁-antitrypsin (200 µg/ml) blocked NE’s ability to increase PGE₂ release (data not shown). The induction of PGE₂ release required proteolytic activity. Preincubation of NE (20 µg/ml) with α₁-antitrypsin (200 µg/ml) blocked NE’s ability to increase PGE₂ release (data not shown).

COX-2 mRNA expression. To determine how PGE₂ synthesis was related to regulation of the amount of COX-2, a reverse transcription-polymerase chain reaction (RT-PCR) was employed. Although PGE₂ release increased 10 min after the addition of NE, a significant induction of COX-2 mRNA expression was detectable at only 1 h (Fig. 3A). After stimulation of HAEC with NE (20 µg/ml) for 3 h, the COX-2 mRNA expression relative to that for the APRT housekeeping gene was approximately eight times that for unstimulated cells (Fig. 3B). Dexamethasone (10 µM) inhibited the gene transcription for COX-2 to a substantial extent (Fig. 4).

**DISCUSSION**

Our findings have demonstrated that NE stimulates PGE₂ production through COX-2 upregulation and that the activation of p44/42 MAP kinases is involved. Stimulated, airway epithelium may play an important role in modulating airway inflammation rather than act as a passive barrier. The NE-induced bronchoprotective activities of bronchial epithelium may represent a natural defense mechanism of the airways to regulate inflammatory processes in chronic neutrophilic inflammation.

Within the range of concentrations that could be detected in patients with asthma or cystic fibrosis

**NE-induced p44/42 MAP kinase phosphorylation.** We further investigated whether NE induced PGE₂ release through MAP kinase phosphorylation, a step necessary for MAP kinase activation. This was confirmed by the detection of phosphorylated forms for MAP kinases by Western blot analysis using specific phospho-MAP kinase antibodies. After NE stimulation, p44/42 MAP kinases were rapidly phosphorylated, with the concentration of phosphorylated p44/42 MAP kinases peaking at 10 min and declining at 90 min (Fig. 5A). For resting cells, p44/42 phosphorylation was detectable, whereas no p38 phosphorylation was observed in either the presence or absence of NE stimulation (Fig. 5B). To determine the effect of UO126 on p44/42 MAP kinase activation, we examined the phosphorylation status of these enzymes. Coincubation of cells with NE and UO126 resulted in an inhibition of p44/42 MAP kinase phosphorylation in a dose-dependent pattern (Fig. 6).

**Effect of UO126 on NE-induced COX-2 expression and PGE₂ release.** We have observed that the NE-induced PGE₂ release from HAEC involved p44/42 MAP kinase activation and that the phosphorylation was abolished by UO126. Pretreatment of cells with UO126 (10 µM) caused a substantial suppression of COX-2 mRNA expression (Fig. 7A) and a complete inhibition of NE-induced PGE₂ production (Fig. 7B), which confirmed that p44/42 MAP kinase was involved in NE-induced PGE₂ release. UO126 alone did not affect the levels of PGE₂.

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**Fig. 1. Effect of neutrophil elastase (NE) on PGE₂ generation by human airway epithelial cells (HAEC).** PGE₂ level is determined in supernatants of epithelial cells incubated for 10 min, 30 min or 1, 3, 6, or 24 h with buffer alone or with various concentrations of NE at 1, 5, 10, or 20 µg/ml. Mean PGE₂ values (± SE) are shown for 4–6 experiments performed in duplicate. *P < 0.05, **P < 0.005 compared with cells incubated with buffer alone.

**Fig. 2. The inhibition of NE-induced PGE₂ release by cells coincubated with dexamethasone, indomethacin, or celecoxib.** Cells, stimulated by NE (10 µg/ml), were coincubated with dexamethasone (10 µM), indomethacin (1 µM), or celecoxib (1 µM) (Fig. 2). Dexamethasone, indomethacin, or celecoxib alone had no effect on PGE₂ release (data not shown). The induction of PGE₂ release required proteolytic activity. Preincubation of NE (20 µg/ml) with α₁-antitrypsin (200 µg/ml) blocked NE’s ability to increase PGE₂ release (data not shown). The induction of PGE₂ release required proteolytic activity. Preincubation of NE (20 µg/ml) with α₁-antitrypsin (200 µg/ml) blocked NE’s ability to increase PGE₂ release (data not shown).
(27 ± 11 vs. 466 ± 121 µg/ml) (6), NE (20 µg/ml) significantly increased PGE₂ release into the cell culture supernatants. This increase was abrogated by dexamethasone and nonsteroid anti-inflammatory drugs such as indomethacin and the selective COX-2 inhibitor celecoxib. To identify the upstream enzyme that is responsible for PGE₂ production, we used semi-quantitative RT-PCR to examine expression of COX-2. COX-1 is constitutively expressed, whereas COX-2 is highly inducible by a number of cytokines and is associated with inflammatory processes (21, 23). We observed that COX-1 expression appeared to be constant at a very low level and not inducible (data not shown). By contrast, COX-2 expression following NE stimulation rose in a time- and dose-dependent pattern, which was mimicked by subsequent PGE₂ release. Recent evidence has suggested that COX-2-dependent PGE₂ release may play a role in the resolution of inflammation (10). The transcription of COX-2 gene was completely blocked by dexamethasone, and this blockage resulted in substantial reduction of PGE₂ production. The mechanism and therapeutic effects of steroids for treatment of inflammatory airway disorders (such as asthma) appear to be well understood. However, the beneficial or deleterious consequences of dexamethasone resulting in the suppression of COX-2 expression and PGE₂ production remain to be elucidated.

At a concentration of >40 µg/ml, NE elicited cell detachment. The NE-induced shedding of airway epi-

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A: representative ethidium bromide-stained gels are shown for cells incubated with buffer alone or NE at a concentration of 5 (NE 5), 10 (NE 10), or 20 (NE 20) µg/ml. The PCR products were 769 bp in size for COX-2 and 246 bp for APRT. B: densitometry readings derived from PCR gels. The mean band density (± SE) relative to that of the APRT control is shown for 3 separate experiments. *P < 0.05, **P < 0.005 compared with cells incubated with buffer alone.
thelium is thought to be due to proteolytic cleavage of the extracellular matrix. This may result in goblet cell metaplasia and an impaired mucociliary clearance, which have been observed in several inflammatory lung diseases (11). Proteolytic activity was required for NE to induce PGE2 production as this activity was abolished by coincubation of cells with α1-antitrypsin.

The culture medium has some growth factors as described previously. However, PGE2 levels in the control groups did not change significantly within 24 h of incubation. The only difference between control and treatment groups was the presence of NE. It is unlikely that some protein, released following protease reaction, stimulates PGE2 production in an autocrine manner within a few minutes. It is possible that NE stimulates PGE2 production through activation of protease-activated receptors (PAR), especially PAR-2, which is specific for serine proteases such as NE. A blocking antibody for PAR-2 will need to be developed to clarify whether NE stimulates epithelial cells through the activation of PAR-2 or via other mechanisms that subsequently enhance phosphorylation of MAP kinases and COX-2 gene transcription.

It has been reported that IL-1β can induce PGE2 production in human bronchial epithelial cells (22) and gastric epithelial cells (8) through the activation of p38 and p44/42 MAP kinases and upregulation of COX-2. Proinflammatory mediators, such as TNF-α, IL-1α, and platelet-activating factor, can induce IL-8 expression within bronchial epithelial cells via a p38 MAP kinase-dependent pathway (19). To identify the mechanism of NE-induced PGE2 production, we have investigated the signal pathways including p38 and p44/42 MAP kinases. Our results have shown that p44/42, but not p38, MAP kinases were rapidly phosphorylated following NE stimulation, and this phosphorylation was inhibited by UO126. UO126, specific inhibitor of p44/42 MAP kinases, suppressed NE-induced COX-2 mRNA expression and PGE2 release. However, p38 phosphorylation was not observed even in the presence of NE stimulation. Rather than using bronchial epithelium-derived cell lines, cells employed in this study were primary epithelial cells explanted from human bronchus. The characteristics and responses are believed to be more authentic than those of cell lines. Our preliminary experiments have indicated that most of the stimulated release of PGE2 was in the direction of the submucosa (data not shown). This observation would appear to be consistent with a previous study suggesting that >95% of the PGE2 produced by dog trachea epithelium is released in the submucosal direction following eosinophil major basic protein stimulation (14). This provides the opportunity for epithelium-released PGE2 to influence the underlying tissues, such as nerves, smooth muscle, and inflammatory cells.

PGE2 possesses potential therapeutic properties in vitro and may elicit bronchodilation when introduced into asthmatic airways in vivo (24). PGE2, the so-called “epithelium-derived relaxing factor,” may play an important role in regulating airway tone. Airway epithelium removal prevented PGE2 production and thus increased the contractile response of smooth muscles evoked by acetylcholine, histamine, and PGF2α (1). Furthermore, PGE2 is able to inhibit the release of mediators from lung mast cells (26) and inhibit eosinophil chemotaxis and cytokine-stimulated eosinophil survival as well as

![Image](http://ajplung.physiology.org/)

**Fig. 6.** Effect of UO126 on p44/42 MAP kinase phosphorylation.

Cells were treated with UO126 (10 μM) alone, NE (20 μg/ml) alone, or with NE (20 μg/ml) and various concentrations of UO126 indicated. After incubation for 10 min, cells were harvested for Western blotting and subjected to analysis with specific antibodies to phosphorylated p44/42 (p44/42-p) or pan-p44/42 (p44/42) MAP kinases. Blots representative of 3 experiments are shown.

**Fig. 7.** Effect of UO126 on NE-induced COX-2 mRNA expression and PGE2 release.

Cells were treated with buffer (control) or NE at 10 (NE10) or 20 (NE20) μg/ml or with NE (20 μg/ml) and 10 μM UO126 (NE20 + UO10) for 3 h. RT-PCR was performed for analysis of COX-2 mRNA. A representative gel of 3 separate experiments is shown. B: supernatants were collected for PGE2 release measurement following incubation of cells with buffer (control) or UO126 (10 μM) alone or with NE (20 μg/ml) and 5 (NE + UO-5) or 10 (NE + UO-10) μM UO126 for 3 h. Mean values (± SE) are shown for 3 separate experiments. A significant inhibition (*P < 0.05) compared with cells treated with NE is indicated.
IL-2 production by T lymphocytes and IL-4-induced IgE production by B lymphocytes (2, 4, 25).

In summary, bronchial epithelium is an effector and regulator of airway inflammation and smooth muscle tone. The ways to enhance bronchoprotection by bronchial epithelium may be of great value in the future treatments of chronic inflammatory disorders of the airways.

DISCLOSURES

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