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Interleukin-1β–Induced Mucin Production in Human Airway Epithelium Is Mediated by Cyclooxygenase-2, Prostaglandin E_2 Receptors, and Cyclic AMP-Protein Kinase A Signaling

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ABSTRACT

We reported recently that interleukin (IL)-1β exposure resulted in a prolonged increase in MUC5AC mucin production in normal, well differentiated, human tracheobronchial epithelial (NHTBE) cell cultures, without significantly increasing MUC5AC mRNA (*Am J Physiol* **286:**L320–L330, 2004). The goal of the present study was to elucidate the signaling pathways involved in IL-1β–induced MUC5AC production. We found that IL-1β increased cyclooxygenase-2 (COX-2) mRNA expression and prostaglandin (PG) E_2 production and that the COX-2 inhibitor celecoxib suppressed IL-1β–induced MUC5AC production. Addition of exogenous PGE_2 to NHTBE cultures also increased MUC5AC production and IL-1β–induced Muc5ac hypersecretion in tracheas from wild-type but not from COX-2$-/-$ mice. NHTBE cells expressed all four E-prostanoid (EP) receptor subtypes and misoprostol, an EP2 and EP4 agonist, increased MUC5AC production, whereas sulprostone, an EP1 and EP3 agonist, did not. Furthermore, specific protein kinase A (PKA) inhibitors blocked IL-1β and PGE_2-induced MUC5AC production. However, neither inhibition of epidermal growth factor receptor (EGFR) activation with the tyrosine kinase inhibitor 4-(3-chloroanilino)-6,7-dimethoxyquinazoline HCl (AG-1478) or EGFR blocking antibody nor inhibition of extracellular signal-regulated kinase/P-38 mitogen activated protein kinases with specific inhibitors blocked IL-1β stimulation of MUC5AC mucin production. We also observed that tumor necrosis factor (TNF)-α, platelet activating factor (PAF), and lipopolysaccharide (LPS) induced COX-2 and increased MUC5AC production that was blocked by celecoxib, suggesting a common signaling pathway of inflammatory mediator-induced MUC5AC production in NHTBE cells. We conclude that the induction of MUC5AC by IL-1β, TNF-α, PAF, and LPS involves COX-2–generated PGE_2, activation of EP2 and/or EP4 receptor(s), and cAMP-PKA–mediated signaling.

Mucins are heavily glycosylated, high molecular weight glycoproteins produced by the epithelia of the respiratory, gastrointestinal, and reproductive tracts. They are responsible for the viscoelastic properties and hydrophilicity of secreted mucus and provide lubrication and protection of the epithelial lining (Rose and Gendler, 1997). The airways are constantly exposed to a variety of environmental toxicants, including particulate matter and microbial pathogens. Mucociliary clearance involves trapping inhaled particles and bacteria in the mucus layer and transporting them from the lung via the actions of ciliary beating. Dysregulation of any component of the mucociliary clearance mechanism can lead to airway damage. Increased production of mucus, which commonly occurs in diseases of the respiratory tract involving inflammation, can cause airway narrowing and obstruction, thus impeding airflow.

Thirteen mucin genes have been reported (Rose and Gendler, 1997). However, in airway secretions, the products of the *MUC5AC* and *MUC5B* genes seem to be the predominant polymeric mucins (Kirkham et al., 2002). In the adult human

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ABBREVIATIONS: IL, interleukin; COX, cyclooxygenase; PG, prostaglandin; EP, E prostanoid receptor; NHTBE, normal human tracheobronchial epithelial; PKA, protein kinase A; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TNF-α, tumor necrosis factor-α; PAF, platelet activating factor; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TGF-α, transforming growth factor-α; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1-pyrazole; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene; RT, reverse transcriptase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; EGFr, epidermal growth factor receptor; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; WT, wild type; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; AG-1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; FP, prostanoid F receptor.

airway, MUC5B mucin is mainly produced in mucous cells of the submucosal glands and in the glandular ducts, whereas MUC5AC mucin is considered to be the product of goblet cells lining the airway (Buisine et al., 1999). During the course of airway inflammatory disease, different cell types invade the airway, releasing a variety of cytokines, growth factors, eicosanoids, and free radicals, resulting in a marked increase in the number of goblet cells, an increase in MUC5AC mRNA, and overproduction of mucin (Fahy, 2001).

Many studies have been conducted to elucidate the role of various cytokines in mucus hypersecretion (for review, see Jeffery and Zhu, 2002). IL-1β is a proinflammatory cytokine that is secreted by immune cells in response to bacterial or viral challenges (Dinarello, 1997) and has been shown to play a role in airway diseases characterized by increased mucus production (Kadota et al., 1996). IL-1β regulates a variety of genes and gene products including the gene encoding the COX-2 enzyme that results in the production of bioactive prostaglandins (Smith et al., 2000). Prostaglandins (PGs) are lipid mediators that are involved in many physiological and pathophysiological processes, including inflammatory airway diseases (Sugimoto et al., 2000). PGE_2, one of the major PGs produced, exerts its biological effects by binding to specific cell surface receptors, designated EP (for E-prostanoid). Four different EP-receptors have been identified, named EP1 to EP4, and several splice variants of the EP3-receptor are known (Narumiya and Fitzgerald, 2001). The EP3 and EP4 receptors seem to be the major subtypes in lung tissue (Sugimoto et al., 2000). Activation of EP receptors leads to well defined alterations in intracellular calcium and cAMP concentrations; e.g., cAMP is raised by EP2 and EP4, whereas intracellular calcium is increased after EP1 and EP3 activation. In addition, activation of the EP3 receptor may also decrease cAMP (Sugimoto et al., 2000). Thus, many different physiological processes are regulated by PGE_2 activation of specific receptor subtypes.

Recent studies from our laboratory showed that IL-1β increased MUC5AC (but not MUC5B) mucin production and coordinately increased fluid secretion in well differentiated, normal human tracheobronchial epithelial (NHTBE) cell cultures (Gray et al., 2004). In these studies, it was also found that IL-1β did not significantly increase *MUC5AC* mRNA levels but caused a prolonged, substantial increase in MUC5AC mucin production. We would like to emphasize that the present study as well as previous studies were not designed to measure the secretagogue effects of IL-1β and other inflammatory mediators; rather, they were designed to measure cytokine-stimulated sustained production and release of MUC5AC mucin.

In the present study, NHTBE cell cultures were exposed to IL-1β and the signaling pathway between COX-2 induction and MUC5AC production was investigated. While this work was in progress, Kim et al. (2002), using the human, tumorigenic lung cell line, NCI H-292, described the IL-1β signaling pathway leading to COX-2 induction and subsequent production of MUC5AC. Aside from our work, emphasizing events downstream of COX-2, and that of Kim et al. (2002), focusing on events upstream of COX-2, it seems that significant differences between NHTBE cells and NCI H-292 cells also exist.

Our studies demonstrate that in cultured NHTBE cells, IL-1β stimulates MUC5AC production by a COX2/PGE_2-dependent mechanism. We show that NHTBE cells express the four known EP receptors and that the EP2/EP4 receptors (but not the EP/EP3 receptors), cAMP, and PKA are involved in the IL-1β–stimulated MUC5AC production. An important finding of this study is that the EGF receptor, which has been reported to play a critical role in the up-regulation of MUC5AC expression induced by several inflammatory mediators (Nadel, 2001), is not involved in IL-1β–stimulated MUC5AC mucin production of NHTBE cells. This finding is further supported by the fact that ERK and p38 MAP kinase inhibitors did not block the IL-1β effect. Finally, we present evidence that TNF-α, PAF, and LPS also stimulate MUC5AC mucin production by a COX2/PGE_2-dependent mechanism.

Materials and Methods

Materials

TGF-α, IL-1β, TNF-α, PAF, IL-4, IL-9, IL-13, IFN-γ, and LPS were purchased from R&D Systems (Minneapolis, MN). Forskolin and indomethacin were obtained from Sigma (St Louis, MO) and PGE_2, $PGF_{2\alpha}$, misoprostol, sulprostone, and SC-560 were obtained from Cayman (Ann Arbor, MI), and celecoxib was purchased from LKT Labs (St. Paul, MN). AG-1478 and the ERK inhibitors PD98059 and U0126 were purchased from Calbiochem (LaJolla, CA). Anti-EGFR blocking antibody and anti-EGFR and anti-phospho EGFR antibodies were purchased from Upstate Biotechnology (Lake Placid, NY).

Cell Culture

Air-Liquid Interface Cultures. Passage 2, NHTBE cells (from two independent donors were purchased from Clonetics Corp. (LaJolla, CA) were seeded onto 24-mm, uncoated, semipermeable, Transwell clear membranes (Corning Costar, Cambridge, MA) at 2×10^4 cells/cm^2 in serum-free, hormone, and growth factor supplemented medium (all media supplements were purchased from Sigma, St. Louis, MO), containing all-*trans*-retinoic acid (Sigma). For the complete medium formulation, see Gray et al. (1996). Cultures were grown submerged for the first 7 days, at which time the air-liquid interface was created. Media were changed daily, and the cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Thirty-five-day-old cultures, with a fully developed mucociliary phenotype, were used in all experiments. Cultures were exposed basally to either IL-1β (2.5 ng/ml; this concentration was found to yield maximal stimulation of MUC5AC production; see Gray et al., 2004) or to prostaglandins, cytokines, inhibitors of COX, PKA, ERK, and P-38 at concentrations chosen based on preliminary dose response studies.

Immunodetection and Quantitation of Mucins. Quantitation of MUC5AC and MUC5B produced by NHTBE cultures and the immunoblotting methods used to detect them have been previously reported (Gray et al., 1996, 2004; Thornton et al., 2000). To monitor MUC5AC and MUC5B mucin production, the apical surfaces of the cultures (triplicate cultures per group) were vigorously washed with PBS at indicated times after treatment. The mucin collected in the wash was assayed by dot blotting as described previously (Gray et al., 1996, 2004). Diluted apical secretions were applied to nitrocellulose membranes, which were incubated with 1:250 dilutions of either the mouse monoclonal anti-MUC5AC antibody 45M1 (Neomarkers, Freemont) or the rabbit polyclonal anti-MUC5B antibody (a generous gift from Dr. David Thornton, Manchester, UK), followed by reaction with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. The signal was detected by chemiluminescence (ECL kit; Amersham Biosciences, Buckinghamshire, UK) and the levels of MUC5AC and MUC5B reactivity were measured densitometrically. Mucin levels per culture were calculated (optical density $\times$ dilution factor of the sample) and the data shown as -fold increase.

The mouse monoclonal antibody 45M1 (Neomarkers) has been shown to react with gastric MUC5AC (Nordham et al., 2002), MUC5AC produced by NCI H-292 cells (Koo et al., 2002), and normal airway epithelia (Vermeer et al., 2003; Gray et al., 2004). We confirmed the specificity of the Neomarkers' antibody by comparing its reactivity with that of the MAN-5AC-1 (Thornton et al., 1996) against human airway MUC5AC mucin. We found that the 45M1 and MAN-5AC-1 antibodies have quantitatively very similar reactivity with purified MUC5AC mucin as well as with secretions from control and IL-1β–stimulated NHTBE cultures. Furthermore, neither antibody cross-reacted with purified MUC5B.

COX-1 and COX-2 mRNA Levels

Detailed methods to detect and quantitate COX-1 and COX-2 mRNA levels by RT-PCR have been reported previously (Subbarayan et al., 2001). In brief, total RNA was isolated from separate, triplicate cultures. Oligonucleotide primer pairs used for COX-1 were 5′ CAATGCCACCTTCATCCGA-3′ (forward) and 5′-GAGCCGCAGTTGATACTGA-3′ (reverse) (Funk et al., 1991), which generated a 430-base pair product. COX-2 primer pairs were 5′-CGAGGTGTATGTATGAGTGTG-3′ (forward) and 5′-TCTAGCCAGAGTTTCACCGTA-3′ (reverse) (Hla and Nielson, 1992) and generated a 540-base pair product. PCRs were performed within the linear amplification range, and the amplification efficiency was verified by determining the amount of cDNA produced after various numbers of PCR cycles. The normalization RNA control used was β2M (BD Biosciences Clontech, Palo Alto, CA). Specific amplification of COX-1 and COX-2 was confirmed by sequencing. PCR products were separated on 2% agarose gels (containing ethidium bromide), and the resulting bands were analyzed using a digital imaging system (Alpha Innotech, San Eleandro, CA). The ratios of the signal intensities of the cDNAs from cytokine compared with control cultures were determined.

Detection of EP and FP mRNA Expression

Total RNA was isolated from triplicate, individual cultures. RT-PCR was performed using an ABI 7700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA) and the PCR core reagent kit (Applied Biosystems) in accordance with the manufacturer's instructions. PCR primers were designed using Primer Express software (Applied Biosystems Inc.): the sequences for primers are shown in Table 1. Reverse transcription reactions and PCR were performed in the same tube according to methods described previously (Hamadeh et al., 2002). All reactions were performed in duplicate, and at least two RT-PCR runs were made. $MgCl_2$ (4 mM), deoxy-nucleotide triphosphates with dUTP (0.8 mM), buffer (1×), total RNA (50 ng in 2.5 μl of water), forward and reverse primers (20 μM), RNasin (0.4 μl), Amplitaq Gold DNA polymerase (0.25 μl), and reverse transcriptase (0.25 μl; Superscript, Invitrogen, Carlsbad, CA) were added to the tube, and the volume was brought to 50 μl with water. The reverse transcriptase reactions were carried out for 30 min at 48°C followed by 10 min at 95°C to activate Amplitaq Gold. PCRs were performed at 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. PCR products were resolved by gel electrophoresis and yielded the predicted sizes. The specificity of the amplified products was monitored by melting curves as well as by DNA sequencing.

TABLE 1
Primers

EP1	
Forward	5′-ATGGTGGGCCAGCTTGTC-3′
Reverse	5′-GCCACCAACACCAGCATTG-3′
EP2	
Forward	5′-CGACCTTCTACACGCTGGTATG-3′
Reverse	5′-CCGGGCTCACCAACAAAGT-3′
EP3	
Forward	5′-GGGCCTGATGGAAGGTGTTT-3′
Reverse	5′-TCTATCATGAGAACTGCACCAAGTC-3′
EP4	
Forward	5′-CGACCTTCTACACGCTGGTATG-3′
Reverse	5′-CCGGCTCACCAACAAAGT-3′
Trfr[a]	
Forward	5′-CAGGAACCGAGTCTCCAGTGAG-3′
Reverse	5′-GGTGAAGTCTGTGCTGTCCAGTT-3′

[a] Ordonez et al. (2001).

PGE_2 Determinations

Cultures were treated for 24 h with IL-1β (0 or 2.5 ng/ml). After the treatment, the basal media were collected (triplicate wells per treatment group) and the levels of PGE_2 were determined by standard radio-immune assay (Biotrak radioimmunoassy kit; Amersham Biosciences).

Ex Vivo IL-1β and PGE_2 Treatments of Mouse Tracheas

COX-2–deficient mice were maintained on an out-bred genetic background of C57BL/6J and 129/Ola (Morham et al., 1995). Wild-type and COX-2-deficient mice were generated by mating COX-2 heterozygous mutant female and male mice. Mice were genotyped by PCR as described previously (Loftin et al., 2001). All experiments were conducted in accordance with the National Institute of Environmental Health Sciences Animal Care and Use Program. Animals were euthanized by CO_2 asphyxiation, and tracheas (larynx to bifurcation) were removed from the thorax after inserting a polyethylene cannula in the laryngeal end. After rinsing with PBS, the tracheas (three or four tracheas per treatment group) were filled with 50 μl of either IL-1β (2.5 ng/ml), PGE_2 (150 nM), or control media; the ends of the tracheas were ligated and the tracheas were incubated for 24 h in a humidified atmosphere of 5% CO_2 in air at 37°C. After incubation, each trachea was flushed with PBS (0.5 ml), and the levels of Muc5ac mucin in the rinse were determined using the human anti-MUC5AC antibody 45M1, which cross-reacts with mouse Muc5ac mucin.

Immunodetection of EGFR

NHTBE cell cultures were deprived of EGF and bovine pituitary extract for 24 h before a 10-min treatment with either TGF-α (10 ng/ml) or IL-1β (2.5 ng/ml). In EGFR inhibition studies, after 24 h EGF deprivation, indicated cultures were pretreated for 1 h with EGFR blocking antibody (4 μg/ml) and then stimulated with TGF-α for 10 min. After treatments, cultures were washed with PBS, resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml each of apoprotinin, leupeptin, and pepstatin) and centrifuged at 13,000 rpm. Fifteen microliters of lysate and 5 μl of loading buffer (Invitrogen) per sample was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blocked for 2 h using 5% milk in Tris-buffered saline/Tween 20. The blot was incubated overnight at 4° with either anti-phospho-EGFR or anti-EGFR polyclonal antibodies at concentrations recommended by the supplier, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000 dilution). The signal was detected by enhanced chemiluminescence and quantitated by densitometric scanning.

ERK and P-38 MAP Kinase Inhibition Studies

NHTBE cell cultures were deprived of EGF and bovine pituitary extract for 24 h. Cultures were pretreated for 1 h with PD98059 (50 μM), U0126 (25 μM), or SB203580 (50 μM). The levels of MUC5AC mucin were determined after an 8-h cotreatment with IL-1β (2.5 ng/ml).

Statistical Analysis

Data were analyzed using analysis of variance, taking into account the variability caused by multiple experiments and, where appropriate, that caused by replicate number. All analyses were performed using PROC GLM in the statistical software package SAS

(version 8.2; SAS Institute, Cary, NC), and all multiple comparisons were performed using the LSMEANS option available within PROC GLM. To ensure homoscedasticity of variances (i.e., homogeneity of variances), we log-transformed the data before appealing to analysis of variance. The mean data reported are based on the least square mean provided by the LSMEAN option and not the raw sample means.

Results

IL-1β Induces MUC5AC Production, COX-2 Expression, and PGE_2 Production. Thirty-five-day-old NHTBE cultures with a fully developed, mucociliary phenotype were used in all experiments. Such cultures contain 5 to 10% goblet cells identified by their typical morphology as well as by Alcian Blue-Periodic acid/Schiff-positive staining (Gray et al., 2004). We showed previously that IL-1β stimulated sustained (72 h) MUC5AC mucin production without significantly increasing MUC5AC mRNA levels (Gray et al., 2004). It has been reported that IL-1β induces expression of *COX-2* both in vivo and in a variety of in vitro culture systems (Smith et al., 2000). To examine the possible role of *COX-2* and its products in IL-1β induction of mucin production, cultures were exposed to IL-1β (0 or 2.5 ng/ml) and the levels of MUC5AC and MUC5B mucins, COX-1 and -2 mRNAs, and secreted PGE_2 were measured at different times after treatment. As seen in Fig. 1A, MUC5AC mucin was significantly increased in 8 h, reaching a peak at 24 h after IL-1β treatment, whereas MUC5B mucin levels were not significantly altered. IL-1β treatment resulted in a 2- to 3-fold induction of COX-2 mRNA levels (Fig. 1, B and C), which seemed to peak by 4 h and decrease thereafter. COX-1 mRNA levels were unchanged after 24 h of treatment. The levels of secreted PGE_2 present in the basal media after 24 h of IL-1β treatment were increased approximately 30-fold compared with the levels in control cultures (Fig. 1D).

COX-2 Inhibitors Block IL-1β Induced MUC5AC Mucin Production. To determine whether inhibition of the COX enzymes abolishes the IL-1β stimulation of MUC5AC mucin, cultures were treated for 24 h with IL-1β alone or in combination with indomethacin (20 μM), a nonselective COX-1 and -2 inhibitor, celecoxib (1 and 10 μM), a COX-2–selective inhibitor, or SC-560 (10 μM), a COX-1–selective inhibitor. As seen in Fig. 2, IL-1β increased MUC5AC mucin levels >2-fold. Indomethacin and celecoxib inhibited IL-1β–induced MUC5AC production whereas the COX-1–selective inhibitor SC-560 had no effect. The addition of the inhibitors to cultures not stimulated with IL-1β had no effect on MUC5AC mucin levels (data not shown). These data suggest that IL-1β–induced MUC5AC overproduction is dependent on the induction of COX-2 and increased PGE_2 production.

Dose-Dependent Induction of MUC5AC Production by PGE_2. When a series of PGs, including PGE_2, $PGF_{2\alpha}$, PGD_2, and PGI (all at 1500 nM) were tested for their ability to stimulate MUC5AC mucin production, only PGE_2 and $PGF_{2\alpha}$ treatment resulted in increased levels of MUC5AC (data not shown). To determine whether physiological concentrations of PGE_2 and $PGF_{2\alpha}$ were capable of inducing MUC5AC mucin production, cultures were treated for 24 h with various concentrations of PGE_2 or $PGF_{2\alpha}$. As seen in Fig. 3A, PGE_2 stimulated MUC5AC mucin levels in a dose-dependent manner. The first significant increase over control levels was observed at 0.015 nM. In contrast, $PGF_{2\alpha}$ increased MUC5AC mucin only at the highest concentration (1500 nM) tested. Higher concentrations of $PGF_{2\alpha}$ were not tested because they were not considered physiologically relevant.

The addition of celecoxib to PGE_2-treated cultures did not suppress MUC5AC mucin levels (Fig. 3B) indicating that inhibition of IL-1β–induced MUC5AC production by celecoxib resulted from the specific inhibition of COX-2 dependent PGE_2 synthesis.

IL-1β Does Not Induce Muc5ac Mucin Production in Tracheas of COX-2–Deficient Mice. To substantiate our findings that IL-1β–induced MUC5AC mucin production was

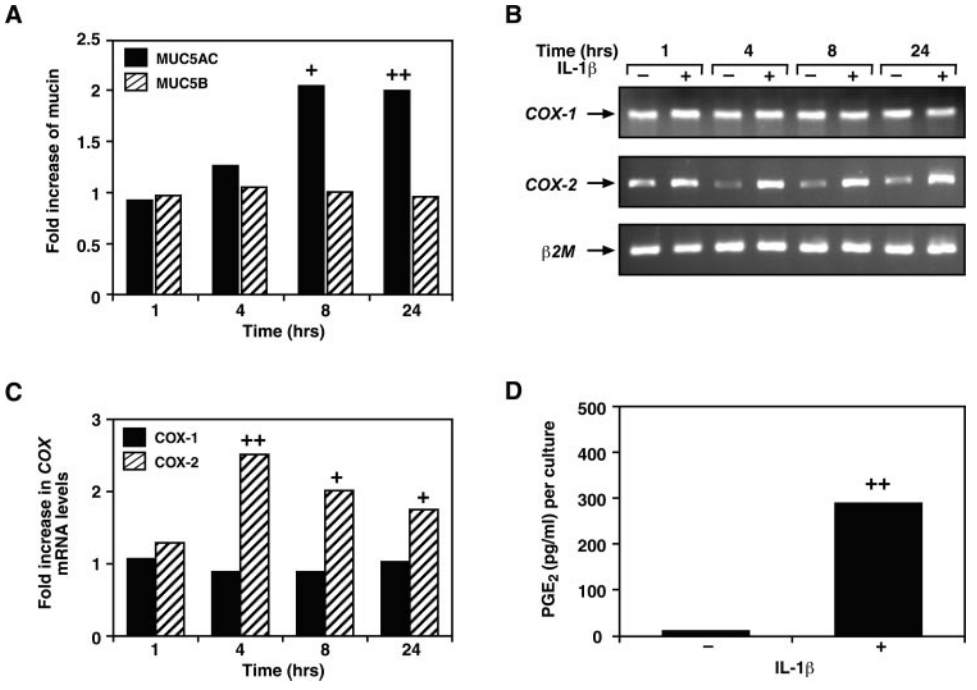

Fig. 1. The effect of IL-1β treatment on mucin production, COX-1 and COX-2 mRNA levels, and PGE_2 production. A, cultures were treated with IL-1β (2.5 ng/ml) or control media for 24 h, and the levels of MUC5AC (■) and MUC5B (▨) mucins were determined. The data are reported as the mean fold increase in mucin compared with sham-treated control cultures obtained from three independent experiments. +, $p < 0.01$; ++, $p < 0.0001$. In the same experiment, COX-1 and COX-2 mRNA levels were determined by RT-PCR (B) and quantitated by densitometric scanning (C). The data represent COX-1 (■) and COX-2 (▨) mRNA levels in the IL-1β–treated cultures compared with control cultures from three independent experiments. +, $p < 0.01$; ++, $p < 0.0001$. D, secreted levels of PGE_2 (triplicate cultures per treatment group) after IL-1β treatment were determined. The data are presented as the mean levels of PGE_2 (pg/ml) obtained from three independent experiments. ++, statistical significance at $p < 0.0001$.

mediated by COX-2 metabolites, tracheas were obtained from COX-2–deficient [COX-2−/−) and wild-type (WT) mice] and were filled with control media or media containing either IL-1β (2.5 ng/ml) or PGE_2 (150 nM). After a 24-h incubation, the contents of the tracheas were collected and the amount of luminal Muc5ac mucin was determined. We observed that amounts of Muc5ac mucin in tracheas of WT and COX-2−/− mice instilled with media alone were nearly identical (data not shown). However, as shown in Fig. 4, IL-1β significantly increased the amount of Muc5ac mucin in tracheas of WT but not in tracheas from COX-2−/− mice. In contrast, instillation of PGE_2 induced similar increases of Muc5ac mucin in tracheas from both WT and COX-2−/− mice. These findings support the results obtained with NHTBE cultures that COX-2–generated PGE_2 mediates IL-1β–induced MUC5AC mucin production.

Agonist of EP2/EP4 but Not EP1/EP3 Induces MUC5AC Production. PGE_2 exerts its action through four distinct membrane-associated G-protein-coupled receptors termed EP1, EP2, EP3, and EP4 (Narumiya and Fitzgerald, 2001). To determine whether EPs were expressed by NHTBE cells, total RNA was collected, and the presence of receptor mRNAs was detected by RT-PCR. As seen in Fig. 5A, all four EP receptor subtypes as well as the $PGF_{2\alpha}$ receptor (FP) were expressed.

To identify the PGE_2 receptors involved in PGE_2-mediated induction of MUC5AC mucin production, NHTBE cultures were treated for 24 h with the EP2/EP4-selective agonist, misoprostol, or the EP1/EP3-selective agonist sulprostone at concentrations ranging from 0.5 to 5000 nM. Misoprostol stimulated MUC5AC mucin levels in a dose-dependent manner, whereas sulprostone, over the concentration range tested, had no significant effect (Fig. 5B). This suggests that PGE_2 induced MUC5AC mucin production occurs via activation of EP2 and/or EP4 receptor signaling.

IL-1β–Induced MUC5AC Mucin Production Involves cAMP-PKA and Not EGFR Signaling. Biological effects that result from the binding of PGE_2 to EP2/EP4 receptors involve downstream cAMP-dependent PKA signaling pathways (Narumiya and Fitzgerald, 2001). We therefore examined the effect of forskolin, an inducer of cAMP on mucin production and of two inhibitors of PKA on IL-1β and PGE_2-induced MUC5AC mucin overproduction. As shown in Fig. 6A, after a 24-h treatment, forskolin increased MUC5AC mucin production to the same degree as IL-1β, indicating that adenylate cyclase-cAMP activation stimulated MUC5AC mucin production. We also found that after cotreatment of cultures with IL-1β and either of the PKA antagonists H89 or myristoylated PKA inhibitor (both at 100 nM) for 8 h (a time when IL-1β induced MUC5AC ~2-fold

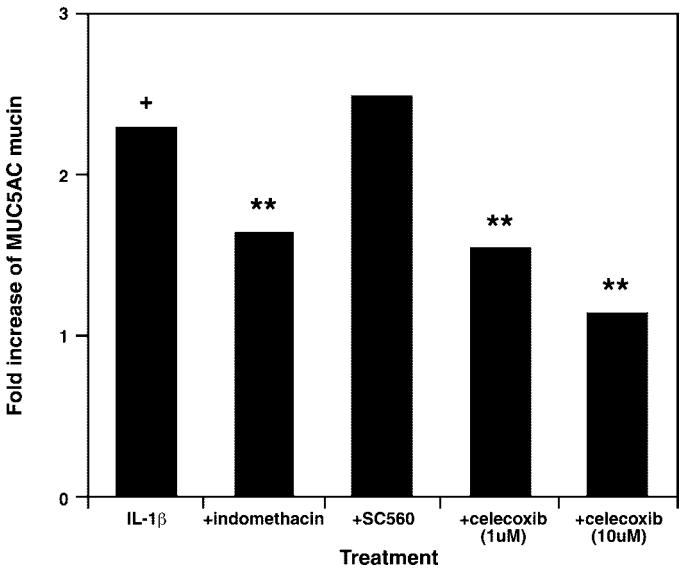

Fig. 2. The effect of COX inhibitors on IL-1β–stimulated MUC5AC mucin production. MUC5AC mucin levels (triplicate cultures per treatment group) were determined in untreated cultures or after 24 h treatment with either IL-1β (0 or 2.5 ng/ml) alone or IL-1β together with either indomethacin (20 mM), SC-560 (10 mM), or celecoxib (1 or 10 mM). The data from three independent experiments are reported as the mean fold change of MUC5AC mucin levels in control cultures compared with IL-1β treatment alone or IL1β plus cotreatment with COX inhibitors. +, statistically significant stimulation ($p < 0.0001$); **, significant inhibition ($p < 0.0001$) of IL-1β–induced MUC5AC mucin production.

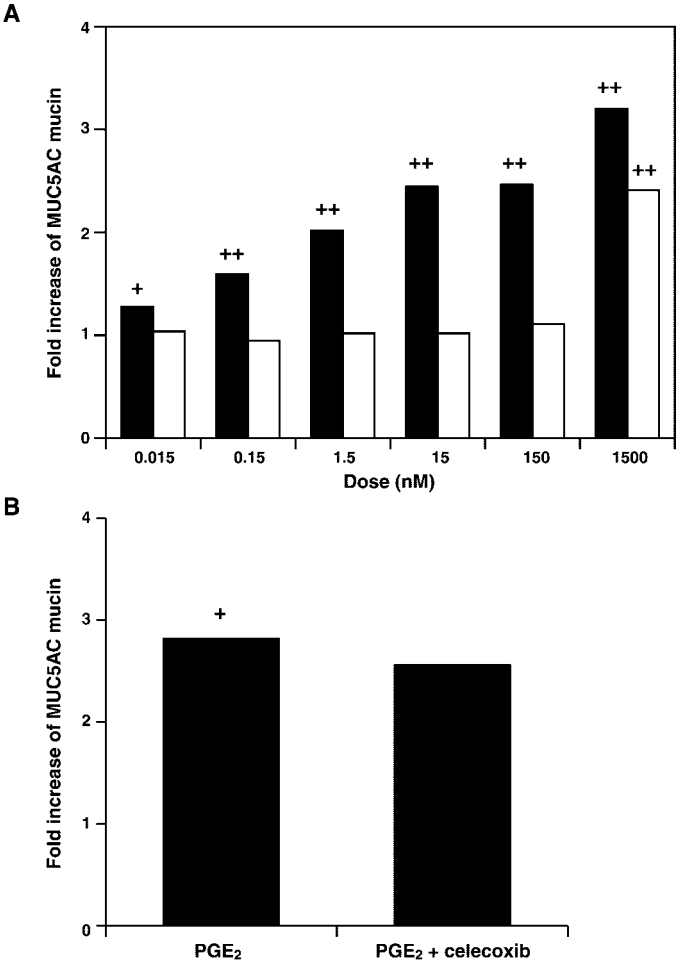

Fig. 3. Effects of PGE_2 and $PGF_{2\alpha}$ on MUC5AC production. A, cultures were exposed to the indicated concentrations of PGE_2 (■) or $PGF_{2\alpha}$ (□) for 24 h (triplicate cultures per condition per experiment), and the levels of MUC5AC mucin were determined. The data are expressed as the mean fold increase of MUC5AC mucin from three independent experiments in PG-treated cultures compared with untreated control cultures. +, statistically significant stimulation of MUC5AC production ($p < 0.001$); ++, $p < 0.0001$. B, cultures were treated for 24 h with PGE_2 (0 or 150 nM) alone or with celecoxib. The data from three independent experiments are reported as mean fold increase of MUC5AC in groups treated with PGE_2 and PGE_2 + celecoxib compared with control cultures. ++, statistically significant increases of MUC5AC mucin production ($p < 0.0001$). There was no significant inhibition of MUC5AC mucin in the celecoxib-cotreated cultures.

above control levels; see Fig. 1A), resulted in inhibition of IL-1β–stimulated MUC5AC production (Fig. 6B). As seen in Fig. 6C, mPKi also inhibited PGE_2-induced MUC5AC mucin production. Taken together, these findings indicate that cAMP and PKA signaling are involved in the stimulation of MUC5AC production by IL-1β and PGE_2.

A number of recent studies using the neoplastically transformed cell line NCI H-292 suggest that EGFR-mediated events play a crucial role in the stimulation of MUC5AC overproduction by inflammatory mediators as well as bacterial products (for review, see Nadel, 2001). Therefore, we decided to determine whether the EGFR plays a similar role in IL-1β–induced MUC5AC mucin production in NHTBE cultures. EGFR ligands were removed from NHTBE culture media 24 h before treatment with graded amounts of TGF-α (0.5–100 ng/ml), an EGFR agonist, or PGE_2 (150 nM, used as a positive control). As seen in Fig. 7A, after a 24-h treatment, TGF-α did not cause increased MUC5AC production in NHTBE cultures, whereas PGE_2 did. Blocking EGFR signaling in NHTBE cells with either the tyrosine kinase inhibitor AG-1478 (250 nM) or an anti-EGFR (4 μg/ml) blocking antibody did not suppress the stimulation of MUC5AC by IL-1β or PGE_2 (Table 2). However, the anti-EGFR blocking antibody inhibited TGF-α–induced EGFR phosphorylation (Fig. 7B), indicating that EGFR is functional in NHTBE cultures. We also found that IL-1β treatment did not result in EGFR activation, whereas TGF-α treatment did (Fig. 7C). These data indicate that IL-1β/PGE_2 stimulation of MUC5AC mucin production by NHTBE cells does not require EGFR signaling.

ERK and P-38 MAPK, downstream mediators of EGFR signaling (Rice et al., 2002), have been implicated in IL-1β–induced MUC5AC induction in NCI H-292 cells (Kim et al., 2002). To determine whether this was also the case in NHTBE cells, after EGFR ligand deprivation, cultures were pretreated for 1 h with either PD98059 (50 μM) or U1026 (25 μM), both inhibitors of ERK signaling, or with SB203580 (20 μM), a P-38 MAPK inhibitor. The cultures were then co-treated for 8 h with IL-1β. As seen in Table 3, neither the ERK nor the P-38 MAPK inhibitors blocked IL-1β–induced MUC5AC mucin production. These data indicate that in NHTBE cultures, IL-1β stimulation of MUC5AC mucin production does not involve ERK or P-38 MAPK signaling as was described for the NCI H-292 cell line.

TNF-α, LPS, and PAF Stimulation of MUC5AC Production Involves Induction of COX-2. To determine whether other inflammatory mediators implicated in airway inflammation and mucus hypersecretion can also induce MUC5AC mucin, NHTBE cultures were treated for 24 h with either IL-1β (2.5 ng/ml), TNF-α (10 ng/ml), LPS (10 μg/ml), PAF (10 nM), or IL-4, IL-9, IL-13, and IFN-γ (all at concentrations from 1–100 ng/ml). We found that only IL-1β, TNF-α, PAF, and LPS significantly increased MUC5AC, but not MUC5B, production (Fig. 8A). TNF-α, PAF, and LPS also increased COX-2 mRNA levels to the same extent as IL-1β (Fig. 8B), and celecoxib blocked the induction of MUC5AC

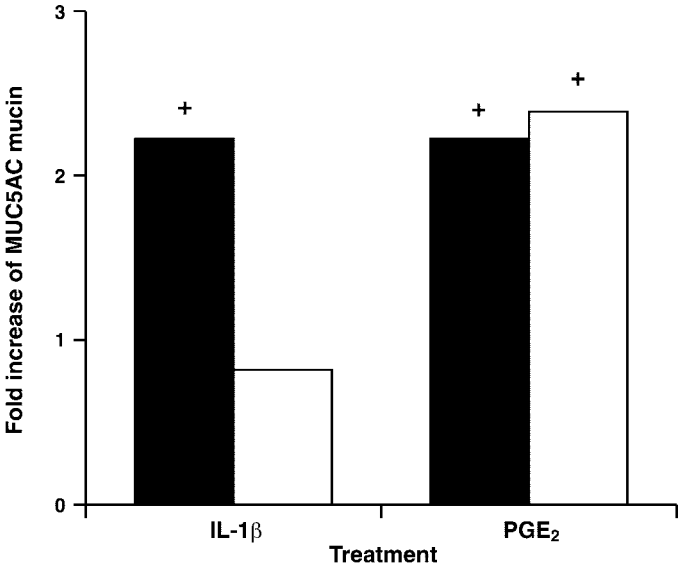

Fig. 4. The effect of IL-1β and PGE_2 on Muc5ac mucin production by tracheas from wild-type and COX-2–deficient mice. The experiment was repeated twice. Tracheas (three tracheas per treatment group) obtained from WT (■) and COX-2−/− (□) mice were filled with media alone or with media containing either IL-1β (2.5 ng/ml) or PGE_2 (150 nM). After 24-h treatment, the intratracheal levels of Muc5ac mucin were determined. The data are reported as mean fold increase of Muc5ac mucin from the two independent experiments in IL-1β- or PGE_2-treated tracheas compared with the respective controls. +, statistically significant increases over sham-treated control cultures ($p < 0.0001$).

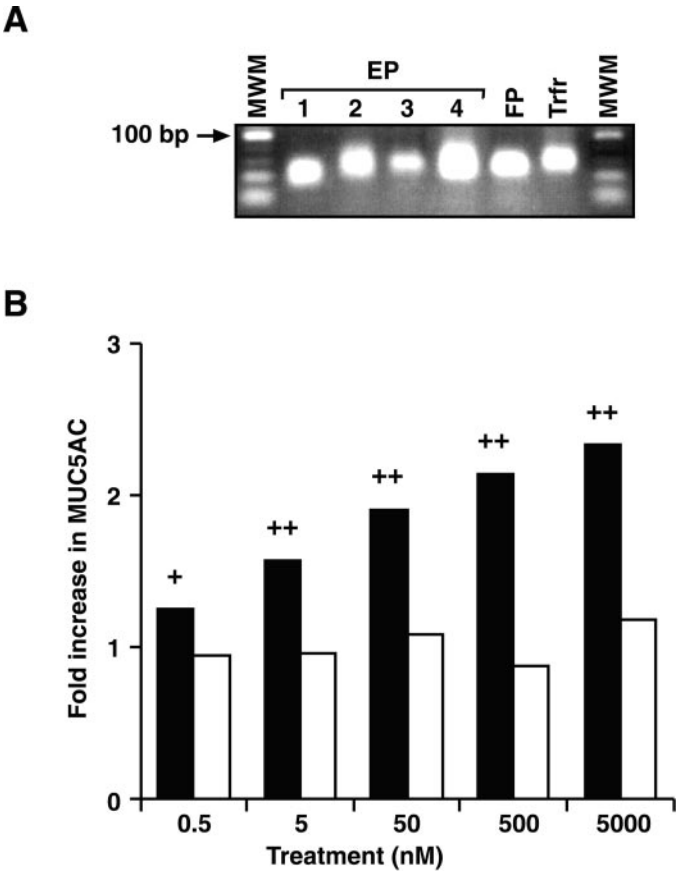

Fig. 5. Prostanoid receptor expression and the effect of EP receptor agonists on MUC5AC production. A, the presence of EP and FP mRNAs was detected by RT-PCR and the products visualized on an ethidium stained gel. Lanes 1 and 8, molecular weight markers (MWM); lanes 2 to 5, EP1–4, respectively; lane 6, FP; lane 7, *Trfr*. B, MUC5AC mucin levels (triplicate cultures per treatment group) were determined after 24-h treatments with the indicated concentrations of misoprostol (■) or sulprostone (□). The data are reported from the two independent experiments as mean fold increase of MUC5AC mucin in treated cultures compared with sham-treated control cultures. +, statistical significance at $p < 0.001$; ++, $p < 0.0001$.

mucin by all four mediators (Fig. 8C). None of the other mediators, at the concentrations tested, increased either MUC5AC or MUC5B mucin levels or COX-2 mRNA levels (data not shown). These data indicate that the stimulation of MUC5AC production by IL-1β, as well as by TNF-α, PAF, and LPS, is dependent on the induction of COX-2.

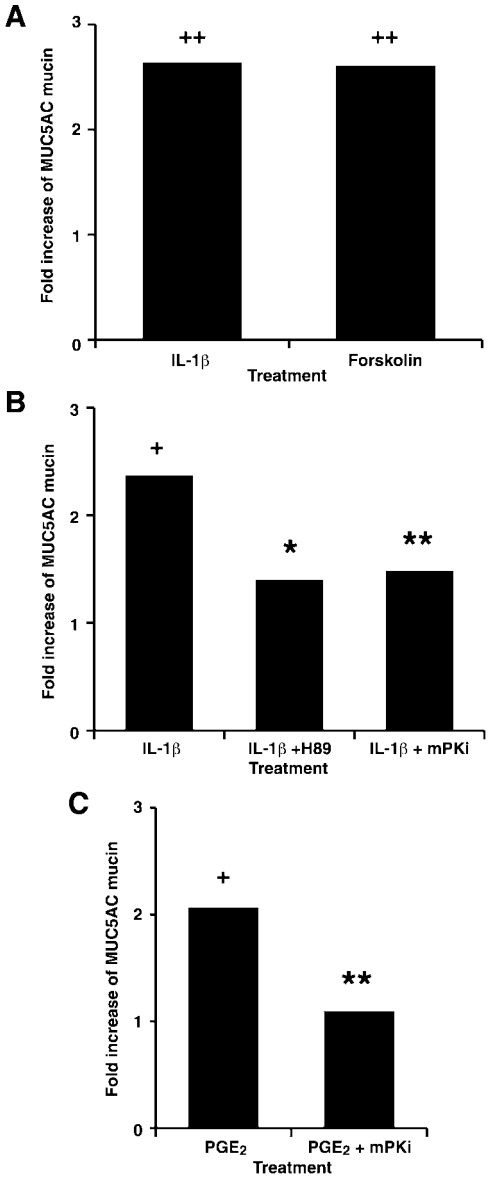

Fig. 6. The role of PKA signaling in the stimulation of MUC5AC mucin production. A, cultures were treated for 24 h (triplicate cultures per treatment group) with either IL-1β (0 or 2.5 ng/ml) or forskolin (20 μM), and the levels of MUC5AC mucin were determined. The data from the three independent experiments are reported as the mean fold increase of MUC5AC in treated groups compared with controls. ++, statistically significant increase ($p < 0.0001$). B, cultures (triplicate cultures per group) were treated for 8 h with IL-1β (0 or 2.5 ng/ml) alone or IL-1β together with either H-89 (100 nM) or myristoylated PKA inhibitor (mPKi; 100 nM), and the levels of MUC5AC mucin were determined. The data from three independent experiments are reported as the mean fold change of MUC5AC in IL-1β–treated cultures and those cotreated with PKA inhibitors compared with controls. +, statistically significant stimulation of MUC5AC mucin production; *, statistically significant inhibition of IL-1β stimulation ($p < 0.001$). C, after an 8-h treatment (triplicate cultures per condition) with PGE_2 (0 or 150 nM) alone or cotreatment with myristoylated PKA inhibitor (mPKi; 100 nM), the levels of MUC5AC were determined. The data from two independent experiments are reported as the mean fold increase of MUC5AC mucin production compared with controls. +, statistically significant stimulation ($p < 0.0001$); **, statistically significant inhibition of stimulation ($p < 0.001$).

Discussion

Mucus hypersecretion is a common feature of many airway inflammatory diseases, including asthma and chronic bron-

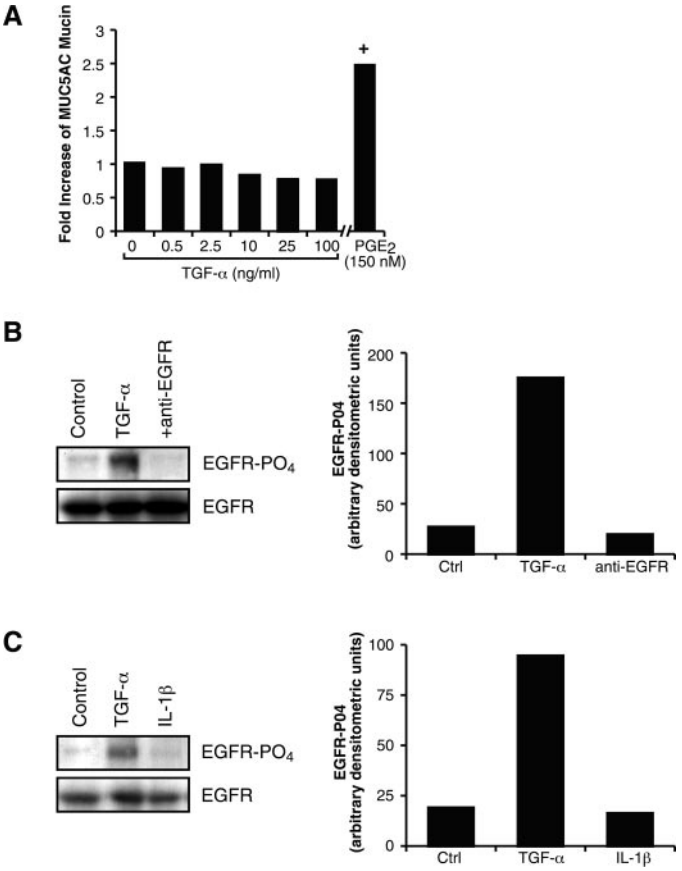

Fig. 7. The role of EGFR activation on induced MUC5AC mucin production. A, after removal of EGFR ligands from NHTBE culture media for 24 h, graded amounts (0.5–100 ng/ml) of TGF-α, an EGFR agonist, or PGE_2 (150 nM, positive control) were added to the cultures for a 24-h period. The data from three independent experiments are reported as the mean fold-increase of MUC5AC in treated groups compared with controls. +, statistically significant stimulation ($p < 0.001$). B, cultures were pretreated for 1 h with either media alone or media containing anti-EGFR blocking antibody (4 μg/ml) followed by cotreatment with TGF-α (10 ng/ml) for 10 min. Cell lysates were harvested, and EGFR phosphorylation was determined by Western blotting (left) and quantitated by densitometry (right). C, Western blots depict EGFR phosphorylation (left) after 30-min treatment with TGF-α (10 ng/ml) or IL-1β (2.5 ng/ml) and quantitation of the signals by densitometric scanning (right).

TABLE 2

EGFR signaling is not involved in IL-1β or PGE_2 stimulated MUC5AC mucin production

Cultures were pretreated for 1 h with either media alone or media containing anti-EGFR blocking antibody (4 μg/ml) or AG-1478 (250 nM) after which time IL-1β (0 or 2.5 ng/ml) or PGE_2 (150 nM) was added. After a 24-h treatment, apical secretions were collected (triplicate cultures per treatment), and the levels of MUC5AC were determined. The data are expressed as the amount of MUC5AC mucin per culture ± S.D. (arbitrary densitometric units).

Treatment	EGFR inhibitors		
	None	AG-1478	Anti-EGFR
Control	550 ± 99	517 ± 81	404 ± 115
IL-1β	1067 ± 71	1123 ± 156	1013 ± 81
PGE_2	1131 ± 238	1094 ± 109	1030 ± 67

chitis (Fahy, 2001; Ordonez et al., 2001). In vivo studies carried out over the last 10 years clearly indicate the complexity of the mechanisms involved in mucus overproduction caused by various inflammatory conditions in the lungs. Using cultures of fully differentiated human (Jayawickreme et al., 1999; Gray et al., 2004) and rodent (Guzman et al., 1995) airway epithelium, we have focused our previous and present studies on the stimulation of mucus overproduction by direct interaction of specific inflammatory mediators with the airway epithelium. The well organized, polarized, pseudostratified, mucociliary epithelium that forms in these cultures secretes both MUC5AC and MUC5B mucins (Thornton et al., 2000), which are the principal gel-forming mucins found in in vivo airway secretions and are elevated in sputums obtained from patients with asthma, chronic bronchitis, or cystic fibrosis (Davies et al., 1999).

In the present study, we showed that IL-1β increased MUC5AC production in NHTBE cells by a mechanism involving COX-2 induction and PGE_2 release. Several lines of evidence suggest that lipid inflammatory mediators may play a role in mucus hypersecretion (for review, see Christie and Henderson, 2002). It has been shown that exposure of airway epithelium to exogenous eicosanoids can stimulate mucus secretion in vitro as well as in vivo (Maron et al., 1983). Furthermore, it is well established that eicosanoid-producing enzymes, such as cyclooxygenases and lipoxygenases, are expressed in airway epithelium (Holtzman et al., 1994) and are regulated by various factors, including cytokines (Mitchell et al., 1994; Hill et al., 1998; Jayawickreme et al., 1999). Because IL-1β has been shown to stimulate COX-2 activity and gene expression (Kadota et al., 1996), we hypothesized that COX-2 products may be involved in mediating the IL-1β–induced increase in MUC5AC mucin production. Our studies showed that IL-1β rapidly induced COX-2 mRNA expression in NHTBE cultures, whereas COX-1 mRNA levels were not affected. The levels of endogenously produced PGE_2 increased ~30-fold because of COX-2 induction. We also showed that PGE_2 added to the culture media increased MUC5AC mucin production in a dose-dependent manner and that the COX-2 selective inhibitor celecoxib, but not the COX-1 selective inhibitor SC-560, blocked the IL-1β–induced mucin production. We also found that in tracheas from COX-2–deficient mice, PGE_2 stimulated Muc5ac mucin production but IL-1β did not. In contrast, both PGE_2 and IL-1β induced Muc5ac mucin production in tracheas from wild-type mice, thus strengthening the evidence that COX-2–generated PGE_2 plays a critical role in IL-1β–stimulated mucin production.

The biological actions of PGE_2 are mediated by transmembrane EP receptors (Narumiya and Fitzgerald, 2001). Using RT-PCR, we found that EP1, -2, -3, and -4 subtype mRNAs, as well as FP mRNAs, were expressed by cultured NHTBE cells. PGE_2 binds with high affinity to all four EP receptors. To identify the EP receptors involved in PGE_2 stimulation of MUC5AC production, we used the PGE_2 analog misoprostol,

TABLE 3

ERK/P-38 MAPK signaling is not involved in IL-1β stimulated MUC5AC mucin production in NHTBE cell cultures

EGFR ligands were removed from the cultures (triplicate cultures per treatment group for each experiment) 24 h before treatment. Cultures were pretreated for 1 h with PD98059 (50 μM), U0126 (25 μM) or SB203580 (50 μM). The levels of MUC5AC mucin were determined after 8-h cotreatment with IL-1β. Concentrations of inhibitors were chosen based on preliminary dose response experiments and on previously published results (Li et al., 1998). The data represent the mean fold-change of MUC5AC mucin in treated cultures compared with untreated control cultures determined from two independent experiments.

Treatment	Inhibitor	Fold change of MUC5AC Mucin
IL-1β	None	2.22*
	PD98059	2.58
	U0126	2.71
	SB203580	2.25

*, $P < 0.05$, statistically significant induction of MUC5AC.

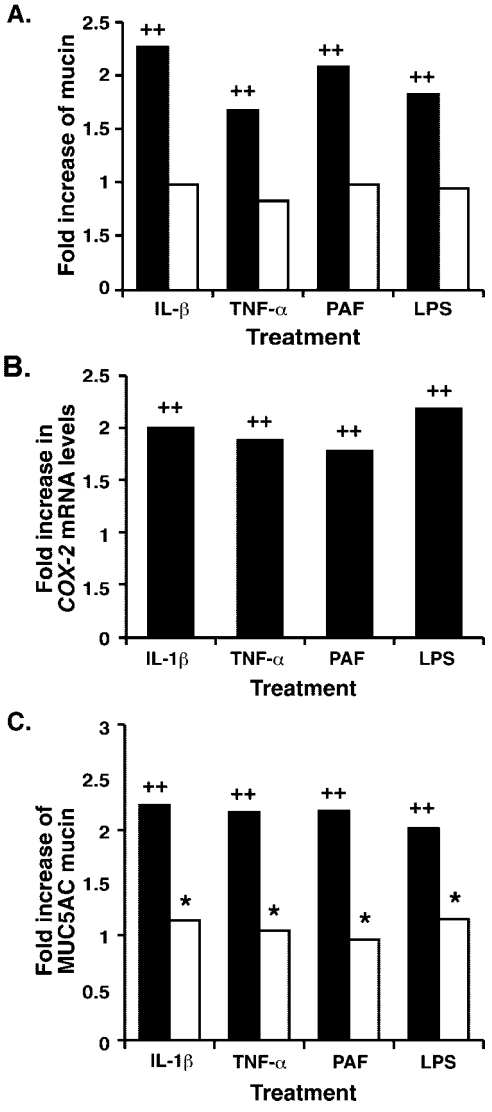

Fig. 8. The effect of different cytokine treatments on MUC5AC and MUC5B mucin production and the role of COX-2. A, the levels of MUC5AC (■) and MUC5B (□) mucin were determined 24 h after exposure to inflammatory mediators and cytokines (triplicate cultures per group). The data from three independent experiments represent the mean fold increase of mucin in treated cultures compared with control cultures. ++, statistically significant increase ($p < 0.0001$). B, after 8-h treatment with either IL-1β, TNF-α, PAF, or LPS, COX-2 mRNA levels were determined and quantitated from three independent experiments and are reported as the mean fold increase in treated compared with control cultures. ++, statistically significant increase ($p < 0.0001$). C, the levels of MUC5AC mucin were determined from cultures (triplicate cultures per group) treated for 24 h with inflammatory mediator alone (■) or in combination with celecoxib (10 μM; □). The data from three independent experiments are reported as the mean fold change of MUC5AC mucin per culture compared with controls. ++, statistically significant increase of MUC5AC mucin ($p < 0.001$); *, statistically significant inhibition of stimulation of MUC5AC ($p < 0.01$).

which binds with high affinity to EP2 and EP4, and the analog, sulprostone, which binds with high affinity to EP1 and EP3 (Narumiya and Fitzgerald, 2001). We found that the EP2/EP4 agonist stimulated MUC5AC over-production whereas the EP1/EP3 agonist did not, suggesting that PGE_2-stimulated MUC5AC production is mediated via EP2/EP4. It is noteworthy that $PGF_{2\alpha}$ also induced MUC5AC mucin production but only at the highest level tested. Differences in the capacity of PGE_2 and $PGF_{2\alpha}$ to induce MUC5AC production might indicate differences in prostanoid receptor levels in airway epithelial cells but suggest that PGE_2 is the PG responsible for the induction of MUC5AC in NHTBE cells.

EP2 and EP4 are G-protein coupled receptors that activate adenylate cyclase upon ligand binding, resulting in increased cAMP levels and activation of cAMP-dependent protein kinases (Negishi et al., 1995; Narumiya and Fitzgerald, 2001). It is well established that IL-1β can increase intracellular cAMP levels in a variety of cell types and that inducers of cAMP such as forskolin or cAMP analogs can mimic IL-1β effects (Dinarello, 1997). Indeed, we showed that forskolin treatment increased MUC5AC production and that specific PKA inhibitors blocked both IL-1β- and PGE_2–stimulated MUC5AC production, indicating that cAMP-PKA signaling mediates PGE_2-induced MUC5AC mucin production in NHTBE cultures.

Several lines of evidence reported in the literature suggest that the EGF receptor plays an important role in mediating the up-regulation of MUC5AC mRNA (Takeyama et al., 1999; Lamjabbar and Basbaum, 2002) or protein (Burgel et al., 2001) induced by several inflammatory mediators. These studies were conducted either with permanent cell lines derived from malignant lung tumors (e.g., Takeyama et al., 1999; Perrais et al., 2002), in cultured rat tracheal epithelial cells (Guzman et al., 1995), or in rat tracheas in vivo (Takeyama et al., 1999). We reported previously that in well differentiated rat tracheal epithelial cell cultures, EGF increases MUC5AC mRNA and stimulates mucus production and mucous cell hyperplasia (Guzman et al., 1995). In contrast, NHTBE cells grown under very similar conditions did not respond to EGF (Gray et al., 1996, 2001) or TGF-α (Fig. 7A) with increased mucus production. Therefore, we conclude that in NHTBE cell cultures, the induction of sustained overproduction of MUC5AC mucin by IL-1β is not dependent on EGFR activation. Therefore, the role of the EGFR in the regulation of mucus production seems to be different in different cell systems and seems to be species-dependent.

Kim et al. (2002) reported the induction of MUC5AC mucin by IL-1β in NCI H-292 cells. Several important differences exist between our studies and those reported by Kim et al. (2002). Our studies were conducted using normal, well differentiated, polarized human tracheobronchial cell cultures, whereas Kim et al. (2002) used neoplastically transformed NCI H-292 cell cultures. Kim et al. (2002) showed that in NCI H-292 cells, IL-1β–induced MUC5AC production involved activation of the ERK/p38-MAPK pathway, whereas in NHTBE cells, this pathway did not seem to be involved (Table 3). Furthermore, we have shown previously that in NHTBE cells, IL-1β causes no significant increase in MUC5AC mRNA levels (Gray et al., 2004), although it does increase MUC5AC mRNA levels in NCI H-292 cells (Kim et al., 2002; Koo et al., 2002). It is interesting that studies investigating the stability of MUC5AC mRNA also suggested that major differences exist in some critical regulatory systems between the two cell systems (Gray et al., 2001; Koo et al., 2002). In the present studies, we focused on the stimulation of MUC5AC mucin production caused by signaling events downstream of COX-2 induction, including the roles of the PGE_2 EP receptors and cAMP-PKA signaling, whereas Kim et al. (2002) focused on signaling events involved with COX-2 induction.

We showed previously that IL-1β induced only a marginal, transient increase in *MUC5AC* mRNA in the normal human bronchial cell cultures but a sustained increase in MUC5AC mucin (Gray et al., 2004). We speculate that this IL-1β effect may be a result of a cAMP/PKA-mediated post-transcriptional/post-translational mechanism(s), modifying either MUC5AC synthesis or stability. IL-1β was recently shown to increase the level of the transcription factor HIF-1α by reducing its degradation (Jung et al., 2003). At present, we can only speculate that IL-1β may increase MUC5AC mucin in the NHTBE cultures by also increasing protein stability. Post-transcriptional/post-translational control of protein synthesis and degradation are currently the subject of intensive investigation by a number of laboratories (Kisselev et al., 2003).

Our data indicate that several sequential steps are involved in IL-1β stimulation of MUC5AC mucin production by normal tracheobronchial epithelium: 1) IL-1β induction of COX-2, 2) increased PGE_2 biosynthesis, followed by 3) autocrine/paracrine EP2/EP4 receptor activation and 4) cAMP-dependent PKA-mediated signaling. Furthermore, TNF-α, PAF, and LPS also seem to stimulate MUC5AC mucin production in NHTBE cultures via a COX-2/PGE_2 pathway.

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