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CREB Mediates Prostaglandin F$_{2\alpha}$-Induced MUC5AC Overexpression$^1$

Wen-Cheng Chung,$^2*$ Seung-Hee Ryu,$^2*$ Hongxia Sun,* Darryl C. Zeldin, † and Ja Seok Koo$^3*$‡

Mucus secretion is an important protective mechanism for the luminal lining of open tubular organs, but mucin overproduction in the respiratory tract can exacerbate the inflammatory process and cause airway obstruction. Production of MUC5AC, a predominant gel-forming mucin secreted by airway epithelia, can be induced by various inflammatory mediators such as prostaglandins. The two major prostaglandins involved in inflammation are PGE$_2$ and PGF$_{2\alpha}$. PGE$_2$-induced mucin production has been well studied, but the effect of PGF$_{2\alpha}$ on mucin production remains poorly understood. To elucidate the effect and underlying mechanism of PGF$_{2\alpha}$ on MUC5AC production, we investigated the signal transduction of PGF$_{2\alpha}$ associated with this effect using normal human tracheobronchial epithelial cells. Our results demonstrated that PGF$_{2\alpha}$ induces MUC5AC overproduction via a signaling cascade involving protein kinase C, ERK, p90 ribosomal S6 protein kinase, and CREB. The regulation of PGF$_{2\alpha}$-induced MUC5AC expression by CREB was further confirmed by cAMP response element-dependent MUC5AC promoter activity and by interaction between CREB and MUC5AC promoter. The abrogation of all downstream signaling activities via suppression of each signaling molecule along the pathway indicates that a single pathway from PGF$_{2\alpha}$ receptor to CREB is responsible for inducing MUC5AC overproduction. As CREB also mediates mucin overproduction induced by PGE$_2$ and other inflammatory mediators, our findings have important clinical implications for the management of airway mucus hypersecretion. The Journal of Immunology, 2009, 182: 2349–2356.

Mucins are macroglycoproteins produced by the epithelia of the respiratory, reproductive, and gastrointestinal tracts. They furnish mucus with viscoelastic and hydrophilic properties to protect and lubricate the luminal lining (1). While mucus constitutes part of the first line of immune defense, its overproduction can exacerbate inflammation by deferring the clearance of inflammatory mediators. Conversely, the uncontrolled inflammatory mediators can further stimulate mucin production, and such a mutual aggravating process can reel into a vicious cycle and cause lethal airway obstruction or, to a lesser extent, worsen the airway infection. Therefore, mucin hypersecretion is a critical manifestation of airway inflammation and several other related diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and asthma (2). Among more than 20 mucins that have been identified (1), MUC5AC is one of the major gel-forming mucins found in airway secretions (3, 4). It is mainly produced by goblet cells of airway epithelia in healthy individuals.

The expression of MUC5AC and the number of goblet cells are markedly increased during airway inflammation (1, 2, 5, 6). Cytokines and other inflammatory mediators, such as TNF-α, IL-1β, LPS, and neutrophil elastase, are known to stimulate airway mucin hypersecretion either directly or indirectly. IL-1β is one of the most important multifunctional proinflammatory cytokines with an active role in both acute and chronic airway inflammation (7, 8). IL-1β has been reported to induce MUC5AC gene expression and mucin hypersecretion in cultured normal human tracheobronchial epithelial (NHBTE) cells and in human airway epithelial cell line NCI-H292 (6, 9–14). Such effects of IL-1β were found to be mediated by prostanoids, which are increased via the induction of cyclooxygenase 2 (COX2) expression (12). Prostaglandins are a series of lipid autocoids derived from the metabolism of arachidonic acid by COX and PG synthases. They have been shown to be involved in modulating lung inflammation (15–18). Two important prostanoids, PGE$_2$ and PGF$_{2\alpha}$, exert their effects by activating G protein-coupled receptors EP1–4 and FP, respectively (16, 18). Activation of EP2 and EP4 has been shown to induce expression of MUC5AC and of another mucin gene, MUC8, through a signaling cascade involving ERK MAPK, p90 ribosomal S6 protein kinase (RSK), and CREB (12, 19). On the other hand, although the potent secretagogue effect of PGF$_{2\alpha}$ on bronchi and trachea was described decades ago (20–22), the underlying molecular mechanism of PGF$_{2\alpha}$-induced mucin gene expression remains poorly understood. Besides being a potent stimulator of mucus secretion, PGF$_{2\alpha}$ also causes contraction of smooth muscle, and thus its secretagogue effect is even more detrimental under pathological conditions. To date, only one functional form of PGF$_{2\alpha}$ receptor, FP, which couples to G$_s$ protein (23), has been reported in humans (18). Aside from the potent secretagogue activity, PGF$_{2\alpha}$ also causes contraction of smooth muscle, and thus its secretagogue effect is even more detrimental under pathological conditions. To date, only one functional form of PGF$_{2\alpha}$ receptor, FP, which couples to G$_s$ protein (23), has been reported in humans (18).
from the conventional phosphatidylinositol 3-kinase C-protein kinase C (PKC) pathway (23, 24), activation of mitogenic pathways has also been reported for FP receptor. Via the MAPK pathway, PGF2α has been shown to up-regulate the expression of several genes, resulting in hypertrophy of the vascular smooth muscle (25, 26). However, in previous studies, mucin secretion has not been exploited as an end point of signal transduction. Thus, how FP activation leads to mucin secretion remains to be elucidated.

Cyclic AMP response element (CRE) binding protein is an important nuclear-resident transcriptional activator, which regulates the expression of a spectrum of genes. It can be activated by several upstream pathways, such as the conventional protein kinase A (PKA) and MAPK pathways (27). Previously, we have shown that MUC5AC contains CRE motif in its promoter region and can be regulated through the activation of CREB by various stimuli (14, 28). Recently, the inflammatory status of bronchi of asthmatic patients has been associated with a higher level of active CREB (phospho-CREB, pCREB) (29). We hypothesize that CREB may be the hub that conveys the proinflammatory signaling of PGF2α stimulation to mucin overproduction. In the present study, we demonstrate the stimulation of MUC5AC production by PGF2α using NHTBE cells as a model system and further elaborated the signaling linkage between PGF2α stimulation and the regulation of mucin gene expression. By delineating the signaling of PGF2α-induced mucin production, we aim to close the gap of research on PG-induced mucin secretion and to better our understanding about the interplay between inflammation and mucin production.

Materials and Methods

Cell culture and reagents

NHTBE cells were purchased from Clonetics. PGF2α, AL-8810, and flu-prostenol were from Cayman Chemical. Go6976, U0126, and H89 were from Calbiochem. Second-passage NHTBE cells (1 × 105) were seeded on a 24-mm transwell plates (Corning) and grown in serum-free growth medium (bronchial epithelial growth medium). Cells at 70% confluence were transfected with CRE promoter-luciferase reporter plasmid (Stratagene) and confirmed by sequencing. The primers used to introduce point mutations of the putative CRE site within human MUC5AC promoter with exonuclease (Erase-a-Base System; Promega) and cloned into the pGL3-Basic luciferase vector (Promega). Site-directed mutations of the putative CRE site within human MUC5AC promoter were made in the reporter construct, MUC5AC-LUC (~1366/+), using the QuickChange site-directed mutagenesis kit (Stratagen) and confirmed by sequencing. The primers used to introduce point mutations are: M1, 5'-CTCATTACTGTAATGCGCCC-3', M2, 5'-CCATGAGGATTGCTGACTGCCC-3', M3, 5'-CCATGAGGATTGCTGACTGCCC-3', M4, 5'-CCATGAGGATTGCTGACTGCCC-3'. The putative CRE site is underlined; boldface indicates the mutation sites (14).

Quantitative RT-PCR

Total RNA was extracted from NHTBE cells after 4-day treatment with 10−6 or 10−7 M PGF2α using RNAeasy Mini kits (Qiagen). Extracted RNA was converted to cDNA using a random hexamer primer (GeneAmp RNA PCR Core kit; Applied Biosystems). PCR reaction was performed using SYBR Green PCR Core kit (Applied Biosystems) according to the manufacturer’s instructions. Primer sequences for MUC5AC were forward, 5'-TTGTCGGCGGAAAGACGAC-3', and reverse, 5'-CTCTTCTATGCTTTAGCTTCAGC-3', as described previously (12, 28). Results are normalized with the expression level of human GAPDH and expressed as fold induction against untreated controls.

Flow cytometry

The 14 day-culture of NHTBE cells in transwell plates was treated with 10−6 or 10−7 M PGF2α for 4 days. After releasing from the plate by trypsinization, cells were fixed in 1% paraformaldehyde at 4°C overnight. The fixed cells were permeabilized with PBS containing 0.1% Triton X-100 and 5% BSA for 30 min, washed with 4°C, and labeled with 1/250 dilution of monoclonal anti-MUC5AC (clone 45M1) in blocking solution at room temperature for 1 h. After washing, cells were stained with Alexa Fluor 488 goat anti-mouse IgG (1/250; Invitrogen) for 40 min at room temperature and washed in 1% BSA/PBS containing 0.1% Tween 20 (PBST) twice. The stained cells were resuspended in PBS, and MUC5AC-positive cells were measured using FACScan flow cytometer equipped with a 488-nm argon laser (BD Biosciences). Cells stained with secondary Ab only were used as a control for basal signal. Data from 10,000 events per sample were recorded and processed using CellQuest software (BD Biosciences).

Immunofluorescence analysis

NHTBE cells were grown on coverslips for 7 days. After treatment with PGF2α, the cells were fixed in a methanol/aceton mixture (1:1, v/v), washed with PBS, and blocked with 5% preimmune serum for 30 min. The cells were then incubated with rabbit polyclonal RSK Ab (1/100 dilution) for 1 h at room temperature. The coverslips were washed with PBST, incubated with an Alexa Fluor 488-tagged secondary Ab (Molecular Probes) for 1 h at room temperature, and counterstained for nuclei with 4',6-diamidino-2-phenylindole (DAPI) for 30 min. After washing with PBS, slides were mounted using the SlowFade antifade kit (Molecular Probes). The stained cells were visualized under a fluorescence microscope (Axioskop 40; Zeiss), and the images were captured at a magnification of ×400 and stored using the AxioVision software program (Zeiss) as described in the manufacturer’s instructions.

RNA interference

RNA interference was performed on NHTBE cells using the siIMPORTER siRNA transfection reagent (Upstate Biotechnology) as described previously (32). For target gene silencing, SMARTpool–sequenced small interfering RNAs (siRNAs) targeting human PKCα (GenBank accession no. NM_002953), PKCδ (GenBank accession no. NM_002953), ERK (GenBank accession no. NM_002745), CREB (GenBank accession no. NM_004379), and a nonspecific control pool (siRNA-negative control; Dharmacon) were diluted and stored according to the manufacturer’s instructions. NHTBE cells at 60% or 70% confluence were transfected with a final concentration of 100 nM of target SMARTpool siRNA or the nonspecific control pool. Cells were analyzed 72 h after transfection. After 72 h of transfection, when target protein levels had been reduced >80% as assessed by Western blot analysis, the cells were treated with or without PGF2α for another 30 min. Then whole-cell lysates were prepared for Western blot analysis.

Preparation of luciferase reporter constructs for MUC5AC promoter

Measurement of MUC5AC promoter activity using a luciferase reporter vector has been reported previously (33). Fragments of 5’ flanking region of MUC5AC ranging from 3.7 kb (nucleotide from −3752/+7) to 0.29 kb (nucleotide from −2960/+7) were generated by digestion of the 3.7-kb fragment of the MUC5AC promoter with exonuclease (Erase-a-Base System; Promega) and cloned into the pGL3-Basic luciferase vector (Promega). Site-directed mutations of the putative CRE site within human MUC5AC promoter were made in the reporter construct, MUC5AC-LUC (~1366/+7), using the QuickChange site-directed mutagenesis kit (Stratagen) and confirmed by sequencing. The primers used to introduce point mutations are: M1, 5'-CTCATTACTGTAATGCGCCC-3', M2, 5'-CCATGAGGATTGCTGACTGCCC-3', M3, 5'-CCATGAGGATTGCTGACTGCCC-3'. The putative CRE site is underlined; boldface indicates the mutation sites (14).

Transient transfection and luciferase assays

NHTBE cells were transfected as described previously (32). Briefly, NHTBE cells (1 × 104 cells/well) were plated in 12-well plates using bronchial epithelial growth medium. Cells at 70% confluence were transfected with CRE promoter-luciferase reporter plasmid (Stratagen) and β-galactosidase (β-gal) reporter plasmid (BD Biosciences/Clontech) using Lipofectamine 2000 transfection reagent (Invitrogen). Similarly, NCI-H292 cells (1 × 104 cells/well) were plated in 12-well plates and grown in RPMI 1640 medium with 10% FBS. When reaching 70% confluence, cells were transiently transfected with CRE reporter construct along with β-gal.
reporter plasmid. To determine the promoter regions of the MUC5AC gene activated by PGF2\(_\alpha\) stimulation, cells were cotransfected with reporter construct containing deletion mutants or point-mutated CRE sites of MUC5AC promoter and the \(\beta\)-gal reporter plasmid. Four hours after transfection, cells were treated with PGF2\(_\alpha\) and cultured for another 48 h. Luciferase activity was measured using a luminometer (Lumat LB 9507; EG&G Berthold). \(\beta\)-gal activity was measured using a \(\beta\)-gal enzyme assay system (Promega) and used to normalize transfection efficiency.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as described elsewhere (28). Briefly, NHTBE cells were activated with PGF2\(_\alpha\) for 4 h and then incubated with 1% formaldehyde for 10 min at 37°C to cross-link the DNA with proteins. The cells were then washed with cold PBS and resuspended in lysis buffer (1% SDS, 100 mM NaCl, 50 mM Tris-HCl (pH 8.1), and 5 mM EDTA), and the DNA was fragmented with sonication to an average length of 500-1000 bp. Anti-pCREB Ab or normal rabbit IgG was added to each sample, which was then incubated in a rotary mixer overnight at 4°C. The immunocomplex was precipitated by protein A beads and incubated with 200 mM NaCl overnight at 65°C to reverse the formaldehyde cross-linking, and then the DNA was collected and analyzed with PCR. Primers for MUC5AC promoter sequences are: 5'-AAGGTCTTCGGCAAGTTCC-3' (forward) and 5'-TTCTCTCCCCACTAACAC-3' (reverse). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

**Statistical analysis**

Statistical analysis was performed with a Prism program (GraphPad Software) using one-way ANOVA, followed by Dunnett’s test for comparing experiment groups against a single control for multiple comparisons, or a paired \(t\) test when comparing between two groups.

**Results**

**PGF\(_{2\alpha}\) induces overexpression of MUC5AC and increases MUC5AC-producing cells**

In earlier studies (12, 20), it was shown that PGF2\(_\alpha\) stimulates mucus secretion; however, the chronic effect of PGF2\(_\alpha\) on the production of a specific mucin has not been fully described. To determine the effect of PGF2\(_\alpha\) on the production of mucin protein MUC5AC, we treated NHTBE cells with different concentrations of PGF2\(_\alpha\) for 4 days and mucin secretion was collected daily (Fig. 1A). After a 4-day treatment with PGF2\(_\alpha\) (0.1 and 1 \(\mu\)M), MUC5AC secretion in NHTBE cells was significantly increased, and such effect was attenuated by the pretreatment with FP-specific antagonist AL-8810 (10 \(\mu\)M) (Fig. 1B). A similar level of MUC5AC secretion was induced by fluprostenol, a selective and metabolically stable FP agonist (Fig. 1B). The effect of PGF2\(_\alpha\) on MUC5AC overproduction was further examined at different dimensions using RT-PCR and immunocytochemistry staining. PGF2\(_\alpha\) treatment significantly increased the MUC5AC mRNA

**FIGURE 1.** PGF2\(_\alpha\) induces MUC5AC overproduction in NHTBE cells. Fourteen-day cultures of NHTBE cells in air-liquid-interface system were treated with 0.1 or 1 \(\mu\)M PGF2\(_\alpha\) for 4 days. A, The secreted MUC5AC was collected each day and assayed with immunodot blotting. B, Twenty-four hour MUC5AC secretion was also collected from cells treated with PGF2\(_\alpha\) for 4 days in the absence or presence of FP antagonist AL-8810 (10 \(\mu\)M) and from cells treated with FP agonist fluprostenol. C, MUC5AC gene expression was assayed using RT-PCR. D, The percentage of cells expressing MUC5AC in each culture was measured with flow cytometry using Ab against MUC5AC. Data are shown as means ± SE from at least three experiments. \*, \(p < 0.05\) and **, \(p < 0.01\).

**FIGURE 2.** PGF2\(_\alpha\) activates CREB in a time- and concentration-dependent manner. NHTBE cells were treated with 1 \(\mu\)M PGF2\(_\alpha\) for the indicated periods of time (A) or with various concentrations of PGF2\(_\alpha\) for 30 min (B). After treatment, whole-cell lysates were prepared and subjected to Western blot analysis using anti-CREB and anti-pCREB Abs. Equal loading of samples was confirmed by stripping the blot and reprobing it with \(\beta\)-actin Ab. The ratio of the band intensity between CREB and pCREB was plotted below the gel image.
level (Fig. 1C) and the percentage of MUC5AC-positive cells in the NHTBE cell culture (Fig. 1D). The sustained elevation in MUC5AC expression level together with the increase in the number of MUC5AC-positive cells reveals a prolonged effect of PGF$_{2\alpha}$ on mucin overproduction.

**CREB is activated by PGF$_{2\alpha}$**

Although CREB is not a component of the conventional signaling pathway for a $G_q$ protein-coupled receptor like FP, CREB was previously shown to mediate the transcriptional regulation of some mucin proteins (14, 19). We examined whether CREB can also be activated by PGF$_{2\alpha}$ stimulation. NHTBE cells were treated with 1 $\mu$M PGF$_{2\alpha}$ for different lengths of time or with different concentrations of PGF$_{2\alpha}$ for 30 min. The activation of CREB was measured by detecting the phosphorylation of CREB at Ser 133 (pCREB) with Western blot. PGF$_{2\alpha}$ induced CREB activation in a time-dependent manner, while the level of CREB remained unchanged (Fig. 2A). CREB activation was detected as early as 5 min after the beginning of PGF$_{2\alpha}$ treatment and reached a maximum at 1 h; the pCREB level remained above the control level for >4 h (data not shown). A concentration-dependent effect on activation of CREB was also observed for PGF$_{2\alpha}$ treatment (Fig. 2B). CREB activation can be induced by PGF$_{2\alpha}$ at a concentration as low as 0.1 $\mu$M; the effect reaches a maximum at 1 $\mu$M concentration of PGF$_{2\alpha}$.

**PGF$_{2\alpha}$-induced CREB activation is mediated by PKC/MEK/ERK/RSK pathway**

As the FP receptor pathway does not lead to the production of cAMP, we sought an alternative pathway for the mediation of PGF$_{2\alpha}$-induced CREB activation. Based on our observation that the PKC pathway is one of the upstream signaling pathways leading to the activation of CREB (32), we tested whether this is also the case for PGF$_{2\alpha}$-induced CREB activation. NHTBE cells were pretreated for 60 min with 10 $\mu$M of various selective inhibitors targeting different signaling proteins. CREB activation was examined after subsequent treatment of cells with PGF$_{2\alpha}$ (1 $\mu$M, 30 min). The PKC inhibitor Go6976 and the MEK1/2 inhibitor U0126 abolished PGF$_{2\alpha}$-induced CREB activation, whereas the PKA inhibitor H89 had no effect on such activation (Fig. 3A). These results demonstrated that PKC and MEK/ERK, but not PKA, mediate PGF$_{2\alpha}$-induced CREB activation.

To confirm the involvement of the PKC pathway in PGF$_{2\alpha}$-induced CREB phosphorylation and to further delineate the PGF$_{2\alpha}$-induced signaling pathways leading to CREB activation, we used RNA interference to knock down the expression of signaling components along the PKC/ERK/RSK/CREB pathway and examined the effect of PGF$_{2\alpha}$ on the activation of these components after each specific knockdown. We focused on the PKC pathway because it is the predominant conventional PKC isozyme in NHTBE cells (34). NHTBE cells were transfected with pools of SMARTpool siRNAs that target PKC$\alpha$, ERK, RSK, and CREB, respectively. The maximal silencing of protein expression was achieved 3 days after transfection (data not shown). Depletion of PKC$\alpha$ completely abolished PGF$_{2\alpha}$-induced activation of its downstream signaling molecules (ERK, RSK, and CREB) without affecting their protein levels (Fig. 3B). Similar effects were observed with the silencing of ERK and RSK; only the propagation of the downstream signaling was blocked, whereas the response to PGF$_{2\alpha}$ stimulation was not affected for upstream signaling molecules.

As the activation of CREB by RSK requires the translocation of RSK from the cytoplasm to the nucleus where CREB resides, we used immunofluorescence staining to demonstrate this phenomenon in

**FIGURE 3.** PGF$_{2\alpha}$-induced activation of CREB is mediated through the PKC$\alpha$/ERK/RSK signal transduction pathway. A, NHTBE cells were preincubated with various signal transduction inhibitors (10 $\mu$M) for 60 min and then treated with 1 $\mu$M PGF$_{2\alpha}$ or with vehicle control for 30 min. Whole-cell lysates were prepared and subjected to Western blot analysis using anti-CREB and anti-pCREB Abs. Equal loading of samples was confirmed by noting equal amounts of $\beta$-actin in each lane. B, NHTBE cells were transfected with siRNA of PKC$\alpha$, ERK, RSK, or a nonspecific control pool (NS-siRNA) alone. Three days after transfection, the cells were treated with or without PGF$_{2\alpha}$ for 30 min. After treatment, equal amounts of whole-cell lysates were isolated and analyzed by immunocytofluorescence. Cells were incubated with a vehicle control (upper panels) or with 1 $\mu$M PGF$_{2\alpha}$ (lower panels) for 30 min before fixation. After fixation, the cells were stained with RSK Ab followed by anti-rabbit Alexa Fluor 488 Abs (green), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) and then the two images were merged.
**FIGURE 4.** PGF$_{2a}$ induces CRE-dependent transactivation, and CRE in MUC5AC promoter is indispensable for PGF$_{2a}$-induced MUC5AC promoter activity. NCI-H292 (A) and NHTBE (B) cells were transiently cotransfected with a CRE promoter-driven luciferase containing plasmid and a β-gal reporter plasmid, or transfected with a luciferase reporter vector containing various 5’-deleted MUC5AC promoter constructs (C) or transfected with the −1366/+7 region of the MUC5AC promoter construct containing various mutated CRE sites (D). After transfection, the cells were further incubated with 1 μM PGF$_{2a}$ or with vehicle control for 48 h. The data are normalized to the change in luciferase activity (expressed as fold change) relative to the untreated control and expressed as the means ± SE of three independent experiments, with each performed in triplicate. *, p < 0.05 and **, p < 0.01.

**FIGURE 5.** PGF$_{2a}$ induces binding of pCREB to CRE site on MUC5AC promoter. NHTBE cells were treated with or without Ctl μM PGF$_{2a}$ for 4 h and then were assayed by ChIP using anti-pCREB to precipitate CRE-containing chromatin DNA. The presence of MUC5AC promoter in CRE-containing chromatin was detected using PCR with primers framing MUC5AC promoter regions (nt −980 to nt −708) as described in Materials and Methods. A portion of the pre-immunoprecipitation chromatin was assayed to verify equal loading (Input). Chromatin precipitated with nonspecific Ab (control IgG Ctl, PGF$_{2a}$ treatment control) was assayed under otherwise identical conditions as a negative control. Results shown are representative of three independent experiments.

PGF$_{2a}$-treated NHTBE cells to confirm that RSK mediates PGF$_{2a}$-induced CREB activation. As shown in Fig. 3C, most of the RSK proteins were present in the cytoplasm of untreated controls. After PGF$_{2a}$ treatment, most RSK proteins were detected in the nucleus.

**CRE in MUC5AC gene promoter is required for PGF$_{2a}$-induced overexpression of MUC5AC**

The effect of PGF$_{2a}$ on CRE-dependent transcriptional activation was determined by transiently transfecting NHTBE cells with a luciferase reporter containing CRE promoter. Treatment of NHTBE cells with PGF$_{2a}$ for 48 h resulted in an increase in luciferase activity to 1.8-fold that of untreated control (Fig. 4A). These results suggest that by binding to its cognate CRE site in the promoter, the PGF$_{2a}$-activated CREB induces its transcriptional activity. It has been observed that primary epithelial cells usually have low transfection efficiency. To verify our finding in NHTBE cells, we performed the same reporter transfection and analysis in a lung cancer cell line, H292. PGF$_{2a}$ increased the promoter activity in H292 cells to 2.3-fold that of controls (Fig. 4B).

To further identify the promoter region of MUC5AC that is critical for PGF$_{2a}$-induced expression of MUC5AC, luciferase reporter constructs with progressive 5’ deletion along the MUC5AC promoter were prepared, and the PGF$_{2a}$-stimulated promoter activities were analyzed in NHTBE cells. As shown in Fig. 4C, PGF$_{2a}$ increased the luciferase activity of the cells transfected with the construct containing the −3752/+7 region of MUC5AC promoter (1.9-fold of untreated control). Deletion of the MUC5AC
PKC/CREB pathway mediates the PGF2α-induced MUC5AC production

To demonstrate the importance of PKC/CREB pathway in PGF2α-induced expression of MUC5AC, NHTBE cells were pretreated with inhibitors against PKC (GF109203X, 10 μM) or ERK (U0126, 10 μM) or PKA (H89, 10 μM) before PGF2α stimulation (A), and MUC5AC secretion was collected after 24 h of treatment. NHTBE cells grown on regular plastic wells were transfected with siRNA against CREB or nontarget control (B) (filled bar) or vehicle (open bar). Expression levels of MUC5AC were measured with RT-PCR. The data are expressed as means ± SE of fold change relative to the nontarget siRNA-transfected untreated control. †, p < 0.05 and ††, p < 0.01 (vs PGF2α-stimulated without inhibitor or CREB siRNA).

Discussion

Mucin hypersecretion is not only a sign of airway inflammation but also a life-threatening symptom of airway diseases in severe conditions. This is especially true for MUC5AC mucin owing to its gel-forming multimeric structure, which contributes significantly to the viscous property of mucus (4). Additionally, MUC5AC is a predominant airway mucin whose production can be induced during respiratory diseases (1). Therefore, understanding the regulation of MUC5AC gene expression is even more crucial under clinical consideration.

The production of MUC5AC and some other mucins has been reported to be regulated by several inflammatory mediators (1, 6). Interestingly, all these inflammatory mediators seem to induce MUC5AC overproduction via the COX2/PGE2 pathway (12); however, the role of another major product of COX2, PGF2α, was less clear. Although the potent secretagogue effect of PGF2α on trachea was reported decades ago, the effect of PGF2α on the overproduction of specific mucins has not been studied in detail. This is the first report demonstrating the effect of PGF2α on MUC5AC overproduction at the secretion, gene expression, and cell phenotype levels. The results of our study further established the PGF2α signal transduction pathway leading from activation of its cell-surface receptor to transcription activity in the nucleus.

In the present study, we observed that 4 days of PGF2α treatment increased expression of MUC5AC mRNA (2- to 2.5-fold) and MUC5AC secretion (4- to 6-fold), while PGF2α increased the MUC5AC-positive cells by ~50% (Fig. 1D). These results indicate that PGF2α-induced mucus hypersecretion is a combination of mucin gene overexpression and mucous cell hyperplasia. Differentiation of epithelial cells into MUC5AC-secreting cells is a complex and time-consuming process; however, once the cells are committed, production of MUC5AC seems to be long-lasting. Our time-course results also offer, at least in part, an explanation for the long-term process of mucin induction, PGF2α-induced prolonged activation of CREB (Fig. 2A) compared with that induced by PGE2 (19). This prolonged CREB activation might be required to facilitate the
Inflammatory mediators seem to induce mucin overproduction. As mentioned above, inflammatory mediators are also positively associated with pCREB level (29). Hence, the importance of CREB in the progression of inflammation cannot be overemphasized. In addition to binding to CRE, CREB can also interact with other transcription factors and coactivators (36). It has been suggested that the duration of CREB phosphorylation may account for the different degree of transcriptional activity effected by CREB activation (36). The prolonged CREB activation induced by PGF2α may further potentiate the transcription activation initiated by other factors.

In conjunction with our previous studies, which elucidated the signaling of IL-1β and PGF2α-induced mucin overproduction (12, 19), we conclude the signaling network for prostaglandins and IL-1β-induced mucin production in our present study (Fig. 7). Inside this network, CREB plays a pivotal role in executing the inflammatory responses, such as mucin and COX2 gene expression, which not only propels the positive feedback cycle of prostaglandin signaling, but also facilitates both the short- and long-term production of mucin protein to further affect the overall consequence of inflammation. Aside from playing an important role in inflammatory diseases, prostaglandins are also involved in other critical physiological and pathological processes, such as angiogenesis (37). The results of the present study, which establish the linkage between PGF2α-induced MUC5AC overproduction and CREB activation, not only increase our understanding about the signal transduction of IL-1β and prostaglandins, but they also have important clinical implications for the management of airway inflammation and other pathological conditions.

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**Disclosures**

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