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CREB Mediates Prostaglandin $F_{2\alpha}$-Induced MUC5AC Overexpression

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Abstract

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 prostaglandin (PG) E_2 and $F_{2\alpha}$. 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, extracellular signal-regulated kinase, p90 ribosomal S6 protein kinase, and cAMP response element binding protein (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 implication for the management of airway mucus hypersecretion.

Keywords

Lung; Inflammation; Signal Transduction; Transcription Factors; Human; Gene Regulation

Mucins are macro glycoproteins 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

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of inflammatory mediators. Conversely, the uncleared inflammatory mediators can further stimulate mucin production, and such 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 (COPD), and asthma (2). Among more than 20 mucins that have been identified (1), MUC5AC is one of the major gel-forming mucin 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, interleukin-1β (IL-1β), lipopolysaccharide (LPS), and neutrophil elastase (NE), 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 (NHTBE) cells and in human airway epithelial cell line NCI-H292 (6,9–14). Such effects of IL-1β were found to be mediated by prostaglandins (PGs), which are increased via the induction of cyclooxygenase 2 (COX2) expression (12). PGs 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 PGs, 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 extracellular signal-regulated kinase mitogen-activated protein-kinase (ERK MAPK), p90 ribosomal S6 protein kinase (RSK), and cAMP response element binding protein (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_q protein (23)—has been reported in humans (18). Aside from the conventional phospholipase C (PLC)-protein kinase C (PKC) pathway (23,24), activation of mitogenic pathways has also been reported for FP receptor. Via the MAPK pathway, $PGF_{2\alpha}$ 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 (CREB) 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 $PGF_{2\alpha}$ stimulation to mucin overproduction. In the current study, we demonstrated the stimulation of MUC5AC production by $PGF_{2\alpha}$ using NHTBE cells as a model system and further elaborated the signaling linkage between $PGF_{2\alpha}$ stimulation and the regulation of mucin gene expression. By delineating the signaling of $PGF_{2\alpha}$ 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 (San Diego, CA). $PGF_{2\alpha}$, AL-8810, and fluprostenol were from Cayman Chemical (Ann Arbor, Michigan). Go6976, U0126, and H89 were from Calbiochem (San Diego, California). Second-passage NHTBE cells (1×10^5) were seeded on a 24-mm Trans-well plate (Corning, Acton, MA) and grown in serum-free growth factor- and hormone-supplemented culture medium as described previously (30–32). After 7 days under immersed culture conditions, the cell culture was switched to an air-liquid interface. Cells were incubated with bronchial epithelial cell basal medium for 24 h prior to treatment. To study the effect of chemical inhibitors on signal transduction pathways, cells were pretreated with each inhibitor 1 h prior to treatment with $PGF_{2\alpha}$. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Immunoblotting Analysis

Whole-cell extracts were prepared using 2× SDS Laemmli lysis buffer. Equal amounts of total protein (20 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies used were mouse monoclonal antibody against β-actin (clone AD-15; Sigma-Aldrich, St. Louis, MO), rabbit polyclonal antibodies against CREB, pCREB (Ser-133 phosphorylated), phospho-PKC_α ($pPKC_\alpha$; Ser-659 phosphorylated; Upstate Biotechnology, Waltham, MA), ERK, phosphor-ERK (pERK; Thr-202 and Tyr-204 phosphorylated), RSK, phosphor-RSK (pRSK; Ser-380 phosphorylated; Cell Signaling Technology, Cambridge, MA), and PKC_α (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins reactive with primary antibody were visualized with a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (Amersham Bioscience, Arlington Heights, IL). Equal sample loading was verified by stripping the blots and reprobing them with an anti-β-actin antibody. For MUC5AC secretion, the apical wash of NHTBE cell culture was blotted onto the nitrocellulose membrane using a filtration manifold (Schleicher & Schuell, Keene, NH) and then probed with anti-MUC5AC (clone 45M1; NeoMarker, Fremont, CA) and visualized using the same method described previously (6).

Quantitative Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from NHTBE cells after 4 day treatment with 10^{-6} or 10^{-7} M $PGF_{2\alpha}$ using RNeasy mini-kits (Qiagen, Valencia, CA). Extracted RNA was converted to cDNA using a random hexamer primer (GeneAmp RNA PCR Core kit; Applied Biosystems, Foster City, CA). PCR reaction was performed using SYBR Green PCR Core kit (Applied Biosystem, Foster City, CA) according to manufacturer's instruction. Primer sequences for *MUC5AC* were forward, 5′-TGTGGCGGGAAAGACAGC-3′; and reverse, 5′-CCTTCCTATGGCTTAGCTTCAGC-3′, as described previously (12,28). Results are normalized with the expression level of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed as fold induction against untreated controls.

Flow Cytometry

The 14 day-culture of NHTBE cells in trans-well plates were treated with 10^{-6} or 10^{-7} M $PGF_{2\alpha}$ 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 phosphate-buffered saline (PBS) containing 0.1% triton X 100 and 5% BSA for 30 min at 4°C and labeled with 1:250 dilution of monoclonal anti-MUC5AC (clone 45M1) in blocking solution at room temperature (RT) for 1 hr. After washing, cells were stained with Alexa Fluor 488 goat anti-mouse IgG (1:250; Invitrogen, Carlsbad CA) for 40 min at RT and washed in 1% BSA/PBST twice. The stained cells were re-suspended in PBS and MUC5AC positive cells were measured

using FACScan flow cytometer equipped with a 488 nm argon laser (Becton Dickinson Instruments, Franklin Lakes, NJ). Cells stained with secondary antibody only were used as a control for basal signal. Data from 10,000 events per sample were recorded and processed using CellQuest software (Becton Dickinson Instuments).

Immunofluorescence Analysis

NHTBE cells were grown on a coverslips for 7 days. After treatment with $PGF_{2\alpha}$, the cells were fixed in a methanol:acetone 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 antibody (1:100 dilution) for 2 h at room temperature. The coverslips were washed with PBS containing 0.1% Tween 20 (PBST), incubated with an AlexaFluor 488-tagged secondary antibody (Molecular Probes, Eugene, OR) 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; Carl Zeiss, Thornwood, NY), and the images were captured at a magnification of 400× and stored using the AxioVision software program (Carl 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 siRNAs targeting human PKC_{α} (GenBank accession no. NM_002953), RSK (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 RNA Technologies, Lafayette, CO) 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 more than 80% as assessed by western blot analysis, the cells were treated with or without $PGF_{2\alpha}$ 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 have 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 −296/+7) in size were generated by digestion of the 3.7 kb fragment of the *MUC5AC* promoter with exonuclease (Erase a Base System; Promega Corp., Madison, WI) and cloned into the pGL3-Basic luciferase vector (Promega Corp). Site-directed mutations of the putative CRE site within human *MUC5AC* promoter were made in the reporter construct, MUC5AC-LUC (−1366/+7), using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. The primers used to introduce point mutations are: M1, 5′-CCATCAAG***ACT***CTTGAACTGGCCC-3′; M2, 5′-CCATCAAG*TG**GTG**TGA*ACTGGCCC-3′; M3, 5′-CCATCAAG*TGACT**GAC***ACTGGCCC-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 (1×10^4 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 (Stratagene)

and β-galactosidase (β-gal) reporter plasmid (BD Biosciences Clontech, Palo Alto, CA) using Lipofectamine 2000 transfection reagent (Invitrogen). Similarly, NCI-H292 cells (1×10^4 cells/well) were plated in 12-well plates, and grown in RPMI-1640 medium with 10% fetal bovine serum. 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 $PGF_{2\alpha}$ stimulation, cells were cotransfected with reporter construct containing deletion mutants or point-mutated CRE sites of *MUC5AC* promoter and the β-gal reporter plasmid. Four hours after transfection, cells were treated with $PGF_{2\alpha}$ and cultured for another 48 h. Luciferase activity was measured using a luminometer (Lumat LB 9507 luminometer; EG&G, Berthold, Germany). β-gal activity was measured using a β-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 $PGF_{2\alpha}$ 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 to 1,000 base pairs. Anti-pCREB antibody or normal rabbit IgG was added to each sample, which was then incubated in a rotary mixer overnight at 4°C. The immuno-complex was precipitated by protein A beads and incubated with 200 mM NaCl overnight at 65°C to reverse the formaldehyde cross-linking, then the DNA was collected and analyzed with PCR. Primers for MU5AC promoter sequences are: 5′-AAGGTCTTCGGCAAGTTCC-3′ (forward) and 5′-TTCTCTCCCCCACGTAACAC-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 Prism program (GraphPad Software, San Diego, CA) using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing experiment groups against a single control for multiple comparisons, or 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 $PGF_{2\alpha}$ stimulates mucus secretion, however, the chronic effect of $PGF_{2\alpha}$ on the production of a specific mucin has not been fully described. To determine the effect of $PGF_{2\alpha}$ on the production of mucin protein MUC5AC, we treated NHTBE cells with different concentrations of $PGF_{2\alpha}$ for 4 days and mucin secretion was collected daily (Fig. 1A). After 4 day treatment with $PGF_{2\alpha}$ (0.1 and 1 μM), MUC5AC secretion in NHTBE cells was significantly increased, and such effect was attenuated by the pretreatment with FP specific antagonist, AL-8810 (10 μM) (Fig. 1B). A similar level of MUC5AC secretion was induced by fluprostenol, a selective and metabolically stable FP agonist (Fig. 1B). The effect of $PGF_{2\alpha}$ on MUC5AC overproduction was further examined at different dimensions using RT-PCR and immunocytochemistry staining. $PGF_{2\alpha}$ treatment significantly increased the MUC5AC mRNA 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 μM $PGF_{2\alpha}$ 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 over 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 μM; the effect reaches a maximum at 1 μM 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. NHTEB cells were pre-treated for 60 min with 10 μM of various selective inhibitors targeting different signaling proteins. CREB activation was examined after subsequent treatment of cells with $PGF_{2\alpha}$ (1μM, 30min). The PKC inhibitor Go6976 and the MEK 1/2 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 PKCα because it is the predominant conventional PKC ioszyme in NHTBE cells (34). NHTBE cells were transfected with pools of SMARTpool siRNAs that targets PKCα, ERK, RSK and CREB respectively. The maximal silencing of protein expression was achieved 3 d after transfection (data not shown). Depletion of PKCα 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 $PGF_{2\alpha}$-treated NHTBE cells to confirm that RSK mediates $PGF_{2\alpha}$-induced CREB activation. As shown in Fig. 3C, most of the RSK proteins were present in the cytoplasm of untreated controls. After $PGF_{2\alpha}$ treatment, the majority of RSK proteins were detected in the nucleus.

CRE in *MUC5AC* Gene Promoter is Required for $PGF_{2\alpha}$-Induced Overexpression of *MUC5AC*

The effect of $PGF_{2\alpha}$ on CRE-dependent transcriptional activation was determined by transiently transfecting NHTBE cells with a luciferase reporter containing CRE promoter. Treatment of NHTBE cells with $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$-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. $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$-induced expression of *MUC5AC*, luciferase reporter constructs with progressive 5′-deletion along the *MUC5AC* promoter were prepared, and the $PGF_{2\alpha}$-stimulated promoter activities were analyzed in NHTBE cells. As shown in Fig. 4C, $PGF_{2\alpha}$ 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* 5′-flanking sequence from nucleotide (nt) −3752 to −929 had no apparent effect on the promoter activity induced by $PGF_{2\alpha}$. However, such $PGF_{2\alpha}$-induced activation was completely abolished when the deletion proceeded from nt −929 to −596. Our previous study showed that a CRE motif is present at nt −878 (unpublished data). To determine the role of this CRE motif in $PGF_{2\alpha}$-induced *MUC5AC* regulation, we introduced a series of mutations into this CRE sequence using site-directed mutagenesis and then analyzed the promoter activity in NHTBE cells. The $PGF_{2\alpha}$ responsiveness of the *MUC5AC* promoter was abolished in the mutant constructs (Fig. 4D; M1, M2, and M3). These results strongly suggest that the CRE motif located between nt −878 and −871 on the *MUC5AC* promoter is critical for $PGF_{2\alpha}$-induced expression of *MUC5AC*.

The results of our promoter analysis led us to examine the *in vivo* DNA-protein binding by ChIP assay, to confirm the interaction of CREB with the putative CRE element on native *MUC5AC* promoter. As shown in Figure 5, a single PCR band (273 bp) was detected in both $PGF_{2\alpha}$-treated and untreated NHTBE cells when immunoprecipitation was performed using anti-pCREB. In contrast, no PCR product was detected when non-immune control IgG was used. Interestingly, the binding of pCREB to this fragment of the *MUC5AC* promoter occurred as early as 30 min after the initiation of $PGF_{2\alpha}$ treatment (data not shown) and was sustained until 4 h after. These results confirm that the CRE site of the *MUC5AC* promoter is recognized by CREB *in vivo*.

PKC-CREB Pathway Mediates the $PGF_{2\alpha}$-Induced MUC5AC Production

To demonstrate the importance of PKC-CREB pathway in $PGF_{2\alpha}$-induced expression of *MUC5AC*, NHTBE cells were pretreated with inhibitors against PKC (GF109203X), ERK (U0126), and PKA (H89) respectively before $PGF_{2\alpha}$ stimulation (Fig. 6A). Mucin MUC5AC secretion was collected after 24 h treatment to minimize the potential cytotoxicity from these inhibitors. Inhibition of signaling components of PKC-CREB pathway (either PKC or ERK) significantly reduced $PGF_{2\alpha}$ induced MUC5AC secretion to a level similar to the un-stimulated control, whereas inhibition of PKA only slightly reduced such secretion. We further examined whether CREB is required for $PGF_{2\alpha}$-induced expression of *MUC5AC* gene by depriving NHTBE cells of CREB protein using RNA interference. As expected, before knockdown of CREB expression, $PGF_{2\alpha}$ was able to induce expression of *MUC5AC*, but after knockdown of CREB expression, $PGF_{2\alpha}$-induced expression of *MUC5AC* was abolished (Fig. 6B). This result clearly demonstrated that CREB is required for $PGF_{2\alpha}$-induced expression of *MUC5AC*.

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/PGE_2 pathway (12); however, the role of another major product of COX2, $PGF_{2\alpha}$, was less clear. Although the potent secretagogue effect of $PGF_{2\alpha}$ on trachea was reported decades ago, the effect of $PGF_{2\alpha}$ on the overproduction of specific mucins has not been studied in detail. This is the first report demonstrating the effect of $PGF_{2\alpha}$ on MUC5AC overproduction at the secretion, gene expression, as well as cell phenotype levels. The results of our study further established the $PGF_{2\alpha}$ signal transduction pathway leading from activation of its cell-surface receptor to transcription activity in the nucleus.

In the current study, we observed that 4 days of $PGF_{2\alpha}$ treatment increased expression of *MUC5AC* mRNA (2–2.5 fold) and MUC5AC secretion (4–6 fold), while $PGF_{2\alpha}$ increased the MUC5AC-positive cells by about 50% (Fig. 1D). These results indicate that $PGF_{2\alpha}$-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 previous observation that $PGF_{2\alpha}$ was less potent than PGE2 in stimulating mucin production in a relatively shorter term treatment (12). Other possible reason for that could be the lability of $PGF_{2\alpha}$, as we also observed that a smaller amount of the stable analog of $PGF_{2\alpha}$, fluprostenol, was needed for inducing a similar level of mucin secretion. In concert with the long-term process of mucin induction, $PGF_{2\alpha}$ induced prolonged activation of CREB (Fig. 2A) compared with that induced by PGE_2 (19). This prolonged CREB activation might be required to facilitate the transformation of epithelial cells into mucin-secreting cells and to further perpetuate the elevation of mucin production.

The $PGF_{2\alpha}$ receptor FP is a serpentine receptor coupling to G_q proteins. The presence of FP in NHTBE cells has previously been demonstrated (12). Intuitively, the activation of a G_q protein-coupled receptor should be able to stimulate the mucus secretion by an increase in intracellular Ca^{2+}. However, we found that the critical pathway leading to MUC5AC overproduction is mediated through sequential activation of the PKC/ERK/RSK/CREB pathway (Fig. 7), which does not involve the PKA/CREB pathway (Fig. 3A and 6A). Previously, more than one MAP kinase has been reported to be involved in the PGE_2-induced signaling pathway (14,35), and two different preceding activators of CREB, RSK and MSK have been indicated in different reports (14,19). This discrepancy may be due to differences in cell types and/or to additional signaling derived from secondary pathways. We were not able to verify all of the possible causes in the present study. However, the nearly complete abrogation of the downstream signaling observed with the knockdown of each individual signaling component along the pathway (Fig. 3B) indicates that $PGF_{2\alpha}$/FP/PKC/ERK/RSK/CREB linear signaling pathway plays a major role in the $PGF_{2\alpha}$-induced activation of CREB and that no other essential alternative pathway or feed-back loop is involved in the activation. Additionally, the $PGF_{2\alpha}$- induced translocation of RSK from cytoplasm to nucleus (Fig. 3C) provides strong support for our proposed pathway.

Our results unequivocally demonstrate the regulatory and indispensable role of CREB in $PGF_{2\alpha}$-induced overproduction of MUC5AC (Fig. 4, 5, and 6). Since both the signaling pathways of PGE_2 and $PGF_{2\alpha}$ converge on CREB (Fig. 7), CREB plays a pivotal role in signaling of inflammatory prostaglandins. As mentioned above, inflammatory mediators seem to induce mucin overproduction via the induction of COX2 and PGE_2 pathway. The expression

of COX2 and MUC5AC are both regulated by CREB. Moreover, it was recently demonstrated that the number of inflammatory CD45+ cells in human asthmatic bronchial submucosa is highly correlated to p-CREB expression in the same region, and the production of inflammatory mediators are also positively associated with p-CREB level (29). Hence, the importance of CREB in the progression of inflammation cannot be over emphasized. In addition to binding to CRE, CREB can also interact with other transcription factors and co-activators (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 $PGF_{2\alpha}$ may further potentiate the transcription activation initiated by other factors.

In conjunction with our previous studies which elucidated the signaling of IL-1β- and PGE2-induced mucin overproduction (12,19), we conclude the signaling network for PGs and IL-1β-induced mucin production in our current 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 feed back cycle of PGs signaling, but also facilitates both the short term 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 $PGF_{2\alpha}$-induced MUC5AC overproduction and CREB activation, not only increase our understanding about the signal transduction of IL-1β and prostaglandins but also have important clinical implications for the management of airway inflammation and other pathological conditions.

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ABBREVIATIONS

ChIP
: Chromatin immunoprecipitation

COX2
: cyclooxygenase 2

NHTBE
: normal human tracheobronchial epithelial

PKA
: Protein Kinase A

PKC
: protein kinase C

RSK
: p90 ribosomal S6 protein kinase

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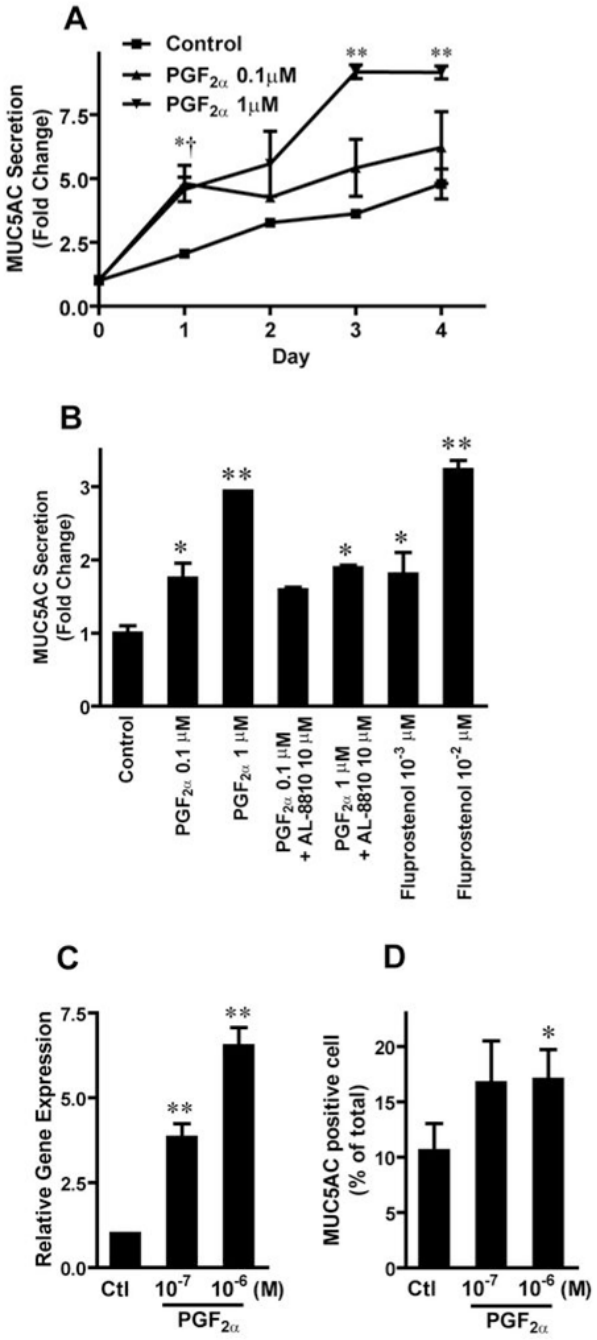

Fig. 1. $PGF_{2\alpha}$ induces MUC5AC overproduction in NHTBE cells

Fourteen-day culture of NHTBC cells in air-liquid-interface system were treated with 0.1 or 1 μM $PGF_{2\alpha}$ for 4 days. The secreted MUC5AC was collected each day and assayed with immuno-dot blotting (A). Twenty four hour MUC5AC secretion was also collected from cells treated with $PGF_{2\alpha}$ for 4 days in the absence or presence of FP antagonist AL-8810 (10 μM) and from cells treated with FP agonist fluprostenol (B). *MUC5AC* gene expression was assayed using RT-PCR (C). The percentage of cells expressing MUC5AC in each culture was measured with flow cytometry using antibody against MUC5AC (D). Data are shown as means ± S.E. from at least three experiments. *: $p<0.05$, **: $p<0.01$.

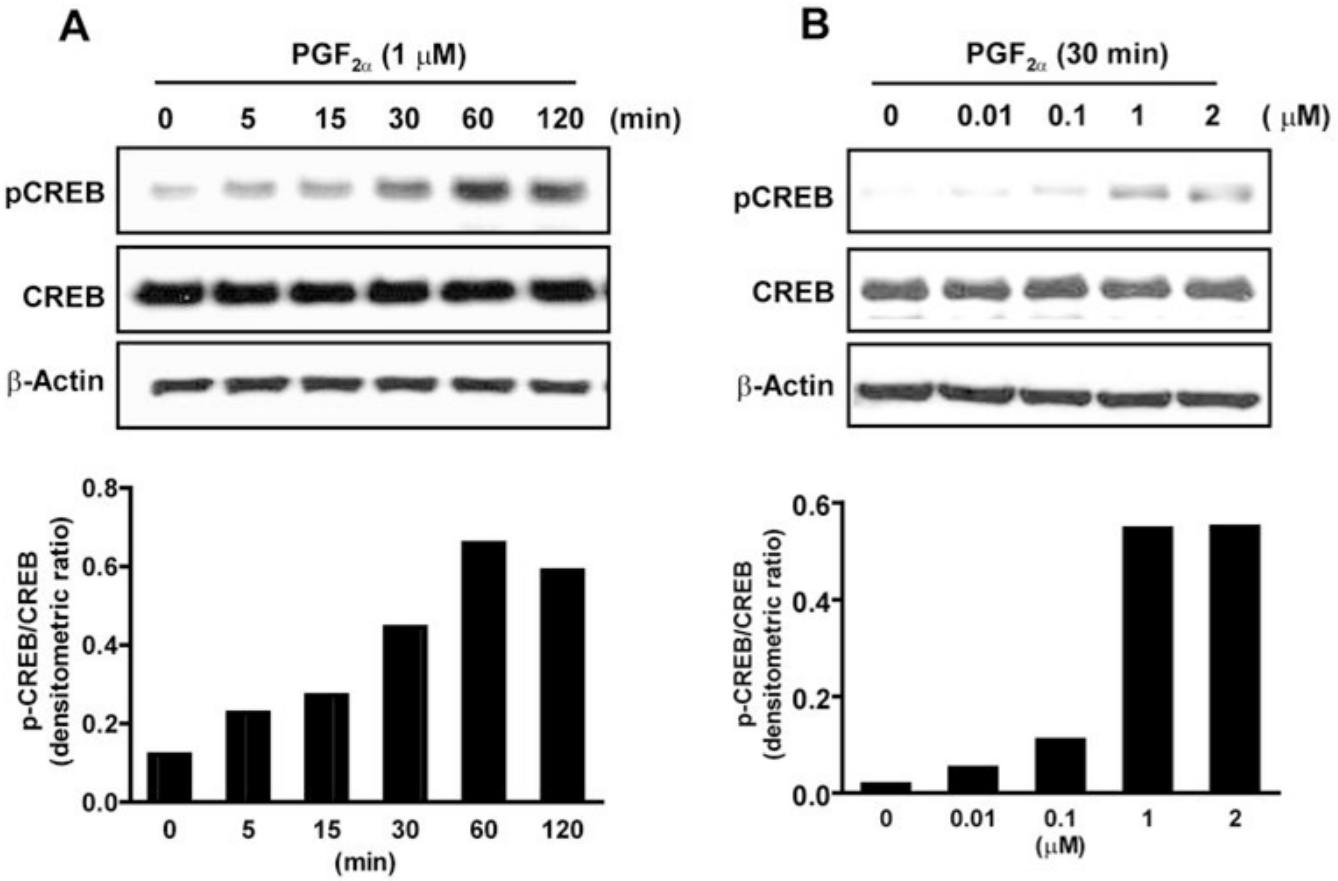

Fig. 2. $PGF_{2\alpha}$ activates CREB in a time- and concentration-dependent manner

NHTBE cells were treated with 1 μM $PGF_{2\alpha}$ for the indicated periods of time (A) or with various concentrations of $PGF_{2\alpha}$ for 30 min (B). After treatment, whole-cell lysates were prepared and subjected to western blot analysis using anti-CREB and anti-pCREB antibodies. Equal loading of samples was confirmed by stripping the blot and re-probing it with β-actin antibody. The ratio of the band intensity between CREB and pCREB was plotted below the gel image.

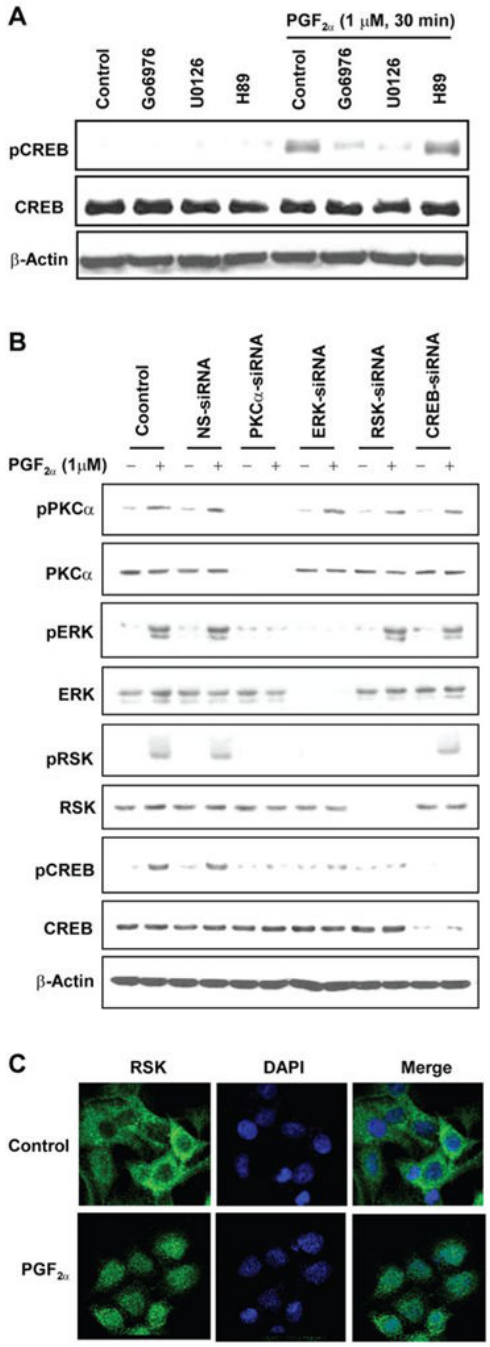

Fig. 3. $PGF_{2\alpha}$-induced activation of CREB is mediated through the PKCα/ERK/RSK signal transduction pathway

(A) NHTBE cells were preincubated with various signal transduction inhibitors (10 μM) for 60 min and then treated with 1 μM $PGF_{2\alpha}$ or with vehicle control for 30 min. Whole-cell lysates were prepared and subjected to western blot analysis using anti-CREB and anti-pCREB antibodies. Equal loading of samples was confirmed by noting equal amounts of β-actin in each lane. (B) NHTBE cells were transfected with siRNA of PKCα, ERK, RSK, or a non-specific 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 western blot using the indicated antibodies. (C) NHTBE cells grown

on coverslips for 7 days were analyzed by immunocytofluorescence. Cells were incubated with a vehicle control (upper panel) or with 1 μM $PGF_{2\alpha}$ (bottom panel) for 30 min before fixation. After fixation, the cells were stained with RSK antibody followed by anti-rabbit AlexaFluor 488 antibodies (green), and nuclei were stained with 4′6′-diamidino-2-phenylindole (DAPI; blue) and then the two images merged.

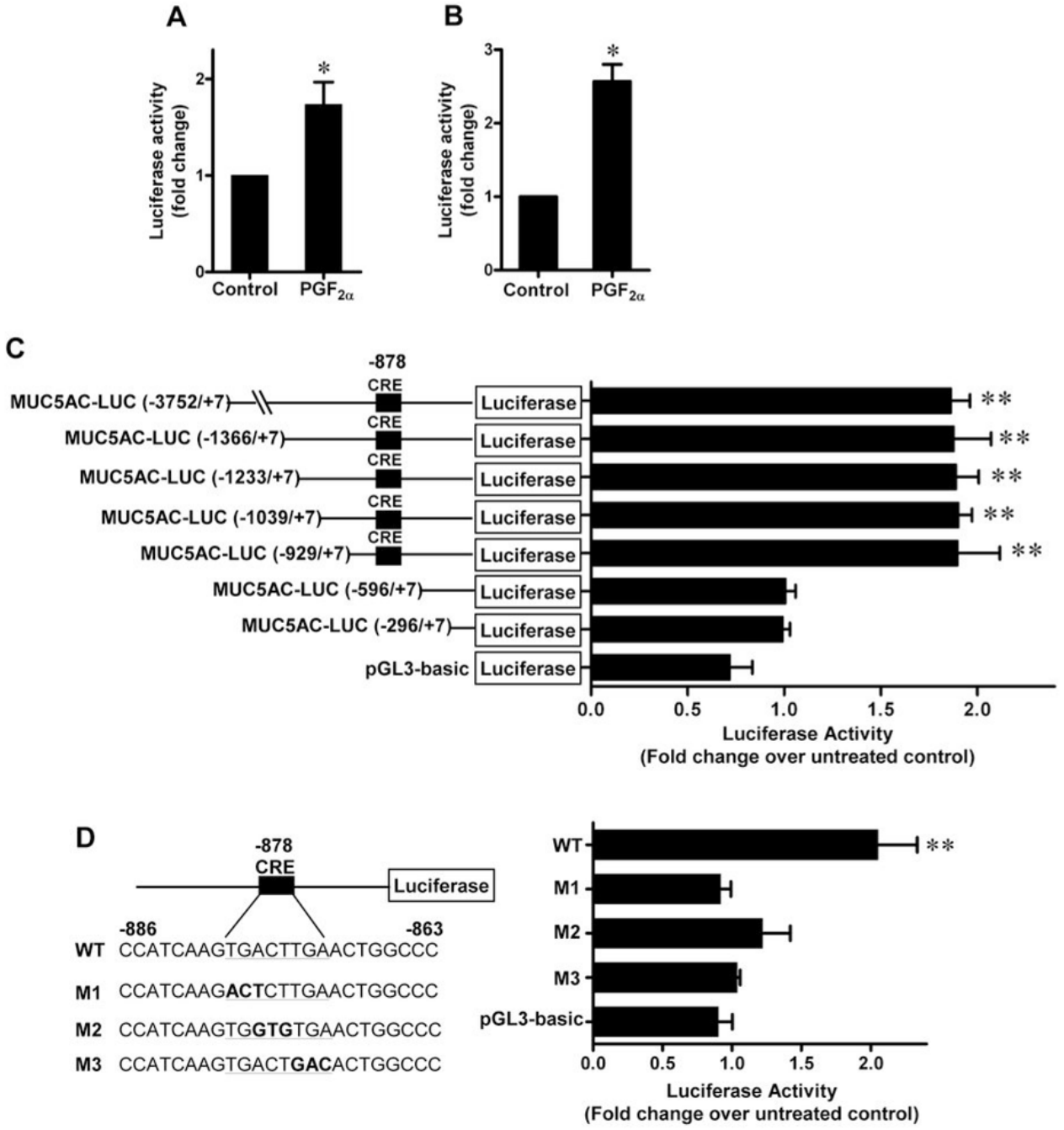

Fig. 4. $PGF_{2\alpha}$ induces CRE-dependent transactivation, and CRE in *MUC5AC* promoter is indispensable for $PGF_{2\alpha}$-induced *MUC5AC* promoter activity

NCI-H292 (A) and NHTBE (B) cells were transiently co-transfected 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 $PGF_{2\alpha}$ or with vehicle control for 48 h. The data are normalized to the change in luciferase activity (expressed as -fold) relative to the untreated control and expressed as the means ± S.E. of three independent experiments, each performed in triplicate. *: $p<0.05$, **: $p<0.01$.

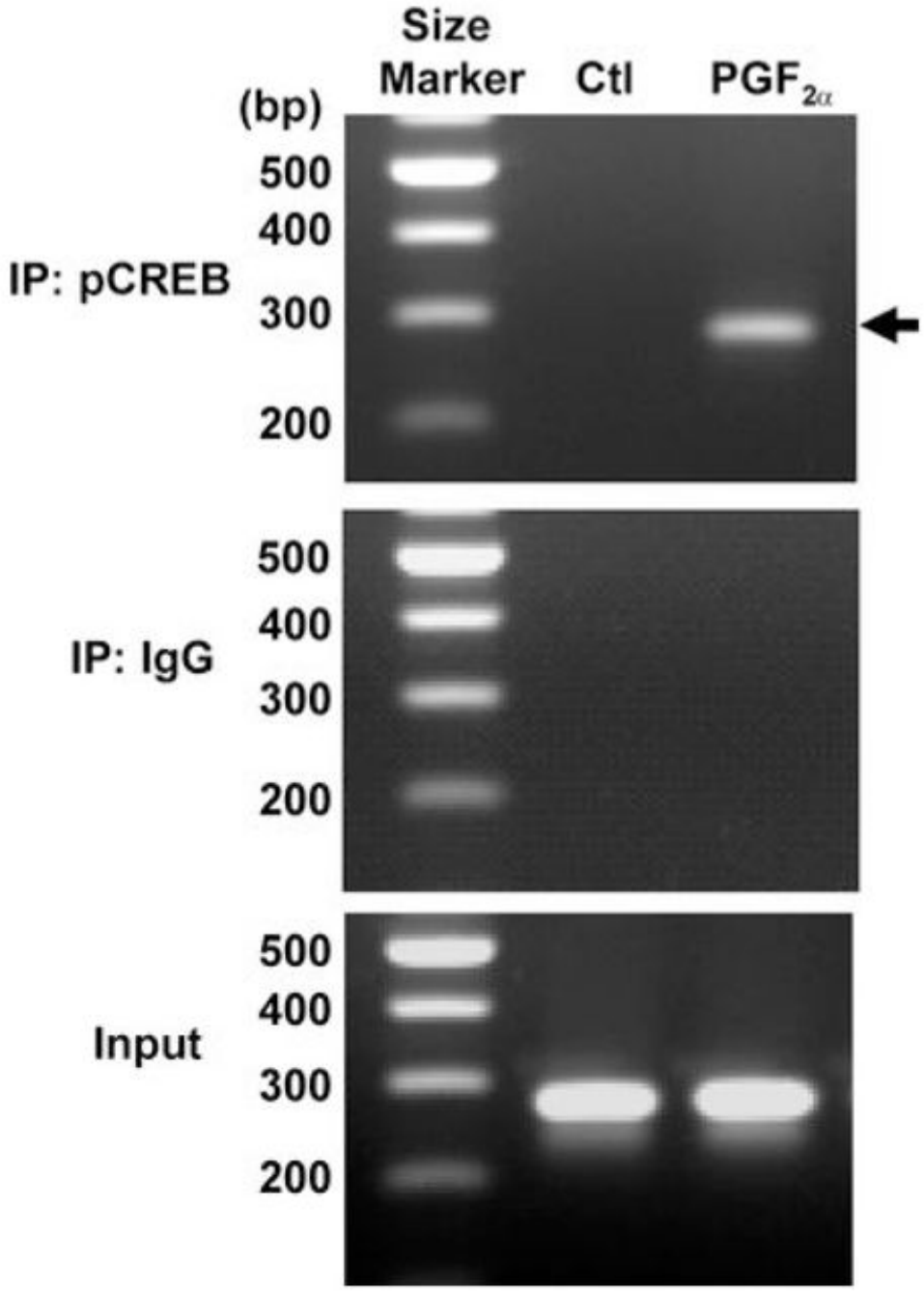

Fig. 5. $PGF_{2\alpha}$ induces binding of phospho-CREB to CRE site on *MUC5AC* promoter

NHTBE cells were treated with 1 μM $PGF_{2\alpha}$ for 4 h, and then were assayed by Chromatin immunoprecipitation (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 antibody (Control IgG) was assayed under otherwise identical conditions as a negative control. Results shown are representative of three independent experiments.

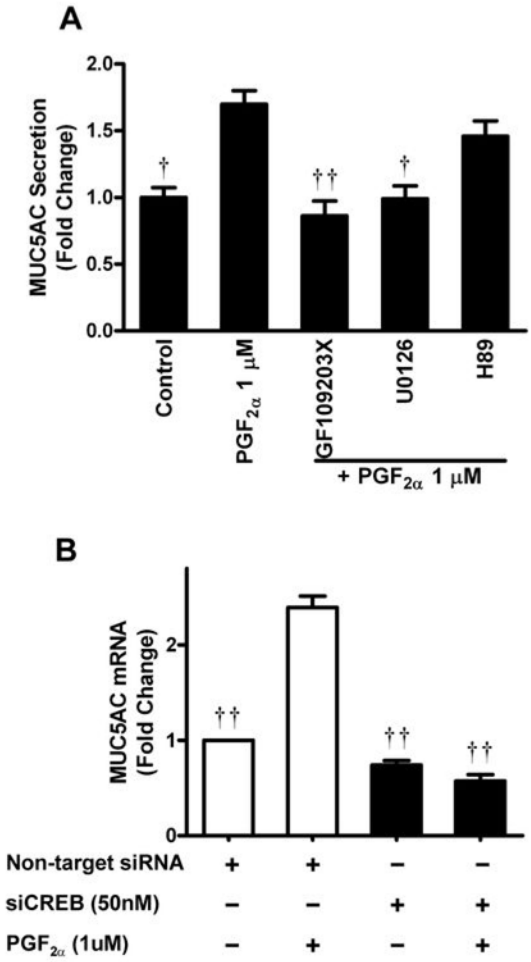

Fig. 6. Disruption of PKC-CREB pathway abolishes $PGF_{2\alpha}$-induced *MUC5AC* expression

NHTBE cells grown on trans-wells were treated with inhibitor against PKC (GF109203X, 10 μM) or ERK (U0126, 10 μM) or PKA (H89, 10 μM) before $PGF_{2\alpha}$ stimulation (A), MUC5AC secretion was collected after 24 h treatment. NHTBE cells grown on regular plastic well were transfected with siRNA against CREB or non-target control (B) (closed bar) or vehicle (open bar). Expression levels of *MUC5AC* were measured with RT-PCR. The data are expressed as means ± S.E.of fold change relative to the non-target siRNA-transfected untreated control. †: $p<0.05$, ††: $p<0.01$ (versus $PGF_{2\alpha}$ stimulated without inhibitor or CREB siRNA).

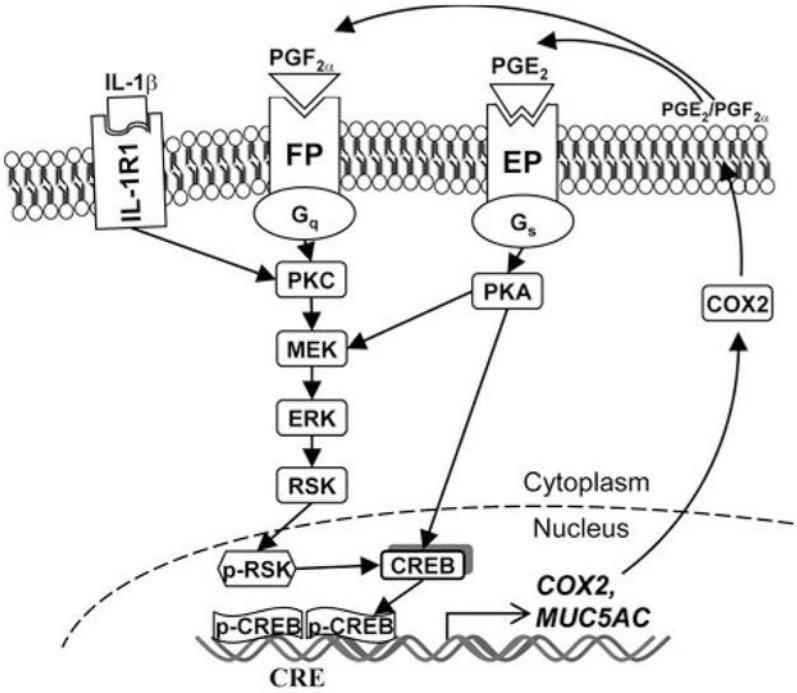

Fig. 7. IL-1β and prostaglandins induce MUC5AC overexpression

IL-1β, $PGF_{2\alpha}$ and PGE_2 activate CREB-regulated *COX2* and *MUC5AC* gene expression through PKA or PKC mediated MAPK/RSK pathway. When synthesized, COX2 catalyzes the production of prostanoids, including PGE_2 and $PGF_{2\alpha}$, which further stimulate their respective receptors and subsequent production of mucin and COX2 through the same pathways. After activation of IL-1β and $PGF_{2\alpha}$ receptors (IL-1R1 and FP), the cytosolic signaling molecules are subsequently phosphorylated to the active state in the order of PKC, MEK, ERK, RSK, and followed by translocation of RSK into nucleus to activate CREB. The stimulation of PGE_2 receptor (EP), which activates PKA pathway, also leads to CREB activation. The binding of activated CREB to CRE motif on *MUC5AC* and *COX2* promoters triggers their expression.