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Inhibition of acrolein-stimulated MUC5AC expression by *Platycodon grandiflorum* root-derived saponin in A549 cells

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ABSTRACT

Mucin overproduction is a hallmark of chronic airway diseases such as chronic obstructive pulmonary disease. In this study, we investigated the inhibition of acrolein-induced expression of mucin 5, subtypes A and C (MUC5AC) by Changkil saponin (CKS) in A549 cells. Acrolein, a known toxin in tobacco smoke and an endogenous mediator of oxidative stress, increases the expression of airway MUC5AC, a major component of airway mucus. CKS, a *Platycodon grandiflorum* root-derived saponin, inhibited acrolein-induced MUC5AC expression and activity, through the suppression of NF-κB activation. CKS also repressed acrolein-induced phosphorylation of ERK1/2, JNK1/2, and p38MAPK, which are upstream signaling molecules that control MUC5AC expression. In addition, the MAPK inhibitors PD98059 (ERK1/2), SP600125 (JNK1/2), and SB203580 (p38 MAPK), and a PKC delta inhibitor (rottlerin; PKCδ) inhibited acrolein-induced MUC5AC expression and activity. CKS repressed acrolein-induced phosphorylation of PKCδ. Moreover, a reactive oxygen species (ROS) inhibitor, *N*-acetylcysteine, inhibited acrolein-induced MUC5AC expression and activity through the suppression of PKCδ and MAPK activation, and CKS repressed acrolein-induced ROS production. These results suggest that CKS suppresses acrolein-induced MUC5AC expression by inhibiting the activation of NF-κB via ROS–PKCδ–MAPK signaling.

1. Introduction

Chronic obstructive pulmonary disease (COPD) involves airway inflammation and constriction, and is the fourth leading cause of mortality and morbidity worldwide (Kliber et al., 2010). Chronic exposure to inhaled allergens such as mites, fungi, infections, cigarette smoke, oxidants, and chlorinated irritants is a major cause of COPD. Cigarette smoke is the most significant risk factor for COPD and leads to structural changes during airway remodeling, inflammation of the airway, and a decline of lung function (Aoshiba and Nagai, 2004; Bracke et al., 2006).

Acrolein (CH_2=CH—CHO) is a major component of cigarette smoke and is the most reactive α,β-unsaturated aldehyde found in the environment. Acrolein is produced by a wide variety of both natural and synthetic processes, including the incomplete combustion of organic material. The International Agency for Research on Cancer (IARC) reported that the acrolein concentration in cigarette smoke ranged from 3 to 220 μg/cigarette, depending on the type of cigarette. Inhaled acrolein induces hyperresponsiveness of bronchial smooth muscle (Ben-Jebria et al., 1994). Recent studies have reported that increased acrolein levels are associated with many diseases, including atherosclerosis, Alzheimer's disease, and diabetes, suggesting that acrolein may be involved in the pathogenesis of these conditions (Daimon et al., 2003; Lovell et al., 2001; Uchida et al., 1998).

Acrolein has also been reported as a biological marker of oxidative stress (Kehrer and Biswal, 2000). It was shown to increase the intracellular level of reactive oxygen species (ROS) (Park et al., 2005; Uchida et al., 1998) in PC12 cells (Luo et al., 2005), bronchial epithelial cells (Nardini et al., 2002), and umbilical vein endothelial cells (Park et al., 2005). ROS-mediated cell damage is an important etiological factor in the pathogenesis of chronic inflammatory airway disease (Leopold and Loscalzo, 2005) and contributes to mucin production in the airway epithelium.

Mucin is a complex dilute aqueous solution of lipids, glycoconjugates, and proteins. Airway mucus forms a protective barrier between the respiratory tract epithelium and the environment. At least 19 mucin (MUC) genes are expressed in the human airway. Among these, MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6–9, 11–13, and 16–20 (Higuchi et al., 2004; Moniaux et al., 2001), four of which are

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gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC19), are expressed in the lung. MUC5AC is a major respiratory mucin present in secretions from goblet cells, and its production is enhanced by a variety of stimuli such as neutrophil elastase (Voynow et al., 1999), air pollutants (Longphre et al., 2000), and bacterial products (Wang et al., 2002). The oxidants in cigarette smoke trigger mitogen-activated protein kinase (MAPK) signaling cascades in lung epithelial cells (Mossman et al., 2006). Airway MUC5AC is transcriptionally upregulated by cigarette smoke via a process mediated by NF-κB, which is composed of the response element binding proteins p65 and p50 (Liu et al., 2009). Furthermore, PKC is involved in the induction of MUC2 and MUC5AC over-expression in airway and goblet cells by TNFα or bacterial components (Levine et al., 1995).

Platycodi radix, the root of *Platycodon grandiflorum* (Campanulaceae family), has been used as a traditional oriental medicine for the treatment of bronchitis, asthma, pulmonary tuberculosis, and inflammation, in addition to its use as a sedative (Lee, 1973). In previous studies, Changkil (CK), an aqueous extract of the roots of 20-year-old *P. grandiflorum* plants, prevented hypercholesterolemia and hyperlipidemia (Kim et al., 1995). Recently, it was shown that *P. grandiflorum* root-derived saponin (Changkil saponin; CKS) has protective effects against acetaminophen- and carbon tetrachloride-induced hepatotoxicity (Lee et al., 2001; Lee and Jeong, 2002) and can inhibit anaphylactic reactions, IgE-mediated allergic responses in mast cells (Han et al., 2009), and TNFα-induced expression of adhesion molecules in human endothelial ECV 304 cells (Kim et al., 2006). In addition, we reported that CK inhibits ovalbumin-induced airway inflammation in a murine model of asthma (Choi et al., 2009).

In the present study, we examined the inhibitory effect of CKS on acrolein-induced MUC5AC expression and NF-κB-mediated signaling in a human lung carcinoma cell line. We demonstrate that CKS represses acrolein-induced MUC5AC expression by inhibiting NF-κB activation via the ROS–PKCδ–MAPK signaling pathway in A549 cells.

2. Materials and methods

2.1. Chemicals

Acrolein, *N*-acetyl-cysteine (NAC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO); lactate dehydrogenase (LDH), from Roche (Indianapolis, IN); RPMI1640, fetal bovine serum (FBS), sodium pyruvate, and penicillin–streptomycin, from Gibco BRL (Grand Island, NY); the luciferase assay system, from Promega (Madison, WI); and LipofectAMINE 2000 and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), from Invitrogen (San Diego, CA). PD98059, SP600125, SB203580, rottlerin, JSH-23 and phospho-PKCδ antibody were obtained from Calbiochem (La Jolla, CA). Mucin 5AC (45M1), NF-κB p65, IκBα, lamin B1, and β-actin (C4) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against phospho-ERKl/2, phospho-JNK1/2, and phospho-p38 MAPK, and HRP-linked anti-rabbit IgG secondary antibody were purchased from Cell Signaling Technologies (Beverly, MA). Peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated streptavidin were from Jackson ImmunoResearch Laboratories, Inc. (Baltimore Pike, PA). TMB substrate reagent was obtained from BD Biosciences (San Diego, CA), and the enhanced chemiluminescence system and polyvinylidene difluoride (PVDF) membranes were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Daejeon, Korea).

2.2. Preparation of CKS

CKS refers to an aqueous extract obtained from 20-year-old roots of *P. grandiflorum*, supplied by Jang Saeng Doraji Co., Jinju, South Korea. The composition of *P. grandiflorum* root has been reported elsewhere (Kim et al., 1995). CK and CKS were prepared as previously described (Lee et al., 2001, 2004; Lee and Jeong, 2002). CK was subjected to column chromatography over amberlite XAD-2, Diaion MCl Gel HP20, or Kogel BG4600. Saccharides and amino acids were eluted from the column with water, and then CKS was obtained by elution with methanol (Tada et al., 1975).

2.3. Cell culture

The A549 human lung carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin, at 37 °C in a humidified chamber with 5% CO_2. To avoid the interference of growth factor in serum, A549 cells were serum-starved for 24 h and then stimulated with CKS or acrolein.

2.4. Measurement of cell cytotoxicity

A549 cells were plated at a density of 5×10^4 cells/500 μl in 48-well plates, and cell viability was determined by MTT and LDH leakage assays. After incubation, the cells were treated with MTT solution (final concentration, 1 mg/ml) for 1 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO, and the absorbance at 570 nm was measured with a microplate reader (Thermo Electron Co., Waltham, MA). The level of LDH release was measured in the supernatants using a colorimetric test based on the level of NADH. CKS did not interfere with the absorbance measurements at the wavelengths used in these assays.

2.5. Semi-quantitative RT-PCR

Total RNA was extracted from A549 cells using RNAiso reagent (Takara, Kyoto, Japan) and stored at −80 °C. The RNA was reverse transcribed to form cDNA, which was used as a template for the polymerase chain reaction (PCR), performed using a Takara thermal cycler (Takara, Seoul, Korea) according to the manufacturer's instructions. The primer sets for PCR amplification were as follows: MUC5AC forward (5′-CGA CAA CTA CTT CTG CGG TGC-3′) and reverse (5′-GCA CTC ATC CTT CCT GTC GTT-3′); and GAPDH forward (5′-ACC ACA GTC CAT GCC ATC AC-3′) and reverse (5′-TCC ACC ACC TGT TGC TGT-3′). The PCR conditions for MUC5AC amplification were 38 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; the conditions for GAPDH were 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The amplified products were resolved by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and imaged using a gel documentation system (UVP, Cambridge, UK).

2.6. MUC5AC protein measurement by ELISA

MUC5AC protein levels were measured by enzyme-linked immunosorbent assay (ELISA) as previously reported (Song et al., 2007). Briefly, culture supernatant (50 μl/well) was added to 96-well ELISA plates and dried at 44 °C. The plates were washed three times with phosphate-buffered saline (PBS) and blocked in 2% bovine serum albumin (BSA) for 1 h, followed by incubation with 50 μl of anti-mucin 5AC (45M1) antibody (1:100 dilution) for 1 h. The plates were washed, peroxidase-conjugated goat anti-mouse IgG (1:2500 dilution in PBS containing 15% FBS) was added to the wells (100 μl/well), and the plate was incubated for 4 h. Peroxidase-conjugated streptavidin was added (100 μl/well), followed by the addition of TMB as a chromogen. Immunoreactive proteins were detected by measuring the absorbance at 450 nm using a microplate reader (Thermo Electron Co., Waltham, MA). The results are expressed as a percentage of the baseline control.

2.7. Western blot analysis

After treatment, the cells were washed with cold PBS and lysed on ice for 30 min in 100 μl of lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% NP40), followed by centrifugation at 13,000×*g* for 20 min. Lysate samples (50 μg of protein) were electrophoresed in 10% SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in 5% non-fat dry milk at room temperature for 1 h, incubated with the indicated primary antibodies for 2 h, and then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h. Immunoreactive proteins were detected by enhanced chemiluminescence. Band intensity was quantified by densitometry using Image J program (Image Processing and Analysis in Java, NIH, USA). Quantitation of protein activation and expression levels was expressed as relative folds compared to control group.

2.8. Transient transfection and luciferase activity assay

The 3.7-kb 5′ flanking region of the human MUC5AC gene in a luciferase reporter vector pGL4 was amplified from human genomic DNA through PCR as already described (Li et al., 1998; Wang et al., 2002). The DNA sequence of MUC5AC-wt promoter was confirmed by dideoxynucleotide sequencing. Deletional mutants (MUC5AC-NF-κB mt and MUC5AC-AP-1 mt) of the 5′-flanking region DNA were obtained by combining restriction digestion of the upstream region of the gene and PCR amplification (Li et al., 1998; Wang et al., 2002). The restriction DNA fragments or PCR-amplified fragments were ligated into a luciferase reporter gene. For transient transfections, A549 cells were seeded at 5×10^4 cells/500 μl/well in a 48-well plate. At 70–80% confluence, the cells were washed with PBS and incubated with RPMI 1640 without serum or antibiotics for 3 h. The cells were then transfected with the MUC5AC, MUC5AC-NF-κB mt, MUC5AC-AP-1 mt, NF-κB (5′-GGGGACTTTCC-3′)$_n$ ($n = 5$), or AP-1 (5′-TGACTAA-3′)$_n$ ($n = 7$) reporter vector

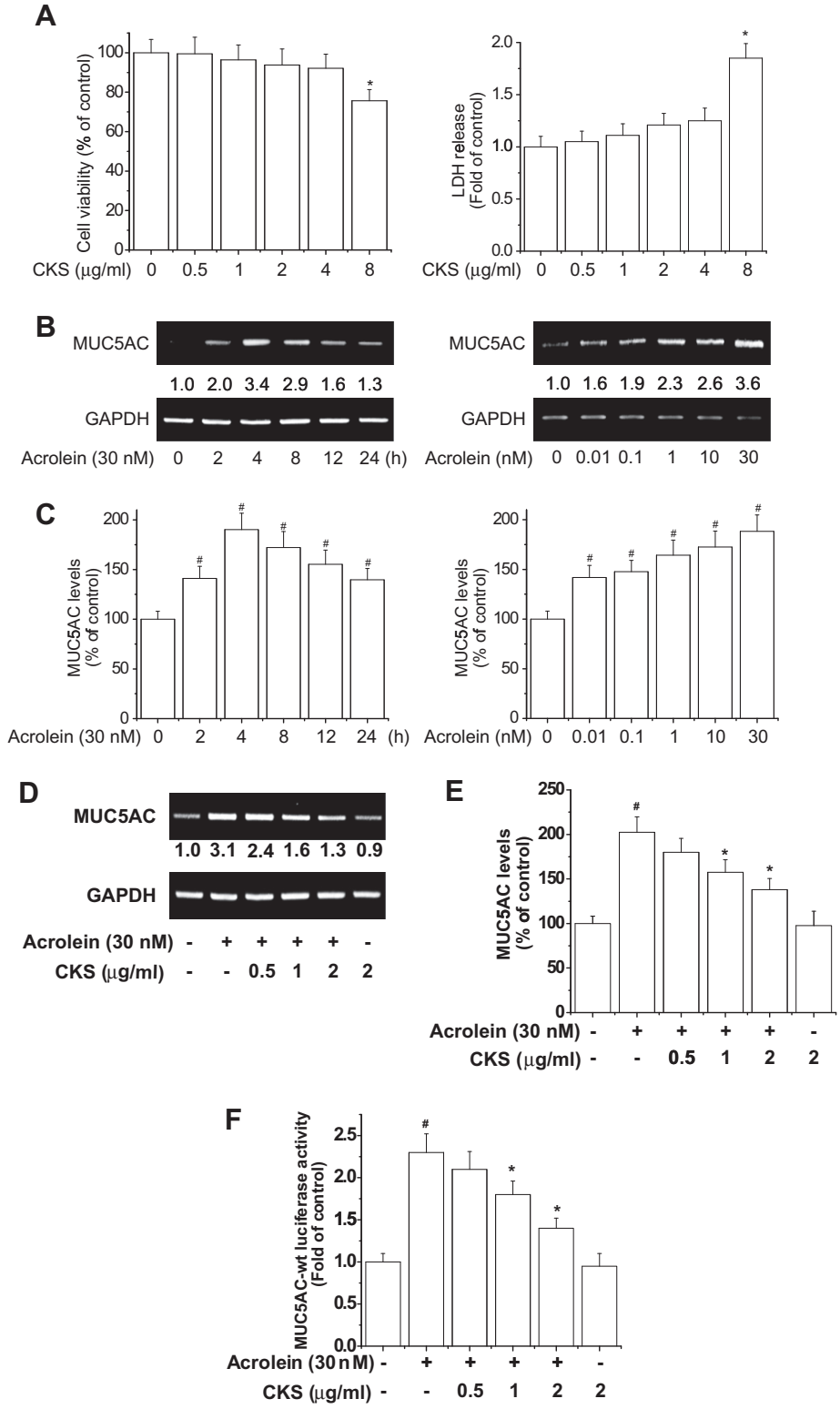

Fig. 1. Effects of CKS on acrolein-induced MUC5AC expression in A549 cells. (A) Cells were treated with 0, 0.5, 1, 2, and 4 μg/ml CKS for 24 h in serum-free medium. Cell cytotoxicity was assessed by MTT and LDH assays. (B) Following 24-h serum starvation, the cells were exposed to 30 nM acrolein for 0, 2, 4, 8, 12, or 24 h, or to 0, 0.01, 0.1, 1, 10, and 30 nM acrolein for 4 h. The cells were lysed, and total RNA was analyzed by semi-quantitative RT-PCR. (C) MUC5AC protein levels in the culture supernatants were measured by ELISA. (D) After 24-h serum starvation, the cells were incubated with CKS for 24 h, and then acrolein was added for 4 h. The cells were lysed, and total RNA was analyzed by semi-quantitative RT-PCR. (E) After 24-h serum starvation, the cells were incubated with CKS for 24 h, and then acrolein was added for 4 h. MUC5AC protein levels in the culture supernatants were measured by ELISA. (F) Cells were transfected with MUC5AC-Luc reporter plasmid. Cells were pretreated with CKS (0.5–2 μg/ml) for 24 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group.

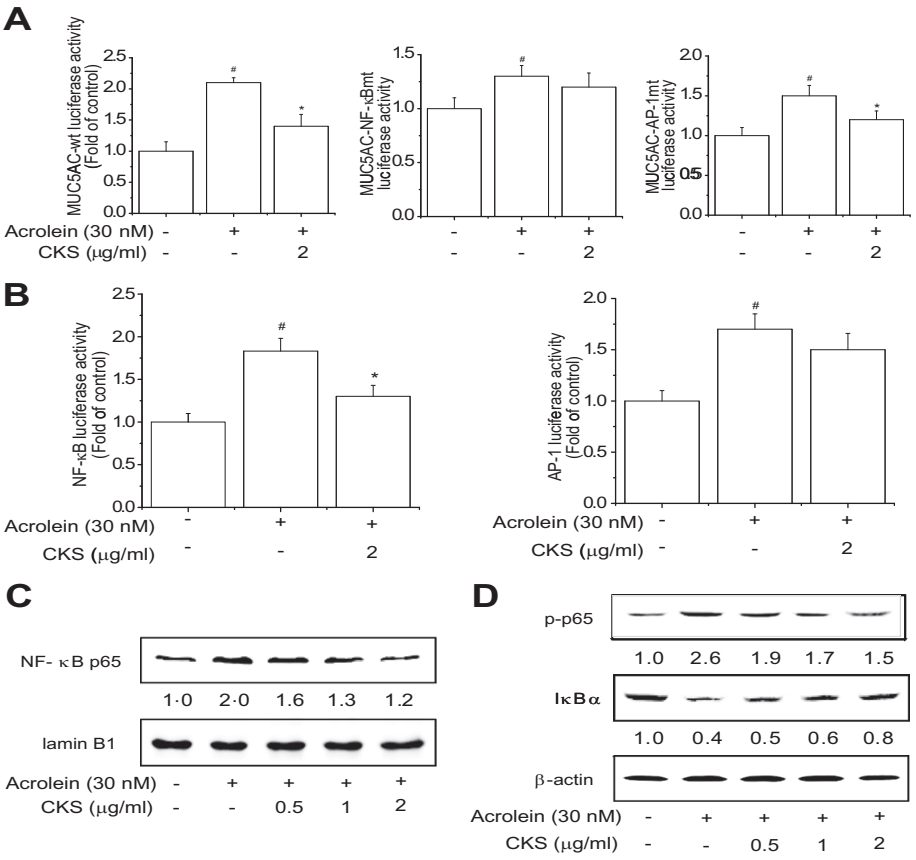

Fig. 2. Effects of CKS on acrolein-induced luciferase activity in A549 cells. (A) A549 cells were transfected with MUC5AC-Luc, MUC5AC-NF-κB mt-Luc, or MUC5AC-AP-1 mt-Luc reporter plasmids. (B) Cells were transfected with reporter plasmids containing tandem elements for NF-κB or AP-1 binding sites. Cells were pretreated with CKS (2 μg/ml) for 24 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. (C) Cells were pretreated with CKS (0.5–2 μg/ml) for 1 h and subsequently treated with 30 nM acrolein for 1 h. Nuclear extracts were subjected to SDS–PAGE, followed by Western blot analysis with anti-NF-κB p65 antibody. (D) Cells were pretreated with CKS (0.5–2 μg/ml) for 1 h and treated with 30 nM acrolein for 1 h. Whole-cell extracts were subjected to SDS–PAGE, followed by Western blot analysis with anti-phospho-NF-κB p65 and anti-IκBα antibodies. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group.

(1 μg; Stratagene, La Jolla, CA) and a β-galactosidase reporter vector (0.5 μg) using LipofectAMINE 2000 reagent according to the manufacturer's protocol. After 5 h, the medium was replaced with basal medium. The cells were incubated with CKS for 24 h, then acrolein was added, and the cells were incubated for another 4 h. After incubation, the cells were lysed, and luciferase activity was measured using a luminometer (Thermo Electron Co.). Luciferase activity was normalized to β-galactosidase activity in the cell lysates and expressed as an average of three independent experiments.

2.9. Measurement of intracellular ROS production

A fluorescent probe, CM-H_2DCFDA, was used to determine the intracellular generation of ROS by acrolein, as previously described (Kim et al., 2006). Briefly, the cells in 48-well plates were pretreated with CKS for 24 h, the supernatant was removed, and the cells were incubated with 25 μM DCFDA for 30 min. Then, the cells were stimulated with acrolein for 30 min. Intracellular ROS was detected as the fluorescence at excitation and emission wavelengths of 485 and 530 nm, respectively, and was measured using a fluorescence spectrophotometer (Biotek, Winooski, VT).

2.10. Statistical analyses

All experiments were performed in triplicate. The results are expressed as means ± S.E.M. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test. A significant value was defined as $P < 0.05$.

3. Results

3.1. Inhibitory effects of CKS on acrolein-stimulated MUC5AC mRNA and protein levels

The MUC5AC gene expression is regulated by epidermal growth factor (EGF) in the lung. MUC5AC mRNA expression was increased after ligand binding to the EGF receptor and activation of the MAPK cascade (Takeyama et al., 2000). EGF signaling may be involved in mucin production in lung adenocarcinoma as well as normal goblet cells. To avoid the interference of growth factor in serum, A549 cells were cultured in RPMI1640 medium without FBS. For serum deprivation, confluent cells were washed twice with PBS and cultured in RPMI1640 without FBS for 24 h. We first determined the concentration dependence of the cytotoxic effects of CKS for 24 h in A549 cells using the MTT and LDH assays (Fig. 1A). CKS at concentrations lower than 4 μg/ml had no cytotoxic effect on the cells and CKS at 8 μg/ml showed about a 25% decreases in cell viability (Fig. 1A). Thus, CKS had no significant cytotoxicity in A549 cells at these concentrations.

Acrolein, a potent inducer of MUC5AC activation, was used to induce MUC5AC mRNA and protein expression (Deshmukh et al., 2005; Pokharel et al., 2008). The time and concentration appropri-

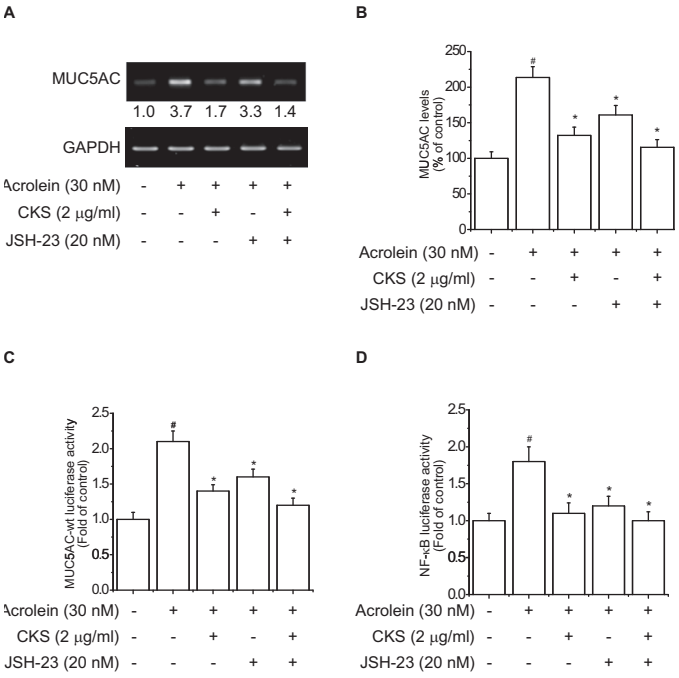

Fig. 3. Effects of CKS on the acrolein-induced activation of NF-κB. (A) Following 24-h serum starvation, cells were treated with CKS and/or JSH-23 for 1 h, and then acrolein was added for 4 h. The cells were lysed, and total RNA was analyzed by semi-quantitative RT-PCR. (B) MUC5AC protein levels in the culture supernatants were measured by ELISA. (C) Cells were transfected with MUC5AC-Luc reporter plasmids. Cells were pretreated with CKS (0.5–2 μg/ml) for 24 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. (D) Cells were transfected with reporter plasmids containing tandem elements for NF-κB binding sites. Cells were pretreated with CKS (0.5–2 μg/ml) for 24 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group.

ate for investigating acrolein induction of MUC5AC were initially determined. Acrolein stimulated rapid, transient, and concentration-dependent increases in MUC5AC mRNA and protein levels in A549 cells. The levels were elevated at 2 h and maximal at 4 h, after which the levels decreased (Fig. 1B and C). We then examined the inhibition of acrolein-induced MUC5AC expression by CKS. Pretreatment of A549 cells with CKS decreased the acrolein-induced MUC5AC mRNA and protein expression, in a concentration-dependent manner (Fig. 1D and E). The effect of CKS on MUC5AC promoter activity was investigated in cells transiently transfected with a luciferase reporter gene linked to the MUC5AC promoter sequence. As shown in Fig. 1F, luciferase expression was activated by up to 2.4-fold in cells treated with acrolein compared with untreated cells. Pretreatment of the cells with CKS decreased the acrolein-induced luciferase activity, in a concentration-dependent manner. Treatment with CKS alone has no effect the basal expression of MUC5AC, basal level of MUC5AC and MUC5AC promoter activity. These results suggest that CKS inhibits MUC5AC expression at the transcriptional level and that the MUC5AC promoter contain a CKS-responsive element.

3.2. Inhibitory effects of CKS on acrolein-stimulated AP-1 and NF-κB activities

MUC5AC gene expression is regulated at the transcriptional level through interactions of the AP-1 and NF-κB binding sequences in the MUC5AC gene promoter (Kato et al., 2006). Treatment with acrolein is known to enhance MUC5AC mRNA and protein expression through the activation of NF-κB and AP-1 (Pokharel et al., 2008). To investigate the roles of these transcription factors in regulating MUC5AC gene expression, A549 cells were transiently transfected with a reporter gene construct containing the wild-type MUC5AC promoter, a NF-κB site-mutated promoter, or an AP-1 site-mutated promoter. Treatment of the cells with CKS in the presence of acrolein decreased the transcription of the reporter gene in the construct harboring the AP-1 site mutation, but had no effect on the reporter activity of the construct with the NF-κB site mutation, suggesting that NF-κB is the target of CKS (Fig. 2A). The luciferase activity in cells transfected with the wild-type NF-κB reporter was significantly reduced by treatment with CKS, whereas CKS had no significant effect on the luciferase activity of cells transfected with a reporter vector containing tandem AP-1 binding sites (Fig. 2B).

3.3. Inhibitory effects of CKS on acrolein-stimulated MUC5AC activation through the suppression of acrolein-stimulated NF-κB activity

A549 cells were incubated with different concentrations of CKS in the presence of acrolein for 1 h, and nuclear extracts were evaluated by Western blot analysis. As shown in Fig. 2C, CKS reduced the acrolein-induced nuclear translocation of NF-κB p65, in a concentration-dependent manner. Measurements of NF-κB p65 phosphorylation and IκBα degradation in whole-cell extracts revealed that CKS reduced the acrolein-induced phosphorylation of NF-κB p65 and degradation of IκBα (Fig. 2D). We used a selective NF-κB activation inhibitor, JSH-23 (Hwang et al., 2010), to determine whether NF-κB activation is involved in acrolein-induced MUC5AC expression in A549 cells. Cells pretreated with JSH-23 (20 nM) for 1 h were stimulated with 30 nM acrolein for 4 h in the presence or absence of CKS (2 μg/ml). Treatment with JSH-23 inhibited the acrolein-induced MUC5AC mRNA and protein expression, and promoter activity (Fig. 3A–C). In addition, JSH-23 inhibited the acrolein-induced NF-κB activity (Fig. 3D). JSH-23 alone did not alter cell viability or expression of MUC5AC mRNA, protein expression, promoter activity, and NF-κB activity in control treated cells (data not shown).

3.4. Inhibitory effects of CKS on acrolein-stimulated MAPK

MAPK signaling is an important upstream regulator of MUC5AC expression and NF-κB signaling (Gensch et al., 2004). We therefore investigated the effects of CKS on EKR1/2, JNK1/2, and p38 MAPK activities. Fig. 4A demonstrates that pretreatment of A549 cells with CKS for 1 h prior to the addition of acrolein decreased the acrolein-induced activation of EKR1/2, JNK1/2, and p38 MAPK. Furthermore, pretreatment with the specific kinase inhibitors PD98059 (ERK1/2), SP600125 (JNK1/2), and SB203580 (p38 MAPK) decreased the acrolein-induced MUC5AC mRNA and protein expression and the acrolein-induced luciferase reporter activity (Fig. 4B–D). In addition, MAPK inhibitor inhibited the acrolein-induced NF-κB activity (Fig. 4E). MAPK inhibitor alone did not alter cell viability or expression of MUC5AC mRNA, protein expression, promoter activity, and NF-κB activity in control treated cells (data not shown).

3.5. Inhibitory effects of CKS on acrolein-stimulated PKCδ

Acrolein activates PKCδ within 10 min, and the activation gradually decreases 30 min after stimulation (Park et al., 2007). Pretreatment of A549 cells with CKS for 1 h prior to the addition of acrolein decreased the acrolein-induced activation of PKCδ (Fig. 5A). In addition, treatment with the PKCδ inhibitor rottlerin decreased the acrolein-induced MUC5AC mRNA and protein

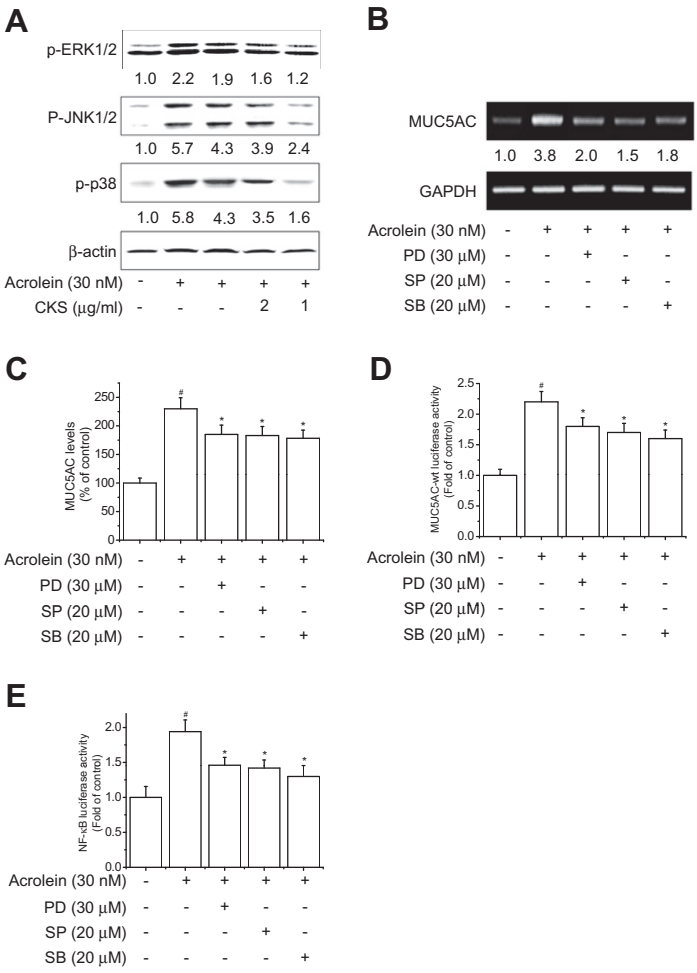

Fig. 4. Effects of CKS on the acrolein-induced activation of MAPK signaling. (A) Cells were pretreated with CKS for 1 h and subsequently stimulated with acrolein in the presence of CKS in serum-free medium for 10 min. Phosphorylation levels of ERK1/2, JNK1/2, and p38 MAPK were determined by Western blot analysis. (B) Cells were pretreated for 1 h with the MAPK inhibitors PD98059 (PD; ERK1/2), SP600125 (SP; JNK1/2) and SB203580 (SB; p38 MAPK), followed by acrolein stimulation for 4 h. The cells were lysed, and total RNA was analyzed by semi-quantitative RT-PCR. (C) MUC5AC protein levels in the culture supernatants were measured by ELISA. (D) Cells were transfected with MUC5AC-Luc reporter plasmids. Cells were pretreated with MAPK inhibitors for 1 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group. (E) Effects of MAPK inhibitor on NF-κB activation. Cells were transfected with NF-κB-Luc reporter plasmids. Cells were pretreated with MAPK inhibitors for 1 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group.

expression and the acrolein-induced luciferase reporter activity (Fig. 5B–D). We further evaluated the inhibitory effect of rottlerin on the MAPK pathway. As seen in Fig. 5E, rottlerin inhibited the acrolein-induced phosphorylation of ERK1/2, JNK1/2, and p38 MAPK. In addition, PKCδ inhibitor rottlerin inhibited the acrolein-induced NF-κB activity (Fig. 5F).

PKCδ inhibitor alone did not alter cell viability or expression of MUC5AC mRNA, protein expression, promoter activity, MAPK activity, and NF-κB activity in control treated cells (data not shown).

3.6. Inhibitory effects of CKS on acrolein-stimulated ROS production

Cigarette smoke has been shown to stimulate ROS production in lung epithelial cells (Lemjabbar et al., 2003), and ROS play an important role in the activation of MAPK signaling (Ranganna et al., 2002). CKS inhibited the acrolein-induced ROS production in A549 cells, in a concentration-dependent manner (Fig. 6A). Moreover, pretreatment of A549 cells with the ROS inhibitor *N*-acetylcysteine (NAC) inhibited the acrolein-induced MUC5AC mRNA and protein expression (Fig. 6B and C), acrolein-induced luciferase reporter activity (Fig. 6D), and acrolein-induced phosphorylation of PKCδ, ERK1/2, JNK1/2, and p38 MAPK (Fig. 6E). In addition, ROS inhibitor NAC inhibited the acrolein-induced NF-κB activity (Fig. 6F). ROS inhibitor alone did not alter cell viability or expression of MUC5AC mRNA, protein expression, promoter activity, MAPK activity, and NF-κB activity in control treated cells (data not shown).

4. Discussion

COPD is a major health problem with increasing global prevalence (Bracke et al., 2006). The majority of COPD patients display mucus hypersecretion that blocks the airways, further aggravating their COPD. According to the World Health Organization, 210 million people have COPD, which is moderate to severe in approximately 40% of those individuals (Ebbert et al., 2009). Chronic exposure to inhaled cigarette smoke, viruses, bacteria, noxious gases, and particles is associated with COPD development (Rabe et al., 2007). The respiratory symptoms of COPD include chronic cough, shortness of breath, and airway mucus hypersecretion. COPD produces more rapid deterioration of lung function in

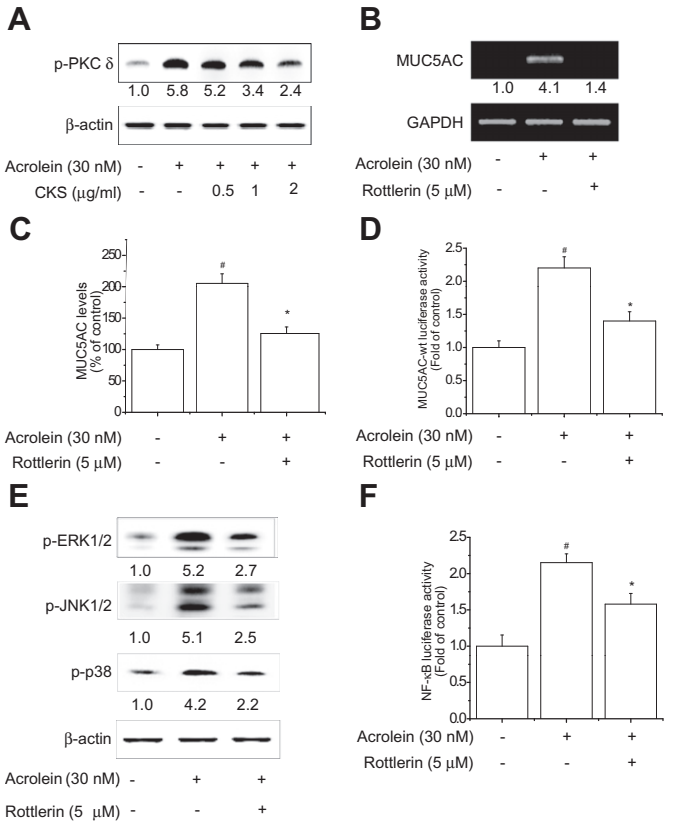

Fig. 5. Effects of CKS on acrolein-induced activation of PKCδ signaling pathways. (A) Cells were pretreated with CKS for 1 h and then stimulated with acrolein in the presence of CKS in serum-free medium for 10 min. Levels of phosphorylated PKCδ were determined by Western blot analysis. (B) Cells were pretreated for 1 h with the PKCδ inhibitor rottlerin, followed by acroelin stimulation for 4 h. The cells were lysed, and total RNA was analyzed by semi-quantitative RT-PCR. (C) MUC5AC protein levels in the culture supernatants were measured by ELISA. (D) Cells were transfected with MUC5AC reporter plasmid and cultured with rottlerin and/or acrolein for 4 h. The luciferase activity was determined in the cell extract. (E) Cells were pretreated with rottlerin for 1 h and subsequently stimulated with acrolein in the presence of rottlerin in serum-free medium for 10 min. Levels of phosphorylated ERK1/2, JNK1/2, and p38 MAPK were determined by Western blot analysis. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group. (F) Effects of PKCδ inhibitor on NF-κB activation. Cells were transfected with NF-κB-Luc reporter plasmids. Cells were pretreated with PKCδ inhibitor for 1 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group.

smokers, with cigarette smoke being the most significant risk factor for COPD (Lokke et al., 2006). Cigarette smoke contains more than 4000 chemicals, many of which combine to produce negative effects in the lungs (Park et al., 2007). However, the precise mechanisms of cigarette smoke-mediated injury to airway epithelial cells have not been fully elucidated. In the present study, we demonstrate that acrolein, a major toxic component of cigarette smoke, contributes to the development of airway mucus production through the induction of MUC5AC expression. We also examined the inhibitory effect of CKS on MUC5AC expression and its mechanism in human lung carcinoma A549 cells.

Acrolein, an α, β-unsaturated aldehyde, is a ubiquitous environmental pollutant. It is generated as a product of incomplete combustion during forest fires, during the combustion of plastics, and during the cooking of food. A major source of acrolein is cigarette smoke; the burning of one cigarette generates 50–90 ppm of acrolein. Acrolein is also a by-product of cellular lipid peroxidation associated with oxidative stress (Kehrer and Biswal, 2000). It is a highly reactive electrophilic compound, can cause rapid depletion of the antioxidant glutathione, and is able to initiate lipid peroxidation (Beauchamp et al., 1985). Inhaled acrolein has been associated with many lung diseases, including asthma and COPD (Hogg, 2001; Samet and Cheng, 1994). Acrolein also increases MUC5AC mRNA and protein expression in A549 cells. Here, we demonstrated that CKS inhibits the acrolein-induced MUC5AC expression, in a concentration-dependent manner.

Mucin synthesis in epithelial layers is controlled via transcriptional and post-transcriptional regulation (Li et al., 1998; Manna et al., 1995). Various transcription factors such as NF-κB, AP-1, and SP1 are involved in MUC5AC expression in airway epithelial cells in response to various stimulants (Chen et al., 2004; Perrais et al., 2002). In the present study, we showed that CKS reduces the acrolein-induced MUC5AC expression by suppressing the transcriptional activation of the MUC5AC promoter. Mutational analysis of the promoter revealed that the major target of CKS was NF-κB, a finding that was further confirmed by the use of reporter vectors containing synthetic elements specific for this transcription factor. We have previously reported that CKS inhibits TNFα- or PMA-induced NF-κB activation and IκBα degradation in these cells (Kim et al., 2006; Lee et al., 2008). In the present study, CKS prevented the acrolein-induced translocation of NF-κB p65 to the nucleus, attenuated the phosphorylation of NF-κB p65, and inhibited the degradation of IκBα in A549 cells. Pretreatment of A549 cells with JSH-23, a potent inhibitor of NF-κB transcriptional activation, reduced the acrolein-induced MUC5AC RNA and protein expression. Furthermore, CKS and JSH-23 treatment of A549 cells transfected with luciferase reporter vectors reduced the acrolein-induced luciferase activity of a reporter vector containing NF-κB binding sites and that of a reporter vector containing the MUC5AC promoter. These results indicate that CKS inhibits the acrolein-induced expression of MUC5AC in A549 cells by suppressing NF-κB activation.

We also investigated the activation of MAPK signaling, which is an important regulator of MUC5AC expression. Western blot analysis showed that acrolein-induced MUC5AC expression was associated with the activation of ERK1/2, JNK1/2, and p38 MAPK and that CKS decreased the acrolein-induced activation of these kinases. Moreover, acrolein-induced MUC5AC expression, kinase activation, and NF-κB activation were decreased in the presence of the MAPK inhibitors PD98059 (ERK1/2), SP600125 (JNK1/2), and SB203580 (p38 MAPK). These findings suggest that CKS regulates acrolein-induced MUC5AC expression through the suppression of the MAPK and NF-κB pathways in A549 cells.

PKC is a serine/threonine-specific protein kinase family with at least 10 different isoforms (Hofmann, 1997), which are categorized into three types: classical (α, β1, β2, and γ isoforms), novel (δ, ε, η, θ, and μ isoforms), and atypical (ξ, and ι/λ isoforms). The classical isoforms are calcium and PMA activated; the novel isoforms are calcium insensitive, but are activated by PMA; and the atypical isoforms are both calcium and PMA inactivated (Dartt et al., 2000). PKC activation has been suggested to be a key event in the signaling cascade that leads to MUC5AC expression in human epithelial cells (Song et al., 2007). In the current study, we showed that acrolein induces the activation of PKCδ in A549 cells and that CKS decreases the acrolein-induced activation of PKCδ, in a concentration-dependent manner. In addition, the PKC δ inhibitor rottlerin decreased the acrolein-induced MUC5AC expression and activity, as well as the acrolein-induced MAPK and NF-κB activation. Taken together, our results suggest that CKS inhibits the acrolein-activated PKCδ-mediated MUC5AC expression via ERK1/2, JNK1/2, and p38 MAPK.

Cigarette smoke leads to elevated cytoplasmic ROS, and the smoke itself contains ROS (Pryor and Stone, 1993). ROS production

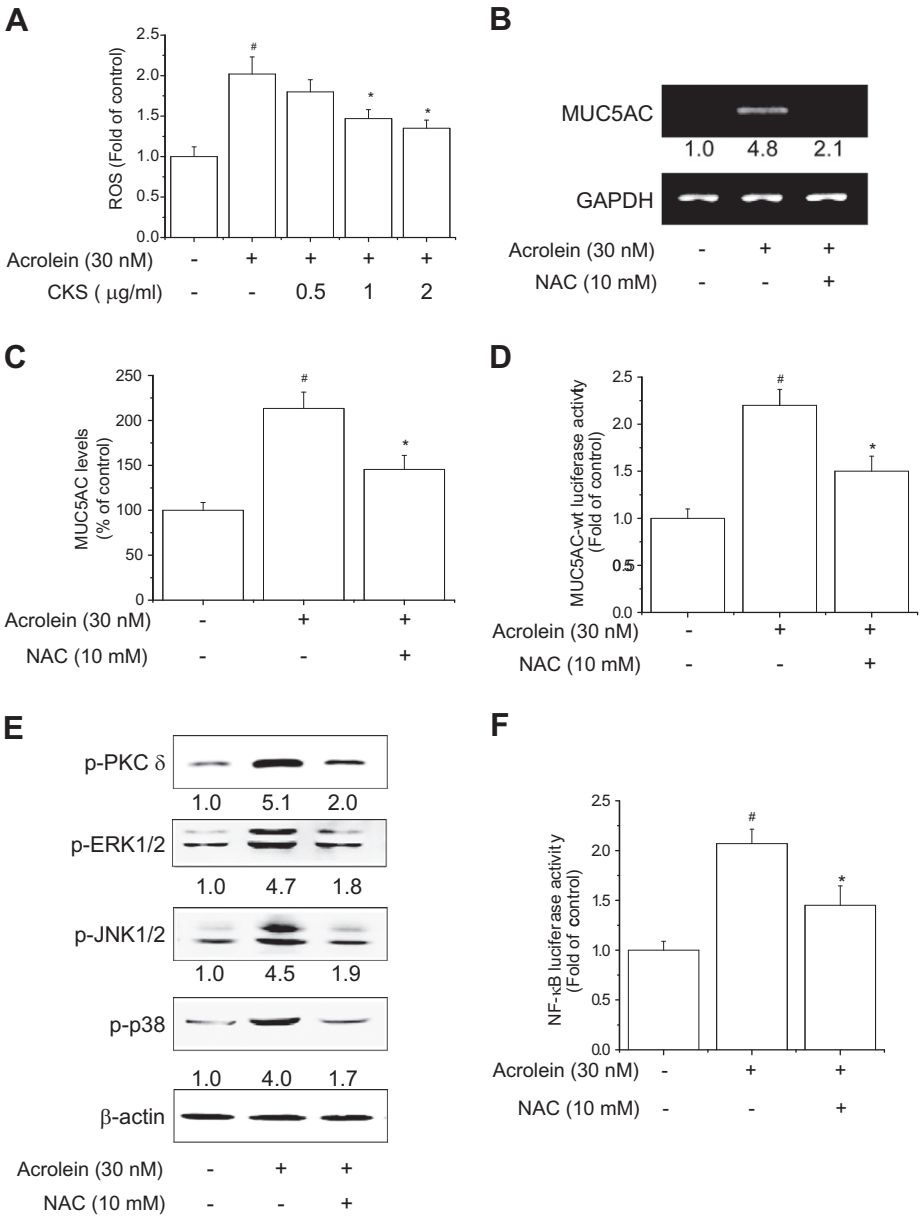

Fig. 6. Effects of CKS on the acrolein-induced activation of ROS signaling pathways. (A) Cells were pretreated with CKS for 24 h and incubated with DCFDA for 30 min. The medium was then removed, and the cells were stimulated with acrolein for 30 min. The fluorescence intensity of cells was measured using a FL600 fluorescence spectrophotometer. (B) Cells were pretreated for 1 h with the ROS inhibitor NAC, followed by acroelin stimulation for 4 h. The cells were lysed, and total RNA was analyzed by semi-quantitative RT-PCR. (C) MUC5AC protein levels in the culture supernatants were measured by ELISA. (D) Cells were transfected with MUC5AC reporter plasmid and cultured with NAC and/or acrolein for 4 h. The luciferase activity was determined in the cell extract. (E) Cells were pretreated with NAC for 1 h and subsequently stimulated with acrolein in the presence of rottlerin in serum-free medium for 10 min. Levels of phosphorylated PKCδ, ERK1/2, JNK1/2, and p38 MAPK were determined by Western blot analysis. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group. (F) Effects of ROS inhibitor on NF-κB activation. Cells were transfected with NF-κB-Luc reporter plasmids. Cells were pretreated with ROS inhibitor for 1 h and subsequently treated with 30 nM acrolein for 4 h, and the relative luciferase activity in cell extracts was determined. The results are expressed as means ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, versus the control group. $^{*}P < 0.05$, versus the acrolein-treated group.

is one of the early effects of cigarette-smoke exposure in lung epithelial cells and plays an important role in stress-induced signaling. Recent studies have reported that ROS increases MUC5AC expression (Casalino-Matsuda et al., 2009; Shao and Nadel, 2005). Here, we showed that acrolein increases ROS production in A549 cells and that this is inhibited in the presence of CKS, in a concentration-dependent manner. Previously, we have reported that CKS inhibits TNFα- or PMA-induced intracellular ROS production (Kim et al., 2006; Lee et al., 2008). A ROS inhibitor, NAC, decreased acrolein-induced MUC5AC expression and activity, and decreased acrolein-induced PKCδ, MAPK and NF-κB activation. These results indicate that the induction of ROS by acrolein leads to the activation of MUC5AC expression.

In this study, we showed that acrolein, a known toxin in cigarette smoke, stimulates the expression of MUC5AC in A549 cells through the activation of the transcriptional factor NF-κB, MAPK signaling via ERK1/2, JNK1/2, and p38 MAPK, PKCδ signaling, and ROS production (Fig. 7). We also demonstrated that CKS suppresses acrolein-induced MUC5AC expression by inhibiting the activation of NF-κB via the ROS–PKCδ–MAPK signaling pathways. Our results suggest the potential of CKS as a therapeutic candidate for respiratory disease.

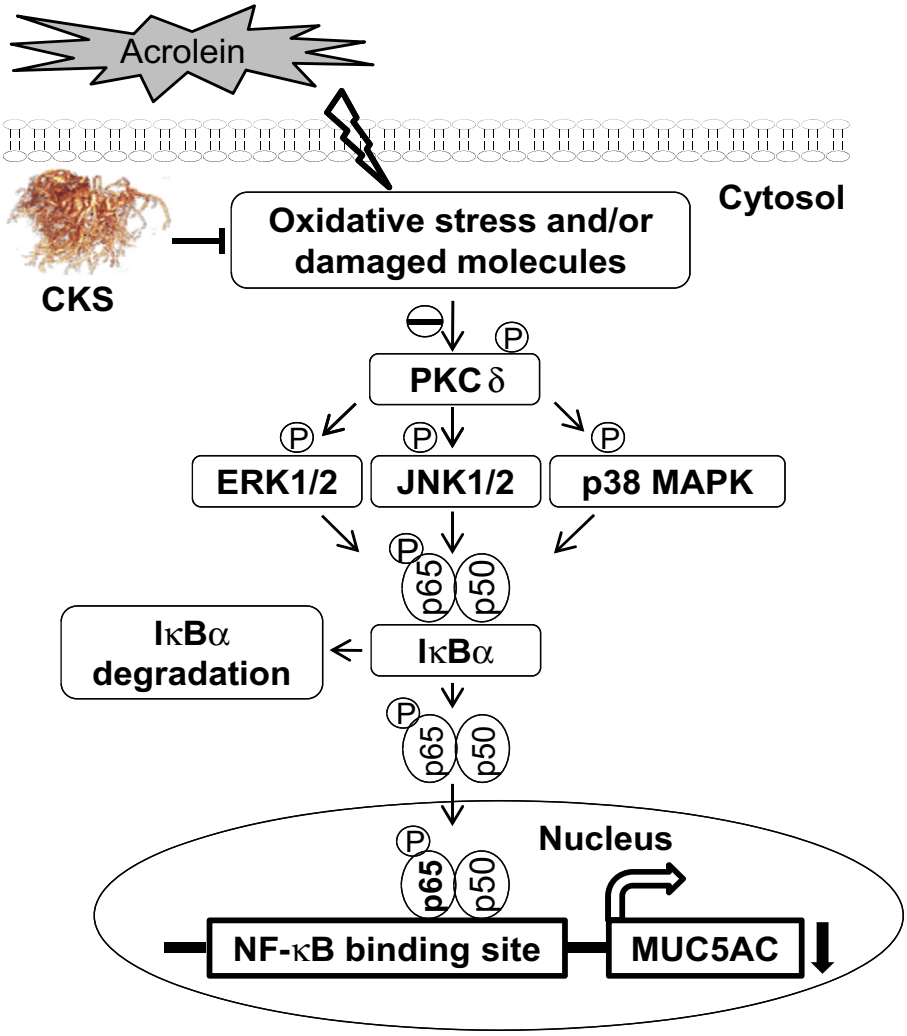

Fig. 7. Schematic showing the mechanisms of CKS inhibition of acrolein-induced signaling in A549 cells. First, CKS inhibits the acrolein-induced ROS production. Second, CKS inhibits the acrolein-activated phosphorylation of PKCδ and ERK1/2, JNK1/2, and p38 MAPK. Finally, CKS inhibits the acrolein-induced expression of MUC5AC through the activation of NF-κB. CKS decreases acrolein-induced airway inflammation through the inhibition of MUC5AC expression in A549 cells.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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References

Aoshiba, K., Nagai, A., 2004. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. Clin. Rev. Allergy Immunol. 27, 35–43.

Beauchamp Jr., R.O., Andjelkovich, D.A., Kligerman, A.D., Morgan, K.T., Heck, H.D., 1985. A critical review of the literature on acrolein toxicity. Crit. Rev. Toxicol. 14, 309–380.

Ben-Jebria, A., Marthan, R., Rossetti, M., Savineau, J.P., Ultman, J.S., 1994. Human bronchial smooth muscle responsiveness after in vitro exposure to acrolein. Am. J. Respir. Crit. Care Med. 149, 382–386.

Bracke, K.R., D'hulst, A.I., Maes, T., Moerloose, K.B., Demedts, I.K., Lebecque, S., Joos, G.F., Brusselle, G.G., 2006. Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. J. Immunol. 177, 4350–4359.

Casalino-Matsuda, S.M., Monzon, M.E., Day, A.J., Forteza, R.M., 2009. Hyaluronan fragments/CD44 mediate oxidative stress-induced MUC5B up-regulation in airway epithelium. Am. J. Respir. Cell Mol. Biol. 40, 277–285.

Chen, R., Lim, J.H., Jono, H., Gu, X.X., Kim, Y.S., Basbaum, C.B., Murphy, T.F., Li, J.D., 2004. Nontypeable Haemophilus influenzae lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKbeta-IkappaBalpha-NF-kappaB signaling pathways. Biochem. Biophys. Res. Commun. 324, 1087–1094.

Choi, J.H., Hwang, Y.P., Lee, H.S., Jeong, H.G., 2009. Inhibitory effect of Platycodi Radix on ovalbumin-induced airway inflammation in a murine model of asthma. Food Chem. Toxicol. 47, 1272–1279.

Daimon, M., Sugiyama, K., Kameda, W., Saitoh, T., Oizumi, T., Hirata, A., Yamaguchi, H., Ohnuma, H., Igarashi, M., Kato, T., 2003. Increased urinary levels of pentosidine, pyrraline and acrolein adduct in type 2 diabetes. Endocr. J. 50, 61–67.

Dartt, D.A., Rios, J.D., Kanno, H., Rawe, I.M., Zieske, J.D., Ralda, N., Hodges, R.R., Zoukhri, D., 2000. Regulation of conjunctival goblet cell secretion by Ca(2+)and protein kinase C. Exp. Eye Res. 71, 619–628.

Deshmukh, H.S., Case, L.M., Wesselkamper, S.C., Borchers, M.T., Martin, L.D., Shertzer, H.G., Nadel, J.A., Leikauf, G.D., 2005. Metalloproteinases mediate mucin 5AC expression by epidermal growth factor receptor activation. Am. J. Respir. Crit. Care Med. 171, 305–314.

Ebbert, J.O., Wyatt, K.D., Zirakzadeh, A., Burke, M.V., Hays, J., 2009. Clinical utility of varenicline for smokers with medical and psychiatric comorbidity. Int. J. Chron. Obstruct. Pulmon. Dis. 4, 421–430.

Gensch, E., Gallup, M., Sucher, A., Li, D., Gebremichael, A., Lemjabbar, H., Mengistab, A., Dasari, V., Hotchkiss, J., Harkema, J., Basbaum, C., 2004. Tobacco smoke control of mucin production in lung cells requires oxygen radicals AP-1 and JNK. J. Biol. Chem. 279, 39085–39093.

Han, E.H., Park, J.H., Kim, J.Y., Chung, Y.C., Jeong, H.G., 2009. Inhibitory mechanism of saponins derived from roots of *Platycodon grandiflorum* on anaphylactic reaction and IgE-mediated allergic response in mast cells. Food Chem. Toxicol. 47, 1069–1075.

Higuchi, T., Orita, T., Nakanishi, S., Katsuya, K., Watanabe, H., Yamasaki, Y., Waga, I., Nanayama, T., Yamamoto, Y., Munger, W., Sun, H.W., Falk, R.J., Jennette, J.C., Alcorta, D.A., Li, H., Yamamoto, T., Saito, Y., Nakamura, M., 2004. Molecular cloning, genomic structure, and expression analysis of MUC20, a novel mucin protein, up-regulated in injured kidney. J. Biol. Chem. 279, 1968–1979.

Hofmann, J., 1997. The potential for isoenzyme-selective modulation of protein kinase C. Faseb J. 11, 649–669.

Hogg, J.C., 2001. Chronic obstructive pulmonary disease: an overview of pathology and pathogenesis. Novartis Found. Symp. 234, 4–19.

Hwang, Y.P., Yun, H.J., Kim, H.G., Han, E.H., Lee, G.W., Jeong, H.G., 2010. Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKCalpha/Raf/MAPKs and NF-kappaB/AP-1-dependent mechanisms. Biochem. Pharmacol. 79, 1714–1726.

Kato, S., Hokari, R., Crawley, S., Gum, J., Ahn, D.H., Kim, J.W., Kwon, S.W., Miura, S., Basbaum, C.B., Kim, Y.S., 2006. MUC5AC mucin gene regulation in pancreatic cancer cells. Int. J. Oncol. 29, 33–40.

Kehrer, J.P., Biswal, S.S., 2000. The molecular effects of acrolein. Toxicol. Sci. 57, 6–15.

Kim, J.Y., Kim, D.H., Kim, H.G., Song, G.Y., Chung, Y.C., Roh, S.H., Jeong, H.G., 2006. Inhibition of tumor necrosis factor-alpha-induced expression of adhesion molecules in human endothelial cells by the saponins derived from roots of *Platycodon grandiflorum*. Toxicol. Appl. Pharmacol. 210, 150–156.

Kim, K.S., Ezaki, O., Ikemoto, S., Itakura, H., 1995. Effects of *Platycodon grandiflorum* feeding on serum and liver lipid concentrations in rats with diet-induced hyperlipidemia. J. Nutr. Sci. Vitaminol. 41, 485–491.

Kliber, A., Lynd, L.D., Sin, D.D., 2010. The effects of long-acting bronchodilators on total mortality in patients with stable chronic obstructive pulmonary disease. Respir. Res. 11, 1–13.

Lee, E.B., 1973. Pharmacological studies on *Platycodon grandiflorum* A DC. IV. A comparison of experimental pharmacological effects of crude platycodin with clinical indications of platycodi radix. Yakugaku Zasshi 93, 1188–1194.

Lee, K.J., You, H.J., Park, S.J., Kim, Y.S., Chung, Y.C., Jeong, T.C., Jeong, H.G., 2001. Hepatoprotective effects of *Platycodon grandiflorum* on acetaminophen-induced liver damage in mice. Cancer Lett. 174, 73–81.

Lee, K.J., Jeong, H.G., 2002. Protective effect of Platycodi radix on carbon tetrachloride-induced hepatotoxicity. Food Chem. Toxicol. 40, 517–525.

Lee, K.J., Kim, J.Y., Jung, K.S., Choi, C.Y., Chung, Y.C., Kim, D.H., Jeong, H.G., 2004. Suppressive effects of *Platycodon grandiflorum* on the progress of carbon tetrachloride-induced hepatic fibrosis. Arch. Pharm. Res. 27, 1238–1244.

Lee, K.J., Hwang, S.J., Choi, J.H., Jeong, H.G., 2008. Saponins derived from the roots of *Platycodon grandiflorum* inhibit HT-1080 cell invasion and MMPs activities: regulation of NF-kappaB activation via ROS signal pathway. Cancer Lett. 268, 233–243.

Lemjabbar, H., Li, D., Gallup, M., Sidhu, S., Drori, E., Basbaum, C., 2003. Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor alpha-converting enzyme and amphiregulin. J. Biol. Chem. 278, 26202–26207.

Leopold, J.A., Loscalzo, J., 2005. Oxidative enzymopathies and vascular disease. Arterioscler. Thromb. Vasc. Biol. 25, 1332–1340.

Levine, S.J., Larivée, P., Logun, C., Angus, C.W., Ognibene, F.P., Shelhamer, J.H., 1995. Tumor necrosis factor-alpha induces mucin hypersecretion and MUC-2 gene expression by human airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 12, 196–204.

Li, J.D., Feng, W., Gallup, M., Kim, J.H., Gum, J., Kim, Y., Basbaum, C., 1998. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for Pseudomonas aeruginosa-induced mucin overproduction in epithelial cells. Proc. Natl. Acad. Sci. USA 95, 5718–5723.

Liu, D.S., Liu, W.J., Chen, L., Ou, X.M., Wang, T., Feng, Y.L., Zhang, S.F., Xu, D., Chen, Y.J., Wen, F.Q., 2009. Rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, attenuates acrolein-induced airway mucus hypersecretion in rats. Toxicology 260, 112–119.

Lokke, A., Lange, P., Scharling, H., Fabricius, P., Vestbo, J., 2006. Developing COPD: a 25 year follow up study of the general population. Thorax 61, 935–939.

Longphre, M., Li, D., Li, J., Matovinovic, E., Gallup, M., Samet, J.M., Basbaum, C.B., 2000. Lung mucin production is stimulated by the air pollutant residual oil fly ash. Toxicol. Appl. Pharmacol. 162, 86–92.

Lovell, M.A., Xie, C., Markesbery, W.R., 2001. Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. Neurobiol. Aging 22, 187–194.

Luo, J., Robinson, J.P., Shi, R., 2005. Acrolein-induced cell death in PC12 cells: role of mitochondria-mediated oxidative stress. Neurochem. Int. 47, 449–457.

Manna, B., Ashbaugh, P., Bhattacharyya, S.N., 1995. Retinoic acid-regulated cellular differentiation and mucin gene expression in isolated rabbit tracheal-epithelial cells in culture. Inflammation 19, 489–502.

Moniaux, N., Escande, F., Porchet, N., Aubert, J.P., Batra, S.K., 2001. Structural organization and classification of the human mucin genes. Front. Biosci. 6, D1192–D1206.

Mossman, B.T., Lounsbury, K.M., Reddy, S.P., 2006. Oxidants and signaling by mitogen-activated protein kinases in lung epithelium. Am. J. Respir. Cell Mol. Biol. 34, 666–669.

Nardini, M., Finkelstein, E.I., Reddy, S., Valacchi, G., Traber, M., Cross, C.E., Van der Vliet, A., 2002. Acrolein-induced cytotoxicity in cultured human bronchial epithelial cells. Modulation by alpha-tocopherol and ascorbic acid. Toxicology 170, 173–185.

Park, J.A., Crews, A.L., Lampe, W.R., Fang, S., Park, J., Adler, K.B., 2007. Protein kinase C delta regulates airway mucin secretion via phosphorylation of MARCKS protein. Am. J. Pathol. 171, 1822–1830.

Park, J.A., He, F., Martin, L.D., Li, Y., Chorley, B.N., Adler, K.B., 2005. Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C{delta}-mediated mechanism. Am. J. Pathol. 167, 651–661.

Perrais, M., Pigny, P., Copin, M.C., Aubert, J.P., Van Seuningen, I., 2002. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J. Biol. Chem. 277, 32258–32267.

Pokharel, Y.R., Yoon, S.Y., Kim, S.K., Li, J.D., Kang, K.W., 2008. Inhibition of acrolein-stimulated MUC5AC production by fucoidan in human bronchial epithelial cells. Pharmazie 63, 757–759.

Pryor, W.A., Stone, K., 1993. Oxidants in cigarette smoke Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite.. Ann. NY Acad. Sci. 686, 12–27.

Rabe, K.F., Hurd, S., Anzueto, A., Barnes, P.J., Buist, S.A., Calverley, P., Fukuchi, Y., Jenkins, C., Rodriguez-Roisin, R., Van Weel, C., Zielinski, J., 2007. Global initiative for chronic obstructive lung disease – global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am. J. Respir. Crit. Care Med. 176, 532–555.

Ranganna, K., Yousefipour, Z., Nasif, R., Yatsu, F.M., Milton, S.G., Hayes, B.E., 2002. Acrolein activates mitogen-activated protein kinase signal transduction pathways in rat vascular smooth muscle cells. Mol. Cell. Biochem. 240, 83–98.

Samet, J.M., Cheng, P.W., 1994. The role of airway mucus in pulmonary toxicology. Environ. Health Perspect. 102, 89–103.

Shao, M.X., Nadel, J.A., 2005. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme. J. Immunol. 175, 4009–4016.

Song, J.S., Kang, C.M., Yoo, M.B., Kim, S.J., Yoon, H.K., Kim, Y.K., Kim, K.H., Moon, H.S., Park, S.H., 2007. Nitric oxide induces MUC5AC mucin in respiratory epithelial cells through PKC and ERK dependent pathways. Respir. Res. 8, 1–12.

Tada, A., Kaneiwa, Y., Shoji, J., Shibata, S., 1975. Studies on the saponins of the root of *Platycodon grandiflorum* A. De Candolle I. Isolation and the structure of platycodin-D.. Chem. Pharm. Bull. 23, 2965–2972.

Takeyama, K., Dabbagh, K., Jeong Shim, J., Dao-Pick, T., Ueki, I.F., Nadel, J.A., 2000. Oxidative stress causes mucin synthesis via transactivation of epider-mal growth factor receptor: role of neutrophils. J. Immunol. 164, 1546–1552.

Uchida, K., Kanematsu, M., Morimitsu, Y., Osawa, T., Noguchi, N., Niki, E., 1998. Acrolein is a product of lipid peroxidation reaction Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. J. Biol. Chem. 273, 16058–16066.

Voynow, J.A., Young, L.R., Wang, Y., Horger, T., Rose, M.C., Fischer, B.M., 1999. Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. Am. J. Physiol. 276, L835–L843.

Wang, B., Lim, D.J., Han, J., Kim, Y.S., Basbaum, C.B., Li, J.D., 2002. Novel cytoplasmic proteins of nontypeable Haemophilus influenzae up-regulate human MUC5AC mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. J. Biol. Chem. 277, 949–957.