BIOMOLECULES
& THERAPEUTICS

Involvement of IKK/IkBα/NF-kB p65 Signaling into the Regulative Effect of Engeletin on MUC5AC Mucin Gene Expression in Human Airway Epithelial Cells

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Abstract

In this study, we examined whether engeletin exerts an effect on the gene expression of MUC5AC mucin, in human pulmonary epithelial NCI-H292 cells. The cells were pretreated with engeletin for 30 min and stimulated with phorbol 12-myristate 13-acetate (PMA), for the following 24 h. The effect of engeletin on PMA-induced nuclear factor kappa B (NF-kB) signaling pathway was also investigated. Engeletin suppressed the mRNA expression and production of MUC5AC mucin, induced by PMA through the inhibition of degradation of inhibitory kappa Bα (IkBα) and NF-kB p65 nuclear translocation. These results suggest engeletin inhibits the gene expression of mucin through regulation of NF-kB signaling pathway, in human airway epithelial cells.

Key Words: MUC5AC, Pulmonary mucin, Engeletin

INTRODUCTION

Mucus in the pulmonary system is a thin layer of gels present in the luminal surface of airway. It consists of various molecules, ions, water, and those molecules exert anti-oxidative and anti-microbial activities (Lillehoj and Kim, 2002). Mucins (mucous glycoproteins) assigning mucus the viscoelasticity is the major macromolecular biochemical constituent of mucus. In the physiological conditions of the pulmonary system, mucus in the airway is well-known to play a critical role in a physical defense against damage of pulmonary epithelium, provoked by irritating gases, inhaled particles, and many viruses and bacteria (Adler and Li, 2001). However, the overproduction and/or oversecretion of airway mucus resulting from the changes in quantity or quality of mucins have been reported to compromise the host defense system and, eventually, provoke the increase of mortality and morbidity, in the pulmonary diseases like asthma, chronic obstructive pulmonary diseases (COPD), and cystic fibrosis (CF) (Rose and Voynow, 2006).

Although glucocorticoids, expectorants, and mucolytics have been utilized clinically to regulate the abnormal secretion of pulmonary mucus, these agents might provoke the irritation of luminal wall of airway, and various negative side effects like the rebounding hypersecretion of mucus (Li *et al*., 2020). Therefore, it is very promising to develop a novel agent controlling the production and/or secretion of mucins through affecting the degradation and/or biosynthesis of them.

In this context, we suggest it is encouraging to examine the potential activity of regulating the pathological production and/or secretion of mucins, using natural products isolated from a multitude of medicinal plants used empirically for controlling the inflammatory pulmonary diseases. We have reported that diverse natural compounds modulated the production of airway mucous glycoprotein (mucin), resulting from its gene expression (Kim *et al*., 2012; Seo *et al*., 2014; Choi *et al*., 2019; Li *et al*., 2021).

In accordance with many reports, Engeletin (Fig. 1) is a flavonoid compound contained in the leaves of Engelhardia roxburghiana and white grapes. Engeletin has been reported to show various biological activities including anti-inflammatory effect (Wu *et al*., 2016; Tian *et al*., 2019). Engeletin exerts moderate inhibitory activity on TNF-α-provoked NF-κB activation (Xu *et al*., 2005). It exhibits a protective effect on LPS-stimulated liver injury via decreasing the level of IL-1β, TNF-α,
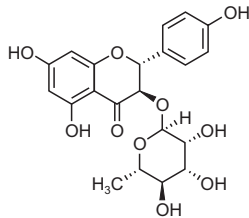
Fig. 1. Chemical structure of engeletin.

as well as the activation of NF-κB (Tian *et al*., 2019). In acute lung injury (ALI), engeletin significantly inhibits the expression of cytokines including TNF-α, IL-6, and IL-1β, in the BALF, and also reversed LPS-stimulated activation of NF-κB (Jiang *et al*., 2018). Besides, engeletin attenuates the NF-κB p65 nuclear translocation in the LPS-induced endometritis mouse model (Wu *et al*., 2016).

However, there is no study relating to the effect of engeletin on the gene expression of mucin in airway epithelial cells. Of the many subtypes of human mucins, MUC5AC mucin is known to be the major type of secreted mucin from airway (Lillehoj and Kim, 2002). Here, based upon this information, we examined the effect of engeletin on mRNA expression and glycoprotein production of MUC5AC mucin, provoked by phorbol ester, in NCI-H292 cells. A human pulmonary mucoepidermoid cell line, NCI-H292 cells, is frequently used for specifying the signaling pathways involved into the gene expression of airway mucin (Li *et al*., 1997; Takeyama *et al*., 1999; Shao *et al*., 2003). Also, phorbol ester stimulates the gene expression of airway MUC5AC mucin, and intracellular nuclear factor kappa B (NF-kB) signaling is involved into the activity of phorbol ester in airway epithelial cells (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Kim *et al*., 2012; Choi *et al*., 2019; Jin *et al*., 2020). Thus, to elucidate an action mechanism of engeletin, we investigated whether engeletin affects the activation of NF-kB signaling pathway stimulated by phorbol ester, in NCI-H292 cells.

MATERIALS AND METHODS

Materials

Engeletin (AV-H08006, purity: 98.0%) (Fig. 1) was purchased from Avention (Incheon, Korea). Anti-β-actin (sc-8432), anti-NF-κB p65 (sc-8008), and anti-inhibitory kappa Bα (IκBα) (sc-371) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-inhibitory kappa B kinase (IKK) α/β (Ser176/180, #2687) and phospho-specific anti-IκBα (serine 32/36, #9246) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-nuclear matrix protein p84 (ab-487) antibody was purchased from Abcam (Cambridge, MA, USA). Either Goat Anti-mouse IgG (#401215) or Goat Anti-rabbit IgG (#401315) was purchased from Calbiochem (Carlsbad, CA, USA) and used as the secondary antibody. The other chemicals used in the current experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Culture of NCI-H292 cells

NCI-H292 cells were purchased from the American Type

Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of pen-strep mixture (penicillin (100 units/mL) plus streptomycin (100 μg/mL)) and HEPES (25 mM) at 37°C in a humidified, 5% CO_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and then cultured in RPMI 1640 with 0.2% FBS, for 24 h.

Treatment of cells with engeletin

After serum deprivation, cells were pretreated with varying concentrations of engeletin for 30 min and then treated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) for 24 h in serum-free RPMI 1640. Engeletin was dissolved in dimethyl sulfoxide and treated in culture medium (final concentrations of dimethyl sulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethyl sulfoxide did not affect mucin gene expression, and expression and activity of molecules involved into NF-kB signaling pathway, in NCI-H292 cells. After 24 h, cells were lysed with buffer solution containing 20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA and protease inhibitor cocktail (Roche Diagnostics, IN, USA) and collected to measure the production of MUC5AC glycoproteins (in a 24-well culture plate). The total RNA was extracted to check the expression of MUC5AC gene (in a 6-well culture plate) using RT-PCR. For the western blot analysis, cells were treated with engeletin for 24 h and then with PMA for 30 min.

Cell viability assay

NCI-H292 cells were seeded at a density of 2×10^5/mL (0.1 mL/well) in a 96-well microtiter plate and allowed to attach for 24 h to keep the log phase growth at the time of drug treatment. After incubation with the indicated concentrations of engeletin for 72 h, cell proliferation was determined using the sulforhodamine B assay (Skehan *et al*., 1990).

Quantitative analysis of MUC5AC mucin

Airway MUC5AC mucin production was measured using enzyme-linked immunosorbent assay (ELISA). Cell lysates were prepared with PBS at 1:10 dilution, and 100 μL of each sample was incubated at 42°C in a 96-well plate, until it would be dry. Plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) (fraction V) for 1 h at room temperature. Plates were washed another three times with PBS and then incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, USA), which was diluted with PBS containing 0.05% Tween 20, and dispensed into each well. After 1 h, the wells were washed three times with PBS, and 100 μL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3,000) was dispensed into each well. After 1 h, plates were washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1 N H_2SO_4. Absorbance was read at 450 nm.

Isolation of total RNA and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc., Gyeonggi, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. Two μg of total RNA was primed with 1 μg of oligo

(dT) in a final volume of 50 μL (RT reaction). Two μL of RT reaction product was PCR-amplified in a 25 μL by using Thermorprime Plus DNA Polymerase (ABgene, Rochester, NY, USA). Primers for MUC5AC were (forward) 5′-TGA TCA TCC AGC AGG GCT-3′ and (reverse) 5′-CCG AGC TCA GAG GAC ATA TGG G-3′. Primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used as quantitative controls. Primers for Rig/S15 were (forward) 5′-TTC CGC AAG TTC ACC TAC C-3′ and (reverse) 5′-CGG GCC GGC CAT GCT TTA CG-3′. The PCR mixture was denatured at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. After PCR, 5 μL of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

Whole cell extract preparation

NCI-H292 cells (confluent in 100 mm culture dish) were pretreated for 24 h at 37°C with 1, 5, 10 or 20 μM of engeletin, and then stimulated with PMA (50 ng/mL) for 30 min, in serum-free RPMI 1640. After the treatment of the cells with engeletin, media were aspirated, and the cells washed with cold PBS. For the cell collection, the cells were scraped and centrifuged at 3,000 rpm for 5 min. After the supernatant was discarded, the cell pellet was mixed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min with continuous agitation. The lysate was centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4°C. The supernatant was either used, or was immediately stored at –80°C. The amount of protein in extract was quantified by Bradford method.

Nuclear and cytosolic extracts preparation

After the treatment with engeletin as stated, the cells were harvested using Trypsin-EDTA solution and then centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). After the supernatant was discarded, the cell pellet was washed by suspending in PBS. The cytoplasmic and nuclear protein fractions were extracted using NE-PER® nuclear and cytoplasmic extraction reagent (Thermo-Pierce Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Both extracts were stored at –20°C. The amount of protein in extracts was quantified by Bradford method.

Western blotting for the detection of proteins

Whole cell, cytosolic, and nuclear extracts containing proteins (each 50 μg as proteins) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto the polyvinylidene difluoride (PVDF) membrane. The blots were blocked using 5% skim milk and probed with appropriate primary antibody in blocking buffer overnight at 4°C. The membrane was washed with PBS and then probed with the secondary antibody conjugated with horseradish peroxidase. Immunoreactive bands were detected by an enhanced chemiluminescence kit (Pierce ECL western blotting substrate, Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The means of individual groups were converted to percent control and expressed as mean ± SEM. The difference between groups was assessed using a one-way ANOVA and the Holm-Sidak test as a post-hoc test. A p-value of <0.05 was considered significantly different.
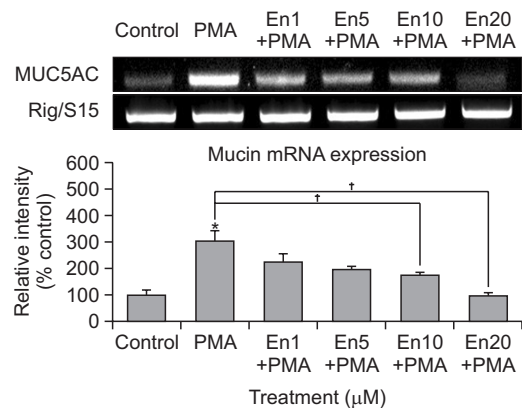

Fig. 2. Effect of engeletin on PMA-induced MUC5AC mucin mRNA expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of engeletin for 30 min and then stimulated with PMA (10 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin mRNA expression using RT-PCR. Three independent experiments were performed, and the representative data were shown. Cont: control, En: engeletin, concentration unit is μM.
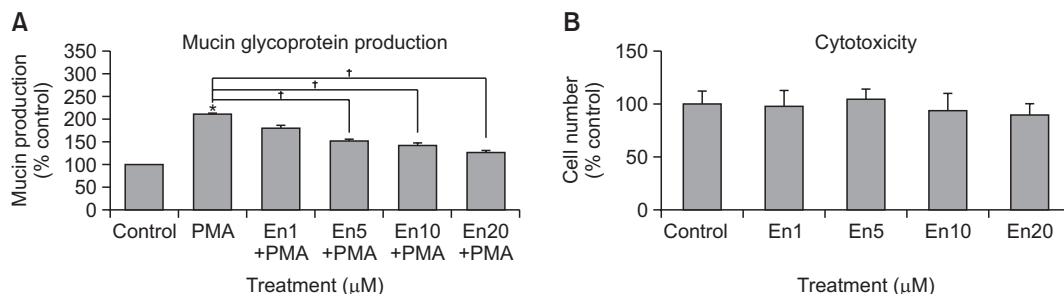

Fig. 3. Effect of engeletin on cell viability and PMA-induced MUC5AC mucin glycoprotein production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of engeletin for 30 min and then stimulated with PMA (10 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin glycoprotein production by ELISA. Each bar represents a mean ± SEM. of three culture wells compared to the control set at 100%. Three independent experiments were performed, and the representative data were shown (A). For the estimation of potential cytotoxicity of engeletin, after incubation with the indicated drug concentrations for 72 h, cell proliferation was determined using a sulforhodamine B assay (B). *Significantly different from control (p<0.05). †Significantly different from PMA alone (p<0.05). Cont: control, En: engeletin, concentration unit is μM.

RESULTS

Effect of engeletin on PMA-induced mRNA expression and glycoprotein production of MUC5AC mucin

Engeletin suppressed PMA-induced MUC5AC mucin mRNA expression (Fig. 2). Also, engeletin suppressed PMA-induced MUC5AC mucin glycoprotein production, dose-dependently. The amounts of MUC5AC mucin in the cells of engeletin-treated cultures were 100 ± 1% (control), 211 ± 3% (10 ng/mL of PMA alone), 180 ± 5% (PMA plus engeletin 1 μM), 152 ± 4% (PMA plus engeletin 5 μM), 142 ± 5% (PMA plus engeletin 10 μM) and 127 ± 3% (PMA plus engeletin 20 μM), respectively (Fig. 3A). Cell viability was checked by sulforhodamine B (SRB) assay and there was no cytotoxic effect of engeletin, at 1, 5, 10 or 20 μM concentration. The cell numbers of engeletin-treated cultures were 100 ± 12% (control), 98 ± 14% (engeletin 1 μM), 105 ± 9% (engeletin 5 μM), 94 ± 16% (engeletin 10 μM) and 90 ± 10% (engeletin 20 μM), respectively (Fig. 3B).

Effect of engeletin on PMA-induced phosphorylation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα

For the activation of NF-κB, PMA gives rise to the phosphorylation of IKK and this phosphorylated IKK sequentially phosphorylates the IκBα. The phosphorylated IκBα dissociates from NF-κB and degraded. Therefore, we tested whether engeletin affects the phosphorylation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα, induced by PMA. As can be seen in Fig. 4, engeletin attenuated PMA-stimulated phosphoryaltion of IKKα/β, through controlling the phosphorylation of the serine 176/180 moiety of IKKα/β. PMA increased the phosphorylation of IκBα, whereas engeletin suppressed its phosphorylation. Also, PMA increased the degradation of
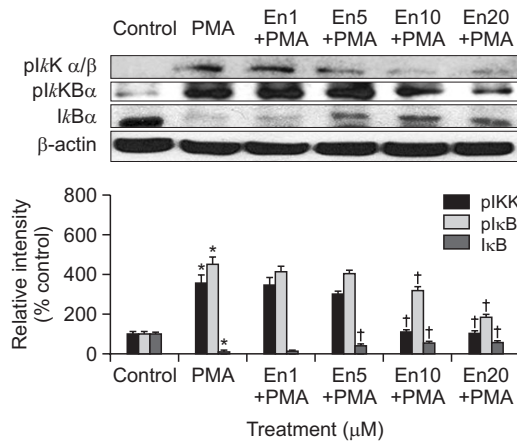
IκBα, whereas engeletin suppressed its degradation.

Effect of engeletin on PMA-induced phosphorylation and nuclear translocation of NF-κB p65

After the activation of NF-κB, it translocates from the cytosol to the nucleus, followed by being combined to the specific site of DNA. This assembly of NF-κB /DNA recruits the RNA polymerase and then the resulting mRNA is translated into the specific proteins, including MUC5AC mucins. The transcriptional activity of NF-κB p65 is dependent on its phosphorylation. As shown in Fig. 5, PMA increased the phosphorylation of p65, whereas engeletin suppressed its phosphorylation. Eventually, engeletin decreased the nuclear translocation of NF-κB p65, stimulated by PMA.

DISCUSSION

Clinically, N-acetyl L-cysteine (NAC), bromhexine, glucocorticoids, ambroxol, dornase-α, azithromycin, erdosteine, hypertonic saline solution, glyceryl guaiacolate, letocysteine, 2-mercaptoethane sulfonate sodium (MESNA), mannitol, S-carboxymethyl cysteine, myrtol, thymosin β-4, and sobrerol have been applied to the pharmacotherapy of pulmonary diseases showing pulmonary mucus hypersecretion, although these drugs are not successful to manifest the eminent clinical efficacy in managing such diseases and elicited various side effects (Li *et al*., 2020). In other words, no specific clinical medicine may regulate the production and/or secretion of MUC5AC mucin in airway mucus.

Therefore, it is very promising to develop a novel agent controlling the production and/or secretion of pulmonary mucins, through affecting the degradation and/or biosynthesis of them. Simultaneously, the regulation of inflammatory response might be the first goal, in order to control the inflammatory pulmonary diseases efficiently. In this context, we suggest it
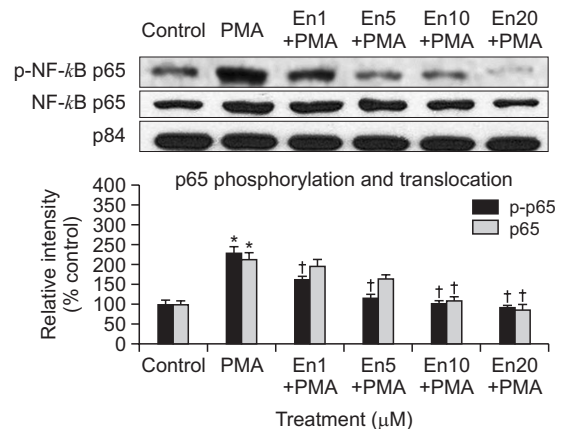

Fig. 4. Effect of engeletin on PMA-induced IKK phosphorylation, IkBα phosphorylation, and IkBα degradation, in NCI-H292 cells. NCI-H292 cells were incubated with varying concentrations of engeletin for 24 h and treated with 50 ng/mL PMA for 30 min. Cytoplasmic extracts were fractionated and then subjected to western blot analysis using phospho-specific IκBα (Ser 32/36) antibody or antibody against anti-IκBα. Whole cell lysates were prepared and then subjected to western blot analysis using phospho-specific IKKα/β (Ser 176/180) antibody. Equal protein loading was evaluated by β-actin levels. *Significantly different from control (*p*<0.05). †Significantly different from PMA alone (*p*<0.05). Cont: control, En: engeletin, IkBa: inhibitory kappa Bα, IKK: inhibitory kappa B kinase, concentration unit is μM.

Fig. 5. Effect of engeletin on PMA-induced phosphorylation and translocation of NF-kB p65, in NCI-H292 cells. Nuclear protein extracts were prepared and subjected to western blot analysis using phospho-specific p65 (Ser 536) antibody and antibody against p65. As a loading control, p84 levels were analyzed. The results shown are the representative of three independent experiments. *Significantly different from control (*p*<0.05). †Significantly different from PMA alone (*p*<0.05). Cont: control, En: engeletin, concentration unit is μM.

is encouraging to examine the potential activity of regulating the pathological production and/or secretion of mucins, using natural products isolated from a multitude of medicinal plants used empirically for controlling the inflammatory pulmonary diseases.

In the current study, we showed that engeletin, a flavonoid compound with anti-inflammatory activity, decreased the expression of MUC5AC mucin mRNA and the production of MUC5AC mucin glycoprotein, stimulated by PMA (Fig. 2, 3). These results suggest that engeletin might regulate the gene expression of pulmonary mucin, by directly acting on airway epithelial cells. As far as we know, this is the first report about the regulatory activity of engeletin on the gene expression of pulmonary mucin, in the steps of transcription and translation.

It is well-known that flavonoids including engeletin are polyphenolic natural products contained in vegetables, fruits, and various edible plants, manifesting diverse pharmacological effects such as anticancer, anti-inflammatory, antimicrobial antioxidative, and radical scavenging activities. Especially, the anti-inflammatory effects of flavonoids involve the suppression of the activities and synthesis of a multitude of pro-inflammatory mediators including cytokines, eicosanoids, C-reactive protein, and adhesion molecules. Molecular action of flavonoids might be manifested through controlling the activities of transcription factors like activating protein-1 (AP-1) and NF-kB (Serafini *et al.*, 2010).

As a multifunctional transcription regulator, NF-κB signaling commonly exists in eukaryotic cells. The activation of NF-κB occurs by preferentially provoking the phosphorylation of the IKKs. IKKs will then activate IκBα phosphorylation. Consequently, the dissociated form of phosphorylated IκBα is accumulated and degraded by the proteasome. NF-κB subunits (p65 and p50) are then released and entered into the nucleus, binding with DNA (Nie *et al.*, 2012; Liu *et al.*, 2020). NF-κB signaling pathway plays a crucial role in mediating the biological functions relating to immunity and inflammations (Huang *et al.*, 2018; Lee *et al.*, 2019; Shang *et al.*, 2019). Under the pathological conditions of airway epithelial cells, NF-κB is involved in hypersecretion of mucin and regulation of activity of cytokine (Liu *et al.*, 2020). Also, in patients suffering from asthma, activation of NF-κB can be abnormal (Huang *et al.*, 2018). It has been reported that the gene expression of MUC5AC mucin might be evoked by the inflammatory mediators which induce the activity of the transcription factors including NF-κB (Fujisawa *et al.*, 2009; Kurakula *et al.*, 2015; Garvin *et al.*, 2016). It has been well-known that PMA provokes the gene expression of MUC5AC mucin, and NF-kB signaling is reported to be involved into the effect of PMA (Ishinaga *et al.*, 2005; Laos *et al.*, 2006; Wu *et al.*, 2007; Kim *et al.*, 2012).

In the current study, as can be seen in results, engeletin suppressed the phosphorylation and nuclear translocation of NF-κB p65 via affecting the steps of the phosphorylation and degradation of IκBα, in human airway epithelial cells (Fig. 4, 5). Thus, the pharmacological action of engeletin on MUC5AC mucin gene expression might be exerted, at least partly, via mitigating IkBα degradation and NF-kB p65 nuclear translocation.

Collectively, the inhibitory activity of engeletin on airway mucin gene expression might be mediated by controlling PMA-stimulated IkBα degradation and NF-kB p65 nuclear translocation. These results suggest a potential of utilizing engeletin as a novel mucoregulator for inflammatory pulmonary diseases. It is desirable to modify the chemical structure of engeletin, so as to show the optimal regulating effect on the secretion and/or production of pulmonary mucus, by the optimally modified compound.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGMENTS

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