INTRODUCTION

Mucus present in the human respiratory system functions as the first line of defensive action against diverse noxious inhaled particles. It is a viscoelastic and gel-like complex substance containing water, salts, and a multitude of macromolecules. This gel-like characteristic of mucus is known to be due mainly to the high molecular weight component, mucins (mucous glycoproteins). The quality and quantity of production of mucins are critical for physicochemical property of mucins that is pivotal in efficient mucociliary clearance of pulmonary inflammatory cells, pathogenic microbes, cell debris, and inhaled particles. The hypersecretion or hyperproduction of sticky mucus, a specific pathological change in the normal quantity or quality of mucins, destructs the physiological defensive mechanisms of respiratory system and provoke diverse respiratory pathologic status as exemplified in cystic fibrosis, bronchiectasis, asthma, and chronic bronchitis (Voynow and Rubin, 2009).

In order to regulate such an abnormal production or secretion of airway mucins, development of a specific pharmacological agent controlling the gene expression, production, and secretion can be an ideal solution. Clinically, it has been reported that glucocorticoids suppress the hyperproduction and/or hypersecretion of airway mucins. However, they showed various adverse effects in the course of pharmacotherapy (Rogers, 2007; Sprenger et al., 2011). Therefore, we suggest it is promising to check the potential activity of regulating the abnormal secretion and/or production of mucins, using compounds isolated from various medicinal plants that were used as folk remedy for alleviating inflammatory respiratory diseases. To develop the novel candidate to control the secretion and/or production of airway MUC5AC mucin, we have tried to examine the potential effect of diverse natural products and reported that a multitude of natural products affected the expression of MUC5AC mucin gene and production of mucin protein, during the last two decades (Heo et al., 2007, 2009; Lee et al., 2011; Kim et al., 2012; Ryu et al., 2013, 2014; Seo et al., 2015).
et al., 2014; Sikder et al., 2014; Lee et al., 2015; Kim et al., 2016; Choi et al., 2018, 2019). According to the literature, kaempferol (Fig. 1), 3,4',5,7-tetrahydroxyflavone, is a flavonol, a secondary metabolite found in various edible plants (Devi et al., 2015) and was reported to be isolated from Polygonati Rhizoma, a folk medicine utilized for controlling inflammatory airway diseases (Park et al., 2012b). Kaempferol exhibits anti-oxidative and anti-inflammatory effects (Kwon et al., 2009; Park et al., 2012a; Podder et al., 2014; Park et al., 2015). Kaempferol showed anti-inflammatory activity in gastrointestinal tract (Park et al., 2012a). Some researchers have been reported that kaempferol affected the gene expression and/or production of airway mucins (Kwon et al., 2009; Podder et al., 2014; Park et al., 2015). Kaempferol suppressed mucus hypersecretion in experimental model for asthma by mitigating airway epithelial endoplasmic reticulum (ER) stress (Park et al., 2015). Podder and his colleagues reported that kaempferol decreased the gene expression of mucin induced by paraquat, a toxic herbicide, in airway epithelial BEAS-2B cells (Podder et al., 2014). Also, kaempferol suppressed the gene expression of airway mucin induced by interleukin-1β (Kwon et al., 2009).

However, as far as we perceive, there is no report about the potential effect of kaempferol on mucin production and mucin gene expression provoked by phorbol ester or epidermal growth factor, in airway epithelial cells. Of the many subtypes of human mucins, MUC5AC subtype of mucin consists of the major type of human airway mucin (Rogers and Barnes, 2006; Voynow and Rubin, 2009). Therefore, we investigated the effect of kaempferol on phorbol 12-myristate 13-acetate (PMA)- or epidermal growth factor (EGF)-induced MUC5AC mucin production and gene expression from NCI-H292 cells. A human pulmonary mucociliated cell line, NCI-H292 cells, is frequently used for specifying the signaling pathways involved in airway mucin production and gene expression (Li et al., 1997; Takeyama et al., 1999; Shao et al., 2003). It has been reported that PMA induces airway MUC5AC mucin gene expression and production, and nuclear factor kappa B (NF-κB) signaling is involved into the activity in airway epithelial cells (Ishinaga et al., 2005; Laos et al., 2006; Wu et al., 2007; Kim et al., 2012; Choi et al., 2018). EGF stimulates epidermal growth factor receptor (EGFR) signaling pathway, one of the major regulatory mechanism of airway mucin production. On the basis of this information, to suggest a specific action mechanism of kaempferol, we investigated if kaempferol had any effect on PMA-induced NF-κB signaling pathway or EGF-induced EGFR signaling pathway, in airway epithelial NCI-H292 cells.

**MATERIALS AND METHODS**

**Materials**

All the chemicals including kaempferol (purity: 95.0%) used in this experiment were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Anti-NF-κB p65 (sc-8008), anti-specificity protein-1 (Sp1) (sc-17824), anti-inhibitory kappa Bα (κBα) (sc-371), and anti-β-actin (sc-8432) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nuclear matrix protein p84 (ab-487) antibody was purchased from abcam (Cambridge, MA, USA). Anti-phospho-EGFR (Y1068), phospho-specific anti-kBα (serine 32/36, #9246), anti-EGFR, anti-phospho-IκKα/β (Ser176/180, #2687), anti-MEK1/2, anti-phospho-mitogen-activated protein kinase (MAPK)extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 (Ser221), anti-phospho-p38 MAPK (T180/ Y182), anti-p38 MAPK, anti-phospho-p44/42 MAPK (T202/ Y204), and anti-p44/42 MAPK antibodies were purchased from Cell Signaling Technology Inc (Danvers, MA, USA). Either Goat Anti-rabbit IgG (#401215) or Goat Anti-mouse IgG (#401215) was used as the secondary antibody and purchased from Calbiochem (Carlsbad, CA, USA).

**NCI-H292 cell culture**

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 penicillin (100 units/mL), streptomycin (100 µg/mL) and HEPES (25 mM) at 37°C in a humidified, 5% CO2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640 with 0.2% fetal bovine serum for 24 h.

**Treatment of cells with kaempferol**

After 24 h of serum deprivation, cells were pretreated with varying concentrations of kaempferol for 30 min and then treated with EGF (25 ng/mL) or PMA (10 ng/mL) for 24 h in serum-free RPMI 1640. Kaempferol 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 serum-free RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100 µg/mL) and HEPES (25 mM) at 37°C in a humidified, 5% CO2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640 with 0.2% fetal bovine serum for 24 h.

**Quantitation of MUC5AC mucin contents**

MUC5AC airway mucin production was measured using 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 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...
the representative data were shown. *Significantly different from control (lysates were collected for measurement of MUC5AC gene expression using RT-PCR. Three independent experiments were performed, and treated with varying concentrations of kaempferol for 30 min and then stimulated with PMA (10 ng/mL) or EGF (25 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC gene expression using RT-PCR. Three independent experiments were performed, and the representative data were shown. *Significantly different from control (p<0.05). †Significantly different from PMA (A) or EGF (B) alone (p<0.05). cont: control, K: kaempferol, concentration unit is µM.

Fig. 2. Effect of kaempferol on PMA- or EGF-induced MUC5AC mucin gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of kaempferol for 30 min and then stimulated with PMA (10 ng/mL) or EGF (25 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC gene expression using RT-PCR. Three independent experiments were performed, and the representative data were shown. *Significantly different from control (p<0.05). †Significantly different from PMA (A) or EGF (B) alone (p<0.05). cont: control, K: kaempferol, concentration unit is µM.
**RESULTS**

**Effect of kaempferol on PMA- or EGF-induced MUC5AC mucin gene expression and production from NCI-H292 cells**

Kaempferol inhibited PMA- or EGF-induced MUC5AC mucin gene expression from NCI-H292 cells (Fig. 2A, 2B). Also, kaempferol significantly inhibited PMA- or EGF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of cultures were 100 ± 8% (control), 257 ± 9% (10 ng/mL of PMA alone), 232 ± 11% (PMA plus kaempferol 1 µM), 180 ± 6% (PMA plus kaempferol 5 µM), 135 ± 5% (PMA plus kaempferol 10 µM) and 98 ± 4% (PMA plus kaempferol 20 µM), respectively (Fig. 3A). The amounts of mucin in the cells of cultures were 100 ± 5% (control), 218 ± 9% (25 ng/mL of EGF alone), 206 ± 6% (EGF plus kaempferol 1 µM), 163 ± 3% (EGF plus kaempferol 5 µM), 121 ± 4% (EGF plus kaempferol 10 µM) and 105 ± 5% (EGF plus kaempferol 20 µM), respectively (Fig. 3B). Cell viability was checked using the sulforhodamine B (SRB) assay and there was no cytotoxic effect of kaempferol at 1, 5, 10, and 20 µM (data not shown).

**Effect of kaempferol on PMA-induced phosphorylation and degradation of IκBα**

In order for NF-κB to be activated, PMA provokes the phosphorylation of IκK and this phosphorylated IκK, in turn, phosphorylates the IκBα. The phosphorylated IκBα dissociates from NF-κB and degraded. Thus, we checked whether kaempferol affects the phosphorylation of IκBα and degradation of IκBα, provoked by PMA. As can be seen in Fig. 4, kaempferol mitigated PMA-stimulated phosphorylation of IκBα. Also, PMA provoked the degradation of IκBα, whereas kaempferol inhibited the IκBα degradation.

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

The activated NF-κB translocates from the cytosol to the nucleus and then connects to the specific site of DNA. This complex of DNA/NF-κB recruits the RNA polymerase and then the resulting mRNA is translated into the specific proteins, including MUC5AC mucins. Also, the transcriptional activity of NF-κB p65 has been known to be dependent upon its phosphorylation. As can be seen in Fig. 5, PMA stimulated

**Statistics**

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 post-hoc. A *p*-value of <0.05 was considered significantly different.
the phosphorylation of p65, whereas kaempferol suppressed its phosphorylation. Finally, kaempferol blocked the nuclear translocation of NF-κB p65, provoked by PMA.

**Effect of kaempferol on PMA-induced phosphorylation and translocation of NF-κB p65 in NCI-H292 cells**

EGFR signaling pathway is known to be one of the major regulatory mechanisms of the production of MUC5AC mucin. As can be seen in Fig. 6, EGF (25 ng/mL, 24 h) stimulates the expression and phosphorylation of EGFR. Kaempferol inhibited EGF-stimulated expression of and phosphorylation of EGFR, as shown by western blot analysis. Also, EGF stimulated the phosphorylation of MEK1/2, whereas kaempferol suppressed the phosphorylation MEK1/2, in NCI-H292 cells.

**Effect of kaempferol on the phosphorylation of p38 and p44/42, and the nuclear expression of Sp1, in NCI-H292 cells**

EGF stimulated the phosphorylation of p38 and p44/42, whereas kaempferol suppressed the phosphorylation of p38 and p44/42 (ERK 1/2) MAPK (Fig. 7), as shown by western blot analysis. Lastly, EGF stimulated the nuclear expression of Sp1, a transcription factor provoking the gene expression of MUC5AC mucin, in NCI-H292 cells. Kaempferol suppressed the nuclear expression of Sp1 (Fig. 7). This, in turn, led to the down-regulation of the production of MUC5AC mucin protein, in NCI-H292 cells.

**DISCUSSION**

In the present, glucocorticoids, N-acetyl L-cysteine (NAC), 2-mercaptoethane sulfonate sodium (MESNA), letocysteine, ambroxol, bromhexine, azithromycin, domanse alfa, glyceryl guaiacolate, hypertonic saline solution, myrtol, erdosteine, mannitol, sobrerol, S-carboxymethyl cysteine, and thymosin β-4 are utilized for the pharmacotherapy of respiratory diseases manifesting airway mucus hypersecretion. However, these agents failed to exert the remarkable clinical efficacy in controlling such diseases and provoked the various side effects (Li et al., 2020). In other words, there is no specific pharmacological agent that can regulate the production and/or secretion of airway MUC5AC mucin in respiratory mucus. Thus, it is very urgent to develop a specific agent through preclinical and clinical study, in order to control the hyperproduction and/or hypersecretion of sticky, pathologically-transformed mucus in the airway of respiratory diseases.

In order to control the diverse inflammatory pulmonary diseases effectively, the regulation of inflammatory response can be the first goal. Our results demonstrated that kaempferol, an anti-inflammatory natural product, suppressed the production of MUC5AC mucin protein and the expression of MUC5AC mucin gene, induced by PMA or EGF (Fig. 2, 3). These results suggest that kaempferol can regulate the production and gene expression of mucin, by directly acting on airway...
epithelial cells. As aforementioned in Introduction, Kwon et al. (2009) reported that kaempferol inhibited MUC5AC mucin gene expression. However, they used interleukin-1β, a pro-inflammatory cytokine stimulating gene expression of various biomolecules including mucin, as an inducer and did not elucidate the molecular mechanism involved in suppression of MUC5AC mucin gene expression. In this study, we selected PMA and EGF as inducers of the gene expression and production of MUC5AC mucin and tried to elucidate the underlying mechanism at the molecular level. There are many reports concerning the usefulness of PMA and EGF as a tool for stimulating airway MUC5AC mucin production and gene expression, in experimental models simulating human asthma and chronic obstructive pulmonary disease (COPD) (Lemmon and Schlessinger, 1994; Takeyama et al., 1999, 2000). Therefore, we examined whether kaempferol affects the EGFR signaling cascade. It has been reported that EGFR-MEK-MAPK-Sp1 signaling cascade plays an important role in the gene expression of MUC5AC mucin (Hewson et al., 2004). Also, inhibitors for EGFR tyrosine kinase suppressed the EGFR-stimulated gene expression and protein production of MUC5AC mucin, suggesting that the hyperproduction of MUC5AC mucin is a result of the activation of EGFR signaling cascade (Mata et al., 2005). At the same time, transcriptional regulation of the eukaryotic gene has been reported to be a complicated process and Sp1 is a well-characterized transcription factor showing multiple activities in the transcription of diverse genes including MUC5AC (Briggs et al., 1986; Kadonaga et al., 1987).

We found that EGFR is constitutively expressed in NCI-H292 cells and kaempferol inhibited EGFR-stimulated expression of EGFR (Fig. 6). Wetzker and Bohmer (2003) reported that EGFR induced the protein tyrosine kinase activity of EGFR and activated the MAPK cascade including p38 MAPK and p44/42 MAPK. Also, inhibition of activity of p38 MAPK and p44/42 MAPK was reported to suppress the EGFR-induced MUC5AC gene expression (Mata et al., 2005). In our results, we demonstrated that kaempferol decreased the phosphorylation of MEK1/2, p38 MAPK, and p44/42 MAPK (Fig. 6, 7). We found that kaempferol suppressed the nuclear expression of Sp1, a transcription factor provoking the gene expression of MUC5AC mucin (Fig. 7). Consequently, this activity of kaempferol, in turn, led to the down-regulation of the production of MUC5AC mucin protein, in NCI-H292 cells.

In summary, the inhibitory activity of kaempferol on airway mucin gene expression and production might be mediated by regulating PMA-induced degradation of IκBα and nuclear translocation of NF-κB p65 and/or affecting EGFR-induced EGFR-MEK-MAPK-Sp1 signaling cascade. These results suggest a potential of utilizing kaempferol as an efficacious mucocative agent for inflammatory respiratory diseases. Through further study, it should be essential to modify the structure of kaempferol so that the optimal compound shows the best controlling effect on the secretion and/or production of mucus.
CONFLICT OF INTEREST

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

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