Kaempferol Regulates the Expression of Airway MUC5AC Mucin Gene via IκBα-NF-κB p65 and p38-p44/42-Sp1 Signaling Pathways

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Abstract

In the present study, kaempferol, a flavonoidal natural compound found in Polygonati Rhizoma, was investigated for its potential effect on the gene expression and production of airway MUC5AC mucin. A human respiratory epithelial NCI-H292 cells was pretreated with kaempferol for 30 min and stimulated with epidermal growth factor (EGF) or phorbol 12-myristate 13-acetate (PMA), for the following 24 h. The effect on PMA-induced nuclear factor kappa B (NF-κB) signaling pathway or EGF-induced mitogen-activated protein kinase (MAPK) signaling pathway was investigated. Kaempferol suppressed the production and gene expression of MUC5AC mucins, induced by PMA through the inhibition of degradation of inhibitory kappa B α (IκBα), and NF-κB p65 nuclear translocation. Also, kaempferol inhibited EGF-induced gene expression and production of MUC5AC mucin through regulating the phosphorylation of EGFR, phosphorylation of p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2 (p44/42), and the nuclear expression of specificity protein-1 (Sp1). These results suggest kaempferol regulates the gene expression and production of mucin through regulation of NF-κB and MAPK signaling pathways, in human airway epithelial cells.

Key Words: MUC5AC, Pulmonary mucin, Kaempferol
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 mucins 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 mucocylinderoid 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 B α (kBa) (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-IKKα/β (Ser176/180, #2667), anti-MEK1/2, anti-phospho-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 (S221), 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 (#401315) 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 0.5% dimethyl sulfoxide did not affect mucin gene expression, mucin production, and expression and activity of molecules involved in NF-κB or EGFR 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 protein (in a 24-well culture plate). The total RNA was extracted in order to measure the expression of MUC5AC gene (in a 6-well culture plate) using RT-PCR. For the western blot analysis, cells were treated with kaempferol for 24 h and then with PMA or EGF for the indicated periods.

**Quantification 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
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 H2SO4. Absorbance was read at 450 nm.

**Total RNA isolation 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 Thermoprime 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 were (forward) 5′-TTC CGC AAG ATA TGG G-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. 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 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.

**Preparation of whole cell extract**

NCI-H292 cells (confluent in 150 mm culture dish) were pretreated for 24 h at 37°C with 1, 5, 10 or 20 µM of kaempferol, and then stimulated with PMA (50 ng/mL) for 30 min, in serum-free RPMI 1640. Also, the cells were pretreated with 1, 5, 10 or 20 µM of kaempferol for 15 min or 24 h and treated with EGF (25 ng/mL) for 24 h or the indicated periods. After the treatment of the cells with kaempferol, media were aspirated, and the cells washed with cold PBS. The cells were collected by scraping and were centrifuged at 3,000 rpm for 5 min. The supernatant was discarded. The cells were 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. Protein content in extract was determined by Bradford method.

**Preparation of nuclear and cytosolic extracts**

After the treatment with kaempferol as outlined, the cells were harvested using Trypsin-EDTA solution and then centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). The supernatant was discarded, and 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. Protein content in extracts was determined by Bradford method.

**Detection of proteins by western blot analysis**

Cytoplasmic, nuclear, and whole cell 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). 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′.
**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 IKK and this phosphorylated IKK, 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

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**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.
Effect of kaempferol on the expression and phosphorylation of EGFR and the phosphorylation of MEK1/2 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) stimulated 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 of 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), letecysteine, ambroxol, bromhexine, azithromycin, domase alfa, glycerol 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 such 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...
H292 cells were pretreated with varying concentrations of kaempferol and the nuclear expression of Sp1 in NCI-H292 cells. NCI-H292 alone (control) showed significantly different from control (p<0.05). Therefore, the pharmacological effect of kaempferol on MUC5AC production and gene expression might be manifested, at least partly, through inhibiting the degradation of IκBα and nuclear translocation of NF-κB p65.

On the other hand, EGFR provokes EGFR signaling pathway and MUC5AC mucin gene expression and production, in NCI-H292 cells and EGFR has been reported to be up-regulated in asthmatic airways (Burgel et al., 2001). EGFR plays a pivotal role as a primary regulator of epithelial function, transducing extracellular signals from its activating ligand into intracellular signaling cascades including MEK-MAPK. The activated MAPK, in turn, activates Sp1 transcription factor to initiate the expression of MUC5AC mucin gene. (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 mucocactive 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.

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; Ishinaga et al., 2005; Laos et al., 2006; Wu et al., 2007; Kim et al., 2012; Choi et al., 2018).

Several studies revealed that MUC5AC mucin gene expression and production can be increased by the inflammatory mediators which activate the transcription factors including NF-κB (Fujisawa et al., 2009; Kurakula et al., 2015; Garvin et al., 2016). PMA stimulates 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). In our results, kaempferol inhibited the phosphorylation and nuclear translocation of NF-κB p65 through acting on the steps of the phosphorylation and degradation of IκBα, in human airway epithelial cells (Fig. 4, 5). Therefore, the pharmacological effect of kaempferol on MUC5AC production and gene expression might be manifested, at least partly, through inhibiting the degradation of IκBα and nuclear translocation of NF-κB p65.
CONFLICT OF INTEREST

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

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