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Effects of Caffeic Acid, Myristicin and Rosemarinic Acid on the Gene Expression and Production of Airway MUC5AC Mucin

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Abstract – *Perilla frutescens* was empirically used for controlling airway inflammatory diseases in folk medicine. We investigated whether caffeic acid, myristicin and rosemarinic acid derived from *Perilla frutescens* significantly affect the gene expression and production of mucin from airway epithelial cells. Confluent NCI-H292 cells were pretreated with caffeic acid, myristicin or rosemarinic acid for 30 min and then stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h. The MUC5AC mucin gene expression and production were measured by reverse transcription - polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Additionally, we examined whether caffeic acid, myristicin or rosemarinic acid affects MUC5AC mucin production indued by epidermal growth factor (EGF) and tumor necrosis factor-α (TNF-α), the other two stimulators of production of airway mucin. The results were as follows: (1) Caffeic acid, myristicin and rosemarinic acid inhibited the gene expression and production of MUC5AC mucin induced by PMA from NCI-H292 cells, respectively; (2) Among the three compounds derived from *Perilla frutescens*, only rosemarinic acid inhibited the production of MUC5AC mucin induced by EGF or TNF-α, the other two stimulators of production of airway mucin. These results suggest that rosemarinic acid derived from *Perilla frutescens* can regulate the production and gene expression of mucin, by directly acting on airway epithelial cells and, at least in part, explains the traditional use of *Perilla frutescens* as remedies for diverse inflammatory pulmonary diseases.
Keywords – Airway mucin, Caffeic acid, Myristicin, Rosemarinic acid

Introduction

Airway mucus is very important in defensive action against invading pathogenic microbes, noxious chemicals and diverse environmental particles. The protective function of airway mucus is attributed to the viscoelasticity of mucins. However, any abnormality in the quality or quantity of mucins not only causes altered airway physiology but may also impair host defenses often leading to severe airway pathology as exemplified in chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis. On the other hand, hyperproduction and/or hypersecretion of airway mucus might be strongly related to airway inflammation.[1] Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excessive mucin secretion (production) by various anti-inflammatory medicinal plants. We have investigated the possible activities of some natural products on mucin secretion from airway epithelial cells. As a result of our trial, we previously reported that several natural products affected mucin secretion and/or production from airway epithelial cells.[2-4] According to traditional oriental medicine, *Perilla frutescens* has been utilised for controlling airway inflammatory diseases.[5] Also, caffeic acid, myristicin and rosemarinic acid - its components - were reported to have diverse biological effects including antioxidant and anti-inflammatory effects.[6-9] However, to the best of our knowledge, there are no reports about the potential effects of caffeic acid, myristicin and rosemarinic acid on the gene expression and production of mucin from airway epithelial cells. Among the twenty one or more MUC genes coding human mucins reported up to now, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium.[1,10] Therefore, we examined the effect of caffeic acid, myristicin or rosemarinic acid on PMA-induced MUC5AC mucin gene expression and production from NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are

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frequently used for the purpose of elucidating intracellular signaling pathways involved in airway mucin production and gene expression.[11-13]

Experimental

General experimental procedures – All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified.

Preparation of caffeic acid, myristicin and rosemarinic acid – Caffeic acid (purity: 98.0%), myristicin (purity: 98.0%) and rosemarinic acid (purity: 98.0%) (Fig.1) were isolated, purified and identified by analytical chemists in the Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University (Suwon, Korea). Briefly, the aerial parts of *P. frutescens* (L.) var. *acuta* (25 kg) were collected on Nam Won, Korea in January 2012, and authenticated by Prof. J. H. Lee (Dongguk University, Gyoungju, Korea). A voucher specimen (SKKU NPL 1207) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University. The dried leaves of *P. frutescens* (L.) var. *acuta* (500.0 g) were extracted with water and 70% EtOH under reflux (2 × 3 h) and then filtered. The filtrates were evaporated under reduced pressure to give water extract (68.0 g, 13%) and EtOH extract (57.0 g, 11%). For an isolation of active constituents, the dried leaves of *P. frutescens* (L.) var. *acuta* (25.0 kg) were extracted with petroleum ether, and methanol, successively and evaporated under reduced pressure to give residues (264 g and 2 kg, respectively). The methanol extract (1 kg) was dissolved in water (800 ml) and partitioned with solvent to give hexane (190.0 g), $CHCl_3$ (134.0 g), EtOAc (60.0 g), and BuOH (87.0 g) soluble portions. The petroleum ether extract (130.0 g) was chromatographed over a silica gel column with Hexane-EtOAc (1:0 - 1:1) as the eluent to give three fractions (P1 – P3). The P2 fraction (37 g) was subfractionated with a silica gel column with Hexane-EtOAc (1:0 - 1:1) as the eluent to give seven fractions (P21 – P27). The P25 fraction (8.7 g) was separated by silica gel column chromatography using a solvent system of Hexane-EtOAc (30:1 - 1:1) as the eluent to yield five fractions (P251 – 255). The P253 fraction (1.0 g) was also subjected to a RP-C_{18} silica gel column with 100% MeCN and purified by preparative normal-phase HPLC with solvent system of Hexane-EtOAc (20:1) to yield myristicin (40 mg). The EtOAc fraction (33.0 g) was chromatographed over a silica gel column with $CHCl_3$- MeOH (20:1 - 1:1) as the eluent to give seven fractions (E1 – E7). The E5 fraction (14 g) was also subjected to a RP-C_{18} silica gel column with 50% MeOH as the eluent to afforded eight fractions (E51 – E58). The E51 fraction (7.9 g) was subfractionated with a silica gel column with $CHCl_3$-MeOH (20:1 - 1:1) as the eluent to give eight fractions (E511 – E518). Subfraction E515 (750 mg) was subjected to a Sephadex LH-20 (80% MeOH) and purified by RP-C_{18} prep. HPLC (30% MeOH) to give caffeic acid (80 mg). Subfraction E518 (4.2 g) was subjected to a Sephadex LH-20 (80% MeOH) and purified by RP-C_{18} prep. HPLC (30% MeCN, 60% MeOH) to give rosmarinic acid (1.9 g). These compounds were identified to be myristicin, rosmarinic acid and caffeic acid by comparison of their spectroscopic and physical data with previously reported values.

NCI-H292 cell culture – NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and 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 ºC 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 recultured in RPMI 1640 with 0.2% FBS for 24 h.

Treatment of cells with caffeic acid, myristicin or rosemarinic acid – After 24 h of serum deprivation, cells were pretreated with varying concentrations of caffeic acid, myristicin or rosemarinic acid for 30 min and treated with PMA (10 ng/mL), EGF (25 ng/mL) or TNF-α (0.2 nM), for 24 h in serum-free RPMI 1640. Caffeic acid, myristicin and rosemarinic acid were dissolved in dimethylsulfoxide and treated in culture medium (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulfoxide did not affect mucin gene expression and production from 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, U.S.A.) and collected to measure the production of MUC5AC protein (in 24-well culture plate). The total RNA was extracted for measuring the expression of MUC5AC gene (in 6-well culture plate) by using RT-PCR.

Total RNA isolation and RT-PCR – Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc. Kyung-gi-do, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. 2 μg of total RNA was primed

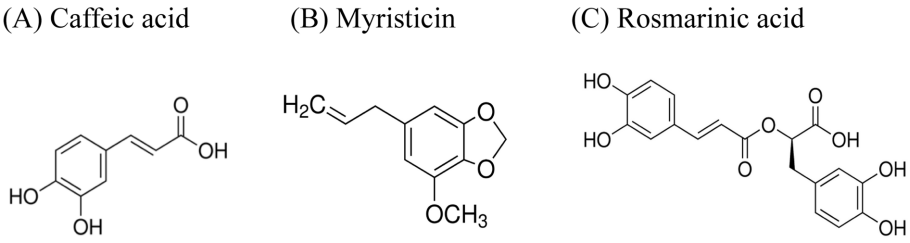

Fig. 1. Chemical structure of caffeic acid, myristicin or rosemarinic acid.

with 1 μg of oligo(dT) in a final volume of 50 μL (RT reaction). 2 μL of RT reaction product was PCR amplified in a 25 μL by using Thermorprime Plus DNA Polymerase (ABgene, Rochester, NY, U.S.A.). 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'. As quantitative controls, primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used. 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 ºC for 2 min followed by 40 cycles at 94ºC for 30 s, 60 ºC for 30 s and 72 ºC 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.

MUC5AC mucin analysis – MUC5AC airway mucin production was measured by ELISA. Cell lysates were prepared with PBS at 1:10 dilution, and 100 μL of each sample was incubated at 42 ºC in a 96-well plate, until dry. Plates were washed three times with PBS and blocked with 2% BSA (fraction V) for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, U.S.A.), 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.

Statistics – Means of individual group were converted to percent control and expressed as mean ± S.E.M. The difference between groups was assessed using one-way ANOVA and Holm-Sidak test as a post-hoc test. $p < 0.05$ was considered as significantly different.

Result and Discussion

PMA has been reported to stimulate the endogenous activator of protein kinase C (PKC), diacylglycerol (DAG)[14] and to be an inflammatory stimulant that can control a gene transcription[15], cell growth and differentiation[16]. PMA also can induce MUC5AC gene expression in NCI-H292 cells.[17] PMA activates a type of protein kinase C (PKC) isoforms. This activates matrix metalloproteinases (MMPs), which cleave pro-EGF receptor (EGFR) ligands from the cell surface to become mature EGFR ligands. These ligands bind to the EGF receptor, provoking the phosphorylation of its intracellular tyrosine kinase. This leads to activation of MEK leading to ERK activation. Following is the activation of the transcription factor, Sp1, and binding of the factor to specific sites with the MUC5AC gene promoter. Finally, the promoter is activated and produced the gene transcription and translation to MUC5AC mucin protein.[14]

Based upon these reports, we investigated whether caffeic acid, myristicin or rosemarinic acid affects PMA-induced MUC5AC mucin gene expression and production from NCI-H292 cells. As can be seen in Fig. 2, MUC5AC gene expression induced by PMA from NCI-H292 cells was inhibited by pretreatment with caffeic acid, myristicin and rosemarinic acid, respectively (Fig. 2). Cytotoxicity was checked by lactate dehydrogenase (LDH) assay and there was no remarkable cytotoxic effect of caffeic acid, myristicin or rosemarinic acid, at the treatment concentrations (data were not shown). At the same time, caffeic acid, myristicin or rosemarinic acid suppressed PMA-induced production of MUC5AC mucin protein (Fig. 3). Caffeic acid significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of caffeic acid-treated cultures were 100 ± 4%, 204 ± 15%, 140 ± 7%, 129 ± 9%, and 105 ± 6% for control, 10 ng/mL of PMA alone, PMA plus caffeic acid 10^{-6} M, PMA plus caffeic acid 10^{-5} M and PMA plus caffeic acid 10^{-4} M, respectively (Fig. 3 (A)). Myristicin significantly inhibited PMA-induced MUC5AC

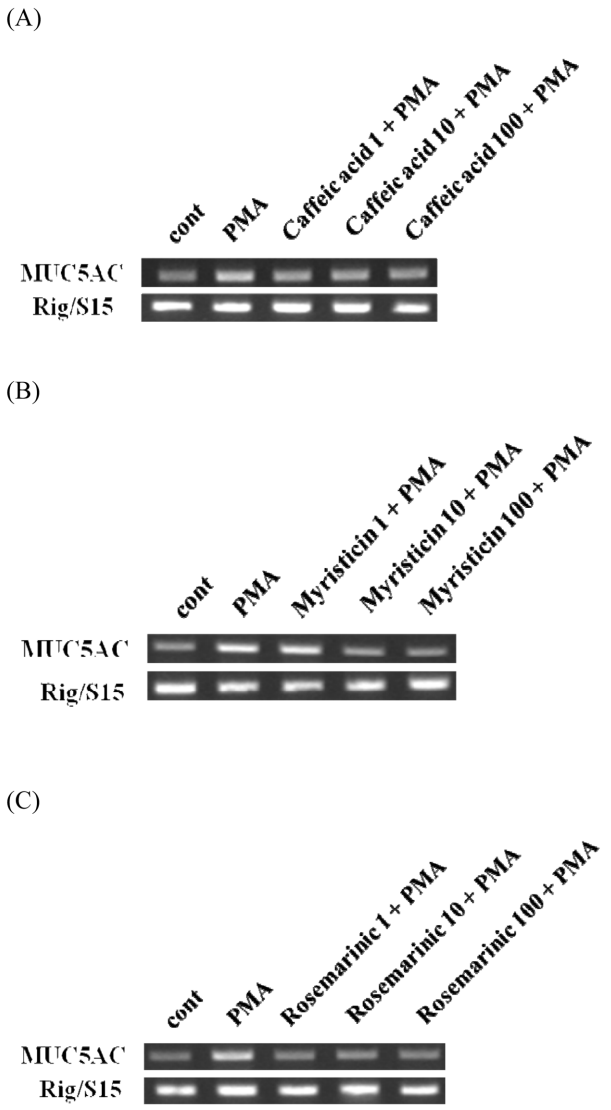

Fig. 2. Effect of caffeic acid, myristicin or rosemarinic acid on PMA-induced MUC5AC gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of caffeic acid, myristicin or rosemarinic acid for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. MUC5AC gene expression was measured by RT-PCR. Three independent experiments were performed and the representative images were shown. (cont: control, concentration unit is μM.).

production from NCI-H292 cells. The amounts of mucin in the cells of myristicin-treated cultures were 100 ± 6%, 210 ± 21%, 131 ± 16%, 109 ± 8% and 91 ± 15% for control, 10 ng/mL of PMA alone, PMA plus myristicin 10^{-6} M, PMA plus myristicin 10^{-5} M and PMA plus myristicin 10^{-4} M, respectively (Fig. 3 (B)). Also, rosemarinic acid significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of rosemarinic acid-treated cultures were 100 ± 4%, 204 ± 15%, 142 ± 7%, 115 ± 10% and 99 ±

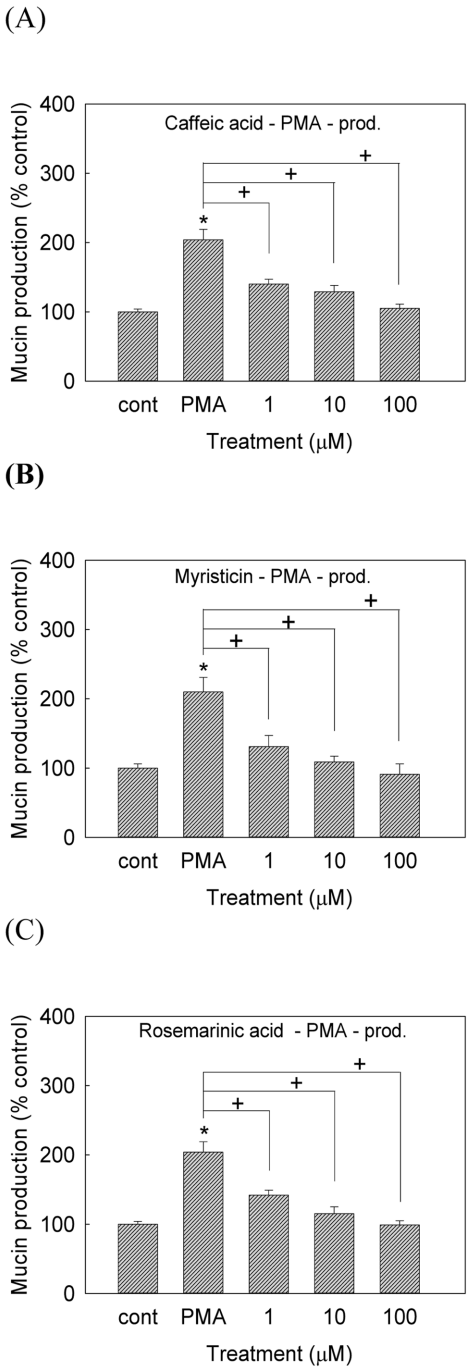

Fig. 3. Effect of caffeic acid, myristicin or rosemarinic acid on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of caffeic acid, myristicin or rosemarinic acid for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100% (A, B, C). Three independent experiments were performed and the representative data were shown.
*significantly different from control ($p < 0.05$).
+significantly different from PMA alone ($p < 0.05$).
(cont: control, concentration unit is μM.)

6% for control, 10 ng/mL of PMA alone, PMA plus rosemarinic acid 10^{-6} M, PMA plus rosemarinic acid 10^{-5} M and PMA plus rosemarinic acid 10^{-4} M, respectively (Fig. 3 (C)).

These results suggest that caffeic acid, myristicin or rosemarinic acid can regulate the gene expression and production of MUC5AC mucin induced by PMA, by directly acting on airway epithelial cells.

Next, we tried to investigate whether caffeic acid, myristicin or rosemarinic acid affects MUC5AC production induced by EGF or TNF-α, the other well-known stimulator of mucin production from airway epithelial cells.

TNF-α was reported to stimulate the secretion and gene expression of airway mucin.[13,18,19] TNF-α converting enzyme (TACE) provoked MUC5AC mucin expression in cultured human airway epithelial cells[13] and TNF-α induced MUC5AC gene expression in normal human airway epithelial cells.[19] TNF-α level in sputum was reported to be increased, with further increases during exacerbation of pulmonary diseases.[20,21] It also induced mucin secretion from guinea pig tracheal epithelial cells.[18]

On the other hand, EGF has been reported to regulate MUC5AC gene expression in the lung. MUC5AC mRNA expression was reported to increase after ligand binding to the EGF receptor and activation of the mitogen-activated protein kinase (MAPK) cascade.[12,20]

As can be seen in results, caffeic acid did not affect EGF- or TNF-α-induced production of MUC5AC mucin protein (Fig. 4). The amounts of mucin in the cells of caffeic acid-treated cultures were 100 ± 5%, 325 ± 21%, 308 ± 4%, 305 ± 18%, and 359 ± 15% for control, 25 ng/mL of EGF alone, EGF plus caffeic acid 10^{-6} M, EGF plus caffeic acid 10^{-5} M and EGF plus caffeic acid 10^{-4} M, respectively (Fig. 4 (A)). The amounts of MUC5AC mucin in the cells of caffeic acid-treated cultures were 100 ± 7%, 405 ± 44%, 404 ± 52%, 459 ± 35% and 482 ± 94% for control, 0.2 nM of TNF-α alone, TNF-α plus caffeic acid 10^{-6} M, TNF-α plus caffeic acid 10^{-5} M and TNF-α plus caffeic acid 10^{-4} M, respectively (Fig. 4 (B)). Myristicin suppressed EGF-induced production of MUC5AC mucin protein, although it did not affect TNF-α-induced production of MUC5AC mucin protein (Fig. 5). The amounts of mucin in the cells of myristicin-treated cultures were 100 ± 9%, 345 ± 21%, 342 ± 4%, 298 ± 18% and 141 ± 15% for control, 25 ng/mL of EGF alone, EGF plus myristicin 10^{-6} M, EGF plus myristicin 10^{-5} M and EGF plus myristicin 10^{-4} M, respectively (Fig. 5 (A)). The amounts of MUC5AC mucin in the cells of myristicin-treated cultures were 100 ± 22%, 221 ± 18%, 218 ± 12%, 210 ± 45% and 252 ± 12% for control, 0.2 nM of TNF-α alone, TNF-α plus myristicin 10^{-6} M, TNF-α plus myristicin 10^{-5} M and TNF-α plus myristicin 10^{-4} M, respectively (Fig. 5 (B)). However, rosemarinic acid inhibited EGF- and TNF-α-induced production of MUC5AC mucin protein, respectively (Fig. 6). Rosemarinic acid significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of rosemarinic acid-treated cultures were 100 ± 13%, 361 ± 1%, 371 ± 3%, 338 ± 6% and 53 ± 14% for control, 25 ng/mL of EGF alone, EGF plus rosemarinic acid 10^{-6} M, EGF plus rosemarinic acid 10^{-5} M and EGF plus rosemarinic acid 10^{-4} M, respectively (Fig. 6 (A)). Rose-

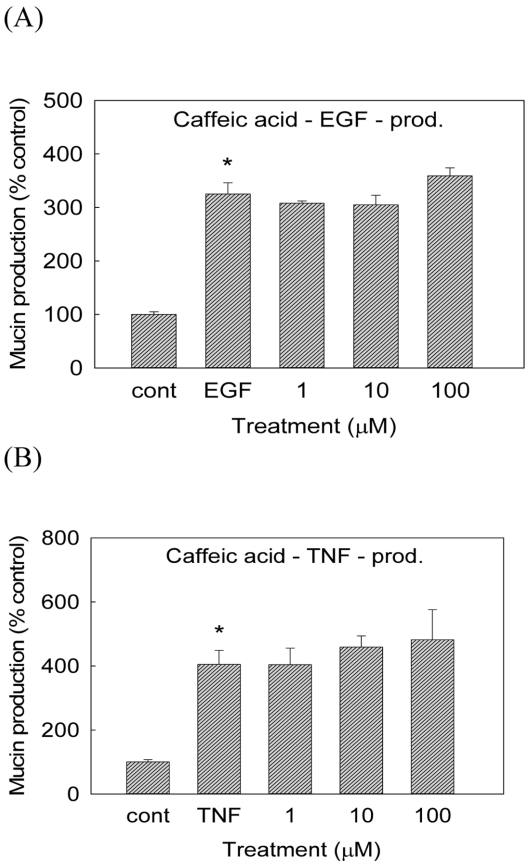

Fig. 4. Effect of caffeic acid on EGF- or TNF-α-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of caffeic acid for 30 min and then stimulated with EGF (25 ng/mL) or TNF-α (0.2 nM, 10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100% (A, B). Three independent experiments were performed and the representative data were shown.

*significantly different from control ($p < 0.05$).

(cont: control, concentration unit is μM.)

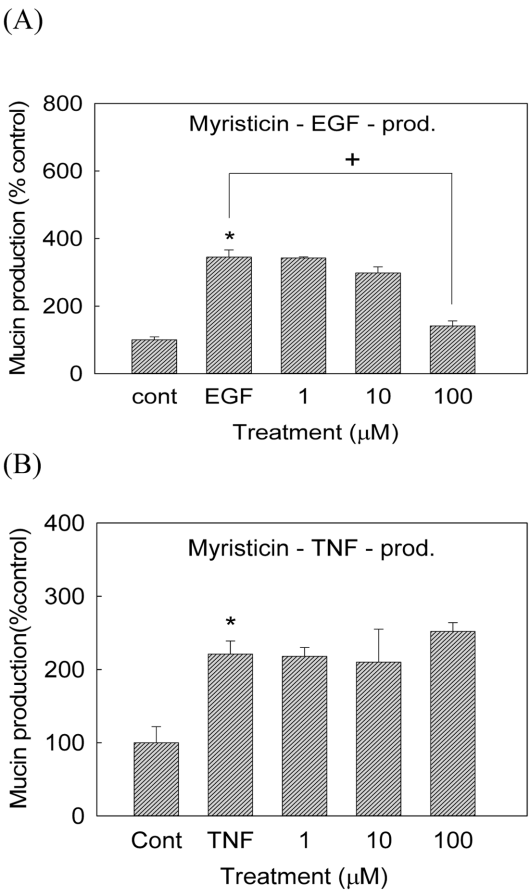

Fig. 5. Effect of myristicin on EGF- or TNF-α-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of myristicin for 30 min and then stimulated with EGF (25 ng/mL) or TNF-α (0.2 nM, 10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100% (A, B). Three independent experiments were performed and the representative data were shown.

*significantly different from control ($p < 0.05$).

+ significantly different from EGF alone ($p < 0.05$).

(cont: control, concentration unit is μM.)

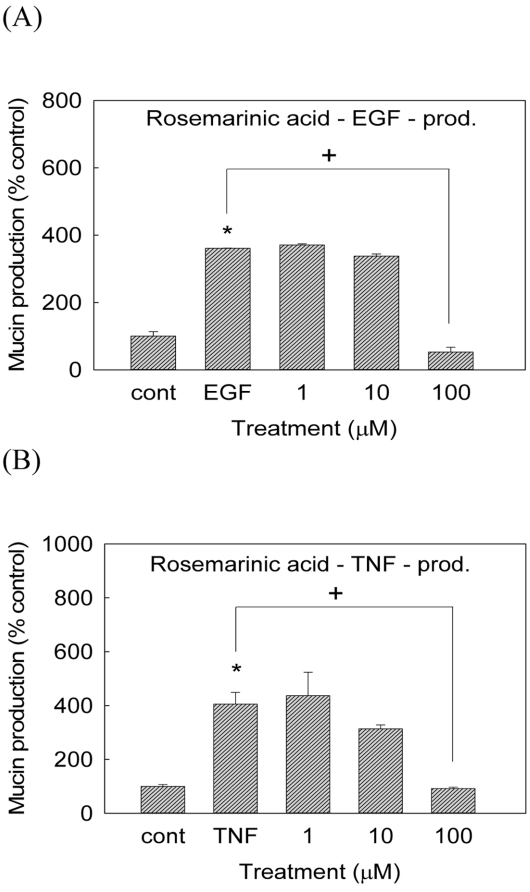

Fig. 6. Effect of rosemarinic acid on EGF- or TNF-α-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of rosemarinic acid for 30 min and then stimulated with EGF (25 ng/mL) or TNF-α (0.2 nM, 10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100% (A, B). Three independent experiments were performed and the representative data were shown.

*significantly different from control ($p < 0.05$).

+significantly different from EGF or TNF-α alone ($p<0.05$).

(cont: control, concentration unit is μM.)

marinic acid also inhibited TNF-α-induced MUC5AC mucin production. The amounts of MUC5AC mucin in the cells of rosemarinic acid-treated cultures were 100 ± 7%, 405 ± 44%, 437 ± 87%, 314 ± 14% and 92 ± 5% for control, 0.2 nM of TNF-α alone, TNF-α plus rosemarinic acid 10^{-6} M, TNF-α plus rosemarinic acid 10^{-5} M and TNF-α plus rosemarinic acid 10^{-4} M, respectively (Fig. 6 (B)).

Taken together, among the three compounds derived from *Perilla frutescens*, only rosemarinic acid inhibited the production of MUC5AC mucin induced by PMA, EGF or TNF-α. Yet, we could not suggest the reason why the responses of the three compounds to EGF- and TNF-α-induced mucin production and gene expression are not consistent compared to those induced by PMA, based on the present result of the study. The underlying mechanisms of action of rosemarinic acid and the other two natural products on MUC5AC mucin gene expression and production are not clear at present, although we are investigating whether rosemarinic acid and the other two natural products act as potential regulators of NF-kB signaling pathway and/or the MAPK cascade after ligand binding to the TNF or EGF receptor, in mucin-producing NCI-H292 cells.

In summary, the inhibitory action of caffeic acid,

myristicin or rosemarinic acid on airway mucin production and gene expression might explain, at least in part, the traditional use of *Perilla frutescens* as an anti-inflammatory mucoregulator for pulmonary inflammatory diseases, in folk medicine. We suggest it is valuable to find the natural products that have specific inhibitory effects on mucin production and gene expression - in view of both basic and clinical sciences - and the result from this study suggests a possibility of using rosemarinic acid as a new efficacious mucoregulator for pulmonary diseases, although further studies are essentially required.

Acknowledgements

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