Natural Product Sciences
20(3) : 196-201 (2014)

Pyunkang-hwan (Pyunkang-tang) Regulates Hypersecretion of Pulmonary Mucin from Rats with Sulfur Dioxide-Induced Bronchitis and Production and Gene Expression of MUC5AC Mucin from Human Airway Epithelial Cells

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Abstract – Pyunkang-hwan (Pyunkang-tang) extract (PGT) is a traditional folk medicine for controlling diverse pulmonary diseases including bronchitis, tonsiltis and pneumonitis. We investigated whether PGT significantly affects secretion, production and gene expression of airway mucin using *in vivo* and in vitro experimental models reflecting the hypersecretion and/or hyperproduction of mucus observed in inflammatory pulmonary diseases. For *in vivo* experiment, effect of PGT was checked on hypersecretion of pulmonary mucin in sulfur dioxide-induced bronchitis in rats. For in vitro experiment, confluent NCI-H292 cells were pretreated with PGT for 30 min and then stimulated with EGF (epidermal growth factor), PMA (phorbol 12-myristate 13-acetate) or TNF-α (tumor necrosis factor-α) for 24 h. The MUC5AC mucin gene expression and mucin protein production were measured by RT-PCR and ELISA. The results were as follows: (1) PGT inhibited the expression of MUC5AC mucin gene induced by EGF, PMA or TNF-α from NCI-H292 cells, respectively; (2) PGT also inhibited the production of MUC5AC mucin protein induced by the same inducers from NCI-H292 cells, respectively; (3) PGT inhibited secretion of mucin in sulfur dioxide-induced bronchitis rat model. This result suggests that PGT can regulate secretion, production and gene expression of airway mucin.
Keywords – Airway, Mucin, Natural products

Introduction

Airway mucus plays pivotal role in defense against invading pathogenic microorganisms, chemicals and 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 cause 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.[1] Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excess mucin secretion (production) by traditionally-used folk medicine. We have tried to investigate the possible activities of some natural products on mucin secretion from cultured 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.[2-5]

PGT, a traditional herbal preparation in the form of water extract comprising the six herbs (Table 1), has been used for controlling the hypersecretion of airway mucus observed in bronchitis, tonsiltis and pneumonitis.[6] Therefore, in this study, we checked whether PGT significantly affects secretion, production and gene expression of airway mucin, in order to elucidate the mode of action of PGT, using *in vivo* and in vitro experimental models reflecting the hypersecretion and/or hyperproduction of mucus observed in inflammatory pulmonary diseases. For *in vivo* experiment, effect of PGT was checked on hypersecretion of pulmonary mucin in sulfur dioxide-induced bronchitis in rats. For in vitro experiment, effect of PGT was checked on production and gene expression of airway mucin from NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of elucidating intracellular signaling pathways involved in airway mucin production and gene expression.[7-9]

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Table 1. Composition of PGT

Herbs	Amounts (g)
Lonicerae Flos (*Lonicerae japonica* Thunberg, Caprofoliaceae)	4
Liriopis Tuber (*Liriope platyphylla* Wang et Tang, Liliaceae)	4
Adenophorae Radix (*Adenophora triphilla* var. *japonoica* Hara, Campanulaceae)	10
Xanthii Fructus (*Xantium strumarinum* Linne, Compositae)	10
Selaginellae Herba (*Selaginella tamariscina* Spring, Selaginellaceae)	10
Rehmanniae Radix Preparata (*Rehmannia glutinosa* Liboschitz var. *purpurea* Making, Scrophulariaceae)	2

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 PGT – PGT is an herbal preparation in the form of water extract comprising the six herbs (Table 1). The six herbs were purchased from Dae-won-dang Oriental Drug Store (Seoul, Korea) and identified by emeritus professor of Herbology, Chang Soo Yook (College of Pharmacy, Kyung Hee University, Seoul, Korea).The voucher specimens were deposited at the Herbarium of the College of Pharmacy, Kyung Hee University, Seoul, Korea: 4 g of Lonicerae Flos (*Lonicerae japonica* Thunberg, Caprofoliaceae), KHUOPS 2013-75; 4 g of Liriopis Tuber (*Liriope platyphylla* Wang et Tang, Liliaceae), KHUOPS 2013-76; 10 g of Adenophorae Radix (*Adenophora triphilla* var. *japonoica* Hara, Campanulaceae), KHUOPS 2013-77; 10 g of Xanthii Fructus (*Xantium strumarinum* Linne, Compositae), KHUOPS 2013-78; 10 g of Selaginellae Herba (*Selaginella tamariscina* Spring, Selaginellaceae), KHUOPS 2013-79; 2 g of Rehmanniae Radix Preparata (*Rehmannia glutinosa* Liboschitz var. *purpurea* Making, Scrophulariaceae), KHUOPS 2013-80. The six herbs were soaked with 500 mL of double-distilled deionized water and decocted for 150 min at 100 ºC. The extract was filtered through sterile gauze, concentrated in a rotary vacuum evaporator and lyophilized. 1 g of PGT was prepared from 65 g of mixture of the six herbs (yield: 1.54%) and stored at −70 ºC until assayed for its biological action.

Animals – Pathogen-free male Sprague-Dawley rats (Daehan Biolink, Seoul, Korea), 5 weeks of age weighing 200 - 220 g, were used. The animals were housed five per cage and were provided with the distilled water and food *ad libitum*. They were kept under a 12 h light/dark cycle (light on 08:00 - 20:00) at constant temperature (22.5 ºC) and humidity (55%). Animals were cared through all of the experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals regulated by Chungnam National University, Daejeon, Korea.

Experimental design- Thirty five rats were randomly divided into the following seven groups: normal control; sulfur dioxide (SO_2)-only exposure; SO_2 exposure - PGT 15.7 mg/kg; SO_2 exposure - PGT 157 mg/kg; SO_2 exposure - PGT 314 mg/kg; SO_2 exposure - PGT 785 mg/kg; SO_2 exposure - dexamethasone 0.5 mg/kg. SO_2 was exposed to rats by inhalation and PGT was administered per oral. A positive control, dexamethasone, was administered to rats via intraperitoneal injection. A 10% solution of sodium metabisulfite was aerosolized into a Plexiglas exposure chamber, using an ultrasonic humidifier (Samsung Electronics Inc., Seoul, Korea). The concentration of sulfur dioxide (SO_2) gas generated by this apparatus was measured to be 150 ppm. Rats were exposed to SO_2 for 3 h per day, 5 days per week, 3 weeks and PGT was administered during the last 2 weeks out of 3 weeks in total. Normal control group were exposed to fresh air in a similar environment without SO_2 exposure.

Bronchoalveolar lavage fluid (BALF) collection and quantitation of *in vivo* mucins in BALF – Rats were euthanized on the last day of experiment and the trachea was cannulated by using sterile polyethylene tube. BAL was performed four times with 5.0 mL of ice-cold PBS (pH 7.4) with 80% of recovery rate. Floating cells and cell debris were removed by centrifugation of BALF at 12,000 × g for 5 min. The BALF samples were stored at −70 ºC until assayed for their mucin contents. The amount of mucins in each BALF sample was measured by using enzyme-linked immunosorbent assay (ELISA). The BALF samples 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% bovine serum albumin (BSA) (fraction V) for 1 h at room temperature. Plates were again washed three times with phosphate-buffered saline (PBS) and then incubated with 100 μL of 45M1 (Neo Markers, CA, U.S.A.), a mouse monoclonal MUC5AC antibody (1 : 200), 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.

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 PBS and recultured in RPMI 1640 with 0.2% FBS for 24 h.

Treatment of cells with PGT – After 24 h of serum deprivation, cells were pretreated with varying concentrations of PGT for 30 min and treated with EGF (25 ng/mL), PMA (10 ng/mL) or TNF-α (0.2 nM) for 24 h in serum-free RPMI 1640. PGT was dissolved in dimethylsulfoxide and treated in culture medium (final concentrations of dimethylsulfoxide were 0.5%). 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.

MUC5AC mucin analysis – MUC5AC mucin protein was measured by using 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.

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 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 94 ºC 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.

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.

Results and Discussion

PGT has been traditionally used for controlling diverse pulmonary diseases including bronchitis, tonsilitis and pneumonitis by oriental medical doctors in folk medicine.[6] Therefore, as aforementioned above, we examined whether PGT significantly affects secretion, production and gene expression of airway mucin, in order to elucidate the mode of action of PGT, using *in vivo* and in vitro experimental models reflecting the hypersecretion and/or hyperproduction of mucus observed in inflammatory pulmonary diseases. As shown in Fig. 1, SO_2 exposure through aerosolized sodium metabisulfite to rats for 3 weeks resulted in profound increases in mucin secretion, compared with the normal control group. However, orally-administered PGT significantly inhibited mucin secretion in BALF, in a dose-dependent fashion. The amounts of mucin in the BALF samples were 100 ± 9%, 225 ± 8%, 181 ± 6%, 154 ± 2%, 142 ± 7%, 131 ± 3% and 97 ± 4% for control, SO_2 alone, SO_2 plus PGT 15.7 mg/kg, SO_2 plus PGT 157 mg/kg, SO_2 plus PGT 314 mg/kg, SO_2 plus

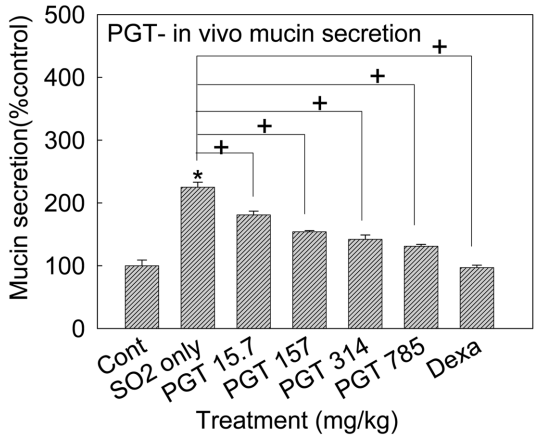

Fig. 1. Effect of PGT on secretion of *in vivo* airway mucin from rats exposed to sulfur dioxide. Rats were exposed to sulfur dioxide and effect of orally-administered PGT on secretion of *in vivo* airway mucin was investigated as described in Materials and methods. Each bar represents a mean ± S.E.M. from 5 rats (cont: control, Dexa: dexamethasone, SO_2: sulfur dioxide).

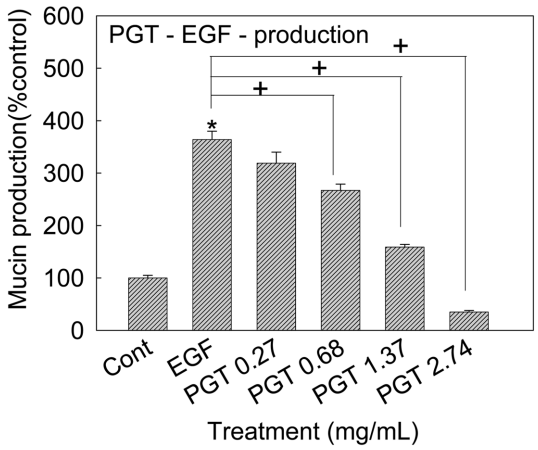

Fig. 2. Effect of PGT on EGF-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of PGT for 30 min and then stimulated with EGF (25 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100%.

*significantly different from control ($p < 0.05$). + significantly different from EGF alone ($p < 0.05$) (cont: control, concentration unit is mg/mL).

PGT 785 mg/kg and SO_2 plus dexamethasone 0.5 mg/kg, respectively. To the best of our knowledge, there is no report on the inhibitory activity of natural products on *in vivo* mucin secretion, as we showed the efficacy of PGT in inhibition of mucin secretion from animals with pulmonary inflammatory disease, in the present study. The positive control, dexamethasone, a corticosteroidal compound which is used as clinical therapeutics for mucus hypersecretion showing potent antiinflammatory

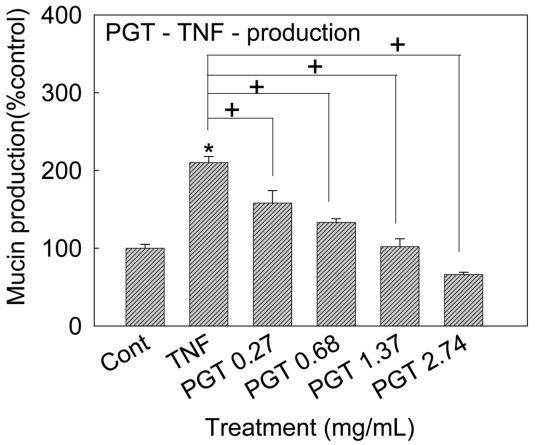

Fig. 3. Effect of PGT on TNF-α-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with various concentrations of PGT for 30 min and then stimulated with TNF-α (0.2 nM, 10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100%.

*significantly different from control ($p < 0.05$). + significantly different from TNF-α alone ($p < 0.05$) (cont: control, concentration unit is mg/mL).

effect, might suppress the overproduction of *in vivo* airway mucin and resultantly might decrease the amount of secretion of mucin to the level of mucin secretion from normal control. This result can explain, at least in part, the traditional use of PGT as a folk remedy for treating several pulmonary inflammatory diseases that are accompanied by hypersecretion of sticky mucus.

Next, since PGT showed the inhibitory action on airway mucin secretion in *in vivo* model, we tried to investigate mucin production from human mucoepidermoid cell line by various inducers. As shown in Figs. 2, 3 and 4, production of MUC5AC mucin was significantly inhibited by pretreatment of PGT. The amounts of mucin in the cells of PGT-treated cultures were 100 ± 5%, 364 ± 16%, 319 ± 21%, 267 ± 12%, 159 ± 5% and 35 ± 3% for control, 25 ng/mL of EGF alone, EGF plus PGT 0.27 mg/mL, EGF plus PGT 0.68 mg/mL, EGF plus PGT 1.37 mg/mL and EGF plus PGT 2.74 mg/mL, respectively (Fig. 2). The amounts of MUC5AC mucin in the cells of PGT-treated cultures were 100 ± 5%, 210 ± 8%, 158 ± 16%, 133 ± 5%, 102 ± 10% and 66 ± 3% for control, 0.2 nM of TNF-α alone, TNF-α plus PGT 0.27 mg/mL, TNF-α plus PGT 0.68 mg/mL, TNF-α plus PGT 1.37 mg/mL and TNF-α plus PGT 2.74 mg/mL, respectively (Fig. 3). The amounts of mucin in the cells of PGT-treated cultures were 100 ± 5%, 350 ± 10%, 306 ± 11%, 305 ± 22%, 204 ± 16% and 53 ± 1% for control, 10 ng/mL of PMA alone, PMA plus PGT 0.27 mg/mL, PMA plus PGT 0.68 mg/mL,

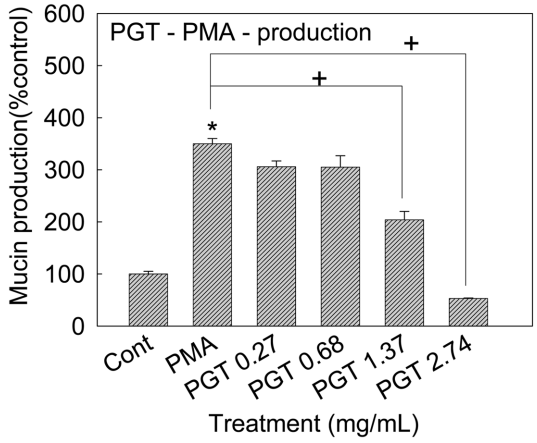

Fig. 4. Effect of PGT on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of PGT for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3 culture wells in comparison with that of control set at 100%.
*significantly different from control ($p < 0.05$). + significantly different from PMA alone ($p < 0.05$) (cont: control, concentration unit is mg/mL).

PMA plus PGT 1.37 mg/mL and PMA plus PGT 2.74 mg/mL, respectively (Fig. 4). There is no remarkable sign of cytotoxicity in the range of treatment concentrations (data were not shown).

On the other hand, MUC5AC mucin gene has been reported to be expressed mainly in goblet cells existing in the airway surface epithelium, among the twenty one or more MUC genes coding human mucins reported to now.[1,10] TNF-α is a well-known stimulant for secretion and gene expression of airway mucin.[9,11,12] TNF-α level in sputum was reported to be increased, with further increases during exacerbation of pulmonary diseases.[8-13] TNF-α converting enzyme (TACE) mediated MUC5AC mucin expression in cultured human airway epithelial cells[9] and TNF-α induced MUC5AC gene expression in normal human airway epithelial cells.[12] It also induced mucin secretion from guinea pig tracheal epithelial cells.[11] Takeyama et al. reported that EGF regulated MUC5AC gene expression in the lung. According to their reports, MUC5AC mRNA expression was increased after ligand binding to the EGF receptor and activation of the MAPK (mitogen-activated protein kinase) cascade.[8,14] PMA acts as an alternative stimulus to the endogenous activator of protein kinase C (PKC), diacylglycerol (DAG)[15] and a model inflammatory stimulant that can modulate a variety of cellular events, including gene transcription,[16] cell growth and differentiation.[17] It also plays a significant role in the induction of MUC5AC gene expression in NCI-H292 cells.[16] As shown in Fig. 5., PGT suppressed the expression of MUC5AC mucin gene induced by EGF, PMA or TNF-α, respectively. At the same time, PGT inhibited the production of MUC5AC mucin protein induced by the three inducers. These results suggest that PGT can regulate mucin gene expression and production of mucin protein induced by EGF, PMA or TNF-α, by directly acting on airway epithelial cells. The underlying mechanisms of action of PGT on MUC5AC production and gene expression are not clear at present, although we are investigating whether PGT act as potential regulators of the MAPK (mitogen-activated protein kinase) cascade after ligand binding to the EGF receptor and/or potential regulators of NF-κB signaling pathway, in mucin-producing NCI-H292 cells.

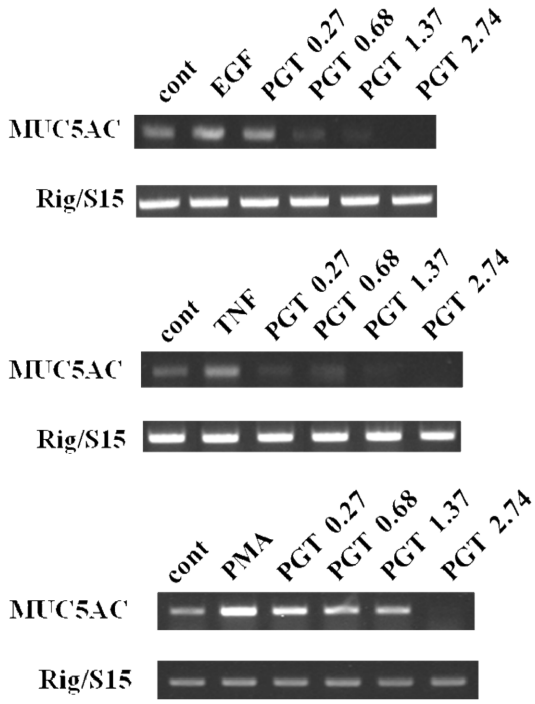

Fig. 5. Effect of PGT on EGF-, PMA- or TNF-α-induced MUC5AC gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of PGT for 30 min and then stimulated with PMA (10 ng/mL), EGF (25 ng/mL) or TNF-α (0.2 nM, 10 ng/mL) for 24 h. MUC5AC gene expression was measured by RT-PCR. Three independent experiments were performed and the representative data were shown (cont: control, concentration unit is mg/mL).

In summary, the inhibitory actions of PGT on airway mucin secretion, production and gene expression might explain, at least in part, the traditional use of PGT as an anti-inflammatory agent and mucoregulator for pulmonary inflammatory diseases, in oriental medicine. We suggest it is valuable to find the folk medicine and related natural products that have specific inhibitory effects on mucin

secretion, production and/or gene expression - in view of both basic and clinical sciences - and the result from this study suggests a possibility of using PGT as a new efficacious mucoregulator for pulmonary diseases, although further studies are essentially required.

Acknowledgements

This research was supported by a grant from Pyunkang Medical Foundation in 2012.

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Received May 25, 2014
Revised June 25, 2014
Accepted June 25, 2014