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Effects of Lobetyolin, Lobetyol and Methyl linoleate on Secretion, Production and Gene Expression of MUC5AC Mucin from Airway Epithelial Cells

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Background: In this study, we investigated whether lobetyolin, lobetyol, and methyl linoleate derived from *Codonopsis pilosula* affect MUC5AC mucin secretion, production, and gene expression from airway epithelial cells.
Methods: Confluent NCI-H292 cells were pretreated with lobetyolin, lobetyol, or methyl linoleate for 30 minutes and then stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 hours. The *MUC5AC* mucin gene expression, and mucin protein production and secretion were measured by reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.
Results: Lobetyolin, lobetyol, and methyl linoleate inhibited the gene expression of MUC5AC mucin induced by PMA; lobetyolin did not affect PMA-induced MUC5AC mucin production. However, lobetyol and methyl linoleate inhibited the production of MUC5AC mucin; lobetyolin and lobetyol did not significantly affect PMA-induced MUC5AC mucin secretion from NCI-H292 cells. However, methyl linoleate decreased the MUC5AC mucin secretion.
Conclusion: These results suggest that among the three compounds, methyl linoleate can regulate gene expression, production, and secretion of MUC5AC mucin by directly acting on the airway epithelial cells.

Keywords: Mucins; MUC5AC Protein, Human; Methyl Linoleate

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Introduction

Pulmonary mucus is pivotal in defensive action against invading pathogenic microorganisms, noxious chemicals and particles. This defensive action of mucus in the pulmonary system is attributed to the physicochemical property of mucins, i.e., viscoelasticity. Mucins are high molecular weight glycoproteins present in the airway mucus and produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, hypersecretion of airway mucus is one of the major symptoms associated with severe pulmonary diseases including asthma, chronic bronchitis, cystic fibrosis, and bronchiectasis[1,2]. Therefore, we suggest it is valuable to find the potential activity of regulating the excessive mucin secretion and/or production by the compounds

derived from various medicinal plants. We have tried to investigate the possible activities of some natural products on mucin secretion and/or production from cultured airway epithelial cells. As a result of our trial, we previously reported that several natural compounds affected mucin secretion and/or production from airway epithelial cells[3-5]. According to folk medicine, *Codonopsis pilosula* has been used for controlling respiratory inflammatory diseases[6]. Major components derived from *C. pilosula* were reported as lobetyol, lobetyolin and methyl linoleate. Lobetyol and lobetyolin were reported to have antioxidative effect[7]. Also, methyl linoleate was reported to have antimicrobial effect[8]. However, to the best of our knowledge, no other studies on methyl linoleate, lobetyol and lobetyolin on mucin secretion, production and gene expression from airway epithelial cells have been carried out. Therefore, in this study, we checked whether methyl linoleate, lobetyol, and lobetyolin affect MUC5AC mucin secretion, production and gene expression from NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of studying the airway mucin production and gene expression[9-11].

Materials and Methods

1. Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. Lobetyolin (purity, 98.0%), lobetyol (purity, 98.0%) and methyl linoleate (purity, 98.0%) from *C. pilosula* were isolated, purified and identified by analytical chemist, Professor Dr. Seungho Lee, in the Laboratory of Pharmacognosy, Department of Pharmacy, College of Pharmacy, Yeungnam University (Daegu, Korea). Briefly, the root of *C. pilosula* (7.0 kg) was extracted with 80% MeOH (45 L×3) for 1 week at room temperature. The solution was evaporated to dryness to afford 3.0 kg of extract. The MeOH extract was loaded on a MCI gel CHP-20 column and first eluted with 100% of H_2O then washed with 100% of MeOH to yield two subfractions (CPW and CPM, respectively). CPM (100 g) was subjected to silica gel column chromatography, and eluted with a gradient mixture of MC/MeOH (1:0 to 0:1) to yield fifteen subfractions (CPM1–CPM15). CPM1 was then subjected loaded to preparative high-performance liquid chromatography (HPLC; 20×250 mm, Shim-pack Prep-ODS kit; Shimazu Co., Kyoto, Japan) with a gradient of MeOH-H_2O (90% to 100%, 6.0 mL/min, 80 minutes) to afford methyl linoleate (15 mg). CPM-6 was subjected to reverse phased column chromatography, eluted with a gradient mixture of MeOH/H_2O (2:8 to 10:0) to yield seven sub-fractions (CPM6-1 to CPM6-7). CPM6-6 (64.9 mg) was purified by preparative HPLC with isocratic condition of acetonitrile/H_2O (85%, 6.0 mL, 80 minutes) to afford lobetyol (16.5 mg). CPM10 (4.1 g) was loaded to reverse phased column chromatography, eluted with a gradient mixture of MeOH/H_2O (2:8 to 10:0) to yield six sub-fractions (CPM10-1 to CPM10-6). CPM10-5 (350 mg) was further purified by preparative-HPLC with a gradient solvent system of decreasing polarity starting from 18% MeOH in H_2O to 28% MeOH in H_2O (6.0 mL, 80 minutes) to afford lobetyolin (700 mg).

2. 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, USA) and cultured (seeding density: 1×10^4 cells per well in 24-well plate) 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% CO_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells (5×10^5 cells per well in 24-well plate) were washed twice with phosphate-buffered saline (PBS) and re-cultured in RPMI 1640 with 0.2% FBS for 24 hours.

3. Treatment of cells with lobetyolin, lobetyol and methyl linoleate

After 24 hours of serum deprivation, cells were pretreated with lobetyolin, lobetyol or methyl linoleate (1, 10, and 100 μM), for 30 minutes and then treated with phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) for 24 hours in serum-free RPMI 1640. Lobetyolin, lobetyol, and methyl linoleate were dissolved in dimethylsulfoxide, diluted in PBS 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 in medium did not affect mucin secretion, production and gene expression from NCI-H292 cells. After 24 hours, the spent media were collected to measure the secretion of MUC5AC protein and 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, Indianapolis, IN, USA) 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 reverse transcription polymerase chain reaction (RT-PCR).

4. MUC5AC mucin analysis using enzyme-linked immunosorbent assay

MUC5AC protein was measured by using enzyme-linked immunosorbent assay. Spent media (for secretion) or cell lysates (for production) 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 for 1 hour 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, Fremont, CA, USA), which was diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 hour, 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 hour, plates were washed three times with PBS. Color reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxide solution and stopped with 1N H_2SO_4. Absorbance was read at 450 nm.

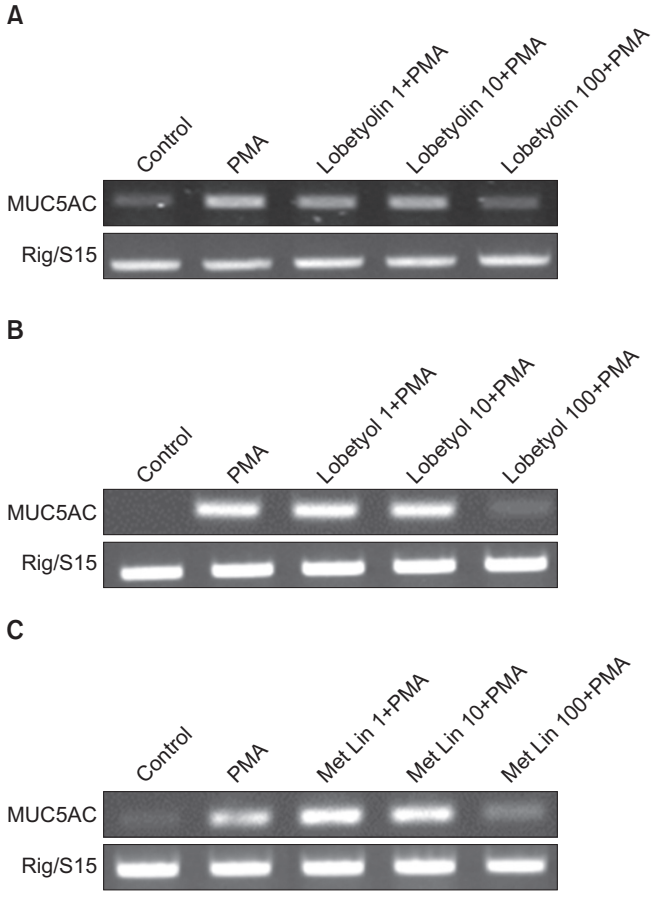

Figure 1. Effect of lobetyolin (A), lobetyol (B), or methyl linoleate (C) on phorbol 12-myristate 13-acetate (PMA)–induced *MUC5AC* gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of lobetyolin, lobetyol, or methyl linoleate for 30 minutes and then stimulated with PMA (10 ng/mL) for 24 hours. *MUC5AC* gene expression was measured by reverse transcription polymerase chain reaction. Three independent experiments were performed and the representative data are shown. Met Lin: methyl linoleate.

5. Total RNA isolation and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology Inc., Seongnam, Korea) and reverse transcribed by using AccuPower RT Premix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Two micrograms of total RNA was primed with 1 μg of oligo (dT) in a final volume of 30 μL (RT reaction). Two microliters of RT reaction product was PCR amplified in a 20 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′. 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°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds and for final extension, 1 cycle at 72°C for 10 minutes. After PCR, 15 μL of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

6. Statistics

Means of individual group were converted to percent control and expressed as mean±SEM. The difference between groups was assessed using one-way ANOVA and Duncan's multiple range test as a *post-hoc* test. $p<0.05$ was considered as significantly different.

Results

1. Effect of lobetyolin, lobetyol or methyl linoleate on PMA-induced *MUC5AC* gene expression from NCI-H292 cells

As can be seen in Figure 1, *MUC5AC* gene expression induced by PMA from NCI-H292 cells was inhibited by pretreatment with lobetyolin, lobetyol or methyl linoleate (Figure 1A-C). Possible cytotoxicity was checked by lactate dehydrogenase assay and there was no remarkable cytotoxic effect of each compound, at 1, 10, and 100 μM (data not shown).

2. Effect of lobetyolin, lobetyol or methyl linoleate on PMA-induced MUC5AC production from NCI-H292 cells

Lobetyolin did not affect PMA-induced MUC5AC production from NCI-H292 cells. However, lobetyol and methyl

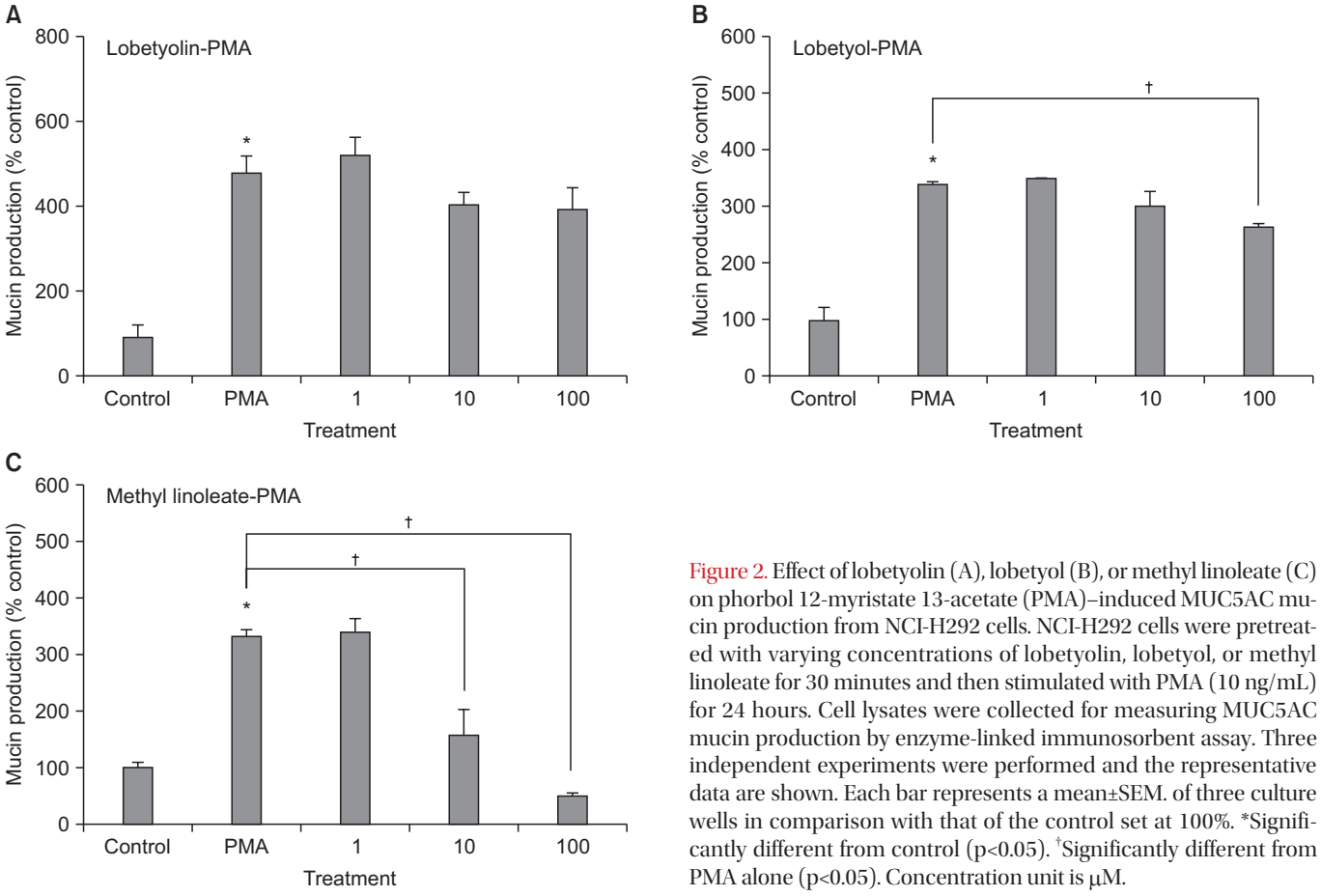

Figure 2. Effect of lobetyolin (A), lobetyol (B), or methyl linoleate (C) on phorbol 12-myristate 13-acetate (PMA)–induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of lobetyolin, lobetyol, or methyl linoleate for 30 minutes and then stimulated with PMA (10 ng/mL) for 24 hours. Cell lysates were collected for measuring MUC5AC mucin production by enzyme-linked immunosorbent assay. Three independent experiments were performed and the representative data are shown. Each bar represents a mean±SEM. of three culture wells in comparison with that of the control set at 100%. *Significantly different from control ($p<0.05$). †Significantly different from PMA alone ($p<0.05$). Concentration unit is μM.

linoleate inhibited MUC5AC production, respectively. The amounts of mucin in the cells of lobetyolin-treated cultures were 100±25%, 487±33%, 524±38%, 411±24%, and 402±45% for control, 10 ng/mL of PMA alone, PMA plus lobetyolin 10^{-6} M, PMA plus lobetyolin 10^{-5} M, and PMA plus lobetyolin 10^{-4} M, respectively (Figure 2A). The amounts of mucin in the cells of lobetyol-treated cultures were 100±21%, 338±4%, 348±5%, 305±22%, and 264±5% for control, 10 ng/mL of PMA alone, PMA plus lobetyol 10^{-6} M, PMA plus lobetyol 10^{-5} M, and PMA plus lobetyol 10^{-4} M, respectively (Figure 2B). The amounts of mucin in the cells of methyl linoleate-treated cultures were 100±10%, 332±14%, 341±22%, 160±45%, and 51±6% for control, 10 ng/mL of PMA alone, PMA plus methyl linoleate 10^{-6} M, PMA plus methyl linoleate 10^{-5} M, and PMA plus methyl linoleate 10^{-4} M, respectively (Figure 2C).

3. Effect of lobetyolin, lobetyol or methyl linoleate on PMA-induced MUC5AC secretion from NCI-H292 cells

As can be seen in Figure 3, methyl linoleate decreased PMA-induced MUC5AC secretion from NCI-H292 cells, dose-dependently. However, lobetyolin and lobetyol did not affect PMA-induced MUC5AC secretion. The amounts of mucin in the spent medium of lobetyolin-treated cultures were 100±12%, 306±15%, 294±26%, 302±17%, and 289±35% for control, 10 ng/mL of PMA alone, PMA plus lobetyolin 10^{-6} M, PMA plus lobetyolin 10^{-5} M, and PMA plus lobetyolin 10^{-4} M, respectively (Figure 3A). The amounts of mucin in the spent medium of lobetyol-treated cultures were 100±26%, 357±18%, 380±9%, 328±26%, and 324±2% for control, 10 ng/mL of PMA alone, PMA plus lobetyol 10^{-6} M, PMA plus lobetyol 10^{-5} M, and PMA plus lobetyol 10^{-4} M, respectively (Figure 3B). The amounts of mucin in the spent medium of methyl linoleate-treated cultures were 100±12%, 298±27%, 265±32%, 198±28%, and 158±17% for control, 10 ng/mL of PMA alone, PMA plus methyl linoleate 10^{-6} M, PMA plus methyl linoleate 10^{-5} M, and PMA plus methyl linoleate 10^{-4} M, respectively (Figure 3C).

Discussion

Lobetyolin, lobetyol and methyl linoleate were reported to be derived from *C. pilosula*[6] and there are some reports with regard to the biological effects of lobetyolin, lobetyol

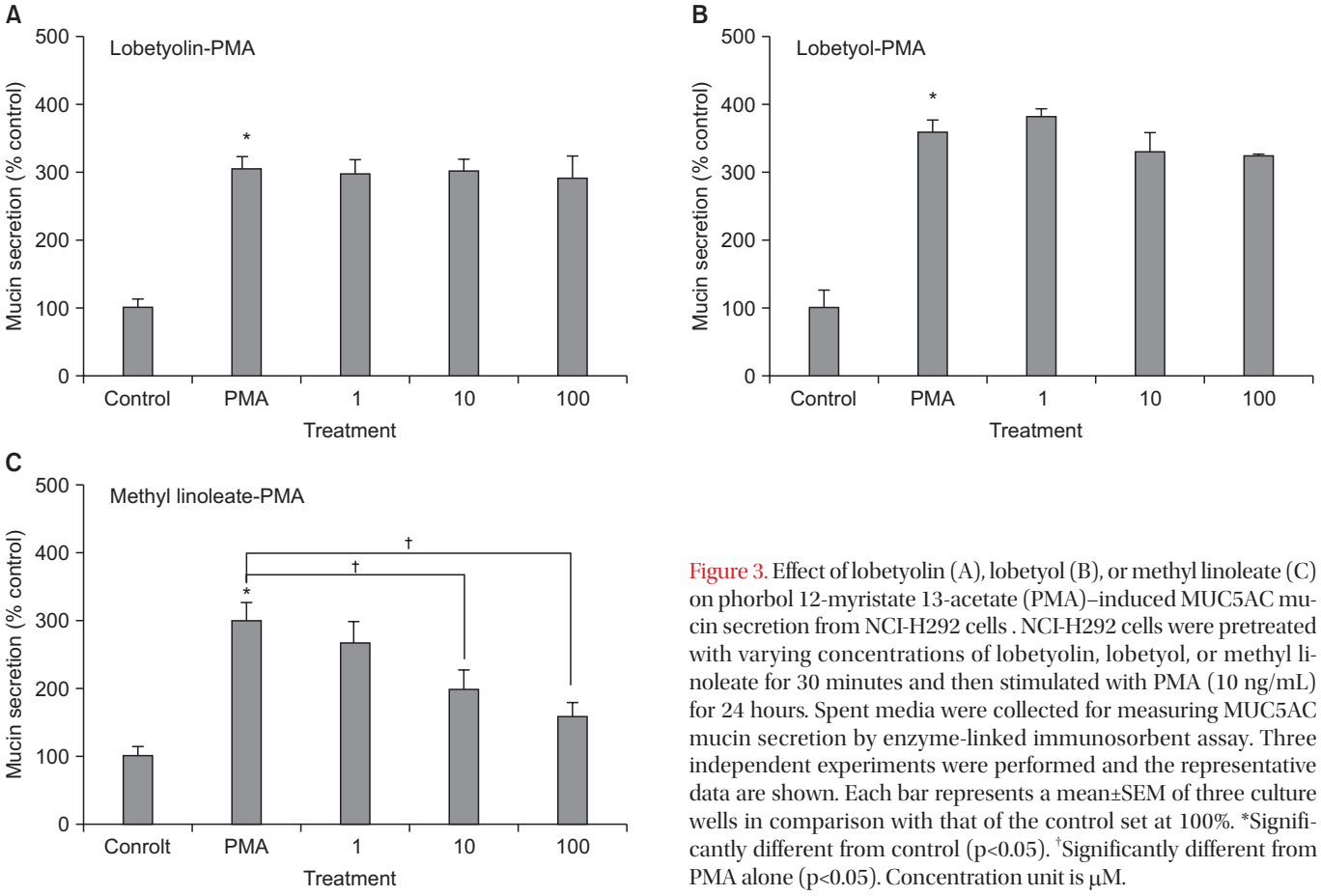

Figure 3. Effect of lobetyolin (A), lobetyol (B), or methyl linoleate (C) on phorbol 12-myristate 13-acetate (PMA)–induced MUC5AC mucin secretion from NCI-H292 cells . NCI-H292 cells were pretreated with varying concentrations of lobetyolin, lobetyol, or methyl linoleate for 30 minutes and then stimulated with PMA (10 ng/mL) for 24 hours. Spent media were collected for measuring MUC5AC mucin secretion by enzyme-linked immunosorbent assay. Three independent experiments were performed and the representative data are shown. Each bar represents a mean±SEM of three culture wells in comparison with that of the control set at 100%. *Significantly different from control ($p<0.05$). †Significantly different from PMA alone ($p<0.05$). Concentration unit is μM.

and methyl linoleate[7,8]. However, as aforementioned in introduction, there are no reports about the potential effects of lobetyolin, lobetyol and methyl linoleate on mucin secretion, production and gene expression from airway epithelial cells. Among the twenty one or more MUC genes coding human mucins reported, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium[2,12]. PMA was reported to stimulate the endogenous activator of protein kinase C (PKC), diacylglycerol[13] and to be an inflammatory stimulant that can control a gene transcription[14], cell growth and differentiation[15]. PMA also can induce *MUC5AC* gene expression in NCI-H292 cells. In brief, PMA activates a type of PKC isoforms. This activates matrix metalloproteinases, which cleave pro-epidermal growth factor receptor (pro-EGFR) ligands from the cell surface to become mature EGFR ligands. These ligands bind to the EGFR, 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. Eventually, the promoter is activated and produced the gene transcription and translation to MUC5AC mucin protein[14]. Based on these reports, we investigated the effects of lobetyolin, lobetyol and methyl linoleate on PMA-induced MUC5AC mucin secretion, production and gene expression from NCI-H292 cells, a human pulmonary mucoepidermoid cell line. As shown in results, lobetyolin, lobetyol and methyl linoleate decreased MUC5AC mucin gene expression stimulated by PMA (Figure 1). Next, we tried to examine whether the three compounds regulate the production of MUC5AC mucin proteins, since the three compounds affect the transcriptional level of gene expression. As a result, lobetyol and methyl linoleate suppressed PMA-induced MUC5AC mucin production from NCI-H292 cells. However, lobetyolin did not affect mucin production (Figure 2). Finally, we checked whether the three compounds affect the secretion step of MUC5AC mucin, because the production and secretion of airway mucin can be controlled separately. As can be seen in Figure 3, methyl linoleate suppressed the secretion of MUC5AC mucin induced by PMA. However, lobetyolin and lobetyol did not affect mucin secretion. These results suggest that, among the three natural products, only methyl linoleate showed the consistent inhibitory activities on secretion, production and gene expression of airway MUC5AC mucin, by directly acting on airway epithelial cells. The underlying mechanism of action of methyl linoleate on MUC5AC secretion, production and gene expression are not

clear at present, although we are investigating whether methyl linoleate act as potential regulators of the mitogen-activated protein kinase cascade after ligand binding to the EGFR and/or potential regulators of nuclear factor kB signaling pathway, in mucin-producing NCI-H292 cells. Taken together, the inhibitory action of methyl linoleate on airway mucin secretion, production and gene expression might explain, at least in part, the folk use of *C. pilosula*, as an anti-inflammatory and anti-allergic agent for pulmonary inflammatory diseases, in traditional folk medicine. We suggest it is valuable to find the natural products that have specific inhibitory effects on mucin secretion, production and gene—in view of both basic and clinical sciences—and the result from this study suggests a possibility of developing methyl linoleate as a candidate for the new efficacious mucoregulators for pulmonary diseases, although further studies are required.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgements

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