Verticine, ebeiedine and suchengbeisine isolated from the bulbs of *Fritillaria thunbergii* Miq. inhibited the gene expression and production of MUC5AC mucin from human airway epithelial cells

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Abbreviations: EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription - polymerase chain reaction; TNF-α, tumour necrosis factor-α.

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**Introduction**

Mucus in respiratory system is pivotal for defensive action against invading particles, noxious chemicals and pathogenic microorganisms. The protective function of airway mucus is due to the physicochemical properties e.g. viscoelasticity of mucins. Mucins are multimillion-dalton glycoproteins present in respiratory mucus that are produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, any abnormality in the quality or quantity of mucins not only cause altered airway physiology but may also impair host defences often leading to severe airway pathology as exemplified in asthma, chronic bronchitis, cystic fibrosis, and bronchiectasis (Voynow and Rubin 2009). Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excessive mucin secretion (production) by various medicinal plants. We investigated the activities of some natural products derived from several medicinal plants on mucin production and/or secretion from airway epithelial cells (Heo et al. 2007; Heo et al. 2009; Lee et al. 2011; Kim et al. 2012). According to traditional oriental medicine, the bulbs of *Fritillaria thunbergii* has been utilised as mucoregulators and expectorants for controlling the airway inflammatory diseases (Jang 2003). Among various compounds reported to be isolated and purified from the bulbs of *Fritillaria thunbergii*, verticine was reported to have an inhibitory activity on angiotensin converting enzyme (ACE), antitussive and antiinflammatory effects (Oh et al. 2003; Wang et al. 2011). However, to the best of our knowledge, there...
is no report about the potential effects of verticine, ebeiedine and suchengbeisine, the compounds isolated from the bulbs of *Fritillaria thunbergii*, 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 (Rogers and Barnes 2006; Voonow and Rubin 2009). Therefore, we examined the effects of verticine, ebeiedine and suchengbeisine on EGF-, PMA- or TNF-α induced MUC5AC mucin gene expression and production in NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of elucidating mechanisms involved in airway mucin production and gene expression (Li et al. 1997; Takeyama et al. 1999; Shao et al. 2003).

**Materials and methods**

**Materials**

All chemicals and reagents used in this experiment were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.) unless otherwise specified. The bulbs of *Fritillaria thunbergii* (11 kg) were collected from Yeongam Province, Korea in January 2012, and authenticated by Prof. Dr. Je-Hyun Lee in Department of Herbolology, College of Oriental Medicine, Dongguk University (Kyung-ju, Korea). A voucher specimen (SKKU NPL 1201) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. For a preparation of the aqueous and 70% ethanol (EtOH) extracts, the bulbs of *F. thunbergii* (500 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 residues (967 g). The methanol extract (750 g) was dissolved in water (800 ml) and partitioned with CHCl₃ and n-BuOH after pretreatment with 1 N hydrochloric acid (HCl), yielding a CHCl₃-fraction (210 g) and n-BuOH fraction (230 g), respectively. The CHCl₃-fraction (210 g) was chromatographed over a silica gel column with CHCl₃-MeOH (20:0–1:1) as the eluent to give ten fractions (C₁–C₁₀). The C₆ fraction (1.5 g) was subjected to a silica gel column with CHCl₃–MeOH (20:1–1:1) and purified by RP-C₁₈ prep. HPLC (60% MeOH in 0.05% TFA) to give suchengbeisine (25 mg). The C₇ fraction (1.2 g) was subjected to a Sephadex LH-20 (CH₂Cl₂ : MeOH = 1:1) and purified by RP-C₁₈ prep. HPLC (60% MeOH in 0.05% TFA) to give verticine (3.0 g). These compounds were identified to be verticine ([3β,5α,6α]-Cevane-3,6,20-triol) (Kaneko et al. 1979, ebeiedine ([3β,5α,6β]-Cevane-3,6-diol) (Lee et al. 1988) and suchengbeisine ([22R,25R]-13α,21-epoxy-5,6,12,13-tetrahydro-3β-hydroxy-5α-veratramamine-6-one) (Huang et al. 2013) (Fig. 1A) by comparison of their spectroscopic and physical data with previously reported values (Fig. 1B).

**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% foetal 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% foetal bovine serum for 24 h.

**Treatment of cells with aqueous extract, EtOH extract, verticine, ebeiedine and suchengbeisine**

After 24 h of serum deprivation, cells were pretreated with varying concentrations of aqueous extract, EtOH extract, verticine, ebeiedine, suchengbeisine or verticine for 30 min and then treated with EGF (epidermal growth factor) (25 ng/ml), PMA (phorbol 12-myristate 13-acetate) (10 ng/ml) or TNF-α (tumour necrosis factor-α) (0.2 nM) for 24 h in serum-free RPMI 1640. Aqueous extract, EtOH extract, verticine, ebeiedine and suchengbeisine were dissolved in dimethylsulphoxide and treated in culture medium (final concentrations of dimethylsulphoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulphoxide did not affect mucin gene expression and production 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, 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.

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**Fig. 1.** Chemical structure of verticine, ebeiedine and suchengbeisine and their purity analysed by 1H, 13C NMR. The isolated compounds were identified to be verticine ([3β,5α,6α]-Cevane-3,6,20-triol), ebeiedine ([3β,5α,6β]-Cevane-3,6-diol) and suchengbeisine ([22R,25R]-13α,21-epoxy-5,6,12,13-tetrahydro-3β-hydroxy-5α-veratramine-6-one) (Fig. 1A) by comparison of their spectroscopic and physical data with previously reported values (Fig. 1B).
Fig. 1. Continued
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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°C in a 96-well plate, until dry. Plates were 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. Colour 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., 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 Thermoprime 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 GAT ATA TGG G-3’. As a quantitative control, 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 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.

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 Holm–Sidak test as a post-hoc test. p < 0.05 was considered as significantly different.

Results

Effects of aqueous extract and EtoH extract of Fritillaria thunbergii on PMA-induced MUC5AC gene expression and mucin production from NCI-H292 cells

As can be seen in Fig. 2, aqueous extract of Fritillaria thunbergii did not affect PMA-induced MUC5AC gene expression and mucin production from NCI-H292 cells Fig. 2(A). The amounts of mucin in the cells of aqueous extract of Fritillaria thunbergii-treated cultures were 100 ± 12%, 227 ± 23%, 209 ± 21%, 218 ± 17% and 198 ± 18% for

![Fig. 2. Effects of aqueous extract and EtoH extract of Fritillaria thunbergii on PMA-induced MUC5AC gene expression and mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of aqueous extract and EtoH extract of Fritillaria thunbergii for 30 min and then stimulated with PMA (10 ng/ml) for 24 h. MUC5AC gene expression was measured by RT-PCR. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± SEM of 3 culture wells in comparison with that of control set at 100%. Three independent experiments were performed and the representative data were shown (Fig. 2A and B). * Significantly different from control (p < 0.05). + Significantly different from PMA (p < 0.05). (cont: control, concentration unit is μg/ml.)](image-url)
Fig. 3. Effect of verticine, ebeiedine or suchengbeisine on EGF-, PMA- or TNF-α-induced MUC5AC gene expression in NCI-H292 cells.

NCI-H292 cells were pretreated with varying concentrations of verticine, ebeiedine or suchengbeisine for 30 min and then stimulated with EGF (25 ng/ml), PMA (10 ng/ml) or TNF-α (0.2 nM) for 24 h. MUC5AC gene expression was measured by RT-PCR. Three independent experiments were performed and the representative data were shown (Fig. 3A–C). (cont: control, concentration unit is μM.)

**Effect of verticine, ebeiedine or suchengbeisine on EGF-, PMA- or TNF-α-induced MUC5AC gene expression in NCI-H292 cells**

As can be seen in Fig. 3, MUC5AC gene expression induced by EGF-, PMA- or TNF-α in NCI-H292 cells was inhibited by pretreatment with verticine, ebeiedine or suchengbeisine, respectively (Fig. 3A–C). Cell viability was checked by sulforhodamine B (SRB) assay and there was no cytotoxic effect of verticine, ebeiedine or suchengbeisine, at 1, 2, 5 and 10 μM (data were not shown).

**Effect of verticine on EGF-induced MUC5AC mucin production from NCI-H292 cells**

Verticine significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells, the highest concentration. The amounts of mucin in the cells of verticine-treated cultures were 100 ± 2%, 283 ± 21%, 210 ± 3%, 173 ± 7%, 120 ± 14% and 104 ± 8% for control, 25 ng/ml of EGF alone, EGF plus verticine 10−6 M, EGF plus verticine 2 × 10−6 M, EGF plus verticine 5 × 10−6 M and EGF plus verticine 10−5 M, respectively (Fig. 4A).

**Effect of ebeiedine on EGF-induced MUC5AC mucin production from NCI-H292 cells**

Ebeiedine significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells, the highest concentration. The amounts of mucin in the cells of ebeiedine-treated cultures were 100 ± 2%, 283 ± 21%, 210 ± 3%, 173 ± 7%, 120 ± 14% and 104 ± 8% for control, 25 ng/ml of EGF alone, EGF plus ebeiedine 10−6 M, EGF plus ebeiedine 2 × 10−6 M, EGF plus ebeiedine 5 × 10−6 M and EGF plus ebeiedine 10−5 M, respectively (Fig. 5A).

**Effect of verticine on PMA-induced MUC5AC mucin production from NCI-H292 cells**

Verticine inhibited PMA-induced MUC5AC production from NCI-H292 cells, dose-dependently. The amounts of mucin in the cells of verticine-treated cultures were 100 ± 2%, 283 ± 21%, 210 ± 3%, 173 ± 7%, 120 ± 14% and 104 ± 8% for control, 10 ng/ml of PMA alone, PMA plus verticine 10−6 M, PMA plus verticine 2 × 10−6 M, PMA plus verticine 5 × 10−6 M and PMA plus verticine 10−5 M, respectively (Fig. 4B).

**Effect of verticine on TNF-induced MUC5AC mucin production from NCI-H292 cells**

Verticine significantly inhibited TNF-induced MUC5AC production from NCI-H292 cells, the highest concentration. The amounts of mucin in the cells of verticine-treated cultures were 100 ± 2%, 283 ± 21%, 210 ± 3%, 173 ± 7%, 120 ± 14% and 104 ± 8% for control, 0.2 nM of TNF-α alone, TNF-α plus verticine 10−6 M, TNF-α plus verticine 2 × 10−6 M, TNF-α plus verticine 5 × 10−6 M and TNF-α plus verticine 10−5 M, respectively (Fig. 4C).

**Effect of ebeiedine on EGF-induced MUC5AC mucin production from NCI-H292 cells**

Ebeiedine significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells, the highest concentration. The amounts of mucin in the cells of ebeiedine-treated cultures were 100 ± 2%, 283 ± 21%, 210 ± 3%, 173 ± 7%, 120 ± 14% and 104 ± 8% for control, 25 ng/ml of EGF alone, EGF plus ebeiedine 10−6 M, EGF plus ebeiedine 2 × 10−6 M, EGF plus ebeiedine 5 × 10−6 M and EGF plus ebeiedine 10−5 M, respectively (Fig. 5A).
**Fig. 4.** Effect of verticine on EGF-, PMA- or TNF-\(\alpha\)-induced MUC5AC mucin production NCI-H292 cells.

NCI-H292 cells were pretreated with varying concentrations of verticine for 30 min and then stimulated with EGF (25 ng/ml), PMA (10 ng/ml) or TNF-\(\alpha\) (0.2 nM) 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%. Three independent experiments were performed and the representative data were shown (Fig. 4 A–C).

* Significantly different from control (\(p < 0.05\)).
+ Significantly different from EGF, PMA or TNF-\(\alpha\) alone (\(p < 0.05\)).
(cont: control, concentration unit is \(\mu\)M.)

**Effect of ebeiedine on PMA-induced MUC5AC mucin production from NCI-H292 cells**

Ebeiedine significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of ebeiedine-treated cultures were 100 ± 1%, 335 ± 9%, 333 ± 9%, 308 ± 5%, 184 ± 3% and 101 ± 10% for control, 10 ng/ml of PMA alone, PMA plus ebeiedine \(10^{-6}\) M, PMA plus ebeiedine \(2 \times 10^{-6}\) M, PMA plus ebeiedine \(5 \times 10^{-6}\) M and PMA plus ebeiedine \(10^{-5}\) M, respectively (Fig. 5B).
Effect of ebiedine on TNF-induced MUC5AC mucin production from NCI-H292 cells

Ebeiedine significantly inhibited TNF-induced MUC5AC production from NCI-H292 cells, at the highest concentration. The amounts of mucin in the cells of ebeiedine-treated cultures were 100 ± 4%, 229 ± 5%, 254 ± 7%, 249 ± 8%, 234 ± 12% and 151 ± 15% for control, 0.2 nM of TNF-α alone, TNF-α plus ebeiedine 10^{-6} M, TNF-α plus ebiedine 2 × 10^{-6} M, TNF-α plus ebeiedine 5 × 10^{-6} M and TNF-α plus ebiedine 10^{-5} M, respectively (Fig. 5C).

Effect of suchengbeisine on EGF-induced MUC5AC mucin production from NCI-H292 cells

Suchengbeisine significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells, at the highest concentration. The amounts of mucin in the cells of suchengbeisine-treated cultures were 100 ± 8%, 190 ± 6%, 188 ± 14%, 184 ± 4%, 168 ± 7% and 82 ± 2% for control, 25 ng/ml of EGF alone, EGF plus suchengbeisine 10^{-6} M, EGF plus suchengbeisine 2 × 10^{-6} M, EGF plus suchengbeisine 5 × 10^{-6} M and EGF plus suchengbeisine 10^{-5} M, respectively (Fig. 6A).

Effect of suchengbeisine on PMA-induced MUC5AC mucin production from NCI-H292 cells

Suchengbeisine inhibited PMA-induced MUC5AC production from NCI-H292 cells, dose-dependently. The amounts of mucin in the cells of suchengbeisine-treated cultures were 100 ± 3%, 363 ± 4%, 316 ± 12%, 234 ± 11%, 184 ± 13% and 96 ± 1% for control, 10 ng/ml of PMA alone, PMA plus suchengbeisine 10^{-6} M, PMA plus suchengbeisine 2 × 10^{-6} M, PMA plus suchengbeisine 5 × 10^{-6} M and PMA plus suchengbeisine 10^{-5} M, respectively (Fig. 6B).

Effect of suchengbeisine on TNF-induced MUC5AC mucin production from NCI-H292 cells

Suchengbeisine significantly inhibited TNF-induced MUC5AC production from NCI-H292 cell. The amounts of mucin in the cells of suchengbeisine-treated cultures were 100 ± 2%, 205 ± 10%, 189 ± 7%, 182 ± 4%, 172 ± 5% and 167 ± 2% for control, 0.2 nM of TNF-α alone, TNF-α plus suchengbeisine 10^{-6} M, TNF-α plus suchengbeisine 2 × 10^{-6} M, TNF-α plus suchengbeisine 5 × 10^{-6} M and TNF-α plus suchengbeisine 10^{-5} M, respectively (Fig. 6C).

Discussion

As aforementioned in Introduction, verticine was reported to have an inhibitory activity on angiotensin converting enzyme (ACE), antitussive and antiinflammatory effects (Oh et al. 2003; Wang et al. 2011). However, to the best of our knowledge, there is no report about the potential effects of verticine, ebiedine and suchengbeisine, the compounds isolated from the bulbs of Fritillaria thunbergii, on the gene expression and production of mucin from airway epithelial cells. Of the twenty one or more mucin genes which identified until now, MUC5AC has been known as a major type of airway gel-forming mucin because it is highly expressed in the goblet cells (Rogers and Barnes 2006; Voynow and Rubin 2009; Song et al. 2003) and is regulated by proinflammatory cytokines (Takeyama et al. 2000). TNF-α converting enzyme (TACE) mediated MUC5AC mucin expression in cultured human airway epithelial cells (Shao et al. 2003) and TNF-α induced MUC5AC gene expression in normal human airway epithelial cells (Song et al. 2003). TNF-α is a well-known inducer for secretion and gene expression of airway mucin (Shao et al. 2003; Song et al. 2003; Fisher et al. 1999). TNF-α level in sputum was reported to be increased, with further increases during exacerbation of diseases (Takeyama et al. 1999; Cohn et al. 2002).

On the other hand, epidermal growth factor (EGF) was reported to regulate MUC5AC gene expression in the lung. MUC5AC mRNA expression was increased after binding to the EGF receptor and activation of the MAPK (mitogen-activated protein kinase) cascade.

![Fig. 6. Effect of suchengbeisine on EGF-, PMA- or TNF-α-induced MUC5AC mucin production NCI-H292 cells.](image-url)

NCI-H292 cells were pretreated with varying concentrations of suchengbeisine for 30 min and then stimulated with EGF (25 ng/ml), PMA (10 ng/ml) or TNF-α (0.2 nM) 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%. Three independent experiments were performed and the representative data were shown (Fig. 6A-C).

* Significantly different from control (p < 0.05).
+ Significantly different from EGF, PMA or TNF-α alone (p < 0.05).
(cont: control, concentration unit is μM.)
Regulate the gene expression and production of mucin induced by the three inducers (Figs. 3–5). These compounds inhibited the production of MUC5AC protein induced by 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 (Hewson et al. 2004).

Based on the above reports, we investigated whether verticine, ebeiedine and suchengbeisine isolated from the bulbs of *Fritillaria thunbergii* suppress the gene expression and production of airway MUC5AC mucin induced by EGF, PMA or TNF-α. As can be seen in Results, verticine, ebeiedine and suchengbeisine suppressed the expression of MUC5AC mucin gene induced by EGF, PMA or TNF-α, respectively (Fig. 2). At the same time, verticine, ebeiedine and suchengbeisine inhibited the production of MUC5AC mucin protein induced by the three inducers (Figs. 3–5). These results suggest that verticine, ebeiedine and suchengbeisine can regulate the gene expression and production of mucin induced by EGF, PMA or TNF-α, by directly acting on airway epithelial cells.

Taken together, findings in this study might explain the traditional use of the bulbs of *Fritillaria thunbergii* as a folk remedy for treating several pulmonary inflammatory diseases that are accompanied by hypersecretion of sticky mucus. The underlying mechanisms of action of verticine, ebeiedine or suchengbeisine on MUC5AC gene expression and production are not clear at present, although we are investigating whether verticine, ebeiedine or suchengbeisine acts as potential regulator of the MAPK (mitogen-activated protein kinase) cascade after binding to the EGFR receptor and/or potential regulator of NF-κB signalling pathway, in mucin-producing NCI-H292 cells. We suggest it is valuable to find new efficacious mucoregulator for pulmonary diseases, although further studies are essentially required.

Conflict of interest

The authors have declared that there are no conflicts of interest.

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References


