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Effects of Baicalin, Baicalein and Schizandrin on Airway Mucin Production Induced by Epidermal Growth Factor and Phorbol Ester

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Abstract − We conducted this study to investigate whether baicalin, baicalein or schizandrin significantly affect MUC5AC mucin production induced by epidermal growth factor (EGF) or phorbol ester (PMA) in human airway epithelial cells. Confluent NCI-H292 cells were pretreated with varying concentrations of baicalin, baicalein or schizandrin for 30 min and then stimulated with EGF or PMA for 24 h, respectively. MUC5AC mucin protein production was measured by ELISA. The results were as follows: (1) Baicalin was found to inhibit the production of MUC5AC mucin protein induced by both EGF and PMA. (2) Baicalein, the aglycone of baicalin, also inhibited MUC5AC mucin production. (3) Schizandrin, derived from Schizandrae Fructus, inhibited MUC5AC mucin production by the same inducers. These results suggest that baicalin, baicalein and schizandrin can regulate the production of mucin protein by directly acting on human airway epithelial cells.

Keywords: Airway mucin, Baicalin, Baicalein and schizandrin

INTRODUCTION

Mucus present in the human respiratory system is very important in defense against invading pathogenic microorganisms, chemicals and particles. This defensive action of airway mucus is due to the viscoelasticity of mucins. Mucins are multimillion dalton glycoproteins which are present in the airway mucus and produced by goblet cells in the surface epithelium and mucous cells in the submucosal gland. Hypersecretion of airway mucus, however, is one of the major symptoms associated with severe pulmonary diseases including asthma, chronic bronchitis, cystic fibrosis and bronchiectasis (Voynow and Rubin, 2009). Therefore, we suggest it is valuable to determine the potential activities of compounds derived from various medicinal plants for inhibiting excess mucin production. We investigated the possible activities of some natural products on mucin secretion in cultured airway epithelial cells. As a result of our study, we previously reported that several natural compounds affected mucin secretion by airway epithelial cells (Lee *et al*., 2003; Heo *et al*., 2006; Heo *et al*., 2007). According to a number of reports, Scutellariae Radix has been used to control airway allergic or inflammatory diseases, and their components, baicalin and baicalein, were reported to have diverse biological effects (Chou *et al*., 2003; Dong *et al*., 2005; Van Leyen *et al*., 2006; Hsieh *et al*., 2007). Baicalein was reported to be a potent antioxidant and free radical scavenger and has been regarded as a 12/15-lipoxygenase inhibitor and xanthine oxidase inhibitor (Van Leyen *et al*., 2006). Baicalein has been shown to antagonize the expression of adhesion molecules induced by interleukin-β1 (IL-β1) and tumor necrosis factor (TNF-α) (Hsieh *et al*., 2007). Baicalin demonstrated anti-inflammatory and analgesic effects through the inhibition of important inflammatory mediators and proinflammatory cytokines, as well as through neutrophil infiltration at sites of inflammation (Chou *et al*., 2003). Inhalation of baicalin showed an inhibition of airway hyperresponsiveness (Dong *et al*., 2005). Also, Schizandrae Fructus and one of its components (schizandrin), were re-

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ported to have various biological effects including free a radical scavenging effect (Li *et al*., 1990), hepatoprotective effect (Liu, 1989; Chiu *et al*., 2003) and to offer protection of neuronal cells from excitotoxicity (Kim *et al*., 2004). On the other hand, it was previously reported that baicalin and schizandrin inhibited ATP-stimulated mucin release from airway goblet cells, although baicalein did not affect ATP-stimulated airway mucin release in studies conducted by our group (Lee *et al*., 2003; Heo *et al*., 2006; Heo *et al*., 2007). However, to the best of our knowledge, there are no reports about the effects of baicalin, bacalein or schizandrin on mucin production induced by epidermal growth factor or phorbol ester, in human airway epithelial cells. Therefore, in this study, we investigated whether baicalin, bacalein or schizandrin affect the production of mucin induced by epidermal growth factor or phorbol ester, in the NCI-H292 human pulmonary mucoepidermoid cell line.

MATERIALS AND METHODS

Materials

All chemicals and reagents used in this study, including baicalin (purity: 98.0%) and baicalein (purity: 98.0%), were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified. Schizandrin (purity: 95.0%) was isolated, purified and identified by analytical chemists at the Research Institute of Natural Products of Seoul National University (Seoul, Korea).

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°C 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% fetal bovine serum for 24 h.

Treatment of cells with agents

After 24 h of serum deprivation, cells were pretreated with baicalin, baicalein or schizandrin (1, 10 and 100 μM) for 30 min and then treated with EGF (25 ng/ml) or PMA (10 ng/ml) for 24 h in serum-free RPMI 1640, respectively. 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 a 24-well culture plate.

MUC5AC mucin analysis using ELISA

MUC5AC 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°C until dry in a 96-well plate, Plates were washed three times with PBS and blocked with 2% BSA (fraction V) for 1h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μl of 45M1, a mouse monoclonal MUC5AC antibody (NeoMarkers, CA, U.S.A.) (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 reactions were developed using 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1N H2SO4. Absorbance was measured at 450 nm.

Statistics

Means of individual groups were converted to percent control and expressed as mean ± S.E.M. The difference between groups was assessed using one-way ANOVA and Student's t-test for unpaired samples. $p < 0.05$ was considered as significantly different.

RESULTS

Effect of baicalin on EGF-induced MUC5AC production

Fig. 1 shows that baicalin significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells at the highest concentration. The amounts of mucin in the cells of baicalin-treated cultures were 100 ± 7%, 150 ± 9%, 193 ± 10%, 145 ± 5% and 104 ± 6% for control, 25 ng/ml of EGF alone, EGF plus baicalin 10-6 M, EGF plus baicalin 10^{-5} M and EGF plus baicalin 10^{-4} M, respectively (Fig. 1).

Effect of baicalin on PMA-induced MUC5AC production

Fig. 2 shows that baicalin significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells at concentrations between 10^{-5} M and 10^{-4} M. The amounts of mucin in the cells of baicalin-treated cultures were 100 ± 8%, 303 ± 20%, 330 ± 15%, 54 ± 8% and 57 ± 7% for control, 10 ng/ml of PMA alone, PMA plus baicalin 10^{-6} M, PMA plus baicalin 10^{-5} M and PMA plus baicalin 10^{-4} M, respectively (Fig. 2).

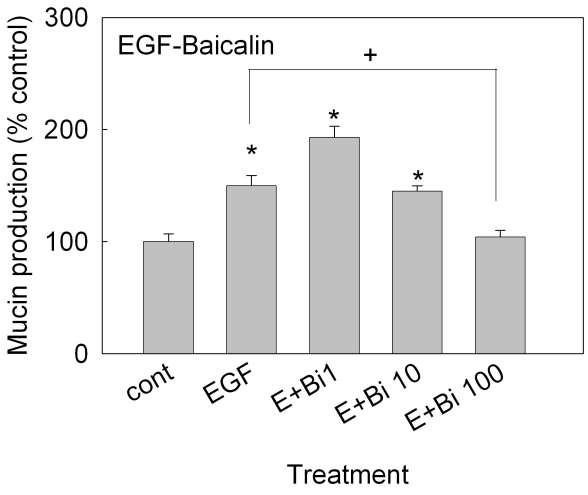

Fig. 1. Effect of baicalin on EGF-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of baicalin for 30min and then stimulated with EGF (25 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of a control set at 100%. *Significantly different from control ($p<0.05$). +Significantly different from EGF alone ($p<0.05$) (cont: control, Bi: baicalin, concentration unit is μM.)

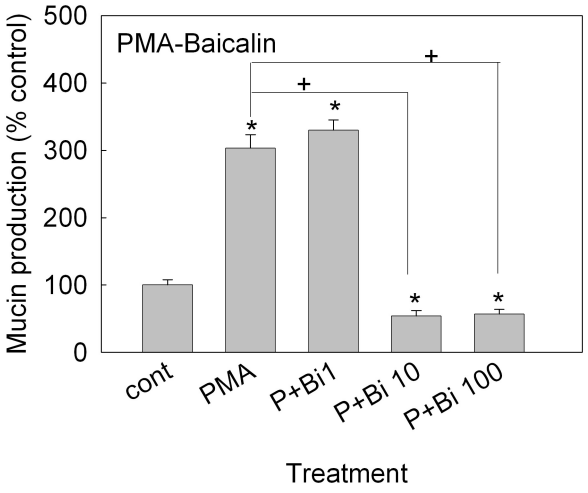

Fig. 2. Effect of baicalin on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of baicalin for 30min and then stimulated with PMA (10 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of a control set at 100%. *Significantly different from control ($p<0.05$). +Significantly different from PMA alone ($p<0.05$) (cont: control, Bi: baicalin, concentration unit is μM.)

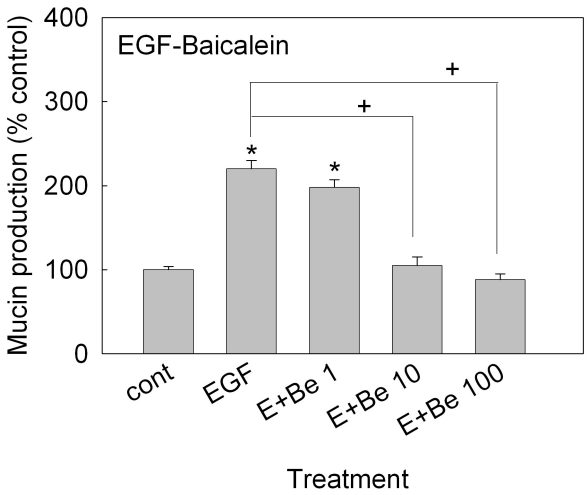

Fig. 3. Effect of baicalein on EGF-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of baicalein for 30min and then stimulated with EGF (25 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of a control set at 100%. *Significantly different from control ($p<0.05$). +Significantly different from EGF alone ($p<0.05$) (cont: control, Be: baicalein, concentration unit is μM.)

Effect of baicalein on EGF-induced MUC5AC production

Fig. 3 shows that baicalein significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells at concentrations between 10^{-5} M and 10^{-4}M. The amounts of mucin in the cells of baicalein-treated cultures were 100 ± 4%, 220 ± 10%, 198 ± 9%, 105 ± 10% and 88 ± 7% for control, 25 ng/ml of EGF alone, EGF plus baicalein 10^{-6} M, EGF plus baicalein 10^{-5} M and EGF plus baicalein 10^{-4} M, respectively (Fig. 3).

Effect of baicalein on PMA-induced MUC5AC production

Fig. 4 shows that baicalein significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells at concentrations between 10^{-5} M and 10^{-4} M. The amounts of mucin in the cells of baicalein-treated cultures were 100 ± 9%, 173 ± 6%, 160 ± 8%, 100 ± 7% and 67 ± 8% for control, 10 ng/ml of PMA alone, PMA plus baicalein 10^{-6} M, PMA plus baicalein 10^{-5} M and PMA plus baicalein 10^{-4} M, respectively (Fig. 4).

Effect of schizandrin on EGF-induced MUC5AC production

Fig. 5 shows that schizandrin significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells at concentrations between 10^{-5} M and 10^{-4} M. The amounts

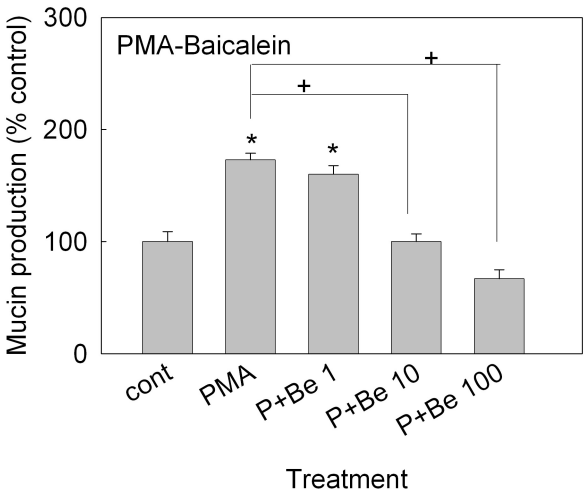

Fig. 4. Effect of baicalein on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of baicalein for 30min and then stimulated with PMA (10 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA.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

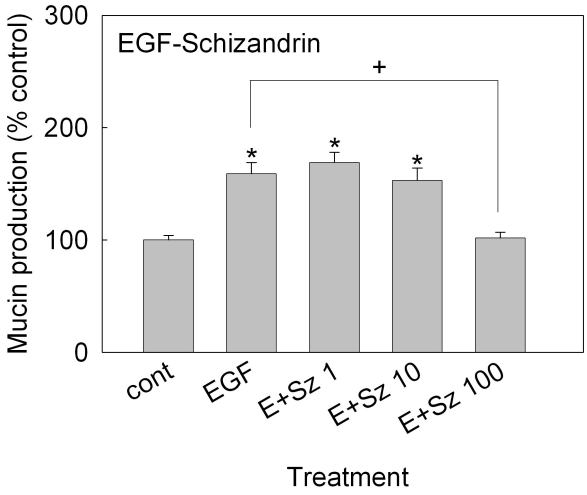

Fig. 5. Effect of schizandrin on EGF-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of schizandrin for 30min and then stimulated with EGF (25 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of a control set at 100%. *Significantly different from control ($p<0.05$). +Significantly different from EGF alone ($p<0.05$) (cont: control, Sz: schizandrin, concentration unit is μM.)

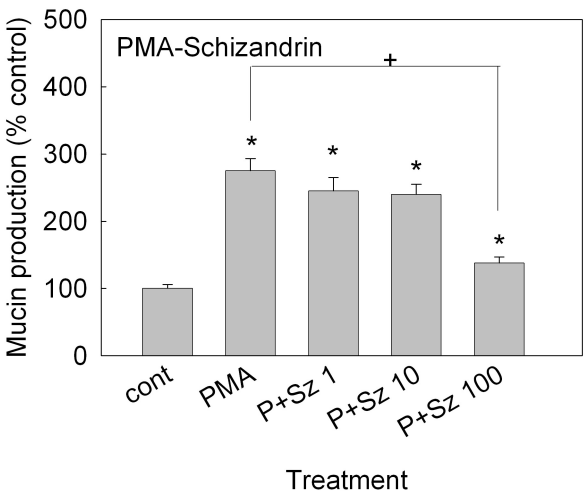

Fig. 6. Effect of schizandrin on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of schizandrin for 30min and then stimulated with PMA (10 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of a control set at 100%. *Significantly different from control ($p<0.05$). +Significantly different from PMA alone ($p<0.05$) (cont: control, Sz: schizandrin, concentration unit is μM.)

of mucin in the cells of schizandrin-treated cultures were 100 ± 4%, 159 ± 10%, 169 ± 9%, 153 ± 11% and 102 ± 5% for control, 25 ng/ml of EGF alone, EGF plus schizandrin 10^{-6} M, EGF plus schizandrin 10^{-5} M and EGF plus schizandrin 10^{-4} M, respectively (Fig. 5).

Effect of schizandrin on PMA-induced MUC5AC production

Fig. 6 shows that schizandrin significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells at concentrations between 10^{-5} M and 10^{-4} M. The amounts of mucin in the cells of schizandrin-treated cultures were 100 ± 6%, 275 ± 18%, 245 ± 20%, 240 ± 15% and 138 ± 9% for control, 10 ng/ml of PMA alone, PMA plus schizandrin 10^{-6} M, PMA plus schizandrin 10^{-5} M and PMA plus schizandrin 10^{-4} M, respectively (Fig. 6).

DISCUSSION

One important approach to the effective control of severe pulmonary diseases involving the hyperproduction and hypersecretion of airway mucin to develop a potential pharmacological tool for regulating production and/or secretion of mucus. Mucins are macromolecular glycoproteins present in the airway mucus and have peptide backbones

and carbohydrate branches (Voynow and Rubin, 2009). Currently, 20 MUC genes have been reported as coding the peptide backbone of human mucins and, among them, MUC5AC is strongly expressed in airway goblet cells (Rogers and Barnes, 2006; Yuan-Chen Wu *et al.*, 2007). Also, epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA) were reported to regulate MUC5AC mucin gene expression in the lung (Takeyama *et al.*, 1999; Hewson *et al.*, 2004). MUC5AC mRNA expression was increased after ligand binding to the EGF receptor and activation of the MAPK (mitogen-activated protein kinase) cascade (Takeyama *et al.*, 1999; Takeyama *et al.*, 2000). Phorbol 12-myristate 13-acetate (PMA) is an inflammatory stimulant affecting gene transcription, cell growth and differentiation and induces MUC5AC gene expression in NCI-H292 cells (Park *et al.*, 2002; Hewson *et al.*, 2004). Based on these reports, we investigated whether baicalin, baicalein or schizandrin affect EGF- and PMA-induced MUC5AC mucin production by NCI-H292 cells, a human pulmonary mucoepidermoid cell line which is frequently used for studying intracellular signaling pathways involved in airway mucin production (Li *et al.*, 1997; Takeyama *et al.*, 1999; Shao *et al.*, 2003). Results showed that baicalin, baicalein and schizandrin each inhibited the production of MUC5AC mucin protein induced by EGF and PMA (Fig. 1-6). In Figs. 2 and 5, although mucin production at 1 μM of test compound with the inducer was slightly higher than that in cells treated with EGF or PMA inducer alone, there was no statistical significance between the two groups. However, in Fig. 1, mucin production by treatment with 1 μM baicalin plus the inducer (EGF) was significantly higher than production in cells treated with EGF alone. However, 1 μM baicalin itself seems to have no stimulating effect on mucin production, based on the observation that the treatment using 1 μM baicalin with PMA, did not show any difference from the treatment using PMA alone (Fig. 2). Therefore, there is the possibility that, in the 1 μM baicalin plus inducer (EGF) group, EGF itself might show more potent action than in the EGF alone group, although we do not suggest the exact cause based on data from the current study. Taken together, these results suggest that baicalin, baicalein and schizandrin can control production of mucin protein induced by EGF and PMA, by directly acting on human airway epithelial cells. This result also suggests the possibility of using baicalin, baicalein and schizandrin as mucoregulators for pulmonary diseases showing the pathologic hyperproduction and hypersecretion of mucus, since baicalin and schizandrin have already been reported to inhibit the stimulated release (secretion) of airway mucin during conditions of cellular inflammation (Lee *et al.*, 2003; Heo *et al.*, 2006; Heo *et al.*, 2007). Although the underlying mechanism of action of the three natural compounds on MUC5AC mucin production is currently clear, we are trying to examine whether the three natural compounds act as potential regulators of the MAPK cascade after ligand binding to the EGF receptor in mucin-producing NCI-H292 cells.

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