

## Regulation of Tumor Necrosis Factor- $\alpha$ -induced Airway Mucin Production and Gene Expression by Carbenoxolone, Prunetin, and Silibinin

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**Background:** In this study, we tried to investigate whether carbenoxolone, prunetin, and silibinin affect tumor necrosis factor (TNF)- $\alpha$ -induced MUC5AC mucin production and gene expression from human airway epithelial cells.

**Methods:** Confluent NCI-H292 cells were pretreated with each agent (carbenoxolone, prunetin, and silibinin) for 30 min and then stimulated with TNF- $\alpha$  for 24 hours. The MUC5AC mucin gene expression and mucin protein production were measured by reverse transcription-polymerase chain reaction and enzyme linked immunosorbent assay, respectively.

**Results:** Carbenoxolone, prunetin and silibinin inhibited the production of MUC5AC mucin protein induced by TNF- $\alpha$ ; the 3 compounds also inhibited the expression of MUC5AC mucin gene induced by TNF- $\alpha$ .

**Conclusion:** This result suggests that carbenoxolone, prunetin and silibinin can inhibit mucin gene expression and production of mucin protein induced by TNF- $\alpha$ , by directly acting on airway epithelial cells.

**Key Words:** MUC5AC protein, human; Carbenoxolone; Prunetin; Silibinin

### Introduction

Mucus in the airway epithelium plays a pivotal role in defensive mechanisms against airborne chemicals, particles and pathogenic microorganisms. The protective function of airway mucus is due mainly to the viscoelastic property of mucous glycoproteins or 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 serious airway pathology as exemplified in chronic

bronchitis, cystic fibrosis, asthma, and bronchiectasis<sup>1</sup>. Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excess mucin secretion (production) by the components from medicinal plants that have been used for the management of airway diseases. 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 compounds affected mucin secretion and/or production from airway epithelial cells<sup>2,3</sup>. According to folk medicine, *Glycyrrhiza glabra L.* (licorice) has been used for regulating diverse inflammatory diseases including pulmonary diseases<sup>4</sup>. Carbenoxolone, a steroid-like compound derived from *Glycyrrhiza glabra L.*, was reported to have various biological effects including anti-inflammatory effect<sup>5-8</sup>. Prunetin, a flavonoid derived from *Glycyrrhiza glabra L.*, showed antioxidant, inhibition of

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phosphodiesterase, inhibition of aldehyde dehydrogenase and alcohol dehydrogenase activities<sup>9-12</sup>. Also, according to a number of reports, flavonoids derived from milk thistle, *Carthus marianus L.*, showed hepatoprotective, antioxidant, anti-inflammatory, anti-cancer and immunomodulatory effects<sup>13-19</sup>. However, to the best of our knowledge, there are no reports about the potential effects of carbenoxolone, prunetin and silibinin on tumor necrosis factor (TNF)- $\alpha$ -induced MUC5AC mucin production and gene expression from human airway epithelial cells. Therefore, in this study, we checked whether carbenoxolone, prunetin and silibinin affect airway mucin production and gene expression stimulated by TNF- $\alpha$  from NCI-H292 cells, a human pulmonary mucoepidermoid cell line.

## Materials and Methods

### 1. Materials

All the chemicals and reagents used in this experiment including carbenoxolone (purity, 98.0%), prunetin (purity, 98.0%) and silibinin (purity, 97.0%) were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

### 2. 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 in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100  $\mu$ g/mL) and HEPES (25 mM) at 37°C in a humidified, 5% CO<sub>2</sub>/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 hours.

### 3. Treatment of cells with carbenoxolone, prunetin, and silibinin

After 24 hours of serum deprivation, cells were pre-treated with carbenoxolone (1, 10, 100  $\mu$ M), prunetin

(1, 10, 100  $\mu$ M), and silibinin (1, 10, 100  $\mu$ M) for 30 minutes and treated with TNF- $\alpha$  (0.2 nM) for 24 hours in serum-free RPMI 1640. After 24 hours, 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 ELISA

MUC5AC protein was measured by using enzyme-linked immunosorbent assay (ELISA). Cell lysates were prepared with PBS at 1 : 10 dilution, and 100  $\mu$ L 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% BSA (fraction V) for 1 hour at room temperature. Plates were again washed three times with PBS and then incubated with 100  $\mu$ L of 45M1, a mouse monoclonal MUC5AC antibody (NeoMarkers, Fremont, CA, USA) (1 : 200), 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  $\mu$ L 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 1 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm.

### 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 Co., Daejeon, Korea) according to the manufacturer's instructions. 2  $\mu$ g of total RNA was primed with 1  $\mu$ g of oligo (dT) in a final volume of 50  $\mu$ L (RT reaction). 2  $\mu$ L of RT reaction product was PCR amplified in a 25  $\mu$ L 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'. The size of expected fragment amplified by PCR was 458 bp. 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 size of expected fragment amplified by PCR was 361 bp. The PCR mixture was denatured at 94°C for 2 minutes followed by 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds. After PCR, 5  $\mu$ L 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  $\pm$  SEM. 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

### 1. Effect of carbenoxolone on TNF- $\alpha$ -induced MUC5AC mucin production from NCI-H292 cells

As can be seen in Figure 1, carbenoxolone inhibited TNF- $\alpha$ -induced MUC5AC mucin production. The amounts of MUC5AC mucin in the cells of carbenoxolone-treated cultures were 100  $\pm$  10%, 1,250  $\pm$  98%, 1,235  $\pm$  108%, 1,124  $\pm$  76%, and 500  $\pm$  48% for control, TNF- $\alpha$  0.2 nM only, carbenoxolone 10<sup>-6</sup> M+TNF- $\alpha$ , carbenoxolone 10<sup>-5</sup> M+TNF- $\alpha$  and carbenoxolone 10<sup>-4</sup> M+TNF- $\alpha$ , respectively (Figure 1).

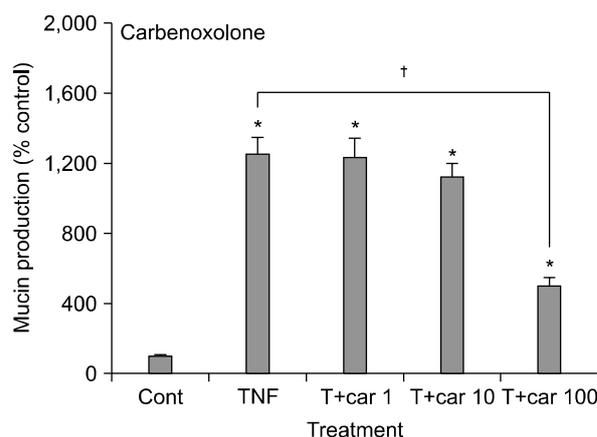
### 2. Effect of prunetin on TNF- $\alpha$ -induced MUC5AC mucin production from NCI-H292 cells

As can be seen in Figure 2, prunetin also inhibited TNF- $\alpha$ -induced MUC5AC mucin production, dose-dependently. The amounts of MUC5AC mucin in the cells

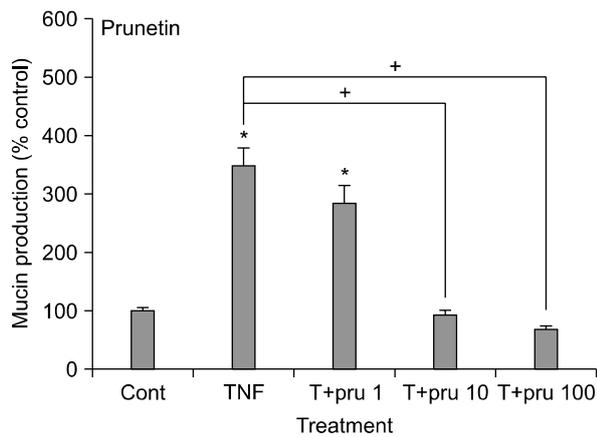
of prunetin-treated cultures were 100  $\pm$  6%, 349  $\pm$  29%, 284  $\pm$  30%, 92  $\pm$  9%, and 69  $\pm$  5% for control, TNF- $\alpha$  0.2 nM only, prunetin 10<sup>-6</sup> M+TNF- $\alpha$ , prunetin 10<sup>-5</sup> M+TNF- $\alpha$  and prunetin 10<sup>-4</sup> M+TNF- $\alpha$ , respectively (Figure 2).

### 3. Effect of silibinin on TNF- $\alpha$ -induced MUC5AC mucin production from NCI-H292 cells

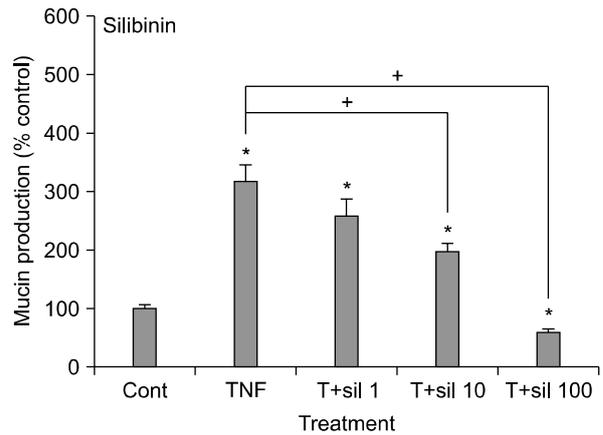
As can be seen in Figure 3, silibinin inhibited TNF- $\alpha$ -induced MUC5AC mucin production, dose-dependently. The amounts of MUC5AC mucin in the cells of silibinin-treated cultures were 100  $\pm$  7%, 317  $\pm$  29%, 257  $\pm$  30%, 198  $\pm$  13%, and 59  $\pm$  5% for control, TNF- $\alpha$  0.2 nM only, silibinin 10<sup>-6</sup> M+TNF- $\alpha$ , silibinin 10<sup>-5</sup> M+TNF- $\alpha$  and silibinin 10<sup>-4</sup> M+TNF- $\alpha$ , respectively (Figure 3).



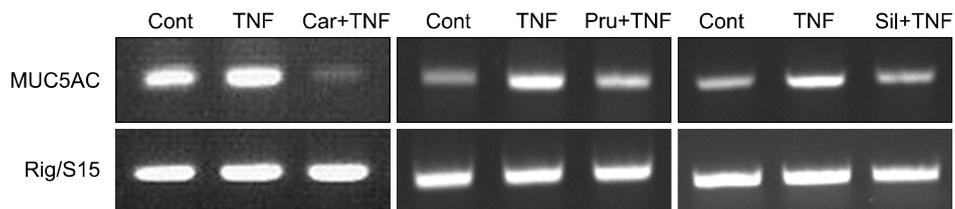
**Figure 1.** Effect of carbenoxolone on tumor necrosis factor (TNF)- $\alpha$ -induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with various concentrations of carbenoxolone for 30 minutes and then stimulated with TNF- $\alpha$  (0.2 nM) for 24 hours. Cell lysates were collected for measurement of MUC5AC mucin production by enzyme-linked immunosorbent assay. Means of individual group were converted to percent control and expressed as mean  $\pm$  SEM. 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. Each bar represents a mean  $\pm$  SEM of 3~4 culture wells in comparison with that of control set at 100%. con: control; Car: carbenoxolone. \*Significantly different from control ( $p < 0.05$ ), †Significantly different from TNF- $\alpha$  alone ( $p < 0.05$ ).



**Figure 2.** Effect of prunetin on tumor necrosis factor (TNF)- $\alpha$ -induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with various concentrations of prunetin for 30 minutes and then stimulated with TNF- $\alpha$  (0,2 nM) for 24 hours. Cell lysates were collected for measurement of MUC5AC mucin production by enzyme-linked immunosorbent assay. Means of individual group were converted to percent control and expressed as mean $\pm$ SEM. 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. Each bar represents a mean $\pm$ SEM of 3~4 culture wells in comparison with that of control set at 100%. cont: control, Pru: prunetin. \*Significantly different from control ( $p < 0,05$ ), †Significantly different from TNF- $\alpha$  alone ( $p < 0,05$ ).



**Figure 3.** Effect of silibinin on tumor necrosis factor (TNF)- $\alpha$ -induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with various concentrations of silibinin for 30 minutes and then stimulated with TNF- $\alpha$  (0,2 nM) for 24 hours. Cell lysates were collected for measurement of MUC5AC mucin production by enzyme-linked immunosorbent assay. Means of individual group were converted to percent control and expressed as mean $\pm$ SEM. 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. Each bar represents a mean $\pm$ SEM of 3~4 culture wells in comparison with that of control set at 100%. cont: control; Sil: silibinin. \*Significantly different from control ( $p < 0,05$ ), †Significantly different from TNF- $\alpha$  alone ( $p < 0,05$ ).



**Figure 4.** Effects of carbenoxolone, prunetin and silibinin on tumor necrosis factor (TNF)- $\alpha$ -induced MUC5AC mucin gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with carbenoxolone, prunetin, and silibinin (100  $\mu$ M), respectively, for 30 minutes and then stimulated with TNF- $\alpha$  (0,2 nM) for 24 hours. MUC5AC gene expression was measured by reverse transcription-polymerase chain reaction. cont: control; Car: carbenoxolone; Pru: prunetin; Sil: silibinin.

#### 4. Effects of carbenoxolone, prunetin and silibinin on TNF- $\alpha$ -induced MUC5AC gene expression from NCI-H292 cells

As can be seen in Figure 4, carbenoxolone, prunetin and silibinin inhibited TNF- $\alpha$ -induced MUC5AC gene expression at the concentration of  $10^{-4}$  M, respectively.

### Discussion

MUC5AC was reported to be mainly expressed in goblet cells existing in the airway surface epithelium among the twenty genes coding human mucins (MUC)<sup>20</sup>. Also, TNF- $\alpha$  is a well-known stimulator of secretion and gene expression of airway mucin<sup>21-23</sup>. TNF- $\alpha$  level in sputum was reported to be increased, with further increases during exacerbation of diseases<sup>24,25</sup>. TNF- $\alpha$  converting enzyme (TACE) mediated MUC5AC mucin expression in cultured human airway epithelial cells<sup>22</sup> and TNF- $\alpha$  induced MUC5AC gene expression in normal human airway epithelial cells<sup>23</sup>. It also induced mucin secretion from guinea pig tracheal epithelial cells<sup>21</sup>. On the basis of these reports, in this study, we tried to test the possible effects of carbenoxolone, prunetin and silibinin on TNF- $\alpha$ -induced MUC5AC mucin production and gene expression 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<sup>22,26</sup>. As can be seen in results, carbenoxolone, prunetin and silibinin inhibited the production of MUC5AC mucin protein induced by TNF- $\alpha$ . In the result of experiment shown in Figure 1, TNF- $\alpha$  induced MUC5AC mucin production more potently than mucin productions in the other two results of experiments shown in Figures 2 and 3. Actually, based on the preliminary experiments performed by our group, the efficacy of stimulation of mucin production by TNF- $\alpha$  varied depending on the experimental conditions, although the exact cause of this phenomenon is unclear at present. On the other hand, the three compounds inhibited the expression of MUC5AC mucin gene induced

by TNF- $\alpha$ . This result suggests that carbenoxolone, prunetin and silibinin can regulate mucin gene expression and production of mucin protein, by directly acting on airway epithelial cells. The underlying mechanisms of action of these three compounds on MUC5AC production and gene expression are not clear at present, although we are trying to investigate whether these three compounds act as regulators of NF- $\kappa$ B signaling pathway activated by TNF- $\alpha$  in mucin-producing NCI-H292 cells. Taken together, the inhibitory actions of carbenoxolone, prunetin and silibinin on airway mucin production and gene expression might explain, at least in part, the traditional use of *G. glabra* L. as anti-inflammatory agent and mucoregulator for airway inflammatory diseases, in oriental medicine and the folk use of *Carduus marianus* L. as anti-inflammatory agent. We suggest it is valuable to find the natural products that have specific inhibitory effects on mucin production and/or gene expression - in view of both basic and clinical sciences - and the result from this study suggest a possibility of using carbenoxolone, prunetin and silibinin as new efficacious mucoregulators for respiratory diseases, although further studies are essential.

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