

Effects of Short-term Treatment of Daidzein, Puerarin, Genistein and Tumerone on Mucin Secretion from Cultured Airway Epithelial Cells

Ho Jin HEO¹, Hyun Jae LEE¹, Cheolsu KIM¹, Jae Sue CHOI², Jung Joon LEE³, Young Sik KIM⁴, Sam Sik KANG⁴, Yun Hee KIM⁵, Yang Chun PARK⁵, Jeong Ho SEOK¹ and Choong Jae LEE^{1*}

¹Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon

²Faculty of Food Science and Biotechnology, Pukyong National University, Busan

³Korea Research Institute of Bioscience and Biotechnology, Daejeon

⁴Department of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul

⁵Department of Oriental Medicine, College of Oriental Medicine, Daejeon University, Daejeon, Korea

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Abstract – In this study, we investigated whether daidzein, puerarin, genistein and (+)-ar-tumerone affect mucin secretion from cultured airway epithelial cells and compared with the inhibitory action of poly-L-lysine (PLL) and the stimulatory action of adenosine triphosphate (ATP) on mucin secretion. Confluent primary hamster tracheal surface epithelial (HTSE) cells were metabolically radiolabeled using ³H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent to assess the effects on ³H-mucin secretion. The results were as follows: daidzein, puerarin, genistein and (+)-ar-tumerone did not affect mucin secretion at the highest concentrations (10⁻³M), during 30 min of treatment period. Basically, this finding suggests that daidzein, puerarin and genistein - 3 components derived from *Puerariae Radix* - and (+)-ar-tumerone derived from *Curcumae Rhizoma* might not function as a mucoregulator in various inflammatory respiratory diseases showing mucus hypersecretion, although further studies are needed.

Key words □ Airway mucin, Daidzein, Puerarin, Genistein, (+)-ar-Tumerone

INTRODUCTION

Mucus lining the airway luminal surface plays a pivotal role in defense mechanisms against airborne chemicals, particles and invading microorganisms through mucociliary clearance. Its protective function is due mainly to the viscoelastic property of mucous glycoproteins or mucins. Mucins are high molecular weight glycoproteins produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. 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 e.g. chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis (Newhouse and Biennenstock, 1983). Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excess mucin secretion by the components from oriental

herbs that have been used for the management of respiratory diseases. We have tried to investigate the possible activities of some components from medicinal plants on mucin secretion from airway epithelial cells using a primary hamster tracheal surface epithelial (HTSE) cell culture - an established in vitro model for secretory cell metaplasia (Wasano *et al.*, 1988). As a result, we previously reported that a few natural compounds affected mucin secretion from cultured airway epithelial cells (Lee *et al.*, 2003, Lee *et al.*, 2004a, Lee *et al.*, 2004b). According to traditional oriental medicine, *Puerariae Radix* has been used as a therapeutic for respiratory allergic or inflammatory diseases (National Association of Professor of Herbology in Oriental Medical School, 2005a) and their components, daidzein, puerarin and genistein, were reported to have various biological effects including inhibition of activation of airway mucin gene (Jang, 2003, Dohrman *et al.*, 1998, Li *et al.*, 1997). We also investigated the possible effect of (+)-ar-tumerone, derived from *Curcumae Rhizoma*, on airway mucin secretion and tried to compare the possible activities of these agents with the inhibitory action on mucin secretion by PLL, a non-steroi-

*Corresponding author

Tel: +82-42-580-8255, Fax: +82-42-585-6627

E-mail: LCJ123@cnu.ac.kr

dal polycationic inhibitor of mucin secretion (Ko *et al.*, 1999) and the stimulatory action by ATP, a stimulator of mucin secretion (Kim *et al.*, 1997).

MATERIALS AND METHODS

Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified. Daidzein, puerarin, genistein and (+)-ar-tumerone were isolated, purified and identified by analytical chemists in Pukyong National University (Busan, Korea), Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) and Research Institute of Natural Products of Seoul National University (Seoul, Korea).

Primary hamster tracheal surface epithelial (HTSE) cell culture

Tracheas were obtained from male Golden Syrian hamsters, 8 weeks of age (Harlan Sprague Dawley, Indiana, U.S.A.). HTSE cells were harvested and cultured on a thick collagen gel substratum as previously reported (Wasano *et al.*, 1988). Briefly, animals were euthanized in a CO₂ chamber and the tracheas were exposed under aseptic conditions. The tracheas were cannulated using a polyethylene tube through which the tracheal lumen was filled with 0.1% pronase (Type XIV) prepared in Ca⁺⁺, Mg⁺⁺ free Minimum Essential Medium (MEM, GIBCO) and incubated at 4°C for 16 h. The luminal contents were flushed, and cells were washed twice with MEM containing 10% fetal bovine serum by centrifuging at 200×g. The washed cell pellets were dissociated in a growth medium containing Medium 199 and Dulbecco's Modified Eagle's medium (DME) (1:1) supplemented with insulin (5 µg/ml), epidermal growth factor (12.5 ng/ml), hydrocortisone (0.1 µM), fetal bovine serum (5% v/v, Hyclone, Logan, UT, U.S.A.), sodium selenite (0.01 mM), retinoic acid (0.1 µM), Penicillin G (100 U/ml, GIBCO), Streptomycin (100 µg/ml, GIBCO), and Gentamicin (50 µg/ml) ("complete" medium). At this stage, most of the cells were in small aggregates and plated at a density of 10⁴ cells/cm² into tissue culture dishes containing a thick collagen gel (0.15 ml/cm²) using collagen type I (Regenmed, Seoul, Korea). Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ and culture medium were changed on day 1, 3, 5 and 7.

Metabolic labeling of mucins and treatment of cultures

Mucins were metabolically radiolabeled for 24 h by incubating confluent cultures (24 well plate, 5×10⁵ cells/well) with 0.2 ml/well of the "complete" medium containing 10 µCi/ml of [³H] glucosamine (39.2 Ci/mmol, New England Nuclear) for 24 h, as previously reported (Kim *et al.*, 1987). At the end of the 24 h incubation, the spent media (the pretreatment sample) were collected, and the labeled cultures were washed twice with Dulbecco's phosphate-buffered saline (PBS) without Ca⁺⁺ and Mg⁺⁺ before chasing for 30 min in PBS containing varying concentrations of each agent (the treatment sample). PLL (average molecular weight 7,500) and ATP were prepared and administered to cultures in PBS. Daidzein, puerarin, genistein and (+)-ar-tumerone were dissolved in dimethylsulfoxide and administered in PBS (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. PBS solution between this range and 0.5% dimethylsulfoxide did not affect mucin secretion from HTSE cells. Floating cells and cell debris were removed by centrifugation of samples at 12,000×g for 5 min. The samples were stored at -80°C until assayed for their ³H-mucin contents.

Quantitation of ³H-mucins

High molecular weight glycoconjugates excluded after Sepharose CL-4B gel-filtration column chromatography and resistant to hyaluronidase were defined as mucins and measured by the column chromatography as previously reported (Kim *et al.*, 1985). Media samples were adjusted to pH 5.0 with 0.1 M citric acid and treated with 100 U/ml of testicular hyaluronidase (Type VI-S) at 37°C for 16 h. At the end of the incubation, the digestion mixture were neutralized to pH 7.4 using 0.2 M NaOH, boiled for 2 min and centrifuged. The supernatants were applied to Sepharose CL-4B columns (1×50 cm) equilibrated with PBS containing 0.1% (w/v) Sodium Dodecyl Sulfate (SDS). Columns were eluted with the same buffer at a constant flow rate of 0.336 ml/min and fractions of 0.42 ml were collected. Void volume fractions (4 peak fractions) were mixed with 4 ml of scintillation cocktail and the radioactivity of fractions were counted using a liquid scintillation counter (LSC). The sum of radioactivity in four peak fractions will be defined as the amount of mucin in the sample. The effect of agents on mucin secretion will be measured as follows : the amount of mucin secreted during the treatment period were divided by the amount of mucin secreted during the pretreatment period and the ratio were expressed as a secretory index.

Means of secretory indices of each group were compared and the differences were assessed using statistics.

Statistics

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

RESULTS AND DISCUSSION

Daidzein, puerarin and genistein were reported to occur in *Puerariae Radix* and have various biological effects including antioxidative, antipyretic and anticancer effect (Jang, 2003). Genistein, an isoflavonoid derived from *Puerariae Radix*, inhibited the upregulation of airway mucin genes - MUC2 and MUC5AC - induced by pathogens (Li *et al.*, 1997, Dohrman *et al.*, 1998). However, to the best of our knowledge, there is no report about a trial of investigating the effect of compounds derived from *Puerariae Radix* on hypersecretion of mucus observed in diverse respiratory diseases. Thus, we tried to test the potential effects of daidzein, puerarin and genistein on mucin secretion from cultured airway epithelial cells, under the conditions of short-term treatment with comparably high doses (10^{-3} M). However, as shown in Fig.1, 2 and 3, daidzein, puerarin and genistein did not provoke any change in quantity of secretion of airway mucin, during 30 min of treatment period, even at 1mM. The amounts of mucin in the spent media of daidzein-treated cultures were $100 \pm 3\%$, $111 \pm 7\%$, $106 \pm 2\%$ and $114 \pm 7\%$ for control, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively (Fig. 1). The amounts of mucin in the spent media of puerarin-treated cultures were $100 \pm 2\%$, $117 \pm 20\%$, $104 \pm 6\%$, $127 \pm 9\%$ and $132 \pm 14\%$ for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively (Fig. 2). The amounts of mucin in the spent media of genistein-treated cultures were $100 \pm 3\%$, $96 \pm 4\%$, $106 \pm 1\%$, $112 \pm 2\%$ and $100 \pm 2\%$ for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively (Fig. 3). In fact, we expected daidzein, puerarin and genistein would have inhibitory activity on mucin secretion, based on traditional therapeutic use as remedies for respiratory disease in oriental medicine. On the contrary to this expectation, they did not show the possibility of controlling hypersecretion of respiratory mucus.

Also, *Curcumae Rhizoma* was reported to show the anti-inflammatory effect (Jang, 2003) and there is a possibility that it affects mucus hypersecretion from airway, through anti-inflammatory effect on airway epithelial cells. Therefore, we

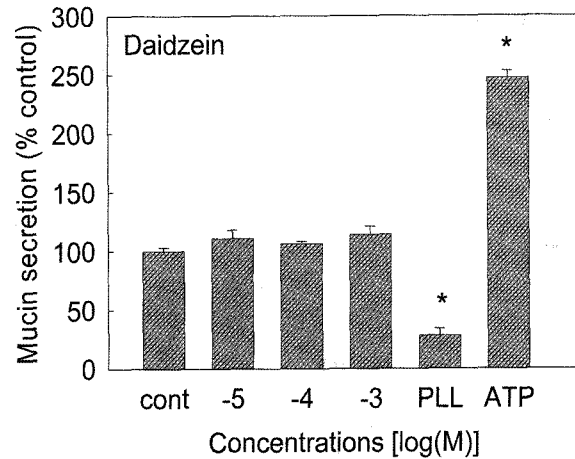


Fig. 1. Effect of daidzein on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of daidzein. For comparison, both $200 \mu\text{M}$ of ATP, a well-known mucin secretagogue and $10 \mu\text{M}$ of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^3H -mucins in the spent media were measured as described in Materials and Methods. Each bar represents a mean±S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$) (ATP : adenosine triphosphate, PLL : poly-L-lysine)

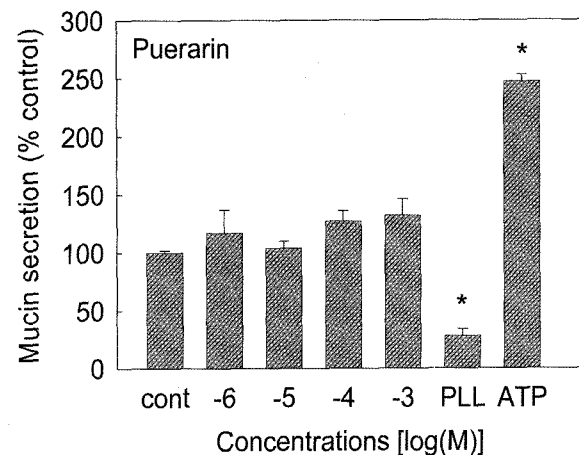


Fig. 2. Effect of puerarin on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of puerarin. For comparison, both $200 \mu\text{M}$ of ATP, a well-known mucin secretagogue and $10 \mu\text{M}$ of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^3H -mucins in the spent media were measured as described in Materials and Methods. Each bar represents a mean±S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$) (ATP : adenosine triphosphate, PLL : poly-L-lysine)

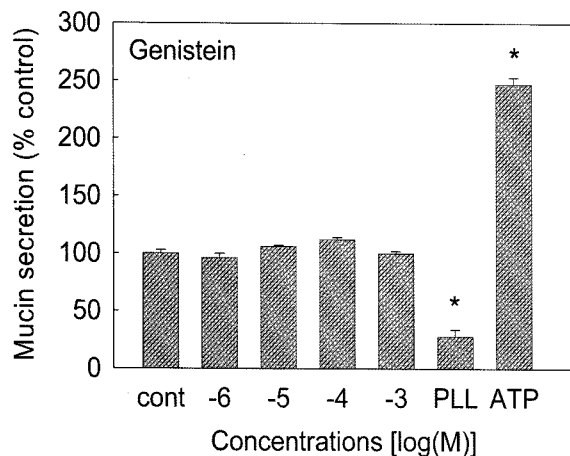


Fig. 3. Effect of genistein on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of genistein. For comparison, both 200 μM of ATP, a well-known mucin secretagogue and 10 μM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^3H -mucins in the spent media were measured as described in Materials and Methods. Each bar represents a mean \pm S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$) (ATP: adenosine triphosphate, PLL: poly-L-lysine)

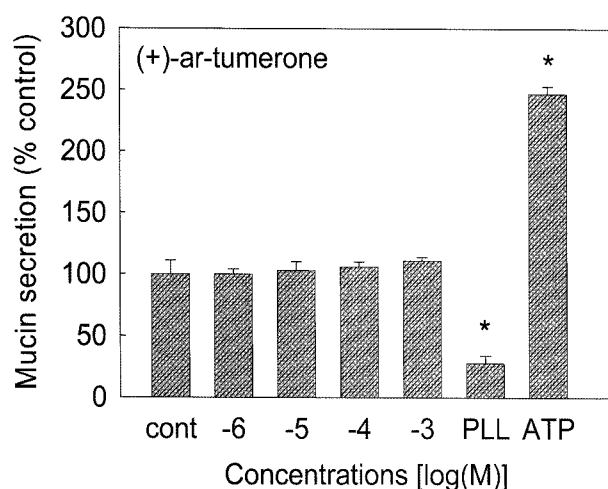


Fig. 4. Effect of (+)-ar-tumerone on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of (+)-ar-tumerone. For comparison, both 200 μM of ATP, a well-known mucin secretagogue and 10 μM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^3H -mucins in the spent media were measured as described in Materials and Methods. Each bar represents a mean \pm S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$) (ATP: adenosine triphosphate, PLL: poly-L-lysine)

tried to test the potential effect of (+)-ar-tumerone - a compound derived from *Curcumae Rhizoma* - on airway mucin secretion. However, as shown in Fig. 4, (+)-ar-tumerone did not affect airway mucin secretion, at all. The amounts of mucin in the spent media of (+)-ar-tumerone-treated cultures were $100 \pm 11\%$, $100 \pm 4\%$, $103 \pm 7\%$, $106 \pm 4\%$ and $111 \pm 3\%$ for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively (Fig. 4). In summary, daidzein, puerarin, genistein and (+)-ar-tumerone could not affect mucin secretion by directly acting on airway mucin-secreting cells whereas the two positive controls, PLL and ATP respectively inhibited and stimulated mucin secretion, from the same cells. This result suggests that just 30 min of treatment period may be too short to provoke mucin secretion by daidzein, puerarin, genistein and (+)-ar-tumerone. Thus, prolonging drug-treatment period should be tried and effects on stimulated mucin secretion by proinflammatory factors should be investigated, through future studies.

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