

Phellopterin Suppresses Airway Mucin Secretion induced by Adenosine Triphosphate

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Abstract – *Angelicae Dahiricae Radix* has been used for controlling inflammatory respiratory diseases in oriental medicine and their components, phellopterin, isoimperatorin and byakangelicol were reported to have various biological effects. In this study, we investigated whether phellopterin, isoimperatorin and byakangelicol affect adenosine triphosphate (ATP)-induced mucin secretion from cultured airway epithelial cells. 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: 1) phellopterin significantly inhibited ATP-induced mucin secretion; 2) However, isoimperatorin and byakangelicol did not affect ATP-induced mucin secretion, significantly. This result suggests that phellopterin can regulate 'mucin secretion induced by ATP' - a phenomenon simulating mucus overproduction from inflamed airway epithelial cells - by directly acting on airway mucin-secreting cells. Therefore, phellopterin should further be investigated for the possible use as mucoregulators in the treatment of inflammatory airway diseases.

Key words □ Airway, Mucin, Phellopterin, Isoimperatorin, Byakangelicol

INTRODUCTION

Airway mucus is the first-line barrier and plays an important 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 (Newhouse and Biennestock, 1983). However, any abnormality in the quality or quantity of mucins causes not only 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 (Ellis, 1985). Therefore, it seems to be highly valuable if we could find an activity to control (inhibit) the excess mucin secretion by the components from

medicinal plants that have been used for the management of airway diseases. We have tried to investigate the effects of some components from herbal medicines 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 HTSE cells (Lee *et al.*, 2003; Lee *et al.*, 2004a; Lee *et al.*, 2004b). According to oriental medicine, *Angelicae Dahiricae Radix* has been used for controlling respiratory inflammatory diseases and their components, phellopterin, isoimperatorin and byakangelicol respectively, were reported to have diverse biological effects (Choi *et al.*, 2005; Lin *et al.*, 2002; Oh *et al.*, 2002; Ban *et al.*, 2004; Marquez *et al.*, 2004; Matsuda *et al.*, 2005; Piao *et al.*, 2004; Stavri and Gibbons, 2005; Vimal and Devaki, 2004). However, to the best of our knowledge, there are no reports about the effect of the components from *Angelicae Dahiricae*

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Radix on stimulated mucin secretion from airway. Thus, in the present study, we examined whether phellopterin, isoimperatorin and byakangelicol affect ATP-induced mucin secretion from cultured HTSE cells and also compared the activities of these agents with that by PLL, a non-steroidal polycationic inhibitor of mucin secretion (Ko *et al.*, 1999).

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. Phellopterin, isoimperatorin and byakangelicol were isolated, purified and identified by analytical chemists in Research Institute of Natural Products of Seoul National University (Seoul, Korea).

Primary hamster tracheal surface epithelial (HTSE) cell culture

The animals were cared in accordance with the Guide for the Care and Use of Laboratory Animals regulated by Chungnam National University. 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 mM), Penicillin G (100 U/ml, GIBCO), Streptomycin (100 mg/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 media 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 [6-³H] 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 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. Phellopterin, isoimperatorin and byakangelicol were dissolved in dimethylsulfoxide and administered in PBS (final concentrations of dimethylsulfoxide were 0.5%). Floating cells and cell debris were removed by centrifugation of samples at 12,000×g for 5 min. The samples were stored at -80 until assayed for their ³H-mucin contents.

Quantitation of ³H-mucins

High molecular weight glycoconjugates excluded by 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.*, 1987). 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 mixtures 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, Hydrofluor (National Diagnostic, U.S.A.) and the radioactivity of fractions was counted using a liquid scintillation counter (LSC). The sum of radioactivity in four peak fractions was defined as the amount of mucin in the sample. The effect of agents on mucin secretion was measured as follows: the amount of mucin secreted during the treatment period was divided by the amount of mucin secreted during the pretreatment period, and the ratio was 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 \pm 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

Effect of phellopterin on stimulated mucin secretion

As shown in Fig. 1, phellopterin suppressed ATP-induced mucin secretion, significantly. The amounts of mucin in the spent media of drug-treated cultures were $100 \pm 4\%$, $103 \pm 7\%$, $162 \pm 5\%$, $133 \pm 3\%$ and $28 \pm 6\%$ for control, phellopterin 10^{-3}M , $2 \times 10^{-4}\text{M}$ of ATP alone, $2 \times 10^{-4}\text{M}$ of ATP plus phellopterin 10^{-3}M and PLL 7,500 10^{-5}M , respectively (Fig. 1). 10^{-5}M of PLL (MW 7,500) which was reported to be an inhibitor of mucin secretion (Ko et al, 1999) was used as positive control.

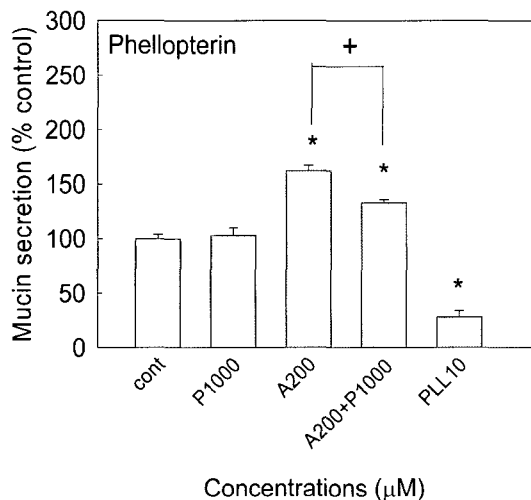


Fig. 1. Effect of phellopterin on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent. For comparison, 10 mM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion was used as a positive control. 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 3 - 4 culture wells in comparison with that of control set at 100%.

* significantly different from control ($p < 0.05$).

+ significantly different from 200 mM of ATP alone ($p < 0.05$).

(cont : control, A : ATP, adenosine triphosphate, P : phellopterin, PLL : poly-L-lysine)

Effect of isoimperatorin on stimulated mucin secretion

As shown in Fig. 2, isoimperatorin did not affect ATP-induced mucin secretion, significantly. The amounts of mucin in the spent media of drug-treated cultures were $100 \pm 4\%$, $106 \pm 5\%$, $162 \pm 5\%$, $148 \pm 10\%$ and $28 \pm 6\%$ for control, isoimperatorin 10^{-3}M , $2 \times 10^{-4}\text{M}$ of ATP alone, $2 \times 10^{-4}\text{M}$ of ATP plus isoimperatorin 10^{-3}M and PLL 7,500 10^{-5}M , respectively (Fig. 2). 10^{-5}M of PLL (MW 7,500) which was reported to be an inhibitor of mucin secretion (Ko et al, 1999) was used as positive control.

Effect of byakangelicol on stimulated mucin secretion

As shown in Fig. 3, byakangelicol did not affect ATP-induced mucin secretion, significantly. The amounts of mucin in the spent media of drug-treated cultures were $100 \pm 4\%$, $95 \pm 12\%$, $162 \pm 5\%$, $138 \pm 8\%$ and $28 \pm 6\%$ for control, byakangelicol 10^{-3}M , $2 \times 10^{-4}\text{M}$ of ATP alone, $2 \times 10^{-4}\text{M}$ of ATP plus byakangelicol 10^{-3}M and PLL 7,500 10^{-5}M , respectively (Fig. 3). 10^{-5}M of PLL (MW 7,500) which was reported to be an inhibitor of mucin secretion (Ko et al, 1999) was used as positive control.

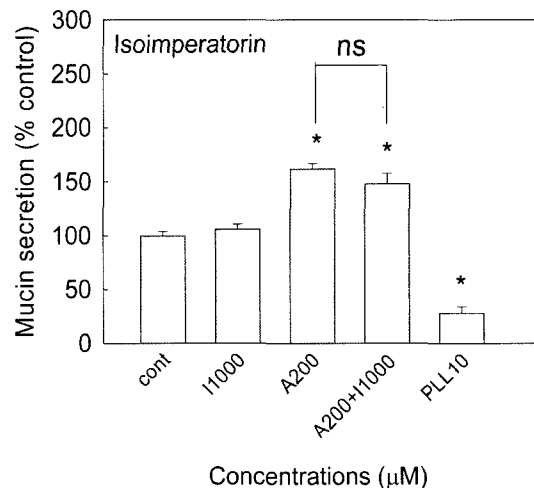


Fig. 2. Effect of isoimperatorin on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent. For comparison, 10 mM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion was used as a positive control. 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 3 - 4 culture wells in comparison with that of control set at 100%.

*significantly different from control ($p < 0.05$).

(cont : control, A : ATP, adenosine triphosphate, I : isoimperatorin, PLL : poly-L-lysine)

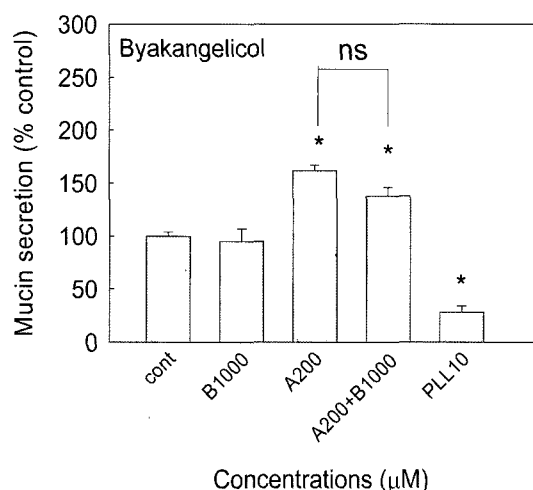


Fig. 3. Effect of byakangelicol on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^3H -glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent. For comparison, 10 mM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion was used as a positive control. 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 3 - 4 culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$). (cont : control, A : ATP, adenosine triphosphate, B : byakangelicol, PLL : poly-L-lysine)

DISCUSSION

Gordon reported that, during airway inflammation, it is expected that local extracellular ATP concentrations in the airway can reach high levels from lysed epithelial or inflammatory cells, since intracellular ATP concentrations are greater than 5 mM (Gordon, 1986). Also, ATP has been reported to stimulate mucin secretion from airway (Kim *et al.*, 1997). Therefore, we tried to examine the possible effects of phellopterin, isoimperatorin and byakangelicol on mucin secretion induced by ATP. *Angelicae Dahuricae Radix* and its components were reported to have diverse biological effects including inhibition of GABA-transaminase, inhibition of cyclooxygenase-2 in human pulmonary epithelial cells, hepatoprotective activity, antimycobacterial activity, antioxidative activity, inhibition of prostaglandin E2 production, inhibition of nitric oxide production, inhibition of lipid peroxidation and inhibition of T-cell proliferation (Choi *et al.*, 2005; Lin *et al.*, 2002; Oh *et al.*, 2002; Ban *et al.*, 2004; Marquez *et al.*, 2004; Matsuda *et al.*, 2005; Piao *et al.*, 2004; Stavri and Gibbons, 2005; Vimal and Devaki, 2004). However, to the best of our knowledge, there

are no reports about the effect of components derived from *Angelicae Dahuricae Radix* such as phellopterin, isoimperatorin and byakangelicol on airway mucin. As shown in results, phellopterin significantly suppressed ATP-induced mucin secretion at the highest concentration (10^{-3}M). However, isoimperatorin and byakangelicol did not affect ATP-induced mucin secretion. This result suggests that phellopterin can regulate 'mucin secretion stimulated by ATP' - a phenomenon simulating mucus overproduction from inflamed airway epithelial cells - by directly acting on airway mucin-secreting cells. The underlying mechanisms of action of these compounds on mucin secretion are not clear at present, and should be investigated through future studies. In summary, the inhibitory action of phellopterin on induced mucin secretion might explain, at least in part, the traditional use of *Angelicae Dahuricae Radix* as a folk remedy for airway inflammatory diseases in oriental medicine. We suggest it is valuable to find the natural products that have specific inhibitory effects on mucin secretion - in view of both basic and clinical sciences - and the result from this study suggest a possibility of using phellopterin as a mucoregulator for respiratory diseases showing hypersecretion of mucus, although further studies are essential.

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