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Effect of Erythromycin on Basal and Stimulated Mucin Release from Cultured Hamster Tracheal Surface Epthelial Cells

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Abstract – In the present study, we investigated whether lipopolysaccharide induce mucin release and erythromycin affect basal and adenosine triphosphate-induced (stimulated) mucin release, from airway goblet cells. Confluent primary hamster tracheal surface epithelial cells were metabolically radiolabeled and chased for 30 min or 24 hr in the presence of varying concentrations of lipopolysaccharide or erythromycin to assess the effects on ^{3}H-mucin release. The results were as follows : 1) Lipopolysaccharide failed to induce mucin release, 2) Erythromycin showed no effect on both basal and stimulated mucin release during 30 min or 24 hr treatment period. We conclude that lipopolysaccharide and erythromycin can not affect mucin release by direct acting on airway mucin-secreting cells.

Keywords □ airway, mucin, lipopolysaccharide, erythromycin

INTRODUCTION

The mucus hypersecretion in the airway is one of the major symptoms associated with asthma, chronic bronchitis, cystic fibrosis and bronchiectasis (Ellis, 1985). Mucins are multimillion dalton glycoproteins present in the airway mucus and produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. Also, mucins are mainly responsible for the physicochemical property of mucus and thus have been used as a biochemical marker for mucus hypersecretion (Kim *et al.*, 1997). Since the remarkable hyperplasia of airway goblet cells has been observed in patients with airway disease e.g. chronic bronchitis, goblet cells may play an important role in airway hypersecretion under such a condition. The secretion of airway mucin is generally stimulated by various inflammatory agents (Kim *et al.*, 1997). On the other hand, there are some reports about the potential regulator of mucin gene expression or release of mucin from airway epithelial cells. Takeyama and colleagues suggested that the inhibitors of epidermal growth factor receptor potentially function as a regulator in hypersecretory diseases of airways (Takeyama *et al.*, 1999). Gray *et al.* reported that thyroid hormone suppressed mucin gene expression at the transcriptional level in normal human tracheobronchial epithelial cells (Gray *et al.*, 2001). However, the agents aforementioned have diverse limitations in the application for pharmacotherapy of human diseases with airway mucus hypersecretion. Thus, we suggest it is valuable to find the possible activity of inhibiting the excessive mucin release by the drugs that have been clinically used for the management of airway diseases. We hypothesized that the bacterial endotoxin, lipopolysaccharide (LPS) might induce mucin release in vitro as observed in vivo, and the basal and possible LPS-induced mucin release might be regulated by direct actions of such a drug. Long-term administration of macrolide antibiotics e.g. erythromycin was reported to reduce sputum production in patients with chronic airway disease, probably by inhibiting airway inflammation (Tagaya *et al.*, 2002). However, to the best of our knowledge, there are no reports about direct effect of erythromycin on mucin release from airway goblet cells. Therefore, we tried to investigate whether erythromycin might affect mucin release from airway goblet cells using a primary hamster tracheal surface epithelial (HTSE) cell culture - an established in vitro model for secretory cell metaplasia (Wasano *et al.*, 1988) - and tried to compare the possible inhibitory activities of these drugs with the inhibitory action on mucin release by poly-L-lysine (PLL), a newly-known non-ste-

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roidal polycationic inhibitor of mucin release (Ko *et al.*, 1999) and the stimulatory action by adenosine triphosphate (ATP), a well-known stimulator of mucin release (Kim *et al.*, 1997).

MATERIALS AND METHODS

Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO) unless otherwise specified.

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 as promulgated and 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 CO_2 chamber and the tracheas were exposed under aseptic conditions. The tracheas were canulated 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 hr. The luminal contents were flushed, and cells were washed twice with Ca^{++}, Mg^{++}-free Minimum Essential Medium 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 μM), 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 10^4 cells/cm^2 into tissue culture dishes containing a thick collagen gel (0.15 ml/ cm^2) using collagen type I (Regenmed, Seoul, Korea). Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 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 hr by incubating confluent cultures (24 well plate, 5×10^5 cells/well) with 0.2 ml/well of a "complete" medium containing 10 μCi/ml of [6-^{3}H] glucosamine (39.2 Ci/mmol, New England Nuclear) for 24 hr, as previously reported (Kim *et al.*, 1987). At the end of the 24 hr 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^{++} (pH 7.2) before chasing for 30 min or 24 hr in PBS containing varying concentrations of each agent (the treatment sample). LPS, erythromycin, ATP and PLL (average molecular weight 7,500) were prepared in PBS. 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 ^{3}H-mucin contents.

Quantitation of ^{3}H-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 hr. 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 each 0.42 ml fractions were collected. Void volume fractions (4 peak fractions) were mixed with 4 ml of scintillation cocktail, Hydrofluor (National Diagnostic) 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 release was measured as follows : The amount of mucin released during the treatment period was divided by the amount of mucin released 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 ± 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 LPS on basal mucin release during 30 min treatment period

As shown in Fig. 1, LPS did not affect mucin release during 30 min treatment period. The amounts of mucin in the spent media of LPS-treated cultures were 100 ± 2%, 98 ± 5% and 104 ± 8% for control, 0.01 mg/ml and 0.1 mg/ml, respectively. For comparison, 10^{-5}M of PLL (35 ± 3%) which was reported to be an inhibitor of mucin release were used as positive control (Fig. 1).

Effect of erythromycin on basal mucin release during 30 min treatment period

As shown in Fig. 2, erythromycin did not affect basal mucin release during 30 min treatment period. The amounts of mucin in the spent media of erythromycin-treated cultures were 100 ± 10%, 97 ± 5%, 105 ± 9% and 114 ± 10% for control, 10^{-5} M, 10^{-4}M and 10^{-3}M, respectively. For comparison, 10^{-5}M of PLL (30 ± 7%) was reported to be an inhibitor of mucin release were used as positive control (Fig. 2).

Effect of erythromycin on basal mucin release during 24 hr treatment period

As shown in Fig. 3, erythromycin did not affect basal mucin release at the highest concentration (10^{-3}M) during 24 hr treatment period. The amounts of mucin in the spent media of erythromycin-treated cultures were 100 ± 6%, 113 ± 12% and

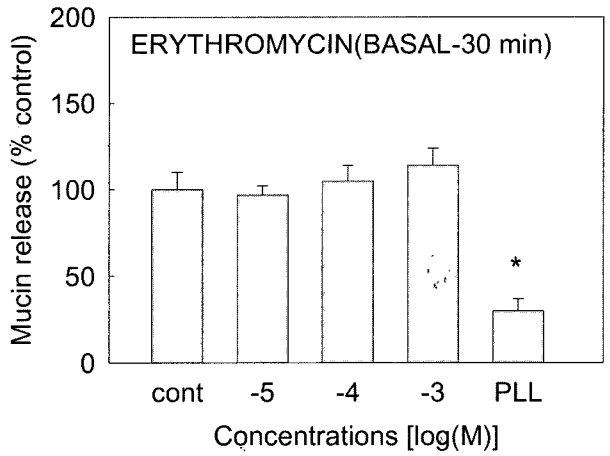

Fig. 2. Effect of erythromycin on basal mucin release during 30 min treatment period. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min in the presence of varying concentrations of erythromycin. For comparison, 10^{-5}M of PLL (MW 7,500) which was reported to be an inhibitor of mucin release were used as positive control. The amounts of ^{3}H-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 ($p< 0.05$).

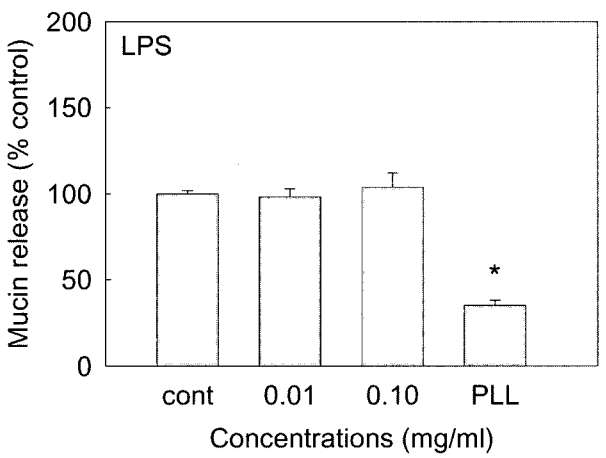

Fig. 1. Effect of LPS on mucin release during 30 min treatment period. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min in the presence of varying concentrations of LPS. For comparison, 10^{-5} M of PLL (MW 7,500) which was reported to be an inhibitor of mucin release were used as positive control. The amounts of ^{3}H-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 ($p<0.05$).

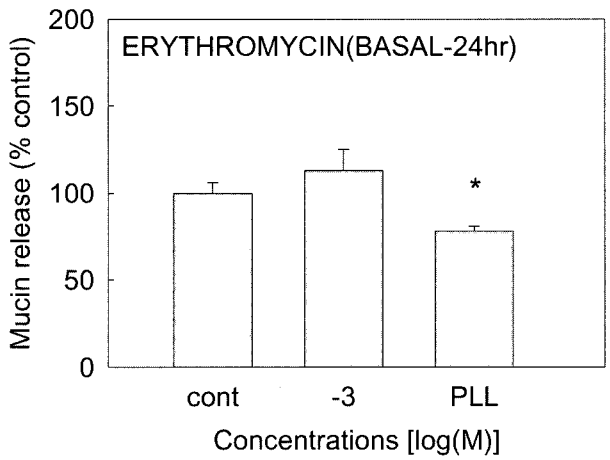

Fig. 3. Effect of erythromycin on basal mucin release during 24 hr treatment period. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 24hr in the presence of 10^{-3}M of erythromycin. 10^{-5}M of PLL (MW 7,500) which was reported to be an inhibitor of mucin release were used as positive control. The amounts of ^{3}H-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 ($p<0.05$).

78 ± 3% for control, 10^{-3}M and 10^{-5}M of PLL, respectively. (Fig. 3).

Effect of erythromycin on stimulated mucin release during 30 min treatment period

As shown in Fig. 4, erythromycin showed no effect on ATP-induced (stimulated) mucin release at the highest concentration (10^{-3}M) during 30 min treatment period. The amounts of mucin in the spent media of treated cultures were 100 ± 10%, 110 ± 10% and 245 ± 7% and 238 ± 12% for control, 10^{-3}M of erythromycin, 2.5×10^{-4}M of ATP and 2.5×10^{-4}M of ATP+10^{-3}M of erythromycin, respectively. (Fig. 4).

DISCUSSION

In this study, we used a primary Hamster Tracheal Surface Epithelial (HTSE) cell culture system to elucidate the effects of LPS and Erythromycin on mucin release. Hamster Tracheal Surface Epithelial cells grown on a thick collagen gel synthesize and secrete mucins at confluence, which are indistinguishable from in vivo mucins in terms of both size and charge. Using this cell culture system, an optimum condition was established to study the pharmacology of airway goblet cell

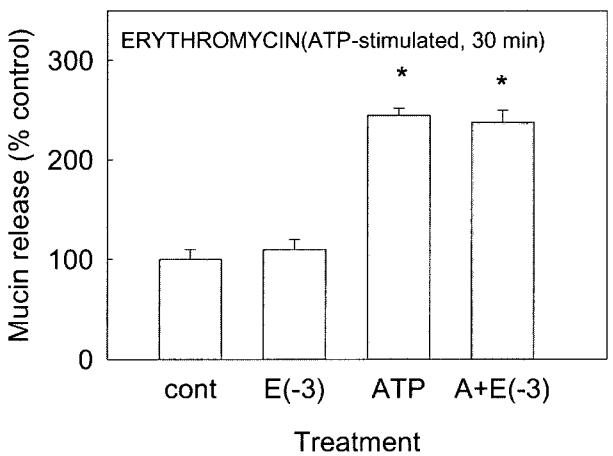

Fig. 4. Effect of erythromycin on stimulated mucin release during 30 min treatment period. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min in the presence of 10^{-3}M of erythromycin, 2.5×10^{-4}M of ATP and 2.5×10^{-4}M of ATP + 10^{-3}M of erythromycin. 10^{-5}M of PLL (MW 7,500) which was reported to be an inhibitor of mucin release were used as positive control. The amounts of ^{3}H-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 ($p<0.05$).

mucin release (Kim *et al.*, 1985). Bacterial endotoxin, a lipopolysaccharide (LPS) complex, increased concurrently the release and storage of mucosubstances in rat airway epithelium (Steiger *et al.*, 1995). LPS (0.1 mg/ml) stimulated the production of IL-8, a major contributor to inflammatory responses associated with various lung diseases, from normal human bronchial epithelial cells (Palmberg *et al.*, 1998). Thus, the possible LPS-induced mucin release might simulate the hypersecretion of mucin in airway inflammatory diseases. However, as seen in Fig. 1, LPS did not induce mucin release from Hamster Tracheal Surface Epithelial cells during 30 min treatment period. Although the possible cause of the failure of induction of mucin release in vitro by LPS should be elucidated through the separate studies, it can be suggested that IL-8 produced by LPS can stimulate neutrophils in the airway in vivo and subsequent inflammation and mucus hypersecretion might be occurred, whereas, in vitro, LPS-induced IL-8 can not stimulate neutrophils and subsequent hypersecretion of mucin, due to the absence of neutrophils in HTSE cells, if the mechanisms of hypersecretion of mucin in vivo and in vitro are similar to each other. On the other hand, macrolide antibiotic e.g. erythromycin showed various biologic effects which are not via antimicrobial actions. Macrolide suppressed the electrolyte secretion from airway epithelial cells (Tamaoki *et al.*, 1992), glycoprotein secretion from submucosal glands (Goswami *et al.*, 1990) and inhibited the migration of neutrophils (Nelson *et al.*, 1987). Macrolide antibiotics inhibit nitric oxide (NO) generation by inhibiting type II NO synthase gene expression of rat pulmonary alveolar macrophages (Kohri *et al.*, 2002). Also, macrolides protected the LPS-induced vascular leakage and neutrophil recruitment in rat trachea and this inhibition on vascular permeability was removed by depletion of neutrophils (Tamaoki *et al.*, 1994). In diseases associated with airway hypersecretion, a huge amount of neutrophils and high concentrations of IL-8 are induced in the airway (Richman-Eisenstat *et al.*, 1992). Inhalation of IL-8 caused a time-dependent increase in goblet cell secretion of guinea pig trachea and macrolides inhibited the effects of IL-8, dose-dependently (Tamaoki *et al.*, 1996). On the basis of these reports, it remains to be proved whether macrolides manifested their anti-secretory actions by inhibiting neutrophil function or by acting on goblet cells. As seen in Fig. 2, 3 and 4, erythromycin did not significantly affect both basal and stimulated mucin release from HTSE cells even at the highest concentration (10^{-3}M) during either 30 min or 24 hr treatment period. Although the possible cause of the difference in results should be studied through ongoing experiments,

we suggest that the action of macrolide on the secretion of airway mucus might be related more to the effect on recruitment of neutrophils than the direct action on mucin-secreting cells, based on the absence of neutrophils in HTSE cells. Of course, it is impossible for us to clarify the cause of this phenomenon based solely on the result obtained from the present study. Taken together, both LPS and erythromycin could not affect mucin release by direct acting on airway mucin-secreting cells whereas the two positive controls, poly-L-lysine and adenosine triphosphate respectively inhibited and stimulated mucin release from the same cells, in spite of the short treatment period -30 min. We could not find that erythromycin showed a remarkable inhibitory action on mucin release. However, it is of great value to find clinically-used drugs that have, as another pharmacological activity, possible inhibitory effects on mucin release from the viewpoint of management of hypersecretion of airway mucus.

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