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Effects of Phorbol Ester, Gö-6976, Ro-31-8220 and Röttlerin on Basal Mucin Release from Airway Goblet Cells

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Abstract – In the present study, we tried to investigate whether protein kinase C (PKC) activator, phorbol 12-Myristate 13-Acetate (PMA), and PKC inhibitors, Gö-6976, Ro-31-8220 and röttlerin significantly affect basal mucin relesed from cultured airway goblet cells. Confluent primary hamster tracheal surface epithelial (HTSE) cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min in the presence of each agent to assess the effects on ^{3}H-mucin release. The results were as follows : (1) PMA increased mucin release from cultured HTSE cells, during 30 min of treatment period; (2) However, Gö-6976, Ro-31-8220 and röttlerin did not significantly affect mucin release, during 30 min of treatment period. This finding suggests, at least in part, that PKC might play a minor role in the signaling pathways involved in basal - physiological or constitutive - mucin release from airway goblet cells, although further studies are needed.

Key words □ airway, mucin, PKC inhibitor

INTRODUCTION

Airway mucus hypersecretion 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 potential activity of inhibiting the excessive mucin release by the agents that might regulate the activity of molecules involved in signaling pathways of release(secretion) of airway mucin. Ko et al. reported that PMA-stimulated mucin release was completely blocked by PKC inhibitors such as sphingosine and calphostin C, whereas adenosine triphosphate (ATP)-induced mucin release was blocked, only in part, by these inhibitors (Ko *et al.*, 1997). Shin et al. reported that ATP stimulated mucin release by activation of PKC following activation of phospholipase A_2 (PLA_2) and phospholipase C (PLC) coupled to the P2 receptor via G-proteins (Shin *et al.*, 2001). However, to the best of our knowledge, there are no reports about direct effects of PKC inhibitors such as Gö-6976, Ro-31-8220 and röttlerin on basal - physiological or constitutive - mucin release and its signaling pathway in airway goblet cells. Thus, we tried to investigate whether, Gö-6976, Ro-31-8220 and röttlerin might affect basal

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mucin release and its signaling pathway, using a primary hamster tracheal surface epithelial (HTSE) cell culture - an established in vitro model for mucin-secreting cell metaplasia (Wasano *et al.*, 1988).

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 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 mM), 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 hrs 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 mins in PBS containing varying concentrations of each agent (the treatment sample). 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 hrs. At the end of the incubation, the digestion mixtures were neutralized to pH 7.4 using 0.2 M NaOH, boiled for 2 mins 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 PMA on mucin release

PMA significantly increased mucin release at 10^{-6} M, during 30 mins of treatment period. The amounts of mucin in the spent media of PMA-treated cultures were 100±9%, 107±5%, 132± 10% and 190±9% for control, 10^{-8} M, 10^{-7} M and 10^{-6} M, respectively (Fig. 1).

Effect of Gö-6976 on mucin release

Gö-6976 did not affect mucin release during 30 mins of treatment period. The amounts of mucin in the spent media of Gö-6976-treated cultures were 100 ± 3%, 86 ± 15%, 103 ± 8% and 118 ± 7% for control, 10^{-6} M, 10^{-5} M and 10^{-4} M, respectively. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M poly-l-lysine (PLL) (MW 7,500) were used as positive controls (Fig. 2).

Effect of Ro-31-8220 on mucin release

Ro-31-8220 did not affect mucin release during 30 mins of treatment period. The amounts of mucin in the spent media of Ro-31-8220-treated cultures were 100 ± 10%, 107 ± 5%, 103 ± 12% and 105 ± 7% for control, 10^{-6} M, 10^{-5} M and 10^{-4} M, respectively. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 3).

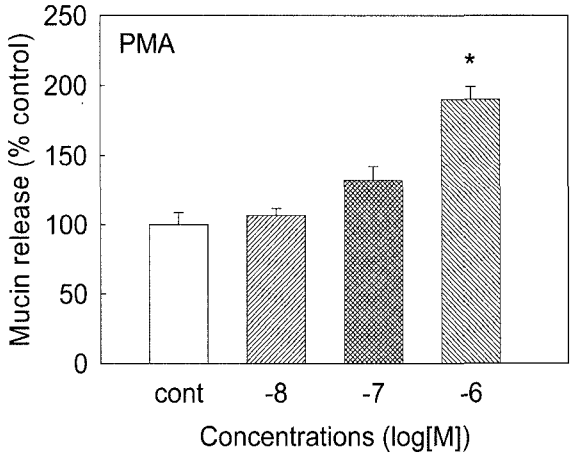

Fig. 1. Effect of PMA on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of PMA and the amount of ^{3}H-mucins in the spent media was measured as described in Materials and Methods. Each bar represents a mean±S.E.M. from 3 - 4 culture wells in comparison with that of control set at 100%. *Significantly different from control ($p<0.05$).

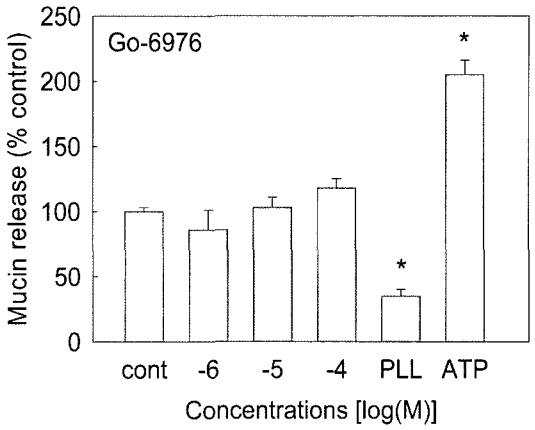

Fig. 2. Effect of Gö-6976 on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of Gö-6976 and the amount of ^{3}H-mucins in the spent media was measured as described in Materials and Methods. Each bar represents a mean ± S.E.M. from 3 - 4 culture wells in comparison with that of control set at 100%. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls. *significantly different from control ($p<0.05$).

Effect of röttlerin on mucin release

Röttlerin did not affect mucin release during 30 mins of treatment period. The amounts of mucin in the spent media of röttlerin-treated cultures were 100 ± 4%, 87 ± 8%, 98 ± 4% and

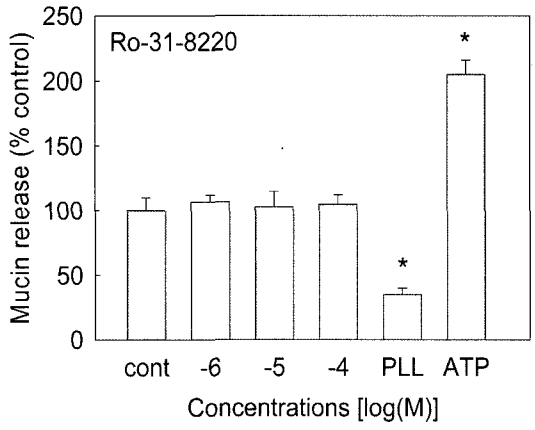

Fig. 3. Effect of Ro-31-8220 on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of Ro-31-8220 and the amount of ^{3}H-mucins in the spent media was measured as described in Materials and Methods. Each bar represents a mean±S.E.M. from 3 - 4 culture wells in comparison with that of control set at 100%. For comparison, both 2.0×10^{-4} M ATP and 10^{-5}M PLL (MW 7,500) were used as positive controls. *significantly different from control ($p<0.05$).

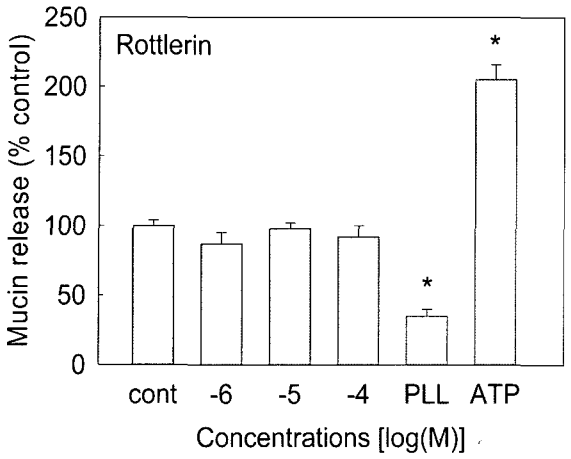

Fig. 4. Effect of röttlerin on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of röttlerin and the amount of ^{3}H-mucins in the spent media was measured as described in Materials and Methods. Each bar represents a mean±S.E.M. from 3 - 4 culture wells in comparison with that of control set at 100%. For comparison, both 2.0×10^{-4} M ATP and 10^{-5}M PLL (MW 7,500) were used as positive controls. * significantly different from control ($p<0.05$).

92 ± 8% for control, 10^{-6} M, 10^{-5} M and 10^{-4} M, respectively. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 4).

DISCUSSION

In this study, we used a primary Hamster Tracheal Surface Epithelial (HTSE) cells to elucidate the effects of three inhibitors of PKC on mucin release from airway goblet cells and tried to compare the activities of these agents with the inhibitory action on mucin release by PLL, an inhibitor of mucin release (Ko *et al.*, 1999, Lee *et al.*, 2002) and the stimulatory action by ATP, a well-known stimulator of mucin release (Kim *et al.*, 1997). HTSE 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 mucin release (Kim *et al.*, 1985). As shown in Fig. 1, PMA, the PKC activator, could stimulate basal mucin release from airway goblet cells. Therefore, specific inhibitors of PKC might be able to affect basal mucin release. 3-[1-[3-(Amidinothio)propyl- 1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, named as Ro-31- 8220, is a red solid compound which is competitive and selective inhibitor of PKC and PKA (Beltman *et al.*, 1996). 2-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo (2,3-a)pyrrolo (3,4-c)-carbazole, named as Gö-6976, is a solid compound which also inhibits PKC. It selectively inhibits Ca^{2+}-dependent PKC and does not affect the kinase activity of the Ca^{2+} -independent PKC (Martiny-Baron et al., 1993). Röttlerin is another PKC inhibitor that exhibits greater selectivity for specific types of PKCs (Villalba *et al.*, 1999). Therefore, in this study, we intended to examine the effect of these three PKC inhibitors on basal mucin release. However, as shown in Fig. 2, 3, and 4, Ro-31-8220, Gö-6976 and röttlerin did not significantly affect basal mucin release from airway goblet cells. In fact, we expected these three inhibitors of PKC would have inhibitory activity on basal mucin release. On the contrary to this expectation, they did not show any activity on basal mucin release, at all. In summary, Ro-31-8220, Gö-6976 and röttlerin could not affect basal - physiological or constitutive - mucin release by directly acting on airway mucin-secreting cells whereas the two positive controls, poly-L-lysine and ATP respectively inhibited and stimulated mucin release from the same cells. This result suggests us that it is too short for PKC inhibitors to affect basal mucin release during just 30 min of treatment period and be further studied through prolonging drug-treatment period. Although we could not find the specific agent which inhibits basal mucin release from airway goblet cells, we suggest it is very important and significant to try to find the specific compounds that have inhibitory effects on mucin release - in view of both basic and clinical sciences.

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