Korean J Physiol Pharmacol
Vol 9: 155–158, June, 2005

Effects of Sphingosine-1-phosphate, Furosemide and Indomethacin on Mucin Release from Airway Goblet Cells

Ju-Ock Kim[1], Hyung Soo Kim[2], Sun-Young Kim[1], Seong-Soo Jeong[1], Ho Jin Heo[2], Jeong Ho Seok[2], and Choong Jae Lee[2]

[1]Pulmonology Section, Department of Internal Medicine, Chungnam National University Hospital, Daejeon 301–721, [2]Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon 301–303, Korea

In this study, we investigated whether sphingosine-1-phosphate, furosemide, and indomethacin affect 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 the above agents to assess the effects on ^{3}H-mucin release. Sphingosine-1-phosphate stimulated mucin release during 30 min of treatment period in a dose-dependent manner. However, furosemide and indomethacin showed no effect on both basal and stimulated mucin release during 30 min or 24 hr of treatment period. We conclude that sphingosine-1-phosphate can affect mucin release by directly acting on airway mucin-secreting cells.

Key Words: Airway, Mucin, Furosemide, Indomethacin, Sphingosine-1-phosphate

INTRODUCTION

Airway mucus plays an important role in host defense against airborne chemicals, paticles and invading microorganisms through a mechanism called the mucociliary clearance. Its protective function is mainly due to the viscoelastic property of mucous glycoproteins or mucins (Ellis, 1985). Mucins are high molecular weight glycoproteins produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. Therefore, any abnormality in the quality or quantity of mucins not only causes altered airway physiology, but also impairs host defenses, often leading to serious airway pathology as exemplified in chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis (Kim et al, 1997). To remove an excess mucus from the airway, there could be two ways; i) getting rid of the mucus by physical means, that is to say, aspiration after dilution of mucus, and ii) suppression of secretion and/or production of mucus by pharmacological means. However, clinically, the physical method induces irritation of airway luminal wall and leads to hypersecretion of mucus through a reflex mechanism. Thus, the pharmacological means to inhibit mucin secretion and/or production has become an important approach to regulate the hypersecretion of airway mucus (Mutschler et al, 1995). Secretion of airway mucin is generally stimulated by various agents whereas glucocorticoids inhibits the hypersecretion of airway mucins (Mutschler et al, 1995; Lee et al, 2002). However, since glucocorticoids have diverse limitations in the application to pharmacotherapy of human diseases with airway mucus hypersecretion, it is highly desirable to find a way to inhibit the excessive mucin release by agents that have clinically been used for the management of airway diseases. Furosemide, a diuretic, may affect respiratory mucus either directly through chloride transport on the basolateral surface of airway epithelium or indirectly through increased diuresis and dehydration (Prandota, 2001). Prophylactic administration of indomethacin, a non-steroidal anti-inflammatory drug, inhibited mucous secretory cell hyperplasia and mucus hypersecretion induced by cigarette smoking (Jeffery, 1986). However, to the best of our knowledge, there are no reports about direct effects of furosemide and indomethacin on mucin release from airway goblet cells. Therefore, we attempted to investigate whether these agents might affect basal and stimulated 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). Additionally, we tried to investigate whether sphingosine-1-phosphate might regulate mucin release from airway goblet cells. Sphingosine-1-phosphate has been shown to modulate human airway smooth muscle contraction, cell growth and proinflammatory cytokine production that promote bronchoconstriction, airway inflammation and remodeling in asthma (Ammit et al, 2001). Indeed, we found that sphingosine-1-phosphate can stimulate mucin release in a dose-dependent manner.

Corresponding to: Choong Jae Lee, Department of Pharmacology, College of Medicine, Chungnam National University, 6 Munhwa-dong, Jung-gu, Daejeon 301-131, Korea. (Tel) 82-42-580-8255, (Fax) 82-42-585-6627, (E-mail) LCJ123@cnu.ac.kr

ABBREVIATIONS: PLL, poly-L-lysine; ATP, adenosine triphosphate; HTSE, hamster tracheal surface epithelial; PBS, phosphate-buffered saline.

METHODS

Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO. USA), 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 Golden Syrian male hamsters, 8 weeks of age (Harlan Sprague Dawley, Indiana, USA). Hamster tracheal surface epithelial 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 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, USA), 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 thick collagen gel (0.15 ml/cm^2) made of 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 was 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 of incubation, the spent media (the pretreatment sample) were collected, and the labeled cultures were washed twice with Dulbecco's phosphate-buffered saline without Ca^{++} and Mg^{++} before chasing for 30 min or 24 hr in Dulbecco's phosphate-buffered saline containing varying concentrations of each agent (the treatment sample). Furosemide, indomethacin, ATP and poly-L-lysine (average molecular weight 7,500) were dissolved and administered in Dulbecco's phosphate-buffered saline. Sphingosine-1-phosphate was dissolved in methanol and administered in Dulbecco's phosphate-buffered saline (final concentration of methanol was 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Dulbecco's phosphate-buffered saline in this range did not affect mucin release from cultured hamster tracheal surface epithelial cells (Kim et al, 1997). Floating cells and cell debris were removed by centrifugation 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 through 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 Dulbecco's phosphate-buffered saline containing 0.1% (w/v) sodium dodecyl sulfate. 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 radioactivity was counted using a liquid scintillation counter. 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±SEM. 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 furosemide on mucin release

As shown in Fig. 1, furosemide did not affect basal and ATP-induced (stimulated) mucin release during 30 min of treatment period. The amounts of mucin in the spent media of treated cultures were 100±7%, 107±5%, 90±12%, 95±6%, 256±17% and 267±20% for control, 10^{-5} M furosemide, 10^{-4} M furosemide, 10^{-3} M furosemide, 2.5×10^{-4} M ATP and 2.5×10^{-4} M ATP + 10^{-3} M furosemide, respectively. Also, even during 24 hr of treatment period at the highest concentration (10^{-3} M), furosemide did not affect basal mucin release. The amounts of mucin in the spent media of furosemide-treated cultures were 100±6% and 110±7% for control and 10^{-3} M, respectively. For comparison, 10^{-5} M poly-L-lysine which is reported to be an inhibitor of mucin release (Ko et al, 1999) were used as positive controls during both 30 min and 24 hr of treatment (Fig. 1).

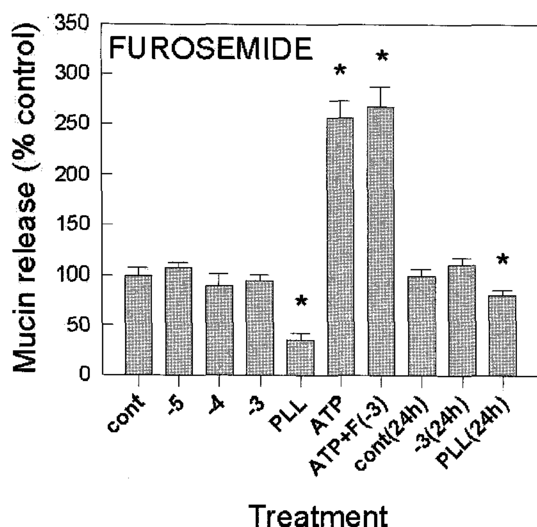

Fig. 1. Effect of furosemide on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min or 24 hr in the presence of varying concentrations of furosemide (marked as log [molar concentartion]). For comparison, 10^{-5} M poly-L-lysine (MW 7,500) that was reported to be an inhibitor of mucin release were used as positive controls. The amounts of ^{3}H-mucins in the spent media were measured as described in Materials and Methods. Each bar represents mean S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p<0.05$).

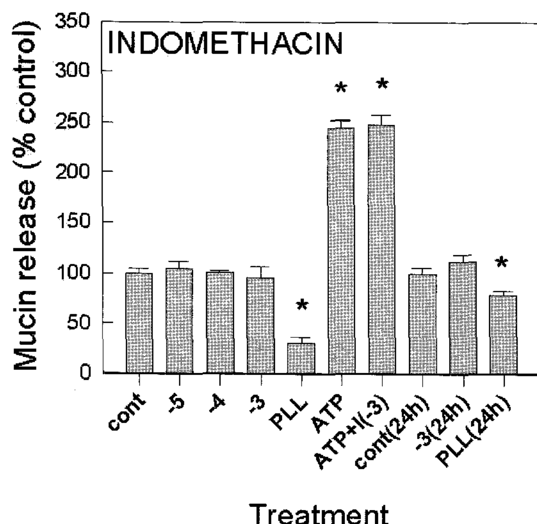

Fig. 2. Effect of indomethacin on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min or 24 hr in the presence of varying concentrations of indomethacin (marked as log [molar concentartion]). For comparison, 10 ^{5} M poly-L-lysine (MW 7,500) that was reported to be an inhibitor of mucin release were used as positive controls. The amounts of ^{3}H-mucins in the spent media were measured as described in Materials and Methods. Each bar represents mean S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p<0.05$).

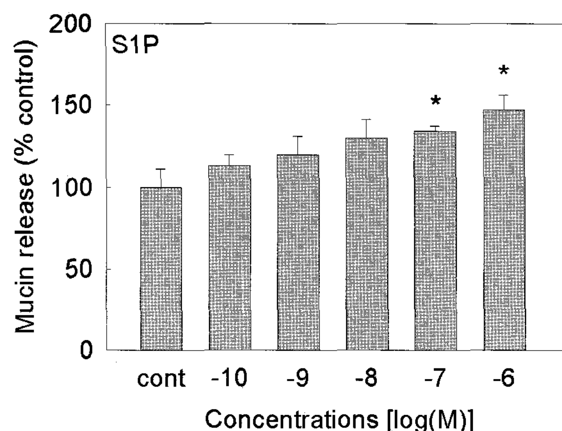

Fig. 3. Effect of sphingosine-1-phosphate on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hr and chased for 30 min in the presence of varying concentrations of sphingosine-1-phosphate. The amount of ^{3}H-mucin in the spent media was measured as described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

Effect of indomethacin on mucin release

As shown in Fig. 2, indomethacin did not affect basal and ATP-induced (stimulated) mucin release during 30 min treatment period. The amounts of mucin in the spent media of treated cultures were 100±5%, 105±7%, 101±2%, 96±11%, 245±7% and 248±9% for control, 10^{-5} M indomethacin, 10^{-4} M indomethacin, 10^{-3} M indomethacin, 2.5×10^{-4} M ATP and 2.5×10^{-4} M ATP + 10^{-3} M indomethacin, respectively. Also, even during 24 hr treatment period at the highest concentration (10^{-3} M), indomethacin did not affect basal mucin release. The amounts of mucin in the spent media of indomethacin-treated cultures were 100±6% and 112±7% for control and 10^{-3} M, rspectively. For comparison, 10^{-5} M poly-L-lysine which is reported to be an inhibitor of mucin release were used as positive controls during both 30 min and 24 hr of treatment (Fig. 2).

Effect of sphingosine-1-phosphate on mucin release

As shown in Fig. 3, during 30 min of treatment period, sphingosine-1-phosphate increased mucin release. The amounts of mucin in the spent media of sphingosine-1-phosphate-treated cultures were 100±11%, 113±7%, 120±11%, 130±11%, 134±3% and 147±9% for control, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M, respectively (Fig. 3).

DISCUSSION

In this study, we used a primary Hamster Tracheal Surface Epithelial cell culture system to elucidate the effects of furosemide, indomethacin and sphingosine-1-phosphate 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 establi-

shed to study the pharmacology of mucin release of airway goblet cells (Kim et al, 1985).

When furosemide is nebulized into the airway of patient with bronchial asthma, the anti-asthmatic effects by furosemide have been observed (Bianco et al, 1989). Furosemide has been reported to be effective in the management of bronchoconstriction accompanying chronic lung disease and narrowing of the upper respiratory airways (Prandota, 2001). Asthma is defined as an inflammatory disease characterized by mucus hypersecretion as one of its major manifestations (Ellis, 1985). Therefore, if furosemide has anti-bronchoconstricting effect with anti-hypersecretory effect, it can be used as an ideal drug for the management of bronchial asthma. On the basis of these reports and hypothesis, we tested the possible effects of furosemide on airway mucin release. However, as shown in Fig. 1, furosemide at the highest concentration (10^{-3} M) did not significantly affect basal mucin release from HTSE cells during either 30 min or 24 hr of treatment period. On the other hand, inhibition of cyclooxygenase by non-steroidal anti-inflammatory drug such as indomethacin might suppress mucus and respiratory tract fluid, because endogenous prostaglandins may play a role in the regulation of airway secretions. In clinical trial, indomethacin reduced the bronchorrhea sputum in patients with chronic airway disease (Tamaoki et al, 1992). Therefore, we investigated the possible effects of indomethacin on airway mucin release. In our results, as shown in Fig. 2, indomethacin did not inhibit basal mucin release from HTSE cells at the concentrations of 10^{-3} M during either 30 min or 24 hr of treatment period. Thus, it is highly likely that both furosemide and indomethacin do not regulate mucin release by acting directly on mucin-secreting cells, however, if they show anti-asthmatic and/or anti-hypersecretory effects, it should be via different mechanisms.

There are a few studies to demonstrate the effects of sphingosine-1-phosphate on secretion of important endogenous materials (Rabano et al, 2003; Cummings et al, 2002). Sphingosine-1-phosphate stimulated secretion of cortisol from adrenocortical cells (Rabano et al, 2003) and regulated secretion of interleukin-8 from airway epithelial cells (Cummings et al, 2002). Similarly, as can be seen in Fig. 3, sphingosine-1-phosphate stimulated mucin release from airway goblet cells, dose-dependently. This result implies that endogenous sphingosine-1-phosphate may be involved in regulation of mucus secretion in physiological situations and/or pathological ones such as asthma, chronic bronchitis, bronchiectasis and emphysema. Since sphingosine-1-phosphate augments hyperreactivity of asthmatic airways (Ammit et al, 2001; Jolly et al, 2001), we suggest that sphingosine-1-phosphate is an important mediator that can both induce mucus hypersecretion and modulate contraction of airway smooth muscle in asthma.

Based on our results, we failed to find a drug that showed a remarkable inhibitory action on mucin release. However, from the viewpoint of management of airway mucus hypersecretion, it is of a great interest to find the possible inhibitory effects of clinically-used drugs on mucin release as another pharmacological activity. Additionally, inhibition of sphingosine-1-phosphate production by inhibitors of sphingosine kinase (sphingosine-1-phosphate-producing enzyme) could be a strategy to regulate mucus hypersecretion in asthma, although the signaling pathway involved in stimulation of mucin release by sphingosine-1-phosphate should be elucidated before application of strategy.

ACKNOWLEDGEMENT

This work was supported by Chungnam National University Hospital Research fund 2004.

REFERENCES

Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymska VP. Sphingosine-1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* 15: 1212–1214, 2001

Bianco S, Pieroni MG, Refini RM, Rottoli L, Sestini P. Protective effect of inhaled furosemide on allergen-induced early and late asthmatic reactions. *N Engl J Med* 321: 1069–1073, 1989

Cummings RJ, Parinandi NL, Zaiman A, Wang L, Usatyuk PV, Garcia JG, Natarajan V. Phospholipase D activation by sphingosine-1-phosphate regulates interleukin-8 secretion in human bronchial epithelial cells. *J Biol Chem* 277: 30227–30235, 2002

Ellis EF. Asthma in childhood. *J Allergy Clin Immunol* 72 (suppl): 526–539, 1985

Jeffery PK. Anti-inflammatory drugs and experimental bronchitis. *Eur J Respir Dis* 146: 245–257, 1986

Jolly PS, Rosenfeldt HM, Milstien S, Spiegel S. The roles of sphingosine-1-phosphate in asthma. *Mol Immunol* 38: 1239–1245, 2001

Kim KC, McCracken K, Lee BC, Shin CY, Jo MJ, Lee CJ, Ko KH. Airway goblet cell mucin: its structure and regulation of secretion. *Eur Respir J* 10: 2644–2649, 1997

Kim KC, Rearick JI, Nettesheim P, Jetten AM. Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture. *J Biol Chem* 260: 4021–4027, 1985

Kim KC, Wasano K, Niles RM, Schuster JE, Stone PJ, Brody JS. Human neutrophil elastase releases cell surface mucins from primary cultures of hamster tracheal epithelial cells. *Proc Natl Acad Sci USA* 84: 9304–9308, 1987

Ko KH, Lee CJ, Shin CY, Jo M, Kim KC. Inhibition of mucin release from airway goblet cells by polycationic peptides. *Am J Physiol* 277: L811–L815, 1999

Lee CJ, Paik SH, Ko KH, Kim KC. Effects of polycationic peptides on mucin release from airway goblet cells: relationship between polymer size and activity. *Inflamm Res* 51: 490–494, 2002

Mutschler E, Derendorf H. *Drug actions*. Boca Raton, Florida, CRC Press, p 410–411, 1995

Prandota J. Clinical pharmacology of furosemide in children: a supplement. *Am J Ther* 8: 275–289, 2001

Rabano M, Pena A, Brizuela A, Marino A, Macarulla JM, Trueba M, Gomez-Munoz A. Sphingosine-1-phosphate stimulates cortisol secretion. *FEBS Lett* 535: 101–105, 2003

Tamaoki J, Chiyotani A, Kobayashi K, Sakai N, Kanemura T, Takizawa T. Effect of indomethacin on bronchorrhea in patients with chronic bronchitis, diffuse panbroncjiolitis, or bronchiectasis. *Am Rev Respir Dis* 145: 548–552, 1992

Wasano K, Kim KC, Niles RM, Brody JS. Membrane differentiation markers of airway epithelial secretory cells. *J Histochem Cytochem* 36: 167–178, 1988