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Effects of Proinflammatory Cytokines and Natural Products on Mucin Release from Cultured Hamster Tracheal Surface Epithelial Cells

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In this study, we investigated whether TNF-alpha, IL-1beta, CTMA (carboxymethyl trimethylammonium) and LPD (Lup-20[29]-ene-3beta,28-diol) affect mucin release from airway goblet cells and compared the activities of these agents with the inhibitory action of PLL and the stimulatory action of ATP on mucin release. Confluent primary hamster tracheal surface epithelial (HTSE) cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent to assess the effects on ^{3}H-mucin release. The results were as follows: TNF-alpha, CTMA and LPD increased mucin release at the highest concentration, but IL-1beta did not. We conclude that CTMA and LPD can stimulate mucin release by directly acting on airway mucin-secreting cells, and suggest that these agents should be further investigated for the possible use as mild expectorants during the treatment of chronic airway diseases.

Key Words: Airway, Mucin, Natural products, TNF-alpha

INTRODUCTION

Mucus lining in the airway plays an important role in host defenses against airborne chemicals, paticles and invading microorganisms through a mechanism called the mucociliary clearance. Its protective function is due mainly to the viscoelastic property of mucous glycoproteins or mucins (Newhouse & Biennenstock, 1983). 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 cause altered airway physiology, but also may impair host defenses, often leading to serious airway pathology as exemplified in chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis (Ellis, 1985). To remove the excess mucus from the airway, there are 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, the physical method induces irritation of airway luminal wall and leads to hypersecretion of mucus through a reflex mechanism, clinically. Therefore, the pharmacological mean to inhibit mucin secretion and/or production has become an important approach to regulate the hypersecretion of airway mucus (Mutschler & Derendorf, 1995). Secretion of airway mucin is generally stimulated by various inflammatory agents while glucocorticoids could inhibit the hypersecretion of airway mucins (Mutschler & Derendorf, 1995; Kim et al, 1997). Since glucocorticoids have various limitations in the application for pharmacotherapy of human diseases with airway mucus hypersecretion, we suggest that it would be valuable to find components from herbs or herbal preparations, which have been used for the management of airway diseases in traditional oriental medicine, to inhibit the excess mucin release. Although a number of oriental herbs or herbal preparations are known to be effective in regulating the secretion and production of thick and tenacious mucus in the airway, either clinical or pharmacological data obtained from well-controlled and established studies are not available to date. Therefore, we tried to investigate the effects of some components from medicinal plants on 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). CTMA is a component derived from *Lycium chinense Miller* and LPD is a triterpenoid compound originated from *Betula platyphylla var japonica*, respectively. These two plants have been used for controlling airway allergic or inflammatory diseases in oriental medicine, and their components, CTMA and LPD, were reported to have various biological effects including

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ABBREVIATIONS: PLL, poly-L-lysine; HTSE, hamster tracheal surface epithelial; PBS, phosphate-buffered saline; IL-1beta, interleukin-1beta; TNF-alpha, tumor necrosis factor-alpha; CTMA, carboxymethyl trimethylammonium; LPD, Lup-20[29]-ene-3beta, 28-diol.

anti-inflammatory action (Research institute of natural products in Seoul National University, 2003). On the other hand, proinflammatory cytokines including TNF-alpha and IL-1beta have been shown to stimulate either mucin release or expression of mucin gene (Fischer et al, 1999; Ki et al, 2002; Shao et al, 2003; Song et al, 2003). Thus, in the present study, we examined whether CTMA and LPD inhibit (decrease) either basal mucin release or mucin release stimulated by TNF-alpha and IL-1beta from cultured HTSE cells. Additionally, we tried to compare the activities of these agents with the inhibitory action on mucin release by PLL, a newly-known non-steroidal polycationic inhibitor of mucin release (Ko et al, 1999) and the stimulatory action by ATP, a well-known stimulator of mucin release (Kim et al, 1997).

METHODS

Materials

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

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 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 h. The luminal contents were flushed, and cells were washed twice with MEM containing 10% fetal bovine serum by centrifuging at 200 × g, for 5 min. 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 (5μg/ml), epidermal growth factor (12.5 ng/ml), hydrocortisone (0.1μM), fetal bovine serum (5% v/v, Hyclone, Logan, UT, U.S.A.), sodium selenite (0.01μM), retinoic acid (0.1μM), Penicillin G (100 U/ml, GIBCO), Streptomycin (100μg/ml, GIBCO), and Gentamicin (50μg/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 was 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, 5×10^5 cells/well) with 0.2 ml/well of the "complete" medium containing 10μCi/ml of [6-^{3}H] glucosamine (39.2 Ci/mmol, New England Nuclear) for 24 h, as previously reported (Kim et al, 1987). At the end of 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 the case of CTMA, IL-1beta, LPD) or 24 h (in the case of TNF-alpha) in PBS containing varying concentrations of each agent (the treatment sample). CTMA, TNF-alpha, IL-1beta, PLL (average molecular weight 7,500) and ATP were prepared and administered to cultures in PBS. LPD was dissolved in ethanol and administered in PBS (final concentrations of ethanol were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Either PBS solution with this range [4] or 0.5% ethanol did not affect mucin release 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 ^{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, 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 with 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 0.42 ml fractions each were collected. Void volume fractions (4 peak fractions) were mixed with 4 ml of scintillation cocktail, Hydrofluor (National Diagnostic) and the radioactivity of fractions were 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 defined as 'secretory index'. Means of secretory indices of each group were compared and 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 TNF-alpha on mucin release

As shown in Fig. 1, TNF-alpha significantly increased mucin release at 500 U/ml during 24h treatment period. The amounts of mucin in the spent media of TNF-alpha-treated cultures were 100±7% and 156±6% for control and 500 U/ml, respectively. For comparison, both 2×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive

controls (Fig. 1).

Effect of IL-1beta on mucin release

As shown in Fig. 2, IL-1beta did not affect mucin release at 1,000 U/ml. The amounts of mucin in the spent media of IL-1beta-treated cultures were 100±6%, 96±8% and 95 ±8% for control, 100 U/ml and 1,000 U/ml, respectively. For comparison, both 2×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 2).

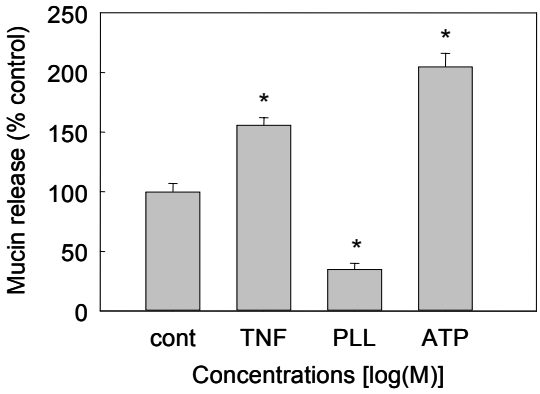

Fig. 1. Effect of TNF-alpha on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 24h in the presence of TNF-alpha. For comparison, both 200μM ATP and 10μM PLL (MW 7,500) were used as positive controls. The amounts of ^{3}H-mucins in the 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 ($p<0.05$).

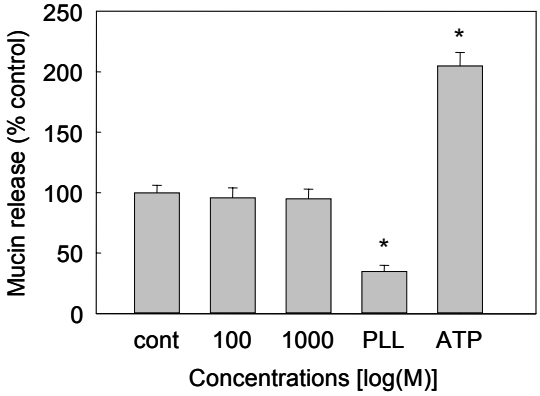

Fig. 2. Effect of IL-1beta on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of IL-1beta. For comparison, both 200μM ATP and 10μM PLL (MW 7,500) were used as positive controls. The amounts 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 ($p<0.05$).

Effect of CTMA on mucin release

As shown in Fig. 3, CTMA increased mucin release, significantly. The amounts of mucin in the spent media of CTMA-treated cultures were 100±5%, 103±9%, 97±12%, 112±10% and 142±7% for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. For comparison, both 2×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 3).

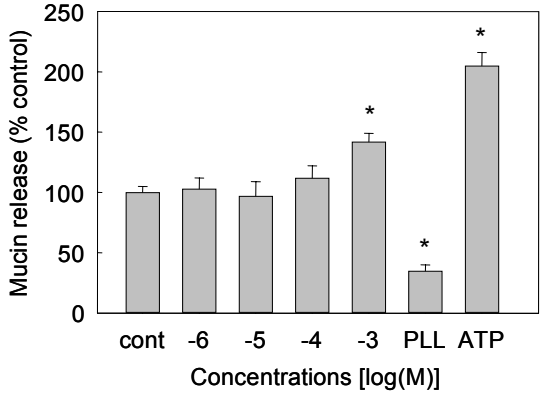

Fig. 3. Effect of CTMA on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of CTMA. For comparison, both 200μM ATP and 10μM PLL (MW 7,500) were used as positive controls. The amounts of ^{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 ($p<0.05$).

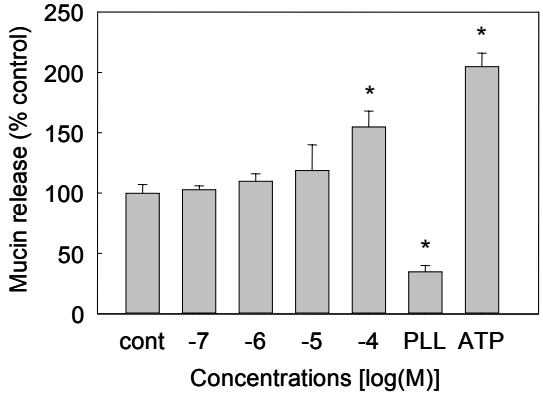

Fig. 4. Effect of LPD on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of LPD. For comparison, both 200μM ATP and 10μM PLL (MW 7,500) were used as positive controls. The amounts of ^{3}H-mucins 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 ($p<0.05$).

Effect of LPD on mucin release

As shown in Fig. 4, LPD also increased mucin release, significantly. The amounts of mucin in the spent media of LPD-treated cultures were 100±7%, 103±3%, 110±6%, 119±21% and 155±13% for control, 10^{-7} M, 10^{-6} M, 10^{-5}M and 10^{-4} M, respectively. For comparison, both 2×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 4).

DISCUSSION

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 HTSE cell culture system, an optimum condition was established to study the pharmacology of airway goblet cell mucin release (Kim et al, 1987; Ko et al, 1999). Therefore, in this study, we used this HTSE cells to elucidate the effects of TNF-alpha, IL-1beta, CTMA and LPD on mucin release from airway goblet cells. There are several reports about the activity of TNF-alpha and IL-1beta on airway epithelial cells: Shao et al. reported that TNF-alpha converting enzyme mediated MUC5AC mucin expression in cultured human airway epithelial cells (Shao et al, 2003)., and IL-1beta and TNF-alpha have been reported to induce MUC5AC overexpression in normal human nasal epithelial cells (Song et al, 2003). Fischer et al. also reported that TNF-alpha increased mucin secretion from guinea pig tracheal epithelial cells (Fischer et al, 1999). IL-1beta was shown to increase MUC5AC mucin production in airway epithelial cells (Kim et al, 2002). On the basis of these reports, we investigated whether TNF-alpha and IL-1beta induce mucin release from cultured hamster tracheal epithelial cells, and whether natural products possibly inhibit stimulation of mucin release by these cytokines. As seen in Fig. 1, TNF-alpha increased mucin release, in confirmation of the aforementioned studies using human or guinea pig airway epithelial cells. However, as seen in Fig. 2, IL-1beta failed to increase mucin release from cultured hamster tracheal epithelial cells. The reason IL-1beta did not affect mucin release in our cell culture system was not investigated, and it should be investigated in future research. There are numerous reports on various biological effects of CTMA and LPD, especially in conjunction with anti-inflammatory effects (Stromaier et al, 1990; Stromaier and Schlag, 1993; Recio et al, 1995; Bernard et al, 2001; Yamashita et al, 2002; Ismali et al, 2002; Chiang et al, 2003; Manez et al, 1997). CTMA was reported to affect airway luminal surface after intratracheal instillation (Stromaier et al, 1990; Stromaier and Schlag, 1993). On the basis of these reports, we tried to test the potential inhibitory effect of CTMA on mucin release from airway goblet cells. However, as seen in Fig. 3, CTMA did not inhibit, but rather increased basal mucin release at highest concentration. This result suggests that CTMA may have a direct stimulatory effect on mucin-secreting cells. LPD showed steroid-like anti-inflammatory actions through anti-PLA_2 activity (Recio et al, 1995; Bernard et al, 2001). LPD, isolated from the root of Anemone raddeana which has been used in traditional oriental medicine for controlling rheumatism and neuralgia, inhibited the generation of superoxide (Yamashita et al, 2002). Triterpenoidal compound such as LPD was shown to have an effect on chronic dermal inflammation (Manez et al, 1997). On the basis of these reports, we tried to test the potential inhibitory effect of LPD on airway basal or stimulated mucin release. As seen in Fig. 4, LPD also did not inhibit, but significantly increased mucin release. In fact, we expected LPD would have inhibitory activity on mucin release (like glucocorticosteroids), based on aforementioned reports and traditional oriental medicine. On the contrary to this expectation, it stimulated mucin release. Taken together, CTMA, LPD and TNF-alpha increased mucin release from cultured hamster tracheal epithelial cells. The underlying mechanisms of action of these agents on mucin release are not clear at present and are under investigation through our ongoing research. It would be of great contribution to find natural products that have specific inhibitory effects on mucin release from the view of both basic and clinical sciences. The result in this study suggest a possibility of CTMA and LPD as mild expectorants for respiratory diseases, although further studies are needed.

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REFERENCES

Bernard P, Scior T, Didier B, Hibert M, Berthon JY. Ethnopharmacology and bioinformatic combination for leads discovery: application to phospholipase A2 inhibitors. *Phytochemistry* 58: 865-874, 2003

Chiang LC, Ng LT, Chiang W, Chang MY, Lin CC. Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of Plantago species. *Planta Med* 69: 600-604, 2003

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

Fischer BM, Rochelle LG, Voynow JA, Akley NJ, Adler KB. TNF-alpha stimulates mucin secretion and cyclic GMP production by guinea pig tracheal epithelial cells in vitro. *Am J Respir Cell Mol Biol* 20: 413-422, 1999

Ismali H, Sosa S, Brkic D, Fkih-Tetouani S, llidrissi A, Touati D, Aqin RP et al. Topical anti-inflammatory activity of extracts and compounds from Thymus broussonettii. *J Pharm Pharmacol* 54: 1137-1140, 2002

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, 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

Kim Y, Kwon E, Park D, Song S, Yoon S, Baek S. IL-1beta induces MUC2 and MUC5AC synthesis through cyclooxygenase-2 in NCI-H292 cells. *Mol Pharmacol* 62: 1112−1118, 2002

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

Manez S, Recio MC, Giner RM, Rios JL. Effect of selected triterpenoids on chronic dermal inflammation. *Eur J Pharmacol* 334: 103−105, 1997

Mutschler E, Derendorf H. Drug actions. Boca Raton, CRC press, Florida., p 410−411, 1995

Newhouse MT, Biennenstock J. Respiratory tract defense mechanism. In: Baum GL, Wolinsky E ed, *Textbook of pulmonary disease*. 3rd ed. Little Brown and Company, Boston, Massachusetts, 1983

Recio MC, Giner RM, Manez S, Gueho J, Julien HR, Hostettmann K, Rios JL. Investigations on the steroidal anti-inflammatory activity of triterpenoids from Diospyros leucomelas. *Planta Med* 61: 9−12, 1995

Research institute of natural products in Seoul National University, Treatise on asian herbal medicines. Haksul-pyunsu-kwan, Seoul, p 4517, 2003

Shao MX, Ueki IF, Nadel JA. TNF-alpha converting enzyme mediated MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 100(20):11618−11623, 2003

Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, Yoon JH. IL-1beta and TNF-alpha induced MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinase-MSK1-CREB activation in human airway epithelial cells. *J Biol Chem* 278(26): 23243−23250, 2003

Stromaier W, Redl H, Schlag G. Studies on the potential role of a semisynthetic surfactant preparation in an experimental aspiration trauma in rabbits. *Exp Lung Res* 16(2): 101−110, 1990

Stromaier W, Schlag G. Experimental aspiration trauma: comparison of steroid treatment versus exogenous natural surfactant. *Exp Lung Res* 19(3): 397−405, 1993

Wasano K, Kim KC, Niles RM, Brody JS. Membrane differentiation markers of airway epithelial secretory cells. *J Histochem Cytochem* 36: 167−17, 1988

Yamashita K, Lu H, Lu J, Chen G, Yokoyama T, Sagara Y, Manabe M et al. Effect of three triterpenoids lupeol, betulin and betulinic acid on the stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. *Clinica Chimica Acta* 325: 91−96, 2002