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Effects of Diclofenac, Acetamonophen, Nimesulide and Acetylsalicylic Acid on Mucin Release from Cultured Hamster Tracheal Surface Epthelial Cells

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Abstract – In this study, we tried to investigate whether diclofenac, acetaminophen, nimesulide, acetylsalicylic acid and tumor necrosis factor-alpha (TNF-alpha) significantly affect mucin release 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 or 24 hr in the presence of each agent to assess the effects on ^{3}H-mucin release. The results were as follows : (1) TNF-alpha significantly increased mucin release from cultured HTSE cells during 24 hr of treatment period; (2) However, diclofenac, acetaminophen, nimesulide and acetylsalicylic acid did not affect mucin release, during 30 min of treatment period. Basically, this finding suggests that non-steroidal antiinflammatory drugs (NSAIDs) might not function as a mucoregulator in various inflammatory respiratory diseases showing mucus hypersecretion, although further studies are needed.

Key words □ airway, mucin, NSAIDs

INTRODUCTION

Hypersecretion of airway mucus 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 drugs that have been clinically used for the management of diverse diseases. Proinflammatory cytokines including TNF-alpha, IL-1beta and IL-6 have been shown to stimulate either mucin release or expression of mucin gene (Fischer *et al.*, 1999, Kim *et al.*, 2002, Shao *et al.*, 2003, Song *et al.*, 2003) while mucous secretory cell hyperplasia and mucus hypersecretion were reported to be inhibited by administration of NSAIDs (Jeffery, 1986, Hauber *et al.*, 2005, Roger *et al.*, 2001). However, to the best of our knowledge, there are no reports about direct effects of NSAIDs e.g. diclofenac, acetaminophen, nimesulide and acetylsalicylic acid on mucin release from airway goblet cells. Thus, we hypothesized that NSAIDs might regulate mucin release by directly acting on airway goblet cells and tried to investigate whether diclofenac, acetaminophen, nimesulide and

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acetylsalicylic acid might affect mucin release, 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 (DMEM) (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 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). TNF-alpha, diclofenac, nimesulide, acetylsalicylic acid, ATP and PLL (average molecular weight 7,500) were prepared and administered in PBS. Acetaminophen was dissolved in ethanol and administered in PBS (final concentration of ethanol was 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°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 TNF-alpha on mucin release

TNF-alpha significantly increased mucin release at 500 U/ml during 24h of treatment period. The amounts of mucin in the spent media of TNF-alpha-treated cultures were 100 ± 6% and 150±8% for control and 500 U/ml, respectively. (Fig. 1).

Effect of diclofenac on mucin release

Diclofenac did not affect mucin release during 30 min of treatment period. The amounts of mucin in the spent media of diclofenac-treated cultures were 100 ± 11%, 94 ± 9%, 109 ± 4 % and 121 ± 6% 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. 2).

Effect of acetaminophen on mucin release

Acetaminophen did not affect mucin release during 30 min of treatment period. The amounts of mucin in the spent media of acetaminophen-treated cultures were 100 ± 6%, 105 ± 5%, 103 ± 6% and 111 ± 10% for control, 10^{-6} M, 10^{-5} M and 10^{-4} M, respectively. For comparison, both 2.0×10^{-4} M ATP and

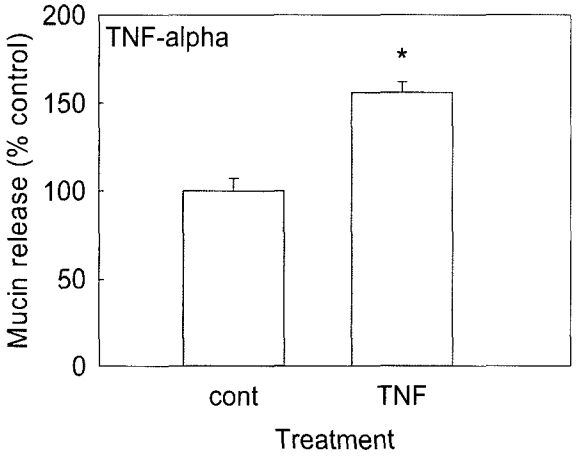

Fig. 1. Effect of TNF-alpha on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 24 hours in the presence of 500 U/ml of TNF-alpha and the amount of ^{3}H-mucins in the spent media was measured as described 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 ($p<0.05$).

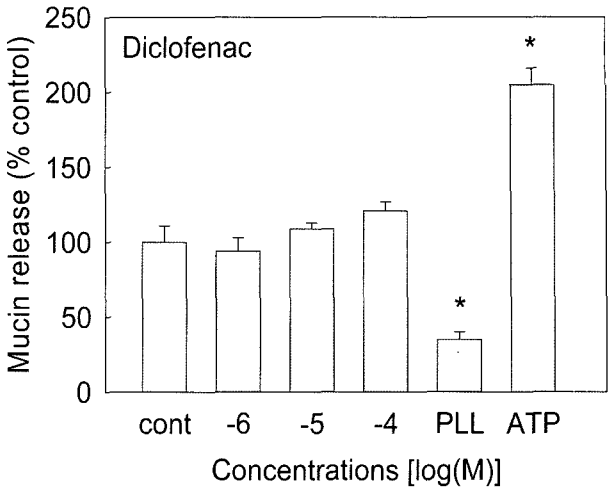

Fig. 2. Effect of diclofenac on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of diclofenac and the amount of ^{3}H-mucins in the spent media was measured 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 ($p< 0.05$).

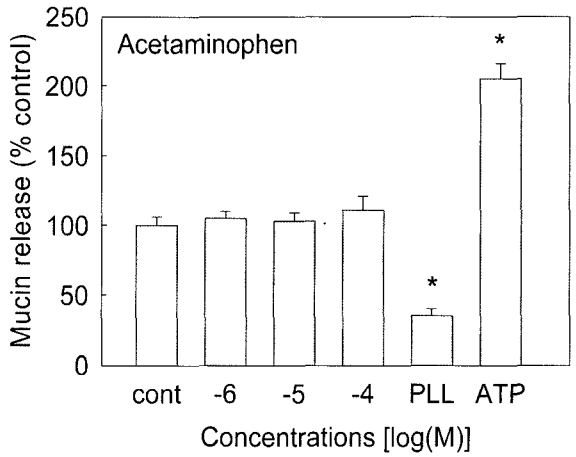

Fig. 3. Effect of actaminophen on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of acetaminophen and the amount of ^{3}H-mucins in the spent media was 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 ($p< 0.05$).

10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 3).

Effect of nimesulide on mucin release

Nimesulide did not affect mucin release during 30 min of treatment period. The amounts of mucin in the spent media of nimesulide-treated cultures were 100 ± 4%, 105 ± 7%, 84 ± 5% and 118 ± 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).

Effect of acetylsalicylic acid on mucin release

Acetylsalicylic acid did not affect mucin release during 30 min of treatment period. The amounts of mucin in the spent media of acetylsalicylic acid-treated cultures were 100 ± 6%, 116 ± 7%, 105 ± 6% and 115 ± 10% 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. 5).

DISCUSSION

As mentioned in introduction, it is of great value to find novel compounds which have the inhibitory or mucoregulative effects on mucin release, from the viewpoint of management of hypersecretion of airway mucus. Therefore, we used a primary Hamster Tracheal Surface Epithelial (HTSE) cells to elucidate the effects of NSAIDs on mucin release from airway goblet cells and tried to compare the activities of NSAIDs 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). Increased levels of TNF-alpha and IL-1beta have been measured in sputum, with further increases during exacerbations (Chung, 2001, Cohn *et al.*, 2002). It was reported that TNF-alpha converting enzyme mediated MUC5AC mucin expression in cultured human airway epithelial cells (Shao *et al.*, 2003) and TNF-alpha increased MUC5AC expression in normal human airway epithelial cells (Song *et al.*, 2003). Also, TNF-alpha increased mucin secretion from guinea pig tracheal epithelial cells (Fischer *et al.*, 1999). On the basis of these reports, we investigated

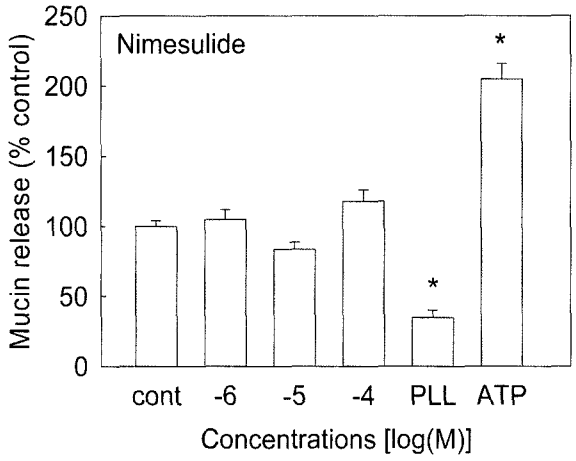

Fig. 4. Effect of nimesulide on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of nimesulide and the amount of ^{3}H-mucins in the spent media was measured 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 ($p < 0.05$).

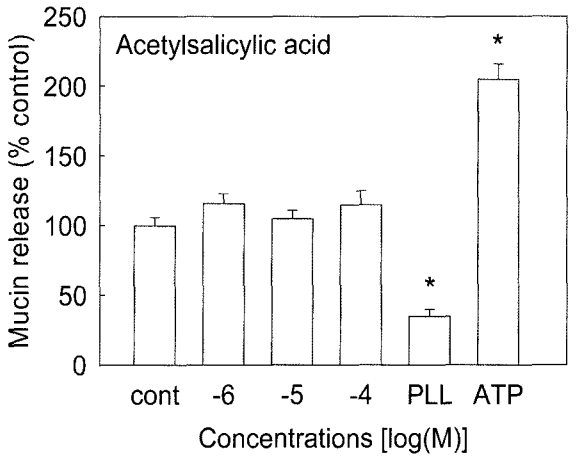

Fig. 5. Effect of acetylsalicylic acid on mucin release from cultured HTSE cells. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 hours and chased for 30 minutes in the presence of varying concentrations of acetylsalicylic acid and the amount of ^{3}H-mucins in the 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 ($p < 0.05$).

whether TNF-alpha affect mucin release from cultured airway goblet cells, by direct action. As shown in Fig. 1, TNF-alpha significantly increased mucin release from cultured HTSE cells, during 24 hr of treatment. If the proinflammatory agents including TNF-alpha can increase release of mucin from airway goblet cells, NSAIDs - the agent that shows anti-inflammatory action - might inhibit or regulate mucin release. The anti-inflammatory activity of NSAIDs is mediated mainly through inhibition of biosynthesis of prostaglandins. NSAIDs blocks COX-1 and COX-2, prostaglandin- producing enzymes (Mutschler *et al.*, 1995). There are reports suggesting that activation of COX-2 by inflammatory stimulations provokes the expression of MUC5AC gene and production of MUC5AC mucin (Kim et al., 2002, Song et al., 2003). If so, the inhibition of COX-2 by NSAIDs can inhibit the synthesis and release (secretion) of airway mucin. However, the direct effect of various NSAIDs on mucin release does not have tried, to the best of our knowledge. Based on these ideas, we investigated the potential effects of several NSAIDs on mucin release. As shown in Fig. 2, 3, 4 and 5, diclofenac, acetaminophen, nimesulide and acetylsalicylic acid did not provoke any change in quantity of release of airway mucin. In fact, we expected NSAIDs would have inhibitory activity on mucin release like glucocorticosteroids, based on aforementioned reports. On the contrary to this expectation, they did not affect mucin release, at all. In summary, NSAIDs could not affect mucin release by directly 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. This result suggests that it is too short for NSAIDs to affect mucin release during just 30 min of treatment period and be further studied through prolonging drug-treatment period. At the same time, effects of NSAIDs on stimulated mucin release by proinflammatory factors should be investigated, through future studies.

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