Effect of Ambroxol on Secretion, Production and Gene Expression of Mucin from Cultured Airway Epithelial Cells

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Abstract
In this study, we investigated whether ambroxol significantly affects secretion, production and gene expression of mucin from cultured airway epithelial cells. Confluent primary rat tracheal surface epithelial (RTSE) cells were pretreated with adenosine triphosphate (ATP) for 5 min and then treated for 30 min with ambroxol to assess the effect on mucin secretion using ELISA. Additionally, confluent NCI-H292 cells were pretreated with ambroxol for 30 min and then stimulated with EGF or PMA for 24 h. The MUC5AC mucin gene expression and mucin protein production were measured by RT-PCR and ELISA. The results were as follows: (1) ambroxol did not significantly affect ATP-induced mucin secretion from cultured RTSE cells; (2) ambroxol inhibited the production of MUC5AC mucin protein induced by EGF and PMA in NCI-H292 cells; (3) ambroxol also inhibited the expression of MUC5AC mucin gene induced by EGF and PMA in NCI-H292 cells. This result suggests that ambroxol can inhibit the production and gene expression of MUC5AC mucin, by directly acting on human airway epithelial cells.

Key Words: Airway, Mucin, Ambroxol

INTRODUCTION

Mucus in the human airway is very important for defense against invading pathogenic microorganisms, chemicals and particles. This defensive action of airway mucus is attributed to the physicochemical properties of mucins. Mucins are multimillion dalton glycoproteins present in the airway mucus and are produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, hypersecretion of airway mucus is one of the major symptoms associated with severe pulmonary diseases including asthma, chronic bronchitis, cystic fibrosis and bronchiectasis (Vojnow and Rubin, 2009). There are two ways to remove excess mucus from the airway; 1) get rid of the mucus by physical means, i.e., aspiration after dilution of mucus, and 2) suppression of secretion and/or production of mucus by pharmacological means. However, clinically, the physical method induces irritation of the airway luminal wall and simulates 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 and Derendorf, 1995). Secretion of airway mucin is generally stimulated by various agents. Whereas glucocorticoids inhibit the hypersecretion of airway mucins (Mutschler and Derendorf, 1995; Lee et al., 2002), they have various limitations in their application to pharmacotherapy of human diseases involving airway mucus hypersecretion. An alternative approach for regulation of airway mucus hypersecretion is to inhibit the excessive mucin secretion and/or production using agents that have already been clinically used for the management of various diseases. According to a number of studies, ambroxol - one of the clinically-used mucolytics and/or expectorants - was reported to demonstrate anti-oxidative and/or anti-inflammatory activity (Stetinova et al., 2004; Beeh et al., 2008). Ambroxol increased the secretion of pulmonary surfactant which is involved in the expectorant action of ambroxol (Miyata et al., 1986). Ambroxol provoked serous and mucous secretion and restored normal production of mucin in patients suffering from chronic bron-
chopneumopathy (Aliperta et al., 1986). However, to the best of our knowledge, there are no reports concerning the potential effect of ambroxol on mucin production, gene expression or secretion by airway epithelial cells. Therefore, in this study, we investigated the effect of ambroxol on mucin production and gene expression in NCI-H292 cells, a human pulmonary mucoepidermoid cell line, and also ambroxol’s effect on mucin secretion as stimulated by adenosine triphosphate (ATP), from primary cultured rat tracheal surface epithelial (RTSE) cells.

MATERIALS AND 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 rat tracheal surface epithelial (RTSE) cell culture

Animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals as regulated by Chongnam National University. Tracheas were obtained from male Sprague Dawley (SD) rats, 5 weeks of age (Hartan Sprague Dawley, Indiana, U.S.A.). RTSE cells were harvested and cultured on a thick collagen gel substratum as previously reported (Wasano et al., 1988; Ko et al., 1999). Briefly, animals were euthanized in a CO₂ chamber and the tracheas were exposed under aseptic conditions. The tracheas were cannulated 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-BRL, U.S.A.) and incubated at 4°C for 16 h. The luminal contents were flushed, and cells were washed twice with Ca**, Mg**-free Minimum Essential Medium containing 10% fetal bovine serum by centrifugation 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-BRL), Streptomycin (100 μg/ml, GIBCO-BRL) and Gentamicin (50 μg/ml) ("complete" medium). At this stage, most of the cells were in small aggregates and were plated at a density of 10⁴ cells/cm² into tissue culture dishes containing a thick collagen gel (0.15 ml/cm²) using collagen type I (Regenmed, Seoul, Korea). Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ and culture medium was changed on days 1, 3, 5 and 7.

Treatment of RTSE cells with ambroxol and quantitation of mucins by ELISA

Confluent RTSE cells were prepared according to the aforementioned method. On culture day 6, spent medium from each culture well was removed and 200 μl of fresh complete medium was added to each well. Following a 24 h incubation, the spent media (designated as the pretreatment (PT) sample) was collected and the cultures were washed twice with Dulbecco’s phosphate-buffered saline (PBS) without Ca** and Mg** before chasing for 30 min in PBS containing varying concentrations of ambroxol (the treatment (T) sample). Ambroxol hydrochloride and ATP were dissolved in double-distilled, deionized water (DDW) and administered in PBS. The final pH values of these solutions were between 7.0 and 7.4. PBS solution in this range did not affect mucin secretion by RTSE cells. Floating cells and cell debris were removed by centrifugation of samples at 12,000×g for 5 min. The samples were stored at −70°C until assayed for their mucin contents. The amount of mucins in each sample was measured by using enzyme-linked immunosorbent assay (ELISA). PT and T samples were diluted with PBS at 1:10 ratio and 100 μl of each sample was incubated at room temperature in a 96-well plate for 2 h. Wells were washed three times with PBS and blocked with 2% BSA for 1 h at room temperature. Wells were again washed three times with PBS and then incubated with 100 μl of 17Q2 (Covance, U.S.A.), a mouse monoclonal anti-total mucin antibody, which was diluted with PBS containing 0.05% Tween 20 (1:1,000), and dispensed into each well. After 1 h, wells were washed three times with PBS, and 100 μl of horse-radish peroxidase-goat anti-mouse IgG conjugate (1:3,000) was dispensed into each well. After 1 h, wells were washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1N H₂SO₄. Absorbance was read at 450 nm. The effect of ambroxol on mucin secretion was measured as follows: the amount of mucin secreted during the pretreatment period and the ratio was expressed as a secretory index. Means of secretory indices from each group were compared and the differences were assessed using statistics.

NCI-H292 cell culture

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/ml), streptomycin (100 μg/ml) and HEPES (25 mM) at 37°C in a humidified, 5% CO₂/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and re-cultured in RPMI 1,640 containing 0.2% fetal bovine serum for 24 h.

Treatment of cells with ambroxol

Following 24 h of serum deprivation, cells were pretreated with ambroxol (1, 10 and 100 μM) for 30 min and then treated with EGF (25 ng/ml) or PMA (10 ng/ml) for 24 h in serum-free RPMI 1640. After 24 h, cells were lysed with buffer solution containing 20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA and protease inhibitor cocktail (Roche Diagnostics, IN, U.S.A.) and collected to measure the production of MUC5AC protein (in 24-well culture plate). The total RNA was extracted for measuring the expression of MUC5AC gene (in 6-well culture plate) by using RT-PCR.

MUC5AC mucin analysis using ELISA

MUC5AC protein was measured by using ELISA. Cell lysates were prepared with PBS at 1:10 dilution, and 100 μl of each sample was incubated at 42°C in a 96-well plate, until dry. Plates were washed three times with PBS and blocked with 2% BSA (fraction V) for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μl of 45M1, a mouse monoclonal MUC5AC antibody
Total RNA isolation and RT-PCR

Total RNA was isolated using an Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc. Kyung-gi-do, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer’s instructions. 2 μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 50 μl (RT reaction). 2 μl of RT reaction product was PCR amplified in a 25 μl volume by using Thermoprime Plus DNA Polymerase (ABgene, Rochester, NY, U.S.A.). Primers for MUC5AC were: (forward) 5’-TGA TCA TCC AGC AGG GCT-3’ and (reverse) 5’-CCG AGC TCA GAG GAC ATA TGG G-3’. The size of expected fragment amplified by PCR was 458 bp. As quantitative controls, primers for RIG/S15 RNA, which encodes a small ribosomal subunit protein (a housekeeping gene that was constitutively expressed), were used. Primers for RIG/S15 were: (forward) 5’-TTC CGC AAG TTC ACC TAC C-3’ and (reverse) 5’-CGG GCC GGC CAT GCT TTA CG-3’. The size of expected fragment amplified by PCR was 361 bp. The PCR mixture was denatured at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. Following PCR, 5 μl of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

Statistics

Means of individual groups were converted to percent control and expressed as mean ± S.E.M. The difference between groups was assessed using one-way ANOVA and Student’s t-test for unpaired samples. p<0.05 was considered as significantly different.

RESULTS

Effect of ambroxol on ATP-induced mucin secretion from cultured RTSE cells

As shown in Fig. 1, ambroxol did not significantly affect ATP-induced mucin secretion from RTSE cells. The amounts of mucin in the spent media of ambroxol-treated cultures were 100 ± 5%, 180 ± 10%, 195 ± 8%, 186 ± 7% and 202 ± 15% for control, 2×10⁻⁴ M of ATP alone, ATP plus ambroxol 10⁻⁶ M, ATP plus ambroxol 10⁻⁵ M and ATP plus ambroxol 10⁻⁴ M, respectively (Fig. 1).

Effect of ambroxol on EGF-induced MUC5AC production

As shown in Fig. 2, ambroxol significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells at the concentration of 10⁻⁴ M. The amounts of mucin in the cells of ambroxol-treated cultures were 100 ± 1%, 200 ± 5%, 230 ± 7%, 243 ± 14% and 154 ± 6% for control, 25 ng/ml of EGF alone, EGF plus ambroxol 10⁻⁶ M, EGF plus ambroxol 10⁻⁵ M and EGF plus ambroxol 10⁻⁴ M, respectively (Fig. 2).

Effect of ambroxol on PMA-induced MUC5AC production

As shown in Fig. 3, ambroxol significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells at the

Fig. 1. Effect of ambroxol on ATP-induced mucin secretion from cultured RTSE cells. Confluent RTSE cells were chased for 30 min in the presence of varying concentrations of ambroxol. The amounts of mucin in spent media were measured as described in Materials and Methods. Each bar represents a mean ± S.E.M. of 4 culture wells in comparison with that of control set at 100%. *Significantly different from control (p<0.05). cont: control, A: ATP 200, Amb: ambroxol, concentration unit is μM.

Fig. 2. Effect of ambroxol on EGF-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of ambroxol for 30 min and then stimulated with EGF (25 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of control set at 100%. *Significantly different from control (p<0.05). cont: control, A: ambroxol, concentration unit is μM.

Fig. 3. Effect of ambroxol on PMA-induced MUC5AC production from NCI-H292 cells at the
Effects of ambroxol on EGF-induced and PMA-induced mucins involving hyperproduction and hypersecretion of airway mucin. Ambroxol (Miyata et al., 1986) and this increase is involved in the expectorant action of ambroxol. Also, ambroxol increased the secretion of pulmonary surfactant and this is involved in the expectorant action of ambroxol (Miyata et al., 1986). Ambroxol provoked serous and mucous secretion and restored normal production of mucin in patients suffering from chronic bronchopneumopathy (Aliperta et al., 1986). Development of a potent pharmacological tool for regulating production and/or secretion of mucus could play a significant role in the efficient control of severe respiratory diseases involving hyperproduction and hypersecretion of airway mucins. Mucins are macromolecular glycoproteins present in the airway mucus and have peptide backbones and carbohydrate branches (Voynow and Rubin, 2009). To date, 20 MUC genes coding peptide backbone of human mucins have been reported and, among them, MUC5AC is highly expressed in airway goblet cells (Rogers and Barnes, 2006; Yuan-Chen Wu et al., 2007). Also, epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA) were reported to regulate MUC5AC mucin gene expression in the lung (Takeyama et al., 1999; Hewson et al., 2004). MUC5AC mRNA expression was increased after ligand binding to the EGF receptor and activation of the MAPK (mitogen-activated protein kinase) cascade (Takeyama et al., 1999; Takeyama et al., 2000). Phorbol 12-myristate 13-acetate (PMA) is an inflammatory stimulant affecting gene transcription, cell growth and differentiation and induces MUC5AC gene expression in NCI-H292 cells (Park et al., 2002; Hewson et al., 2004). Based on these reports, we tried to investigate whether ambroxol affects EGF- or PMA-induced MUC5AC mucin production and gene expression in NCI-H292 cells, a human pulmonary mucocoeiroploderm cell line, which is frequently used for studying intracellular signaling pathways involved in airway mucin production and gene expression (Li et al., 1997; Takeyama et al., 1999; Shao et al., 2003). As shown in results, ambroxol inhibited the production and gene expression of MUC5AC mucin induced by EGF or PMA. This is the first report concerning the action of ambroxol on airway MUC5AC mucin production and gene expression induced by growth factor or phorbol ester. This finding could result in a better understanding of the molecular action mechanism of ambroxol. Although the underlying mechanism of action of ambroxol on MUC5AC mucin production and gene expression is not presently clear, we are trying to examine whether ambroxol acts as a potential regulator of the MAPK cascade after ligand binding to the EGF receptor in mucin-producing NCI-H292 cells. Additionally, our group previously examined whether ambroxol affects mucin release from the airway and found ambroxol failed to show any activity on basal mucin release from airway goblet cells (Heo et al., 2005).

**Fig. 3.** Effect of ambroxol on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of ambroxol for 30 min and then stimulated with PMA (10 ng/ml) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± S.E.M. of 3-4 culture wells in comparison with that of control set at 100%. *Significantly different from control (p<0.05).

**Fig. 4.** Effects of ambroxol on EGF-induced and PMA-induced MUC5AC gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with ambroxol (100 μM) for 30 min and then stimulated with PMA (10 ng/ml) or EGF (25 ng/ml) for 24 h. MUC5AC gene expression was measured by RT-PCR. cont: control, Amb: ambroxol.
Thus, in the present study, we examined the effect of ambroxol on ATP-induced mucin secretion from cultured RTSE cells, on the basis of the following reports. Nucleotides present in the airway surface liquid controlled mucociliary clearance. This phenomenon was mediated by two purinergic receptor subtypes - P2Y2 receptor and A2 adenosine receptor. Stimulation of P2Y2 receptor by an agonist like ATP was reported to increase airway mucin secretion (Kim et al., 1997; Lazarowski and Boucher, 2009). When airway inflammation occurs, local extracellular ATP concentrations from ruptured cells in the airway can reach high levels, because intracellular ATP concentrations are greater than a few millimolar concentration (Gordon, 1986). However, as shown in “Results”, ambroxol did not affect ATP-induced mucin secretion from cultured RTSE cells. This result partially suggests that ambroxol can not affect mucin secretion induced by ATP - a phenomenon reflecting hypersecretion of mucus from airway epithelial cells during inflammation.

In summary, ambroxol inhibited the production and gene expression of MUC5AC mucin without affecting ATP-induced secretion of mucin, from cultured airway epithelial cells. Also, future research should explain why ambroxol can not affect basal and ATP-stimulated mucin secretion (release) despite the fact that it can inhibit production and gene expression of airway mucin.

ACKNOWLEDGMENTS

This study was partly supported by 2009 grant of the Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

REFERENCES


