<table>
<thead>
<tr>
<th>Title</th>
<th>N-acetyl-L-cysteine prevents bacteria-induced hypersecretion of gel-forming mucin from human bronchiolar epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>小泉 ちあき</td>
</tr>
<tr>
<td>Journal</td>
<td>() -</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10130/3621">http://hdl.handle.net/10130/3621</a></td>
</tr>
</tbody>
</table>
N-acetyl-L-cysteine prevents bacteria-induced hypersecretion of gel-forming mucin from human bronchiolar epithelial cells

Chiaki Koizumi*

Department of Removable Prosthodontics and Gerodontology, Tokyo Dental College, Tokyo, Japan

Short title: Transition of bronchiolar epithelial mucus by uptake of NAC

*Address correspondence to:

Chiaki Koizumi, DDS

Department of Removable Prosthodontics & Gerodontology, Tokyo Dental College

2-9-18 Misaki-chou, Chiyoda-ku, Tokyo 101-0061, Japan

Tel: +81 (0)3-6380-9201 Fax: +81 (0)3-6380-9606

E-mail: koizumichiaki@tdc.ac.jp

Keywords:

Antioxidant, arachidonic cascade, aspiration pneumonia, sputum, oxidative stress
Abstract

Objectives: Accumulation of sputum on bronchi via inflammation and oxidative stress in branchial epithelium is associated with morbidity and mortality of chronic inflammatory lung diseases. N-acetyl-L-cysteine (NAC) enhances oxidative stress resistance by cellular uptake and might serve as the preventative agent. The purpose of this in vitro study was to evaluate the effect of cellular uptake of NAC on mucin (MUC) production by human bronchiolar epithelial cells in association with the cellular redox status and proinflammatory cytokine production under co-culture conditions with a pathogen.

Material and Methods: Human bronchiolar epithelial cells preincubated for 3 h with or without 20 mM NAC were cultured for 8 h in bronchiolar epithelial cell growth medium with or without Streptococcus pneumoniae. The attached viable cell number, MUC production, intracellular reactive oxygen species (ROS) level, cellular glutathione level, proinflammatory cytokine and prostaglandin E2 release, and gene expressions of MUCs, cyclooxygenase-2 (COX2) and proinflammatory cytokines were evaluated after 1 day of culture.

Results: Markedly increased intracellular ROS levels and a reduced amount of cellular glutathione were observed in bacterial co-cultures. However, such cellular redox disturbance was not observed in cultures pretreated with NAC under bacterial exposure. Bacterial co-culture increased the release of proinflammatory cytokines such as interleukin-1β, -6, and -8, and prostaglandin E2 from epithelial cells, and enhanced gene expression of COX2 and proinflammatory cytokines, which was prevented
by pretreatment with NAC. Furthermore, NAC pretreatment prevented the increase in non-specific MUC in cultures after bacterial exposure. Moreover, bacterial co-culture enhanced the gene expression and quantitative evaluation of immunostaining of MUC5AC, which was inhibited by pretreatment with NAC. On the other hand, NAC pretreatment enhanced gene expression and quantitative evaluation of immunostaining of MUC4.

**Conclusion:** Cellular uptake of NAC prevents an increase in gel-forming MUC mediated by bacteria-induced proinflammatory cytokine production and oxidative stress in human bronchiolar epithelial cells.
Introduction

Sputum is mucus containing food debris, exfoliated tissue, and/or pathological bacteria originating from oropharyngeal region [1, 2]. Mucus is mainly produced by bronchial epithelium and submucosal glands via production of inflammatory mediators in response to bacteria or chemical substances. Mucus serves as a slimy defensive barrier to trap and transport these foreign materials out of the airway [3]. However, hypersecretion and increased viscoelasticity of mucus beyond ciliary clearance cause bronchiolar obstruction and bacterial infection. In addition, *Streptococcus pneumoniae*, one of representative oral resident bacterial species, is associated with increases of morbidity and mortality of community-acquired pneumonia [4] and has a concern about emergence of drug-resistant strains to various types of antibiotics [5]. In fact, accumulation of sputum contributes to morbidity and mortality of chronic inflammatory lung diseases such as asthma, cystic fibrosis, chronic obstructive pulmonary disease and aspiration pneumonia [3]. Development of an approach to reduce accumulation of sputum might be effective to prevent chronic inflammatory lung diseases [6].

Mucus consists of mucin (MUC) molecules that are high molecular weight glycoproteins. There are two types of MUCs: secretory and membranous. Secretory MUCs, referred to as gel-forming MUCs, are the major components of mucus [7]. MUC5AC is one of the major gel-forming MUCs and increases mucus viscoelasticity [8, 9]. Increased expression of MUC5AC is associated with chronic inflammatory lung diseases [9, 10]. Whereas membranous MUCs, such as MUC1 and 4, are
membrane-tethered glycoproteins that protect cells from pathogenic invasion by acting as decoys shed from the cell surface in response to bacterial adherence [11, 12]. This function suggests that membranous MUCs can compensate for gel-forming MUCs to prevent bacterial infection [13]. Expression of both types of MUCs can be regulated by the cellular redox status. For example, bronchiolar epithelial cells increase MUC5AC production in response to oxidative stress by bacterial adherence and colonization [14]. The MUC4 gene in a non-cancerous human breast epithelial cell line is affected by oxidative stress during nicotine or hydrogen peroxide exposure [15].

N-acetyl-L-cysteine (NAC) is an antioxidant amino acid derivative and membrane-permeable thiol compound with a small molecular weight (163.19). NAC protects cells from oxidative stress by directly scavenging reactive oxygen species (ROS) and enhancing the cellular antioxidant capability via supplementation of the major cellular antioxidant molecule, glutathione [16]. Previous studies have demonstrated the anti-inflammation and anti-infection capabilities of NAC in osteoblasts or gingival fibroblasts. Bone substitutes cause proinflammatory cytokine productions in osteoblasts together with oxidative stress, which are prevented by pre-addition of NAC to the bone substitute material [17]. Loss of viability and cellular dysfunction by infection with *Staphylococcus aureus* or *Streptococcus pyogenes* have been found to be prevented in gingival fibroblasts and osteoblasts on a collagenous scaffold containing NAC in a bacterial cell co-culture model [18]. Invasion of bacteria into cells is completely prevented on a scaffold containing NAC in contrast to abundant bacteria
within the cytoplasm on material without NAC [18]. Such anti-inflammation and anti-infection capabilities of NAC involve reinforcement of the cellular antioxidant capability to resist oxidative stress caused by bacteria or toxins. Moreover, NAC may improve the cellular defensive system to prevent bacterial invasion into cells. Therefore, it has been hypothesized that cellular uptake of NAC prevents mucus hypersecretion in bronchiolar epithelium by inhibition of infection, even in the presence of bacteria. Proving this hypothesis would provide important information for the development of external medications for lower airway to prevent chronic inflammatory lung diseases.

The purpose of this *in vitro* study was to evaluate the effect of cellular uptake of NAC on the production of MUCs by human bronchiolar epithelial cells in association with the cellular redox status and proinflammatory cytokine production under a co-culture condition with a pathogen.

**Materials and methods**

**NAC preparation**

A 1 mol/l NAC stock solution (pH 7.2) was prepared by dissolving NAC powder (Sigma-Aldrich Japan, Tokyo, Japan) in HEPES buffer.

**Prior culture of epithelial cells and bacteria**
Primary human bronchiolar epithelial cells (HBECs) were purchased (Lonza, Walkersville, MD, USA). The cell cluster was obtained from the distal portion of normal human lung tissue in the 1mm bronchiale area (2^6 to 2^23 branches), the epithelium composition of which was reported as 70% of ciliated cells, 7% of secretory cells (Clara or goblet cells), 15% of basal cells and 8% of undifferentiated cells and the others [19]. The cells were cultured in 40-mm polystyrene dishes (Corning Inc., Tewksbury, MA, USA) in small airway epithelial cell growth medium (SAGM, Lonza) containing the ready-to-use supplement. After the first passage, the cells were seeded in a 24-well culture grade polystyrene plate (Corning Inc.) with 0.5 ml SAGM at 5×10^4 cells/cm^2 and incubated at 37°C in a humidified incubator with 5% CO₂.

As a model bacterium, *Streptococcus pneumoniae* GTC261 was employed. Bacteria were grown in a tightly capped 15-ml sterile plastic tube with 3 ml trypticase soy broth (Becton Dickinson, MD) statically at 37°C overnight (16–18 h) before use in co-culture experiments with epithelial cells.

*NAC pretreatment and bacterial co-culture*

At 12 h after seeding, the medium of the epithelial cell cultures was changed to 0.5 ml SAGM with or without 20 mM NAC. The cells were incubated for 3 h at 37°C in a humidified incubator with 5% CO₂. Then, the culture medium was changed to small airway epithelial cell basal medium (SABM, Lonza) with or without 10 μl of a bacterial suspension containing 2×10^6 cells for a final
concentration of $1\times10^6$ bacterial cells/ml. The multiplicity of infection (MOI) in the co-culture was 2.

The epithelial cell cultures were divided into four conditions: untreated or NAC-pretreated HBECs with or without bacterial co-culture. The cultures were incubated for 8 h at 37°C in a humidified incubator with 5% CO$_2$ and then applied to the following analyses.

**Measurement of viable attached cells**

The number of attached cells was evaluated by cell counting. After discarding the supernatant of the cultures, the cultures were gently rinsed with D-PBS twice and then trypsinized with 0.25% trypsin/1 mM EDTA-4Na. After centrifugation, the cells were counted using a hemocytometer to evaluate cellular viability. In other culture, the cells were stained with calcein-AM (Dojindo Molecular Technologies, Rockville, MD). Calcein-AM is selectively taken up by viable animal cells, but not bacterial cells, and hydrolyzed to fluorescent calcein by intracellular esterase. After discarding the supernatant from the cultures, the cells were gently rinsed with D-PBS twice. Then, the cells were incubated in a calcein-AM solution for 30 min at 37°C. After removing the solution, the cells were gently rinsed with D-PBS again. The fluorescence intensity of calcein was measured using a fluorescence plate reader at 535 nm (excitation at 485 nm) against D-PBS as the blank.

**Cellular ROS detection**

The amount of intracellular ROS generation was quantified by fluorometry with
5-(and-6)-carboxy-20,70-dichlorofluorescein diacetate (carboxy-DCF-DA) (Invitrogen, MD, USA) that is absorbed intracellularly and oxidized into fluorescent DCF-DA by intracellular ROS. The staining process was the same as described for calcein, except for usage of a carboxy-DCF-DA solution. The DCF-DA fluorescence intensity was measured using a fluorescence plate reader at 520 nm (excitation at 488 nm) against D-PBS as the blank.

**Cellular GSH detection**

The amount of total cellular glutathione was determined by measuring the concentration of 2-nitro-5-thiobenzoic acid resulting from a chromogenic reaction between DTNB and reduced glutathione in a glutathione recycling system. Oxidized glutathione (GSSG) was detected by masking reduced glutathione with a masking reagent. The cells were washed with D-PBS twice and then lysed in 200 µl of a 0.1 M HCl solution by freeze/thawing. The lysate was then subjected to total glutathione and GSSG detection. The absorbance at 415 nm was measured using a plate reader. The intracellular concentrations were calculated based on a parallel glutathione standard curve. The amount of reduced glutathione (GSH) was calculated by subtracting the amount of GSSG from the amount of total glutathione in the same lysate.

**Alcian blue colorimetry**

The amount of nonspecific MUC production was evaluated by Alcian blue colorimetry. The cells
were fixed with 4% formaldehyde. The cultures were washed with ddH₂O and stained with Alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Tokyo) for 2 h. After staining, the cultures were washed with 3% acetic acid aqueous solution to remove all nonbound dye. Afterwards, 300 µl of one-to-one mixture of 5 mol/L sodium hydrate and 100% ethanol was added to dissolve the staining using a microplate shaker for 30 min at room temperature. Then, color intensity was measured in terms of absorbance at 615 nm using an ELISA reader.

**Cellular proinflammatory cytokine detection**

The amounts of proinflammatory cytokines, such as interleukin (IL)-1β, -6 and -8, in HBECs were quantified using the multiplex cytokine immunoassay system (Veritas, Tokyo, Japan). The cells were washed with D-PBS twice and lysed in 200 µl D-PBS by freeze/thawing. The lysate was incubated with beads coated with antibodies corresponding to the various cytokines according to the manufacturer’s instructions. The bead size and fluorescence were measured on the Luminex 200 system (Luminex Japan, Tokyo, Japan). Data were analyzed using Master Plex® QT (Hitachi Solutions, Tokyo, Japan).

**Production of prostaglandin E2**

The amount of prostaglandin E2 (PGE2) in HBECs was measured by an immunochemical assay (Prostaglandin E2 EIA Kit; Cayman Chemical Company, MI, USA). This assay is based on
competition between PGE2 in a sample and a PGE2-acetylcholineesterase (AChE) conjugate (PGE2 tracer) for the limited amount of an anti-PGE2 monoclonal antibody. The amount of the PGE2 tracer is constant while the concentration of PGE2 varies. The amount of tracer that binds to the anti-PGE2 monoclonal antibody will be inversely proportional to the concentration of PGE2 in the sample.

Cells were washed with D-PBS twice and lysed in 200 µl D-PBS by freeze/thawing. The lysate was then subjected to PGE2 detection. The absorbance at 415 nm was measured using a plate reader. The intracellular concentrations of PGE2 were calculated based a parallel PGE2 standard curve.

To calculate levels or amounts of per unit cell, the data from ROS, GSH, GSSG, Alcian blue, proinflammatory cytokine, and PGE2 detections were divided by the attached cell number in the corresponding replicate culture, which was measured with a hemocytometer. Culture experiments were performed four times for each assay (n=4).

RNA isolation and reverse transcriptase-polymerase chain reaction

Gene expression of HBECs was analyzed by a reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from cells was extracted using TRIzol (Invitrogen, CA, USA) and an RNeasy purification column (Qiagen, Valencia, CA, USA). After DNAse I treatment, reverse transcription of 0.5 µg total RNA was performed using MMLV reverse transcriptase (Clontech, Carlsbad, CA, USA) in the presence of an oligo(dT) primer (Clontech). PCR was performed using EX Taq DNA
polymerase (Takara Bio Inc., Shiga, Japan) to detect IL-6 and -8, MUC5AC and 4, and cyclooxygenase (COX)-2 mRNA. MUC5AC and 4 are representative gel-forming and membrane MUC molecules, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as a housekeeping gene. The primers (synthesized by Sigma-Aldrich) are listed in Table 1. Preliminary PCRs were performed to determine the annealing temperature and optimal cycle number to yield a linear range of PCR amplification for each primer set. PCR products were visualized on 1.5% agarose gels by ethidium bromide staining. Band intensity was detected and quantified under UV light and normalized with reference to GAPDH mRNA.

Immunochemical analysis of MUC5AC and 4

Cells were fixed with 4% formaldehyde for 10 min and then incubated in 0.1% PBS-Tween containing 1% bovine serum albumin, 10% normal goat serum, and 0.3 M glycine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 h to permeabilize the cells and block non-specific protein-protein interactions. The cells were then incubated with anti-MUC5AC or 4 antibodies (Abcam Plc, Cambridge, UK) for 30 min at 22°C. The secondary antibody, DyLight® 488 goat anti-mouse IgG (green) (Abcam), was applied for 30 min at 22°C. 4’-6-diamidino-2-phenylindole (DAPI) (blue) (Vector Laboratories, Inc., CA, USA) was present in the mounting agent to counterstain the cells. Cellular MUC expressions were observed under a fluorescence photomicroscope (Axiophoto2, Carl Zeiss, Co., Ltd. Jena, Germany). To evaluate cellular MUC
expression, three difference fields of cells with typical morphological features were randomly selected for analysis. Expression of MUC5AC and 4 was analyzed by measuring the positive area and the number of cellular nuclei in the selected field using image analysis software (ImageJ; National Institutes of Health, MD, USA). The MUC-positive area per unit nucleus was set as a representative value for each group. Five independent cultures of different cell batches were evaluated in each group.

Statistical analysis

All repeated data were analyzed by Bonferroni multiple comparisons after one-way analysis of variance to determine the differences between each culture condition using statistical software (SPSS standard version 16; International Business Machines Corporation, NY, USA). Statistical significance was set at p < 0.05.

Results

Effects of pretreatment with NAC on HBECs death caused by bacteria

Calcein-based quantification showed that attached cells in HBECs cultures with bacterial co-incubation were half of those in cultures without bacterial co-incubation (Figure 1A) (p < 0.05). On the other hand, the value in cultures pretreated with NAC was higher than those in untreated cultures with bacterial co-incubation, but no significant difference was found without bacterial
co-incubation. The number of attached cells measured with hematocytometer in untreated cultures with bacterial co-incubation was one third of that in cultures without bacterial co-incubation (Figure 1B), which was increased to two thirds by pretreatment with NAC.

**Effects of pretreatment with NAC on the redox status in HBECs in the presence of bacteria**

Compared with cells without bacterial co-incubation, intracellular ROS levels in untreated cells were markedly increased by bacterial co-incubation (Figure 2A). Pretreatment with NAC prevented the increase in ROS levels, even with bacterial co-incubation. The concentration of GSH per unit cell was increased by pretreatment with NAC, regardless of bacterial co-incubation (Figure 2B). Bacterial co-incubation did not change the concentration of GSH per unit cell (Figure 2B) but markedly increased the concentration of GSSG per unit cell in untreated cells (Figure 2C). Regardless of bacterial co-incubation, cells pretreated with NAC did not show a change in the concentration of GSSG per unit cell compared with untreated cells without bacterial co-incubation.

**Effects of pretreatment with NAC on the proinflammatory cytokine production of HBECs to bacteria**

Bacterial co-incubation increased the production of proinflammatory cytokines including IL-1β, -6 and -8 by more than 2-fold in untreated cells (Figure 3A). The bacteria-induced proinflammatory cytokine production was reduced by pretreatment with NAC. In particular, IL-1β per unit cell under bacterial co-incubation was reduced by pretreatment with NAC to the level in cells without bacterial
co-incubation. In addition, PGE2 synthesis was increased by 1.5-fold in untreated cells with bacterial co-incubation compared with that in cells without bacterial co-incubation (Figure 3B). Therefore, pretreatment with NAC prevented the bacteria-induced increase of PGE2 synthesis in the cells.

Effects of NAC on bacteria-induced mucus production in HBECs

Alcian blue colorimetric analysis showed that non-specific MUC production was increased by 2.5-fold in untreated cell with bacterial co-incubation compared with that in cells without bacterial co-incubation (Figure 3C). However, non-specific MUC production in cells pretreated with NAC was increased by 30% even under bacterial co-incubation compared with that in cells without bacterial co-incubation.

Effects of NAC on gene expression of proinflammatory cytokines and MUCs in HBECs

The gene expression of IL-6 and -8 was up-regulated in cells with bacterial co-incubation compared with that in untreated cells without bacterial co-incubation (Figure 3D). There was lower gene expression of IL-6 and -8 in cells pretreated with NAC, regardless of bacterial co-incubation, compared with that in untreated cells with bacterial co-incubation. COX2 gene expression was steady under all of the culture conditions, but its expression was highest in untreated cells with bacterial co-incubation (Figure 3D). MUC5AC gene expression was up-regulated in untreated cells with bacterial co-incubation, whereas its expression in cells pretreated with NAC regardless of
bacterial co-incubation was similar to that in untreated cells without bacterial co-incubation (Figure 3D). In contrast, MUC4 gene expression was up-regulated by pretreatment with NAC, regardless of bacterial co-incubation, in contrast to that in untreated cells with or without the bacterial co-incubation.

**Effects of NAC on expression of MUC5AC and 4 in HBECs**

Immunofluorescence staining of MUC5AC demonstrated strong expression in untreated cells with bacterial co-incubation only (Figure 4, upper left images). However, regardless of bacterial co-incubation, MUC5AC expression in cells pretreated with NAC was similar to that in untreated cells without bacterial co-incubation. In contrast, expression of MUC4 was higher in cells pretreated with NAC, regardless of bacterial co-incubation (Figure 4, upper right images). Untreated cells also expressed MUC4 to some extent, although it appeared to be unrelated to bacterial co-incubation. MUC5AC expression per unit nucleus was 3-fold higher in untreated cells with bacterial co-incubation than in cells without bacterial co-incubation (Figure 4B). The expression of MUC5AC per unit nucleus in NAC-pretreated cells with or without bacterial co-incubation was equal or less than that in untreated cells without bacterial co-incubation. MUC4 expression per unit nucleus was more than 5-fold higher in NAC-pretreated cells than in the untreated cells, regardless of bacterial co-incubation (Figure 4C).
Discussion

This study employed primary cell cluster obtained from human healthy bronchiolar epithelium. The gross-sectional area of lower airway increases toward alveoli. In addition, the progression of chronic inflammatory lung disease is associated with disease state of bronchiole [20, 21]. Major cell types of bronchiolar epithelium (2⁰ to 2¹⁸ branches) was similar to that of bronchial epithelium (2⁰ to 2⁵ branches) composition of which is ciliated, undifferentiated columnar, secretory (goblet), and basal cells [22]. What set bronchiolar epithelium apart from bronchial epithelium histologically is relatively more ciliated cells and shift from goblet cells to the Clara cells. Clara cells are non-ciliated secretory cells and secrete Clara cell secretory protein, surfactant apoproteins A, B, and D, proteases, antimicrobial peptides, cytokines and chemokines, and MUCs [23], hence, have important rule in prevention of lung disease. Moreover, NAC can be applied as an inhalation aerosol [24] which is able to reach bronchiole and alveoli. All of those things were the reasons why the present study employed bronchiolar epithelium.

The ratio of bacteria to epithelial cells (MOI) was 2 in the present study. This value was within the MOI range of a previous study employing a co-culture model (MOI: 0.005–5) [25] in which the number of U2OS osteosarcoma cells co-cultured with Staphylococcus epidermidis for 48 h was reduced by approximately 90% in comparison with cells without bacterial co-incubation. Co-incubation with S. pneumoniae killed half of the attached human bronchiolar epithelial cells in
the present study. This result indicates that the co-culture protocol used in the present study was capable of providing relatively harsh enough conditions for the survival of epithelial cells.

GSH is transformed into GSSG after scavenging intracellular ROS. GSSG is transformed into GSH by glutathione reductase [16]. This cycle maintains the intracellular redox balance. The bacterial co-culture in the present study caused an intracellular redox imbalance and proinflammatory cytokine production, even in the surviving attached epithelial cells. Intracellular ROS and GSSG levels were markedly increased by bacterial co-incubation, indicating that the progression of bacteria-induced oxidative stress was consuming antioxidants in the epithelial cells. Intracellular levels of PGE2 and proinflammatory cytokines such as IL-1β, -6 and -8 were increased by bacterial co-incubation. This phenomenon was supported by upregulation of COX2, and IL-6 and -8 gene expression after bacterial co-incubation. It is well known that ROS increase the release of free arachidonic acid from phospholipids in the plasma membrane via activation of phospholipase A2 [26]. Proinflammatory mediators such as PGE2 are synergistically associated with the production of proinflammatory cytokines [27-29]. Therefore, our results indicated that the oxidative stress caused by the bacteria triggered the arachidonic cascade and activated proinflammatory cytokine production in the surviving HLAEs.

Bacterial co-incubation increased amount of non-specific MUC production, as well as the gene and
protein expression levels of MUC5AC, indicating an increase in the production of gel-forming MUCs in HBECs by bacterial co-incubation. HBECs produce gel-forming MUCs in response to attachment of bacteria to wash out the pathogenic cells [30]. Therefore, the bacteria-induced increase of gel-forming MUC production in HBECs can be regarded as a cellular protection system against infection. However, excessive production of gel-forming MUC results in accumulation of sputum.

In the present study, NAC treatment of HBECs was performed before bacterial co-incubation. This culture condition did not allow coexistence of NAC with bacteria in contrast to a previous study using co-cultured tissue-forming cells with wound pathogens in the presence of NAC, which demonstrated the anti-infection capability of NAC based on its bacteriostatic effect and antioxidant reinforcement against bacteria-induced oxidative stress [18]. Pretreatment with NAC prevented the cellular proinflammatory cytokine production to the bacterial exposure as indicated by unchanged or slightly increased levels of proinflammatory cytokine expression and PGE2 synthesis in cells pretreated with NAC even with bacterial co-incubation. Cells pretreated with NAC exhibited not only avoidance of intracellular ROS elevation, but also a marked increase in cellular GSH levels regardless of bacterial co-incubation. This finding could be supported by biochemical transformation of NAC into GSH within the cytoplasm after cellular uptake of NAC [31]. However, cellular GSH and GSSG levels did not change in cells pretreated with NAC even with bacterial co-incubation, indicating avoidance of bacteria-induced oxidative stress in cells pretreated with NAC. All of these
observations suggested that prevention of the cellular proinflammatory cytokine production to bacterial exposure was attributed to the some cellular defensive system against bacterial infection rather than antioxidant reinforcement by NAC treatment.

The increase in cellular MUC production by bacterial co-incubation was alleviated by pretreatment with NAC. MUC5AC gene and protein expressions did not increase in cells pretreated with NAC even with bacterial co-incubation. This result suggested that these phenomena were attributed to prevention of the cellular proinflammatory cytokine production to bacterial exposure by pretreatment with NAC. Interestingly, regardless of bacterial co-incubation, gene and protein expressions of MUC4, one of the membranous MUCs, was increased only in cells pretreated with NAC. The membranous type of MUC functions as a protective decoy for bacterial infection by shedding the from cell surface [11, 12]. In contrast to gel-forming MUCs, membranous MUCs should not accumulate in sputum as a result of bacterial clearance. Therefore, NAC might reinforce the bacterial clearance system without accumulation of sputum from bronchi. The biomolecular mechanism underlying NAC-mediated enhancement of MUC4 expression in HBECs should be studied further, in addition to the effect of NAC on cellular receptors such as Toll-like receptor, respond to bacterial pathogenic agents.

The potential of NAC as a preventive medication for chronic inflammatory lung diseases should be
interpreted with caution. First, the present study did not determine the speed of cellular uptake of NAC by HBECs. However, a previous study has demonstrated that uptake of NAC by erythrocytes reaches a peak in 3 h [32]. This time about incorporation of NAC into erythrocytes served as a useful reference for the pretreatment time with NAC for the epithelia cells in the present study. NAC is membrane permeable but its cellular uptake is likely to be slower than lipophilic molecules because of its water solubility. In addition, we did not obtain any information concerning the effect of NAC on subepithelial gland tissue or alveoli. Therefore, the delivery method, protocol, and actual efficacy of NAC for airway epithelial tissue should be investigated in a preclinical animal model.

There have been no reports on medication exerting a preventive effect on bacterial infection with avoidance of mucus hypersecretion in airway epithelial tissues. For example, non-steroid anti-inflammatory drugs (NSAIDs), a representative anti-inflammatory drug in common clinical practice, inhibit mucus synthesis in airway epithelial tissue [33] but are unable to prevent infection. In addition, it has been reported that adverse symptoms such as gastrointestinal ulceration result from prolonged application of NSAIDs [34]. NAC enhances the antioxidant property of bronchiolar epithelial cells, as shown in the present study, and reinforces their resistance against bacterial-induced oxidative stress [18]. Moreover, NAC exerts bacteriostatic effects on various types of pathogens and functions as a mucolytic drug [35]. Therefore, NAC might exert multiple unique effects that contribute to prevention of chronic inflammatory lung diseases. Thus the present study
has implication for the development of a novel concept for prevention of chronic inflammatory lung diseases.

**Conclusion**

Uptake of NAC by HBECs prevented the increase of gel-forming MUC production and increased membranous MUC expression in cells under a co-culture condition with *S. pneumoniae* via alleviation of the cellular proinflammatory cytokine production and maintenance of the cellular redox balance.

**Acknowledgments**

The author thanks Professor Dr. Kaoru Sakurai, Associate Professor Dr. Takayuki Ueda, Associate Professor Dr. Ken Ishizaki, and Senior Assistant Professor Dr. Masahiro Yamada for their mentorship.

**References**


[35] Riise GC, Qvarfordt I, Larsson S, Eliasson V, Andersson BA. Inhibitory effect of N-acetylcysteine on adherence of Streptococcus pneumoniae and Haemophilus influenzae to human
Figure 1.
Figure 2.
Figure 3.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Annealing temperature</th>
<th>Number of cycle</th>
<th>Size of PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX2</td>
<td>TCCAGTACCAAAATCGTATTGCT AGTGCTTCCAACCTCAGACACAT</td>
<td>61.2</td>
<td>30</td>
<td>370</td>
</tr>
<tr>
<td>MUC4</td>
<td>CAGCCTCGCCAGCACCTCACCCTGG GGAGGGGTGTTGATGAAAAACCTTGTGGTCGTCTCTCC</td>
<td>74.9</td>
<td>30</td>
<td>236</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>TCCGGCCTCATCTTCTCC ACTTGGGCACCTGGGCTG</td>
<td>60.2</td>
<td>36</td>
<td>683</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCCCCAGGAGAAGATTCCA GGCCTTTGAGGAGAAGGAG</td>
<td>64.2</td>
<td>30</td>
<td>104</td>
</tr>
</tbody>
</table>
| IL-8   | GAATGGGTGTGGCTAGAATGTGATA CAGACTAGGTTGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
Figure 4.
**Figure legends**

**Figure 1.** Calcein-based relative cell numbers (A) and hematocytometer-based cell density (B) of human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Data are shown as the mean ± SD (n=4). *p < 0.05.

**Figure 2.** Levels of cellular ROS (A), GSH (B), and GSSG (C) per unit cell in human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Data are shown as the mean ± SD (n=4). *p < 0.05.

**Figure 3.** Expression of IL-6 and -8, COX2, MUC5AC and MUC4 genes analyzed by RT-PCR (A), PGE2 (B), alcian blue-stained MUC (C) per unit cell, and cellular proinflammatory cytokines (IL-1β, -6 and -8) (D), in human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Data are shown as the mean ± SD (n=4). *p < 0.05.

**Table 1.** Primers used for RT-PCR analyses

**Figure 4.** (A) Representative images after immunochemical fluorescent staining for MUC5AC (left) and MUC4 (right) (green) with DAPI counterstaining (Blue) in human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Histogram of
MUC5AC-(B) and MUC4- (C) detected area per unit nucleus in images presented in panel A are shown. Data represent the mean ± SD (n=5). *p < 0.05.