Neutrophil elastase increases *MUC5AC* mRNA and protein expression in respiratory epithelial cells

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Voynow, Judith A., Lisa Rosenthal Young, Yiqiong Wang, Teresa Horger, Mary C. Rose, and Bernard M. Fischer. Neutrophil elastase increases *MUC5AC* mRNA and protein expression in respiratory epithelial cells. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L835–L843, 1999.—Chronic neutrophil-predominant inflammation and hypersecretion of mucus are common pathophysiological features of cystic fibrosis, chronic bronchitis, and viral- or pollution-triggered asthma. Neutrophils release elastase, a serine protease, that causes increased mucin production and secretion. The molecular mechanisms of elastase-induced mucin production are unknown. We hypothesized that as part of this mechanism, elastase upregulates expression of a major respiratory mucin gene, *MUC5AC*. A549, a human lung carcinoma cell line that expresses *MUC5AC* mRNA and protein, and normal human bronchial epithelial cells in an air-liquid interface culture were stimulated with neutrophil elastase. Neutrophil elastase increased *MUC5AC* mRNA levels in a time-dependent manner in both cell culture systems. Neutrophil elastase treatment also increased MUC5AC protein levels in A549 cells. The mechanism of *MUC5AC* gene regulation by elastase was determined in A549 cells. The induction of *MUC5AC* gene expression required serine protease activity; other classes of proteases had no effect on *MUC5AC* gene expression. Neutrophil elastase increased *MUC5AC* mRNA levels by enhancing mRNA stability. This is the first report of mucin gene regulation by this mechanism.

mucin; messenger ribonucleic acid; protease; airway epithelium

MUCUS HYPERSECRETION is a major pathological feature of several inflammatory airway diseases including cystic fibrosis (CF), chronic bronchitis, and asthma. The excessive mucus in the airways overwhelms the normal mucociliary clearance mechanisms, leading to obstruction and impaired pulmonary function. In addition, in CF, mucus obstruction of airways is associated with recurrent airway inflammation and infection, resulting in pulmonary fibrosis and respiratory failure. In these diseases, the pathological findings of hypertrophy and hyperplasia of mucous cells, mucus obstruction of airways, and neutrophil-predominant inflammation (18, 40, 47) suggest that mucus hypersecretion may be associated with neutrophilic inflammation.

Neutrophils are present in high concentrations in airway surface fluid (ASF) in infants with CF (23) and persist in patients with varying severity of disease (10, 25). During acute exacerbations, asthmatic patients have a high percentage of neutrophils in ASF (12, 18). In addition, exposure to several air pollutants including ozone and fine particulates (16) and cigarette smoke (39) results in increased neutrophil levels in the airway.

Neutrophils release several mediators during inflammation, and one, neutrophil elastase (NE; EC 3.4.21.37), is a serine protease that impairs mucociliary clearance by several mechanisms. NE injures cilia and decreases ciliary function (3), stimulates mucin secretion (19, 24, 31), induces secretory cell hyperplasia and hypertrophy (13, 15), and increases mucin production (14, 15). The molecular mechanism(s) by which NE stimulates mucin production is unknown. We hypothesize that as part of this mechanism, NE upregulates expression of mucin genes, leading to increased production of mucin glycoproteins.

Mucin glycoproteins, the major macromolecular constituents of mucus, impart viscoelastic qualities to mucus. They are large, heavily O-glycosylated molecules and have been difficult to characterize biochemically. By molecular technology, several mucin genes have been identified and are expressed as mRNA in the respiratory tract. Of the mucin genes expressed in respiratory epithelium, *MUC5AC* appears to be one of the major respiratory mucins (reviewed in Ref. 35). *MUC5AC* is expressed at greater levels than *MUC1* or *MUC2* in nasal cells (46), nasal polyp tissue (45), nasal turbinates (7), and primary bronchial epithelium (6). In addition, MUC5AC glycoprotein was recently shown to be a major component of respiratory secretions from a subject with bronchial asthma (32) and normal subjects (42). Therefore, our studies have focused on regulation of *MUC5AC* gene expression.

To examine the effect of NE on *MUC5AC* gene regulation, two different models of airway epithelia were used to induce mucociliary differentiation: A549, a lung adenocarcinoma cell line, which has been used extensively as a model of respiratory epithelium and expresses both MUC5AC mRNA and glycoprotein (9), and normal human bronchial epithelial (NHBE) cells grown in air-liquid interface culture (2, 21). In this report, we demonstrate that NE upregulated *MUC5AC* gene expression in both culture systems, thus providing a link between chronic neutrophilic inflammation and increased mucin production in airway diseases.

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METHODS

Reagents. A549 cells were obtained from the American Type Culture Collection (Manassas, VA). Ham's F-12K medium, DMEM, fetal bovine serum, penicillin, streptomycin, and glutamine were from Biofluids (Rockville, MD). NHBE cells, bronchial epithelial basic medium, and SingleQuot supplements were from Clonetics (San Diego, CA). Rat tail collagen type I was purchased from Collaborative Biochemical (Bedford, MA). Transwell filters were purchased from Corning Costar (Cambridge, MA). Epidermal growth factor and bovine serum albumin were from Intergen (Purchase, NY). Retinoic acid, *N*-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (AAPV-CMK), bovine pancreatic trypsin, papain, actinomycin D, and ribonucleotides were from Sigma (St. Louis, MO). NE (875 U/mg of protein) and methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide were from Elastin Products (Owensville, MO). α_1-Antitrypsin was from Calbiochem (San Diego, CA). Collagenase was from Worthington Biochemical (Freehold, NJ). Nylon filter (Nytran Plus) was from Schleicher & Schuell (Keene, NH). The pBluescript II SK(−) was purchased from Stratagene (La Jolla, CA). X-OMAT AR film was purchased from Kodak (Rochester, NY). The camera used for densitometry was from Fotodyne (Hartland, WI). [α-^{32}P]UTP and [α-^{32}P]dCTP were from Amersham (Arlington Heights, IL). RNasin was obtained from Promega (Madison, WI). RNAzol B was from Cinna/Biotex Laboratories (Friendswood, TX). Biospin columns were from Bio-Rad (Hercules, CA). RNase A, RNase T1, and proteinase K were from Boehringer Mannheim (Indianapolis, IN). Milk blocking agent was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Horseradish peroxidase-conjugated goat anti-rabbit IgG and bicinchoninic acid protein assay were from Pierce (Rockford, IL).

Cell culture. A549, a lung carcinoma cell line, was cultured in Ham's F-12K medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. NHBE cells were seeded on rat tail collagen type I-coated microporous membranes (Transwell filters) in a serum-free 1:1 mixture of bronchial epithelial cell basic medium and DMEM with SingleQuot supplements, bovine pituitary extract (13 mg/ml), epidermal growth factor (0.5 ng/ml), bovine serum albumin (1.5 μg/ml), and all *trans*-retinoic acid (5×10^{-8} M) in place of SingleQuot retinoic acid. When cells were 65% confluent, culture conditions were changed to an air-liquid interface (2, 21). Medium was removed from the apical surface, and medium in the basolateral chamber was changed daily for 7 days. NHBE cells were then used for experiments.

Cell stimulation. All studies were carried out when A549 cells were 90–95% confluent. Cells were changed to serum-free medium. Cells were exposed to NE at doses and times specified in figure legends. A549 cells were treated for 24 h with elastase in the presence and absence of elastase inhibitors α_1-antitrypsin or AAPV-CMK. α_1-Antitrypsin or AAPV-CMK was incubated for 15 min at room temperature with NE and then diluted 1,000-fold in medium for final concentrations of 0.65 U/ml of NE (equivalent to 25 nM), 125 nM α_1-antitrypsin, and 1 μM AAPV-CMK. In addition to the use of elastase inhibitors, NE was boiled for 15 min and added to the cell culture medium for 24 h (final concentration 25 nM). All inhibitors were tested by a spectrophotometric assay with the use of the NE-specific substrate methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide according to the manufacturer's instructions to ensure that elastase activity was ablated. Control conditions included resting A549 cells or cells treated with 50 μM sodium acetate (pH 5)-100 μM sodium chloride (NE buffer) or elastase inhibitors alone. A549 cells were also treated with 0.65–65 U/ml of bovine pancreatic trypsin, 0.65–65 U/ml of collagenase, and 0.65–6.5 of U/ml papain for 24 h. Cell counts were determined for adherent and nonadherent cells, and viability was assessed by trypan blue dye exclusion. Control conditions included resting cells or cells treated with NE buffer or buffers for other proteases: 0.1 μM HCl (bovine pancreatic trypsin) or 50 μM sodium acetate, pH 4.5 (papain).

RNA isolation and Northern analysis. RNA was isolated from cell cultures as previously described (45) with the guanidinium thiocyanate-cesium chloride method. Total RNA (10 μg) was separated by 1.2% agarose-formaldehyde gel electrophoresis and transferred by capillary blot to a nylon filter (Nytran Plus) in 1 M ammonium acetate. After ultraviolet cross-linking, the filters were hybridized at 62°C as previously described with ^{32}P-labeled probes (specific activity $> 10^8$ counts·min^{-1}·μg^{-1}) for *MUC5AC*, γ-actin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (45). Filters were washed twice with 250 ml of 2× saline-sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min and then with 250 ml of 0.1× SSC and 0.1% SDS at 62°C for 15 min. Filters were exposed for autoradiography at −80°C for 4 h (γ-actin or GAPDH) or 24 h (*MUC5AC*). Band density on autoradiographs was determined by digitalization with the Foto/Eclipse camera and quantitation with National Institutes of Health Image software.

Nuclear runoff assay. Nuclei were isolated from 1.2×10^7 A549 cells at rest or stimulated with 25 nM NE for 2, 4, or 24 h. The nuclei were incubated with 2.7 mM ATP, 1.0 mM CTP, 1.0 mM GTP (all from Sigma), 330 μCi of [α-^{32}P]UTP, and 0.2 U of RNase inhibitor in 20 mM HEPES buffer (pH 7.6)-90 mM KCl-5 mM $MgCl_2$ at 37°C for 30 min (8). RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (RNAzol B). ^{32}P-labeled nascent RNA was purified by Bio-Gel P-30 column chromatography (Biospin columns) and hybridized at 52°C for 36 h to DNA targets (10 μg) immobilized on Nytran filter by ultraviolet cross-linking. DNA targets included plasmids containing *MUC5AC* cDNA, human γ-actin cDNA, and, as a negative control, the plasmid pBluescript II SK(−). Filters were washed three times in 2× SSC for 15 min and then exposed to RNase A (5 μg/ml) and RNase T1 (5 U/ml) in 2× SSC and 10 mM Tris·HCl, pH 7.3, at 37°C for 30 min, followed by treatment with 50 μg/ml of proteinase K in 2× SSC, 10 mM Tris·HCl, and 0.5% SDS at 37°C for 45 min. Filters were exposed for autoradiography at −80°C for 3 days.

RNA stability assay. A549 cells were resting or stimulated with NE (50 nM, 16 h), and then transcription was stopped by treatment with actinomycin D (5 μg/ml) for 4, 8, and 24 h (33). Total cellular RNA was extracted, and *MUC5AC* and GAPDH mRNA levels were evaluated by slot blot analysis and quantitated as described in *RNA isolation and Northern analysis*.

Western analysis. A549 cells were changed to serum-free medium for 20 h and then treated with 100 nM NE for 22 h. After addition of 1 μM AAPV-CMK, medium was collected, and cells were washed and then lysed in buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, and 1 mM sodium orthovanadate) at 4°C for 15 min. Cell debris was removed by centrifugation (11,000 *g*) for 10 min at 4°C. Cell lysates and medium protein concentrations were determined by Pierce bicinchoninic acid protein assay. Cell lysates and medium (25 μg of total protein) were separated on a 1% agarose-Tris-acetate-EDTA-1% SDS gel by electrophoresis,

and proteins were transferred under pressure to a polyvinylidene difluoride membrane as previously described (9). The membrane was blocked with milk blocking agent (1:10 dilution) and incubated with a rabbit polyclonal monospecific anti-MUC5AC antibody (1:500 dilution) (9). Membranes were developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution) and 10 mg of 4-chloro-1-naphthol-10 mg of 3,3′-diaminobenzidine tetrahydrochloride-0.006% hydrogen peroxide substrate. To determine whether NE digested MUC5AC glycoprotein, conditioned serum-free medium from A549 cells (48-h culture) was treated with 100 nM NE (22 h) or with vehicle control, and 25 μg of total protein from each treatment condition were evaluated for MUC5AC glycoprotein with Western analysis.

Statistical analysis. Analysis of data was performed with the Kruskal-Wallis one-way nonparametric analysis of variance and post hoc comparisons by Mann-Whitney's rank sum test. Differences were considered significant at $P < 0.05$.

RESULTS

NE increased MUC5AC mRNA levels in a dose- and time-dependent manner. NE (75 nM, 24 h) increased *MUC5AC* mRNA levels in A549 cells approximately sixfold compared with vehicle alone (Fig. 1*A*). NE also upregulated *MUC5AC* in A549 cells in a time-dependent manner (Fig. 2*A*). *MUC5AC* transcript levels started increasing after 4 h of exposure to NE and continued to increase up to 24 h. In contrast, NE treatment caused a decrease in γ-actin mRNA levels over time (Fig. 2*B*). To determine whether NE-induced *MUC5AC* expression occurred in primary airway cells, we examined expression of *MUC5AC* in NHBE cells in culture. Densitometry of Northern analyses showed that 500 nM NE (1 h) upregulated *MUC5AC* mRNA levels approximately two- to fourfold in NHBE cells (Fig. 3*A*). There are differences in the kinetics of the NE effect on *MUC5AC* expression between NHBE and A549 cells. The higher concentration of NE required for induction of *MUC5AC* mRNA expression in NHBE cells compared with that in A549 cells may be due to the collagen substratum required for NHBE culture. The shorter duration of treatment of NHBE cells for NE induction of *MUC5AC* expression may be due to differences in the mechanism of gene regulation or differences in the survival of NHBE cells after NE treatment. Further studies in NHBE cells are needed to clarify the etiology of these differences.

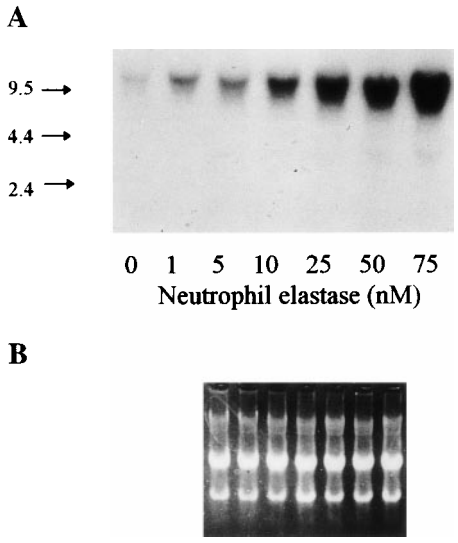

Fig. 1. Northern analyses of A549 cells stimulated with neutrophil elastase (NE). A549 cells were treated with 0–75 nM NE for 24 h, and RNA (10 μg) isolated from stimulated cells was evaluated by Northern analysis and autoradiography for *MUC5AC* expression (*A*). Nos. at *left*, molecular-size markers in kb. *B*: ethidium stain of Northern gel. Data are representative of 4 experiments.

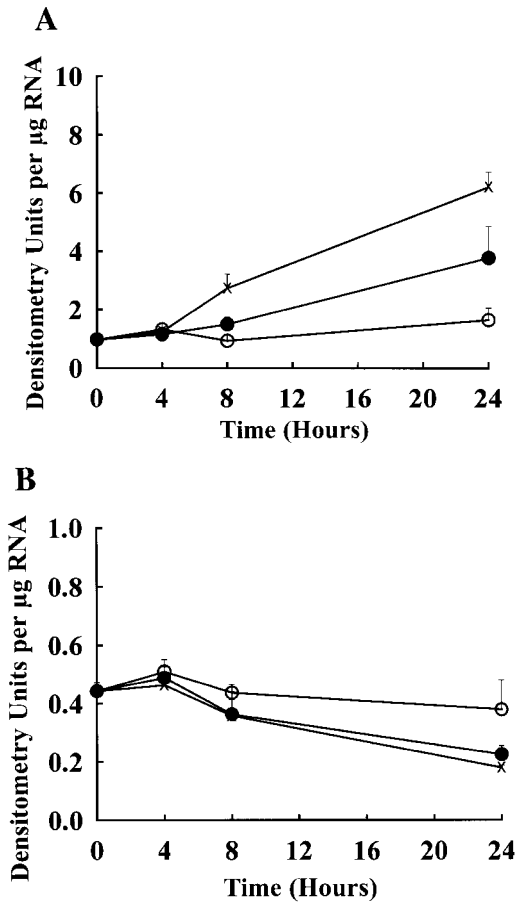

Fig. 2. Time course of *MUC5AC* mRNA levels after NE stimulation. A549 cells were resting (○) or treated with 10 (●) or 50 (×) nM NE for 0, 4, 8, or 24 h. RNA (10 μg) was evaluated by Northern analysis and autoradiography for *MUC5AC* (*A*) and γ-actin (*B*). Data are means ± SE of mRNA levels; $n = 3$ experiments with duplicate samples.

Induction of MUC5AC mRNA expression required proteolytically active NE. To determine whether NE enzymatic activity was required for upregulation of *MUC5AC* transcript levels, A549 cells were incubated with inactivated NE (Fig. 4). NE proteolytic activity was completely ablated by addition of inhibitors α_1-antitrypsin or AAPV-CMK or after boiling for 15 min. NE inactivated by preincubation with inhibitors or by boiling (Fig. 4) did not increase *MUC5AC* mRNA levels in contrast to the increase induced by active NE (Fig. 4). Treatment with elastase inhibitors alone did not change *MUC5AC* mRNA levels compared with resting or vehicle control cells (data not shown).

Human NE is prepared from human sputum and therefore may contain lipopolysaccharide. To determine whether the effect of NE on *MUC5AC* mRNA

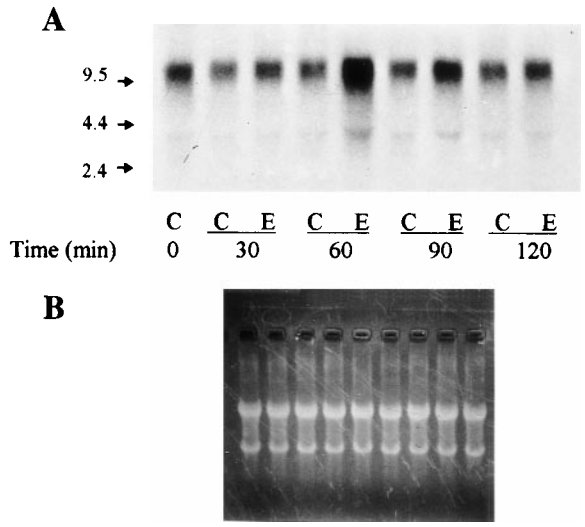

Fig. 3. Northern analysis of normal human bronchial epithelial (NHBE) cells stimulated with NE. NHBE cells grown on a collagen-coated filter in air-liquid interface culture were stimulated with 500 nM NE for 0–120 min. RNA (10 μg) isolated from stimulated cells was evaluated by Northern analysis and autoradiography for *MUC5AC* mRNA expression (*A*). C, control; E, elastase; Nos. at *left*, molecular-size markers in kb. *B*: ethidium staining of Northern gel. Autoradiograph is representative of 3 experiments.

levels was due to potential lipopolysaccharide contamination, we boiled the NE preparation. Boiling the NE preparation would not affect lipopolysaccharide (29). Treatment with boiled NE did not increase *MUC5AC* mRNA levels (Fig. 4), suggesting that the effect of NE was due to proteolytic activity and not to potential lipopolysaccharide contamination.

MUC5AC mRNA expression was regulated by serine protease activity. To determine whether *MUC5AC* regulation by NE was specifically related to serine protease activity, A549 cells were treated with other classes of proteases as well as a second serine protease, and the regulation of *MUC5AC* expression was examined. Bovine pancreatic trypsin, a serine protease, stimulated a significant increase in *MUC5AC* transcript levels in A549 cells (Fig. 5*A*) but required 100-fold higher protease activity (65 U/ml) than NE (0.65 U/ml; Fig. 5*A*, *bar*

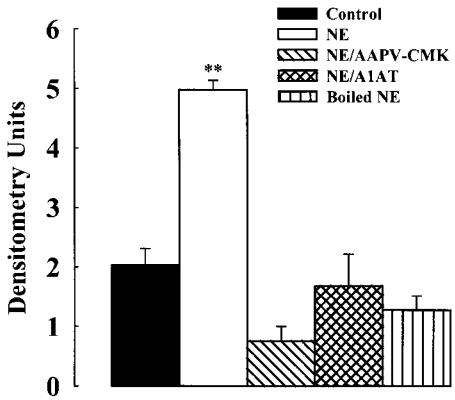

Fig. 4. *MUC5AC* mRNA levels after treatment with NE and/or protease inhibitors. A549 cells were resting or stimulated with 25 nM NE, 1 μM *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (AAPV-CMK) and 25 nM NE, 125 nM α_1-antitrypsin (A1AT) and 25 nM NE, or boiled NE (25 nM). After Northern analysis and autoradiography, *MUC5AC* mRNA levels were quantitated by densitometry and are means ± SE in densitometry units/μg RNA; $n = 4$ experiments with duplicate samples. ** Significant difference in *MUC5AC* mRNA levels between NE treatment and all other conditions, $P < 0.01$.

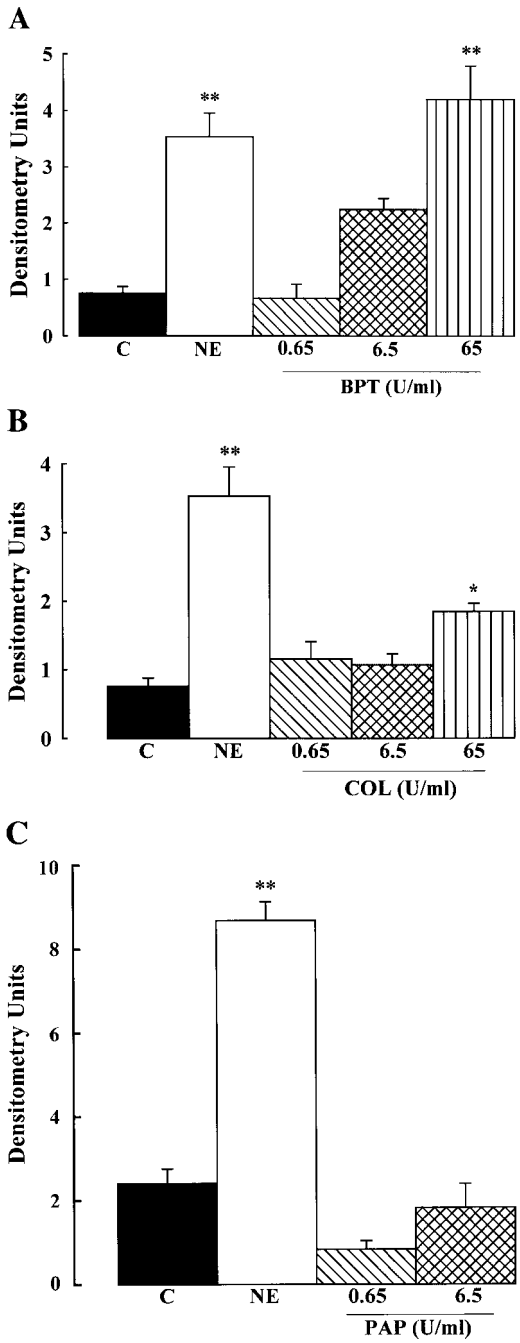

Fig. 5. *MUC5AC* mRNA levels after treatment with proteases for 24 h. A549 cells were resting (C) or treated with NE (25 nM, 0.65 U/ml) or varying concentrations of bovine pancreatic trypsin (BPT; *A*), collagenase (COL; *B*), or papain (PAP; *C*). After Northern analyses and autoradiography, *MUC5AC* mRNA levels were quantitated by densitometry and are means ± SE in densitometry units/μg RNA; $n = 3$ experiments with duplicate samples. *MUC5AC* mRNA levels were significantly greater after NE or BPT (65 U/ml) stimulation compared with all other treatment conditions, $^{**}P < 0.01$. At highest concentration of COL (65 U/ml), there was an increase in *MUC5AC* mRNA levels compared with vehicle control ($^{*}P < 0.01$), but this level was significantly less than NE-stimulated *MUC5AC* mRNA levels ($^{**}P < 0.01$). There was no significant increase in *MUC5AC* after PAP treatment (*C*).

Table 1. *Comparison of cell dissociation with proteases*

Protease	Percentage of Cells Detached
Vehicle	<1 (8)
Neutrophil elastase, 0.65 U/ml	5 (8)
Bovine pancreatic trypsin, 65 U/ml	100 (3)
Collagenase, 65 U/ml	65 (3)
Papain, 6.5 U/ml	74 (2)

Values are mean percentages of A549 cells detached after protease treatment for 24 h or 100 × [cells in medium/(cells in medium + adherent cells)]; nos. in parentheses, no. of experiments. See Fig. 5 for experimental details.

5). At equivalent enzymatic activity (0.65 U/ml), trypsin had no effect on *MUC5AC* mRNA levels (Fig. 5*A*, *bar 3*). In contrast, collagenase, a metalloprotease, caused only a small change in *MUC5AC* mRNA levels at 100-fold higher activity levels (65 U/ml) than NE (Fig. 5*B*, *bar 5*), and papain, a cysteine protease, caused no increase in *MUC5AC* mRNA levels at 10-fold higher activity levels (6.5 U/ml) than NE (Fig. 5*C*, *bar 4*). Importantly, collagenase and papain caused significantly greater cell dissociation than NE (Table 1). Protease treatments caused no change in γ-actin mRNA levels (data not shown) and no change in cell viability (>95% for all conditions). These data suggest that serine proteases regulated *MUC5AC* gene expression by a mechanism distinct from cell dissociation by other proteases.

NE increased MUC5AC mRNA expression by enhancing mRNA stability. To evaluate whether NE regulated *MUC5AC* gene expression by a transcriptional or post-transcriptional mechanism, nuclear runoff and mRNA stability assays were performed. Nuclear runoff studies revealed that NE treatment did not stimulate new transcription of ^{32}P-labeled *MUC5AC* mRNA (Fig. 6). However, RNA stability assays demonstrated an NE-induced increase in *MUC5AC* mRNA half-life from 4.5 h in resting cells to 14.75 h (Fig. 7*A*). In contrast to *MUC5AC*, the half-life of GAPDH mRNA was similar for resting cells (16.5 h) and NE-stimulated cells (21 h; Fig. 7*B*). These experiments are consistent with the concept that NE regulates *MUC5AC* expression by enhancing mRNA stability.

NE treatment stimulated increased MUC5AC glycoprotein production in A549 cells. Western analysis of MUC5AC glycoprotein was performed to determine the

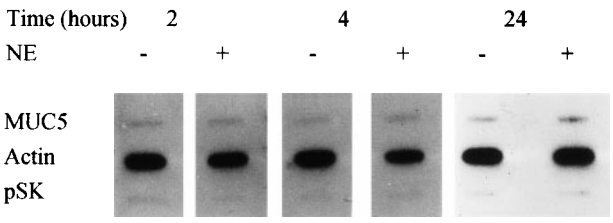

Fig. 6. Modulation of relative rate of *MUC5AC* gene transcription in A549 cells by NE. A549 cells were resting (−) or stimulated with 25 nM NE (+) for 2, 4, or 24 h. Nuclei were isolated, and transcription was allowed to proceed in presence of [^{32}P]UTP. Newly labeled transcripts were hybridized to filter-immobilized cDNA (10 μg) for *MUC5AC* (MUC5), γ-actin, and pBluescript II SK(−) (pSK) as a negative control. Autoradiograph is representative of 2 experiments.

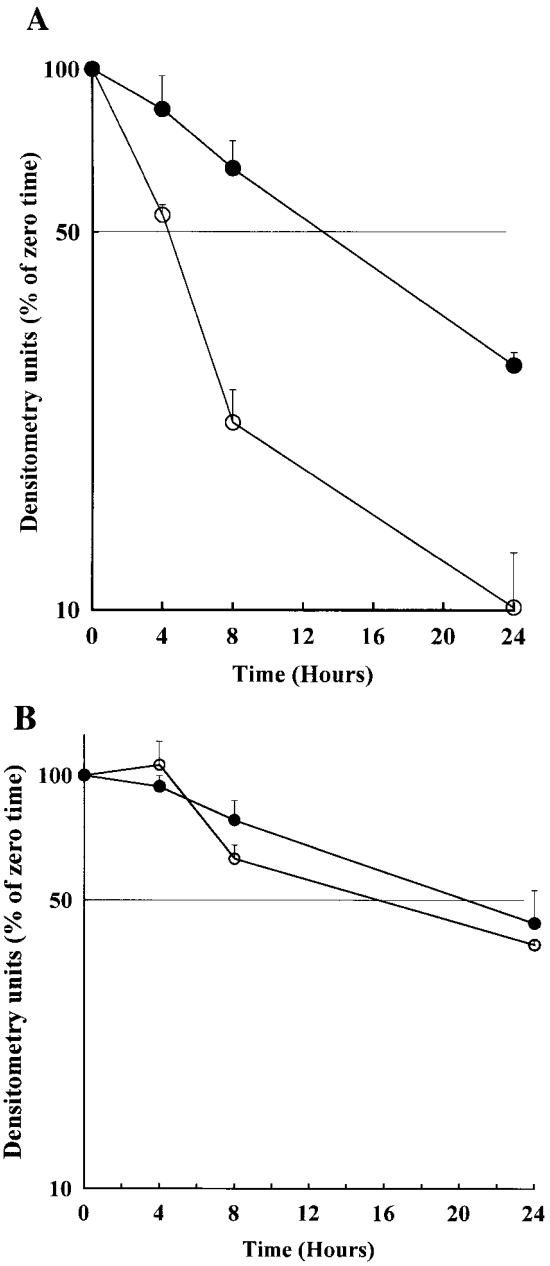

Fig. 7. Stability of *MUC5AC* mRNA transcripts in A549 cells at rest or after NE treatment. A549 cells were treated with 5 μg/ml of actinomycin D for 0, 4, 8, and 24 h after treatment with control vehicle (○) or stimulation with 50 nM NE (●) for 16 h. Data are means ± SE of mRNA levels for *MUC5AC* (*A*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; *B*) expressed as percent starting mRNA levels (% of zero time) after addition of actinomycin D; $n = 4$ experiments with duplicate samples.

effect of NE on MUC5AC production. With the use of a rabbit polyclonal monospecific anti-MUC5AC antibody (9), Western analysis of A549 cell medium revealed a high-molecular-mass polydisperse band representing fully glycosylated MUC5AC mucin glycoprotein (Fig. 8). Western analysis of A549 cell lysate revealed the high-molecular-mass MUC5AC glycoprotein and two additional discrete bands at approximate molecular masses of 400 and 500 kDa. These bands probably represent MUC5AC protein and a partially glycosylated MUC5AC glycoprotein. Treatment with 100 nM

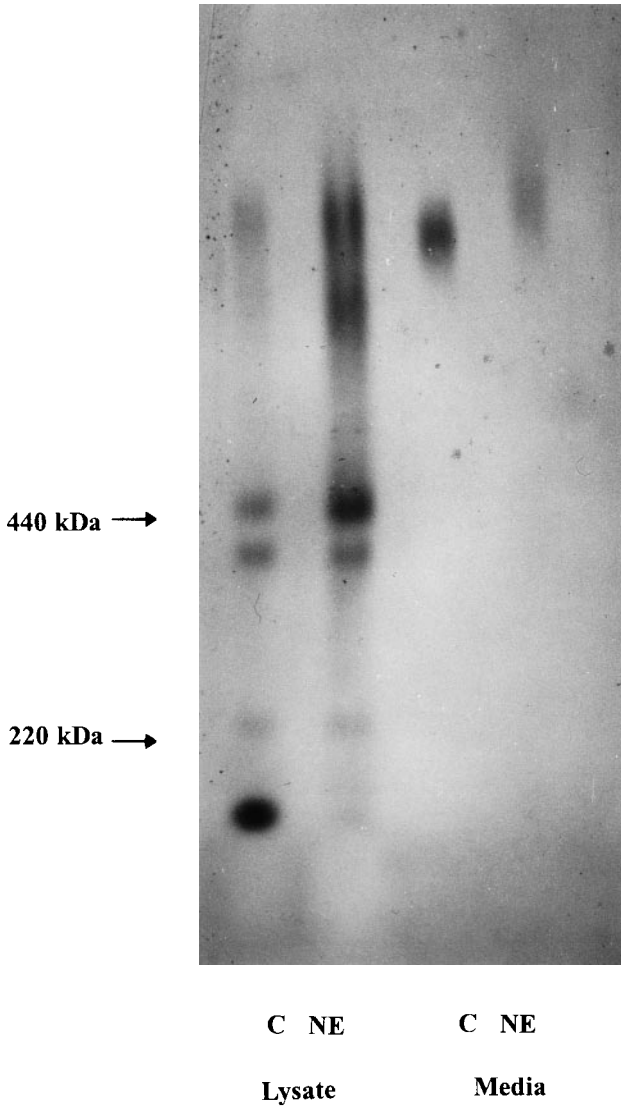

Fig. 8. Western analysis of MUC5AC glycoprotein in A549 cells stimulated with NE. A549 cells were changed to serum-free medium for 20 h and then treated with vehicle control (C) or NE (100 nM) for 22 h. At conclusion of treatment period, AAPV-CMK (1 μM) was added to all plates, medium was removed, and cells were lysed. Control cell lysates (*lane 1*), NE-treated cell lysates (*lane 2*), control medium (*lane 3*), and NE-treated medium (*lane 4*; 25 μg protein/sample) were separated on a 1% agarose-Tris-acetate-EDTA-SDS gel by electrophoresis and evaluated by Western analysis with a rabbit polyclonal monospecific anti-MUC5AC antiserum and development with horseradish peroxidase-conjugated goat anti-rabbit IgG and 10 mg 4-chloro-1-naphthol-10 mg 3,3′-diaminobenzidine tetrahydrochloride-0.006% hydrogen peroxide substrate (9). Nos. at *left*, molecular-mass markers. Western blot is representative of 3 experiments.

NE (22 h) increased MUC5AC protein and glycoprotein levels in A549 cell lysates compared with control cell lysates (Fig. 8, *lanes 1* and *2*). However, MUC5AC glycoprotein levels in the medium of NE-treated A549 cells were decreased compared with those in control cells (Fig. 8, *lanes 3* and *4*). Serine proteases have been reported to digest mucin glycoproteins (24, 34). Therefore, we tested the ability of 100 nM NE (22 h) to digest MUC5AC in cell-free serum-free A549 cell-conditioned medium. MUC5AC glycoprotein was barely detectable in NE-treated medium, whereas MUC5AC glycoprotein was present in control vehicle-treated A549 cell-conditioned medium as shown by Western analysis (Fig. 9). These experiments are consistent with increased MUC5AC glycoprotein production and MUC5AC degradation in the medium because of NE treatment.

DISCUSSION

In this study, we demonstrated that NE increased *MUC5AC* gene expression and glycoprotein production. The concentrations of elastase (25–500 nM) used in this study were within the range found in ASF from CF (10, 20) and asthmatic (18) patients, suggesting that

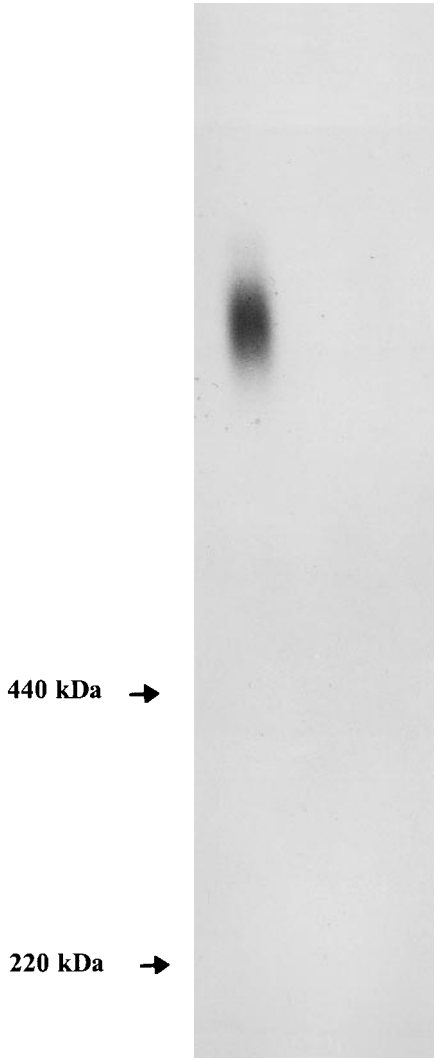

Fig. 9. Western analysis of MUC5AC glycoprotein of A549 cell-conditioned medium. A549 cell-conditioned serum-free medium (48-h culture) was treated with control vehicle or NE (100 nM) for 22 h at 37°C. At end of treatment period, AAPV-CMK (1 μM) was added to each sample, and conditioned medium (25 μg of total protein) was evaluated by Western analysis with a monospecific rabbit polyclonal anti-MUC5AC antibody. Nos. at *left*, molecular-mass markers. Western blot is representative of 3 experiments.

chronic NE exposure to airway epithelium may significantly contribute to mucin overproduction in these diseases.

The effect of NE on *MUC5AC* regulation was investigated in two different in vitro models of airway epithelium: A549 carcinoma cells (reviewed in Ref. 9) and primary respiratory epithelial cells in culture. In both culture systems, NE treatment resulted in increased *MUC5AC* mRNA levels. The A549 cell line was used in this study because it expresses both *MUC5AC* mRNA and glycoprotein (9) and because it is a well-characterized model for investigating molecular and biochemical processes in airway epithelium, including inducible nitric oxide synthase expression (5), arachidonic acid metabolism (17), respiratory viral infection (4), and nuclear factor-κB-mediated gene regulation (48). Other cancer cell lines including NCI-H292 (27–30) and HM3 (28–30) have proven to be useful models for investigating *MUC* gene regulation.

NE has been reported to regulate gene expression of several mammalian genes in addition to *MUC5AC* including IL-8 (33), secretory leukocyte protease inhibitor (1), elastase-specific inhibitor, elafin (37), and intercellular adhesion molecule-1 (49). However, the mechanism of gene regulation by NE is not well understood. In this report, we demonstrated that inactivation of elastase resulted in abrogation of its effect on *MUC5AC* gene regulation. Furthermore, *MUC5AC* gene expression was regulated by serine protease activity (NE and bovine pancreatic trypsin); gene expression was not affected by cysteine protease (papain) or metalloprotease (collagenase) activities. In contrast to the report that NE upregulated IL-8 expression by cell detachment and/or deformation (38), our data suggest that cell detachment alone is not sufficient to regulate *MUC5AC* expression.

A major finding in this report was that in A549 cells, NE increased *MUC5AC* mRNA expression by increasing mRNA stability (Fig. 7). To our knowledge, this is the first report of mucin gene regulation by this mechanism. Another secreted mucin, *MUC2,* has a long half-life in colon cancer cells (22). *MUC2* is posttranscriptionally regulated by forskolin and phorbol ester (43); however, the mechanism of posttranscriptional regulation is not due to increased mRNA stability. These reports support the concept that posttranscriptional regulation of mucin genes may be an important regulatory mechanism in disease states.

The molecular mechanisms for mRNA stability have just begun to be elucidated. The half-lives of some mammalian mRNAs are determined by protein binding to 3′-untranslated regions containing instability sequences such as AU-rich sequences or iron response elements (36). There are three short potential AU-rich sequences in the 3′-untranslated region reported for *MUC5AC* (26, 32) that may be related to mRNA stability. Further studies are needed to explore the role of these sequences in controlling NE enhancement of *MUC5AC* mRNA stability.

Several studies demonstrated that when elastase is introduced into the trachea of rodents, at first there is increased mucin granule secretion, and then over hours to days, there is an accumulation of granules in secretory cells (14, 15) and secretory cell metaplasia (13). In this report, NE treatment increased the intracellular concentration of MUC5AC glycoprotein compared with that in control vehicle-treated A549 cells. The digestion of MUC5AC glycoprotein in A549 cell medium is consistent with previous reports of mucin degradation by elastase in hamster airway cells (24) and CF mucins (34). MUC5AC was detectable in NE-treated A549 cell medium (Fig. 8) but not in NE-treated cell-free A549 cell-conditioned medium (Fig. 9). These experiments suggest that there is replacement of digested MUC5AC in the medium by cell secretion and/or that A549 cells rapidly inactivate NE by producing an anti-protease (37, 44).

There is a growing body of evidence that inflammatory mediators increase expression of mucin genes. Tumor necrosis factor-α upregulates expression of *MUC2* in NCI-H292 cells, a pulmonary mucoepidermoid carcinoma cell line (27). *Pseudomonas aeruginosa* exoproducts increase expression of *MUC2* and *MUC5AC* in two cancer cell lines, HM3 and NCI-H292, by transcriptional regulation (28–30). *MUC5AC* mRNA expression is increased in transgenic mice overexpressing interleukin-4 (41). Both MUC5AC mRNA and glycoprotein are induced in rat airways by exposure to acrolein, an aldehyde found in cigarette smoke (11). In inflammatory airway diseases, a combination of NE and these mediators may be present. Together, these mediators may act synergistically to upregulate expression of several mucin genes, resulting in increased mucin production.

In summary, NE treatment resulted in increased stability of *MUC5AC* mRNA expression in airway epithelial cells by a mechanism requiring serine proteolytic activity. Furthermore, NE treatment increased production of MUC5AC glycoprotein in A549 cell lysates. This study provides an important link in understanding the pathogenesis of NE-induced mucin production.

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