The Signaling Pathway Involved in Neutrophil Elastase–Stimulated *MUC1* Transcription

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We previously reported that neutrophil elastase (NE) stimulated *MUC1* gene expression in A549 lung epithelial cells through binding of Sp1 to the *MUC1* promoter element. The current study was undertaken to elucidate the complete signaling pathway leading to Sp1 activation. Using a combination of pharmacologic inhibitors, dominant-negative mutant, RNA interference, and soluble receptor blocking techniques, we identified a protein kinase C δ (PKC δ) $\rightarrow$ dual oxidase 1 (Duox1) $\rightarrow$ reactive oxygen species (ROS) $\rightarrow$ TNF- α -converting enzyme (TACE) $\rightarrow$ TNF- α $\rightarrow$ TNF receptor (TNFR)1 $\rightarrow$ extracellular signal-regulated kinase (ERK)1/2 $\rightarrow$ Sp1 pathway as responsible for NE-activated *MUC1* transcription. This cascade was identical up to the point of TACE with the signaling pathway previously reported for NE-stimulated MUC5AC production. However, unlike the MUC5AC pathway, TNF- α , TNFR1, ERK1/2, and Sp1 were unique components of the *MUC1* pathway. Given the anti-inflammatory role of *MUC1* during airway bacterial infection, up-regulation of *MUC1* by inflammatory mediators such as NE and TNF- α suggests a crucial role for *MUC1* in the control of excessive inflammation during airway bacterial infection.

Keywords: neutrophil elastase; *MUC1*; signaling; airway

MUC1 (*MUC1* in human and *Muc1* in nonhuman species) is a transmembrane mucin-like glycoprotein expressed on the surface of epithelial cells lining various mucosal surfaces, including the respiratory tract (1–3). We recently showed that mice deficient in *Muc1* expression exhibited both enhanced airway inflammation and bacterial clearance during *Pseudomonas aeruginosa* (PA) airway infection (4), suggesting an anti-inflammatory role for *Muc1* and the importance of *Muc1* levels during airway bacterial infection. The anti-inflammatory activity of *MUC1*/*Muc1* after treatment with bacterial products also was demonstrated in various *in vitro* studies (4). How the levels of *MUC1*/*Muc1* are regulated during airway bacterial infection is unknown, but recent evidence indicates the involvement of neutrophil elastase (NE) (5).

NE is present in micromolar concentrations in airway surface liquid of patients with cystic fibrosis and patients with chronic bronchitis (6, 7), and stimulates mucin release and mucin gene expression by cultured airway epithelial cells through the proteolytic activity (8, 9). Using a co-culture system containing hamster neutrophils and tracheal surface epithelial (TSE) cells, we

CLINICAL RELEVANCE

This article describes, for the first time, a complete signaling pathway involved in neutrophil elastase-induced *MUC1* transcription, which is very important for basic science and highly useful for clinical medicine.

demonstrated that activation of the neutrophils by fMLP or cytochalasin B resulted in the stimulation of mucin release (10), indicating that the concentrations of NE produced by activation of neutrophils are sufficient to release mucins. To date, NE is the most potent mucin secretagogue that has been described (11).

In addition to gel-forming mucins such as MUC5AC, NE also induces the expression of the membrane-bound *MUC1* and *MUC4* mucins (5, 12). Our prior report showed that *MUC1* protein synthesis by A549 cells was enhanced after treatment with NE, and this effect was blocked by pretreatment with actinomycin D or cycloheximide (5). By real-time RT-PCR, *MUC1* mRNA levels, but not transcript stability, were increased by NE. Using a *MUC1* gene promoter-luciferase reporter assay, NE increased the activity of the *MUC1* promoter, and this effect was completely blocked by mithramycin A, an inhibitor of the Sp1 transcription factor. By deletion analysis, an Sp1-binding site located between nucleotides –99 and –90 relative to the transcription initiation site of the *MUC1* promoter was identified as responsible for NE-induced *MUC1* expression. Finally, by electrophoretic mobility shift assay, we demonstrated that NE increased Sp1 binding to this segment of the *MUC1* promoter. Collectively, these observations indicated that increased *MUC1* protein synthesis induced by elastase occurred as a consequence of elevated gene transcription mediated by Sp1 binding to a specific *cis* regulatory element. In support of our studies, Morris and Taylor-Papadimitriou (13) and Kovarik and coworkers (14, 15) reported that the Sp1 site at –99/–90 was crucial for cell- and tissue-specific regulation of *MUC1* gene expression.

The most recent evidence with respect to NE suggests that MUC5AC production is stimulated through a protein kinase C δ (PKC δ) $\rightarrow$ dual oxidase 1 (Duox1) $\rightarrow$ reactive oxygen species (ROS) $\rightarrow$ TNF- α -converting enzyme (TACE) $\rightarrow$ transforming growth factor- α (TGF- α) $\rightarrow$ epidermal growth factor receptor (EGFR) $\rightarrow$ mitogen-activated protein kinase (MAPK) pathway (16–18). In light of the previously determined signaling pathway identified for NE-stimulated MUC5AC production, the current study was undertaken to elucidate the similarities, if any, between the NE-induced *MUC1* and MUC5AC pathways. Our results indicated that the proximal components of the NE-stimulated *MUC1* and MUC5AC pathways were identical (PKC δ $\rightarrow$ Duox1 $\rightarrow$ ROS $\rightarrow$ TACE). However, at the point of TACE, the two pathways diverged, with the *MUC1* branch being mediated by TNF- α $\rightarrow$ TNFR1 $\rightarrow$ ERK1/2 $\rightarrow$ Sp1.

(Received in original form March 3, 2007 and in final form May 16, 2007)

This work was supported by National Heart, Lung, and Blood Institute Grant RO1 HL-47125 (K.C.K.), National Institute of Environmental Health Sciences Grant R21 ES-013483 (E.P.L.), and a research grant from the Cystic Fibrosis Foundation (E.P.L.).

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Am J Respir Cell Mol Biol Vol 37, pp 691–698, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2007-00720C on June 28, 2007

Internet address: www.atsjournals.org

MATERIALS AND METHODS

Materials

All reagents were from Sigma (St. Louis, MO) unless otherwise indicated. NE was from Elastin Products (Owensville, MO). TAPI-1 was from Peptides International (Louisville, KY). SB202190 and SP600125 were from EMD Biosciences (La Jolla, CA). U0126 was from Cell Signaling (Beverly, MA). A Duox1 siRNA and negative control RNA were from Ambion (Austin, TX). SN-50 and a negative control peptide were from Alexis Biochemicals (Lausen, Switzerland). A mutant I κ B- α -pCMV4 plasmid (mI κ B- α) was kindly provided by Dr. R. Srivastava (University of Texas Health Center, Tyler, TX).

Cell Culture

A549 cells (ATCC, Manassas, VA) were seeded at 5.0×10^4 cells/well in 24-well plates in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin (Invitrogen) and incubated at 37°C in a humidified CO₂ incubator. In some experiments, A549 cells were seeded at 3.0×10^4 cells/well in 24-well plates for transfection with the Duox1 siRNA. Normal human bronchial epithelial (NHBE) cells (Cambrex, Walkersville, MD) were cultured in bronchial epithelial growth medium plus supplements (Cambrex) at a density of 2.0×10^4 cells/well in 24-well plates precoated with 5.0 μ g/well of human placenta collagen IV.

Real Time RT-PCR

Quantitative RT-PCR was performed as described (5). Briefly, total RNA was isolated using RNeasy (Qiagen, Valencia, CA), and 2 μ g were reverse transcribed using the iScript cDNA Synthesis kit (BioRad, Hercules, CA) in a total volume of 20 μ l. PCR was performed using the Taqman probes and iQ Supermix (BioRad) according to the manufacturer's instructions. The MUC1 forward primer was 5'-TCAGCTTCTACTCTGGTGACAA-3', the reverse primer was 5'-ATTGAGAA TGGAGTGCTCTTGCT-3', and the probe was 5'-FAM-TCTGCCAG GGCTACCACAACCC-BHQ-1-3' (Integrated DNA Technologies, Inc., Coralville, IA). The GAPDH forward primer was 5'-AGCCTCAA GATCATCAGCAATG-3', the reverse primer was 5'-GTTGTCATG GATGACCTTGGC-3', and the probe was 5'-Hex-CCTGCACCAC CAATGCTTAGCAC-BHQ-1-3' (Integrated DNA Technologies, Inc.). PCR cycles ($n = 40$) consisted of a 15-second melting at 95°C, followed by annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.

Duox1 siRNA and mI κ B- α Transfection of A549 Cells

At 24 hours after seeding, A549 cells were transfected with 100 nM of the Duox1 siRNA (#214819; Ambion) or the scrambled RNA as a negative control (negative control #1; Ambion), or 400 ng/ml of mI κ B- α -pCMV4 or pCMV4 empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, RNAs were mixed with 1.0 μ l of lipid reagent and diluted with OPTI-MEM (Invitrogen) to 100 μ l. After 20 minutes of incubation at room temperature, the RNA–DNA–lipid complexes in a final volume of 400 μ l were added to each well and incubated for 48 hours. Knockdown of *Duox1* gene expression was confirmed by RT-PCR using the following primers: forward, 5'-CGACATTGAGACTGAGTTGA-3'; reverse, 5'-CTGGAATGAC GTTACCTTCT-3'.

MUC1 Promoter-Luciferase Reporter Assay

At 24 hours after seeding, or 48 hours after transfection with the Duox1 siRNA or mI κ B- α plasmid, A549 cells were transfected with 800 ng/well of the pGL2b empty vector or the MUC1 promoter-pGL2b plasmid plus 10 ng of the phRL-TK internal control plasmid (Promega, Madison, WI) encoding *Renilla* luciferase. After transfection, the cells were washed, starved for 24 hours in serum-free DMEM, and treated for 24 hours with PBS or 100 nM (2.6 U/ml) of NE. In some experiments, the transfected cells were pretreated for 1 hour with rottlerin (PKC δ inhibitor), glutathione (ROS inhibitor), TACE protease inhibitor-1 (TAPI-1), AG1478 (EGFR inhibitor), U0126 (MEK inhibitor), SB202190 (p38 inhibitor), or SP600125 (JNK inhibitor) before NE treatment. Luciferase activity was determined using the Dual luciferase assay system

(Promega) according to the manufacturer's instructions and a microplate luminometer (Lmax; Molecular Devices Corp., Sunnyvale, CA). Luciferase activity driven by the MUC1 promoter was normalized to the internal control by calculating the ratio of firefly luciferase activity to *Renilla* luciferase activity of each sample and expressed as the percentage of MUC1 promoter activity relative to control samples.

Immunoprecipitation and Immunoblotting

A549 cells were seeded at 8×10^5 cells/well into 6-well plates, and incubated for 24 hours in DMEM plus 10% FBS. The cells were washed, starved for 1 hour in serum-free DMEM, and treated for 0, 15, 30, or 60 minutes with PBS or 100 nM of NE. At each time point, the cells were washed with PBS, immediately extracted with lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2.0% SDS, 5.0% glycerol, 1.0% protease inhibitor cocktail, 2.0 mM PMSF) at 4°C, and equal protein aliquots (20 μ g) were resolved on 10% SDS-polyacrylamide gels. In some experiments, cell lysates were immunoprecipitated with MUC1 antibody GP1.4 (Biomed, Foster City, CA) or Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) plus protein-A-agarose (Invitrogen) and precipitated proteins applied to SDS-gels. Resolved proteins were transferred to PVDF membrane, and the membrane was blocked for 1 hour at 22°C in TBS-T (10 mM Tris-HCl, pH 7.5, 136 mM NaCl, 2.0 mM KCl, 0.1% Tween 20) containing 5% nonfat dry milk. After blocking, the membrane was washed with TBS-T, and reacted with antibodies to phospho-ERK1/2 (diluted 1:2,000 with TBS-T containing 5% BSA; Cell Signaling), phospho-p38 (1:1,000; Cell Signaling), phospho-JNK (1:1,000; Cell Signaling), phospho-threonine (1:1,000; Research Diagnostics, Concord, MA), β -tubulin (1:10,000; Sigma), or MUC1 (1:1,000). After primary antibody reactions, the membrane was washed with TBS-T, incubated for 1 hour at 22°C with horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:20,000 with TBS-T containing 2% nonfat dry milk; Kirkegaard and Perry Laboratories, Gaithersburg, MD) or anti-rabbit IgG (1:10,000; KPL) and developed with chemiluminescent substrate (Pierce, Rockford, IL). The bands were identified by comigration of prestained protein size markers (BioRad).

Measurement of Cytotoxicity

Cytotoxicity of drug inhibitors was assessed by light microscopy for changes in cell shape and cell floating, and lactic dehydrogenase (LDH) release. Briefly, confluent A549 cells were treated with 5 μ M of rottlerin, 10 mM of glutathione (GSH), 100 μ M of TAPI-1, 10 μ M of AG1478, 10 μ M of U0126, 10 μ M of SB202190, 10 μ M of SP600125, or 50 μ g/ml of SN-50 for 1 hour, the cultures were washed once and replenished with fresh medium containing the same concentrations of the above inhibitors, and the incubation continued for 24 hours before collecting spent media for LDH assay. In the case of transfected cells, 45–50% confluent monolayers were transfected with 100 nM of the Duox1 siRNA or 400 ng/ml of the mI κ B- α plasmid as described above before collecting spent media. LDH activities in both spent media and cell lysates were measured as previously described (5). None of the inhibitors tested showed significant cytotoxicity on the basis of the above three criteria (data not shown).

Statistical Analysis

Differences between means $\pm$ SEM values were assessed using the Student's t test for unpaired samples and considered significant at $P < 0.05$.

RESULTS

NE Increases MUC1 mRNA Expression by Primary NHBE Cells

Previously, we reported that NE treatment of A549 cells induced *MUC1* gene and protein expression (5). To verify a similar effect in primary airway cells, NHBE cells were treated for 24 hours with PBS or 50, 100, or 200 nM of purified NE. MUC1 mRNA levels were determined by real-time RT-PCR, and normalized to GAPDH mRNA. As shown in Figure 1, NE induced a dose-dependent increase in MUC1 transcripts in cultured NHBE cells that was identical to that seen in A549 cells. Maximum levels of MUC1 mRNA were seen with 100 nM of elastase.

Figure 1. Neutrophil elastase (NE) increases *MUC1* transcript levels in primary normal human bronchial epithelial (NHBE) cells. Confluent NHBE and A549 cells were treated for 24 hours with PBS or 50, 100, or 200 nM of NE, and *MUC1* mRNA levels were measured by real-time RT-PCR. *MUC1* mRNA levels were normalized to GAPDH mRNA levels. Each data point represents the mean $\pm$ SEM ($n = 3$). * $P < 0.05$ compared with PBS-treated (0 nM NE) cells. The results were reproduced in separate experiments.

NE Increases *MUC1* Transcription through PKC δ , Duox1, ROS, and TACE

Because prior studies had focused on A549 cells in elucidating the mechanism responsible for NE-stimulated *MUC5AC* expression (9, 16), we focused our efforts on elucidating the *MUC1* pathway using the same cell culture system. PKC δ activation has been linked with NE-stimulated *MUC5AC* production through a Duox1 $\rightarrow$ ROS $\rightarrow$ TACE pathway (18). Therefore, we determined whether or not PKC δ was involved in NE-induced *MUC1* transcription using rottlerin, a specific PKC δ inhibitor (19). A549 cells were transfected with a *MUC1* promoter-luciferase reporter plasmid or the pGL2b empty vector, pretreated with the various concentrations of rottlerin, washed, treated with NE or PBS, and relative luciferase activity was determined. As shown in Figure 2A, 5.0 μ M of rottlerin completely blocked NE-driven luciferase activity, compared with PBS-treated cells. None of the cell cultures transfected with the pGL2b empty vector exhibited luciferase activity (data not shown).

ROS are oxygen-containing molecules that are more reactive than ground-state molecular oxygen (e.g., O_2^- , OH^- , and H_2O_2). ROS are generated by Duox1, a homolog of NADPH oxidase, in human airway epithelial cells (17). Because both Duox1 and ROS were previously described as being involved in NE-stimulated *MUC5AC* production (17, 20), we determined their roles in *MUC1* promoter activation provoked by NE. A549 cells cotransfected with a Duox1 siRNA plus the *MUC1*-luciferase plasmid exhibited significantly reduced luciferase activity compared with cells transfected with a nontargeting control RNA (Figure 2B). Duox1 siRNA showed a greater than 95% inhibition of Duox1 mRNA expression in A549 cells based on RT-PCR, whereas that for control siRNA showed no inhibition (data not shown). In addition, A549 cells were pretreated with the ROS scavengers, reduced GSH or 1,3-dimethyl-2-thiourea (DMTU), and *MUC1* promoter activity was determined. Both compounds effectively inhibited NE-dependent luciferase activity, compared with PBS-treated cells (Figures 2C1 and 2C2).

To determine the role of TACE, A549 cells were transfected with the *MUC1*-luciferase reporter plasmid, pretreated with TAPI-1 (a TACE inhibitor), and NE-stimulated luciferase activity was determined. As shown in Figure 2D, TAPI-1 inhibited NE-induced *MUC1* promoter activity in a dose-dependent manner, such that at the highest concentration tested there was complete inhibition of luciferase activity. Taken together, the results presented in Figure 2 indicated that NE stimulated *MUC1* transcription through a PKC δ $\rightarrow$ Duox1 $\rightarrow$ ROS $\rightarrow$ TACE pathway.

Figure 2. NE-induced *MUC1* transcription involves protein kinase C δ (PKC δ), dual oxidase 1 (Duox1), reactive oxygen species (ROS), and TNF- α -converting enzyme (TACE). (A) Involvement of PKC δ . A549 cells were transfected with the pGL2b empty vector or the *MUC1*-pGL2b plasmid, pretreated for 30 minutes with the indicated concentrations of rottlerin, treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity (firefly/*Renilla*) was determined. (B) Involvement of Duox1. A549 cells were transfected with 100 nM of a Duox1 siRNA or nontargeting control RNA, the cells were incubated for 48 hours, transfected with 800 ng/well of pGL2b empty vector or *MUC1*-pGL2b plasmid, incubated for 24 hours, treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity was determined. (C) Involvement of ROS. A549 cells were transfected with the pGL2b empty vector or the *MUC1*-pGL2b plasmid, pretreated for 30 minutes with the indicated concentrations of glutathione (GSH) (C1) or 1,3-dimethyl-2-thiourea (DMTU) (C2), treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity was determined. (D) Involvement of TACE. A549 cells were transfected with the pGL2b empty vector or the *MUC1*-pGL2b plasmid, pretreated for 30 minutes with the indicated concentrations of TAPI-1, treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity was determined. Open bars, PBS; solid bars, NE. Each bar represents the mean $\pm$ SEM ($n = 3$). * $P < 0.05$ comparing *MUC1*-pGL2b-transfected cells treated with NE with the PBS control. The results were reproduced in separate experiments.

TNFR but Not EGFR Is Involved in NE-Stimulated *MUC1* Transcription

TACE is a Zn $^{+2}$ -dependent metalloprotease that cleaves multiple substrates, including TNF- α and TGF- α , from their membrane-bound to soluble forms (21). TNF- α is a ligand for TNFR, whereas TGF- α is a ligand for EGFR. Takeyama and colleagues (22) showed that treatment of NCI-H292 cells with TGF- α induced *MUC5AC* production. Moreover, AG1478, a selective inhibitor of EGFR tyrosine kinase activity, blocked NE-induced *MUC5AC* gene expression. Therefore, we next conducted a series of experiments to determine the relative roles of EGFR and TNFR in NE-induced *MUC1* expression. To determine whether or not EGFR regulated *MUC1* transcription, A549 cells transfected

with the MUC1-luciferase plasmid were treated with AG1478 and relative luciferase activity determined after NE treatment. Even at the highest concentration tested, AG1478 did not affect NE-induced MUC1 transcription (Figure 3A), suggesting that EGFR was not involved in stimulation of MUC1 gene expression by the elastase. As a control, 10 μ M of AG1487 completely blocked EGF-mediated ERK phosphorylation in A549 cells, indicating that EGFR activity was inhibited by this level of the inhibitor (data not shown).

Based on the above results, we hypothesized that NE stimulated MUC1 expression through TACE-mediated production of TNF- α and activation of TNFR. First, we determined whether or not TNF- α increased MUC1 expression in A549 cells. As shown in Figure 3B, TNF- α treatment significantly increased MUC1 promoter activity after 12 and 24 hours of treatment, compared

with PBS-treated cells. MUC1 protein levels detected by combined immunoprecipitation/immunoblot analysis also were dramatically increased in A549 cells after treatment with TNF- α compared with the PBS control (Figure 3C).

TNF- α interacts with two receptors, TNFR1 and TNFR2, but lung epithelial cells were reported to express only TNFR1 (23). To determine if TNFR1 was involved in NE-stimulated MUC1 expression, A549 cells were transfected with the MUC1-luciferase plasmid, treated with NE in the presence of antagonistic soluble TNFR1, and relative luciferase activity was determined. As shown in Figure 3D, soluble TNFR1 completely inhibited NE-driven MUC1 promoter activity compared with cells treated with the vehicle control.

NE Increases MUC1 Transcription through ERK1/2

Many studies have demonstrated that MAPKs are involved in regulating MUC5AC gene expression (24–28). Therefore, we determined whether or not ERK1/2, p38, and JNK regulated MUC1 transcription using pharmacologic inhibitors of each kinase. Pretreatment of A549 cells transfected with the MUC1-luciferase plasmid with U0126, a specific MEK1/2 inhibitor, completely blocked NE-induced stimulation of MUC1 transcription (Figure 4A). In contrast, SB202190 (p38 inhibitor) and SP600125 (JNK inhibitor) did not diminish NE-stimulated MUC1 promoter activity compared with cells treated with PBS. Moreover, immunoblot analysis of lysates of NE-treated A549 cells revealed increased levels of phospho-ERK1/2 (Figure 4B) compared with PBS-treated cells. In conclusion, MEK1/2 (and by inference ERK1/2), but not p38 or JNK, were involved in NE-induced MUC1 transcription. Finally, to verify that the upstream components of the NE-stimulated MUC1 pathway were connected to downstream ERK1/2 activation, A549 cells were pretreated with rottlerin or GSH, or were transfected with the Duox1 siRNA and NE-induced ERK1/2 phosphorylation determined. As shown in Figure 5, inhibition of PKC δ , Duox1, or

Figure 3. TNF receptor (TNFR) but not epidermal growth factor receptor (EGFR) is involved in NE-stimulated MUC1 transcription. (A) EGFR is not involved in NE-stimulated MUC1 transcription. A549 cells were transfected with the pGL2b empty vector or the MUC1-pGL2b plasmid, pretreated for 30 minutes with the indicated concentrations of AG1478, treated for 24 hours with PBS or 100 nM NE, and relative luciferase activity was determined. (B) TNF- α increases MUC1 transcription. A549 cells were transfected with the pGL2b empty vector or the MUC1-pGL2b plasmid, treated for 12 or 24 hours with PBS or 50 ng/ml of TNF- α , and relative luciferase activity was determined. (C) TNF- α increases MUC1 protein expression. Nontransfected A549 cells were treated for 24 hours with PBS or 50 ng/ml of TNF- α and MUC1 protein in cell lysates analyzed by immunoprecipitation/immunoblotting with MUC1 antibody (left panel). The blot in the left panel of C was stripped and reprobed with peroxidase-conjugated goat anti-mouse IgG antibody alone (i.e., no primary antibody) to demonstrate equal protein loading and membrane transfer efficiencies. (D) NE increases MUC1 transcription through TNFR1. A549 cells were transfected with the pGL2b empty vector or the MUC1-pGL2b plasmid, treated for 24 hours with PBS or 100 nM of NE in the presence of the indicated concentrations of soluble TNFR1, and relative luciferase activity was determined. Open bars, PBS; solid bars, NE. Each bar represents the mean $\pm$ SEM ($n = 3$). * $P < 0.05$ comparing MUC1-pGL2b-transfected cells treated with NE with the PBS control. The results were reproduced in separate experiments.

Figure 4. NE increases MUC1 transcription through ERK1/2. (A) A549 cells were transfected with the pGL2b empty vector or the MUC1-pGL2b plasmid, pretreated for 30 minutes with the indicated concentrations of U0126, SB202190, or SP600125, treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity was determined. Open bars, PBS; solid bars, NE. Each bar represents the mean $\pm$ SEM ($n = 3$). * $P < 0.05$ comparing MUC1-pGL2b-transfected cells treated with NE with the PBS control. (B) A549 cells were treated for 24 hours with PBS or 100 nM of NE and cell lysates were examined by immunoblotting with antibodies against phospho-ERK1/2 or β -tubulin. The results were reproduced in separate experiments.

Figure 5. Inhibition of Duox1, PKC δ , or ROS blocks NE-stimulated ERK1/2 activation. A549 cells were pretreated for the indicated times with PBS, 5.0 μ M of rottlerin (A), 10 mM of GSH (C), or 25 μ M of soluble TNFR1 (D), or were transfected with 100 nM of the PKC δ siRNA (B). After pretreatments, the cells were treated for 24 hours with PBS or 100 nM NE and cell lysates were examined by immunoblotting with antibodies against phospho-ERK1/2 or β -tubulin. The results were reproduced in separate experiments.

ROS decreased phospho-ERK1/2 levels after NE treatment, compared with the respective control-treated cells.

NE Induces Threonine Phosphorylation of Sp1

Our previous report indicated that treatment of A549 cells with NE induced *MUC1* gene expression through binding of Sp1 to the *MUC1* promoter (5), corroborating the observations of previous investigators (13–15). However, other studies have suggested a role for NF- κ B in TNF- α -induced *MUC1* expression (29) as well as *MUC5AC* expression (30). Therefore, we performed a series of experiments to further analyze the roles of Sp1 and NF- κ B in *MUC1* expression induced by NE. First, A549 cells were treated with PBS or 100 nM of NE and cell lysates were examined by immunoblotting for phosphorylation of Sp1 as an indicator of activation of the transcription factor. NE treatment resulted in threonine phosphorylation of Sp1 after 20, 30, and 60 minutes of protease treatment (Figure 6A). Furthermore, NE-induced Sp1 phosphorylation was blocked by pretreatment with U0126, indicating that MAPK activation was upstream of Sp1 activation (Figure 6B). Although it has been shown that other MAPKs such as p38 and JNK also can phosphorylate Sp1 (31, 32), the type (serine versus threonine) and/or the site(s) of Sp1 phosphorylation by these MAPKs seems to be different from

Figure 6. NE increases threonine phosphorylation of Sp1, but not NF- κ B activation. (A) A549 cells were treated for 24 hours with PBS or 100 nM of NE, equal protein aliquots of cell lysates were immunoprecipitated with anti-Sp1 antibody, and immunoblotted with anti-phospho-threonine antibody (upper panel) or anti-Sp1 antibody (lower panel). (B) A549 cells were pretreated for 30 minutes with PBS or 10 μ g/ml of U0126, treated for 24 hours with PBS or 100 nM of NE and phospho-Sp1 levels determined as in A. (C) A549 cells were transfected with the MUC1-pGL2b alone, or cotransfected with MUC1-pGL2b plus mlκB- α -pCMV4 or pCMV4 alone, treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity was determined. (D) A549 cells were transfected with the MUC1-pGL2b plasmid, pretreated for 30 minutes with PBS or 50 μ g/ml of SN-50 or the mock control peptide, treated for 24 hours with PBS or 100 nM of NE, and relative luciferase activity was determined. Open bars, PBS; solid bars, NE. Each bar represents the mean $\pm$ SEM ($n = 3$). * $P < 0.05$ comparing cells treated with NE with the respective PBS controls. The results were reproduced in separate experiments.

that by ERK based on their differential effects on *MUC1* transcription as shown in Figure 4.

Next, to determine the role of NF- κ B, A549 cells were cotransfected with plasmids containing the MUC1-luciferase reporter construct and mlκB- α , a mutant form of the IκB- α that blocks phosphorylation and degradation of wild-type IκB- α , thereby retaining NF- κ B in an inactive complex (33). If NE activated *MUC1* through NF- κ B, we predicted that mlκB- α would decrease *MUC1* expression. However, NE-stimulated *MUC1* promoter activity in cells transfected with mlκB- α was equal to that seen in cells transfected with the empty pCMV4 vector (Figure 6C). Moreover, pretreatment of cells with SN-50 to inhibit NF- κ B translocation to the nucleus had no effect on NE-stimulated *MUC1* expression compared with a negative control peptide (Figure 6D). As positive controls, both mlκB- α and SN-50 inhibited NE-induced IL-8 expression (data not shown). Collectively, the

results presented in Figures 1–7 indicated that NE stimulated *MUC1* transcription through a $\text{PKC}\delta \rightarrow \text{Duox1} \rightarrow \text{ROS} \rightarrow \text{TACE} \rightarrow \text{TNF-}\alpha \rightarrow \text{TNFR1} \rightarrow \text{ERK1/2} \rightarrow \text{Sp1}$ pathway.

DISCUSSION

This study identified a signaling pathway in human airway epithelial cells responsible for NE-stimulated *MUC1* gene expression. While the proximal part of the *MUC1* pathway was identical to that previously reported for NE-induced *MUC5AC* transcription ($\text{PKC}\delta \rightarrow \text{Duox1} \rightarrow \text{ROS} \rightarrow \text{TACE}$), the downstream components of the two pathways distal to TACE were different (*MUC1*: $\text{TNF-}\alpha \rightarrow \text{TNFR1} \rightarrow \text{ERK1/2} \rightarrow \text{Sp1}$; *MUC5AC*: $\text{TGF-}\alpha \rightarrow \text{EGFR} \rightarrow \text{MAPK} \rightarrow \text{NF-}\kappa\text{B}$) (34, 35). Thus, TACE is situated at a branching point leading to either *MUC1* or *MUC5AC* expression (Figure 7). We hypothesize that such bipartite pathways for mucin gene expression containing common and unique signaling components may have important implications for regulating mucus production during inflammatory responses invoked by airway neutrophils.

Neutrophils are major effector cells in the clearance of bacteria from the lungs. Their anti-microbial function is directly mediated through phagocytosis and indirectly by release of bacteriostatic and bactericidal mediators (36). Among these mediators is NE, a serine protease stored in azurophil granules and released extracellularly during the course of an infection (37). Studies using NE knockout ($\text{NE}^{-/-}$) mice showed that elastase is required for host defense against gram-negative bacteria and acts via proteolysis of outer membrane proteins (38, 39). Paradoxically, however, $\text{NE}^{-/-}$ mice are not at increased risk of spontaneous bacterial infection. Some evidence suggests that NE degrades the extracellular matrix, thereby promoting neutrophil migration into the lumen of the lung (40). However, most of our knowledge regarding NE revolves around its capacity to cause lung tissue destruction, as is seen in emphysema that is due to deficiency of α_1 -antitrypsin production. NE inhibitors have been shown to decrease pulmonary inflammation and lung edema in animal models of acute inflammation (41).

Figure 7. Schematic illustration of the NE-stimulated signaling pathways leading to *MUC1* and *MUC5AC* gene expression in airway epithelial cells. The question mark indicates the unknown identity of the cell surface receptor that binds to NE.

An important part of the mechanism through which NE influences respiratory tract inflammation involves up-regulation of mucin secretion. For example, Park and coworkers (42) showed that NE treatment of well-differentiated NHBE cells maintained at an air–liquid interface increased the release of *MUC5AC* and *MUC5B* glycoproteins via an intracellular signaling pathway involving phosphorylation of the MARCKS protein by $\text{PKC}\delta$. Aoshiba and colleagues (43) demonstrated that stimulation of airway epithelial cells with NE produced ROS. Fischer and Voynow (16) reported that NE treatment of NHBE and A549 cells induced ROS production, leading to *MUC5AC* gene expression. The effect of NE was also shown to occur *in vivo* since administration of elastase to Balb/c mice induced *Muc5ac* mRNA and protein expression and goblet cell metaplasia (44). Shao and Nadel (17) showed that siRNA knockdown of Duox1 inhibited the production of ROS. Lemjabbar and coworkers (45) demonstrated that ROS activated TACE in airway epithelial cells. Shao and Nadel (18) also reported that NE induced *MUC5AC* production via a cascade involving PKC, ROS, and TACE. Finally, Kohri and colleagues (46) published that treatment of NCI-H292 human respiratory epithelial cells with NE stimulated the production of *MUC5AC* glycoprotein by $\text{TGF-}\alpha$ -dependent activation of EGFR, and this effect was blocked by NE inhibitors.

While it is clear that *MUC5AC* is the predominant gel-forming mucin induced by NE in airway epithelial cells, and the pathway leading to its induction has been well studied, no reports have described about the transcriptional regulation of the *MUC5AC* gene promoter by NE. Studies by Voynow and coworkers (9) indicated that NE increased the stability of *MUC5AC* gene transcripts, but did not increase transcription of the mucin gene or translation of its mRNA. Investigations of *MUC5AC* gene expression in response to stimulation by other factors, such as bacterial exoproducts, EGF, $\text{TNF-}\alpha$, and PMA, have implicated activation of the $\text{NF-}\kappa\text{B}$ and Sp1 transcription factors (26, 27, 30, 34). In the case of *MUC1*, several previous studies identified positive regulatory elements in its gene promoter (47–49). Our prior report showed that *MUC1* protein synthesis by A549 cells was enhanced after treatment with NE, and this effect was blocked by pretreatment with actinomycin D or cycloheximide, suggesting that the protease acted by increasing new gene expression (5).

What is the significance of upregulation of *MUC1* by NE during airway inflammation resulting from airway bacterial infection? Given the anti-inflammatory role of *MUC1*/*Muc1* during *P. aeruginosa* infection (4), it seems that up-regulation of *MUC1*/*Muc1* by NE may be crucially important in controlling inflammation during bacterial infection. In fact, our recent preliminary data from *in vivo* experiments indicate that the levels of *Muc1* of uninfected mouse lungs were relatively low but increased gradually after *P. aeruginosa* infection (data not shown). The possible roles of NE as well as $\text{TNF-}\alpha$ in controlling airway inflammation in the context of airway *Muc1* levels is currently under investigation in our laboratory.

An important question that remains to be answered is the identity of the cell surface receptor through which NE mediates its effects. Immunocytochemical and immunoelectron microscopic studies revealed that treatment of hamster tracheas with NE resulted in localization of the protease only on the apical surface of the epithelial cells, and not basolaterally or intracellularly (50). These results have led to the proposal that NE-stimulated mucin secretion is initiated by interaction of elastase with a cell surface receptor. Indeed, evidence for the existence of a NE-specific receptor on alveolar macrophages and leukocytes has been described (51, 52). Although some laboratories have presented evidence supporting protease-activated receptors (PARs)

as putative NE receptors (53), convincing and reproducible data are not available. However, several lines of evidence are inconsistent with NE signaling through PARs. First, Dulon and colleagues (54) reported that NE inactivated PAR-2 in A549 and 16HBE14o⁻ cells, and prevented subsequent activation by trypsin, a known agonist for PAR-2. Second, Chokki and coworkers (55) showed that human airway trypsin-like protease, but not a PAR-2-activating peptide, induced *MUC2* and *MUC5AC* gene expression in NCI-H292 cells. Finally, PAR-2 expression was restricted to the basolateral surface of airway epithelial cells (56), which would render it inaccessible to NE released by luminal neutrophils on the apical side of respiratory mucosa. Our recent unpublished data showed that while trypsin also induced *MUC1* transcription, its activity was less than 10% of that generated by NE (I. Kuwahara, unpublished data). Currently, studies in our laboratory are directed at identification of the cell surface receptor that NE activates during *MUC1* and *MUC5AC* gene expression.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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