Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells

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Mucus hypersecretion is a prominent manifestation in patients with chronic inflammatory airway diseases. MUC5AC mucin is a major component of airway mucus, and its expression is modulated by a TNF-α-converting enzyme (TACE)–EGF receptor pathway that can be activated by reactive oxygen species (ROS). Dual oxidase 1 (Duox1), a homologue of glycoprotein p91phox, is expressed in airway epithelium and generates ROS. We hypothesize that Duox1 activates TACE, cleaving pro-TGF-α into soluble TGF-α, resulting in mucin expression. To examine this hypothesis, we stimulated normal human bronchial epithelial cells and NCI-H292 airway epithelial cells with phorbol 12-myristate 13-acetate and with human neutrophil elastase. These stimuli induced TACE activation, TGF-α release, and mucin expression, effects that were inhibited by ROS scavengers, implicating ROS in TACE activation. Inhibition of epithelial NADPH oxidase or knockdown of Duox1 inactivated TACE and mucin expression. Furthermore, the PKC±/PKCθ inhibitor rottlerin prevented the effects induced by phorbol 12-myristate 13-acetate and human neutrophil elastase, suggesting that PKC± and PKCθ are involved in Duox1 activation. From these results, we conclude that Duox1 plays a critical role in mucin expression by airway epithelial cells through PKC±/PKCθ-Duox1-ROS-TACE-pro-ligand-EGF receptor cascade.

EGF receptor | mucus hypersecretion | reactive oxygen species | TNF-α-converting enzyme | type α TGF

Chronic inflammatory airway diseases are associated with mucus hypersecretion (1), which contributes to the morbidity and mortality of these diseases by plugging airways. MUC5AC mucin is a major component of airway mucus, and its expression is modulated by TNF-α-converting enzyme (TACE)-mediated EGF receptor (EGFR) transactivation; activated TACE cleaves EGFR proligand pro-TGF-α into soluble TGF-α, which binds to and activates EGFR, resulting in MUC5AC mucin expression (2–4).

Airway epithelial cells produce reactive oxygen species (ROS) (5). There is increasing evidence showing that ROS are involved in cell signaling (6). Recently, ROS scavengers have been shown to prevent cigarette-smoke-induced TACE activation, EGFR phosphorylation, and mucin production in airway epithelial cells (4), implicating ROS in the activation of the TACE-EGFR pathway.

ROS have long been known to be generated by NADPH oxidase (Nox) of phagocytes (Phox) (7). The core component of Nox is the catalytic subunit glycoprotein p91phox (8). Recently, a homologue of the gp91phox, dual oxidase 1 (Duox1), was identified in human airway epithelial cells and shown to generate ROS (9, 10). We hypothesize that Duox1 activates TACE via ROS generation, cleaving pro-TGF-α into soluble TGF-α, resulting in EGFR activation and mucin expression. To examine this hypothesis, we stimulated normal human bronchial epithelial (NHB) cells and NCI-H292 airway epithelial cells with phorbol 12-myristate 13-acetate (PMA) and human neutrophil elastase (HNE). PMA is a model inflammatory stimulus known to induce mucin via TACE-EGFR pathway in NCI-H292 airway epithelial cells (2), but the mechanism of activation of this pathway is unknown. HNE is a serine protease secreted by neutrophils and exists in high concentrations (1–20 μM) (11, 12) in the airway surface fluid of patients with chronic inflammatory airway diseases. HNE is known to cause mucin production via TGF-α-dependent EGFR activation (13), but it is unknown how this process is initiated. Here, we show that PMA and HNE activate Duox1 to produce ROS, activating TACE, which cleaves pro-TGF-α into soluble TGF-α, resulting in mucin expression in human airway epithelial cells.

Materials and Methods

Materials. Allopurinol, 1,3-dimethyl-2-thiourea (DMTU), n-propyl gallete (nPG), and PMA were purchased from Sigma. Apocynin, diphenyleneiodonium chloride (DPI), G6983, G6976, N1 monoethyl-L-arginine, and rottlerin were purchased from Calbiochem. Monoclonal p47phox and rabbit polyclonal p67phox antibodies were purchased from Santa Cruz Biotechnology. HNE was purchased from Elastin Products (St. Louis).

Cell Culture. Proliferating NHBE cells were cultured from Cambrex (Walkersville, MD). When cells reached 80% confluence, they were seeded at density of 2 × 104 per cm2 onto Costar transwell plate inserts (Fisher), growing in bronchial epithelial growth medium (Cambrex) supplemented with defined growth factors and retinoic acid (0.1 μg/liter) contained in the Single-Quot kit (Cambrex) at 37°C in a humidified, 5% CO2/95% air, water-jacketed incubator. After 7 days in an immersed culture condition, cell culture was switched to an air–liquid interface condition for 2–3 weeks.

NCI-H292 airway epithelial cells were cultured as described in ref. 2. After the cells reached confluence, they were serum-starved for 24 h before experiments to maintain low basal levels of MUC5AC expression.

Culture Conditions of Airway Epithelial Cells with Stimuli and Inhibitors. For inhibitory studies, cells were pretreated with inhibitors for 30 min before exposure to stimuli. In studies of PMA or HNE, the cells were treated with PMA (10 ng/ml) or HNE (100 nM) for 1 h, washed three times with medium, and cultured with the same concentrations of inhibitors as in the pretreatment period. After 6 or 24 h, cell-culture supernatants and cell lysates were collected to analyze MUC5AC expression.

Cytotoxicity Detection and Measurement of Total Protein. Lactate-dehydrogenase activity in supernatants of cell cultures treated with or without inhibitors was measured by using the Cytotoxicity Detection Kit (Roche Diagnostics). Total protein in cell lysates of cell cultures treated with or without inhibitors was measured by the Quot kit (Cambrex) at 37°C in a humidified, 5% CO2/95% air, water-jacketed incubator. After 7 days in an immersed culture condition, cell culture was switched to an air–liquid interface condition for 2–3 weeks.

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Abbreviations: DMTU, 1,3-dimethyl-2-thiourea; DPI, diphenyleneiodonium chloride; Duox, dual oxidase; EGF, EGF receptor; NHBE, normal human bronchial epithelial; HNE, human neutrophil elastase; Nix, NADPH oxidase; Phox, phagocyte Nox; nPG, n-propyl gallete; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; siRNA, small interfering RNA; TACE, TNF-α-converting enzyme; Tip, TACE inhibitory peptide.

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measured by using the BCA Protein Assay kit (Pierce). None of the measurements showed significant cytotoxicity for the inhibitors at the concentrations used in the present studies.

**Measurement of H2O2 Production.** Cells were treated with PMA (10 ng/ml) or HNE (100 nM) for various times. For inhibitory studies, cells were pretreated with inhibitors for 30 min before exposure to stimuli. H2O2 production in the supernatants was measured by using the Amplex Red Hydrogen Peroxide/Superoxide Assay kit (Molecular Probes).

**RNA Isolation, Reverse Transcription, and TaqMan Real-Time Quantitative PCR.** Total RNA was isolated, and reverse transcription was performed as described in ref. 2. TaqMan real-time quantitative PCR analysis of MUCSAC gene expression was achieved by using a sequence detector (Model 7900, Applied Biosystems) according to the manufacturer’s instructions. MUCSAC expression was normalized to 18s rRNA. The following TaqMan primers and probes were used: 18s rRNA, GATCCATTGGAGGGCAAGTCT (forward) and GCAGCAACTTTATATACGCTATTGC (reverse); probe-FAM, TGCCAGGCACTTTGCAGCAC; MUC5AC, GGAGGTGCCCTTCACCA (forward) and CGTGCGGACTCATTCCTTCA (reverse); probe-FAM, AGTGCGGCACTTGCACCAACGAC; Duox1, GCCCTGTACAACCAGGACTT (forward) and CGCACAATTTGTTCAAGGAC (reverse); and probe-FAM, CCAGGGAGGAGCCTCAGGCA.

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**Fig. 1.** Effect of PMA on H2O2 production and the effects of ROS scavengers on TGF-α release, MUCSAC gene expression, and mucin protein production by PMA. (A) NHBE and NCI-H292 cells were treated with PMA (10 ng/ml) for various times to measure H2O2 production, as described in Materials and Methods. (B) NHBE cells were pretreated with an EGFR-neutralizing Ab and either DMTU (20 mM) or nPG (100 µM) for 30 min. Then, the cells were stimulated with PMA for 2 h to measure soluble TGF-α in the supernatants. (C) After pretreatment with DMTU or nPG for 30 min, NHBE cells were stimulated with PMA for 8 h to analyze MUCSAC gene expression by using real-time quantitative PCR or for 24 h to measure mucin protein production by ELISA (D). Mucins were measured in the cell lysate (dark areas) and in the supernatant (light areas). Similar results were obtained in NCI-H292 cells (data not shown). Data are expressed as mean ± SD (n = 3). * P < 0.01, compared with PMA alone.

**Fig. 2.** Effect of inhibitors of NADPH oxidase on PMA-induced responses. (A) NHBE cells were pretreated with DPI (1.0 µM) for 30 min and then exposed to PMA (10 ng/ml) for 30 min to measure H2O2 production. (B) NHBE cells were pretreated with an anti-EGFR-neutralizing Ab and with DPI (1.0 µM) for 30 min and then stimulated with PMA for 2 h to measure TGF-α in the supernatants. (C) NHBE cells were pretreated with DPI (1.0 µM), AP (100 µM), or NMEA (100 µM) for 30 min, then treated with PMA for 8 h to analyze MUCSAC gene expression or for 24 h to measure mucin protein production (D). Similar results were obtained in NCI-H292 cells (data not shown). Data are expressed as mean ± SD (n = 3). * P < 0.01, compared with PMA alone.

**Fig. 3.** Effect of knockdown of Duox1 expression on PMA-induced responses in NCI-H292 cells. (A) The cells were transfected with Duox1 siRNA at various concentrations or with negative control siRNA (NC, 100 nM), cultured for 72 h, and then analyzed for Duox1 mRNA expression by using real-time PCR. (B) At 72 h after transfection with Duox1 siRNA (100 nM) or NC siRNA (100 nM), the cells were exposed to PMA (10 ng/ml) for 30 min to measure H2O2 production. (C) At 72 h after transfection, the cells were preincubated with an EGFR-neutralizing Ab for 30 min and then stimulated with PMA for 2 h to measure TGF-α in the supernatants. (D and E) At 72 h after transfection, the cells were treated with PMA (10 ng/ml) for 8 h to analyze MUCSAC gene expression (D) or for 24 h to measure mucin protein production (E). Data are expressed as mean ± SD (n = 3). * P < 0.01, compared with PMA alone and with NC siRNA.
**Methods**

then stimulated with PMA for 30 min to measure H₂O₂ production. (A) NHE cells were pretreated with or without apocynin (1 mM) for 30 min and then stimulated with PMA (10 ng/ml) for 30 min. Plasma membrane fraction was prepared and subjected to Western blot analysis for detection of p47(phox) with anti-p47(phox) Ab (Upper) and detection of p67(phox) with anti-p67(phox) Ab (Lower) in membranes, as described in Materials and Methods. (B) NHE cells were pretreated with apocynin (1 mM) for 30 min and then stimulated with PMA for 30 min to measure H₂O₂ production. (C) NHE cells were pretreated with an anti-EGFR-neutralizing Ab for 30 min, treated with apocynin for 30 min, and then stimulated with PMA for 2 h to measure TGF-α in the supernatants. (D and E) After pretreatment with apocynin for 30 min, NHE cells were stimulated with PMA for either 8 h to analyze MUC5AC gene expression (D) or 24 h to measure mucin protein production (E). Similar results were obtained in NCI-H292 cells (data not shown). Data in B–E are expressed as mean ± SD (n = 3). * P < 0.01, compared with PMA alone.

**MUC5AC Mucin ELISA.** Production of MUC5AC mucin protein in cell lysates and cell culture supernatants was measured by ELISA, as described in ref. 3. The amount of MUC5AC mucin in each sample was normalized to total protein in cell lysate and was expressed as μg of mucin per μg of total cellular protein.

**Effect of Stimuli on Cleavage and Release of Soluble TGF-α.** Cells were stimulated with or without PMA or HNE for 2 h. For inhibitory studies, cells were pretreated with inhibitors for 30 min before adding a stimulus. To prevent soluble TGF-α from binding to EGFR, an EGFR-neutralizing Ab (4 μg/ml) was added 30 min before adding a stimulus. Cell supernatants were collected, and TGF-α was measured with the TGF-α ELISA kit (Oncogene Science).

**Small Interfering RNA (siRNA) Preparation and Transfection of Cells**

Predesigned human Duox1 siRNAs (nos. 24873 and 24969) were purchased from Ambion (Austin, TX). In our preliminary studies, only siRNA no. 24969 (100 nM) had a significant inhibitory effect on MUC5AC mucin production by PMA. Therefore, this siRNA was used for all studies. The 21-nt sequences of Duox1 (siRNA no. 24969) were (sense) GGACUAUCCUGGCUAGAGtt and (antisense) CUCCUAGCCAG-GAUAAAGUCCgt. Silencer Negative Control no. 1 siRNA (Ambion) was used as a nonspecific siRNA. SiRNA transfection into NCI-H292 cells was carried out by using Lipofectamine 2000 (Invitrogen). Specific silencing of Duox1 was confirmed by using real-time quantitative PCR at 72 h after transfection.

**Preparation of Membrane Fraction and Western Blot Analysis.** Membrane fraction of cells was prepared according to the method described in ref. 14. Briefly, the cells were harvested and washed twice with PBS. The cell pellet was resuspended in relaxation buffer (100 mM KCl/3 mM NaCl/3.5 mM MgCl₂/1 mM EGTA/10 mM Heps/0.5 mM phenylmethylsulfonyl fluoride) and 1:100 dilution of proteinase inhibitors (Complete Mini, Roche), disrupted by two 10-s cycles of sonication on ice, and then centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was then ultracentrifuged at 100,000 × g for 30 min at 4°C. The pellet was resuspended in relaxation buffer with vigorous mixing and again centrifuged at 100,000 × g for 15 min at 4°C. The final pellet, representing the membrane fraction, was resuspended in relaxation buffer and subjected to Western blot analysis, as described in ref. 2.

**Recombinant TACE Cleavage Assay.** Purified recombinant TACE without the prodomain was purchased from Calbiochem.
TACE inhibitory peptide (TIP) was synthesized from TACE prodomain sequence, PKVCGYLK (15), by BioSource International (Camarillo, CA). TACE activity was determined by continuous kinetic assays using the fluorogenic TACE substrate III, DABCYL-LAQAVRSSR-EDANS (Calbiochem). The fluorescence intensity was monitored in a CytoFluor 2350 multiwell fluorescent plate reader (Applied Biosystems) by using wavelengths of 340 and 490 nm for excitation and emission, respectively. All reactions were initiated in 50 mM Tricine buffer (pH 7.5) containing 100 mM NaCl, 10 mM CaCl₂, and 1 mM ZnCl₂. To inhibit TACE, TIP (400 μM) was incubated with TACE (200 nM) for 1 h at 27°C. To test whether HNE can reverse TACE inhibition, HNE was added to the reactions (TACE plus TIP) for final concentrations of 10, 100, and 1,000 nM and incubated for 30 min. H₂O₂ (4 mM), which has been reported to reverse TACE inhibition by TIP (16), was used as a positive control. Finally, TACE substrate III was added to the reactions for a final concentration of 20 μM, and the fluorescence intensity was monitored every 10 min for 6 h.

**Statistical Analysis.** Data are presented as mean ± SD (n = 3). ANOVA was used to determine statistically significant differences (P < 0.01).

**Results**

ROS Mediate PMA-Induced TGF-α Release and MUC5AC Expression. PMA (10 ng/ml) induced ROS production in both NHBE and NCI-H292 cells (Fig. 1A). Pretreatment with ROS scavengers DPI and nPG prevented PMA-induced ROS generation (Fig. 1B), TGF-α release (Fig. 1C), MUC5AC gene expression (Fig. 1D), and mucin protein production (Fig. 1E). To exclude the involvement of other oxidases, we investigated the effect of inhibitors of xan-

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**Fig. 6.** Effect of HNE on TACE activation and roles of PKC/α and Duox1 in HNE-induced ROS generation, TACE activation, MUC5AC gene expression, and mucin protein production. (A) First, recombinant TACE (200 nM) was incubated with or without TIP (400 μM) for 1 h at 27°C and then incubated with TACE substrate III (20 μM) to measure fluorescence intensity for 6 h, as described in Materials and Methods. Second, TACE was preincubated with TIP for 1 h to inactivate TACE, and then H₂O₂ (4 mM) or various concentrations of HNE (10, 100, and 1,000 nM) were added to the reactions for 30 min, followed by TACE substrate, and the fluorescence intensity was monitored for 6 h. (B) NHBE cells were pretreated with rottlerin (3 μM), DPI (1 μM), or apocynin (1 mM) for 30 min and then stimulated with HNE (100 nM) for 30 min to measure H₂O₂ production. (C) NHBE cells were pretreated with an anti-EGFR neutralizing Ab for 30 min and then stimulated with HNE (100 nM) for 2 h to measure TGF-α in the supernatants. (D and E) After pretreatment with or without rottlerin (3 μM) or with DPI (1 μM) or apocynin (1 mM) for 30 min, NHBE cells were stimulated with HNE (100 nM) for 8 h to analyze MUC5AC gene expression (D) or for 24 h to measure mucin protein production (E). (F) NCI-H292 cells were transfected with Duox1 siRNA (100 nM) or NC siRNA (100 nM). After 72 h, the cells were exposed to HNE (100 nM) for 30 min to measure H₂O₂ production. (G) At 72 h after transfection, NCI-H292 cells were preincubated with an EGFR-neutralizing Ab for 30 min and then stimulated with HNE (100 nM) for 2 h to measure soluble TGF-α. (H and I) At 72 h after transfection, the cells were treated with HNE (100 nM) for 8 h to analyze MUC5AC gene expression (H) or for 24 h to measure protein production (I). Data are expressed as mean ± SD (n = 3). *P < 0.01, compared with HNE alone and with negative control transfection.
thine oxidase (allopurinol, 100 μM) and nitric oxide synthase (N\textsuperscript{N}G- monoethyl-L-arginine, 100 μM). None of the inhibitors had a significant inhibitory effect on MUC5AC gene expression (Fig. 2C) and protein production (Fig. 2D) by PMA. These results implicate Nox in PMA-induced effects in airway epithelial cells.

**Duox1 Generates ROS, Inducing TACE Activation and Mucin Expression by PMA.** Duox1 is a gp91phox homologue expressed in human airway epithelial cells. Knockdown of Duox1 expression in NCI-H292 cells with siRNA (Fig. 3A) prevented ROS generation (Fig. 3B), TGF-α release (Fig. 3C), MUC5AC gene expression (Fig. 3D), and protein production (Fig. 3E) by PMA. Transfection of negative control siRNA was without inhibitory effect (Fig. 3). These results implicate Duox1 in PMA-induced responses.

**PKC Isoforms PKC\textsuperscript{δ} and PKC\textsuperscript{θ} Mediate Duox1 Activation and Mucin Expression by PMA.** PKC\textsuperscript{δ} and PKC\textsuperscript{θ} mediate PMA-induced effects (Fig. 5E, data shown for mucin protein production only). These results suggest that PKC\textsuperscript{δ}/θ activates Duox1 to produce ROS, which activate TACE, leading to TGF-α release and mucin expression.

**HNE Induces Mucin Expression via a Signaling Pathway Involving PKC\textsuperscript{δ}/θ-Duox1-ROS-TACE.** Having established the role of Duox1 in mucin expression by PMA, we examined whether this mechanism could be extended to HNE. First, we examined whether HNE has a direct action on TACE. Preincubation of TACE with TIP prevented cleavage of TACE substrate (Fig. 6A). Exogenous H\textsubscript{2}O\textsubscript{2} (4 mM) reversed the TACE inhibition by TIP, implicating a direct action of ROS on TACE. However, various concentrations of HNE (10, 100, and 1,000 nM) failed to reverse the inhibitory effect of TIP on TACE, suggesting that there is no direct action of HNE on TACE. Second, we examined effects of PKC\textsuperscript{δ}/θ inhibitor rottlerin or Nox inhibitors DPI and apocynin on HNE-induced responses. Pretreatment with these inhibitors prevented HNE-induced ROS generation (Fig. 6B), TGF-α release (Fig. 6C), mucin gene expression (Fig. 6D), and mucin protein production (Fig. 6E) in both NHBE and NCI-H292 cells. Next, we confirmed the role of Duox1 in these responses to HNE by knocking down the expression of Duox1 with siRNA. Knockdown of Duox1 prevented all of these effects of HNE in NCI-H292 cells (Fig. 6F–I). Together, we conclude that HNE induces MUC5AC gene expression and protein production via a signaling pathway involving PKC\textsuperscript{δ}/θ-Duox1-ROS-TACE-TGF-α in human airway epithelial cells.
with the catalytic domain via an intramolecular bond between the cysteine residue in the prodomain and the zinc atom in the catalytic domain (16). Processed TACE molecules are transported to cell-surface membranes.

2. Upon cellular stimulation, the cysteine-zinc bond breaks, the cysteine sulfhydryl moiety and release it from coordination with the catalytic zinc, thus activating TACE.

The identification of Nox homologues in epithelial tissues such as bronchial epithelium (e.g., Duox1) implies that the generation of ROS in these tissues is a fine-tuned biological strategy. Three hypotheses concerning the function of ROS generated by Nox/Duox have been proposed: (i) role of host defense in killing invading microbes and cancer cells, (ii) role in signal transduction, and (iii) role in metabolism. In the present study, we focused on the role of ROS in TACE activation. Here, we found that Duox1 activated by PMA and HNE produces ROS, which activate TACE, resulting in TGF-α release and mucin production in airway epithelial cells. Because TACE cleaves many substrates (e.g., TNF-α, TNF receptors, TGF-α, amphiregulin, and L-selectin), the identification of TACE activation by Duox1 may have broad implications concerning the understanding of both physiological and pathophysiological processes in cells.

Nox has been studied extensively in phagocytes (23, 24). The phagocytic Nox consists of a membrane-bound cytochrome, b558; a complex of gp91phox and p22phox; and four cytosolic components, p47phox, p67phox, p40phox, and Rac1/2 (7). Six-transmembrane gp91phox is the catalytic core of Nox. Nox homologues in noninflammatory cells include Nox1 through Nox5 (25, 26) and Duox1 homologues in human airway epithelial cells. Because TACE cleaves many substrates (e.g., TNF receptors, TGF-α, amphiregulin, and L-selectin), the identification of TACE activation by Duox1 may have broad implications concerning the understanding of both physiological and pathophysiological processes in cells.

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