Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor-α-converting enzyme in human airway epithelial (NCI-H292) cells

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Shao, Matt X. G., Takashi Nakanaga, and Jay A. Nadel. Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor-α-converting enzyme (TACE) and subsequent proteolytic shedding, resulting in EGFR phosphorylation and mucin production in human airway epithelial (NCI-H292) cells. Here we hypothesize that cigarette smoke causes EGFR activation by increasing the availability of soluble EGFR ligands, which then bind to and activate EGFR.

Smoking and COPD

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death in the U.S. Because cigarette smoking is so importantly implicated in the pathogenesis of COPD and because mucus hypersecretion plays such an important role in COPD, understanding of the mechanisms of smoking-induced mucus hypersecretion could lead to new therapies for COPD. Cigarette smoke causes mucin overproduction via EGFR (Egfr) in airway epithelial cells, but the cellular mechanism remains unknown. Airway epithelial cells contain EGFR proligands on their surfaces, which can be cleaved by metalloprotease and subsequently bind to EGFR resulting in mucin production. We hypothesize that TNF-α-converting enzyme (TACE) is activated by cigarette smoke, resulting in increased shedding of EGFR proligand, leading to EGFR phosphorylation and mucin induction in human airway epithelial (NCI-H292) cells. We show that cigarette smoke increases MUC5AC production in NCI-H292 cells, an effect that is prevented by an EGFR-neutralizing antibody and by specific knockdown of transforming growth factor-α (TGF-α) using small interfering RNA (siRNA) for TGF-α, implicating TGF-α-dependent EGFR activation in the responses. Cigarette smoke increases TGF-α shedding, EGFR phosphorylation, and mucin production, which are prevented by metalloprotease inhibitors (GM-6001 and TNF-α protease inhibitor-1) and by specific knockdown of TACE with TACE siRNA, implicating TACE in smoking-induced responses. Furthermore, pretreatment with antioxidants prevents smoking-induced TGF-α shedding and mucin production, suggesting that reactive oxygen species is involved in TACE activation. These results implicates TACE in smoking-induced mucin overproduction via the TACE-protigand-EGFR signal pathway in NCI-H292 cells.

Materials and Methods

Materials. AG-1478, GM-6001 (GM), negative control of GM-6001 [GM(-)], tumor necrosis factor-α proteinase inhibitor-1 (TAPI-1), EGFR monoclonal antibody (Ab-3), EGFR monoclonal antibody (Ab-5), and TGF-α monoclonal antibody (Ab-3) were purchased from Calbiochem (San Diego, CA). BIBX 1522 was generously provided by 10.220.33.1 on August 27, 2017 http://ajplung.physiology.org/ Downloaded from

IN THE HUMAN RESPIRATORY TRACT, the airway epithelium provides a barrier against various environmental insults. Normal airway epithelium is coated with a mucus layer. Mucins (e.g., MUC5AC) are major components of mucus (10, 11, 29), which assists in clearance of inhaled foreign materials.

Chronic obstructive pulmonary disease (COPD) is a major health problem, which is the fourth leading cause of death in the U.S. (21). Goblet cell hyperplasia and mucus hypersecretion are prominent features of COPD, especially during exacerbations (1, 12, 25, 34, 35). Cigarette smoking is implicated in the pathophysiology of COPD and is closely associated with mucus hypersecretion (18, 32, 35). However, the cellular mechanisms underlying this association remain unknown.

In 1999, Takeyama et al. (31) discovered that when NCI-H292 cells grow in dense cultures, they undergo mucous cell differentiation and produce mucins via activation of EGF receptor (EGFR). Takeyama et al. (32) also found that cigarette smoke causes mucin production via EGFR activation in vitro and in vivo. However, the cellular mechanisms by which cigarette smoke causes EGFR activation are still unknown. Richter et al. (28) reported that cigarette smoke induces the expression and release of EGFR ligands (e.g., transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF (HB-EGF)) in airway epithelial cells. These studies raise the possibility that cigarette smoke causes EGFR activation by increasing the availability of soluble EGFR ligands, which then bind to and activate EGFR.

Recently, PMA has been reported to induce EGFR phosphorylation and mucin production via activation of tumor necrosis factor-α-converting enzyme (TACE) and subsequent proteolytic shedding of the EGFR ligand pro-TGF-α in NCI-H292 cells (30). Here we hypothesize that cigarette smoke causes EGFR activation and mucin production via activation of TACE, which cleaves the precursor of an EGFR ligand, resulting in EGFR phosphorylation and mucin induction in NCI-H292 cells.

To examine this hypothesis, first we investigated whether cigarette smoke induces ligand-dependent EGFR phosphorylation and mucin production in NCI-H292 cells and, if so, which specific EGFR ligand is involved in these processes. Next, we examined whether metalloprotease activation is required for the ligand release and subsequent EGFR phosphorylation by cigarette smoke and, if so, which specific metalloprotease is involved. Finally, we examined how cigarette smoke activates the metalloprotease. We show that cigarette smoke activates metalloprotease TACE, cleaving pro-TGF-α, leading to shedding of soluble TGF-α, resulting in EGFR phosphorylation and mucin overproduction in NCI-H292 cells. The study suggests that oxygen free radicals are responsible for the activation of TACE by cigarette smoke.

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by Boehringer Ingelheim. 1,3-Dimethyl-2-thiourea (DMTU) and n-propyl gallate (nPG) were purchased from Sigma. EGFR (1005) rabbit polyclonal antibody and p-Tyr (PY99) monoclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were plated at 5 × 10^5 cells in 2 ml in each well of a six-well plate or at 1–2 × 10^5 cells in 1 ml in each well of a 24-well plate (both 6-well and 24-well plates were purchased from BD Falcon, Bedford, MA) and were grown in RPMI 1640 medium containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPE (25 mM) at 37°C in a humidified, 5% CO_2/95% air, water-jacketed incubator. After the cells reached confluence, they were serum-starved for 24 h before experiments to maintain low basal levels of MUC5AC expression.

Preparation of cigarette smoke solution and treatment of cells with cigarette smoke. Standard research cigarettes (code 2R1, products from University of Kentucky Tobacco and Health Research Foundation) were used in the study. Cigarette smoke solution was prepared as described previously (5). In brief, smoke of one cigarette was withdrawn into a polypropylene syringe (30 ml) at a rate of 1 puff/min and bubbled slowly into 25 ml of RPMI 1640 containing 50 mM HEPE buffer (hereafter called “cigarette smoke” solution). The cigarette smoke solution was then sterilized by filtration through a 0.22-μm cellulose acetate sterile syringe filter (Corning, NY), aliquoted, and stored at −20°C. Aliquots of the cigarette smoke solution were thawed at 4°C, and various dilutions were prepared with RPMI 1640 immediately before incubation with cells.

After 24 h of serum starvation, cells were exposed to RPMI 1640 containing various dilutions of the cigarette smoke solution. After 24 h of exposure, cell culture supernatants and cell lysates were collected to measure MUC5AC mucin protein production.

MUC5AC ELISA. Cells were grown in 24-well plates. Production of MUC5AC protein in cell lysates and in cell culture supernatants were measured by ELISA (31). The amount of MUC5AC protein in each sample was normalized to total protein in cell lysate and was expressed as μg/mg cell protein.

Analysis of effect of cigarette smoke on cleavage and release of soluble TGF-α. Cells were grown in 24-well plates. After reaching confluence and being serum-starved for 24 h, cells were either incubated with cigarette smoke or maintained in medium alone for 2 h. For inhibition studies, cells were pretreated with the inhibitors for 30 min before exposure to cigarette smoke. In studies designed to prevent soluble TGF-α from binding to EGFR, we added an EGFR-neutralizing antibody (Ab-3, 4 μg/ml) 30 min before adding each stimulus. Cell supernatants were collected, and TGF-α was measured with the TGF-α ELISA kit (Oncogene, San Diego, CA) according to the manufacturer’s instructions.

RNA isolation and RT-PCR. Total RNA was isolated using RNAqueous-4PCR kit (Ambion, Austin, TX), and 1.0–1.5 μg was primed with Oligo(dT) and reverse transcribed with the RETROscript kit (Ambion) in a final volume of 20 μl (RT reaction), following manual instructions. Two microliters of the RT reaction were PCR-amplified in a 50-μl reaction with SuperTag DNA polymerase (Ambion). Primers were MUC5AC (forward) 5′-TCCGGGCTCATCTTCTCC-3′ and (reverse) 5′-AAGCTTGATTCTTTGCTCTCA; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA, AAATGTAGAATGGGATGCTGTTGCC; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA, AAATGTAGAATGGGATGCTGTTGCC; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA, AAATGTAGAATGGGATGCTGTTGCC; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA, AAATGTAGAATGGGATGCTGTTGCC; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA, AAATGTAGAATGGGATGCTGTTGCC; pro-HB-EGF: AAAGTCCGTGACTTGCAAGAG, AAGGACTTCTGATCAATCGAGGA. As nonspecific siRNA control, a sequence targeting firefly (Photinus pyralis) luciferase gene (X65342) 153–175 was used (CGTACGGGAAATCTCCTGATG). Twenty-one-nucleotide double-stranded RNAs were prepared in vitro by Silencer siRNA Construction kit (Ambion). siRNA transfection into NCI-H292 cells was carried out with the Silencer siRNA Transfection kit (Ambion). Specific silencing of targeted genes was confirmed by RT-PCR analysis at 48 h after transfection.

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

RESULTS

Cigarette smoke upregulates MUC5AC expression in NCI-H292 cells. Cigarette smoke induced MUC5AC gene expression (Fig. 1A) and MUC5AC protein production (Fig. 1B) dose dependently. MUC5AC gene expression and protein production were also induced time dependently by cigarette smoke (Fig. 1, C and D). Cigarette smoke (1:10 dilution) caused a
Cigarette smoke induces ligand-dependent EGFR phosphorylation and MUC5AC expression. Cigarette smoke (1:10 dilution) induced EGFR phosphorylation (Fig. 2A), MUC5AC gene expression (Fig. 2B), and mucin protein production (Fig. 2C). These effects were prevented by pretreatment of the cells with AG-1478 (10 μM) or with BIBX (5 μg/ml) (selective inhibitors of EGFR phosphorylation, Fig. 2, A–C), implicating EGFR phosphorylation in cigarette smoke-induced MUC5AC mucin expression. Preincubation of the cells with an EGFR-neutralizing antibody (4 μg/ml) inhibited cigarette smoke-induced EGFR phosphorylation (Fig. 2D), MUC5AC gene expression (Fig. 2E), and mucin protein production (Fig. 2F). These results implicate ligand-dependent EGFR phosphorylation in MUC5AC mucin expression by cigarette smoke.
EGFR ligand TGF-α mediates cigarette smoke-induced EGFR phosphorylation and MUC5AC expression. Ligands TGF-α, HB-EGF, AR, and Hrg-α are expressed in human airway epithelial cells (26, 33). We examined which ligand was specifically involved in cigarette smoke-induced EGFR phosphorylation and mucin expression by knocking down the expression of pro-TGF-α, pro-HB-EGF, pro-AR, and pro-Hrg-α, respectively, with siRNA for each proligand. The inhibitory effect of siRNA on the expression of each proligand was monitored at the mRNA level by RT-PCR (Fig. 3A). Knockdown of pro-TGF-α prevented cigarette smoke-induced EGFR phosphorylation (Fig. 3B), MUC5AC gene expression (Fig. 3C), and mucin protein production (Fig. 3D). However, knockdown of pro-HB-EGF, pro-AR, and pro-Hrg-α had no significant inhibitory effects on these processes (Fig. 3, B–D).

Preincubation of the cells with a TGF-α-neutralizing antibody (4 μg/ml) prevented cigarette smoke-induced EGFR phosphorylation (Fig. 3E) and MUC5AC gene expression (Fig. 3F) and protein production (Fig. 3G), further confirming the involvement of TGF-α in cigarette smoke-induced EGFR phosphorylation and MUC5AC mucin expression. Cigarette smoke induces shedding of soluble TGF-α via activation of metallopeptase. Pro-TGF-α is constitutively expressed on the plasma membrane surface of NCI-H292 cells. Exposure of the cells to cigarette smoke (1:10 dilution) for 2 h in the presence of an EGFR-neutralizing antibody (4 μg/ml) increased soluble TGF-α in the supernatant measured by ELISA (Fig. 4A, two left bars). GM (10 μM), a broad-spectrum metalloprotease inhibitor, and TAPI-1 (10 μM), a relatively selective metalloprotease TACE inhibitor, inhibited cigarette smoke-induced TGF-α release (Fig. 4A), EGFR phosphorylation (Fig. 4B), MUC5AC gene expression (Fig. 4C), and mucin protein production (Fig. 4D). GM (10 μM) had no inhibitory effect on these processes (Fig. 4, A–D). These results suggest a critical role of a metalloprotease in cigarette smoke-induced TGF-α shedding, EGFR activation, and MUC5AC mucin expression. The fact that TAPI-1 also prevented all of these effects of cigarette smoke on NCI-H292 cells suggests the possible involvement of TACE in cigarette smoke-induced responses in NCI-H292 cells.

TACE mediates cigarette smoke-induced TGF-α release, EGFR phosphorylation, and MUC5AC mucin expression. To study the involvement of TACE in cigarette smoke-induced...
Cell responses, we specifically knocked down TACE expression by transfection of the cells with TACE siRNA. TACE siRNA transfection caused suppression of TACE expression at the mRNA level shown by RT-PCR (Fig. 5A). Knockdown of TACE expression inhibited cigarette smoke-induced TGF-α release (Fig. 5B), EGFR phosphorylation (Fig. 5C), MUC5AC gene expression (Fig. 5D), and mucin protein production (Fig. 5E). These results implicate the metalloprotease TACE in cigarette smoke-induced responses in NCI-H292 cells.

**DISCUSSION**

Our previous studies showed that an EGFR cascade is responsible for mucin induction in response to a wide variety of stimuli including cigarette smoke in airway epithelial cells (31, 32). EGFR phosphorylation initiates a signaling pathway of various stimuli (22). Two different processes may be involved in EGFR activation: 1) ligand-dependent EGFR phosphorylation and 2) ligand-independent EGFR phosphorylation. An increasing body of evidence indicates that EGFR can be transactivated by G protein-coupled receptors via metalloprotease-dependent shedding of EGFR ligands (20, 27, 36). These results suggested that some of the EGFR activation previously thought to involve ligand-independent EGFR phosphorylation is mediated via ligand binding to EGFR. To investigate whether ligand binding is required for EGFR phosphorylation by cigarette smoke, we preincubated cells with a neutralizing anti-EGFR antibody to block the EGFR ligand binding sites on the cell surface. This pretreatment prevented EGFR phosphorylation (Fig. 2D) and mucin production (Fig. 2, E and F) by cigarette smoke, implicating ligand-dependent EGFR activation in mucin production by cigarette smoke.

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COPD is a major cause of death in the U.S. and accounted for 112,584 deaths in 1998 (21). Most patients with COPD have mucus hypersecretion. Overproduced mucus plugs the airways and contributes to exacerbations of COPD. Cigarette smoking is the most important risk factor initiating the development of COPD. The present study shows for the first time that cigarette smoke induces MUC5AC mucin production via activation of TACE and subsequent cleavage of proligand pro-TGF-α in a human airway epithelial cell line (NCI-H292 cells).

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Fig. 5. Effect of knockdown of TNF-α-converting enzyme (TACE) expression on TGF-α release, EGFR phosphorylation, and MUC5AC expression by cigarette smoke. A: cells were transfected with or without TACE siRNA (10 nM) or luciferase siRNA (Luc, 10 nM, as negative control), cultured for 48 h, and then analyzed for TACE mRNA expression by RT-PCR. B: cells were transfected with TACE siRNA (10 nM) or Luc siRNA (10 nM) and cultured for 48 h. After preincubation with an EGFR-neutralizing antibody (4 μg/ml) for 30 min to block EGFR-ligand binding sites, cells were stimulated with vehicle or cigarette smoke (1:10) for 2 h. Supernatants were collected for measurement of soluble TGF-α by ELISA. TACE siRNA inhibited soluble TGF-α release in control states and by cigarette smoke, whereas Luc siRNA had no inhibitory effect. Cells were transfected with TACE siRNA or with Luc siRNA. Forty-eight hours later, cells were treated with cigarette smoke (1:10) for 15 min and analyzed for EGFR phosphorylation (C) or for 8 h and analyzed for MUC5AC gene expression (D) or were treated with vehicle or cigarette smoke (1:10) for 24 h and measured for MUC5AC mucin protein production (E). In nontransfected cells, cigarette smoke increased EGFR phosphorylation, MUC5AC mRNA expression, and mucin protein production. TACE siRNA (10 nM) prevented these processes, whereas Luc siRNA (10 nM) was without inhibitory effect. Results are typical of the results in 3 separate experiments. Data in B and E are expressed as means ± SD (n = 3). Mucins were measured in the cell lysate (dark areas) and in the supernatant (light areas). *P < 0.01, compared with vehicle treatment alone and with Luc transfection. **P < 0.01, compared with cigarette smoke treatment alone and with Luc transfection.

Fig. 6. Role of reactive oxygen species in cigarette smoke-induced TGF-α release, EGFR phosphorylation, and MUC5AC expression. A: cells were pretreated with an anti-EGFR-neutralizing antibody (4 μg/ml) for 30 min to block EGFR-ligand binding sites and then treated with or without free radical scavengers [1,3-dimethyl-2-thiourea (DMTU, 20 mM) or n-propyl gallete (nPG, 100 μM)] for 30 min and then stimulated with vehicle or cigarette smoke (1:10 dilution) for 2 h. Supernatants were collected for measurement of soluble TGF-α by ELISA. After pretreatment with or without DMTU (20 mM) or nPG (100 μM) for 30 min, cells were stimulated with cigarette smoke (1:10 dilution) for 15 min and analyzed for EGFR phosphorylation (B) or for 8 h and analyzed for MUC5AC gene expression (C) or they were stimulated with vehicle or cigarette smoke (1:10 dilution) for 24 h and analyzed for MUC5AC mucin protein production (D). Results are typical of the results in 3 separate experiments. Data in A and D are expressed as means ± SD (n = 3). Mucins were measured in the cell lysate (dark areas) and in the supernatant (light areas). *P < 0.01, compared with vehicle alone; **P < 0.01, compared with cigarette smoke alone.
duced TGF-α release (Fig. 4A), EGFR phosphorylation (Fig. 4B), and MUC5AC gene expression (Fig. 4C) and protein production (Fig. 4D), implicating metalloprotease activity in these processes.

Two members of a disintegrin and metalloprotease (ADAM) family proteases, ADAM10 and TACE (ADAM17), are constitutively expressed in NCI-H292 cells (16, 30). Although ADAM10 cleaves both pro-TGF-α and pro-HB-EGF and has been reported to mediate MUC2 mucin induction by gram-positive bacteria (16, 36), the facts that 1) HB-EGF siRNA did not prevent cigarette smoke-induced EGFR phosphorylation and MUC5AC mucin production (Fig. 3, B–D) and 2) the efficiency of cleavage of pro-TGF-α by ADAM10 is 90-fold less than TACE in vitro (9) mitigate against involvement of ADAM10 in EGFR phosphorylation and mucin production by cigarette smoke in NCI-H292 cells. TACE has recently been reported to be responsible for the ectodomain shedding of TGF-α in various epithelial tissues (24, 30, 39). To examine a possible role of TACE in cigarette smoke-induced TGF-α release, EGFR activation, and mucin induction in NCI-H292 cells, we specifically knocked down TACE expression using TACE siRNA (Fig. 5A). The TACE siRNA used in this study has been reported to inhibit TACE expression efficiently in both NCI-H292 cells (30) and SCC-9 (a squamous carcinoma cell line) cells (8). Knockdown of TACE expression inhibited TGF-α shedding (Fig. 5B), EGFR phosphorylation (Fig. 5C), and mucin expression (Fig. 5, D–E) by cigarette smoke, showing that TACE is responsible for these effects in NCI-H292 cells.

TACE is synthesized in a latent form (2). A thiol group from a cysteine residue in the prodomain interacts with zinc in the catalytic domain and thereby inactivates TACE (2, 3, 37, 38). The mechanisms that activate TACE are not yet clear. Our finding that antioxidants (e.g., DMTU, npG) prevent cigarette smoke-induced TGF-α shedding (Fig. 6A), EGFR phosphorylation (Fig. 6B), and mucin production (Fig. 6, C and D) implicates ROS in TACE activation by cigarette smoke in NCI-H292 cells.

Cigarette smoke has multiple effects on airway epithelial cells. In sparse cell cultures, cigarette smoke is reported to predominantly enhance cell proliferation via shedding of EGFR ligand AR and subsequent EGFR activation (17). However, in dense cell cultures, cigarette smoke induces mucus cell differentiation and mucin production via activation of TACE, shedding of soluble TGF-α, and EGFR phosphorylation (present study). The mechanisms by which cigarette smoke has differential effects on NCI-H292 cells in different microenvironments have not yet been fully explored. Kim et al. (13) reported that in dense cultures NCI-H292 cells undergo contact-dependent growth inhibition, and TGF-α increases MUC5AC protein production markedly in dense, but not in sparse, cultures, suggesting that cell–contacts modulate cell responses to stimuli. Lemjabbar et al. (17) reported that cigarette smoke causes significant AR release, rather than TGF-α, in NCI-H292 cell cultures. We suggest that the level of TGF-α in cell culture supernatant could have been underestimated by the authors, because they did not block the EGFR ligand binding sites on the cell surface before exposing the cells to the stimulus (17). Richter et al. (28) reported that in the presence of an EGFR-neutralizing antibody that prevented ligand binding and internalization of the receptor-bound ligand, there was a marked accumulation of TGF-α, but not AR, in the supernatant of NCI-H292 cell cultures after exposure to cigarette smoke, suggesting that, in the absence of the antibody, TGF-α was rapidly utilized by the cells and that TGF-α is a primary mediator of the cigarette smoke-induced responses in NCI-H292 cells. This is in agreement with our previous report (30) and current studies. All of these results were from studies performed in a cancer cell line (NCI-H292 cells), which may express different phenotypes from those expressed in primary cultures of airway epithelia. Even primary cell cultures may differ from the in vivo state.

In summary, we show that stimulation with cigarette smoke activates TACE, which cleaves pro-TGF-α and releases mature soluble TGF-α that binds to and activates EGFR, resulting in mucin production in human airway epithelial NCI-H292 cells. These findings are especially important because cigarette smoking is so closely linked to COPD. The discovery that cigarette smoke-induced mucin overproduction is mediated by TACE activation suggests new therapies for COPD.

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