RESEARCH ARTICLE

Roles of ROS/TACE in neutrophil elastase-induced mucus hypersecretion in NCI-H292 airway epithelial cells

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ABSTRACT. Complications arise in chronic obstructive pulmonary diseases (COPD) with excessive mucus production, especially during the exacerbation period, which contributes to airway blockage and bacterial infection. Neutrophil elastase (NE) is detected at high levels in airway secretions, and is the primary inducer of mucin production. Understanding the mechanism of NE-induced overproduction of mucin may lead to new therapies for COPD. It is known that activation of epidermal growth factor receptor (EGFR) and its downstream signaling cascade are involved in mucin production. However, the mechanism of NE-induced EGFR activation remains unclear. Tumor necrosis factor-α-converting enzyme (TACE) cleaves pro-transforming growth factor (TGF)-α in airway epithelial cells to release the mature, soluble TGF-α form, which subsequently binds to and activates EGFR. In this investigation, we demonstrate that NE-induced mucin production requires reactive oxygen species (ROS) production, which activates TACE, resulting in TGF-α shedding, and EGFR phosphorylation in NCI-H292 epithelial cells.

Keywords: leukocyte elastase, reactive oxygen species, mucins

Mucus hypersecretion is a prominent manifestation in patients with chronic inflammatory airway disease, contributing to morbidity and mortality by blocking airways and causing recurrent infections. Neutrophil elastase (NE) is a serine protease secreted by neutrophils that is expressed in high concentrations in the airway secretions of these patients [1]. It is known that NE is a potent inducer of mucin-5AC (MUC5AC) production [2]. Previous studies have shown that NE induces MUC5AC production through epidermal growth factor receptor (EGFR) activation cascades [3, 4]. Pechon *et al*. first reported that the action of TNF-α-converting enzyme (TACE) is required in the process. TACE cleaves pro-transforming growth factor (TGF)-α into soluble TGF-α, which then acts as the ligand for EGFR, activating the downstream signaling cascade [5]. Shao and colleagues then described the TACE/TGF-α/EGFR/MAPK signaling pathway involved in MUC5AC gene expression in human airway epithelial cells after stimulation with cigarette smoke or neutrophil elastase [6]. Kohri also showed that neutrophil elastase-induced mucin production is EGFR ligand-dependent, but they did not investigate the MMP-proligand shedding process [1]. It has been confirmed that NE can stimulate reactive oxygen species (ROS) production in normal, human bronchial epithelial cells. Additionally, there is increasing evidence suggesting that ROS acts as an kinase extensively involved in cell signaling. Therefore, we hypothesize that NE activates TACE *via* generation of ROS, resulting in cleavage of pro-TGF-α, EGFR activation and, subsequently, MUC5AC expression in airway epithelial cells.

To test this hypothesis, we investigated whether NE induced ROS production, mature TGF-α shedding, EGFR phosphorylation and MUC5AC production in NCI-H292 airway epithelial cells. Next, we investigated whether metalloproteinase activation was required for ligand release and subsequent EGFR phosphorylation by NE. The specific metalloproteinase involved in this process was also investigated. Finally, we examined whether ROS production was required for metalloproteinase activation and MUC5AC production. These results indicate that NE stimulates cells to produce ROS, which activates TACE, which in turn, cleaves pro-TGF-α into soluble TGF-α, resulting in EGFR activation and MUC5AC expression in NCI-H292 cells.

METHODS AND MATERIALS

Materials

DMEM/Ham's F12, HEPES, Trizol, and calf serum were purchased from Sigma (USA). 1,3-dimethyl-2-thiourea (DMTU), TGF-α, human neutrophil elastase, tumor necrosis factor-α proteinase inhibitor-1 (TAPI-1), EGFR neutralizing antibody, mouse anti-human EGFR

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monoclonal antibody, mouse anti-phosphorylated human EGFR monoclonal antibody were purchased from Calbiochem (USA). Mouse anti-human MUC5AC monoclonal antibody 45M1, and TACE rabbit polyclonal antibody were purchased from Neomarkers (USA). The TGF-α ELISA Kit was purchased from R&D (USA), MUC5AC primers were generously provided by the Medical Department, Gene Research Center, The University of Hong Kong.

Cell lines and cell culture

NCI-H292 cells were plated in a six-well plate with 5×10^5 - 6×10^5 cells per well, cultured in 2 mL of DMEM/Ham's F12 medium containing 10% calf serum at 37°C in 5% CO_2. At 70% - 80% confluence, cells were serum-starved in the presence or absence of additional growth factors for 24 h in the following manner: (all cells were grown under serum-free conditions) (1) the control group: grown in DMEM/Ham's medium in the absence of serum. (2) the NE stimulation group: grown in the presence of NE (25 nM); (3) the TAPI-1 group: pretreated with 5, 10, 20 μM TAPI-1 for 30 min, treated with EGFR neutralizing antibody (4 μg/mL) for 10 min to block the ligand binding site, then NE (25 nM) was added; (4) the DMTU group: pretreated with DMTU (20 μM) for 30 min, treated with EGFR neutralizing antibody (4 μg/mL) for 10 min, then NE (25 nM) was added; (5) the exogenous TGF-α group: treated with TGF-α (10 ng/mL). (6) DMTU, TGF-α treatment group: pretreated with DMTU (20 μM) for 30 min, treated with EGFR neutralizing antibody (4 μg/mL) for 10 min, then TGF-α (10 ng/mL) was added. After a 24-h exposure, the cell culture lysates and cell supernatants in each group were collected and MUC5AC expression were analyzed.

Measurement of ROS generation

Cells were cultured in 24-well plates and incubated with 5, 10, or 25 nM NE for 24 h. At 70% confluence, medium was removed and a ROS detection kit (Genmed, USA) was used, in accordance with the manufacturer's instructions, to measure the amount of ROS generated in each group Absorbance was measured at 650 nm.

RNA isolation and reverse transcription-PCR

Total RNA was extracted from NCI-H292 cells using Trizol, and quality was verified by resolving samples using 1% agarose gel electrophoresis. $A_{260}/_{280}$ was within the range of 1.8-2.0. cDNA was generated from RNA using Oligo (dT) primer and reverse transcribed with the Moloney murine leukemia virus (MMLV) reverse transcriptase kit (Shanghai Sangon Biological Engineering Technology & Services Co., China). Primers: MUC5AC, forward-5'-TGATCATCCAGCAG CAGGGCT-3', reverse- 5'-CCGAGCTCAGAGGACAT ATGGG-3'. GAPDH, forward-5'-AGTGGATATTGTT GCCATCA-3', reverse-5'-GAAGATGGTGATGGGAT TTC-3'. PCR conditions: denatured at 94°C for 10 min; 94°C for 30 s, 57°C for 45 s, 70°C for 45 s, for 30 cycles; 72°C for 7 min. PCR products were resolved using 2% agarose gel electrophoresis and bands visualized by ethidium bromide staining.

MUC5AC ELISA

MUC5AC protein expression levels were measured using an ELISA. Cell lysates were prepared with phosphate-buffered saline (PBS) at multiple dilutions. Fifty μL of each sample were incubated with bicarbonate-carbonate buffer (50 μL) at 40°C, in a 96-well plate until it dried. Cells were washed three times with PBS and blocked

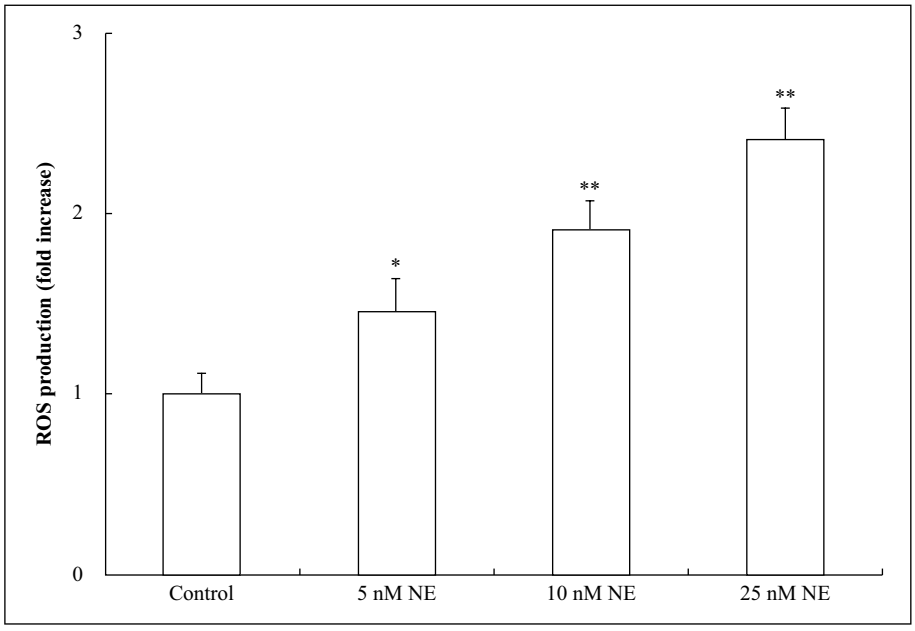

Figure 1

Reactive oxygen species (ROS) production in treated NCI-H292 cells. Cells were untreated or treated with various concentrations of neutrophil elastase (NE) (5, 10, 25 nM) for 24 h. Untreated cells was used as the control group. ROS production was measured. Data are expressed as mean ± SD (n = 3). * $p < 0.05$; ** $p < 0.01$, compared with control group.

with 2% bovine serum albumin (BSA) for 1 h at room temperature. Then, cells were incubated with mouse anti-human MUC5AC monoclonal antibody (45M1) (10 μg/mL, Neomarkers, USA) in PBS containing 0.05% Tween-20. After 1 h, cells were incubated with horseradish peroxidase (HRP) conjugated-goat anti-mouse IgG (1 μg/mL) for 1 h. HRP was developed with 3, 3′, 5, 5′-tetramethylbenzidine (TMB) peroxidase solution, quenched with 1 M H_2SO_4, and color was measured by absorbance at 450 nm.

Soluble TGF-α ELISA

Cell culture supernatants from each group were collected, and TGF-α was measured using the TGF-α ELISA kit (R&D,USA). Briefly, 100 μL of assay diluent and 50 μL of sample were added to each well for a 2-h incubation at room temperature. Then, 200 μL of conjugate were added to each well for 2 h at room temperature, followed by addition of 200 μL of substrate reaction solution for 30 min. Finally, 50 μL of stop solution were added to quench the reaction. Absorbance was measured at 450 nm.

Western blot analysis for EGFR, phosphorylated EGFR and TACE proteins

Cells were lysed in lysate buffer (RIPA buffer, 150 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, and

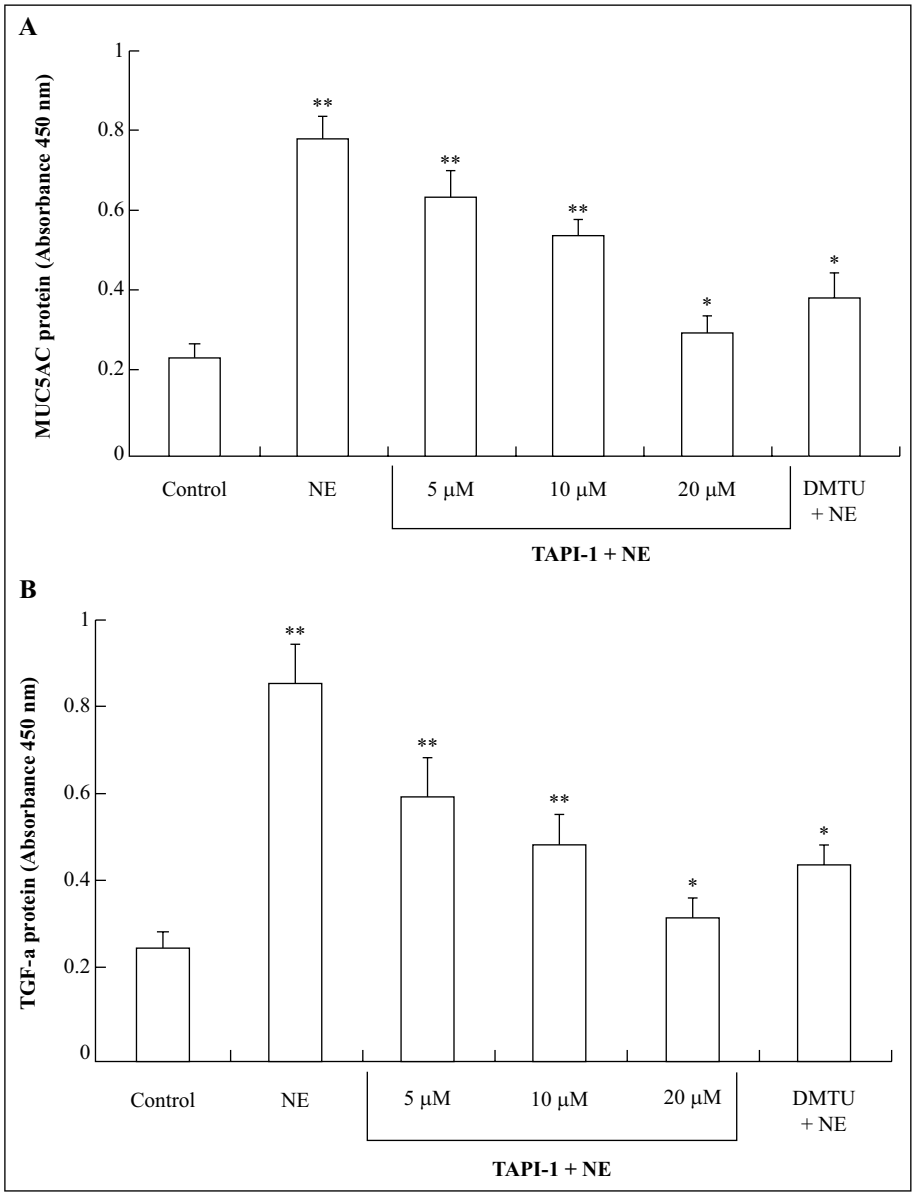

Figure 2

Effects of TACE inhibitor TAPI-1 and ROS scavenger DMTU on neutrophil elastase (NE)-induced MUC5AC and TGF-α protein expression. NCI-H292 cells were left untreated as the normal control group; experimental cells were treated with NE (25 nM) for 24 h or pretreated with inhibitors and then treated with NE (25 nM) for 24 h. Cells were incubated with the indicated concentrations of TAPI-1 (5, 10, 20 μM) or DMTU (20 μM).

A) Quantification of MUC5AC protein using ELISA.

B) Quantification of TGF-α protein. Data are expressed as mean ± SD (n = 3). ** $p < 0.01$; * $p < 0.05$, compared with control group. * $p < 0.01$, compared with the NE-treated group.

2 mM EDTA), disrupted on ice for 20 min, and then centrifuged at 12,000 rpm for 15 min at 4°C to remove nuclei and unbroken cells. Equal amounts of protein were suspended in SDS sample buffer and boiled for 5 min. Proteins were resolved by 8% SDS/PAGE. The resulting gels were equilibrated in the transfer buffer with 25 mM Tris-HCl, 192 mM glycine, and 20% methanol (pH = 8.3). Protein was then transferred by electrophoresis to polyvinylidene difluoride (PVDF) membrane. Membranes were incubated in 5% milk/PBS, 0.05% Tween-20 for 1 h at room temperature, and then incubated with EGFR monoclonal antibody, anti-phosphotyrosine EGFR monoclonal antibody (1 μg/mL), or TACE polyclonal antibody overnight at 4°C. After washing three times, membranes were incubated with HRP-goat anti-mouse IgG or HRP-goat anti-rabbit IgG (1 mg/L) for 2 h at room temperature. Enhanced chemiluminescence (ECL) and autoradiography were performed to visualize EGFR, phosphorylated-EGFR, and TACE protein. Photodensity area calculus was performed in order to quantify protein. Western blot analysis of β-actin protein was performed to ensure equal protein loading.

Statistical methods

Statistical analysis was performed using SPSS 10.0 for Windows, SPSS Inc. All data were presented as mean ± SD (n = 3). ANOVA was used to determine statistically significant differences. A P-value < 0.05 was considered statistically significant.

RESULTS

Effects of NE on ROS generation

NCI-H292 cells were incubated with 5, 15, or 25 nM of NE for 24 h, respectively. ROS production increased in a dose-dependent manner in response to NE treatment, compared to the untreated control cells, which is consistent with previous data, The highest ROS content was detected in 25nM NE-treated cells *(figure 1)*.

Effects of TAPI-1 on NE-induced MUC5AC expression in NCI-H292 cells

As it had previously been shown that NE induced cleavage of pro-TGF-α through induction of TACE, it was

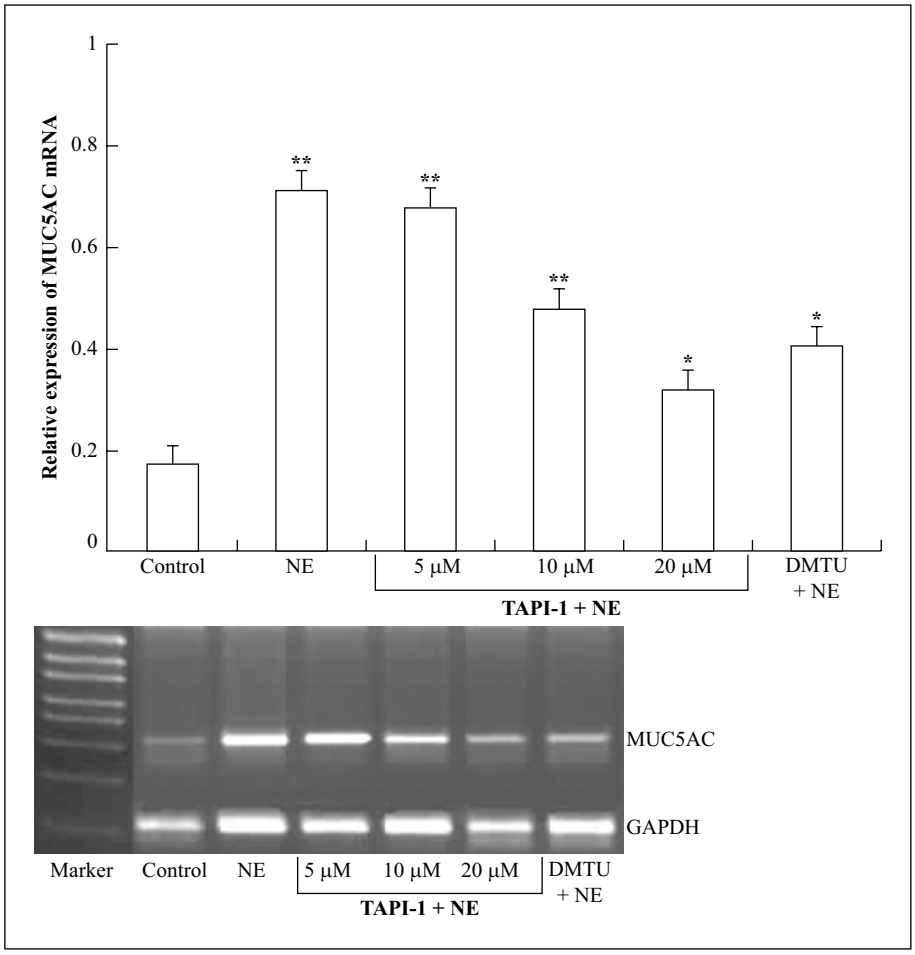

Figure 3

Effects of TAPI-1 and DMTU on NE-induced MUC5AC mRNA expression. NCI-H292 cells were left untreated and constituted the control group, treated with NE (25 nM) for 24 h or pretreated with inhibitors and then treated with NE (25 nM) for 24 h. Cells were incubated with the indicated concentrations of TAPI-1 (5, 10, 20 μM) or DMTU (20 μM). MUC5AC mRNA expression was determined using reverse transcription-PCR analysis. Data are expressed as mean ± SD (n = 3). ** $p < 0.01$; * $p < 0.05$, compared with control group. * $p < 0.01$, compared with the NE-treated group.

determined whether this process was required for increased MUC5AC expression. As shown in *figures 2A* and *3*, NE treatment of NCI-H292 cells increased MUC5AC protein production and gene expression. Pretreatment of cells with an efficient TACE inhibitor, TAPI-1, prevented NE-induced mucin response *(figures 2A and 3)* in a dose-dependent manner. Pretreatment with TAPI-1 (20 μM) reduced NE-induced MUC5AC mucin protein *(figure 2A)* and MUC5AC mRNA *(figure 3)* levels to that of the unstimulated control. It was then confirmed that inhibition of TACE activation by pretreatment with TAPI-1 prevented NE-induced, soluble TGF-α production *(figure 2B)*. These results suggest that NE-induced MUC5AC requires activation of TACE; the release of soluble TGF-α may also play an important part.

Effects of TAPI-1 of EGFR phosphorylation in NCI-H292 cells

It has been demonstrated that soluble TGF-α acts as a ligand for the EGFR. Therefore, we determined whether EGFR was activated by NE treatment and whether soluble TGF-α production was required. Treatment of NCI-H292 cells with NE for 24 h induced the release of soluble TGF-α, as well as phosphorylation of EGFR *(figure 5)* ($p < 0.01$). In addition, pretreatment with TAPI-1 (20 μM) decreased the levels of soluble TGF-α *(figure 2B)* and phosphorylated-EGFR protein *(figure 5)* induced by NE ($p < 0.01$). These results indicate that NE treatment induces phosphorylation of EGFR in NCI-H292 cells and requires soluble TGF-α production.

Effects of DMTU on NE-induced MUC5AC production in NCI-H292 cells

NCI-H292 cells were pretreated with a ROS scavenger, DMTU, followed by treatment with NE. In the normal control group with no stimuli, TACE protein was present mainly in its latent, inactive form, about 120 kDa, very little cleaved, active protein (100 kDa) being observed. Treatment with NE induced an increase in the 100 kDa active form of TACE protein *(figure 4)*. Pretreatment with DMTU decreased NE-induced TACE protein activation, $p < 0.05$, when compared with the NE-stimulated group. In addition, pretreatment with DMTU also prevented the NE-induced increase in MUC5AC protein released in

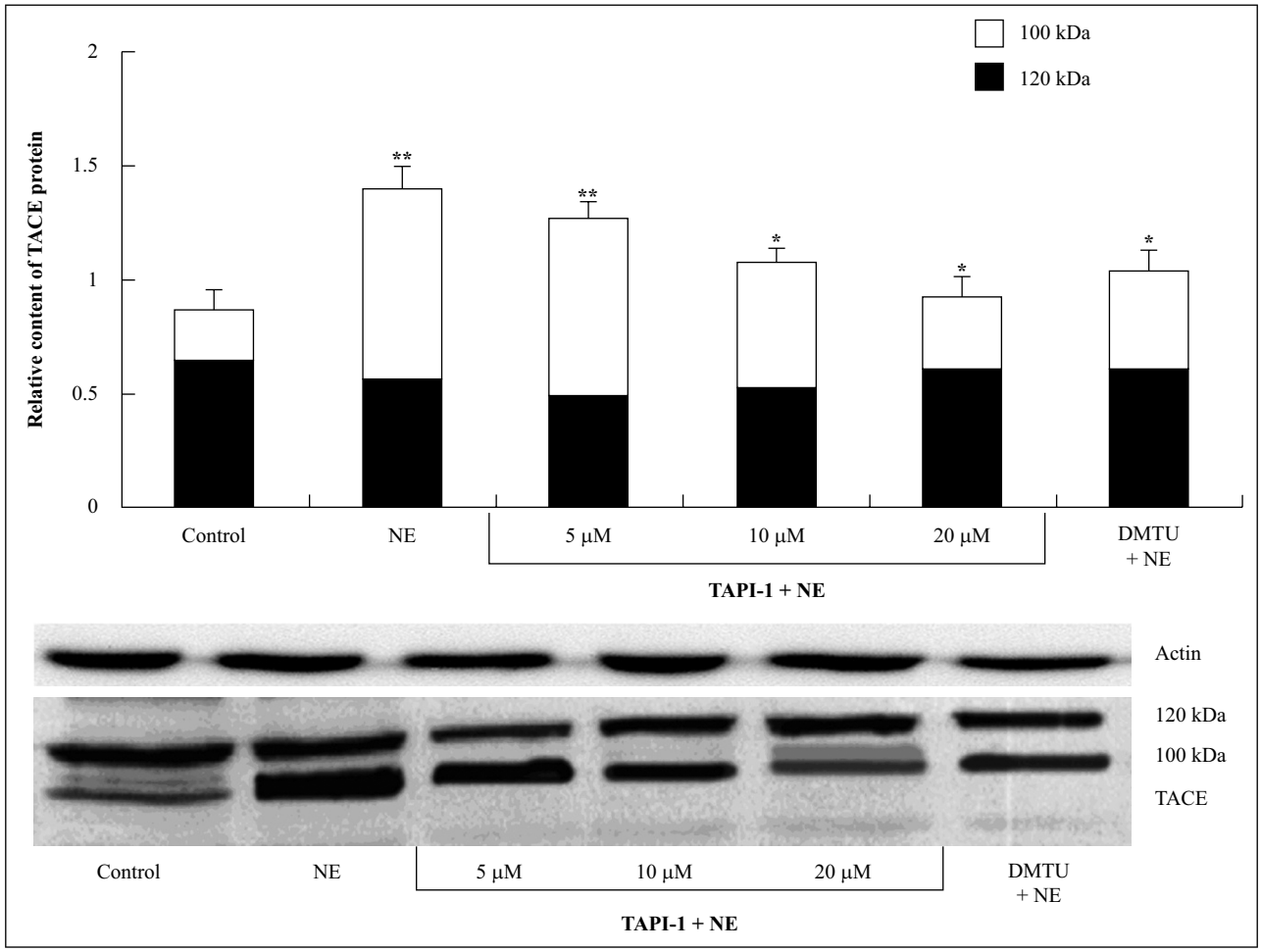

Figure 4

Effects of TAPI-1 and DMTU on NE-induced TACE protein production. NCI-H292 cells were left untreated , treated with NE (25 nM) for 24 h, or pretreated with inhibitors and then treated with NE (25 nM) for 24 h. Cells were incubated with the indicated concentrations of TAPI-1 (5, 10, 20 μM) or DMTU (20 μM). TACE protein was detected by western blot analysis. Data are expressed as mean ± SD ($n = 3$).The active form of TACE protein (100 kDa) increased noticeably, ** $p < 0.01$; * $p < 0.05$, compared with control group. * $p < 0.05$, compared with the NE-treated group. However, the inactive form of TACE protein (120 kDa) showed no statistically significant change.

culture supernatants and mRNA expression in cells *(figures 2A and 3)*, soluble TGF-α protein production in culture supernatants *(figure 2B)*, and phosphorylation of EGFR protein in cells *(figure 5)*.

Effects of DMTU on exogenous TGF-α-induced MUC5AC expression in NCI-H292 cells

Addition of exogenous, soluble TGF-α was also able to increase MUC5AC mRNA and protein expression in NCI-H292 cells, $p < 0.05$ *(figure 6)*. However, in contrast to the NE-induced increase in MUC5AC expression, pretreatment with DMTU had no significant effect on exogenous, soluble TGF-α-induced mucin synthesis. These data indicate that NE-induced ROS production and TACE activation are upstream of TGF-α production.

DISCUSSION

The EGFR signaling pathway has been shown to be a convergent pathway activated by various stimuli that induce mucin synthesis and mucin gene expression [7]. The activation of EGFR transfers the extracellular signal to intracellular signaling molecules, such as the mitogen-activated protein kinase (MAPK) family [8, 9]. These signaling molecules activate transcription factors, such as special protein-1, to initiate MUC5AC gene transcription, promoting mucin synthesis and hypersecretion. However, the mechanism of EGFR signaling cascade activation has not been well-defined. Previous studies have shown that metalloproteinase inhibition can prevent the release of EGFR ligand in stimulated cells [10]. TACE cleaves the extracellular domain of pro-TGF-α, releasing soluble, mature TGF-α, which results in

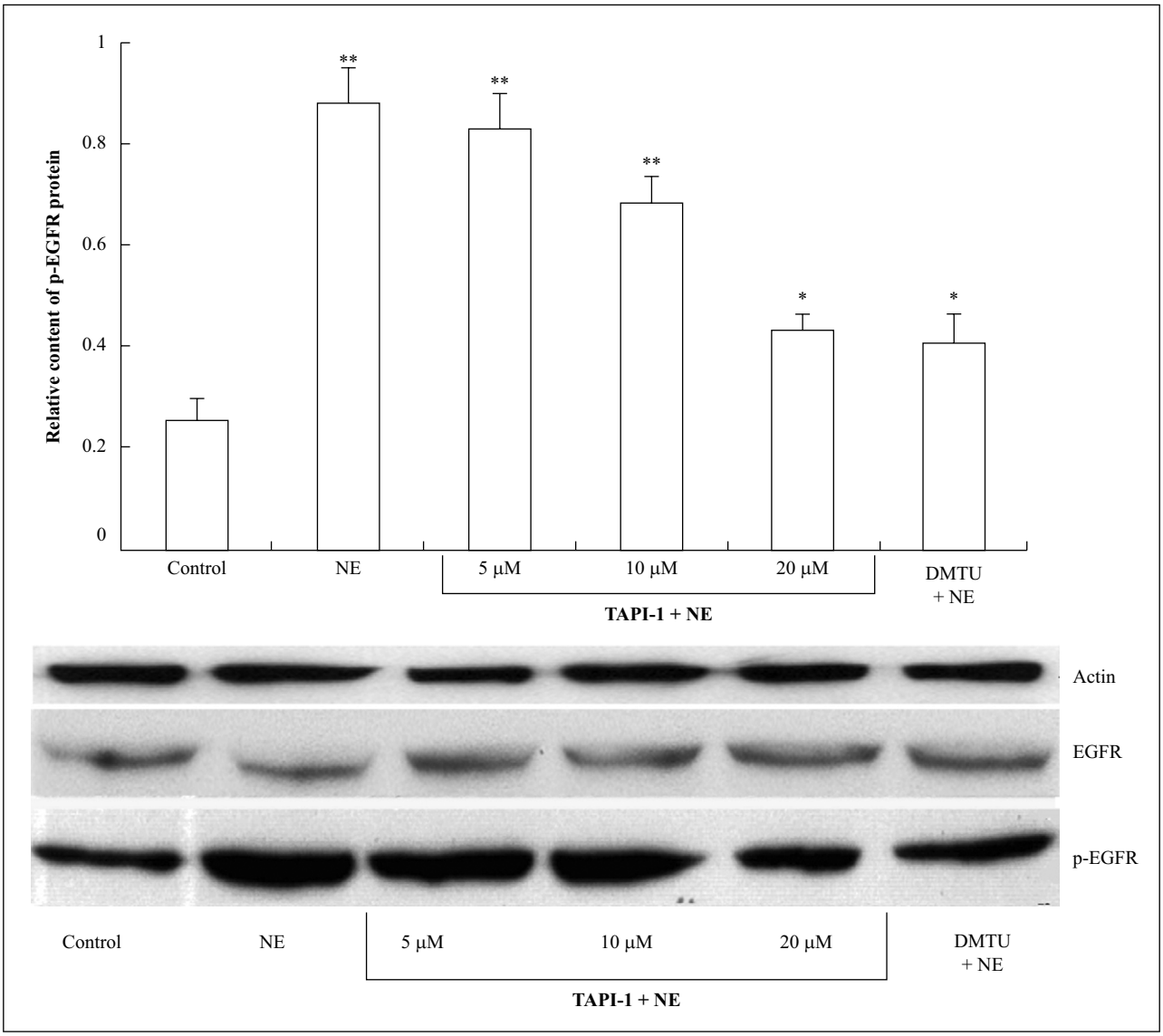

Figure 5
Effects of TAPI-1 and DMTU on EGFR phosphorylated protein. NCI-H292 cells were left untreated as the control group, other cells were treated with NE (25 nM) for 24 h or pretreated with inhibitors and then treated with NE (25 nM) for 24 h. Cells were incubated with the indicated concentrations of TAPI-1 (5, 10, 20 μM) or DMTU (20 μM). EGFR and phosphorylation of EGFR protein were detected using western blot analysis. Data are expressed as mean ± SD (n = 3). ** $p < 0.01$, * $p < 0.05$, compared with control group. * $p < 0.01$, compared with the NE-treated group.

phosphorylation of EGFR and induction of the downstream signaling cascade [11].

Airway epithelial cells produce EGFR and EGFR ligands [12-14]. TGF-α plays a critical role in EGFR phosphorylation [15, 16], leading to MUC5AC production in airways. TGF-α is synthesized as transmembrane pro-TGF-α [17], which is processed and released (ectodomain shedding) from the cell surface by management of metalloproteinases. TACE has been reported to cleave pro-TGF-α into mature soluble TGF-α in diverse epithelial

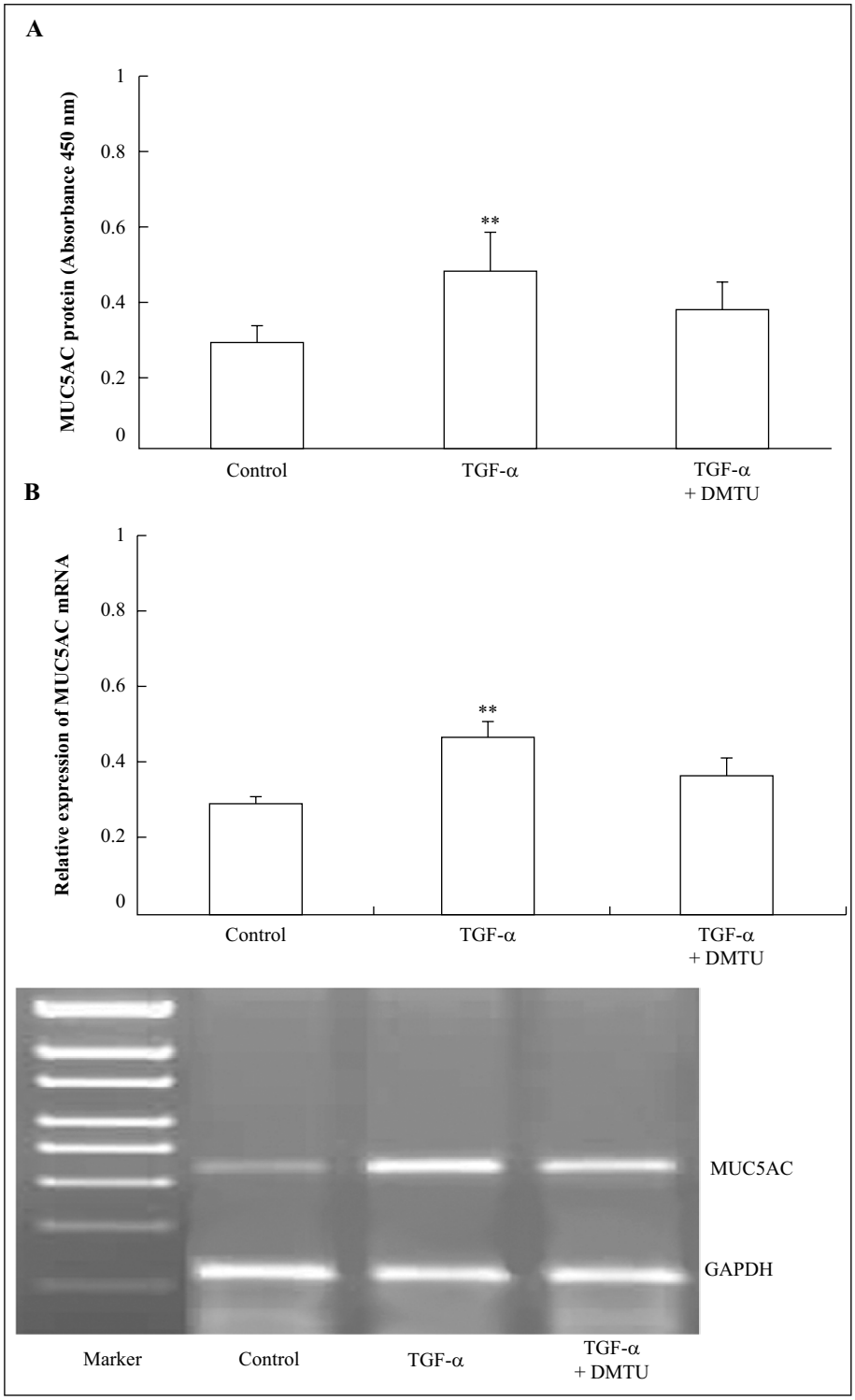

Figure 6

Effects of DMTU on exogenous TGF-α-induced MUC5AC protein production and mRNA expression in NCI-H292 cells. Cells treated with 10 ng/mL exogenous TGF-α or pretreated with DMTU (20 μM), and then treated with TGF-α (10 ng/mL).

A) MUC5AC protein expression measured using ELISA.

B) Relative MUC5AC mRNA expression was detected using reverse transcript-PCR analysis in different groups. Ten ng/mL exogenous TGF-α lead to an increase of MUC5AC protein production and mRNA expression, and these effects were not inhibited by DMTU. ** $p < 0.05$, compared with control group.

cells. TACE, also known as ADAM-17, is a member of "a disintegrin and metalloproteinase" (ADAM) family, a group of zinc-dependent, transmembrane metalloproteinases [18, 19]. TACE plays a critical role in various membrane binding growth factor activation, and their receptor cleavage, including TNF-α, TGF-α and amphiregulin [20]. TACE is synthesized in a latent form that remains in an inactive state, with the thiol group from a cysteine residue in the N-terminal prodomain binding to Zn^{2+} in the catalytic domain. Disruption of this cysteine-zinc bond results in a conformational change and thereby activating TACE. There are two well known, major mechanisms of TACE activation [21]. One involves protein kinase C (PKC) activation by stimuli (*e.g.* phorbol ester, neutrophil elastase), which induces serine phosphorylation in the cytoplasmic domain of TACE causing a conformational change in the extracellular domain [22]. The conformation change breaks the cysteine-zinc bond and unmasks the catalytic site, which binds and cleaves substrates of TACE. The second mechanism is cysteine oxidation in the prodomain, which unmasks the catalytic site of TACE. It has been reported that ROS or nitric oxide can attack the cysteine sulfhydryl moiety in the prodomain of TACE, and release it from binding sites with the catalytic zinc, thereby activating latent TACE [23]. Indeed, the involvement of a PKC-signaling pathway and the oxidation theory are not mutually exclusive because PKC activation has been reported to generate reactive oxygen species, and NE can generate ROS through PKC activation [24]. TACE is activated by ROS, resulting in substrate cleavage, and thus, cleavage of the EGFR pro-ligand into mature, solubleligand. Ligand then binds to and activates EGFR, resulting in MAPK family activation, mucin gene expression and protein production [25].

NE is one of the most common and potent agonists in mucus hypersecretion diseases [26, 27]. It is known to stimulate MUC5AC mucin production by means of the EGFR signaling pathway, as well as oxidant- and retinoic acid receptor-α-dependent mechanisms [28, 29]. Among these pathways, the EGFR signal cascade is one of the most important [30]. Our study was aimed at investigating the upstream signaling mechanisms involved in the NE-induced EGFR signaling cascade in airway epithelial cells. NE-treatment of NCI-H292 cells increased ROS production, TACE activation, soluble TGF-α production, EGFR phosphorylation and MUC5AC expression. Pretreatment of cells with TAPI-1, a TACE inhibitor, prevented cleavage of pro-TGF-α, EGFR phosphorylation and expression of MUC5AC. In addition, pretreatment with DMTU, a ROS scavenger, prevented TACE activation, as well as all the downstream signaling events; NE-induced MUC5AC expression in cells was downregulated by DMTU. However, treatment with DMTU did not prevent the increased MUC5AC expression induced by addition of exogenous TGF-α. These data indicate that ROS do not cleave TGF-α directly, but through the activation of TACE. These results highlight the unique role of ROS/TACE in mucus hypersecretion, and further emphasize the importance of the EGFR signaling cascade involved in this process.

Taken together, these results indicate that NE induces MUC5AC mucin synthesis via ROS production, TACE activation, pro-TGF-α cleavage, and EGFR activation in NCI-H292 cells. It should be emphasized that, as important signaling transducers, ROS/TACE should definitely be taken into consideration in the study of signal transduction mechanisms in mucus hypersecretion caused by various stimuli, and this would deepen our understanding of both the mechanisms of action and the regulation of airway mucus hyperscretion.

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