Neutrophil elastase stimulates MUC5AC expression in human biliary epithelial cells: a possible pathway of PKC/Nox/ROS

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Abstract

Introduction: Bacterial infection and bile flow retardation form a vicious cycle which promotes stone formation and recurrence, and it seems that mucin overexpression plays an important role in this process. However, the mechanism of increased mucus secretion in the biliary tract by bacterial infection and its treatment remain unclear.

Material and methods: Human biliary epithelial cells were induced by neutrophil elastase (NE), and H_2O_2 production in the cell supernatants was detected by a specific kit, and then cells were pretreated with a H_2O_2 inhibitor, and expression of MUC5AC was detected by real-time polymerase chain reaction (PCR), Western blot, and immunohistochemistry. Moreover, selective PKC and Nox inhibitors, apocynin and bisindolylmaleimide I, were used to pretreat cells and detect H_2O_2, MUC5AC mRNA and protein expression. Then, we pretreated cells with selective inhibitors or NE, and detected transforming growth factor α (TGF-α) using an ELISA kit.

Results: H_2O_2 production increased in an NE dose-dependent manner ($p < 0.001$), and NE upregulated MUC5AC expression at both mRNA and protein levels, while DMTU, could reduce this high expression ($p < 0.01$ at mRNA level, $p < 0.001$ at grey analysis for western blot and $p < 0.01$ at mean density for immunohistochemical staining at protein level). Moreover, apocynin and bisindolylmaleimide I could reduce the H_2O_2 production stimulated by NE ($p < 0.05$), and reduce MUC5AC high expression ($p < 0.01$ at mRNA level, $p < 0.001$ at both grey analysis for western blot and mean density for immunohistochemical staining at protein level). In addition, NE induced TGF-α production, and any of the three selective inhibitors could reduce it ($p < 0.05$).

Conclusions: NE-induced reactive oxygen species participated in the upregulation of MUC5AC production. Moreover, protein kinase C and NADPH oxidase (Nox) regulate MUC5AC production in NE-challenged human biliary epithelial cells.

Key words: neutrophil elastase, MUC5AC, reactive oxygen species, protein kinase C, Nox.

Introduction

Hepatolithiasis is a prevalent disease in Asian regions, particularly in China [1]. Despite the therapeutic effects of surgical and nonsurgical procedures [2], stone recurrence is observed in most hepatolithiasis patients, resulting in serious complications, such as cholangitis, biliary

stenosis, biliary fibrosis, and even cholangiocarcinoma, which always necessitates reoperation and significantly limits long-term survival [3]. Therefore, investigating the mechanism underlying stone formation and identifying potential targets to prevent stone recurrence remain a priority.

Although hepatolithiasis is a multifactorial disease, bacterial infection and bile flow retardation form a vicious cycle which promotes stone formation and recurrence, and mucin overexpression plays important roles in this process [4]. Bile is normally sterile, while bacteria, the most common of which are *Escherichia coli* and *Klebsiella*, are frequently found in the bile of patients with hepatolithiasis [5, 6]. Mucin distributed in epithelial mucosae has tissue and cell-specific expression, and functions to protect the epithelial surface and lubricate. To date, more than 20 types of mucin have been reported, six of which have been found in the biliary tract. During hepatolithiasis development, bacterial infection increases mucin 5AC (MUC5AC) expression, which is marginally present in normal bile, resulting in increased mucus viscosity and bile flow retardation, which promotes stone formation and recurrence [7]. Therefore, investigating the regulatory mechanism of MUC5AC overexpression and finding potential targets to inhibit this process might be a strategy for preventing stone recurrence and will improve the long-term survival in cases of hepatolithiasis.

Polymorphonuclear neutrophil (PMN) infiltration is a common phenomenon in the process of bacterial infection. During the process, PMNs release several mediators, and one, neutrophil elastase (NE; EC 3.4.21.37), is a serine protease that is thought an "end-effect" factor of inflammatory pathologies [8]. Neutrophil elastase is considered a significant factor in hepatic ischemia-reperfusion injury (IRI), nonalcoholic fatty liver disease (NAFLD) and many other diseases [9, 10]. Several studies have shown that NE can induce the overexpression of MUC5AC in human airway epithelial cells [11–16]. These studies showed that the PKC-Nox pathway, platelet activating factor (PAF), interleukin (IL), tumor necrosis factor α (TNF-α), and prostaglandin can increase expression of MUC5AC [10, 17, 18]. Studies on airway epithelial cells also showed that NE could activate protein kinase C (PKC) as well as downstream signaling molecules including dual oxidase 1 (Duox1), reactive oxygen species (ROS) and other factors that increase MUC5AC expression [12–14, 16].

As the biliary tract and the respiratory tract harbor similar embryological origins, our current study was undertaken to elucidate whether there exists a similar pathway of NE-induced MUC5AC expression in human intrahepatic biliary epithelial cells (HIBEpiC).

Material and methods

Basic cell culture and passage

Human intrahepatic biliary epithelial cells (HIBEpiC) were purchased from Sciencell (No. 5100) and were cultured in epithelial cell medium (consisting of 500 ml of basal medium, 10 ml of fetal bovine serum, 5 ml of epithelial cell growth supplement, and 5 ml of penicillin/streptomycin solution, all purchased from Sciencell) in a humidified atmosphere of 5% CO_2 at 37°C. The medium was refreshed every 2 days, and cells were passaged at a ratio of 1 : 2–1 : 3 every 3–5 days according to the cell condition.

Cell treatment with neutrophil elastase (NE) and inhibitors

HIBEpiC cells were treated with 50 ng/ml NE (Sigma). For inhibitor studies, cells were pretreated with inhibitors for 30 min before exposing the cells to NE. Concentrations of DMTU (inhibitor of ROS), apocynin (inhibitor of Nox) and bisindolylmaleimide I (inhibitor of PKC) were 25 mM, 1 mM and 5 μM respectively according to our previous work [16].

H_2O_2 measurement

Cells were treated with NE (50 ng/ml, 100 ng/ml, 1 μg/ml or 10 μg/ml) for 2 h, with or without selective inhibitors pretreated for 30 min, and H_2O_2 production in the cell supernatants was measured by using the Hydrogen Peroxide Assay kit (Invitrogen) according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured cells using Trizol reagent (Takara) following the manufacturer's instructions, and RNA was quantified by spectrophotometry. cDNA was prepared using the PrimeScript RT reagent kit with gDNA Eraser (Takara). Real-time PCR was performed using SYBR Green Premix Ex Taq (Takara). Sequences for the primers used were as follows: MUC5AC: (forward) 5′-TGGACACCAAATACGCCAACAAG-3′, (reverse) 5′-CTGCTCACAGATGCCAAAGCC-3′, Duox1: (forward) 5′-ATCGCCACCTACCAGAACATC-3′, (reverse) 5′- GGAGACACTTGAGTTCCGATTG-3′, β-actin: (forward) 5′-CTTAGTTGCGTTACACCCTTTCTTG-3′, (reverse) 5′- CTGTCACCTTCACCGTTCCAGTTT-3′.

For real-time PCR, the PCR mixture was denatured at 95°C for 10 s, annealed at 60°C for 20 s and then extended at 72°C for 30 s. This cycle was repeated for a total of 40 cycles. The fold change in expression of MUC5AC mRNA relative to β-actin was calculated based on the threshold cycle (Ct) values.

Immunohistochemical staining of MUC5AC protein

Cells were fixed in 4% paraformaldehyde, incubated with 0.5% TritonX-100 for 10 min, peroxidase-blocked in 3% H_2O_2 for 15 min to quench endogenous peroxidases, blocked by normal goat serum for 15 min, and then incubated with MUC5AC antibody (1 : 200 Santa Cruz) overnight at 4°C. The next morning, after removing excess antibody by washing with PBS, cells were incubated with biotinylated goat anti-mouse immunoglobulin G (1 : 200 dilution) for 30 min at room temperature, and then the cells were incubated for another 30 min in horseradish peroxidase (HRP, Beyotime). Cells were developed for 3 min with diaminobenzidine as the chromogen substrate, and the cells were counterstained with hematoxylin (Solarbio). Finally, the cells were observed by microscopy.

Western blot

Protein concentrations were measured by using the bicinchoninic acid protein assay. Protein samples were separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, USA). Non-specific binding to the membrane was blocked for 1 h at room temperature with 5% fat-free milk in TBST, and then the membranes were incubated with 1 : 400 MUC5AC primary antibody (Santa Cruz, USA) at 4°C overnight. Then, the membrane was washed 4 times with TBST and then incubated with a 1 : 5000 dilution of the appropriate secondary antibody at room temperature for 45 min. After the membrane was washed twice with TBST, membrane-bound antibody was visualized by using an enhanced chemiluminescent kit (Millipore) according to the manufacturer's instructions.

Transforming growth factor α (TGF-α) ELISA

Cells were treated with 50 ng/ml NE for 4 h with or without pretreatment of inhibitors of ROS, Nox or PKC. The TGF-α level in the cell supernatants was measured using the ELISA kit (R&D) according to the manufacturer's instructions.

Statistical analysis

Values are given as the mean ± SD. Differences between multiple groups were compared using one-way analysis of variance (ANOVA). When statistical significance was identified based on ANOVA, the Student-Newman-Keuls test was used for multiple comparisons. *P*-values < 0.05 were regarded as statistically significant.

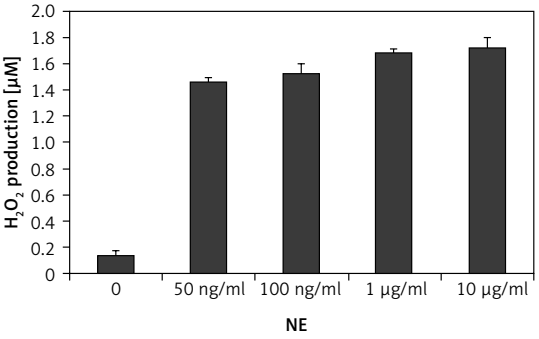

Figure 1. Cells were treated with NE at 0, 50 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml. H_2O_2 production increased as NE concentration increased (0.13 ±0.04, 1.46 ±0.04, 1.52 ±0.08, 1.68 ±0.04 and 1.72 ±0.08 μmol/l respectively

P < 0.001 for each group compared with the appropriate control.

Results

NE-induced H_2O_2 production

Cells were treated with different concentrations of NE (0, 50 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml) for 2 h to determine the optimal dose of NE. H_2O_2 production was 0.13 ±0.04, 1.46 ±0.04, 1.52 ±0.08, 1.68 ±0.04 and 1.72 ±0.08 μmol/l respectively. $P < 0.001$ for each group compared with the appropriate control. Moreover, 50 ng/ml NE showed statistically significant induction of H_2O_2 (Figure 1).

ROS are necessary for NE-induced MUC5AC expression

To determine whether ROS were involved in NE-induced MUC5AC expression, we assessed the effect of altering ROS levels in HIBEpiC cells. DMTU (25 nM), an ROS scavenger, attenuated NE-induced MUC5AC expression at the mRNA level based on real-time PCR as shown in Figure 2 A (1.00 ±0.03, 3.27 ±0.17 and 1.90 ±0.05, $p < 0.01$, expressed in $2^{-\Delta\Delta Ct}$, respectively). It was found that MUC5AC protein increased at 6 h and peaked at 24 h in airway epithelial cells [19]. Therefore, we determined MUC5AC protein expression by western blot analysis (Figures 2 B, C) and immunohistochemistry (Figures 2 D, E) after NE stimulation for 24 h. Figure 2 C shows grey analysis for western blot, and values were 1.00, 2.25 ±0.08, 1.62 ±0.03 respectively, $p < 0.001$ for each group compared with the appropriate control. Figure 2 E shows mean density for MUC5AC, and values were 0.29556 ±0.000573, 0.30828 ±0.0024015 and 0.29898 ±0.000968, $p < 0.01$ for each group compared with the control. Taken together, these data indicate that ROS are involved in NE-induced MUC5AC expression in HIBEpiC cells.

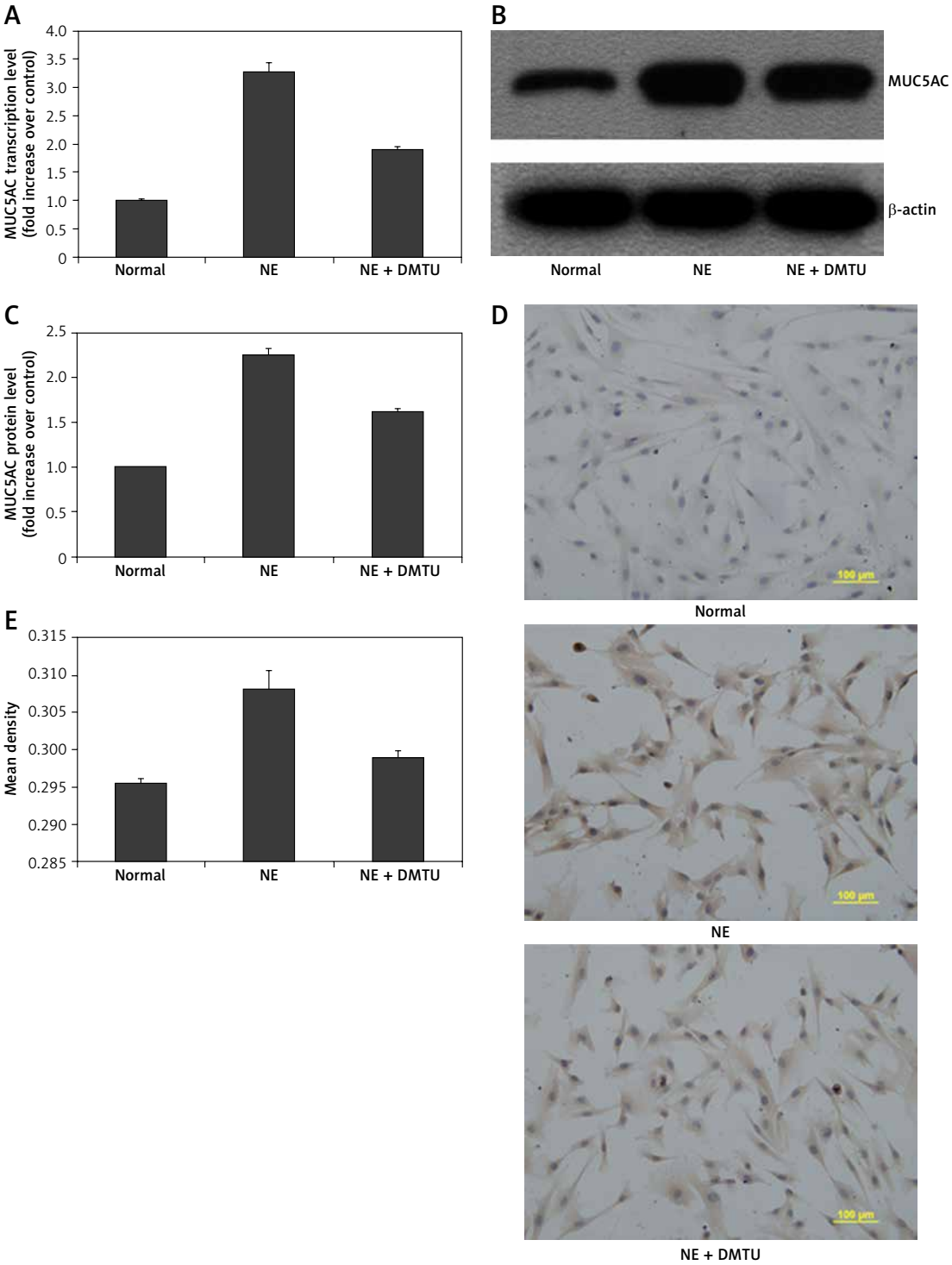

Figure 2. ROS is involved in NE-induced MUC5AC expression in HIBEpiC cells. **A** – HIBEpiC cells were pretreated with DMTU (25 mM) for 30 min and then were stimulated with NE for 12 h. Real-time PCR was performed to measure the changes in gene levels. Transcript levels were calibrated based on β-actin levels. Relative expression of MUC5AC mRNA was measured by the $2^{-\Delta\Delta Ct}$ method. All data are presented as the fold change in MUC5AC gene expression (1.00 ±0.03, 3.27 ±0.17 and 1.90 ±0.05, $p < 0.01$, expressed in $2^{-\Delta\Delta Ct}$, respectively). **B** – HIBEpiC cells were pretreated with DMTU (25 mM, 30 min) and were stimulated with NE for 24 h to determine the effect of DMTU on MUC5AC protein expression by western blot. The protein expression of MUC5AC increased upon NE exposure, and the NE-dependent increase in MUC5AC was attenuated in cells treated with DMTU. **C** – Grey analysis for western blot (1.00, 2.25 ±0.08, 1.62 ±0.03 respectively, $p < 0.001$ for each group compared with the appropriate control). **D** – NE-induced MUC5AC protein expression was inhibited by DMTU. After pre-treatment of cells with DMTU (25 mM for 30 min), HIBEpiC cells were stimulated with NE for 24 h, and MUC5AC protein expression was detected by immunohistochemistry (100×). **E** – Mean density for MUC5AC was 0.29556 ±0.000573, 0.30828 ±0.0024015 and 0.29898 ±0.000968, $p < 0.01$ for each group compared with the control

PKC and NADPH oxidase play important roles in NE-induced upregulation of MUC5AC

As H_2O_2 production is regulated by NADPH oxidase (Nox), and Nox can be activated by PKC to generate ROS [20], we hypothesized that Nox and PKC may be involved in NE-induced MUC5AC expression. Apocynin, a Nox inhibitor, and bisindolylmaleimide I, a PKC inhibitor, were used respectively to determine the involvement of Nox and PKC in NE-mediated MUC5AC expression. Cells were treated with different doses of NE (0, 50 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml) for 2 h, while two of the four groups of cells were pretreated with inhibitors respectively for 30 min. We found that both apocynin and bisindolylmaleimide I inhibit NE-induced ROS generation, as shown in Figure 3 A. H_2O_2 production in the normal group was 0.13 ±0.04 µmol/l, and in the "NE" group was 1.46 ±0.04, 1.52 ±0.08, 1.68 ±0.04 and 1.72 ±0.08 µmol/l respectively as the concentration of NE increased. H_2O_2 production in the "NE + apocynin" group was 1.06 ±0.08, 1.13 ±0.04, 1.26 ±0.10 and 1.35 ±0.10 µmol/l and in the "NE + bisindolylmaleimide I" group was 1.19 ±0.04, 1.21 ±0.08, 1.37 ±0.07 and 1.43 ±0.07 µmol/l respectively as the concentration of NE increased ($p < 0.05$ for each group compared with the control group). Furthermore, both agents blocked NE-induced MUC5AC expression at the mRNA level (Figure 3 B); it was 1.00 ±0.03, 3.27 ±0.17, 2.00 ±0.04 and 2.05 ±0.10, $p < 0.01$, expressed in $2^{-\Delta\Delta Ct}$, respectively. Furthermore, upregulation of MUC5AC protein by NE was inhibited by apocynin and bisindolylmaleimide I treatment (Figures 3 C–F). Figure 3 D shows grey analysis for Western blot, and values were 1.00, 2.25 ±0.08, 1.63 ±0.01 and 1.47 ±0.06, $p < 0.001$ for each group compared with the control. Figure 3 F shows mean density for MUC5AC (values were 0.29556 ±0.000573, 0.30828 ±0.0024015, 0.30692 ±0.0024974 and 0.30508 ±0.0034838, $p < 0.001$ for each group compared with the control). Thus, we believe that PKC and Nox generate ROS in response to NE stimulation, which results in the upregulation of MUC5AC expression.

PKC/Nox/ROS were involved in NE-induced TGF-α release

To determine whether PKC/Nox/ROS were involved in NE-induced TGF-α release in HIBEpiC, we assessed the effect on TGF-α release of altering PKC, Nox and ROS with bisindolylmaleimide, apocynin and DMTU respectively, and determined TGF-α level by ELISA (Figure 4). These data (1.45 ±0.35, 4.58 ±0.35, 2.37 ±1.40, 2.72 ±1.40 and 3.07 ±0.88 pg/ml, $p < 0.05$ for each group compared with the control) indicate that NE induced TGF-α release in HIBEpiC, and PKC/Nox/ROS were involved in this process.

Discussion

Inflammation is a pathophysiological reaction of the host to protect itself from pathogens. This complex and dynamic process is characterized by an innate immune response, which involves coordinated expression of inflammatory cytokines and implication of various cell types particularly immune cells aimed at clearing the pathogenic agent. In the setting of biliary bacterial infections (e.g. *E. coli* or *Klebsiella*), the host innate immune response is characterized by the initial recognition of invading microbes by the host via Toll-like receptors (TLRs) or other pattern recognition molecules [21]. Subsequently, this results in the production of an array of inflammatory mediators including early responsive cytokines. Another hallmark of innate host biliary defense, especially when the first lines of defense – the epithelial barrier and resident macrophages – are breached, is the massive recruitment of polymorphonuclear neutrophils (PMN) to the infected site. The PMNs are efficient phagocytes whose main function upon activation is thought to be the clearance of infecting bacteria. To do so, these cells are equipped with a myriad of antimicrobial molecules grouped into oxidative and nonoxidative systems [22, 23]. The NE, the PMN-specific serine protease, has been identified as a key antimicrobial enzyme [24]. As a cationic glycoprotein, it is stored in a readily active form in PMN primary granules at concentrations exceeding the millimolar range, making it a major component of PMN. Our previous studies have found that exogenous LPS could stimulate HIBEpiC MUC5AC expression [16, 25]. The NE is a 30 kD neutral serine protease, stored in an active form in the azurophilic granules of neutrophils, and can be released when neutrophils are exposed to LPS. Whether NE could also induce MUC5AC expression remains unknown.

Reactive oxygen species (ROS) are generated from biological aerobic metabolism. They play a physiological role in cells, as well as being risk factors for several diseases [26–28]. Fischer *et al.* [12] found that ROS mediated NE-induced MUC5AC gene expression in A549 cells. In this research, we found that NE induced H_2O_2 production in a dose-dependent manner, subsequently upregulating MUC5AC gene and protein expression. 30-min preincubation of cells with DMTU, a ROS scavenger, could inhibit this upregulation. Thus, we thought that ROS may participate in NE-induced MUC5AC expression in HIBEpiC. In studies in NCI-H292 airway epithelial cells, it has been reported that NE-induced ROS production activated tumor necrosis factor-α-converting enzyme (TACE), result-

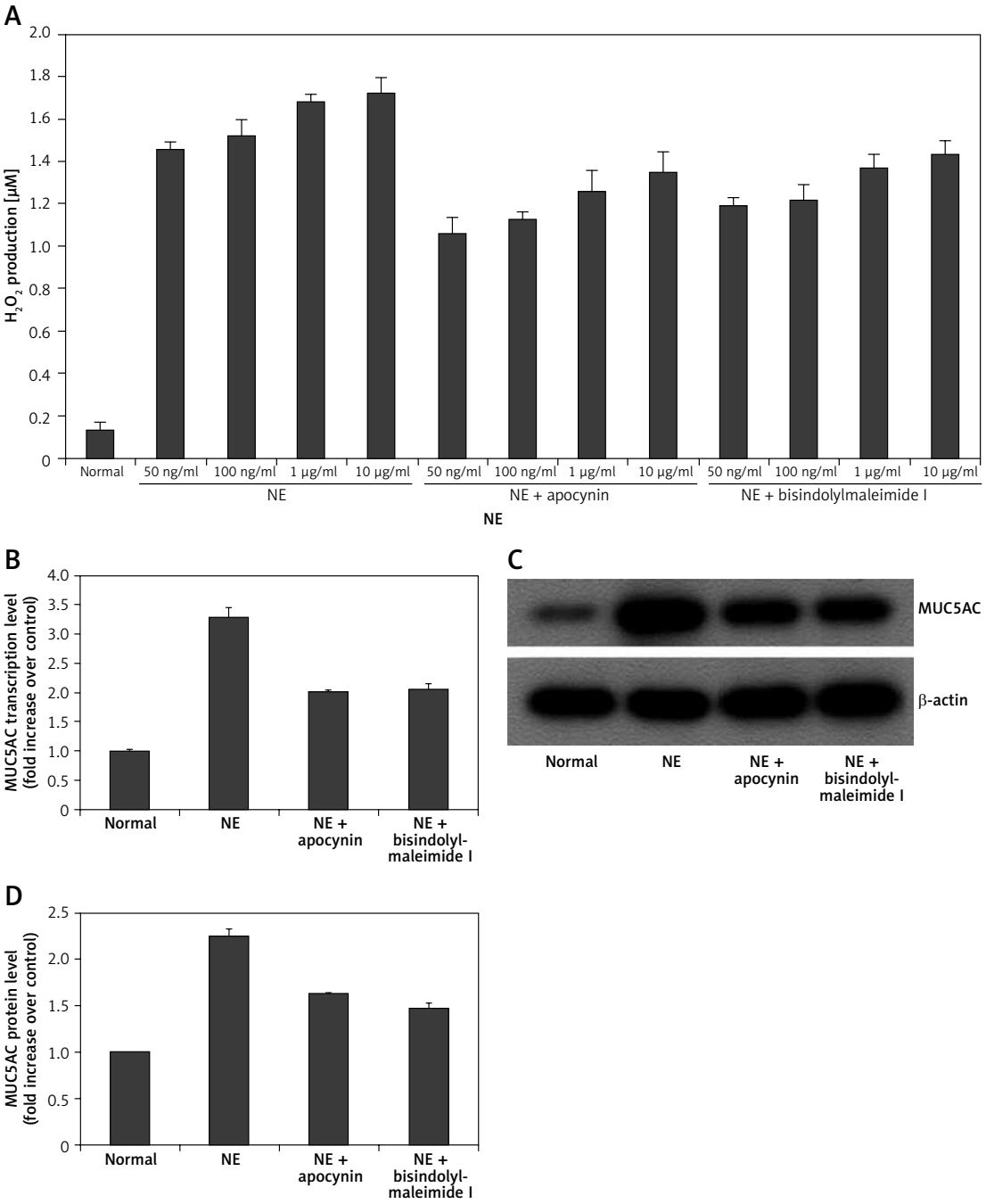

Figure 3. PKC and NADPH oxidase participate in the process of NE upregulation of MUC5AC expression. **A** – Cells were treated with NE at 0, 50 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml in addition to Nox or PKC inhibitor. Pretreatment with apocynin and bisindolylmaleimide I prevents ROS generation. H_2O_2 production in the normal group was 0.13 ±0.04 μmol/l, and in the "NE" group was 1.46 ±0.04, 1.52 ±0.08, 1.68 ±0.04 and 1.72 ±0.08 μmol/l respectively as the concentration of NE increased. H_2O_2 production in the "NE + apocynin" group was 1.06 ±0.08, 1.13 ±0.04, 1.26 ±0.10 and 1.35 ±0.10 μmol/l and in the "NE + bisindolylmaleimide I" group was 1.19 ±0.04, 1.21 ±0.08, 1.37 ±0.07 and 1.43 ±0.07 μmol/l respectively as the concentration of NE increased. $P < 0.05$ for each group compared with the control group. **B** – Apocynin (1 mM) and bisindolylmaleimide I (5 μM) attenuated NE-induced MUC5AC expression at the mRNA level. HIBEpiC cells were pretreated with apocynin or bisindolylmaleimide I for 30 min, and then the cells were stimulated with NE (50 ng/ml) for 12 h, and MUC5AC mRNA levels were determined by real-time PCR. Transcript levels were calibrated based on β-actin expression. Relative expression of MUC5AC mRNA was measured by using the $2^{-\Delta\Delta Ct}$ method. All data are presented as the fold change in MUC5AC gene expression (1.00 ±0.03, 3.27 ±0.17, 2.00 ±0.04 and 2.05 ±0.10, $p < 0.01$, expressed in $2^{-\Delta\Delta Ct}$, respectively). **C** – HIBEpiC cells were pretreated with apocynin (1 mM) or bisindolylmaleimide I (5 μM) respectively for 30 min and then were stimulated with NE (50 ng/ml) for 24 h to determine the effect of these agents on MUC5AC protein expression by western blot. **D** – Grey analysis for western blot (1.00, 2.25 ±0.08, 1.63 ±0.01 and 1.47 ±0.06, $p < 0.001$ for each group compared with the control)

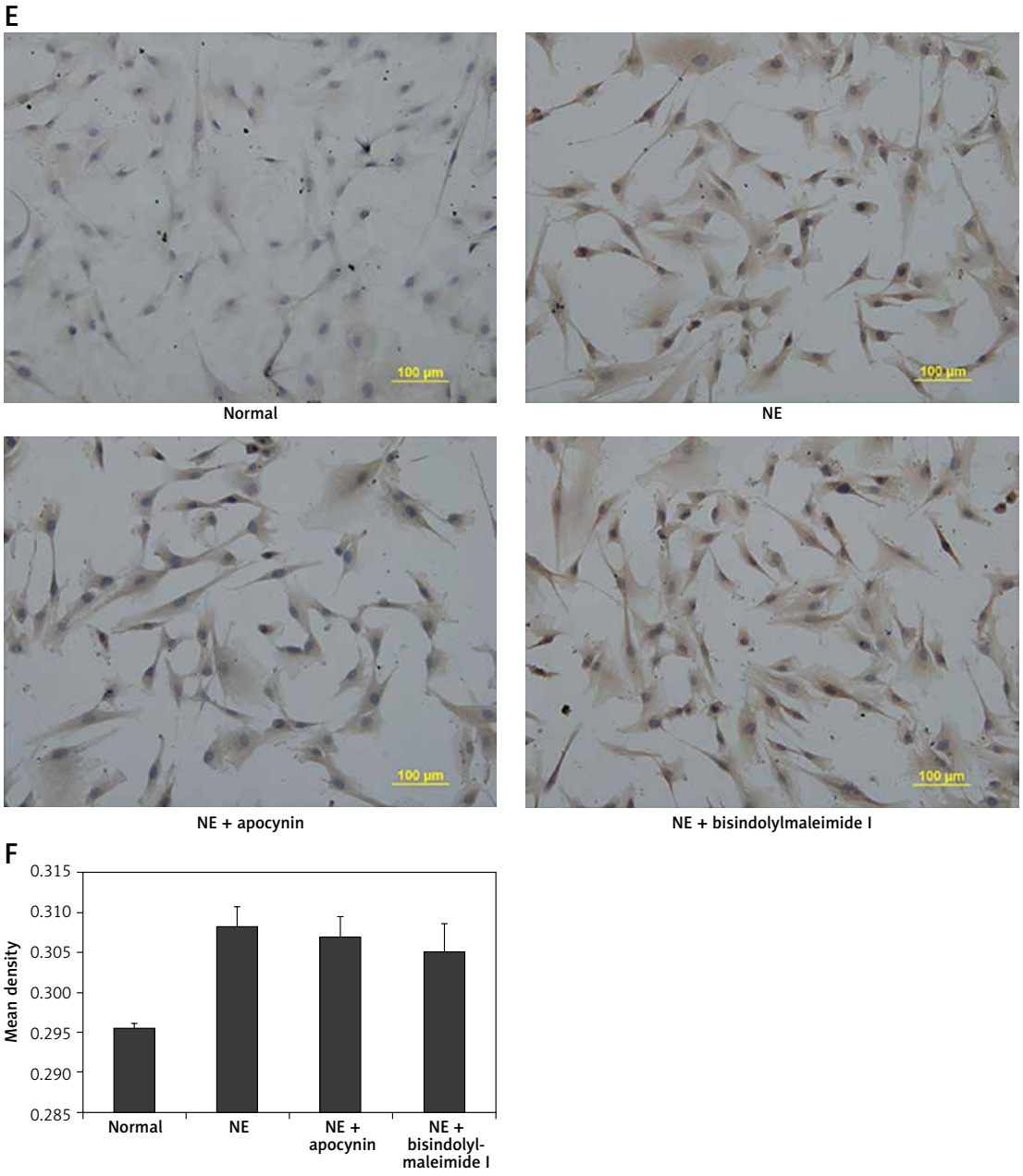

Figure 3. E – NE-induced MUC5AC protein expression was abolished by apocynin and bisindolylmaleimide I treatment. HIBEpiC cells were pretreated with apocynin (1 mM) or bisindolylmaleimide I (5 μM) respectively for 30 min and then were stimulated with NE (50 ng/ml) for 24 h to determine the effect of these agents on MUC5AC protein expression by immunohistochemistry (100×). **F** – Mean density for MUC5AC was 0.29556 ±0.000573, 0.30828 ±0.0024015, 0.30692 ±0.0024974 and 0.30508 ±0.0034838, $p < 0.001$ for each group compared with the control

ing in conversion of pro-TGF-α into soluble TGF-α, and epidermal growth factor receptor (EGFR) phosphorylation [14, 20]. Moreover, the EGFR pathway has been thought to be a convergent pathway activated by various stimuli which induce mucin gene expression and synthesis [29]. In our experiment, we also found that NE could induce TGF-α release and DMTU inhibits TGF-α production (Figure 4), suggesting that TGF-α, produced by activated ROS, may take part in NE-induced MUC5AC expression.

It has been reported that ROS were generated by Nox of phagocytes (Phox), and the catalytic core of Nox was the six-transmembrane glycoprotein $p91^{phox}$. Duox1 is a homologue of $p91^{phox}$, and was first reported in airway epithelial cells [13]. In our experiment, we found that using apocynin to block Nox, the increased H_2O_2, TGF-α as well as MUC5AC gene and protein expression all decreased (Figures 3, 4). Thus, we believed that Nox might active ROS that induced MUC5AC expression.

PKC activation has been implicated in mucin secretion in human epithelial cells [30]. PKC exists as isoforms δ and θ, and it has been found that PKCδ might take part in the NE-induced MUC5AC

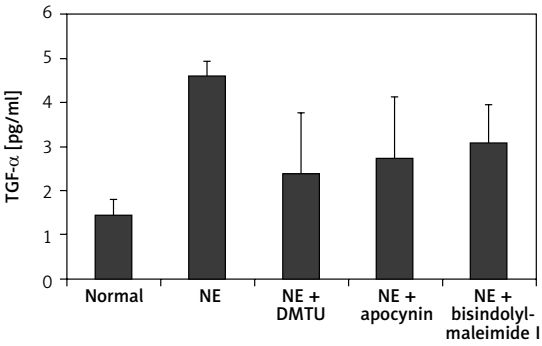

Figure 4. PKC/Nox/ROS were involved in NE-induced TGF-α release. Cells were treated with 50 ng/ml NE for 4 h with or without pretreatment of inhibitors of ROS, Nox or PKC. TGF-α level was determined by ELISA. NE induced TGF-α release and pretreatment with either inhibitor attenuated TGF-α generation (1.45 ±0.35, 4.58 ±0.35, 2.37 ±1.40, 2.72 ±1.40 and 3.07 ±0.88 pg/ml, $p < 0.05$ for each group compared with the control)

expression in airway epithelial cells. The activated PKCδ caused $p47^{phox}$ and $p67^{phox}$ translocated from cytosol to plasma membrane to form the complete enzyme Nox with $gp91^{phox}$ [13, 31]. Then Nox is activated, and consequently other downstream signaling molecules. In this experiment, we found that using a PKC inhibitor, bisindolylmaleimide I, could attenuate the increased H_2O_2, TGF-α and MUC5AC gene and protein expression stimulated by NE (Figures 3, 4). So it seemed that PKC also participates in NE-induced MUC5AC expression. NE is a native ligand for protease-activated receptors (PARs) and Ca^{2+} plays an important role in regulating the final steps of exocytosis in goblet and other secretory cells. Zhou *et al.* [32] found NE acting on PAR2, increasing the cytosolic Ca^{2+} concentration, and subsequently activating PKC. Whether NE has a similar pathway in activating PKC and whether other factors such as annexin II (ANXII) and ezrin [33, 34], which were reported to regulate NE-induced MUC5AC expression in airway epithelial cells, have a similar effect in HIBEpiC requires more intensive research.

Based on our results, we also found that all of the three inhibitors could attenuate the upregulation, while none of them could attenuate the upregulation completely, and it seems that DMTU was more "effective". We suppose there might exist other pathways or cytokines that participate in this process, such as PG2, TNF-α, IL or something else, as in other epithelial cells.

There were some weaknesses in our experiment. First, when we detected H_2O_2 production, we did not collect cells that were treated without NE at different time points. Moreover, we did not use the TUNEL assay or LDH assay or another method to confirm that there had not been any cell death in our experimental set-up.

In conclusion, we have shown that NE could induce H_2O_2 production in a dose-dependent manner. Moreover, using an ROS inhibitor could reduce NE-induced MUC5AC expression. We then found that using a Nox inhibitor could reduce NE-induced MUC5AC expression. As Nox is activated by PKC, we detected that the PKC inhibitor also has the ability to reduce MUC5AC expression. All considered, we concluded that NE-induced ROS participated in the upregulation of MUC5AC production, and, moreover, PKC and Nox have a role in NE-challenged human biliary epithelial cell MUC5AC expression.

Acknowledgments

Yu Tiana and Min Li contributed equally.

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Conflict of interest

The authors declare no conflict of interest.

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