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How GP96 upregulation shields against COPD: mitigating endoplasmic reticulum stress-induced cellular damage *via* p38/ERK pathway activation

Lei Xue^{1,*}, Yihui Wang^{1,*}, Ting Yang¹, Wanling Yang¹, Ying Liu¹ and Jie Tan¹¹ Department of Pulmonary and Critical Care Medicine, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia Autonomous Region, China

Abstract. Chronic obstructive pulmonary disease (COPD) is closely associated with endoplasmic reticulum stress (ERS). We explored the potential role of Glycoprotein 96 (GP96) in mitigating ERS-induced cellular damage in an *in vitro* COPD model. Human bronchial epithelial 16HBE cells were exposed to nicotine to establish a COPD model *in vitro*. The expression of GP96 was manipulated, and the involvement of the extracellular signal-regulated kinase (ERK) signaling pathway was assessed by treatment with the ERK inhibitor PD98059. The effects of GP96 and the p38/ERK pathway on ERS-related markers, apoptosis and its associated proteins, reactive oxygen species (ROS) levels, pro-inflammatory cytokines, and the activation levels of ERK and p38 were evaluated. Nicotine induced GP96 expression in 16HBE cells. Nicotine also repressed cell viability, and promoted apoptosis, ROS release, expressions of ERS-related markers and pro-inflammatory cytokines levels in 16HBE cells, which were reversed by GP96 overexpression. Besides, nicotine elevated p-ERK/ERK and p-P38/P38 levels in 16HBE cells, which was further enhanced by GP96 overexpression. In contrast, GP96 silencing produced effects opposite to those of GP96 overexpression. The ERK inhibitor PD98059 offset the effects of GP96 overexpression, except for its impact on the p-P38/P38 levels, which remained unaffected. GP96 overexpression inhibits ERS-induced apoptosis, oxidative stress and inflammation in nicotine-induced *in vitro* COPD model through potentiating ERK activation.

Key words: Glycoprotein 96 — COPD — Endoplasmic reticulum stress — Inflammation — Extracellular signal regulated kinase activation

Introduction

Chronic obstructive pulmonary disease (COPD) is a common respiratory disorder characterized by progressive airflow obstruction and persistent respiratory symptoms (Labaki and Rosenberg 2020). Smoking constitutes the

primary cause of COPD in high-income countries, being responsible for an estimated 70% of all cases (Lu et al. 2024). As the primary addictive component in tobacco, nicotine drives addiction that increases smoking, thereby promoting the development and progression of COPD (Pérez-Rubio et al. 2019). COPD imposes a substantial global health burden due to its high morbidity and all-cause mortality (Ferrera et al. 2021), ranking as the third leading global cause of death (Brassington et al. 2022). The mechanism underlying COPD is complex and implicates various pathogenic processes, including an abnormal chronic inflammatory response to noxious particles or gases in the lungs (Labaki and Rosenberg 2020; Xu et al. 2024). This chronic inflam-

* These authors contributed equally to this work.

Correspondence to: Jie Tan, Department of Pulmonary and Critical Care Medicine, The Affiliated Hospital of Inner Mongolia Medical University, No. 1, North Tongdao Street, Huimin District, Hohhot, Inner Mongolia Autonomous Region, China
E-mail: tanjie_tj345@yeah.net

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mation entails pro-inflammatory cytokine secretion, which also leads to accumulation of reactive oxygen species (ROS) that can induce oxidative stress (Guo et al. 2022). Oxidative stress activates apoptotic pathways to further enhance the inflammatory response, thus exacerbating the disease (Zuo et al. 2019; Guo et al. 2022). Previous studies have found that endoplasmic reticulum stress (ERS) is one of the main causes of the early development of COPD, and it is involved in the progression of airway inflammation and epithelial cell apoptosis in COPD (Wang et al. 2022; Yu et al. 2022; Peng et al. 2023). In COPD, the altered airway milieu affects the respiratory epithelium and disrupts cellular homeostasis, thus creating stress in the endoplasmic reticulum (ER) (Ribeiro and O'Neal 2012; Kropski and Blackwell 2018). Studies indicate that modulating ERS signaling ameliorates key pathological processes in COPD, such as inflammatory cytokine release and apoptotic cell death, positioning it as a promising therapeutic target (Kropski and Blackwell 2018). Therefore, understanding the regulation of ERS and its downstream pathways remains crucial for developing novel interventions in COPD.

Previous research has reported that heat shock protein 72 (HSP72), a stress-inducible cytosolic molecular chaperone, exerts pro-survival effect in cells under ERS (Gupta et al. 2010). Also, HSP72 has been shown to inhibit the activation of JNK, thereby alleviating COPD *in vitro* (Hao et al. 2021). These findings suggest that HSP72p plays an antagonistic role against ERS-induced damages in COPD. Glycoprotein 96 (GP96), also known as GRP94, has been revealed to positively correlate with HSP72 in colonic carcinomas (Wang et al. 2013). GP96 is a paralog of ER heat shock protein 90, and resides in the ER and the mitochondria, two organelles responsible for protein quality control and metabolic balance (Duan et al. 2021). GP96 can act as a protein chaperone to aid in the folding of client proteins (Yang et al. 2007; Liu et al. 2013; Rachidi et al. 2015), and is induced under stress conditions including ERS (Duan et al. 2021, Xiang et al. 2024). GP96 serves as an effector molecule of the unfolded protein response (UPR), an adaptive mechanism activated to relieve ERS-caused burden of misfolded proteins and restore normal physiologic ER function (Yang and Li 2005; Rachidi et al. 2015). Deletion of GP96 results in accumulation of misfolded proteins and UPR activation; consequently, the failure of the UPR to resolve ERS triggers cell death (Rosenbaum et al. 2014; Rachidi et al. 2015).

Activation of the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway has been recorded to inhibit ERS, thereby attenuating hypoxia-mediated cardiomyocyte damage (Zou and Liu 2021). GP96 can elicit ERK activation in murine macrophages (Reed et al. 2003). GP96 deficiency affects TLR4 functionality and thus impairs the downstream events, ERK and P38 phosphorylation (Cosin-Roger et al. 2018). These data indicate

that GP96 has a direct association with MAPK/ERK signaling activation, through which ERS may be inhibited.

The present study aims to determine whether GP96 alleviates ERS-induced cell damages in an *in vitro* COPD model and whether this potential role of GP96 is related to the activation of p38/ERK signaling pathway.

Material and Methods

Cell culture

Human bronchial epithelial cell lines (16HBE cells) were purchased from American Type Culture Collection (ATCC; PCS-300-010, Manassas, VA, USA), and were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (PM150110, Procell, Wuhan, China) containing 10% fetal bovine serum (FBS; 12103C, Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-Glutamax (A2916801, ThermoFisher, Waltham, MA, USA) and 1% penicillin-streptomycin (TMS-AB2, Sigma-Aldrich, USA) at 37°C in a humidified atmosphere with 5% CO₂. 16HBE cells have been analyzed for short tandem repeat and mycoplasma.

Cell transfection

Sequences of GP96 were amplified by Genepharma (Shanghai, China) and ligated onto pcDNA3.1 vectors (V79520, ThermoFisher, USA) to construct the corresponding over-expression plasmids. The empty vector was assigned as the negative control (NC) for the overexpression plasmids. Small interfering RNA targeting GP96 (Si-GP96, 5'-GAAUGAU-AUCAAACCAAUATT-3') and its negative control (Si-NC, 5'-UUCUCCGAACGUGUCACGUTT-3') were purchased from RIBOBIO (siG081028163030-1-5 and siN00000002-1-5, Guangzhou, China). 16HBE cells were plated in 96-well plates at a density of 1×10^4 cells/well and cultured to reach an 80% confluent monolayer, which were later subjected to transfection of the above plasmids using Lipofectamine 3000 transfection reagent (L3000015, ThermoFisher, USA) according to the manufacturer's protocols. Briefly, after being diluted with Opti-MEM and/or P3000 reagent, the above plasmids and Lipofectamine 3000 transfection reagent were incubated for 10 min at 37°C for the generation of gene-lipid complexes. For transfection, the obtained complexes were added into the monolayer, followed by incubation at 37°C for 48 h. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was used to measure transfection efficiency.

Treatment with nicotine and/or the ERK inhibitor

Nicotine (613215, Sigma-Aldrich, USA) was prepared using dimethyl sulfoxide (DMSO; D1435, Sigma-Aldrich, USA)

and added into the culture medium to obtain a final concentration of 40 $\mu\text{mol/l}$. *In-vitro* COPD models were established by exposing transfected or non-transfected 16HBE cells to 40 $\mu\text{mol/l}$ nicotine for 72 h at 37°C (Lin et al. 2017). The ERK inhibitor, PD98059, was procured from Sigma-Aldrich (P215, USA) and added into the culture medium to reach a final concentration of 10 $\mu\text{mol/l}$ after being dissolved in DMSO. To block ERK, GP96 overexpression plasmid-transfected 16HBE cells were treated with 10 $\mu\text{mol/l}$ PD98059 in the 40 $\mu\text{mol/l}$ nicotine-conditioned culture medium, 12 h before the end of nicotine treatment (Zong et al. 2018).

Cell counting kit (CCK)-8 assay

16HBE cells were plated in 96-well plates at a density of 5×10^3 cells/well, and treated with/without nicotine or transfection plus nicotine combined with/without PD98059 treatment. Later, CCK-8 reagent (C0037, Beyotime, Shanghai, China) was added into the culture medium, followed by 2-h incubation at 37°C. The absorbance, which indicates cell viability, was measured at 450 nm by a microplate reader (EMax Plus, Molecular Devices, Sunnyvale, CA, USA).

QRT-PCR

Total RNA was extracted by Trizol reagent (15596026, ThermoFisher, USA) from 16HBE cells treated as needed. Complementary DNA (cDNA) was synthesized from 1 μg of the total RNA using a Revert Aid first strand cDNA synthesis kit (K1621, ThermoFisher, USA). Real-time PCR detection was implemented on a system (MX3005P, Agilent Technologies, Santa Clara, CA, USA) using TB Green Premix Ex Taq (RR420Q, TaKaRa, Tokyo, Japan) following the thermocycle condition: 95°C for 10 min, followed by 40 circles of 95°C for 15 seconds, and 60°C for 1 min. The primers used in the detection were illustrated as follows: GP96, forward 5'-GCTGACGATGAAGTTGATGTGG-3' and reverse 5'-CATCCGTCCTTGATCCTTCTCTA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-GAAGGCTGGGGCTCATTT-3' and reverse 5'-AGTGATG-GCATGGACTGTGG-3'. Relative expression levels of GP96 were analyzed by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001), with GAPDH employed as the endogenous reference.

Flow cytometry

Flow cytometry was performed to determine cell apoptosis using an AnnexinV-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Double Staining kit (556547, Livning, Beijing, China). Briefly, 16HBE cells treated as needed were digested using ethylenediaminetetraacetic acid (EDTA)-free trypsin (T2600000, 0.25%, Sigma-Aldrich, USA). The cells were collected after centrifugation at $2000 \times g$

for 10 min, and then suspended in phosphate buffered saline (PBS; P4474, Sigma-Aldrich, USA). Centrifugation at $2000 \times g$ for 10 min was again performed to obtain the cells, which were later washed by PBS once and resuspended by Binding Buffer. The resuspended cells were incubated with 5 μl Annexin V-FITC for 15 min. PI (5 μl) was added 5 min before the cells were loaded onto a flow cytometer (Cytotflex, Beckman Coulter, Brea, CA, USA) for apoptosis detection. Flow cytometry was also carried out to determine the ROS release with a ROS assay kit (S0033S, Beyotime, China). In brief, after corresponding treatment as required, 16HBE cells were resuspended in the culture medium containing 10 $\mu\text{mol/l}$ DCFH-DA. Then, 30-min cell incubation was conducted at 37°C, during which fluorescence-emitting DCF was generated via oxidation by ROS. The fluorescent signal was detected by the flow cytometer to indicate the level of ROS in the cells.

Enzyme-linked immunosorbent assay (ELISA)

After treatment as mentioned above, 16HBE cells were harvested and underwent centrifugation at $1000 \times g$ for 10 min at 37°C to obtain the supernatant. ELISA kits (ml027376, ml028583 and ml077385, mlibio, Shanghai, China) were utilized to assess the levels of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF- α) in the supernatant according to the manufacturer's instructions. In short, enzyme-coated plates were added with the supernatant (50 μl), followed by incubation at 37°C for 30 min. Then, biotinylated antibodies (50 μl) and HRP-conjugated enzyme (80 μl) were added at 37°C for 30-min incubation, respectively. Lastly, color development was performed with A substrate (50 μl) and B substrate (50 μl) for 10 min. After the reaction was terminated by stop solution, the absorbance was measured at 450 nm by the microplate reader.

Western blot

Total protein was isolated using radioimmunoprecipitation assay (RIPA) Buffer (R377980, Aladdin, Shanghai, China) with phosphatase inhibitors (P377930, Aladdin, China) from 16HBE cells treated as needed. The concentration of the isolated protein was determined using bicinchoninic acid (BCA) kits (A53227, ThermoFisher, USA), and then the protein (45 μg) was separated by 6% and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (P0676 and P0672, Beyotime, China), followed by being transferred onto polyvinylidene fluoride membranes (P2438, Sigma-Aldrich, USA). Skimmed milk (5%) reconstituted in Tris Buffered Saline with 1% Tween 20 (TBST, TA-125-TT, ThermoFisher, USA) was used to block the membranes at 37°C for 1 h. Thereafter, primary antibodies for B-cell lymphoma 2 (Bcl-2; ab32124, 26 kDa, 1:1000, Abcam, Cambridge, UK), Bcl-2-associated X protein (Bax; ab32503,

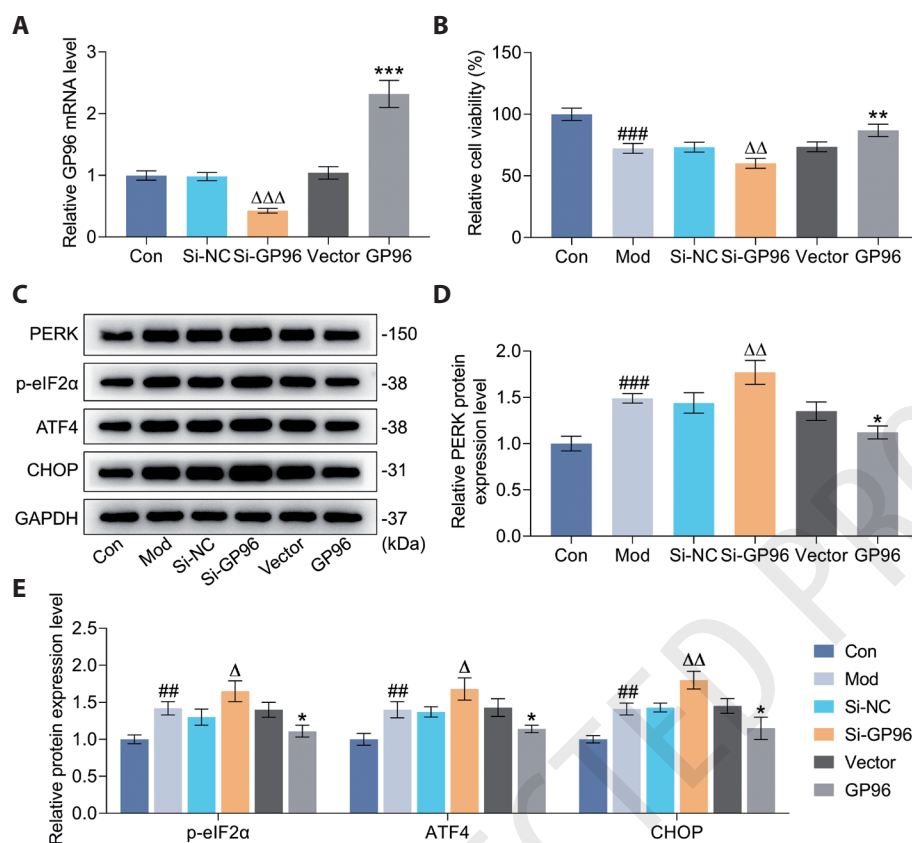


Figure 1. GP96 positively modulated viability and negatively regulated ERS in nicotine-induced 16HBE cells. **A.** The expression of GP96 in 16HBE cells transfected with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids was assessed by qRT-PCR, with GAPDH serving as the endogenous reference. **B.** The viability of 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure was measured by CCK-8 assay. **C-E.** The expressions of PERK, p-eIF2α, ATF4 and CHOP in 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure were assessed by Western blot, with GAPDH serving as the endogenous reference. ^Δ $p < 0.05$, ^{ΔΔ} $p < 0.01$, ^{ΔΔΔ} $p < 0.001$ vs. Si-NC; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. Vector; ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs. Control. GP96, glycoprotein 96; Si-GP96, small interfering RNA targeting GP96; Si-NC, small interfering

RNA targeting negative control; Con, Control group; Mod, Model group; qRT-PCR, quantitative reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PERK, protein kinase RNA-like endoplasmic reticulum kinase; p-eIF2α, phosphorylated-eukaryotic translation initiation factor 2α; ATF4, activating transcription factor 4; CHOP, C/EBP-homologous protein.

21 kDa, 1:1000, Abcam, UK), c-caspase 3 (ab32042, 17 kDa, 1:500, Abcam, UK), phosphorylated (p)-P38 (ab178867, 41 kDa, 1:1000, Abcam, UK), P38 (ab170099, 42 kDa, 1:5000, Abcam, UK), p-ERK (ab201015, 44/42 kDa, 1:1000, Abcam, UK), ERK (ab184699, 44/42 kDa, 1:10000, Abcam, UK), protein kinase RNA-like endoplasmic reticulum kinase (PERK; ab229912, 150 kDa, 1:1000, Abcam, UK), p-eukaryotic translation initiation factor 2α (p-eIF2α; #9721, 38 kDa, 1:1000, Cell signaling technology, Danvers, MA, USA), activating transcription factor 4 (ATF4; ab216839, 38 kDa, 1:1000, Abcam, UK), C/EBP-homologous protein (CHOP; ab11419, 31 kDa, 5 μg/ml, Abcam, UK) and GAPDH (ab8245, 37 kDa, 1:500, Abcam, UK) were applied to probe the membranes overnight at 4°C. Then, the membranes were incubated with HRP-conjugated secondary antibody, Goat anti-Rabbit IgG (31460, 1:10000, ThermoFisher, USA) or Goat anti-Mouse IgG (31430, 1:5000, ThermoFisher, USA) at room temperature for 1 h. Protein signals were developed using ECL reagent kits (ab133409, Abcam, UK) and photographed using an imager (ChemiDoc XRS Plus, Bio-Rad, Hercules, CA, USA), following which protein band intensity was quantified using Image LabTM (Bio-Rad, USA).

Statistical analysis

Graphpad prism (version 8.0, GraphPad Software Inc., San Diego, CA, USA) was utilized for statistical analyses. Data from three independently repeated experiments were expressed as mean ± standard deviation (SD). Independent *t*-tests were performed to compare differences between two groups, while differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). $p < 0.05$ was considered to indicate a statistically significant difference.

Results

GP96 positively modulated viability and negatively regulated ERS in nicotine-induced 16HBE cells.

To examine the role of GP96 in COPD, we manipulated GP96 expression *via* transfection with Si-GP96 or GP96 overexpression plasmids in 16HBE cells, and later, the cells were subjected to COPD modeling. The transfection effi-

ciency was evaluated through qRT-PCR, the results of which showed that Si-GP96 transfection and GP96 overexpression plasmid transfection successfully silenced and overexpressed GP96, respectively, in 16HBE cells ($p < 0.001$, Fig. 1A). Exposure to nicotine was performed to induce COPD in 16HBE cells, and resulted in decreased viability ($p < 0.001$, Fig. 1B). GP96 silencing further enhanced ($p < 0.01$, Fig. 1B), while GP96 overexpression abrogated ($p < 0.01$, Fig. 1B) nicotine-inhibited viability of 16HBE cells. Next, ERS-related protein expressions were assessed in *in-vitro* COPD models undergoing GP96 expression manipulation. The result of Western blot unveiled that exposure to nicotine upregulated

the expression levels of PERK, p-eIF2 α , ATF4 and CHOP in 16HBE cells ($p < 0.01$, Fig. 1C–E), and this upregulation was strengthened after GP96 silencing ($p < 0.05$, Fig. 1C–E), but resisted by GP96 overexpression ($p < 0.05$, Fig. 1C–E).

Upregulation of GP96 reversed the pro-apoptotic effect of nicotine in 16HBE cells

Notably, GP96 expression was observed to be increased in 16HBE cells exposed to nicotine and was successfully silenced or overexpressed by transfection with Si-GP96 or GP96 overexpression plasmids in nicotine-induced 16HBE

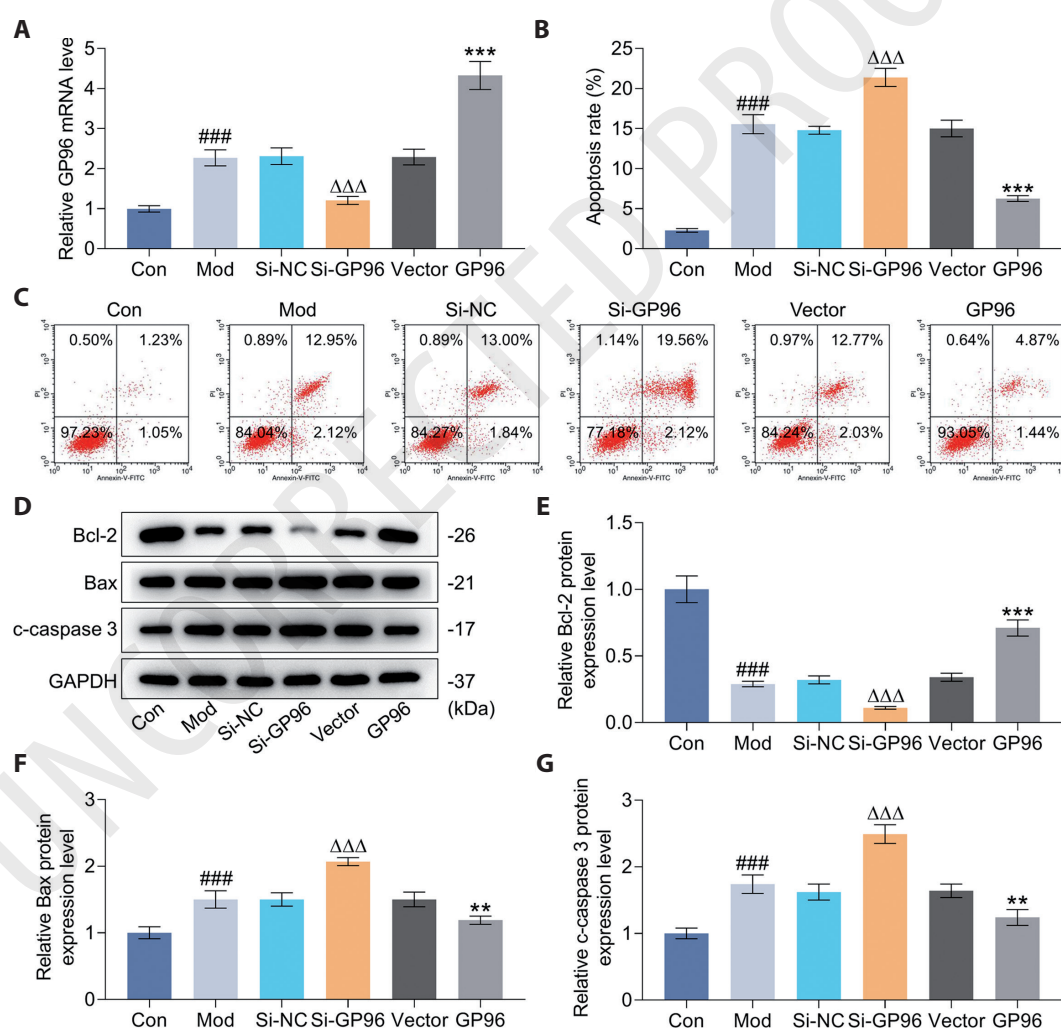


Figure 2. Upregulation of GP96 reversed the pro-apoptotic effect of nicotine in 16HBE cells. **A**. The expression of GP96 in 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure was assessed by qRT-PCR, with GAPDH serving as the endogenous reference. **B,C**. The apoptosis of 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure was determined by flow cytometry. **D–G**. The expressions of Bcl-2, Bax and c-caspase 3 in 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure were evaluated by Western blot, with GAPDH serving as the endogenous reference. ** $p < 0.01$, *** $p < 0.001$ vs. Vector; ΔΔΔ $p < 0.001$ vs. Si-NC, ### $p < 0.001$ vs. Control. PI, propidium iodide; FITC, fluorescein isothiocyanate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein. For more abbreviations, see Figure 1.

cells, as confirmed through qRT-PCR ($p < 0.001$, Fig. 2A). Flow cytometry was then conducted to detect apoptosis and the data showed that nicotine intervention enhanced 16HBE cell apoptosis ($p < 0.001$, Fig. 2B,C), the effect of which was potentiated by GP96 silencing and attenuated by GP96 overexpression ($p < 0.001$, Fig. 2B,C). Besides, ex-

posure to nicotine was found to decrease the expression of Bcl-2 but increase those of Bax and c-caspase 3 ($p < 0.001$, Fig. 2D-G). The trends of these apoptosis-related markers in nicotine-treated cells were enhanced by GP96 silencing ($p < 0.001$, Fig. 2D-G) but reversed by GP96 overexpression ($p < 0.01$, Fig. 2D-G).

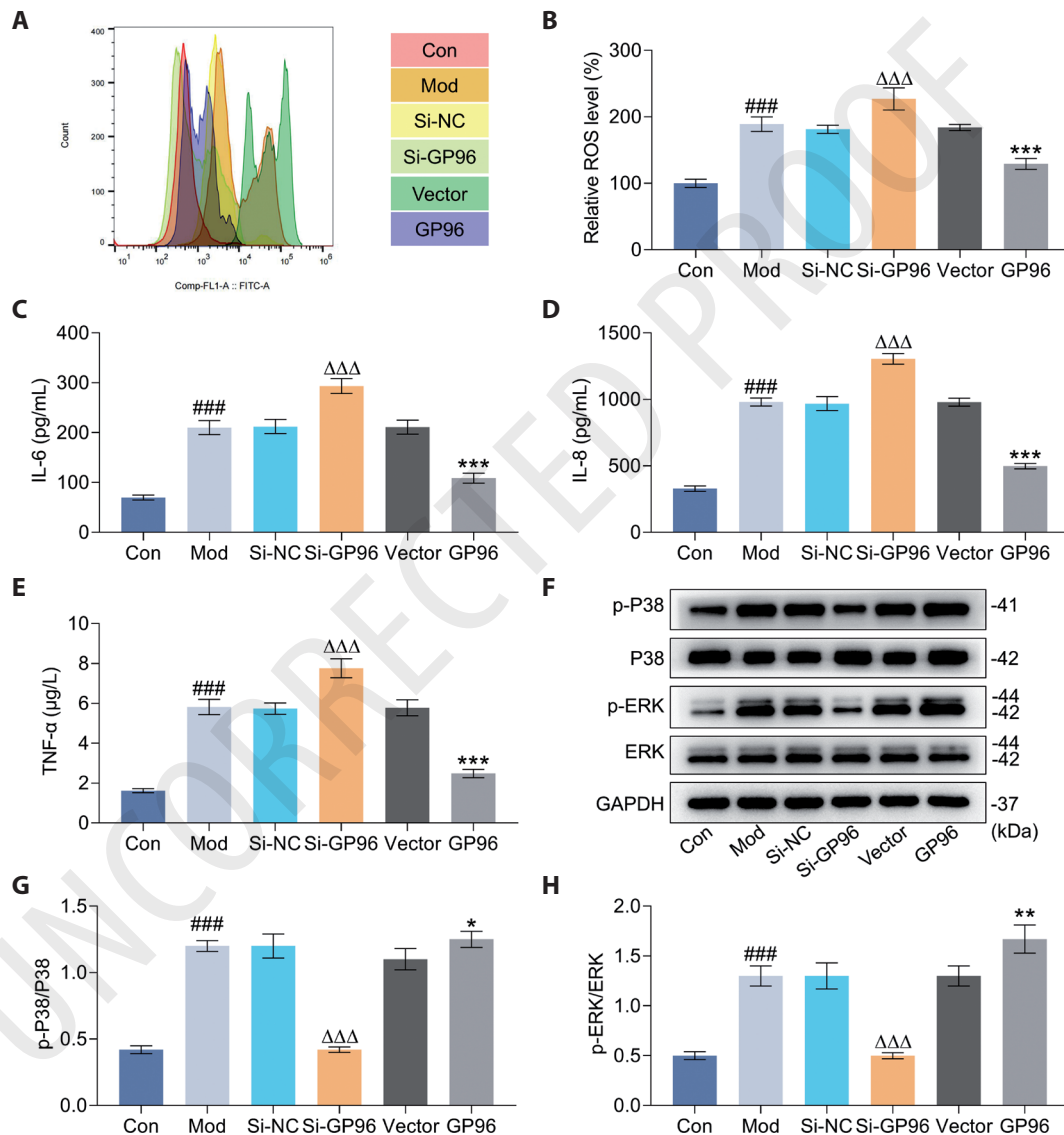


Figure 3. GP96 repressed the ROS release and inflammation and promoted activation of p38 and ERK in nicotine-induced 16HBE cells. **A,B.** The ROS release in 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure was determined by flow cytometry. **C-E.** The release of IL-6, IL-8 and TNF- α from 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine was examined by enzyme-linked immunosorbent assay. **F-H.** The expressions of p-P38/P38 and p-ERK/ERK in 16HBE cells that underwent transfection with Si-NC, Si-GP96, empty vectors or GP96 overexpression plasmids and subsequent nicotine exposure were measured by Western blot, with GAPDH serving as the endogenous reference. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Vector; $\Delta\Delta\Delta$ $p < 0.001$ vs. Si-NC, ### $p < 0.001$ vs. Control. IL-6, interleukin-6; IL-8, interleukin-8; TNF- α , tumor necrosis factor alpha, p-P38, phosphorylated-P38; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated-ERK. For more abbreviations, see Figure 1.

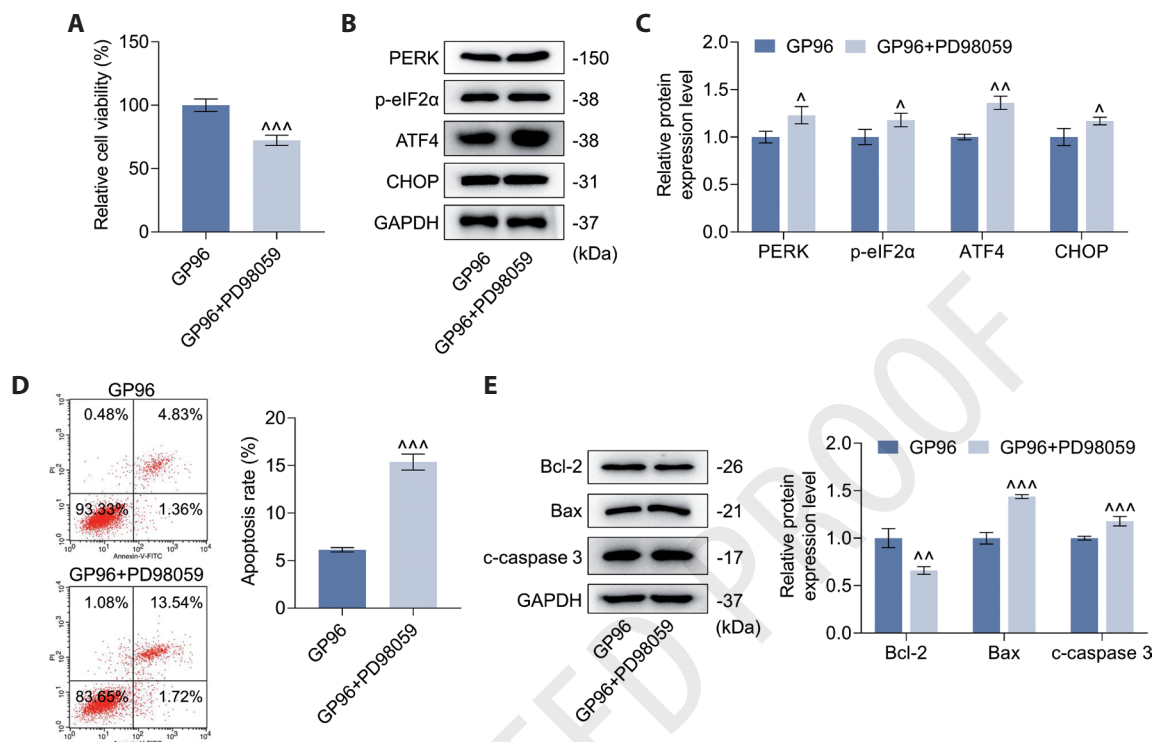


Figure 4. PD98059 reversed the effects of GP96 overexpression on viability, ERS and apoptosis in nicotine-induced 16HBE cells. **A.** The viability of 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment was measured by cell counting kit-8 assay. **B,C.** The expressions of PERK, p-eIF2 α , ATF4 and CHOP in 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment were tested by Western blot, with GAPDH serving as the endogenous reference. **D.** The apoptosis of 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment was determined by flow cytometry. **E.** The expressions of Bcl-2, Bax and c-caspase 3 in 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment were assessed by Western blot, with GAPDH serving as the endogenous reference. [^] $p < 0.05$, ^{^^} $p < 0.01$, ^{***} $p < 0.001$ vs. GP96 group. For abbreviations, see Figure 1.

GP96 dampened the ROS release and inflammation and boosted activation of P38 and ERK in nicotine-induced 16HBE cells

Through flow cytometry, we observed that nicotine-treated 16HBE cells showed overproduction of ROS ($p < 0.001$, Fig. 3A,B), which was intensified by GP96 silencing ($p < 0.001$, Fig. 3A,B) yet suppressed by GP96 overexpression ($p < 0.001$, Fig. 3A,B). The IL-6, IL-8, and TNF- α levels were confirmed, through ELISA, to be upregulated in 16HBE cells due to nicotine treatment ($p < 0.001$, Fig. 3C-E). GP96 silencing further augmented ($p < 0.001$, Fig. 3C-E), while GP96 overexpression inhibited ($p < 0.001$, Fig. 3C-E) these pro-inflammatory markers in the nicotine-induced cells. Previous research has recorded that activation of MAPK/ERK signaling inhibits ERS (Zou and Liu 2021). Through Western blot, we observed that in nicotine-induced 16HBE cells, the expression levels of p-ERK/ERK and p-P38/P38 were both significantly elevated ($p < 0.001$, Fig. 3F-H), which was offset

by GP96 silencing, yet potentiated by GP96 overexpression ($p < 0.05$, Fig. 3F-H).

PD98059 reversed the effects of GP96 overexpression on viability, ERS and apoptosis in nicotine-induced 16HBE cells.

Subsequently, the ERK inhibitors, PD98059, were used to investigate the role of MAPK/ERK signaling in GP96 expression-mediated COPD. PD98059 treatment offset GP96 overexpression-mediated increase in viability of nicotine-exposed 16HBE cells ($p < 0.001$, Fig. 4A). Meanwhile, PERK, p-eIF2 α , ATF4 and CHOP downregulation induced by GP96 overexpression in nicotine-exposed 16HBE cells was reversed by PD98059 treatment ($p < 0.05$, Fig. 4B,C). Moreover, GP96 overexpression-triggered apoptosis inhibition, accompanied by Bcl-2 upregulation and downregulation of Bax and c-caspase 3, in nicotine-exposed 16HBE cells was also counteracted due to PD98059 treatment ($p < 0.01$, Fig. 4D,E).

PD98059 counteracted the effects of GP96 overexpression on the ROS release, inflammation and ERK activation in nicotine-induced 16HBE cells.

PD98059 treatment abrogated GP96 overexpression-induced decrease in ROS production in nicotine-exposed 16HBE cells ($p < 0.01$, Fig. 5A,B). In the meantime, GP96 overexpression-induced downregulation of IL-6, IL-8 and TNF- α was reversed in nicotine-exposed 16HBE cells after PD98059 treatment ($p < 0.001$, Fig. 5C-E). As expected, in nicotine-exposed 16HBE cells, PD98059 treatment offset GP96 overexpression-induced elevation in the expression levels of p-ERK/ERK ($p < 0.01$, Fig. 5H), but exerted no significant effect on the expression level of p-P38/P38 (Fig. 5G).

Discussion

Various pathological mechanisms including oxidative stress, inflammatory responses and cell apoptosis are implicated in the pathogenesis of COPD (Guo et al. 2022; Xu et al. 2024;

Hou 2025). Recently, ERS has emerged as a well-recognized feature of COPD (Janssen-Heininger et al. 2020). The ER environment creates oxidant during disulfide bond formation, which is necessary for protein folding (Janssen-Heininger et al. 2020). The progression of chronic lung diseases including COPD is attributable to the altered redox-based processes like oxidative stress that accompanies ERS (Janssen-Heininger et al. 2020). Besides, in epithelial cells, ERS leads to apoptosis and pro-inflammatory signaling activation (Chen et al. 2018; Kropski and Blackwell 2018; Zhang et al. 2022), which are closely linked to COPD. The above findings imply that inhibition of ERS may suppress COPD progression, serving as a promising therapeutic strategy.

Genetic studies have identified some genes like HSP72 as contributors to ER homeostasis and to adaptation to ERS (Gupta et al. 2010; Chen et al. 2016). GP96, an integral component of the ER homeostatic machinery, exhibits positive correlation with HSP72 in colonic adenocarcinoma (Wang et al. 2008). GP96 can recognize exposed hydrophobic patches to assist in protein folding (Meunier et al. 2002), a process predominantly controlled by the ER (Anelli and Sitia 2008).

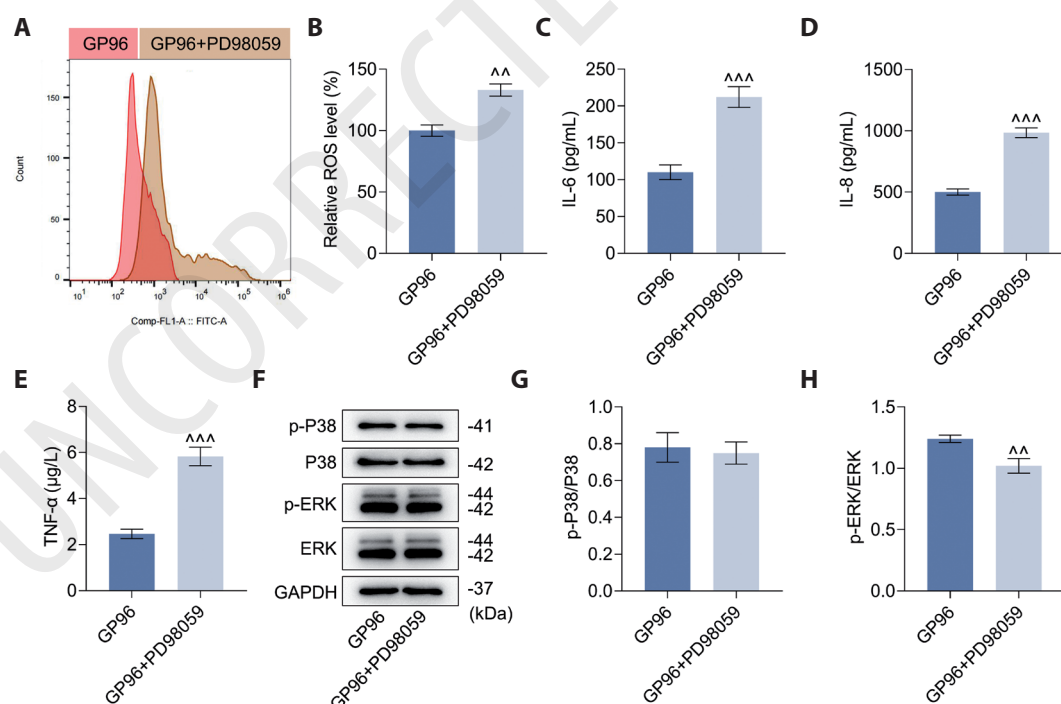


Figure 5. PD98059 reversed the role of GP96 overexpression in the ROS release, inflammation and ERK activation in nicotine-induced 16HBE cells. **A,B.** The ROS release in 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment was determined by flow cytometry. **C-E.** The release of IL-6, IL-8 and TNF- α from 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment was assessed by enzyme-linked immunosorbent assay. **F-H.** The expressions of p-P38/P38 and p-ERK/ERK in 16HBE cells that underwent GP96 overexpression plasmid transfection and subsequent nicotine exposure combined with or without PD98059 treatment were evaluated by Western blot, with GAPDH serving as the endogenous reference. ^^ $p < 0.01$, ^^^ $p < 0.001$ vs. GP96 group. ERK, extracellular signal-regulated kinase. For abbreviations, see Figure 1.

GP96 is induced at the transcriptional level upon ERS and serves as an indicator of the onset of ERS (Lee 2014). GP96 plays a critical role in UPR, an adaptive mechanism that mitigates the detrimental effects of ERS (Yang and Li 2005; Lee 2014). UPR is activated by three ERS transducers, including PERK (Wang and Kaufman 2012). PERK, similar to other transducers, directly or indirectly senses misfolded proteins, when the levels of these proteins are critically high (Oakes and Papa 2015). Then, PERK phosphorylates eIF2 α to inhibit eIF2 α activity, so as to slow down global protein translation and thus offer more time for the folding of proteins backlogged in the ER lumen (Harding et al. 1999). Meanwhile, inhibited eIF2 α activity triggers selective upregulation of ATF4, which can further transcriptionally upregulate CHOP (Oakes and Papa 2015). PERK hyperactivation can upregulate CHOP, which inhibits expression of the gene encoding antiapoptotic Bcl-2 to hasten cell death (Oakes and Papa 2015). Nicotine-induced *in-vitro* COPD models exhibit enhanced ERS, as evidenced by increased levels of CHOP (Lin et al. 2017); notably, the similar trend was observed in our study, accompanied by upregulation of PERK, p-eIF2 α and ATF4, and decreased cell viability. Later, we demonstrated that GP96 reversed the above effects of nicotine, signifying that GP96 may inhibit ERS to protect lung epithelial cells against COPD insults.

ERS induces GP96 expression (Lee 2014), and correspondingly, our data showed that the GP96 level was upregulated in nicotine-induced 16HBE cells. Combined with our findings that GP96 inhibits ERS, it can be inferred that GP96 upregulation in COPD activates an adaptive mechanism to attenuate ERS-induced damages. COPD induces alveolar wall destruction, which is associated with apoptotic cell death of alveolar structural cells (Park et al. 2007). Cell death can be initiated through proapoptotic outputs possessed by the three ERS transducers, if ERS cannot be mitigated (Oakes and Papa 2015). PERK activated upon ERS leads to CHOP upregulation, which represses the expression of Bcl-2, an antiapoptotic protein, thereby hastening cell death (McCullough et al. 2001). Moreover, pro-apoptotic signals from the UPR transducers activate BH3-only proteins to engage the Bcl-2 pathway, thereby indirectly triggering Bax and Bak. This leads to the release of toxic mitochondrial proteins that activate caspase 3 and initiate the intrinsic apoptotic pathway (Oakes and Papa 2015). ERS-mediated apoptosis has been detected in both *in vitro* and *in vivo* COPD models (Lin et al. 2017). Similarly, our nicotine-induced 16HBE cells showed an increased apoptosis rate, as well as Bcl-2 downregulation and upregulation of Bax and c-caspase 3, which were offset by GP96. Since ERS induced cell apoptosis and GP96 inhibited ERS, our results suggested that GP96 impeded ERS-induced apoptosis in COPD.

The oxidant, a key driver in early COPD pathogenesis, stems from destroyed integrity of the membranes and orga-

nelles of epithelial cells (Wiegman et al. 2020), and induces stress responses with the releases of ROS, culminating in oxidative stress (Wiegman et al. 2020). Meanwhile, chronic inflammation in the lung also results in oxidative stress through ROS upregulation (Zuo et al. 2019). Oxidative stress in turn induces inflammation and apoptosis, acting as a contributor to COPD (Wiegman et al. 2020). Our study proved that GP96 expression was negatively correlated with nicotine-induced ROS release in 16HBE cells. Considering our results that GP96 inhibited ERS and previous findings that ERS positively impacts on oxidative stress in COPD (Janssen-Heininger et al. 2020), it is plausible to propose that GP96 dampened ERS to relieve oxidative stress in COPD.

Chronic inflammation in the airways is the most well-characterized mechanism, which underlies the pathogenesis of COPD and results in largely irreversible and progressive airflow limitation (Barnes 2016). This inflammation response is triggered by direct injury of airway epithelial cells (Opitz et al. 2010), causing the release of pro-inflammatory cytokines IL-6, IL-8 and TNF (Decramer et al. 2012). The levels of all these cytokines are elevated in COPD, and TNF- α and IL-6 levels are higher in the symptomatic groups than in the less symptomatic groups (Yazici et al. 2020). ERS has been shown to activate inflammatory signaling and form a forward-feeding loop with inflammation (Hasnain et al. 2012). GP96, which has been revealed to inhibit ERS by our results, was discovered to counteract nicotine-induced release of IL-6, IL-8 and TNF-alpha in 16HBE cells, hinting that GP96 may inhibit ERS to attenuate inflammation in COPD.

MAPK signaling is significantly activated in patients with COPD (Liu et al. 2025). ERK and P38 are two distinct groups of the MAPKs, which mediate the transduction of extracellular stimulations to intracellular responses, thereby modulating cell proliferation, motility, differentiation, and survival (Roux and Blenis 2004). Therein, ERK is activated in most cases by growth factors and phorbol esters, and P38 activation is preferentially provoked by stress stimuli (Roux and Blenis 2004). The activation of these two cascades further activates five subgroups of the MAPK-activated protein kinases, impacting cell phenotypes and nuclear responses, respectively (Roux and Blenis 2004). Previous data have recorded that in murine macrophages, GP96 can elicit ERK activation (Reed et al. 2003), which has been documented to inhibit ERS in hypoxia-mediated cardiomyocytes (Zou and Liu 2021). Conversely, GP96 deficiency impairs both ERK and P38 phosphorylation through affecting TLR4 functionality (Cosin-Roger et al. 2018). Aligned with previous findings (Reed et al. 2003; Cosin-Roger et al. 2018), our outcomes revealed that GP96 expression was positively relevant to ERK and P38 activation induced by nicotine in 16HBE cells. To ascertain whether GP96-mediated activation of ERK and/or P38 is essential in the inhibitory effects of GP96 on ERS and ERS-induced pathological events in COPD, the ERK

inhibitor was applied. The results indicated the effect of GP96 overexpression on all the nicotine-induced changes (except P38 activation) in 16HBE cells was reversed after the application of the ERK inhibitor. This demonstrated that GP96 inhibited ERS-induced pathological events in COPD through activating ERK signaling.

This study has several limitations. The nicotine-exposed 16HBE cell model is a simplified *in vitro* system designed to investigate specific pathways relevant to COPD. However, clinical COPD is a complex multifactorial disease, and this model does not fully recapitulate its overall pathology. Therefore, the observed protective role of GP96 is confined to this defined nicotine-induced stress paradigm, and further validation in more complex models (e.g., cigarette smoke exposure animal models, primary cells from COPD patients) is required. Furthermore, this study primarily demonstrates an association between GP96 and the p38/ERK pathway, leaving the underlying molecular mechanisms to be fully elucidated in future work.

In conclusion, we demonstrate that GP96, which is induced by nicotine, inhibits stress in the ER of 16HBE cells. GP96 overexpression can remarkably suppress ERS and ERS-induced apoptosis, oxidative stress and inflammation in nicotine-induced 16HBE cells, where ERK activation plays a vital role. These findings not only deepen the understanding on the biological complexity of COPD, but also promote the development of effective control strategies for COPD.

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