Journal of Pharmaceutical and Medical Research
ISSN: 2663-1962
DOI: https://doi.org/10.61784/jpmr3034

CIGARETTE SMOKE EXTRACT PROMOTES APOPTOSIS OF HUMAN PULMONARY ARTERIAL ENDOTHELIAL CELLS VIA THE ER STRESS CHOP SIGNALING PATHWAY

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Abstract: Objective: To determine whether cigarette smoke promotes apoptosis of human pulmonary artery endothelial cells (HPAECs) via the CCAAT/enhancer-binding protein homologous protein (CHOP) signaling pathway, and whether 4-phenylbutyric acid (4-PBA) has an inhibitory effect on this apoptosis. **Methods:** CHOP gene expression in HPAECs was silenced by lentiviral transfection of recombinant RNA. Both wild-type and CHOP-silenced HPAECs were divided into four groups: control group (Col group), cigarette smoke extract group (CSE group, with 10% CSE added to the medium), PBA group (PBA group, with 5 mmol/L PBA added to the medium), and CSE + PBA group (with both 10% CSE and 5 mmol/L PBA added to the medium). Cells were treated for 6, 12, and 24 hours. Endoplasmic reticulum morphology was observed by transmission electron microscopy, and cell apoptosis was analyzed by flow cytometry. CHOP protein and mRNA expression levels were detected by Western blot and real-time PCR, respectively. **Results:** CSE induced apoptosis in HPAECs, and the mechanism was associated with the CHOP-mediated apoptosis signaling pathway. 4-PBA reduced CSE-induced apoptosis in HPAECs. **Conclusion:** Cigarette smoke extract can induce apoptosis in HPAECs through the CHOP signaling pathway, and 4-PBA can attenuate this effect.
Keywords: Human pulmonary artery endothelial cells; Endoplasmic reticulum stress; ER stress-induced apoptosis; CCAAT/enhancer-binding protein homologous protein; 4-phenylbutyric acid

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common and frequently occurring disease that can ultimately lead to loss of working capacity and death. Cigarette smoke (CS) can induce apoptosis of pulmonary vascular endothelial cells and alveolar epithelial cells through endoplasmic reticulum stress (ERS) [1], thereby promoting the development and progression of COPD [2]. The CCAAT/enhancer-binding protein homologous protein (CHOP) is upregulated during ERS [3] and serves as a marker protein of ERS [4]. 4-Phenylbutyric acid (4-PBA), an ERS inhibitor [5], can reduce ERS activation [6] and pulmonary vascular remodeling [7], and prevent pulmonary hypertension [5]. Therefore, we hypothesize that 4-PBA may affect apoptosis of pulmonary vascular endothelial cells by modulating CHOP expression. In this study, we established an in vitro lentiviral CHOP-silenced human pulmonary artery endothelial cell (HPAEC) model. We observed the changes in endoplasmic reticulum morphology, apoptosis ratio, and expression levels of CHOP protein and mRNA in both wild-type HPAECs and CHOP-silenced HPAECs (HPAECs-CHOP) after exposure to CSE for different durations. The aim was to determine whether CSE promotes HPAEC apoptosis through the CHOP signaling pathway and whether 4-PBA has an inhibitory effect on this process, providing new insights for the treatment of COPD.

1 MATERIALS AND METHODS

1.1 Materials

HPAECs were purchased from ScienCell Research Laboratories (USA). Furong-brand cigarettes (12 mg tar/cigarette, 1.0 mg nicotine/cigarette) were produced by Hunan China Tobacco Industry Co., Ltd. The cell apoptosis detection kit was obtained from Shanghai Yisheng Biotechnology Co., Ltd. Lentiviruses were purchased from Shanghai GeneChem Co., Ltd. 4-Phenylbutyric acid (4-PBA) was obtained from Sigma (USA). The transmission electron microscope was produced by Tianmei Scientific Instrument Co., Ltd. (China). The BCA protein assay kit, Trizol reagent, reverse transcription kits, and miRNA reverse transcription kits were purchased from Kangwei Century Biotechnology Co., Ltd. (Beijing, China). Mouse anti-, rabbit anti-, normal goat serum, mouse monoclonal anti-CD31 antibody, horse anti-mouse, and goat anti-rabbit secondary antibodies were all obtained from Proteintech (USA). Primers were synthesized by Sangon Biotech (Shanghai). The upstream primer sequence for β-actin was 5′-ACCCTGAAGTACCCCATCGAG-3′, and the downstream primer was 5′-AGCACAGCCTGGATAGCAAC-3′. The upstream primer for CHOP was 5′- ATTGCCTTTCTCCTTCGGGACA-3′, and the downstream primer was 5′-CTCCTCAGTCAGCCAAGCC-3′.

1.2 Preparation of Cigarette Smoke Extract

The preparation of cigarette smoke extract (CSE) was conducted following the methods described in studies [8, 9]. Unfiltered cigarettes were connected to a three-way tube, which was linked on one end to a sealed flask containing 5 mL of phosphate-buffered saline (PBS), and on the other end to a 50 mL syringe. Cigarette smoke was drawn into the flask at a rate of 35 mL per 2 seconds per draw, with 1-minute intervals between each draw, for a total of 10 draws. The flask was shaken to ensure full dissolution of the smoke, resulting in a 100% CSE suspension. The suspension was adjusted to pH 7.4 using 1 mol/L NaOH, sterilized by filtration through a 0.22 μm pore-size filter, and then diluted with serum-free 1640 medium to a final concentration of 10% CSE. The prepared CSE was used in experiments within 30 minutes.

1.3 Cell Culture and Identification

(1) HPAECs were cultured in complete medium at 37 °C in a humidified incubator with 5% CO_2 until the cells reached approximately 80% confluency. Cells were then digested with trypsin and passaged at a 1:2 ratio.
(2) Immunohistochemical identification of HPAECs: Coverslips were prepared using logarithmic-phase HPAECs. When the cells on the coverslips reached about 80% confluency, they were rinsed three times with PBS and fixed with acetone for 15 minutes. Non-specific antigens were blocked with 5% goat serum. Primary antibodies were incubated overnight at 4 °C: mouse monoclonal anti-CD31 antibody (1:100) and rabbit anti-human von Willebrand factor (vWF) antibody (1:600). After three PBS washes, biotin-labeled secondary antibodies (horse anti-mouse and goat anti-rabbit, 1:200) were added and incubated at room temperature for 1 hour. DAB (0.05%) was used for color development. The cells were observed and photographed under an inverted microscope.

1.4 Lentiviral Transfection

HPAECs were incubated in complete culture medium for 24 hours. Once the cells adhered and entered the logarithmic growth phase, the medium was discarded and the cells were rinsed twice with sterile PBS. Cells were then infected with the virus for 8 hours. After infection, the medium was replaced with fresh complete medium, and the cells were cultured for an additional 72 hours before being harvested. CHOP protein expression levels were assessed by Western blot. The cells with the highest transfection efficiency were selected for subsequent experiments.

1.5 Transmission Electron Microscopy Observation of Endoplasmic Reticulum Morphology

Treated HPAECs and HPAECs-CHOP were fixed in 2.5% glutaraldehyde solution for 10 hours. After discarding the fixative, the cells were placed in PBS buffer for 3 hours, followed by post-fixation in 1% osmium tetroxide for 1 hour. The cells were then dehydrated sequentially in 30% ethanol, 50% ethanol, 70% ethanol containing uranyl acetate, 80% ethanol, 95% ethanol, and 100% ethanol for 10 minutes, 15 minutes, 2 hours, 10 minutes, 15 minutes, and 45 minutes, respectively. Samples were embedded in pure epoxy resin and polymerized at 40 °C and 60 °C for 24 hours each. Ultrathin sections of 70 nm were cut, mounted on copper grids, and stained. Endoplasmic reticulum morphology was observed under a Hitachi HT7700 transmission electron microscope at 5000× magnification.

1.6 Flow Cytometry Analysis of Cell Apoptosis

Cells from each group were collected by centrifugation to obtain cell suspensions. Cells were washed twice with PBS, centrifuging at 2000 rpm for 5 minutes each time to remove PBS. The cells were then fixed in pre-cooled 70% ethanol at 4 °C for 1 hour. After centrifugation to discard the fixative, 3 mL of PBS was added to resuspend the cells for 5 minutes. The suspension was filtered once through a 400-mesh sieve, followed by centrifugation at 500–1000 rpm for 5 minutes. PBS was removed, and approximately $1–5 \times 10^5$ cells were collected. The cells were then sequentially resuspended in 500 μL of binding buffer and mixed with 5 μL of Annexin V-FITC. Next, 5 μL of propidium iodide (PI) was added and mixed thoroughly. The samples were incubated in the dark for 15 minutes and analyzed by flow cytometry within 1 hour to detect fluorescence expression in cells.

1.7 Western Blot Detection of CHOP Protein Expression

Proteins were extracted using a total protein extraction kit according to the manufacturer's instructions. Protein concentrations were determined using a BCA protein assay kit following the provided protocol. Western blotting was performed strictly according to standard procedures. Primary antibodies were diluted as follows: CHOP antibody at 1:200 and incubated at room temperature for 90 minutes. Secondary antibodies were diluted at 1:5000 for mouse antibodies and 1:6000 for rabbit antibodies, with incubation at room temperature for 80 minutes. After incubation with ECL chemiluminescent substrate, the membranes were sealed with plastic wrap, exposed, developed, and washed.

1.8 Statistical Analysis

Data analysis was performed using GraphPad Prism 8.0 software. Data are presented as mean ± standard deviation

($\overline{X}$ ±SD). Comparisons between groups were conducted using independent samples t-test or one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Identification of Human Pulmonary Arterial Endothelial Cells

Under the inverted phase-contrast microscope, the cells appeared polygonal or spindle-shaped with clear boundaries. The nuclei were round or oval, with visible nucleoli. The cytoplasm was abundant and contained small granules. The cells grew as a confluent monolayer arranged in a cobblestone-like pattern. Immunohistochemical staining for CD31 and vWF showed nuclei stained blue, while positive signals appeared as varying shades of brown. Over 90% of the cells were positive, confirming that the cultured cells were human pulmonary artery endothelial cells.

2.2 Verification of Lentiviral CHOP Silencing

The infection efficiency of HPAECs was observed under a fluorescence microscope. Cells successfully infected by the lentivirus emitted green fluorescence. Infection efficiency was calculated as the ratio of green fluorescent cells to total cells multiplied by 100%. The results showed an infection efficiency greater than 90%, indicating successful lentiviral transfection.

2.3 Measurement of CHOP Protein Expression in Human Pulmonary Arterial Endothelial Cells after CHOP Silencing

Western blot was used to detect CHOP protein expression levels in different groups of HPAECs after successful lentiviral transfection. The Chop-shRNA#3 group showed significantly lower CHOP protein levels compared to the Chop-shRNA#1 and Chop-shRNA#2 groups ($P < 0.05$), therefore Chop-shRNA#3 was selected for subsequent experiments (**Figure 1**).

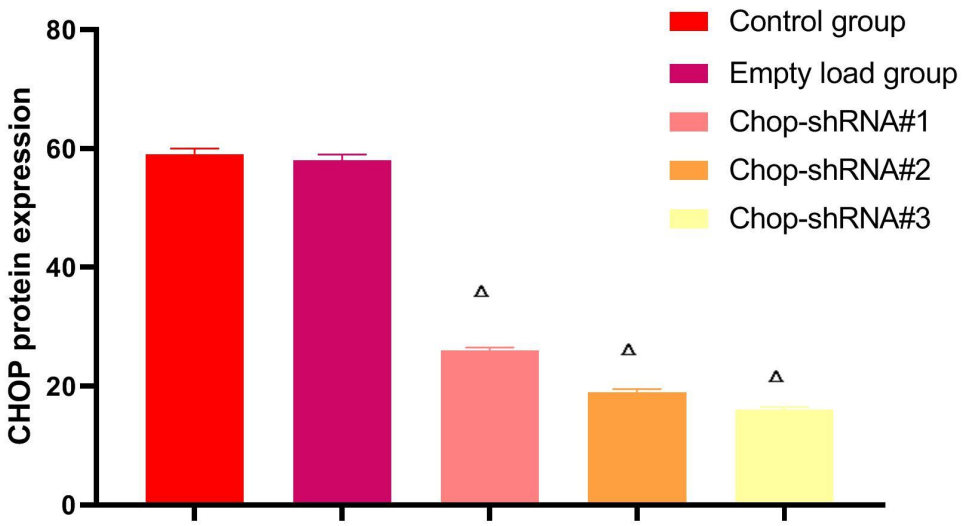

Figure 1 CHOP Protein Expression Levels in Each Group of HPAECs after Lentiviral Interference
Note: △P < 0.01 vs. Control group; ▲P < 0.05 vs. Chop-shRNA#3

2.4 Transmission Electron Microscopy Observation of Endoplasmic Reticulum Morphology

After 24 hours of treatment under different conditions: In wild-type HPAECs, the control (Col) group and the PBA group showed normal morphology. The CSE group exhibited obvious endoplasmic reticulum (ER) swelling, characterized mainly by enlarged volume and flattened, lighter folds. In the CSE + PBA group, ER swelling was alleviated compared to the CSE group. In HPAECs-CHOP, the Col, PBA, and CSE + PBA groups displayed basically normal morphology. The CSE group showed increased electron density of the ER membrane without significant swelling and retained normal folded structures.

2.5 Western Blot Analysis of CHOP Protein Expression Levels

At the same treatment time, there was no significant difference between the PBA group and the Col group in both wild-type HPAECs and HPAECs-CHOP groups. The CSE group showed higher levels than both the Col group and the

CSE+PBA group, while the CSE+PBA group was higher than the Col group. Comparing different treatment times within wild-type HPAECs groups, results at 24 hours were higher than those at 12 hours, and results at 12 hours were higher than those at 6 hours. No significant differences were observed among the HPAECs-CHOP groups. Under the same treatment conditions, the apoptosis rate and CHOP protein expression levels in wild-type HPAECs were higher than those in HPAECs-CHOP. The increase in measured parameters in the wild-type HPAECs CSE group compared to the HPAECs-CHOP CSE+PBA group was more pronounced, exceeding the increase seen between the wild-type HPAECs CSE group and the HPAECs-CHOP CSE group, and also greater than the difference between the wild-type HPAECs CSE group and the wild-type HPAECs CSE+PBA group (**Figures 2–7**).

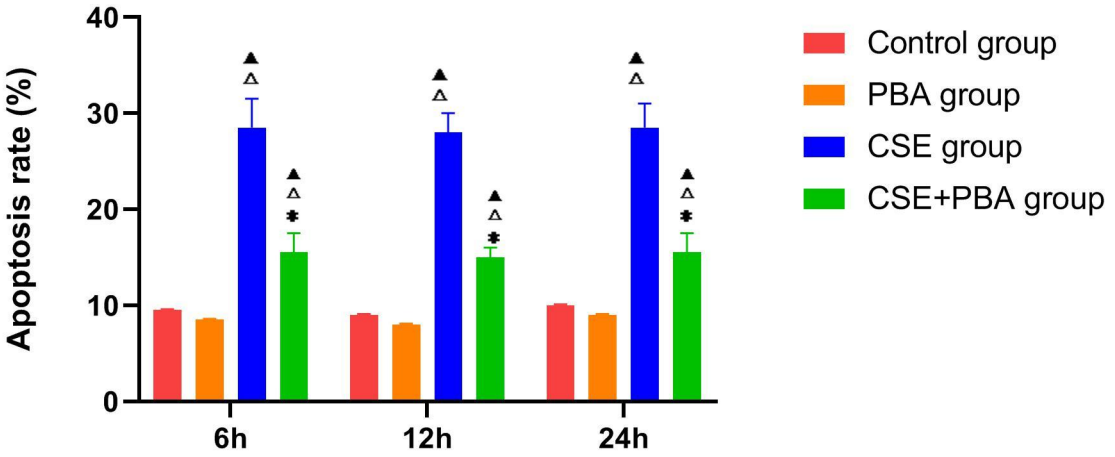

Figure 2 Apoptosis Rates of Wild-type HPAECs at Different Time Points under Various Treatments
Note: ▲P < 0.05 vs. Col group; △P < 0.05 vs. PBA group; *P < 0.05 vs. CSE group

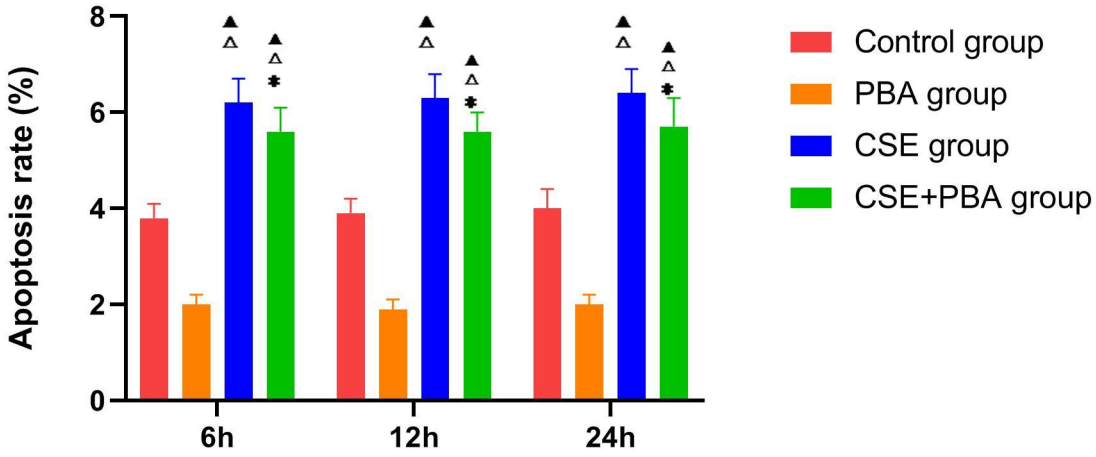

Figure 3 Apoptosis Rates of HPAECs-CHOP at Different Time Points under Various Treatments
Note: ▲P < 0.05 vs. Col group; △P < 0.05 vs. PBA group; *P < 0.05 vs. CSE group

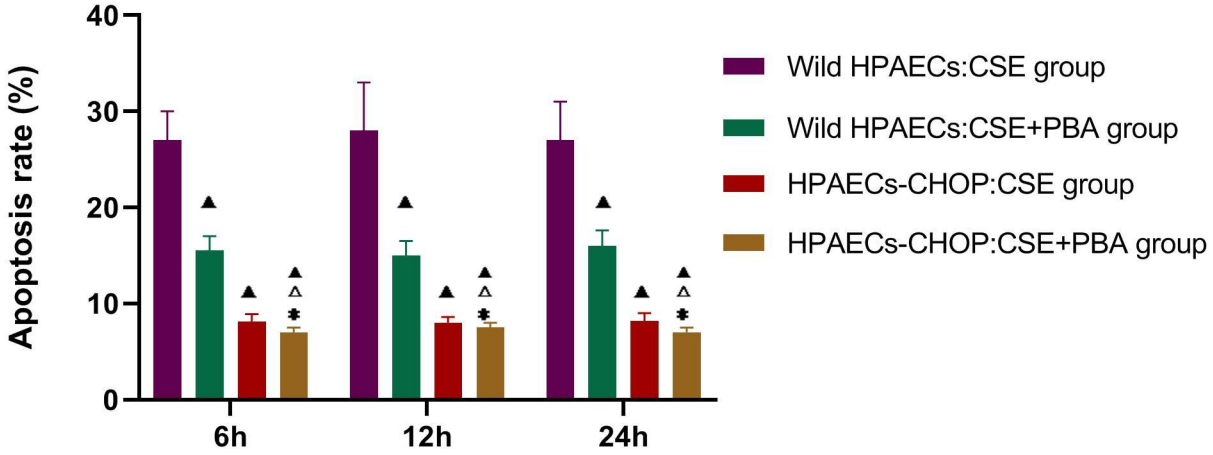

Figure 4 Apoptosis Rates of Wild-type HPAECs and HPAECs-CHOP at Different Time Points under Various Treatments

Note: ▲P < 0.05 vs. Wild-type CSE group; △P < 0.05 vs. Wild-type CSE+PBA group; *P < 0.05 vs. HPAECs-CHOP CSE group

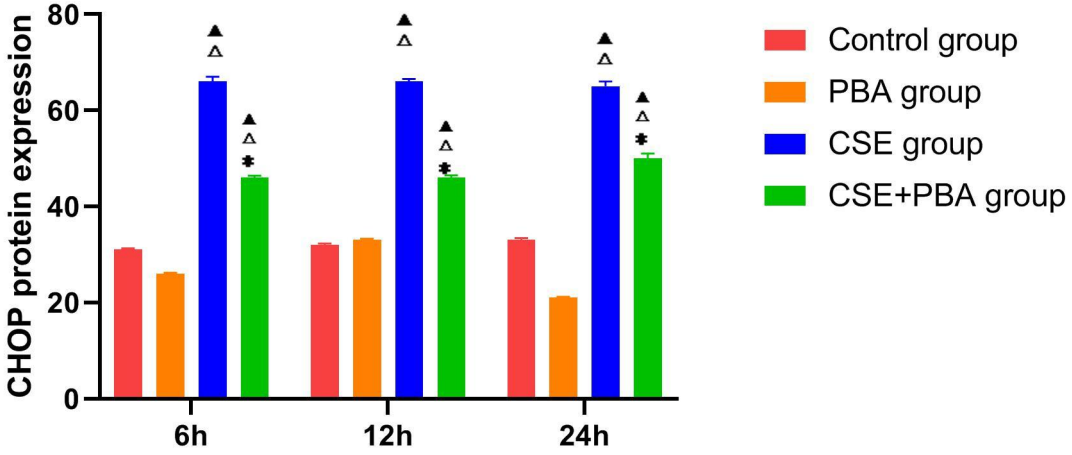

Figure 5 CHOP Protein Expression Levels in Wild-type HPAECs at Different Time Points under Various Treatments

Note: ▲P < 0.05 vs. Col group; △P < 0.05 vs. PBA group; *P < 0.05 vs. CSE group

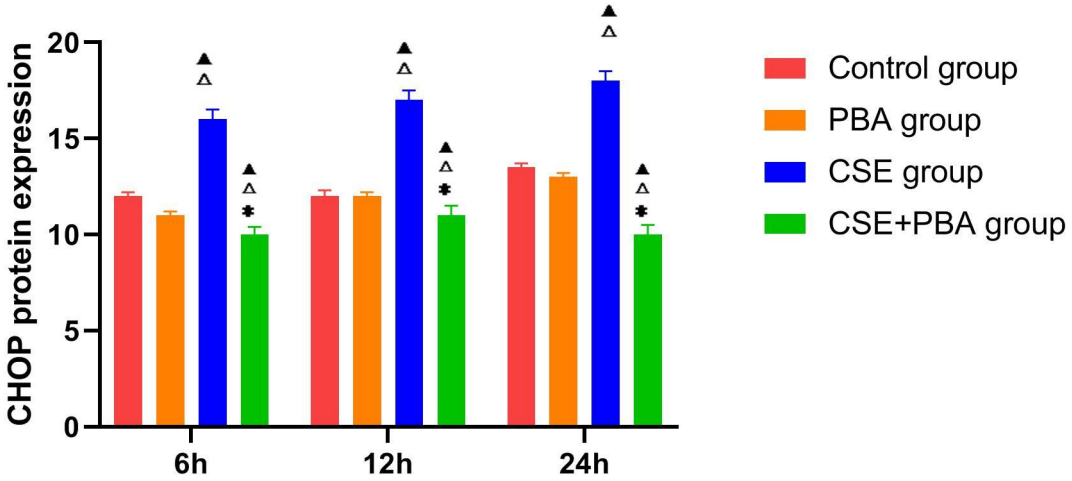

Figure 6 CHOP Protein Expression Levels in HPAECs-CHOP at Different Time Points under Various Treatments

Note: ▲P < 0.05 vs. Col group; △P < 0.05 vs. PBA group; *P < 0.05 vs. CSE group

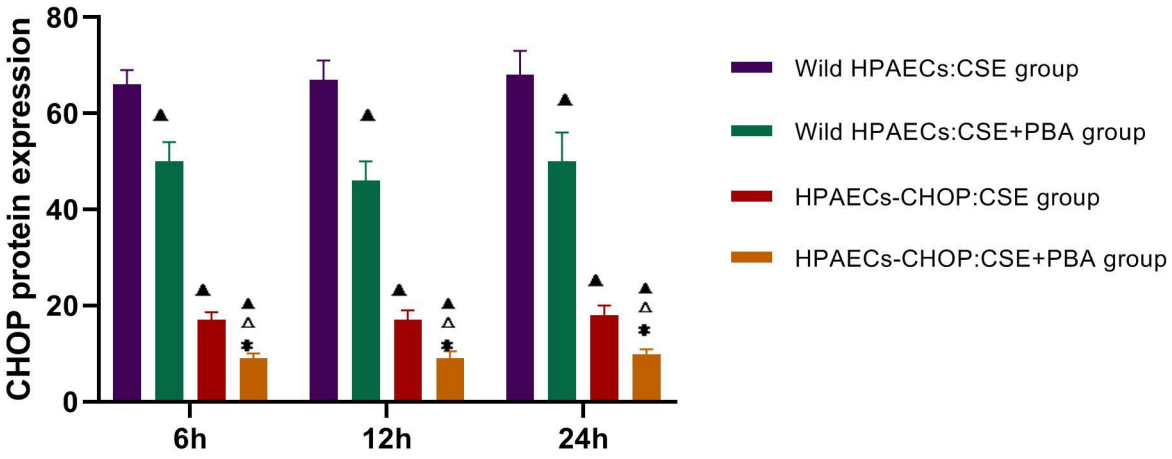

Figure 7 CHOP Protein Expression Levels in Wild-type HPAECs and HPAECs-CHOP at Different Time Points under Various Treatments

Note: ▲P < 0.05 vs. Wild-type CSE group; △P < 0.05 vs. Wild-type CSE+PBA group; *P < 0.05 vs. HPAECs-CHOP CSE group

3 DISCUSSION

Chronic obstructive pulmonary disease (COPD) is a common disease, and smoking is an important risk factor. Cigarette smoke (CS) induces endoplasmic reticulum stress (ERS) in vascular endothelial cells and smooth muscle cells, leading to dysfunction, which may be the initiating factor in the pathological changes of COPD complicated by pulmonary arterial hypertension [10, 11].

Endoplasmic reticulum stress (ERS) is a cellular response mechanism to various pathological and physiological stimuli. Under different stress conditions, the degradation of misfolded and unfolded proteins in the endoplasmic reticulum increases. However, if the stimulus is too intense or prolonged, the endoplasmic reticulum fails to restore normal function, and the cell initiates apoptosis, leading to ERS-induced cell apoptosis [12, 13]. In an in vitro model of HPAECs with lentiviral-mediated CHOP silencing, ultrastructural damage was observed in wild-type HPAECs treated with CSE. The cytoplasm and endoplasmic reticulum were significantly swollen, characterized mainly by increased volume, decreased density, increased rough endoplasmic reticulum, and flattened, lighter ER folding structures. These findings indicate that CSE induces ERS in HPAECs. In contrast, the endoplasmic reticulum morphology in HPAECs-CHOP groups was basically normal. Although the ER membrane electron density increased in the HPAECs-CHOP CSE group, the ER maintained normal folded structures. This suggests that silencing CHOP alleviates CSE-induced ER stress and prevents apoptosis in HPAECs.

A large body of evidence indicates that ERS-induced apoptosis primarily occurs through the following pathways: the apoptosis signal-regulating kinase 1 (ASK1)-JNK signaling pathway, the caspase-12 signaling pathway, and the CHOP signaling pathway [14, 15]. CHOP is a downstream target of ERS [16], with very low expression under physiological conditions, but it is significantly upregulated during ERS and participates in regulating the expression of apoptosis-related genes [17]. In this study, exposure of wild-type HPAECs to CSE resulted in increased apoptosis rates and elevated CHOP mRNA and protein expression levels in a time-dependent manner, suggesting that CSE may promote HPAEC apoptosis by upregulating CHOP expression. Under the same treatment conditions, HPAECs-CHOP exhibited lower apoptosis rates than wild-type cells, indicating that CHOP silencing can alleviate ERS severity in HPAECs and thereby reduce apoptosis.

4-Phenylbutyric acid (4-PBA), a molecular chaperone, is an inhibitor of endoplasmic reticulum stress (ERS) [18]. It can alleviate the severity of ERS by promoting proper protein folding and other mechanisms. In this study, HPAECs treated with 4-PBA showed basically normal endoplasmic reticulum morphology, and the rates of apoptosis as well as CHOP mRNA and protein expression levels were lower compared to cells treated with CSE alone. These results suggest that 4-PBA can prevent apoptosis in HPAECs by mitigating the severity of CSE-induced ERS, consistent with previous reports that 4-PBA protects various cells from apoptosis by reducing ERS [19, 20]. Moreover, this also indicates that 4-PBA may reduce CHOP expression levels [21], thereby decreasing ERS-induced apoptosis in HPAECs.

This study demonstrated at the cellular level that cigarette smoke extract (CSE) promotes apoptosis of human pulmonary arterial endothelial cells (HPAECs) through the CHOP signaling pathway, and that 4-phenylbutyric acid (4-PBA) exerts an anti-apoptotic effect. Moreover, silencing CHOP expression and 4-PBA intervention have a synergistic effect in alleviating endoplasmic reticulum stress (ERS) and reducing cell apoptosis. However, a limitation of this study is that other protein expression levels involved in ERS-induced apoptotic signaling pathways were not examined in the isolated cell experiments, making it impossible to fully assess changes in other relevant factors in the ERS-induced apoptotic pathways of wild-type HPAECs and HPAECs-CHOP under CSE exposure.

4 CONCLUSION

Cigarette smoke extract (CSE) can induce apoptosis in human pulmonary arterial endothelial cells (HPAECs), and this mechanism is related to the CHOP signaling pathway; 4-phenylbutyric acid (4-PBA) can reduce CSE-induced apoptosis in HPAECs.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

FUNDING

This study was funded by the Hunan Provincial Clinical Medical Technology Innovation Project (2021SK50905).

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