

Cigarette smoke extract induces the expression of GRP78 in A549 cells via the p38/MAPK pathway

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Abstract. Apoptosis of alveolar epithelial cells has been implicated in the pathogenesis of chronic obstructive pulmonary disease. To determine the involvement of glucose-regulated protein 78 (GRP78) in the cigarette smoke extract (CSE)-induced apoptosis of alveolar epithelial cells and the potential mechanisms underlying this effect, A549 cells that originate from alveolar type II epithelial cells were exposed to various CSE conditions in the present study. GRP78 expression and its effect on the apoptosis of A549 cells were investigated using techniques such as RT-PCR, western blot analysis, gene knockdown by GRP78 siRNA interference and the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. The activity of the p38/mitogen-activated protein kinase (MAPK) pathway and its involvement in GRP78 expression were also analyzed using SB203580, a p38/MAPK pathway inhibitor. It was demonstrated that GRP78 expression in the cells was significantly upregulated following CSE exposure and a 12-h exposure of 5% CSE was the most efficient in inducing GRP78 expression. This CSE-induced GRP78 expression was significantly attenuated by GRP78 siRNA or by the use of SB203580. The downregulation of GRP78 expression by GRP78 siRNA also led to the increased expression of caspase-3 and an increased apoptotic index (AI, $P < 0.05$ vs. other groups). These results suggested that CSE induced GRP78 expression in A549 cells. This study demonstrated that upregulated GRP78 expression may be anti-apoptotic effects and the p38/MAPK pathway was involved in the process of CSE-induced GRP78 expression in A549 cells.

Introduction

Chronic obstructive pulmonary disease (COPD) is a common illness with increasing incidence in elderly people (1,2). Smoking

is the most significant risk factor contributing to COPD (3,4), as the cigarette smoke-induced alveolar epithelial cell apoptosis is an important cause of COPD emphysema (5-7). Numerous studies have also demonstrated that cigarette smoke induces endoplasmic reticulum stress (ERS) and bronchial epithelial cell apoptosis (8,9). However, the influence of cigarette smoke on alveolar epithelial cells remains poorly understood.

Glucose-regulated protein 78 (GRP78), a chaperone protein of endoplasmic reticulum, is termed ERS protein marker. In the early stage of ERS, the upregulated GRP78 expression (10) is associated with protein folding and maintenance of endoplasmic reticulum calcium homeostasis, which reduces the ERS (11). An increasing number of studies have shown the anti-apoptotic nature of GRP78 (12-14). However, it remains to be demonstrated whether GRP78 is protective in the process of cigarette smoke extract (CSE)-induced apoptosis of alveolar epithelial cells.

Furthermore, the signal transduction pathway to upregulate GRP78 expression remains unknown, regardless of the fact that studies have confirmed its protective nature. Mitogen-activated protein kinase (MAPK) is an important cell response-mediated signaling system. MAPK consists of three predominant families, the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38/MAPK families. The p38/MAPK pathway is important in a variety of physiological and pathological processes, including cell growth, cell cycle, inflammation, cell stress and apoptosis (15). It has been demonstrated that the activation of the p38/MAPK pathway may be involved in the upregulation of GRP78. Shear stress related blood damage upregulates GRP78 levels in vascular endothelial cells through the p38/MAPK pathway, leading to reduced apoptosis of vascular endothelial cells (16). Moreover, lipid-lowering drugs, such as atorvastatin, reduce macrophage apoptosis by increasing GRP78 expression through the p38/MAPK pathway (17). Studies have also shown that cigarette smoke activates the p38/MAPK pathway in COPD (18-20), suggesting that the activation of the p38/MAPK pathway may be associated with the upregulation of GRP78 in COPD.

In conclusion, it was hypothesized that cigarette smoke induces GRP78 upregulation in alveolar epithelial cells through the p38/MAPK pathway and GRP78 upregulation exerts anti-apoptotic effects on alveolar epithelial cells. In the present study, using A549 cells, the CSE-induced GRP78

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expression was investigated at mRNA and protein levels with RT-PCR and western blot analysis. The apoptosis of A549 transfected by GRP78 siRNA was analyzed to investigate the anti-apoptotic properties of GRP78. p38 inhibitor, SB203580, was used to investigate the involvement of the p38/MAPK pathway in CSE-induced GRP78 expression.

Materials and methods

Cell lines and reagents. A549 cells, a cell line widely used *in vitro* for type II pulmonary epithelial cell-related studies, were purchased from the cell center of Xiangya Medical College (Changsha, Hunan, China). Cigarettes (Lotus brand) were provided by China Tobacco Hunan Industrial Co., Ltd. (Changsha, Hunan, China) and Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-BRL (Grand Island, NY, USA). The p38 inhibitor SB203580, was purchased from Alexis Corporation (Lausen, Switzerland). Goat polyclonal antibody against GRP78, GRP78 siRNA and control siRNA (fluorescein conjugated) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Rabbit polyclonal active caspase-3 antibody was obtained from Abcam (Cambridge, UK). The secondary antibodies were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., (Beijing, China), and an In Situ Cell Death Detection kit was purchased from Roche (Basel, Switzerland). The study was approved by the ethics committee of Xiangya Hospital, Central South University (Changsha, China).

Cell culture. A549 cells were cultured in DMEM containing 4.5 mg/ml glucose and supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) in a humidified incubator with 5% CO₂ at 37°C. The cells were passaged into a 6-well culture dish at an optimal density of 2x10⁴ cells/ml and starved for 24 h in high glucose DMEM containing 1% FBS, when the cells had reached ~80% confluence. The cells were then subjected to the designed experiments in this study. The cell number was counted using a trypan blue exclusion assay (Beyotime Institute of Biotechnology, Jiangsu, China).

Preparation of CSE. CSE was prepared using a device made by our laboratory (21) according to the method described previously (22). CSE was freshly produced from three cigarettes by blowing smoke, generated using a vacuum syringe system on a smoking machine, through 3 ml of phosphate buffered saline (PBS) in a siliconised glass tonometer (Vitalograph, Buckingham, UK). The solution was filtered through 0.22- μ m cellulose membranes to achieve sterile smoke extract solutions. Absorbance values were measured by a UV spectrophotometer (DU-640 UV-VIS Biotech Spectrophotometer; Beckman Coulter, Miami, FL, USA) at 270-280 nm. The concentration of the prepared CSE was set as 100%. This solution was applied immediately to the cell cultures when the cells had been cultured for 24 h. CSE with 10% FBS at different concentrations was added to the culture media. The cells were treated with CSE at 37°C in a humidified atmosphere containing 5% CO₂ for various time periods according to the experimental design.

siRNA experiments. GRP78 inhibition was performed using commercially available siRNA kits (human, sc-29338; Santa Cruz Biotechnology, Inc.), in which the siRNA is target-specific, designed to knockdown the gene expression. The minimal levels of GRP78 was assessed by Western blotting. For siRNA experiment, negative controls were performed using the irrelevant siRNA provided in the siRNA kits and using the siRNA buffers. The irrelevant siRNA was a scrambled sequence which did not lead to the specific degradation of any cellular mRNA (Santa Cruz Biotechnology). The transfection was performed according to the manufacturer's instructions. A549 cells were transfected in 6-well plates according to the manufacturer's instructions. The cells were divided into four groups the control, CSE, CSE+GRP78 siRNA and CSE+control siRNA groups.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the Superscript II reverse transcription system (Invitrogen Life Technologies). Primer sequences were as follows: Forward: 5'-TCTGCTTGATGTGTGTCCTCTT-3' and reverse: 5'-GTCGTTACCTTCGTAGACCT-3' for GRP78, PCR product 156 bp; and forward: 5'-GGAAGGTGAAGGTCGGAGT-3' and reverse: 5'-GCTCCTGGAAGATGGTGATGG-3' for GAPDH, PCR product 234 bp (synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd., Shanghai, China). Amplification conditions were as follows: pre-denaturation at 94°C for 5 min; denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec and elongation at 72°C for 1 min for a total of 30 cycles, and finally an elongation step at 72°C for 10 min. The PCR products were separated by electrophoresis using 1.5% agarose gel. The absorbance values of product bands were analyzed with an automatic image analysis system (FluorChem 8900 software system; Alpha Innotech, Witec, Littau, Switzerland).

Western blot analysis. Total protein was extracted and then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene fluoride membranes for western blot analysis. Primary antibodies against GRP78 (1:1,000 dilution), p38 (1:500), P-p38 (1:500), active caspase-3 (1:200) and β -actin (1:2,000) were purchased from Santa Cruz Biotechnology, Inc. The dilution of secondary antibody was 1:2,000. ECL exposure images were captured. Absorbance analysis was conducted using an automatic image analysis system (FluorChem 8900 software system). The ratio of phosphorylated p38 (P-p38) to p38 was taken as the relative expression level of proteins. The ratio of remaining indicators to β -actin bands was taken as the relative expression levels of proteins.

Deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). The TUNEL analysis was performed using the In Situ Cell Death Detection kit (POD, Roche, Basel, Switzerland), according to the manufacturer's instructions. The labeled solution served as the negative control. The cell apoptosis index (AI) was obtained by counting the TUNEL-positive cells and total cells in the selected areas on the slides. AI = TUNEL - positive cells/total cells x 100%.

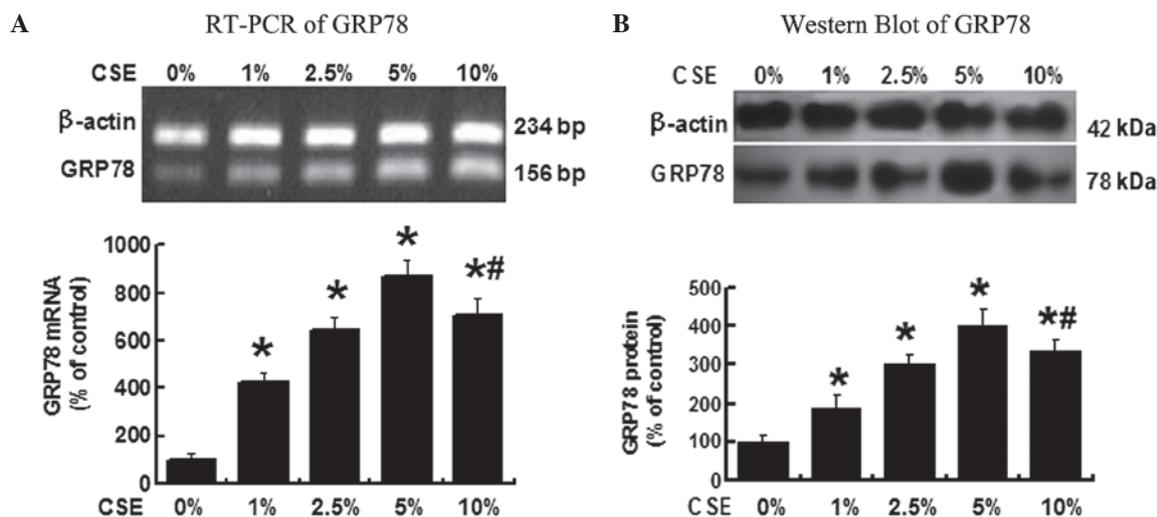


Figure 1. Glucose-regulated protein 78 (GRP78) expression levels in the A549 cells change with varying cigarette smoke extract (CSE) concentrations. (A) Reverse-transcription polymerase chain reaction (RT-PCR) and (B) western blot analysis demonstrated that the expression of GRP78 continuously increases following a rise in the CSE concentration until it reaches 10%. Data are shown as the mean \pm SD (n=3). *P<0.05, vs. the control group and #P<0.05, vs. the 5% CSE group.

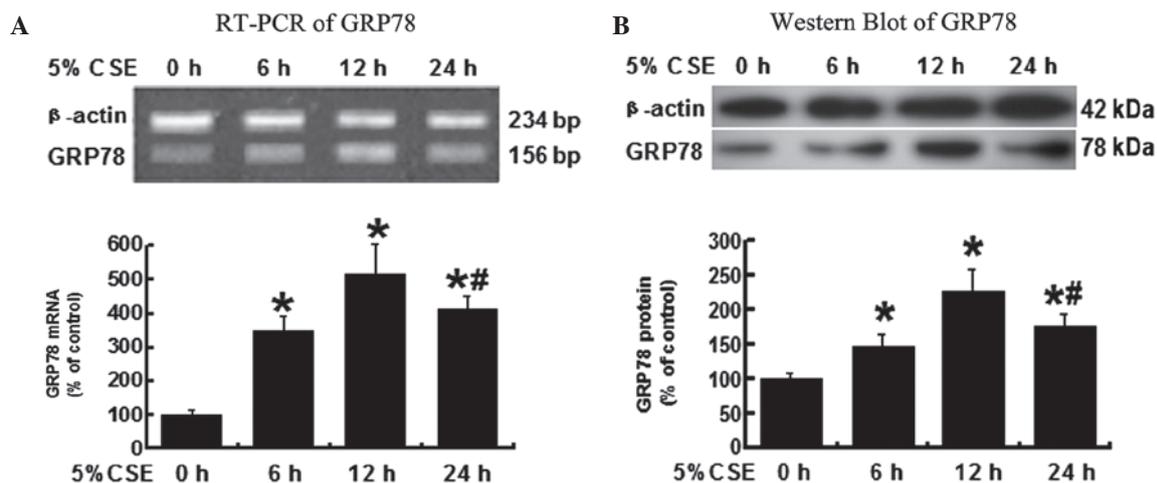


Figure 2. Glucose-regulated protein 78 (GRP78) expression in A549 cells changes with varying cigarette smoke extract (CSE) exposure times. (A) Reverse-transcription polymerase chain reaction (RT-PCR) and (B) western blot analysis demonstrates that the GRP78 expression continuously increases following the rise in CSE exposure time until it reaches the 24 h time point. Data are shown as the mean \pm SD (n=3). *P<0.05, vs. the control group; #P<0.05, vs. 12 h time-point group.

Statistical analysis. Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). The data were presented as the mean \pm SD. Multiple sets of measurement data were compared using single factor analysis of variance. All statistical tests were two-sided using a significance level of $\alpha=0.05$. P<0.05 was considered to indicate a statistically significant difference.

Results

CSE induces GRP78 expression in A549 cells. As shown in Fig. 1A, when the A549 cells were treated with different concentrations of CSE (0, 1, 2.5, 5 and 10%) for 12 h, RT-PCR demonstrated that the GRP78 mRNA levels were significantly increased in the cells treated by 1, 2.5, 5 and 10% CSE compared with the cells treated with 0% CSE, P<0.05.

Among the various CSE concentrations, 5% CSE induced the highest GRP78 expression. The GRP78 mRNA level, however, declined when CSE concentration was increased to 10%, but the level remained greater than that when the cells treated with 2.5% CSE.

The GRP78 protein levels were determined by western blot analysis (Fig. 1B) and demonstrated a similar change in the GRP78 mRNA. GRP78 protein was significantly increased following the CSE treatment, cells treated with 5% CSE exhibited the highest GRP78 protein level, P<0.05 vs. all other groups. These results suggested that CSE induces GRP78 expression in A549 cells and 5% CSE is the concentration which induces the greatest level of GRP78 expression in the cells.

12-h 5% CSE treatment is the most efficient to induce GRP78 expression. To determine the time duration of CSE administra-

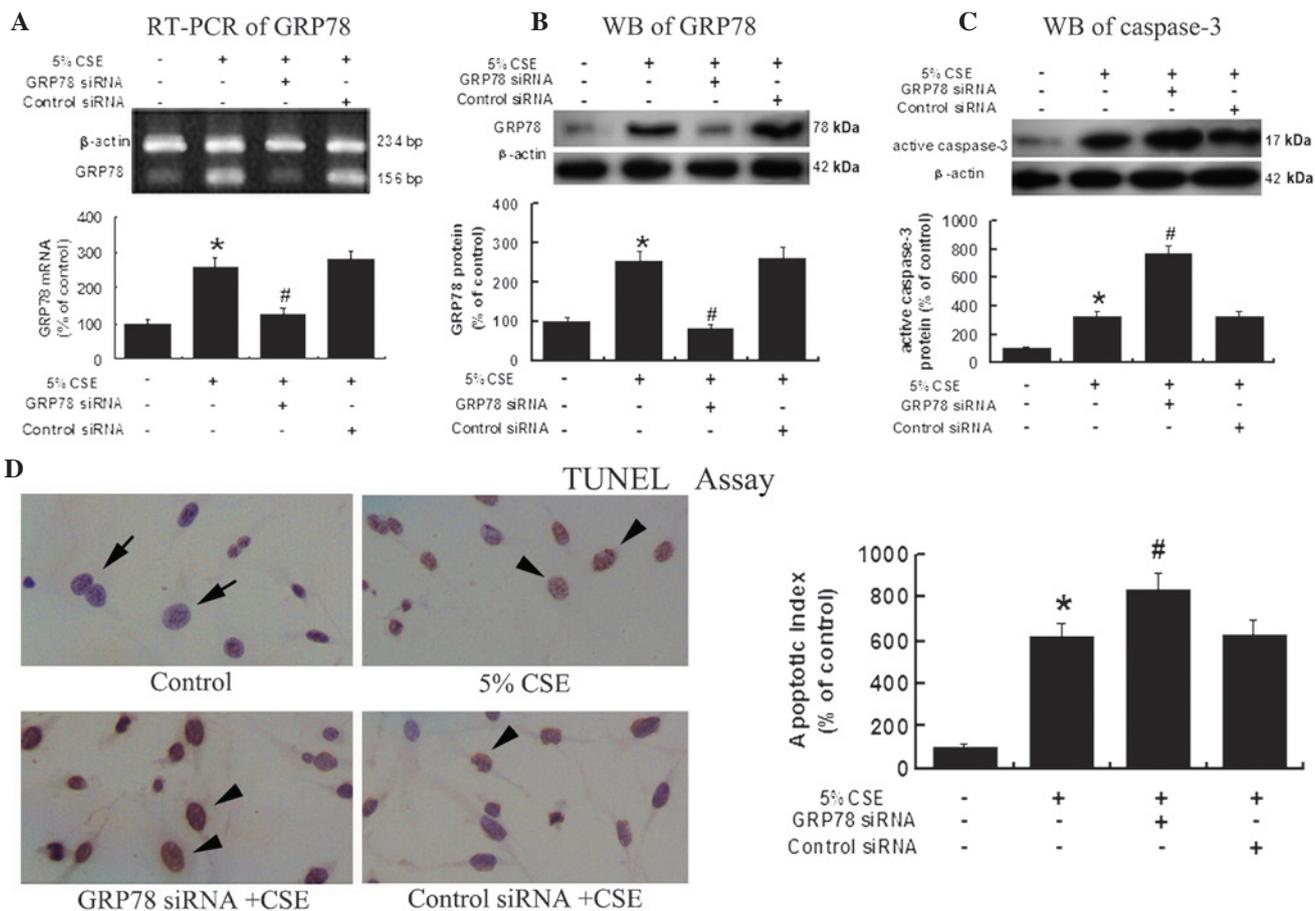


Figure 3. Glucose-regulated protein 78 (GRP78) siRNA inhibits GRP78 expression at the mRNA and protein levels, enhances caspase-3 expression and promotes apoptosis in A549 cells. Transfection of A549 cells with GRP78 siRNA, followed by 5% cigarette smoke extract (CSE) exposure results in a significant decrease in GRP78 expression as detected by (A) reverse-transcription polymerase chain reaction (RT-PCR) and (B) western blot analysis. (C) This GRP78 knockdown also leads to upregulated caspase-3 expression. (D) Deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay demonstrates increased apoptosis due to GRP78 knockdown. In the representative images of the cells (D, left), the arrow heads indicate the apoptotic cells in brown with disordered nuclear and aggregated chromatin. The arrows show the normal cells in purple. The calculated apoptosis index (D, right) shows a significant increase in siRNA treated cells. Data are shown as the mean \pm SD (n=3). *P<0.05, vs. the control siRNA group and #P<0.05, vs. the 5% CSE group.

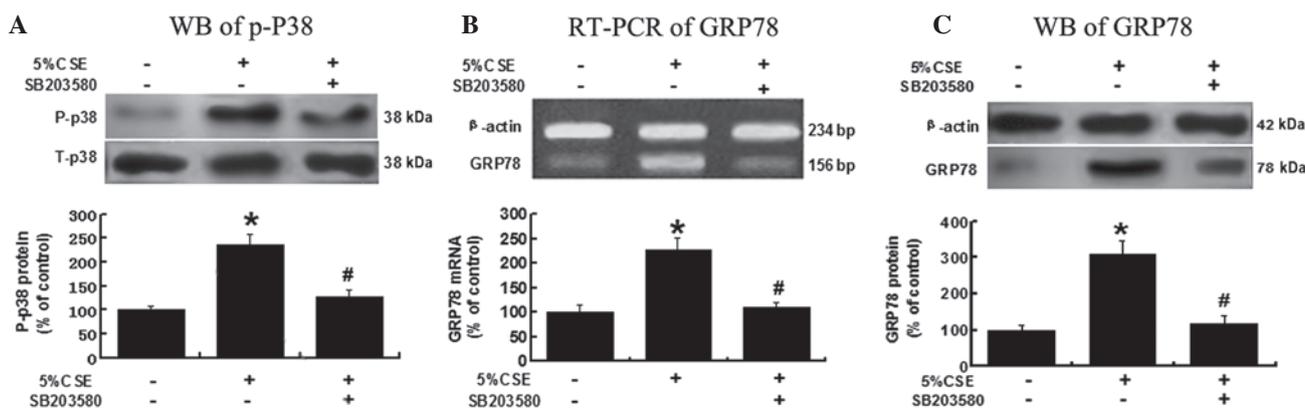


Figure 4. Blockage of p38 inhibits the phosphorylation of p38, inactivating the p38/mitogen-activated protein kinase (MAKP) pathway and repressing glucose-regulated protein 78 (GRP78) expression. Following treatment of the A549 cells with SB203580 for 45 min and exposure to 5% cigarette smoke extract (CSE) for 12 h, the level of phosphorylated p-38, the active form of p-38, is significantly decreased as detected by (A) western blot analysis. GRP78 expression at mRNA and protein levels is decreased as measured with (B) RT-PCR and (C) western blot (WB) analysis. Data are shown as the mean \pm SD (n=3). *P<0.05, vs. the control group and #P<0.05, vs. the 5% CSE group.

tion, which induces the greatest GRP78 expression, the A549 cells were treated with 5% CSE for 0, 6, 12 or 24 h. Cells were collected for RNA isolation and protein extraction. As shown in

Fig. 2A, the GRP78 mRNA levels measured by RT-PCR were significantly higher in the cells treated by 5% CSE for 6, 12 and 24 h, when compared with the untreated cells (0 h), P<0.05.

Similar to the RT-PCR results, the protein levels of GRP78 that were tested by western blot analysis were significantly increased in all the groups except 0 h group (Fig. 2B). The 12 h treatment of 5% CSE induced the most significant expression of GRP78 protein, $P < 0.05$ vs. all the other groups. These results suggest that the 12 h treatment of 5% CSE is the most efficient to induce GRP78 expression in A549 cells.

Upregulated GRP78 expression under CSE insult is protective against CSE-induced apoptosis. To further investigate the anti-apoptotic effects of GRP78, A549 cells were transfected with GRP78 siRNA or control siRNA, then subjected to 5% CSE treatment for 12 h. RT-PCR and western blot analysis results (Fig. 3A and B) showed that the GRP78 expression in the cells from the GRP78 siRNA group was significantly decreased compared with the cells from the control siRNA group, $P < 0.05$.

Notably, western blot analysis (Fig. 3C) also demonstrated a significant increase in the active caspase-3 protein expression in the group pretreated with GRP78 siRNA compared with the group treated with the control siRNA ($P < 0.05$), suggesting that GRP78 exhibits inhibitive effects on caspase-3, a predominant apoptosis activator. Thus suppressing GRP78 may enhance caspase-3 activity.

TUNEL results (Fig. 3D) further confirmed the protective effect of GRP78 against CSE-induced apoptosis. TUNEL showed that the knockdown of GRP78 by GRP78 specific siRNA resulted in a significant increase of apoptosis in the A549 cells following CSE exposure.

CSE induces GRP78 expression via the p38/MAPK pathway. To investigate the possible mechanisms underlying CSE's effects on GRP78 expression, A549 cells were pretreated with SB203580 (3 μ M), a p38/MAPK pathway inhibitor, and then exposed to 5% CSE. As shown in Fig. 4, the activity of the p38/MAPK pathway was significantly reduced, as indicated by a decreased P-p38 level, due to the pretreatment with SB203580, $P < 0.05$ vs. the 5% CSE group (Fig. 4A). The CSE-induced GRP78 expression at the mRNA and protein levels was significantly attenuated in the presence of SB203580, $P < 0.05$ vs. the 5% CSE group (Fig. 4B and C). These results suggested that CSE regulates GRP78 expression through a p38/MAPK-related pathway.

Discussion

Stress signals are transmitted from the endoplasmic reticulum into the nucleus through the unfolded protein response (UPR) signaling pathway (10,23). The ERS, to a certain extent activates protection mechanisms, such as molecular chaperone GRP78 expression that resists stress, exerting cytoprotective effects (16,24). When stress levels are too strong, this compromises or overcomes the protection mechanism, leading to cell damage. Consequently, the stress-induced upregulation of GRP78 expression becomes unsustainable; activating caspase-12 and -3, and eventually leading to apoptosis (25-27).

The involvement of GRP78 in COPD pathogenesis remains unknown. In 2008, Kelsen *et al* (8) demonstrated that smoking initiates the ERS and activates the UPR, leading to overexpression of GRP78 *in vitro* in bronchial epithelial cells. Another

study demonstrated that diesel exhaust chemicals were able to induce upregulated GRP78 expression in bronchial epithelial cells (9). In the present study, A549 cells were treated with CSE and GRP78 expression was observed to increase within a certain time frame (≤ 12 h) and concentration ($\leq 5\%$) of the CSE treatment. However, when CSE concentration was increased to 10% and the exposure time to 24 h, GRP78 expression started to decline. This indicated that when the CSE, as a stressor, is too strong either in its dose or duration, it results in dysfunction of the endoplasmic reticulum, leading to a decreased GRP78 expression.

Although GRP78 is considered to be anti-apoptotic (12-14), its involvement in alveolar epithelial cells remains unclear. In the present study, the anti-apoptotic nature of GRP78 was confirmed by genetic knockdown of GRP78 in the A549 cells. Following GRP78 knockdown, the decreased GRP78 expression was demonstrated to be correlated with increased caspase-3 activity and a higher apoptotic index (AI).

The p38/MAPK signaling pathway is predominantly involved in the inflammatory response of cells and apoptosis regulation under stress conditions. This pathway is demonstrated to be activated by reactive oxygen species in cigarette smoke, such as peroxynitrite, H_2O_2 and O_2^- , in COPD related studies, and the activated p38/MAPK pathway is important in mediating COPD inflammation (19,28,29). Studies have also shown that p38/MAPK pathway is involved in the regulation of GRP78 expression (16,17). In the present study, the expression of P-p38 protein, the activated form of p38, was significantly increased, which was correlated with an increased GRP78 expression when the cells were treated by CSE. Moreover, when SB203580, a p38/MAPK inhibitor, was used prior to CSE exposure, the expression levels of GRP78 and P-p38 significantly decreased. This result suggested that CSE induces GRP78 overexpression predominantly through p38/MAPK pathway.

In conclusion, this study shows that cigarette smoke induces GRP78 expression in A549 cells, and the upregulated GRP78 expression in the cells may exhibit an anti-apoptotic effect. The p38/MAPK pathway is involved in the regulation of GRP78 expression. The A549 cell line originates from alveolar epithelial cells, and therefore, these cells are commonly used in studies related to alveolar epithelial cells. The results obtained in the present study in A549 cells, may therefore be useful in further studies of COPD and alveolar epithelial cells.

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