The Role of Endoplasmic Reticulum Stress in Emphysema Results from Cigarette Smoke Exposure

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Key Words

CCAAT/enhancer-binding protein-homologous protein • Caspase-12 • Emphysema • Apoptosis • Endoplasmic reticulum stress

Abstract

Apoptosis is of considerable importance in the pathogenesis of emphysema, and recent studies show that endoplasmic reticulum (ER) stress is involved in emphysema. In our research, we investigated the role of protein kinase RNA (PKR)-like ER kinase (PERK)/ eukaryotic initiation factor 2 alpha (eIF2 α) pathway, the CCAAT enhancer-binding protein-homologous protein (CHOP) expression, caspase-12 activation and apoptosis in emphysema results from cigarette smoke (CS) exposure. Expression of phosphorylated-PERK (p-PERK), phosphorylated-eIF2 α (p-eIF2 α), CHOP and caspase-12 as well as the apoptosis rate are remarkably increased in rats after exposure to 2 months CS compared with control rats, significantly elevated in rats exposed to 4 months CS over rats exposed only to 2 months CS, and slightly decreased in ex-smoking rats in contrast to rats exposed to 4 months CS. Taken together, our results show that CS induces ER stress in lung epithelial cells, which may subsequently lead to lung in-

jury in rats, and this might be a novel target for protection of pulmonary epithelial cells from ER stress injury in emphysema.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a widespread, prevalent condition that is usually related to CS, which is by far the most undisputed risk factor for COPD [1]. COPD has been listed as the fifth leading cause of death after heart disease, stroke, lower respiratory tract infections and HIV/AIDS worldwide. Pulmonary emphysema is one of the major pathological manifestations of COPD and is characterised by abnormal, permanent airspace enlargement that extends beyond the terminal bronchioles and is accompanied by alveolar wall destruction [1]. Cigarette smoke (CS) induces several pathological changes underlying the development of emphysema, e.g., inflammation, proteolysis, and oxidative stress, all of which may lead to alveoli apoptosis [2-6]. However, the molecular mechanism behind this process is still unclear. The endoplasmic reticulum stress pathway is one of the critical signalling pathways through

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which the cells respond to variously deleterious stimuli. ER stress signalling overactivation can lead to ER-associated apoptosis, which is independent of other apoptotic pathways (such as the mitochondrial pathway) [7].

ER is the major site for synthesis and folding of secretory and membrane-bound proteins. Due to the limited capacity of the ER to process proteins, accumulation of unfolded and misfolded proteins in the ER triggers the ER stress response. ER stress is a double-edged sword that can trigger adaptive survival or apoptosis in a cell. Mild ER stress causes cell to undergo adaptive survival, while excessive and lengthy ER stress induces apoptosis [8]. Further, ER stress-induced apoptosis (ERSIA) has been shown to be involved in the onset and progress of several diseases such as cardiovascular disease, diabetes, neurodegenerative disease, and cancer [9-11]. Recently, it has been shown that ER stress is involved in lung diseases, ischemia, and drug-induced apoptosis for lung epithelial cells [12-17]. Tagawa et al reported that cigarette smoke induced ER stress-associated apoptosis for bronchial epithelial cells *in vitro* [3]. Caspase-12 is an ER-specific caspase which is the key molecule in the death-driving force in ER stress, and the CCAAT enhancer-binding protein-homologous protein (CHOP, also called GADD153) is an important protein that connects ER stress to apoptosis. CHOP overexpression arrests the cell cycle at the G1/S stage and triggers apoptosis [18]. However, the function and mechanism for CHOP in the lung epithelial cell are still elusive. Notably, our study is the first to detect the possible involvement of the protein kinase RNA (PKR)-like ER kinase (PERK)/eukaryotic initiation factor 2 α (eIF2 α) pathway, CHOP expression and proapoptotic gene caspase-12 in the emphysema that results from cigarette smoke exposure.

Materials and Methods

Animals

Forty Wistar rats (male, 180 $\pm$ 20 g, 8-9 weeks old) that were purchased from the Animal Experimental Center of Hunan University of Traditional Chinese Medicine (Changsha, China) were randomly divided into four groups, with ten rats in each group. Group A rats were kept in a filtered air environment, while Group B, Group C and Group D rats were exposed to cigarette smoke twice a day, for one hour in each instance [19], by burning 10 Furong cigarettes (1.0 mg nicotine per cigarette), with 4 cigarettes at first and then 2 cigarettes in each instance [19]. Each cigarette was consumed in approximately 12 min. The total time for CS exposure in Group B, Group C and Group D were 2 months, 4 months, 4 months, and the Group D stopped

smoking for 1 month.

Morphometric analysis

Formalin-fixed, paraffin-embedded lung tissue sections were stained with hematoxylin-eosin and analysed under a light microscope. Emphysema was quantified via measuring the pulmonary mean linear intercept (MLI) and mean alveolar number (MAN) [20]. At least five representative alveoli from each rat were seized and analysed using PIPS-2020 image software (Chongqing Tianhai Company, Chongqing, China).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labelling (TUNEL) staining

TUNEL was performed using the *In Situ* Cell Death Detection Kit (Roche Biotechnology, Switzerland) [21]. According to the manufacturer's instructions, formalin-fixed, paraffin-embedded lung tissue sections were digested using Proteinase K for 20 min at room temperature and then incubated with 3% H₂O₂ in methanol for 30 min at room temperature. The sections were incubated with TDT enzyme solution for 1 h at 37 °C. In negative control studies, the TDT enzyme solution was replaced with phosphate-buffered saline according to the manufacturer's instructions. Peroxidase activity was visualised via a colour reaction using diaminobenzidine. Brown colours indicated positive results [22]. Finally, the sections were counterstained using hematoxylin and then mounted. The apoptosis rate was measured via the PIPS-2020 pathology image analysis system, and at least five representative alveoli that were chosen from each section were independently examined [22].

In situ hybridisation of CHOP mRNA

In situ hybridisation was carried out using an *in situ* hybridisation detection kit (Wuhan Boster Biological technology Co. Ltd., China). The oligonucleotide probes were designed by Beijing Dingguo Biological Technology Company in accordance with the sequences of rat and human CHOP mRNA. The sequence for the probes against CHOP mRNA was 5'-TCC TCA TAC CAG GCT TCC AGC TCC CAG-3'.

Hybridisation was performed on serial tissue sections from the left lung in paraffin and fixed using formalin containing 0.1% diethyl pyrocarbonate (Wuhan Boster Biological technology Co. Ltd., China) according to the manufacturer's instructions. The sections were digested with pepsin for 20 min at 37 °C. After 3.5 h of pre-hybridisation the sections underwent hybridisation with digoxin-labelled, single-stranded oligonucleotide probes for 20 h at 40 °C. In negative control studies, labelled oligo-nucleotide probes were substituted with phosphate-buffered saline. The sections were incubated with rabbit antibodies against digoxin after the unbound probes were washed off and then with biotinylated goat antibodies against rabbit immunoglobulin G. The sections were then incubated with streptavidin-biotin-peroxidase. Peroxidase activity was visualised via a colour reaction using diaminobenzidine (Wuhan Boster Biological technology Co. Ltd., China) as the substrate. Brown and yellow colours indicated positive results. Finally, the sections were counterstained using hematoxylin and mounted. The mRNA expression levels were measured using

the PIPS-2020 pathology image analysis system, and at least five representative alveoli chosen from each section were independently examined [22].

RT-PCR analysis for the CHOP gene and caspase-12 gene

Total RNA was extracted from lung tissues using TRIzol reagent (Molecular Research Center, Cincinnati, Inc., OH, USA). Samples (4 µg) were reversed transcribed using a first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Synthesised cDNA was used for further amplification. Primers were designed using Primer 5 software. The oligonucleotide primers for CHOP: forward primer, 5'-GCATCTTCATACACCACCACA-3' and reverse primer, 5'-TCATTCTCCTGCTCCTTCTC-3', and caspase-12: forward primer, 5'-CCTGGAAGGAATCTGTGG-3' and reverse primer, 5'-AGTTCACCTGGGACCTCA-3', and β-actin forward primer, 5'-GAGACCTTCAACACCCAGC-3' and reverse primer, 5'-ATGTCACGCACGATTTC-3'.

The PCR was performed using the following thermal profiles: For CHOP and β-actin, denaturation at 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C and an extension step at 72 °C for 7 min. For caspase-12, denaturation at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and an extension step at 72 °C for 5 min. The amplified products were electrophoretically separated via 2% agarose gels. The optical density values of the DNA bands were determined using a densitometer and quantified via the Tanon gel image system version 3.74 (Shanghai Tanon Science and Technology, Shanghai, China). mRNA expression was evaluated using the ratio of the intensities of the target band to the β-actin band. RT-PCR analysis was carried out independently, in triplicate for each sample. The primer sequences, optimum annealing temperatures, number of cycles used for amplification, and the predicted length of the amplified products

Immunohistochemical (IHC) analysis for CHOP as well as localization

A commercial SABC kit (Wuhan Boster Biological technology Co. Ltd., China) was used for immunohistochemistry. The formalin-fixed, paraffin-embedded lung tissue sections were digested using 3% H₂O₂ for 7 min at room temperature and then preincubated with 10% non-immunised serum. The sections were incubated with rabbit anti-CHOP specific polyclonal antibodies (1:100) (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4 °C. In negative control studies, the antibodies were replaced with phosphate-buffered saline. The sections were incubated with corresponding biotinylated secondary antibodies against goat after the unbound antibodies were washed off and thereafter incubated with streptavidin peroxidase. Subsequently, peroxidase activity was visualised via a colour reaction using diaminobenzidine. Brown and yellow colours indicated positive results (mainly in the cytoplasm). Finally, the sections were counterstained using hematoxylin (resulting in blue nuclei) and mounted. The protein expression levels were measured using a PIPS-2020 pathology image analysis system that is based on the absorbance of positive signals

in alveoli walls.

Western blot analysis for caspase-12, CHOP, PERK, and p-PERK, eIF2α and p-eIF2α protein in the lung tissue

Total protein was extracted from lung tissues using a total protein extraction kit (Nanjing KeyGen Biotech, Nanjing, China) according to the manufacturer's instructions. Protein concentrations were detected using the BCA protein assay kit (Nanjing KeyGen Biotech).

Thirty micrograms of the total protein was separated via 12% sodium dodecylsulphate-polyacrylamide gel, and transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Piscataway, USA). The membranes were blocked with 5% (w/v) instant non-fat milk in Tris-buffered saline and 0.1% Tween-20 for 1 h at room temperature and then incubated overnight at 4 °C with a 1:500 dilution of specific antibodies for caspase-12 (Abcam, Britain) CHOP, PERK, phosphorylated PERK (p-PERK), eIF2α, phosphorylated (p-eIF2α) and β-actin (Wuhan Boster Biological technology Co. Ltd., China). Membranes were then incubated with a 1:2000 dilution of peroxidase-conjugated anti-rabbit immunoglobulin G (Wuhan Boster Biological technology Co. Ltd., China). An enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, USA) was used for signal detection. Tanon gel image system version 3.74 was used for quantification. Western blot analysis was independently carried out in triplicate for each sample.

Caspase-12 Activity Assay

The activity of Caspase-12 was determined using Fluorometric Assay Kit (Biovision, USA). According to the manufacturer's protocol, lung homogenate was resuspended in cold lysis buffer and then an equal volume of reaction buffer containing ATAD-AFC was added. After incubation at 37 °C for 90 min. The amount of AFC liberated from ATAD-AFC was detected using a spectrofluorometer with 400 nm excitation and 505 nm emission wavelength [23].

Results

The morphological character of lung in COPD model rats

In physiological circumstances, the proliferation and differentiation of lung structure is in balance with its apoptotic activities to maintain its normal function. In this study, we observed that the lung sections from the control rats maintained normal alveolar structure, while upon exposure to CS for 2 months, the lung structure appears damaged, with some alveoli developing into bulla. In rats exposed to 4 months CS, a histological evaluation demonstrated that the pulmonary structure was badly impaired, with thinned or ruptured alveolar walls, enlarged airspaces. In ex-smoking rats, the destruction of the alveolar structure was also obvious (Fig. 1).

Fig. 1. Lung tissue morphological character of rats after exposure to CS ($\times 200$). The rats lung were sectioned and subjected to HE to detect morphological character. Emphysema was quantified via measuring the pulmonary mean linear intercept (MLI) and mean alveolar number (MAN). A: control rats. B: rats exposed to 2 month CS. C: rats exposed to 4 month CS. D: ex-smoking rats. a vs control Group A $P < 0.05$, b vs Group B $P < 0.05$.

Assessment of apoptosis using the TUNEL assay

Apoptosis is not counterbalanced by an increase in proliferation of lung structural cells, which is the important pathogenesis of COPD. There is an increase in apoptotic alveolar epithelial and endothelial cells in the

Fig. 2. Apoptotic cell death in lung tissue of rats after exposure to CS ($\times 200$). The rats lung were sectioned and subjected to TUNEL assay to visualize apoptotic cells (brown). A: control rats. B: rats exposed to 2 month CS. C: rats exposed to 4 month CS. D: ex-smoking rats. a vs control Group A $P < 0.05$, b vs Group B $P < 0.05$.

lungs of rats exposed to CS. TUNEL detection demonstrated that the apoptotic rate was $11.4 \pm 1.7\%$, $22.4 \pm 1.6\%$, $34.8 \pm 3.2\%$, and $31.3 \pm 1.8\%$ for the control rats, 2-month CS exposed rats, 4-month CS exposed rats and ex-smoking rats, respectively (Fig. 2). The TUNEL-positive cells were primarily lung epithelial cells, including AECI and AECII as well as vascular endothelial cells (Fig. 2).

The PERK, p-PERK, eIF α and p-eIF2 α protein levels in lung epithelial cells for control rats and those exposed to CS

ER is the major site for synthesis and folding of secretory and membrane-bound proteins. Response to various stimuli, unfolded or misfolded protein accumulated in the ER triggers ER stress. PERK is an endoplasmic reticulum transmembrane protein, which plays a crucial role in translation control. Upon ER stress, PERK

Fig. 3. Expression of CHOP mRNA and *caspase-12* mRNA in lung tissue of rats after CS exposure. The rats lung were sectioned and subjected to *in situ* hybridisation assay to visualize *CHOP* mRNA cellular distribution. ($\times 200$). The RT-PCR was used to detect the *CHOP* and *caspase-12* mRNA expression levels. M: MARK. A: control rats. B: rats exposed to 2 month CS. C: rats exposed to 4 month CS. D: ex-smoking rats. a vs control Group A $P < 0.05$, b vs Group B $P < 0.05$.

phosphorylates the eIF2 α to attenuate total protein translation. eIF2 α is a 38-kDa protein ubiquitously expressed in various cell types. p-eIF2 α is a highly conserved point of convergence of signaling pathways adapting cells to many stimulus response pathways. In Western blot analysis, the p-PERK and p-eIF2 α protein were in low expression in control group, highly increased in rats exposed to 2 months CS, increased even higher in rats with 4 months CS exposure, and decreased somewhat in ex-smoking rats compared with the rats exposed to 4 months CS. Unlike the expression of p-PERK and p-eIF2 α , the total PERK/eIF2 α levels did not change between the control rats and those exposed to CS (Fig. 4).

CHOP mRNA and protein levels in lung epithelial cells for control rats and those exposed to CS

CHOP, an ER stress-inducible proapoptotic gene, is induced after phosphorylation of eIF2 α . Further, the levels were poorly positive in the control group, slightly positive for the rats exposed to 2 months CS, positive in the rats with 4 months CS exposure and ex-smoking rats (Fig. 3). The RT-PCR results demonstrated that the expression of *CHOP* mRNA was low in the control group, highly

increased in rats exposed to 2 months CS, remarkably elevated in rats with 4 months CS exposure, and then only slightly altered after smoking cessation, which parallels the *in situ* hybridisation results (Fig. 3).

Immunohistochemistry showed that CHOP protein was primarily located in the nucleus, poorly expressed in the control group, slightly positive in rats exposed to 2 months CS, enhanced markedly in rats with 4 months CS exposure and stable for the ex-smoking rats (Fig. 4). In Western-blot analysis, CHOP protein expression was low in the control group, markedly elevated in rats exposed to 2 months CS, significantly increased in rats with 4 months CS exposure, and slightly declined in the ex-smoking rats (Fig. 4).

The mRNA expression, protein and activity levels of caspase-12 in lung epithelial cells for control rats and those exposed to CS

RT-PCR and immunoblotting of caspase-12 validates the upregulation of caspase-12 expression in rats exposed to CS. The activity of caspase-12 is low in control group, increased in rats exposed to 2 month CS, and further elevated in rats with 4 month exposure, and slightly decreased in ex-smoking rats (Fig. 4).

Fig. 4. Activation of the PERK/eIF2 α pathway and the expression of CHOP and caspase-12 in lung of rats after CS exposure. The rats lung were sectioned and subjected to immunohistochemistry to visualize CHOP protein cellular distribution and the CHOP protein was mainly located in nucleus($\times 200$). Lung tissue were subjected to Western blotting using p-PERK, PERK, p-eIF2 α , eIF2 α , CHOP, caspase-12 and β -actin antibodies. The activity of caspase-12 was detected by using Fluorometric Assay Kit. A: control rats. B: rats exposed to 2 month CS. C: rats exposed to 4 month CS. D: ex-smoking rats. a vs control Group A $P < 0.05$, b vs Group B $P < 0.05$.

Statistical analysis

Data are expressed as mean $\pm$ SD. One-way ANOVA was used to determine statistically significant difference among multiple groups, and the Newman-Keuls test was used to analyze statistical significance between two groups. $P < 0.05$ was considered as a statistically significant difference.

Discussion

Cigarette smoke, a complex mixture of nicotine, oxidants, aromatic hydrocarbons, aldehyde, heavy metals and phenolics, directly results in lung damage, which is characterized by destruction of lung structure and loss of alveolar parenchyma [3, 6]. In the present study, we observed that, compared with the normal group, the rats exposed to 2 months cigarette smoke had some alveolar damage. In rats with 4 months smoke exposure, histological evaluation demonstrated enlarged air spaces within the normal alveolar architecture and destruction of the normal alveolar architecture, and, for the ex-smoking rats,

the lung structure was also obviously damaged. Enlargement of the lung airspaces following destruction of the alveolar walls is an important characteristic of emphysema, which is a pathological feature of COPD.

Although the underlying mechanism that leads to the destruction of the lung is still unclear, several mechanisms have been advanced, including a proteolytic/anti-proteolytic activity imbalance, inflammation, and oxidative stress. In addition to these mechanisms, recent studies have demonstrated a forth important mechanism involved in the development of pulmonary emphysema: apoptosis of lung structural cells. Under physiological circumstances, apoptosis of alveolar wall cells is in equilibrium with proliferation and differentiation. When apoptosis is not counterbalanced by an increase in proliferation of lung structural cells, it will lead to the destruction of lung tissue and development of emphysema [2, 6]. Our results showed that compared with control group, the number of apoptotic epithelial cells in the 2-month smoke exposed rats were highly increased and elevated even further in the 4-month smoke exposed rats. Given these results, we can

infer that the increased epithelial apoptosis plays a important role in the destruction of lung structure, which is consistent with the results reported by Aoshiba et al. [2].

It is generally believed that oxidative stress induced by CS is the major cause of alveolar epithelial cell apoptosis in COPD [1]. However, the oxidative stress mechanism that triggers lung epithelial cell apoptosis in COPD is rough, and endoplasmic reticulum (ER)-induced pulmonary epithelial apoptosis is a 'hot spot' for the mechanism behind COPD damage [14, 16]. The ER is the largest cellular organelle that is responsible for folding and sorting proteins. Cellular stress conditions, such as deprivation of oxygen, glucose and oxidative stress, and the accumulation of unfolded or misfolded proteins, impair the function of the ER and activate the cell stress response and unfolded protein response (UPR) to deal with the adverse situation [24, 25]. The UPR is characterised by the activation of three ER-resident proteins: PERK, the activating transcription factor-6 (ATF-6), and the inositol-requiring enzyme 1 (IRE1). The UPR induces translation attenuation via activation of p-eIF2 α by phosphorylate PERK; ER chaperones expression, such as Bip via activation of ATF6 and IRE1; and ER-associated degradation (ERAD) via the ubiquitin-proteasome system [10, 26-31]. In our study, we found that the total PERK and eIF2 α protein levels did not change in the control and CS-exposed rats. However, the expression of p-PERK protein and p-eIF2 α were very low in control group, remarkably increased in 2-month CS-exposed rats, reached its peak expression in rats with 4 months CS exposure, and decreased slightly in rats after smoking cession for 1 month. These results indicate that cigarette smoke induces ER stress in rat lung epithelial cells and ER stress plays an important role in the development of emphysema for rats.

The purpose for UPR is to restore ER homeostasis. However, if the response is unable to rescue cells, ER stress will eventually lead to apoptosis via an increase in CHOP expression as well as activation of the ER-specific caspases and the ASK-JNK pathway [31-33]. CHOP, which belongs to the CCAAT/enhancer binding protein (C/EBP) family, is an ER-residing, apoptosis-promoting factor and plays a crucial role in the signalling pathway for ER stress-induced apoptosis [34, 35]. CHOP inhibits Bcl-2 expression, depletes glutathione, and facilitates translocation of the proapoptotic protein Bax, thereby triggering apoptosis. It has been shown that CS extract selectively induces CHOP expression and, consequently, apoptosis *in vitro* [3]. In the present investiga-

tion we demonstrated that CS induced CHOP expression in lung of rats.

Under the control conditions, CHOP expression is predominantly located in the cytoplasm, but under ER stress its expression is significantly enhanced and it accumulates in the nucleus [34]. We find that CHOP had low expression in the control rats, though it markedly increased in rats that were exposed to 2 months CS and was even higher for rats that were exposed to CS for 4 months. It is postulated that CHOP levels increase in a time-dependent manner for rats exposed to CS and that CS induces ERSIA, which eventually leads to emphysema.

Caspase-12 is an ER-specific caspase, and exists in an inactive procaspase form bound to ER membranes [36]. Upon ER stress, caspase-12 dissociates from the ER membrane, and it is cleaved to a fragment and activated. Once it is initiated, caspase-12 triggers downstream apoptotic pathways [37]. The caspase-12 activation is verified by immunoblot detection of the stress-induced cleaved-caspase-12. ER stress-mediated activation of caspase-12 indicate a important role for caspase-12 in lung cell apoptosis, because it directly cleaves procaspase-9 (independently of the mitochondria), leading to activation of caspase-3 [36, 38], and inducing cell apoptosis. Combined with prior researches, our results suggest that CS induces ER stress-related protein expression and subsequent apoptotic cell death in rats with emphysema.

We examined the role of the PERK/eIF2 α pathway, CHOP expression, caspase-12 activation and apoptosis in the pathological changes induced by cigarette smoke, as they may relate to emphysema development. Our study indicates that CS, as a major risk factor for emphysema, may trigger the ER stress response and initiate apoptosis of lung epithelial cells. The precise mechanism for these factors in inducing ER stress remains to be further investigated. Nevertheless, these activities may represent a novel mechanism in emphysema for lung injury that is induced by CS.

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