

Original Paper

# 14,15-Epoxyeicosatrienoic Acid Suppresses Cigarette Smoke Extract-Induced Apoptosis in Lung Epithelial Cells by Inhibiting Endoplasmic Reticulum Stress

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## Key Words

Cytochrome P450 2J2 • Epoxyeicosatrienoic acids • Apoptosis • Endoplasmic reticulum stress • Cigarette smoke

## Abstract

**Background/Aims:** Epoxyeicosatrienoic acids (EETs), a type of lipid mediators produced by cytochrome P450 epoxygenases, exert anti-inflammatory, angiogenic, anti-oxidative and anti-apoptotic effects. However, the role of EETs in cigarette smoke-induced lung injury and the underlying mechanisms are not fully known. The aim of this study was to explore the effects of CYP2J2-EETs on cigarette smoke extracts (CSE)-induced apoptosis in human bronchial epithelial cell line (Beas-2B) and the possible mechanisms involved. **Methods:** Cytochrome P450 epoxygenase 2J2 (CYP2J2) and its metabolites EETs were assessed by western blotting or LC-MS-MS. Cell viability and apoptosis were determined by MTT assay and AnnexinV-PI staining. Reactive oxygen species (ROS) were assessed by measuring H2DCFDA. Caspase-3, HO-1, MAPK and endoplasmic reticulum (ER) stress-related markers GRP78, p-eIF2a, and CHOP were evaluated by western blotting. **Results:** CSE suppressed expression of both CYP2J2 and EET by Beas-2B cells. CSE also induced apoptosis, the generation of ROS and the ER stress in Beas-2B cells. These changes were abolished by pretreatment with exogenous 14,15-EET while pretreatment with 14,15-EEZE, a selective EET antagonist, abolished the protective effects of 14,15-EET. In addition, EETs increased the expression of antioxidant enzyme HO-1. Furthermore, 14,15-EET reduced CSE-induced activation of p38 and JNK. **Conclusion:** The data suggest that CYP2J2-derived EETs protect against CSE-induced lung injury possibly through attenuating ER stress.

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## Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive, irreversible airflow limitation caused by emphysema and chronic bronchitis. Cigarette smoke (CS) is the primary risk factor for the development of COPD [1]. CS exposure potentially results in chronic airway inflammation, leading to airway obstruction and inflammatory responses of the alveolar walls, which are associated with lung cell death, lung tissue destruction, and emphysema [2].

The endoplasmic reticulum (ER), an important organelle is responsible for the synthesis and folding of proteins which are required for cell survival and normal cellular functions. Excessive and prolonged stress impairs ER functions resulting in an accumulation of misfolded and unfolded proteins, and even ER stress. It has been shown that ER stress and ER stress-induced cell death have important roles in the pathophysiology of several chronic diseases in humans, including cardiovascular diseases and diabetes [3]. Recent studies have shown that CS can cause ER stress in the lungs of patients with COPD [4, 5] and that cigarette smoke extracts (CSE) can induce ER stress and apoptosis in lung epithelial cells [6-8], suggesting that ER stress may contribute to the pathogenesis of COPD.

Epoxyeicosatrienoic acids (EETs) are arachidonic acid metabolites produced by the cytochrome P450 epoxygenase pathway [9, 10]. Human cytochrome P450 2J2 (CYP2J2) is the predominant enzyme responsible for EET biosynthesis [11]. There are four regioisomeric EETs, including 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET. Under action of soluble epoxide hydrolase (sEH), EETs are rapidly hydrolyzed to their corresponding dihydroxyeicosatrienoic acid (DHET) metabolites which are much less biological active than EETs. A number of studies have shown that EETs have anti-inflammatory, antioxidative and antiapoptotic activities [12-16]. In addition, EETs can reduce ER stress signaling in liver, adipose and heart tissues [17, 18]. CYP2J2 mRNA and protein have been detected in the human pulmonary epithelial cells, vascular endothelial cells, vascular smooth muscle cells and alveolar macrophages, either *in vitro* or *in vivo*, [11, 19]. 14,15-EET is the predominant regioisomer of EETs present in the lung. Recently, Wang et al. [20] have reported that sEH-selective inhibitors, which stabilize EETs by preventing their conversion to DHETs, have significant protective effects on tobacco smoke-induced lung injury in a rat model of COPD. Furthermore, the concentrations of 11,12-EET are higher the exhaled breath condensates of patients with COPD than those of control subjects, while the concentrations of 8,9-EET are lower in COPD patients [21]. Although these observations imply that EETs may play an important role in the pathogenesis of COPD, the precise mechanisms of EETs protecting against COPD are still unclear.

In the present study, we assessed the expression of CYP2J2 and the formation of EET in human lung epithelial cell line Beas-2B. We also evaluated the effects of 14,15-EET on the CSE-induced apoptosis in these cells and the possible molecular mechanisms involved.

## Materials and Methods

### Materials

Human bronchial epithelial cells (BEAS-2B) were purchased from the American Type Culture Collection (ATCC, Rockville, MA, USA). 11,12-EET and 14,15-EET were purchased from Cayman Chemical (Ann Arbor, MI, USA). 14,14-EEZE was purchased from Sigma (St. Louis, MO, USA). Solid-phase extraction (SPE) cartridges (Oasis HLB) were from Waters (Milford, MA, USA). Anti-CYP2J2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against GADPH, glucose-regulated protein 78 (GRP78), C/EBP-homologous protein (CHOP), phospho-eukaryotic initiation factor-2 $\alpha$  (P-eIF2 $\alpha$ ), phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, PD98059, SB203580, and SP600125 were obtained from Cell Signaling (Beverly, MA). Annexin V-FITC apoptosis detection kit was obtained from BD (BD, San Diego, CA). All other chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise specified.

### *Preparation of CSE*

Cigarette smoke extract (CSE) was prepared according to previously described methods, with some modifications [22]. Briefly, 2R4F Kentucky reference cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) were smoked using a peristaltic pump. Two cigarettes were bubbled through 25 ml of cell growth medium in 8 min, which the solution was adjusted to a pH of 7.4, considered as 100% strength CSE, and used within 30 min of preparation.

### *Cell culture*

Human bronchial epithelial cells (Beas-2B) were seeded in M199 medium (Gibco, Grand Island, NY, USA) containing 10% FBS (HyClone, Logan, UT, USA), and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 95% air. The medium was refreshed every 2-3 days. Cells were used between passages 5 and 25.

### *Cell viability assay*

Cell viability was assessed using the MTT assay. Briefly, Beas-2B cells were seeded in 96-well plates containing MTT solution (final concentration of 0.5 mg/ml) and incubated for further 4 h at 37 °C. After removing the medium, the intracellular formazan product was dissolved in 250 µl of DMSO. Absorbance of each well was measured at 540 nm using a microplate reader (Model 680, BioRad). The OD values for the wells containing control cells were considered to be 100%, while the OD values for the experimental cells were expressed as the percentages of the control.

### *Flow cytometric analysis with annexin-V/propidium iodide staining*

Annexin V-FITC and PI double staining was performed using an AnnexinV-FITC apoptosis detection kit (BD, San Diego, CA) according to the manufacturer's protocols. Briefly, cells were harvested, centrifuged at 1500 rpm for 5 minutes, and washed twice with ice-cold PBS. After adding 5 µl of Annexin V, 5 µl of PI and 500 µl of PBS to the cells, the samples were incubated at room temperature for 15 minutes in the dark. Cell apoptosis was analyzed on a FACScan flow cytometer (CyAn ADP, Beckman Coulter, USA). Annexin V-FITC-positive, PI-negative cells were scored as apoptotic. Double-stained cells were considered as either necrotic or late apoptotic cells.

### *Western blot analysis*

Cells were lysed on ice with 100 µl of RIPA lysis buffer. The concentrations of total proteins were measured using a BCA protein assay kit (Pierce). Equal amounts of proteins were separated by 8-15% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with primary antibody against CYP2J2 (1:400), caspase-3, GRP78, P-eIF2 $\alpha$ , CHOP, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38 (1:1000) and GAPDH (1:2000) overnight at 4 °C and then with horseradish peroxidase conjugated secondary antibodies (1:4000) for 2 h. After washing with TBST, the specific signals were detected with an enhanced chemiluminescence kit.

### *Measurement of intracellular ROS*

Intracellular ROS generation was determined with the fluorescent probe H2DCFDA, according to the manufacturer's instructions (Vigorous, Beijing, China). Briefly, Beas-2B cells in the presence or absence of EETs, were stimulated with CSE for 6 h, then incubated with H2DCFDA (final concentration 10 µmol/L) at 37 °C for 30 min in the dark. After washing with fresh medium for three times, the levels of intracellular ROS were measured using a fluorescent microplate reader (BioTek, Winooski, VT, USA) at an emission wavelength of 485 nm and an excitation wavelength of 530 nm.

### *Quantification of EETs*

EETs were quantified using the LC/MS/MS method, as previously described [23]. Briefly, cells were harvested and hydrolyzed after adding 200 µl of 10M sodium hydroxide for 20 min at 60 °C. The solution was neutralized by adding 200 µl of 60% acetic acid and 2 ml of 1M sodium acetate buffer, pH 6.0. The hydrolyzed samples were cleared by centrifugation at 23 000 g for 10 min at 0 °C. The supernatants were transferred to fresh tubes and diluted to a concentration of 10% methanol by the addition of 1.8 mL water. The diluted supernatant was loaded onto a 1 mL Oasis HLB SPE cartridge (Waters) on a vacuum manifold.

The SPE cartridge was preconditioned by low flow using the sequences of 1 mL methanol, 1 mL acetone, 2 mL hexane, 1 mL acetone, 1 mL methanol and 2 mL water. The cartridge was washed with 3 mL water and then 1 mL of 10% methanol before being allowed to dry under argon for 10 min. The eicosanoid metabolites were collected by elution with 2 mL anhydrous acetonitrile and then dried under a stream of argon.

10  $\mu$ l of supernatant was injected onto a high resolution column (Zorbax Eclipse Plus-C18, 4.6  $\times$  150 mm, 1.8  $\mu$ m). Acetonitrile was increased within 10 min to 90% and held for a further 10 min with a flow rate of 0.8 ml/min. The HPLC was coupled to the Agilent 6490 triplequad mass spectrometer with electrospray ionization source. Analysis was performed with selective reaction monitoring (SRM) in negative mode, a gas temperature of 350°C, a nitrogen stream of 12 liters/min, and a capillary voltage of 4000 V. The SRM transitions monitored were mass-to-charge ratio ( $m/z$ ) at  $m/z$  319.3  $\rightarrow$  167 for 11,12-EET,  $m/z$  319.3  $\rightarrow$  219 for 14,15-EET,  $m/z$  319.3  $\rightarrow$  257 for 8,9-EET,  $m/z$  319.3  $\rightarrow$  191 for 5,6-EET, respectively.

#### Statistical analysis

The results were analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA). Differences between groups were assessed by one-way ANOVA, while Bonferroni's test was used for multiple comparisons. Data are expressed as the mean  $\pm$  SE. For all tests, p-values less than 0.05 were considered significant.

## Result

### *Effect of CSE on CYP2J2 protein expression and 14,15-EET formation*

Western blotting showed that 5% CSE significantly decreased CYP2J2 protein expression by Beas-2B cells after exposure for 24 h or 36 h (Fig. 1A). Furthermore, we measured the formation of 14,15-EET, the major biologically active metabolite produced by CYP2J2 in the lung. As expected, 5% CSE significantly reduced 14,15-EET formation in Beas-2B cells at the same time points (Fig. 1B). These data indicate that CSE induced a decrease in CYP2J2 expression and 14,15-EET formation.

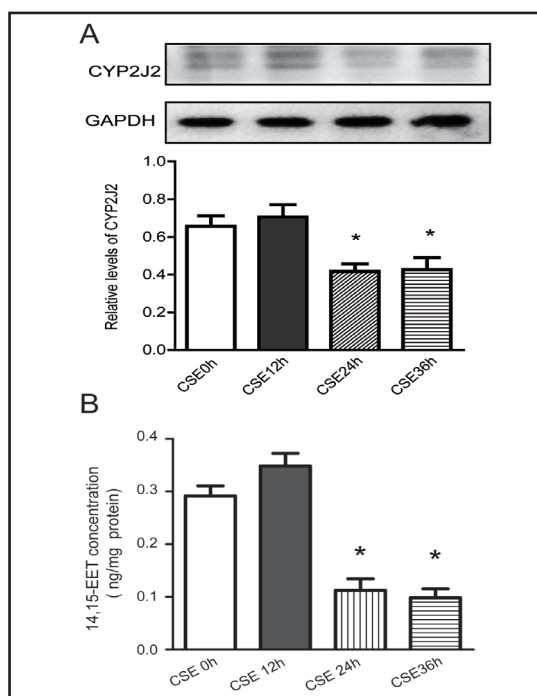
### *14,15-EET increases cell viability and reduces apoptosis*

The MTT assay showed that compared to the control group, CSE exposure significantly reduced cell viability in dose-dependent manner at 24 h (Fig. 2A). Meanwhile, the reduction of cell viability was time-dependent from 18 h to 24 h of exposure. Furthermore, pretreatment with 14,15-EET (1  $\mu$ M), but not 11,12-EET (1  $\mu$ M), significantly mitigated the loss of cell viability induced by 5% CSE (Fig. 2A-B). *In vitro* experiments showed that toxicity of CSE increased in a dose- and time-dependent manner in Beas-2B cells. 10% CSE had severe cytotoxic effect on Beas-2B cells, whereas the concentration of CSE less than 2.5% had little effect on the apoptotic rate and CYP2J2 expression. Thus, exposure of 5% CSE for 24 h was applied in the following experiments.

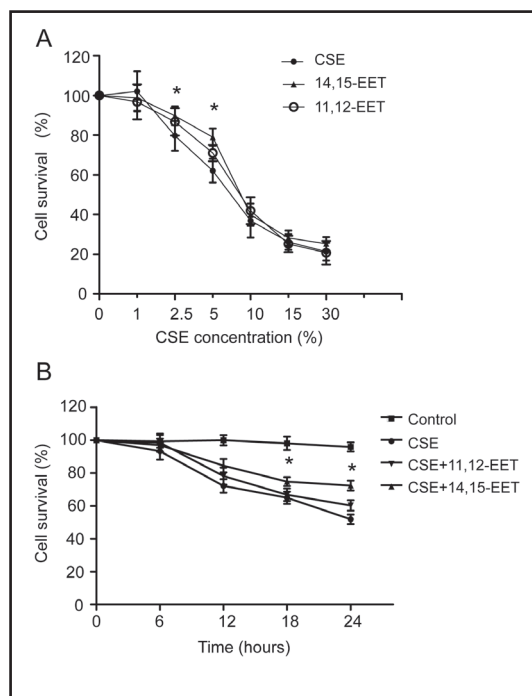
To determine whether this CSE-induced reduction in cell viability was due to apoptosis, flow cytometry using annexin V-FITC and western blot analysis for cleaved caspase-3 were performed. Flow cytometry showed that CSE markedly increased the rate of apoptosis at 24 h compared with vehicle-treated cells (Fig. 3A). Western blotting revealed that CSE significantly increased expression of cleaved caspase-3. Pretreatment with 14,15-EET significantly reduced CSE-induced apoptosis, as measured by annexinV-FITC and caspase-3 staining. We further explored whether 14,15-EEZE, an EET antagonist, could prevent this 14,15-EET-mediated protection against CSE-induced apoptosis. Beas-2B cells were pretreated with 14,15-EEZE (1  $\mu$ M) 30 min before the administration of 14,15-EET. As illustrated in Fig. 3A-B, 14,15-EEZE abolished the protection provided by 14,15-EET. Notably, 14,15-EEZE treatment alone did not affect cell apoptosis. These findings indicate a role for 14,15-EET in inhibiting CSE-induced apoptosis.

### *14,15-EET prevents CSE-induced ER stress*

We investigated whether CSE induces ER stress by measuring the expression levels of GRP78, p-eIF2 $\alpha$ , and CHOP in Beas-2B cells using western blot analysis. As shown in Fig. 4A, following treatment with 5% CSE, the amounts of GRP78, p-eIF2 $\alpha$ , and CHOP significantly



**Fig. 1.** CSE decreased CYP2J2/EETs in Beas-2B cells. (A) Cells were exposed to 5% CSE for the indicated time intervals. CYP2J2 expression was assessed by western blotting. GAPDH served as the standard. (B) Similar to CYP2J2, the concentrations of 14,15-EET were also decreased after exposure to 5% CSE at 24 h and 36 h time points. The concentrations of 14,15-EET in the harvested cells were measured using LC-MS/MS. Values are expressed as the mean  $\pm$  SE (n = 4). \*p < 0.05 vs. control group.



**Fig. 2.** Effects of cigarette smoke extract (CSE) on the survival of human bronchial epithelial cells (Beas-2B). (A) Cells were pretreated with 11,12-EET (1  $\mu$ M) or 14,15-EET (1  $\mu$ M) for 1 h, then incubated with increasing concentrations of CSE for further 24 h. 14,15-EET significantly increased the viability of cells exposed to 2.5% and 5% CSE. (B) Cells were exposed to 5% CSE for the indicated times, and cell viability was evaluated using the MTT assay. Cells pretreated with 14,15-EET showed significant increases in cell viability at 18 and 24 h after CSE exposure. Values are presented as the mean  $\pm$  SE (n = 4). \*p < 0.05 vs. CSE group.

increased in a time-dependent manner. Next, we investigated the effect of 14,15-EET on CSE-induced ER stress. As illustrated in Fig. 4B, pretreatment with 14,15-EET (1  $\mu$ M) significantly decreased the expression of GRP78, p-eIF2 $\alpha$ , and CHOP induced by CSE. Furthermore, 14,15-EEZE reversed the 14,15-EET-mediated protection against CSE-induced ER stress, suggesting that 14,15-EET has a potential protective effect against CSE-induced ER stress.

#### 14,15-EET attenuate ROS generation in Beas-2B

A growing body of evidence has suggested that increased levels of ROS play an important role in CS-induced COPD [24, 25]. In addition, CSE-induced ER stress in lung epithelial cells has been associated with ROS [26]. We sought to determine whether 14,15-EET affects ROS generation after CSE exposure. DCF fluorescence was used to measure ROS in cells exposed to CSE, in the absence or presence of 14,15-EET. In line with published data [27, 28], CSE significantly increased ROS generation in Beas-2B cells 6 hrs after CSE exposure (Fig. 5). Pretreatment with 14,15-EET (1  $\mu$ M) significantly inhibited ROS production, whereas 14,15-EEZE, an antagonist of 14,15-EET blocked 14,15-EET-mediated inhibition (Fig. 5). Cells treated with 14,15-EET or 14,15-EEZE alone did not show a significant change in DCF staining, compared with vehicle-treated cells. These data indicate a role for 14,15-EET in inhibiting CSE-induced ROS production.

**Fig. 3.** 14,15-EET inhibits CSE-induced apoptosis of Beas-2B cells. Cells were pre-treated with 14,15-EET (1  $\mu$ M) or 14,15-EEZE (1  $\mu$ M) for 1 h and then exposed to 5% CSE for further 24 h. Annexin V FACS assay (A) and caspase-3 assay (B) were performed. Values are expressed as the mean  $\pm$  SE (n = 4). \*p<0.05 vs. control group, #p<0.05 vs. CSE group, &p<0.05 vs. CSE +14,15-EET group.

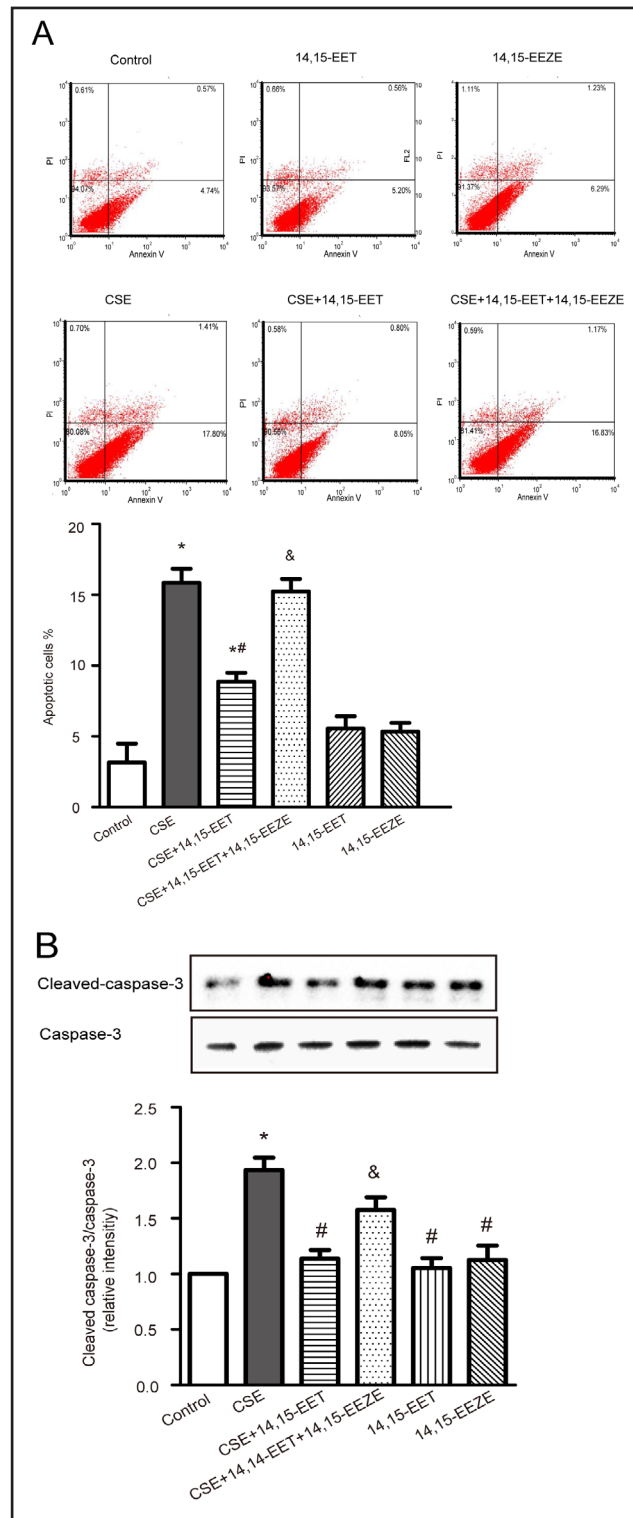
*14,15-EET enhanced the expressions of HO-1*

We next assessed the expression of HO-1, which is an important regulator of lung antioxidant defenses. In accordance with the previous results [22], HO-1 protein was significantly elevated 24 h after exposure to CSE. Interestingly, pretreatment with 14,15-EET caused a further increase HO-1 protein synthesis after CSE exposure (Fig. 6 A). Furthermore, 14,15-EET reduced the expression of Bach-1, a transcriptional suppressor of HO-1 expression, 24 h after exposure to CSE (Fig. 6 B). Previously, HO-1 has been shown to inhibit CSE-induced cell death by decreasing ROS generation [29]. Taken together, these results suggest that 14,15-EET may reduce ROS generation through inhibiting Bach-1 then up-regulating HO-1 expression.

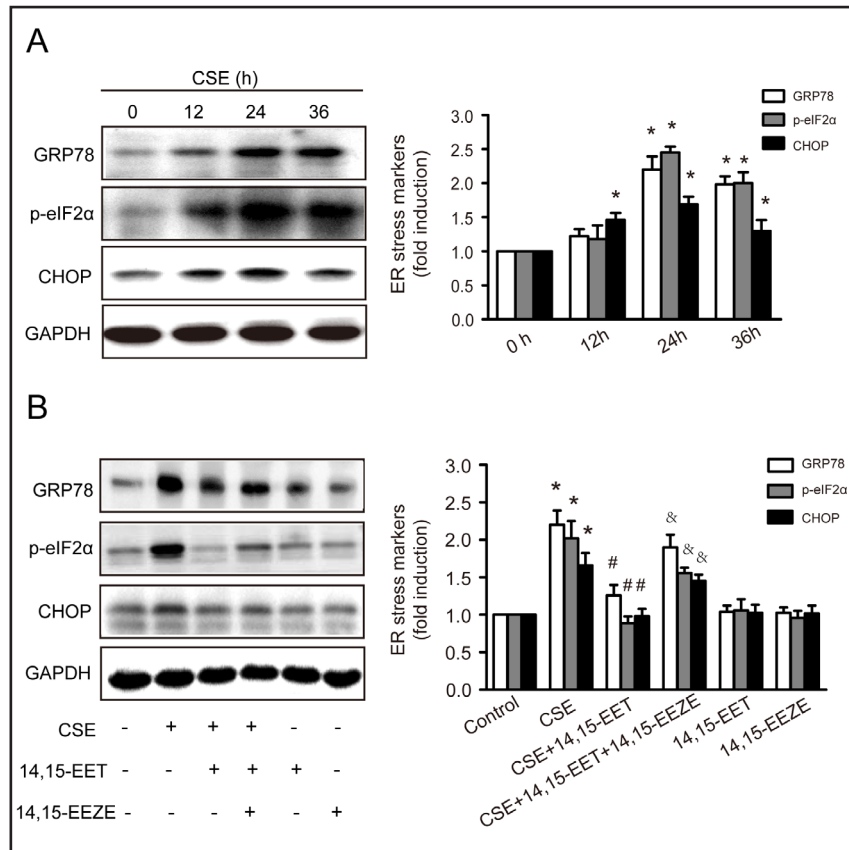
*MAPK pathways are involved in 14,15-EET-mediated protection*

MAPK signaling pathways have been shown to be involved in ER stress-induced apoptosis [30]. To determine whether EETs protect against ER stress-induced apoptosis through MAPK pathways, we evaluated the expression of

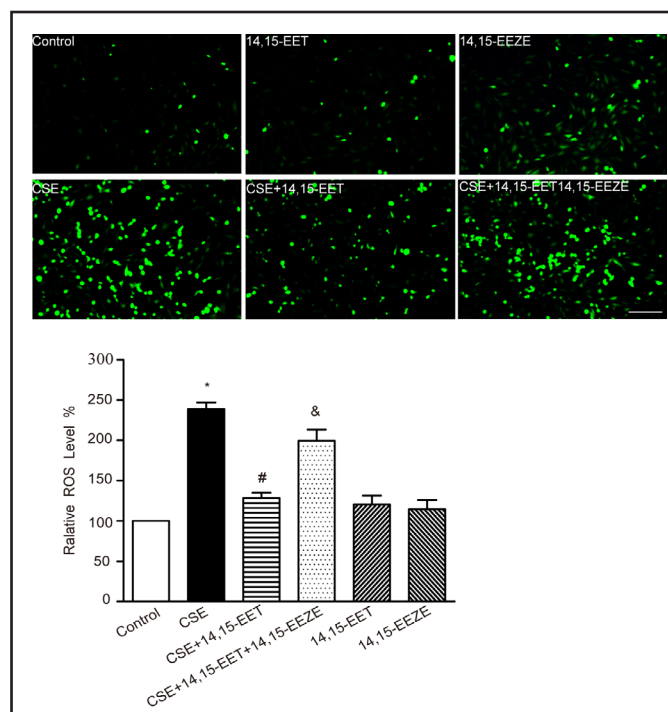
ERK1/2, p38MAPK and JNK by western blot analysis. Consistent with a previous study [31], CSE exposure induced the phosphorylation of ERK1/2, JNK, and p38 MAPK (Fig. 7A-B). Pretreatment with an ERK inhibitor (PD98059), a p38 inhibitor (SB203580) and a JNK inhibitor (SP600125) was able to significantly reduce CSE-induced apoptosis (Fig. 7E). In addition, SB203580 and SP600125 also reduced CSE-induced ER stress (Fig. 7C-D). Importantly, pretreatment with 14,15-EET inhibited the phosphorylation of JNK and



**Fig. 4.** 14,15-EET inhibited CSE-induced ER stress signalling. (A) Time-dependent effects of CSE on ER stress-associated proteins. Beas-2B cells were treated with 5% CSE for the indicated times, and the expression of proteins of GRP78, p-eIF2 $\alpha$ , and CHOP were assessed by western blotting. (B) Effects of 14,15-EET (1  $\mu$ M) and/or 14,15-EEZE (1  $\mu$ M) on ER stress signalling 24 h after CSE exposure. Values are the mean  $\pm$  SE (n = 4). \*p < 0.05, vs. control group, &p < 0.05 vs. CSE+14,15-EET group.

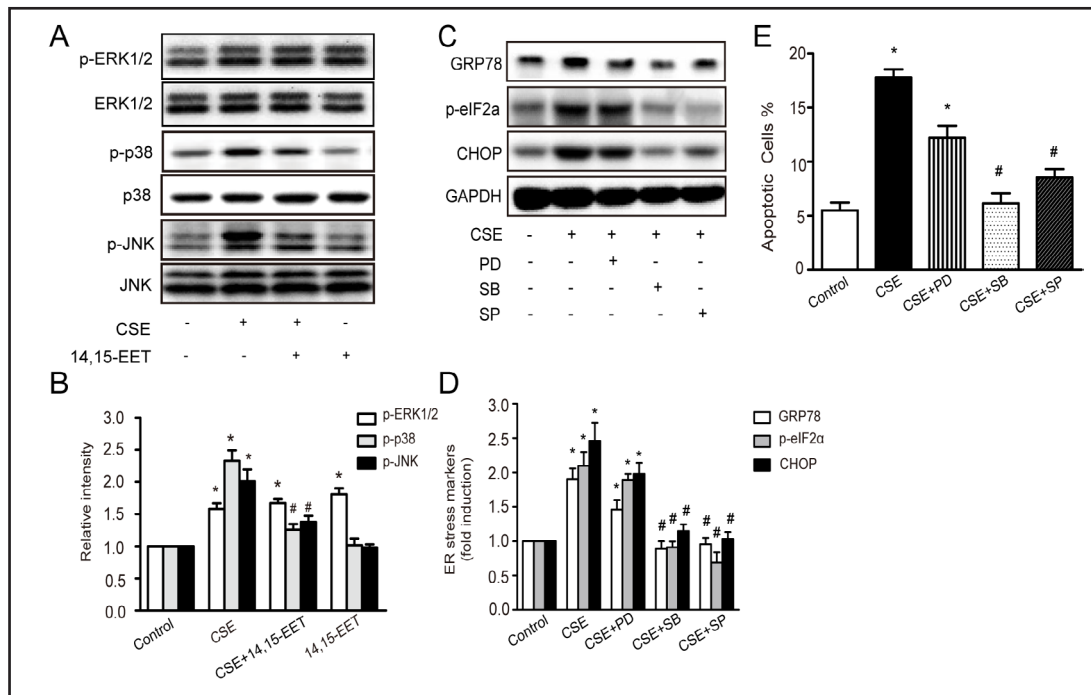
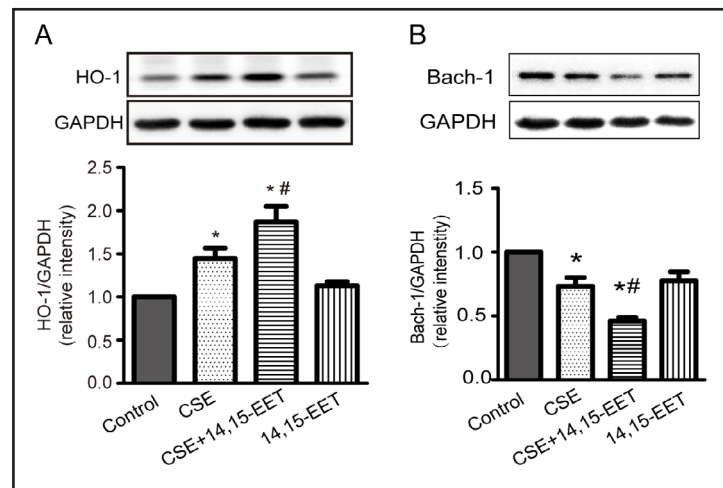


**Fig. 5.** 14,15-EET attenuated CSE-induced ROS production in Beas-2B cells. (A) ROS was measured using H2DCFDA staining by photofluorography. (Scale bar, 25  $\mu$ m, magnification,  $\times$  100). (B) The rate of ROS production was determined by a fluorescence plate reader, and the relative change in fluorescence intensity was expressed as percent change from control at 100%. Values are expressed as the mean  $\pm$  SE (n = 6). \*p < 0.05 vs. control group, #p < 0.05 vs. CSE group, &p < 0.05 vs. CSE+14,15-EET group.



p38 MAPK, but less affected the phosphorylation of ERK1/2 in Beas-2B cells (Fig. 7A-B), suggesting that 14,15-EET may protect CSE-induced apoptosis, at least partially through the p38 MAPK and JNK signalling pathways.

**Fig. 6.** 14,15-EET increased HO-1 and decreased Bach-1 expression after CSE exposure. HO-1 and Bach-1 expressions were assessed by western blotting. GAPDH was served as the standard. Values are presented as the mean  $\pm$  SE (n = 3). \*p < 0.05 vs. control group, #p < 0.05 vs. CSE group.



**Fig. 7.** 14,15-EET suppressed CSE-induced apoptosis by inhibiting MAPK-dependent signalling pathways in Beas-2B cells. (A and B) Effects of 14,15-EET on the activity of phospho-ERK1/2, phospho-p38, and phospho-JNK in Beas-2B cells treated with 5% CSE. (C and D) Cells were incubated with or without pretreatment with MAPK inhibitors (PD98059, an ERK inhibitor, 10  $\mu$ M; SB, SB203580, a p38 inhibitor, 10  $\mu$ M; SP, SP600125, a JNK inhibitor, 10  $\mu$ M) for 30 min and then exposed to 5% CSE for further 24 h. The protein levels were analyzed by western blotting. Values are expressed as the mean  $\pm$  SE (n = 3). \*p < 0.05 vs. control group, #p < 0.05 vs. CSE group.

## Discussion

In the present study, we demonstrated that CSE suppressed CYP2J2 expression and decreased 14,15-EET formation in human pulmonary epithelial cells. Importantly, pretreatment with 14,15-EET attenuated CSE-induced ER stress and cell apoptosis. Moreover, 14,15-EET inhibited ROS production and up-regulated the expression of the antioxidant



HO-1 after CSE exposure. Taken together, these findings suggest that the CYP2J2-EET system might be associated with regulating CSE-induced lung injury and protect against COPD progression by relieving ER stress and oxidative stress.

Human P450 2J2 (CYP2J2) is the predominant CYP epoxygenase that is responsible for the biosynthesis of EETs. It has been reported that CYP2J2 gene expression is repressed in the bronchial biopsies obtained from smokers compared to those of nonsmokers [32]. Our data showed that CSE exposure decreased the expression of CYP2J2 protein and the formation of 14,15-EET in human bronchial epithelial cells. In addition, other CYP epoxygenases like CYP2C8, CYP2C9 are also involved in the synthesis of EETs. Similarly, Deng et al. [33] have shown that increased CYP2J2 and CYP2C8-mediated EET biosynthesis and decreased sEH-mediated EET hydrolysis attenuate vascular inflammatory in lung. Thum et al. [32] have reported that CYP2J2 gene expression was repressed in bronchial biopsies of smokers, but CYP2C9 expression was induced. A recent study has shown that that 2.5% CSE inhibits the expression of CYP2C8 but does not alter the expression of CYP2J2 in NCI-H292 cells [34]. However, our data revealed that 5% CSE exposure decreased the expression of CYP2J2 protein and the formation of 14,15-EET in Beas-2B cells. These disparate observations may regard to differences in the cell types and the concentration of the CSE used. Although the exact mechanisms that down-regulate CYP2J2 expression after CSE exposure are still unclear, there may be some potential mediators that suppress the transcriptional binding factors. It has been shown that ROS affects the metabolism of epoxygenases, thus leading to oxidative stress that may interfere with the balance of cellular redox after exposure to CSE [35, 36]. These changes might cause the activation of the cyclooxygenase mediated metabolism of arachidonic acid and the suppression of the epoxygenase metabolic pathways. It has been suggested that oxidative stress can down-regulate CYP2J2 fatty acid epoxygenase possibly through stimulating the expression of the downstream AP-1 factor c-fos via the p38 and ERK signalling pathways [37]. In addition, inflammatory cytokines may also mediate the suppression of CYP450 gene transcription under inflammatory conditions [38]. Whether regulation of CYP2J2 expression is mediated by the above mechanisms remains to be explored.

On the other hand, the concentrations of cellular EETs are also regulated by the degrading enzymes, especially the soluble epoxide hydrolases (sEH). Thus, inhibition of this pathway might likely increase the EET concentrations. It has been shown that sEH gene expression is up-regulated in the lung of mice after exposed cigarette smoke [39]. Ma and colleagues have also found that cigarette smoking slightly increases the levels of sEH in bronchial epithelial cells [34]. Furthermore, it has been observed that soluble epoxide hydrolase inhibitors (sEHIs) improves lung function and attenuates lung inflammation and emphysematous changes in a rat model after exposure to tobacco smoke [20], suggesting that sEHI action is involved in the stabilization of largely anti-inflammatory lipid epoxides, including EETs, and the reduction in the production of largely proinflammatory lipid-1,2 diols. However, the exact mechanisms of EETs on CSE-induced lung cell damage remain unclear. The apoptosis of lung epithelium cell, alveolar septal and endothelial cells is important in the pathogenesis of COPD [40]. In our best knowledge, it is the first report showing that CSE altered the expression of CYP2J2-EET in lung epithelium cells and that 14,15-EET reduced CSE induced epithelial cell apoptosis by attenuating ER stress. These data support a concept that the CYP2J2-EET system may play an important role in the pathogenesis of COPD.

A growing body of evidence has indicated that exposure to common lung irritants, such as cigarette smoke, may increase the accumulation of misfolded proteins in the endoplasmic reticulum (ER), causing ER stress and ER stress-induced apoptosis and, subsequently, the activation of the unfolded protein response (UPR) signalling cascades [5, 41-43]. It is well known that GRP78 is an ER-chaperon protein and plays a crucial role in the regulation of dynamic homeostasis of the ER. GRP78 is also a marker for ER stress. Phosphorylated eIF2 $\alpha$ , a component of the PERK arm of the UPR, mediates the attenuation of mRNA translation and reduces the burden of protein folding in the ER lumen. CHOP is induced by ER stress at the transcription level and plays a key role in ER stress-induced apoptosis. Data of

the present study showed that CSE induced the expression of GRP78, p $\alpha$ IF-2 and CHOP, whereas pretreatment with 14,15-EET significantly decreased CSE-induced expression of GRP78, p $\alpha$ IF-2 and CHOP proteins, suggesting that EET might protect lung epithelial cells from apoptosis by attenuating the ER stress PERK pathway.

Reactive oxygen species (ROS) are important biological mediators in physiology and pathology. Although ROS are essential for cellular functions, they are also potentially toxic. ROS are capable of damaging DNA, RNA, and proteins and degrading essential cellular molecules. ROS are closely linked with ER stress. High level of ROS can be an essential component of the events leading to protein misfolding in the ER and ER stress-induced apoptosis [26, 27]. Our data showed that CSE exposure increased ROS generation, which was attenuated by pretreatment with 14,15-EET, whereas its antagonist 14,15-EEZE abolished the effects of 14,15-EET, suggesting that EETs might attenuate ER stress by scavenging the accumulation of ROS-induced by CSE.

HO-1, an important antioxidant enzyme, has a key role in ROS generation. Recently, HO-1 has been shown to protect lung epithelial cells against CSE-induced cell death by preserving cellular ATP levels [22] and by down-regulating apoptosis and autophagy-related signaling [44]. In addition, it has been shown that HO-1 mediates ER stress-induced apoptosis by down-regulating CHOP protein expression [45]. In the present study, HO-1 protein was markedly elevated in Beas-2B cells after exposure to CSE. Pretreatment with 14,15-EET caused a further increase in the expression of HO-1 protein. In addition, 14,15-EET reduced the expression of Bach-1, a transcriptional suppressor of HO-1 expression, suggesting EETs may stimulate HO-1 expression via the suppression of Bach 1. These data imply that the EET-mediated protection against CSE-induced ER stress and apoptosis may occur through the upregulation of HO-1, which then suppresses ROS formation. It is known that p-Akt/GSK-3 $\beta$  signaling pathways are participated in the interaction between EET and Bach 1 [33]. In addition, nuclear factor erythroid 2-related factor 2 (Nrf2) is a major regulator of HO-1 induction. It has been reported that Nrf2 plays critical role in regulating transactivation of CYP2J2 by c-Jun/Nrf2 dimers [46]. Goven and colleagues have also shown that CSE affects HO-1 expression and nuclear Nrf2/Bach1 translocation through ERK(1/2) and JNK signalling pathways. Again, the exact mechanism by how EETs regulate HO-1 expression after CSE exposure needs to be further studied.

The mitogen-activated protein kinase (MAPK) pathway is a ubiquitous intracellular signalling pathway consisting of extracellular signal regulated kinase 1/2 (ERK1/2), p38 and c-Jun NH2-terminal kinase (JNK). It regulates cellular proliferation, differentiation and apoptosis, and also plays an important regulatory role in ER stress-induced apoptosis [30]. Our data showed that EET significantly inhibited CSE-induced phosphorylation of p38 and JNK but did not significantly alter the phosphorylation of ERK1/2. These suggest that EET might mediate ER stress-induced apoptosis partially through the inhibition of the p38 and JNK pathway.

In summary, the present study provides insight into the potential protective role of CYP2J2-derived 14,15-EET in CSE-induced lung injury. Specifically, EET protect lung epithelial cells against CSE-induced apoptosis, which is likely mediated through attenuating ER stress. Our data suggest that the CYP2J2-EET system may be a potential target for treating cigarette smoke-induced COPD.

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## Disclosure Statement

The authors declare that they have no conflict of interest

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