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Cigarette smoke-induced lung endothelial apoptosis and emphysema is associated with impairment of FAK and eIF2 α

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Abstract

Lung endothelial cell (EC) apoptosis has been implicated in the pathogenesis of emphysema. However, the mechanism underlying cigarette smoke (CS)-induced lung EC apoptosis and emphysema is not well defined. We have previously shown that cigarette smoke extract (CSE) decreased focal adhesion kinase (FAK) activity via oxidative stress in cultured lung EC. In this study, we compared FAK activation in the lungs of highly susceptible AKR mice and mildly susceptible C57BL/6 mice after exposure to CS for three weeks. We found that three weeks of CS exposure caused mild emphysema and increased lung EC apoptosis in AKR mice (room air: 12.8 $\pm$ 5.6%; CS: 30.7 $\pm$ 3.7%), but not in C57BL/6 mice (room air: 0 $\pm$ 0%; CS: 3.5 $\pm$ 1.7%). Correlated with increased lung EC apoptosis and early onset of emphysema, FAK activity was reduced in the lungs of AKR mice, but not in C57BL/6 mice. Additionally, inhibition of FAK caused lung EC apoptosis, whereas over-expression of FAK prevented CSE-induced lung EC apoptosis. These results suggest that FAK inhibition may contribute to CS-induced lung EC apoptosis and emphysema. Unfolded protein response (UPR) and autophagy have been shown to be activated by CS exposure in lung epithelial cells. In this study, we noted that CSE activated UPR and autophagy in cultured lung EC, as indicated by enhanced eIF2 α phosphorylation and elevated levels of GRP78 and LC3B-II. However, eIF2 α phosphorylation was significantly reduced by three-weeks of CS exposure in the lungs of AKR mice, but not of C57BL/6 mice. Markers for autophagy activation were not significantly altered in the lungs of either AKR or C57BL/6 mice. These results suggest that CS-induced impairment of eIF2 α signaling may increase the susceptibility to lung EC apoptosis and emphysema. Taken together, our data suggest that inhibition of eIF2 α and FAK signaling may play an important role in CS-induced lung EC apoptosis and emphysema.

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Introduction

Emphysema is a common and debilitating lung disease characterized by alveolar airspace enlargement and loss of alveolar capillary septa. Currently, there is no specific treatment available to reverse emphysema due to insufficient understanding of the disease pathogenesis. Protease/anti-protease imbalance has been accepted as a major mechanism for emphysematous lung destruction (Shapiro, 1995; Shapiro, 1999; Shapiro et al., 2003; Taraseviciene-Stewart and Voelkel, 2008). Oxidant stress and immunological injury also play roles in the pathogenesis of emphysema (Taraseviciene-Stewart and Voelkel, 2008). Emerging evidence has highlighted a role of lung endothelial cell (EC) apoptosis in the initiation and progression of emphysema (Giordano et al., 2008; Kasahara et al., 2001; Kasahara et al., 2000). Lung EC apoptosis is significantly increased in emphysematous lungs of human smokers (Kasahara et al., 2001). Cultured pulmonary EC undergo apoptosis after exposure to cigarette smoke extract (CSE) (Damico et al., 2011; Tudor et al., 2000). Additionally, lung EC-specific induction of apoptosis causes emphysematous-like change in mice (Giordano et al., 2008). However, the mechanism underlying cigarette smoke (CS)-induced lung EC apoptosis is not well defined. Focal adhesion kinase (FAK) is a pro-survival factor (Lu and Rounds, 2012). We have previously shown that CSE decreased FAK activation via oxidative stress in cultured lung EC (Lu et al., 2011). We have also demonstrated that three weeks of CS exposure causes mild emphysema and lung EC apoptosis in highly susceptible AKR mice (Lu et al., 2013). In this study, we found that FAK activity was reduced in the lungs of AKR mice exposed to CS for three weeks; an effect associated with lung EC apoptosis and early onset of emphysema. In addition, over-expression of FAK prevented CSE-induced lung EC apoptosis, whereas inhibition of FAK caused lung EC apoptosis. Our results suggest that FAK inhibition contributes to CS-induced lung EC apoptosis, which may play a role in emphysema development.

CS is the major risk factor for emphysema, however, only 10-15% of smokers develop emphysema. The mechanism underlying increased susceptibility to emphysema remains unclear. The unfolded protein response (UPR) is an important mechanism of elimination of endoplasmic reticulum (ER) stress, thereby maintaining ER function and cell survival (Schroder and Kaufman, 2005). CSE induces ER stress and activates UPR in cultured human bronchial epithelial cells and 3T3 cells (Hengstermann and Müller, 2008; Jorgensen et al., 2008). UPR is also activated in the lungs of smokers without evidence of COPD (Kelsen et al., 2008). Nrf2, a redox-sensitive antioxidant transcription factor, can be activated by eIF2 α , a branch of UPR (Digaleh et al., 2013). Nrf2 knockout mice are highly susceptible to CS-induced emphysema (Iizuka et al., 2005). However, it is unknown whether eIF2 α signaling is impaired in emphysematous lungs. In this study, we found that CSE transiently activated UPR in cultured lung EC. However, phosphorylated (active) eIF2 α was significantly reduced in the lungs of AKR mice exposed to CS for three weeks; an effect associated with lung EC apoptosis and early onset of emphysema. These results suggest that CS-induced inhibition of eIF2 α signaling may increase the susceptibility to lung EC apoptosis and emphysema.

Autophagy is a dynamic, continuous process by which cells dispose of damaged or unwanted proteins or organelles via self-digestion, thereby generating intracellular nutrients.

Autophagy has been considered as a protective response to deficiencies of extracellular and intracellular nutrients caused by starvation, hypoxia, or growth factor deprivation. Autophagy is activated in lung epithelial cells and fibroblasts exposed to CSE (Kim et al., 2008). The autophagy marker, LC3B-II is also elevated in patients with COPD, and the degree of LC3B-II elevation correlates positively with clinical severity (Chen et al., 2008). LC3B knockout mice are resistant to CS-induced alveolar cell apoptosis and emphysema (Chen et al., 2010). In this study, we found that CSE activated autophagy in cultured lung EC. However, markers for autophagy activation were not significantly altered in CS-exposed AKR mice with mild emphysema. Our data suggest that activation of autophagy may not be as important in early stage of emphysema.

Materials and Methods

Cells and Reagents

Bovine pulmonary artery endothelial cells (PAEC) and rat lung microvascular endothelial cells (LMVEC) were purchased from VEC Technologies Inc. (Rensselaer, NY) and used between passages 3-8. The bovine PAEC were propagated in MEM medium containing 10% fetal bovine serum (FBS) and sodium pyruvate. The rat LMVEC were propagated in MCDB-131 complete media. Antibodies directed against phospho-eIF2 α , caspase-3, Bax, and LC3B were purchased from Cell Signaling Technology, Inc (Danvers, MA). Antibodies directed against GRP94 and eIF2 α were purchased from Assay Designs (Ann Arbor, MI) and Biolegend (San Diego, CA), respectively. N-acetylcysteine (NAC), PF-573228, and antibodies directed against GRP78 and vinculin were purchased from Sigma Aldrich (St. Louis, MO). Antibody directed against caspase-9 was purchased from Stressgen (San Diego, CA). Antibodies directed against Bcl-2 and Von Willebrand factor (vWF) were purchased from BD Biosciences (San Jose, CA) and DAKO (Carpinteria, CA), respectively. Antibodies directed against Beclin-1, p62, actin, and GAPDH were from Santa Cruz (Santa Cruz, CA). DAPI (Prolong Gold anti-fade reagent with DAPI) were from Invitrogen (Grand Island, NY). ApopTag peroxidase *In Situ* Apoptosis Detection Kit (TUNEL kit) was purchased from EMD Millipore (Billerica, MA). DAB and VIP peroxidase substrates kits were from Vector Lab (Burlingame, CA). pEGFP-C1 construct was purchased from Clontech and GFP-FAK construct was a generous gift from Dr. Caroline Damsky (UCSF, CA).

CS Exposure of Mice

All animal protocols were approved by the Providence VA Medical Center Institutional Animal Care and Use Committee and complied with the Health Research Extension Act and the PHS policy. Male C57BL/6 and AKR mice at age of 6-8 weeks were purchased from Jackson Laboratory. Mice were exposed to room air (RA) or CS for 3 weeks at 6 hours per day and 4 days per week using a TE-10 mouse smoking machine (Teague Enterprises, Woodland, CA) and 3R4F reference cigarettes (University of Kentucky, Tobacco Research Institute, Lexington, KY), as we described previously (Lu et al., 2011). The smoke machine was adjusted to produce a mixture of sidestream (89%) and mainstream (11%) smoke. The smoking chamber atmosphere was monitored for total suspended particles at concentration of 120 mg/m³.

Assessment of Lung Endothelial Apoptosis in Lung Tissue

After assessment of lung mechanics by the FlexiVent system (SCIREQ, Montreal, Canada), lung tissue was fixed with 10% formalin at 25 cm of H₂O pressure. Some lung tissue was used to assess mean linear intercept (MLI). Other lung tissue was used to assess lung EC apoptosis by immunohistochemical analysis of TUNEL staining of apoptotic cells, co-stained with the EC marker, von Willebrand factor (vWF). The total numbers of apoptotic EC, as indicated by dual positive staining of TUNEL and vWF in the entire lung slide, except blood vessels > 500 µm in diameter and the areas of the large airway, were counted and quantitated by an observer blinded to the source of the lungs using Aperio ScanScope (Vista, CA), a bright field whole slide scanner. The data are presented as the number of apoptotic EC per cm² lung section area (TUNEL⁺/vWF⁺ cells/cm²).

Assessment of Bronchoalveolar Lavage (BAL) Cell Count

As we described previously (Lu et al., 2011), mice were euthanized using an overdose of Pentobarbital (120 mg/kg) through i.p injection. An incision was made in the trachea and the lung was lavaged with 600 µl of saline by using 1 ml syringe. The total numbers of white blood cells in the BAL fluid were counted by phase microscopy at 40X.

Preparation of Cigarette Smoke Extract (CSE)

As we described previously (Lu et al., 2011), mainstream smoke from Kentucky reference cigarettes (3R4F) was drawn into 30 ml of pre-warmed PBS by a vacuum. Each cigarette was lit for 5 min, and five cigarettes were used per 30 ml of PBS to generate a CSE-PBS solution. Control solution (sham PBS) was prepared with the same protocol used to generate CSE, except that the cigarettes were unlit. The CSE was diluted with medium and used immediately. Final concentrations of CSE are expressed as percent values (the ratio of volume of CSE-PBS solution to the volume of the final total medium used to treat the cells).

Assessment of Apoptosis in Cultured Lung EC

EC apoptosis was assessed by assaying caspase-3 activation by enzyme activity assay and by immunoblot analysis of the cleaved (active) form of caspase-3 and by nuclear morphological visualization of apoptotic nuclei using fluorescence microscopy after DAPI staining, as we previously described (Lu et al., 2009).

Transfection and Fluorescence Microscopy

As we previously described (Lu et al., 2006), PAEC at 70% confluence were transfected with cDNA encoding GFP (pEGFP-C1) or GFP-FAK, using Lipofectamine™ 2000 transfection reagent. Cells were then incubated with growth medium for 24h before treatment with vehicle or CSE for varying times. Apoptosis was assessed by DAPI staining of apoptotic nuclear. Apoptosis of transfected cells was analyzed by fluorescence microscopy and expressed as the ratio of apoptotic GFP- or GFP-FAK- over-expressing cells to the total counted GFP-or GFP-FAK-over-expressing cells.

Gel Electrophoresis and Immunoblot Analysis

Lysates were solubilized in Laemmli buffer, and proteins were resolved using SDS-PAGE, and then transferred to PVDF membranes and immunoblotted with specific antibodies, as we previously described (Lu et al., 2004).

Data Analysis

For *in vitro* culture studies, all experiments were performed at least in triplicate. Means and standard errors (SE) were calculated based on the values of individual treatments and the numbers of the experiments performed. For animal studies, 3-4 mice per group were used. Means and SE were calculated based on the values of each animal in each group and the numbers of animals used in that group. Data are presented as mean $\pm$ SE. The difference between two means were assessed using Student's T test and the differences among three or more means were assessed using ANOVA followed by Tukey-Kramer post-hoc test. Differences among means are considered significant when $p < 0.05$.

Results

CS-induced emphysema is associated with increased lung EC apoptosis

Lung EC apoptosis has been implicated in CS-induced emphysema (Giordano et al., 2008; Kasahara et al., 2001; Kasahara et al., 2000), among other mechanisms. Humans differ in susceptibility to CS-induced emphysema. Similarly, different strains of mice also differ in susceptibility to CS-induced emphysema (Guerassimov et al., 2004). Compared to C57BL/6 mice which are widely used in research, AKR mice are highly susceptible to CS-induced emphysema, as determined by increased mean linear intercept (Lm), enhanced elastance, elevated Th1 cytokines, and significant inflammation in their lungs after 6 months of CS exposure (Guerassimov et al., 2004). In contrast, C57BL/6 mice only displayed increased Lm without detectable changes in elastance, inflammation, or cytokine profile after exposure to the same period and amount of CS (Guerassimov et al., 2004). We have recently shown that a relatively shorter period of time (3 weeks) of CS exposure causes mild emphysema in AKR mice, but not in C57BL/6 mice, as indicated by a significant increase in lung static compliance and a trend of increase in lung mean linear intercept in AKR mice (Lu et al., 2013). In this study, we investigated if there is a correlation between susceptibility to lung EC apoptosis and susceptibility to emphysema upon CS exposure by comparing lung EC apoptosis and development of emphysema in these two strains of mice. We found that 3 weeks of CS exposure increased BAL inflammatory cells (Fig. 1A) and dramatically elevated lung EC apoptosis (Fig. 1B) in AKR mice, but not in C57BL/6 mice; effects that were associated with early onset of emphysema (Lu et al., 2013).

CSE caused lung macro- and microvascular endothelial cell apoptosis

CSE has been shown to cause pulmonary EC apoptosis (Damico et al., 2011; Tuder et al., 2000). Consistently, we noted that both PAEC and LMVEC exposed to CSE for 6-24h displayed a significant increase in apoptosis, as indicated by caspase-3 cleavage (Fig. 2A) and caspase-3 activity (Fig. 2B). To further elucidate the signaling pathway of apoptosis upon CSE exposure, we examined caspase-9 activation and changes in Bcl-2 family

proteins. We found that CSE decreased pro-caspase-9 levels 24h after CSE exposure (Fig. 2C), suggesting activation of caspase-9. There was no significant change found in the levels of the anti-apoptotic Bcl-2 and the pro-apoptotic Bax (Fig. 2C).

FAK inhibition contributed to CS-induced lung EC apoptosis and emphysema

FAK signaling is important in maintenance of cell survival (Lu and Rounds, 2012). We have previously shown that CSE decreases FAK phosphorylation at tyrosine 397, a modification critical for tyrosine kinase activity of FAK, via oxidative stress (Lu et al., 2011). In this study, we found that the apoptosis-inducing effect of CSE was prevented by the antioxidant, N-acetylcysteine (Fig. 3A-B). To assess the contribution of decreased FAK activity to CSE-induced lung EC apoptosis, we tested if over-expression of FAK could rescue CSE-induced lung EC apoptosis. 24h after transfection, GFP-FAK and GFP (control) were over-expressed in PAEC (Fig. 3C). Furthermore, GFP-FAK over-expressed in PAEC was constitutively auto-phosphorylated at tyrosine-397 (Fig. 3D). Similar to its effect on untransfected cells, CSE significantly increased apoptosis of PAEC over-expressing GFP, however, this apoptosis-inducing effect of CSE was dramatically attenuated in PAEC over-expressing constitutively active GFP-FAK (Fig. 3E-F). On the other hand, inhibition of FAK activity by PF-573228 significantly elevated caspase-3 cleavage (Fig. 3G), suggesting an increase in apoptosis. These results indicate that oxidative stress-mediated FAK inhibition contributes to CSE-induced lung EC apoptosis.

We then examined FAK phosphorylation at tyrosine 397 in the lungs of mice exposed to CS and noted that FAK activation was significantly reduced in the lungs of AKR mice exposed to CS for three weeks (Fig. 3H); an effect that was associated with lung EC apoptosis and early emphysema. Decreased FAK phosphorylation did not occur in C57BL/6 mice exposed to CS for three weeks (Fig. 3H). Our results suggest that FAK inhibition is implicated in CS-induced lung EC apoptosis and onset of early emphysema.

eIF2 α was transiently activated by CSE but impaired in CS-induced emphysematous lungs

CSE has been shown to activate UPR in cultured human bronchial epithelial cells and 3T3 cells (Hengstermann and Müller, 2008; Jorgensen et al., 2008). Similarly, we examined the effect of CSE on activation of UPR in lung EC. PAEC exposed to 5-20% CSE for 4h dramatically enhanced the levels of phosphorylated eIF2 α (Fig. 4A). Increased eIF2 α phosphorylation occurred between 15min and 4h after exposure to 10% CSE (Fig. 4B). Activation of UPR was also indicated by induction of ER molecular chaperone, glucose-regulated proteins (GRP)-78, in PAEC exposed to CSE for 4-24h (Fig. 4C). However, CSE did not alter protein levels of GRP94, another ER resident molecular chaperone (Fig. 4C).

UPR is activated in the lungs of chronic smokers without evidence of COPD (Kelsen et al., 2008). We assessed eIF2 α phosphorylation in the lungs of mice exposed to CS and found that three weeks of CS exposure dramatically reduced both the total and phosphorylated eIF2 α , with more severe reduction of phosphorylated eIF2 α , in the emphysematous lungs of AKR mice, but not of C57BL/6 mice which did not have evidence of emphysema (Fig. 4D-E).

Autophagy was activated by CSE but was not significantly altered in mild emphysematous lungs

CSE has been shown to cause autophagic cell death in HUVEC (Csordas et al., 2011). We assessed the effect of CSE on autophagy in cultured human PAEC, using starvation as a positive control. We found that similar to starvation, CSE increased conversion of LC3B-I to LC3B-II (Fig. 5A), a marker of autophagy activation. Increased autophagy in lungs correlates positively with clinical severity of COPD in patients (Chen et al., 2008). We assessed activation of autophagy by evaluating up-regulation of Beclin-1 and LC3B-II and down-regulation of p62 in the lungs of mice exposed to CS for three weeks. We found that Beclin-1 (Fig. 5B), LC3B-II (Fig. 5C), and p62 (Fig. 5D) were not significantly altered in the lungs of either AKR mice with mild emphysema or C57BL/6 mice without evidence of emphysema.

Discussion

Lung EC apoptosis has been implicated in the development of emphysema (Giordano et al., 2008; Kasahara et al., 2001; Kasahara et al., 2000). In this study, we show that AKR mice, which are more susceptible to CS-induced emphysema (Guerassimov et al., 2004), were more susceptible to CS-induced lung EC apoptosis. Inhibition of FAK has recently been shown to cause emphysema-like changes in rat lungs (Mizuno et al., 2012). Here we show that FAK activity was decreased in the lungs of CS-exposed AKR mice, which had mild emphysema and increased lung EC apoptosis. We also show that over-expression of FAK prevented CSE-induced lung EC apoptosis, whereas inhibition of FAK caused lung EC apoptosis. These results suggest that FAK inhibition contributes to CSE-induced lung EC apoptosis. We noted that CSE activated UPR and autophagy in cultured lung EC. However, both total and phosphorylated eIF2 α were significantly reduced in the lungs of CS-exposed AKR mice with mild emphysema, suggesting that impairment of eIF2 α signaling may play a role in increased susceptibility to CS-induced emphysema.

CS extract (CSE) is an aqueous extract of gaseous CS. CSE exposure is a widely used and acceptable *in vitro* system to determine the mechanisms of CS effect on specific cell types. Our data indicate that CSE caused cultured lung macro- and microvascular EC apoptosis *in vitro*. Consistently, CS also resulted in lung EC apoptosis *in vivo* in highly susceptible AKR mice. However, CS did not induce lung EC apoptosis in less susceptible C57BL/6 mice. This discrepancy between CSE effect on cultured lung EC *in vitro* and CS effect on lung EC of C57BL/6 mice *in vivo* may reflect insensitivity of lung EC of C57BL/6 mice to CS exposure. It may also be due to differences in exposure time and concentrations of CS components between smoke inhalation by intact animals and CSE treatment of cultured cells. In other words, lung EC in mice may have been exposed to lower concentrations of inhaled CS components than cultured EC directly exposed to CSE, resulting in no measurable increased apoptosis in less susceptible intact C57BL/6 mice. In future studies, we will investigate the different susceptibility of lung EC from AKR mice and C57BL/6 mice to CS-induce apoptosis *ex vivo* by isolating lung microvascular EC from these two strains of mice and comparing their susceptibility to CSE-induced apoptosis.

UPR is activated in response to ER stress and activation of the UPR can restore ER function by blocking ER client protein loading, enhancing ER protein folding capacity, and promoting protein degradation thereby enhancing cell survival (Schroder and Kaufman, 2005). It has been shown that CSE activated UPR in cultured human bronchial epithelial cells and 3T3 cells (Hengstermann and Müller, 2008; Jorgensen et al., 2008). UPR is also activated in the lungs of smokers who do not show signs of COPD (Kelsen et al., 2008). However, it is not known whether UPR is altered in emphysematous lungs. In this study, we show that, similar to the effect of CSE on cultured epithelial cells (Hengstermann and Müller, 2008; Jorgensen et al., 2008), CSE transiently increased eIF2 α phosphorylation and increased GRP78 expression in cultured lung EC, indicating activation of at least one branch of UPR. However, prolonged (three weeks) CS exposure decreased UPR, as indicated by reduced levels of both total and phosphorylated eIF2 α in AKR mouse lungs; this effect was associated with lung EC apoptosis and the development of emphysema. In contrast, there was no reduction in eIF2 α phosphorylation in the lungs of C57BL/6 mice exposed to CS for three weeks; this effect was associated with normal lung function without evidence of EC apoptosis. These results demonstrate an association between impairment of eIF2 α signaling and the development of lung EC apoptosis and emphysema. We speculate that CS-induced UPR activation is a compensatory mechanism to protect or limit lung EC injury, rather than increasing injury. Therefore, decreased UPR signaling, particularly reduced eIF2 α in AKR mice after three weeks of CS exposure, may increase the susceptibility to emphysema, as depicted in Figure 6. Activation of eIF2 α signaling may protect against the development of emphysema via Nrf2-mediated production of antioxidants (Iizuka et al., 2005). Future studies are necessary to determine if decreased eIF2 α signaling contributes to CS-induced emphysema due to insufficient production of antioxidants.

Similar to GRP78, GRP94 is another important ER-resident molecular chaperone (Schroder and Kaufman, 2005). Over-expression of GRP94 blunts ER stress-induced apoptosis of neuronal cells (Bando et al., 2004), whereas suppression of GRP94 accelerates ER stress-induced apoptosis (Bando et al., 2004), suggesting that GRP94 protects against ER stress-induced apoptosis. We have previously shown that GRP94 protected against pulmonary EC apoptosis (Lu et al., 2007). To our surprise, CSE did not alter GRP94 protein levels. We do not yet know whether lack of up-regulation of GRP94 contributes to CSE-induced lung EC apoptosis.

FAK provides survival signaling for anchorage-dependent cells. Endothelial cells isolated from FAK-null embryos undergo apoptosis (Ilic et al., 1995). Endothelium-specific deletion of FAK (Cre/FAKflox) causes embryonic lethal and endothelial cell apoptosis (Braren et al., 2006). These results indicate that FAK is critical for EC survival (Lu and Rounds, 2012). Tyrosine 397 has been identified as the major site of FAK auto-phosphorylation (Schaller et al., 1994). In this manuscript we show that inhibition of FAK tyrosine 397 phosphorylation by PF-573228 significantly elevated caspase-3 cleavage (Fig. 3G); this effect was similar to that of CSE exposure (Fig. 2A, 2C). We have previously shown that CSE decreased FAK tyrosine-397 phosphorylation (Lu et al., 2011). In this study, we show that CSE-induced endothelial apoptosis was prevented by over-expression of tyrosine-397 auto-phosphorylated GFP-FAK (Fig. 3C-F). These data indicate that decreased FAK tyrosine-397 phosphorylation plays a role in CSE-induced endothelial apoptosis. Inhibition of FAK

causes emphysema-like change in rat lungs (Mizuno et al., 2012). In this manuscript, we show that FAK activity was decreased in AKR mice exposed to CS for three weeks; effect associated with increased lung EC apoptosis and early emphysema. Taken together, these findings suggest that CS-induced FAK inhibition contributes to lung EC apoptosis and possibly the development of emphysema. Further studies are necessary to address whether reduced FAK activity contributes to CS-induced lung EC apoptosis and emphysema *in vivo*. FAK promotes cell survival via suppression of p53 (Lu and Rounds, 2012). It has been shown that CSE causes pulmonary EC apoptosis via activation of p53 (Damico et al., 2011). We have previously shown that CSE causes lung EC apoptosis via oxidative stress-induced inhibition of FAK (Lu et al., 2011). Thus, we speculate that CSE causes lung EC apoptosis via oxidative stress-mediated inhibition of FAK and subsequent activation of p53, as depicted in Figure 6.

Autophagy is activated in emphysematous human lungs (Chen et al., 2008) and in bronchial epithelial cells exposed to CSE (Kim et al., 2008). Activation of autophagy has been considered as a mechanism of cellular protection against the environmental stress. However, studies using LC3 knockout mice showed that activation of autophagy contributes to the pathogenesis of emphysema (Chen et al., 2010). Our data indicate that autophagy was activated between 1-6h of exposure to CSE and then suppressed at 24h. In contrast to *in vitro* results, we noted that autophagy was not significantly increased in the early emphysematous lungs of AKR mice exposed to CS for three weeks. Additionally, autophagy markers were also not altered in C57BL/6 mice exposed to CS for three weeks when normal lung function was maintained. It has been reported that activation of autophagy is positively correlated with clinical severity of human COPD (Chen et al., 2008). We speculate that autophagy may not be as important in the early stages of emphysema.

In summary, our results demonstrate that lung EC apoptosis is increased in AKR mice correlating temporally with early emphysematous changes. FAK and eIF2 α were inhibited in the lungs of susceptible AKR mice when early emphysema was evident upon CS exposure. Our data suggest that decreased eIF2 α and FAK signaling contributes to CS-induced lung EC apoptosis and early onset of emphysema. Our results also provide further support for the contribution of FAK inhibition and lung EC apoptosis to emphysema development.

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Highlights

- Cigarette smoking (3 weeks) caused lung EC apoptosis and mild emphysema in AKR mice.
- Cigarette smoking (3 weeks) decreased FAK activity in lungs of AKR mice.
- Overexpression of FAK prevented CS extract-induced lung EC apoptosis.
- Lung eIF2 α signaling was reduced in AKR mice exposed to smoking for 3 weeks.
- Decreased eIF2 α and FAK associated with smoking-induced EC apoptosis and emphysema.

Figure 1. Effects of three weeks of CS exposure on lung inflammation and EC apoptosis *in vivo* 6-8 week old male AKR mice and C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 3 weeks at 6 h/d, 4 d/wk, using a TE-10 mouse smoking machine and 3R4F reference cigarettes. Smoking chamber atmosphere was monitored for total suspended particles at 120 mg/m³, which was a mixture of 89% sidestream smoke and 11% mainstream smoke. By the end of CS exposure, static lung compliance (Cst) and lung mean linear intercept (MLI) were assessed (Lu et al., 2013). Lung inflammation was assessed by measuring BAL cell count (panel A). Lung EC apoptosis was assessed by TUNEL co-stained with EC marker, von Willebrand factor (vWF) (panel B). The total number of apoptotic EC was counted by a blinded observer and quantitated using Aperio ScanScope

system. The data is presented as numbers of apoptotic EC (dual positive staining for TUNEL and vWF) per cm² lung tissue section area (TUNEL⁺/vWF⁺ cells/cm²). 3-4 mice per group were used for each panel. * p<0.05 vs. RA exposure. NS: not significant.

A **B**

Figure 2. Effect of CSE on lung EC apoptosis *in vitro*

Panel A: Bovine PAEC were treated with vehicle (V, 10% PBS) or 10% CSE for indicated times and EC apoptosis was assessed by pro-caspase-3 cleavage. Vinculin was used as a protein loading control. Panel B: Rat LMVEC were treated with vehicle (V, 10% PBS) or 10% CSE for 24h and EC apoptosis was assessed by caspase-3 activity. Data are presented as means $\pm$ SE, * $p < 0.05$ v.s. vehicle-treated EC, $n = 3$; Panel C: Bovine PAEC were treated with vehicle (V, 10% PBS) or 10% CSE for indicated times, and cleavage of pro-caspase-3 and pro-caspase-9, protein levels of Bcl-2 family proteins (Bax and Bcl-2) were assessed by immunoblot analysis. Actin was used as a protein loading control. Representative images from three independent experiments for each panel are shown in panels A and C.

A **B**

C **D**

G **H**

Figure 3. Role of oxidative stress and FAK in CS-induced lung EC apoptosis and emphysema
 Panels A-B: Bovine PAEC were pre-incubated with vehicle or 25mM N-acetylcysteine (NAC) for 1h and then exposed to vehicle (V, 10% PBS) or 10% CSE in the absence or presence of 25mM NAC for 24h. Apoptosis was examined by caspase-3 activity (panel A) and by DAPI staining of condensed apoptotic nuclei (panel B). Data are expressed as the ratio of apoptotic cells to the total counted cells $\times 100$ (%). 600 cells in three high power fields for each group in each independent experiment for each panel were analyzed by a blinded observer. Total three independent experiments were performed and included for data

analysis for each panel. Data are presented as means $\pm$ SE, * $p<0.05$ v.s. vehicle-treated EC; ξ $p<0.05$ v.s. CSE-treated EC. Panels C-D: Bovine PAEC were transfected with cDNA encoding for GFP or GFP-conjugated FAK (GFP-FAK) for 24h. Expression of GFP or GFP-FAK in transfected cells was examined by fluorescence and phase-contrast microscopy (panel C). Cells with green demonstrate over-expression of GFP or GFP-FAK. Over-expression of GFP and GFP-FAK was also assessed by immunoblot analysis using antibodies directed against GFP and FAK, respectively (panel D). Phosphorylation of tyrosine-397 in over-expressed GFP-FAK was assessed by immunoblot analysis using antibody directed against phosphorylated FAK-Y397 (panel D). Actin was used as a protein loading control. Panels E-F: Bovine PAEC were transfected with cDNA encoding for GFP or GFP-FAK for 24h and then exposed to vehicle (10% PBS) or 10% CSE for 4h. Apoptosis was assessed by DAPI staining of condensed apoptotic nuclei. Representative images from three independent experiments are shown (panel E). Panel F: Apoptosis was quantitated as the ratio (x 100 %) of the numbers of apoptotic cells over-expressing GFP or GFP-FAK to the numbers of the total counted cells over-expressing GFP or GFP-FAK. Data are presented as means $\pm$ SE. * $p<0.05$ v.s. vehicle-treated EC over-expressing GFP; ξ $p<0.05$ v.s. CSE-treated EC over-expressing GFP. Panel G: Bovine PAEC were treated with vehicle (DMSO) or 20 μ M of FAK inhibitor, PF-573228 for 24h and EC apoptosis was assessed by pro-caspase-3 cleavage. Inhibition of FAK phosphorylation at tyrosine-397 and FAK protein level were also assessed by immunoblot analysis. Actin was used as a protein loading control. Panel H: 6-8 week old male AKR mice and C57BL/6 mice were exposed to RA or CS for 3 weeks at 6 h/d, 4 d/wk, as described in Figure 1 legend. Lung homogenates were collected for determination of FAK activity by assessing FAK phosphorylation at tyrosine 397. Ponceau S staining was used to examine protein loading. Densitometry was performed on immunoblots and the data are presented as the ratio of the densitometry of phosphorylated FAK at tyrosine 397 to the densitometry of protein loading control (Ponceau S staining of the blots). Actin was also used as a protein loading control. 3-4 mice per group were used. * $p<0.05$ vs. AKR mice exposed to RA.

A **B**

C **D**

E

Figure 4. Effects of CS on UPR *in vitro* and *in vivo*

Panels A-C: Bovine PAEC were exposed to vehicle (V, 20% PBS) or varying doses of CSE (5, 10, and 20%) for 4h (panel A), or exposed to vehicle (V, 10% PBS) or 10% CSE (S) for indicated times (panels B, C). The phosphorylated eIF2α (eIF2α~P), GRP78, and GRP94 were assessed by immunoblot analysis. GAPDH and HSP90 were used as protein loading controls for panel A and C, respectively. Ponceau S staining was used to examine protein loading control for panel B. Panels D-E: 6-8 week old male AKR mice and C57BL/6 mice were exposed to RA or CS for 3 weeks at 6 h/d, 4 d/wk, as described in Figure 1 legend. Lung homogenates were collected for evaluation of UPR activation by assessing eIF2α phosphorylation and total eIF2α by immunoblot analysis using vinculin as a protein loading control (panel D). Densitometry was performed on immunoblots and the data are presented as the ratio of the densitometry of phosphorylated eIF2α or total eIF2α to the densitometry of vinculin, as well as the ratio of the densitometry of phosphorylated eIF2α to total eIF2α (panel E). 3-4 mice per group were used. * $p < 0.05$ vs. AKR mice exposed to RA.

A **B**

Figure 5. Effects of CS on autophagy *in vitro* and *in vivo*

Panel A: human PAEC were exposed to vehicle (V, 10% PBS) or 10% CSE (S) for indicated times. Untreated (UT) human PAEC and human PAEC exposed to 100% PBS (starvation) for 15 minutes were used as negative and positive controls, respectively. Autophagy was assessed by measuring conversion of LC3B-I to LC3B-II by immunoblot analysis. Ponceau S staining was used to examine protein loading. The immunoblots are representative of three independent experiments. Panels B-D: 6-8 week old male AKR mice and C57BL/6 mice were exposed to RA or CS for 3 weeks at 6 h/d, 4 d/wk, as described in Figure 1 legend.

Lung homogenates were collected for evaluation of activation of autophagy markers by assessing expression of Beclin-1, LC3B-II, and p62 by immunoblot analysis, using vinculin or actin as a protein loading control. Densitometry was performed on immunoblots and the data are presented as the ratio of the densitometry of proteins interested to the densitometry of protein loading control. 3-4 mice per group were used.* $p<0.05$ vs. AKR mice exposed to RA.

Figure 6. Proposed model of CS-induced lung EC apoptosis and early emphysema

CS: cigarette smoke; CSE: cigarette smoke extract; EC: endothelial cells; FAK: focal adhesion kinase; UPR: unfolded protein response; p-eIF2α: phosphorylated eIF2α.