ORIGINAL RESEARCH

Mitochondrial Fission Mediated Cigarette Smoke–induced Pulmonary Endothelial Injury

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Abstract

Cigarette smoke (CS) exposure increases the risk for acute respiratory distress syndrome in humans and promotes alveolar–capillary barrier permeability and acute lung injury in animal models. However, the underlying mechanisms are not well understood. Mitochondrial fusion and fission are essential for mitochondrial homeostasis in health and disease. In this study, we hypothesized that CS caused endothelial injury via an imbalance of mitochondrial fusion and fission and resultant mitochondrial oxidative stress and dysfunction. We noted that CS altered mitochondrial morphology by shortening mitochondrial networks and causing perinuclear accumulation of damaged mitochondria in primary rat lung microvascular endothelial cells. We also found that CS increased mitochondrial fission likely by decreasing Drp1-S637 and increasing FIS1, Drp1-S616 phosphorylation, mitochondrial translocation,

and tetramerization and reduced mitochondrial fusion likely by decreasing Mfn2 in lung microvascular endothelial cells and mouse lungs. CS also caused aberrant mitophagy, increased mitochondrial oxidative stress, and reduced mitochondrial respiration. An inhibitor of mitochondrial fission and a mitochondria-specific antioxidant prevented CS-induced increased endothelial barrier dysfunction and apoptosis. Our data suggest that excessive mitochondrial fission and resultant oxidative stress are essential mediators of CS-induced endothelial injury and that inhibition of mitochondrial fission and mitochondria-specific antioxidants may be useful therapeutic strategies for CS-induced endothelial injury and associated pulmonary diseases.

Keywords: cigarette smoke; endothelial permeability; mitochondrial fission/fusion; mitophagy; apoptosis

Approximately 1.1 billion of the world's population are active smokers (1). In addition to causing a variety of chronic diseases, cigarette smoke (CS) also increases the incidence of acute respiratory distress syndrome (ARDS) in critically ill patients (2–7) and exacerbates acute lung injury in animal models (8–10). Smokers' lungs may be edematous because explanted lungs from current smokers were heavier than lungs from former or never-smokers (11). Why smokers have increased risk for lung edema and ARDS is not well understood.

Increased lung endothelial barrier permeability and apoptosis are critical components of pathogenesis of ARDS. CS exposure promotes alveolar–capillary permeability (8–10, 12, 13) and lung endothelial apoptosis (14, 15) in human and animals. *In vitro* studies have also shown that CS extract (CSE) increases pulmonary endothelial permeability (8–10, 16, 17) and apoptosis (15, 18, 19) via mechanisms involving oxidative stress (9, 15). Mitochondria are highly susceptible to oxidative injury. Circulating mitochondrial

DNA (mtDNA) has been shown to mediate increased vascular permeability (20). Thus, we hypothesized that CS caused lung microvascular endothelial cell (LMVEC) injury by mitochondrial oxidative stress–mediated mitochondrial dysfunction.

Mitochondrial morphology and health are maintained by the dynamic opposing forces of mitochondrial fusion and fission (21). Mitochondrial fusion enables dilution of potentially injurious content, whereas mitochondrial fission allows segregation and subsequent degradation of damaged

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Clinical Relevance

Previous studies from humans and animal models have demonstrated that cigarette smoke exposure increases the risk for acute respiratory distress syndrome. However, the underlying mechanisms are not well understood. In this study, we showed that cigarette smoke exposure caused lung vascular inner-wall cell injury via imbalance of mitochondrial fusion and fission (fragmentation) and resultant mitochondrial dysfunction. Our data also suggest that the inhibition of mitochondrial fragmentation and mitochondria-specific antioxidants may be useful therapeutic strategies for cigarette smoke-induced lung vascular injury and associated lung diseases.

depolarized mitochondria. Dynamins are large, ubiquitous GTPases that control the dynamics of fission and fusion. Drp 1 (dynamin-related protein 1) is a cytosolic dynamin that upon activation, is translocated to fission sites, where it binds to several outer mitochondrial membrane adaptors, including mitochondrial FIS1 (fission 1 protein), to form fission rings that constrict and scissor the outer and inner mitochondrial membranes. Mitochondrial fusion is controlled by several other GTPases, including Mfn 1 and 2 (mitofusin 1 and 2), which are located at outer mitochondrial membrane. Mice deficient in Drp1 (22) or Mfn1 or Mfn2 (23) are embryonic lethal. Imbalance of mitochondrial fusion and fission has been implicated in a variety of human diseases (24). CS has been shown to increase mitochondrial fission in airway smooth muscle cells (25) and airway epithelial cells (26); little is known about its effects on lung endothelial cells. More importantly, the mechanism by which CS increases mitochondrial fission is not well understood.

Damaged and fragmented mitochondria are eliminated by mitophagy (27). Mitophagy is initiated by increased concentrations of phosphatase and tensin homolog (PTEN)-induced PINK1 (putative protein kinase 1) in the outer mitochondrial membrane and the subsequent recruitment of Parkin, an E3 ubiquitin ligase, to the damaged mitochondria (28). Functional mitophagy is essential for

mitochondria homeostasis, and impaired mitophagy promotes mitochondrial oxidative stress (28). Whether mitophagy and mitochondrial oxidative stress play a pivotal role in CS-induced lung endothelial injury is unknown.

In this study, we show that CS altered mitochondrial morphology by shortening mitochondrial networks and causing perinuclear accumulation of damaged mitochondria in LMVECs. CS increased mitochondrial fission, likely by increasing FIS1, Drp1 phosphorylation at serine-616 (Drp1-pS616), and the ratio of Drp1-pS616:Drp1 phosphorylation at serine-637 (Drp1-pS637); decreasing Drp1-pS637 and Mfn2; and promoting Drp1-pS616 mitochondrial translocation and tetramerization in LMVECs and mouse lungs. CSE also caused aberrant mitophagy, mitochondrial oxidative stress, and mitochondrial dysfunction. Inhibition of mitochondrial fission and a mitochondria-specific antioxidant prevented CSE-induced increased endothelial permeability and apoptosis. Our data suggest that imbalance of mitochondrial fusion and fission and resultant oxidative stress are essential mediators of CS-induced endothelial injury. Thus, our data provide a better understanding of the molecular mechanism by which CS causes mitochondrial dysfunction. Our results also suggest that the inhibition of mitochondrial fission and mitochondria-specific antioxidants may be useful therapeutic strategies for CS-associated pulmonary endothelial injury and associated diseases.

Methods

Cells and Reagents

Rat LMVECs were purchased from VEC Technology and used between passages 2 and 7. MitoTEMPO (mitochondria-targeted antioxidant) and antibodies directed against PINK1, Parkin, FIS1, Drp1, HSP60, Mfn2, and β -actin were purchased from Santa Cruz Biotechnology. Antibodies directed against Tom20, Drp1-pS616, Drp1-pS637, and caspase-3 were purchased from Cell Signaling Technology. Anti- β -catenin antibody was from BD Biosciences. Hoechst 33342, mitoTracker Red kit, mitoSOX Red kit, and Alexa Fluor 405-conjugated second antibody were purchased from Thermo Fisher Scientific. Mdivi-1 was purchased from Enzo Life

Sciences. The MTT assay kit (a colorimetric assay for assessing cell metabolic activity) was purchased from R&D Systems. ECIS (Electric Cell-substrate Impedance Sensing) arrays were from Applied Biophysics. Oligomycin A, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), and a mixture of antimycin A and rotenone were from Seahorse Bioscience. Other chemicals were purchased from Sigma Aldrich unless stated otherwise.

Mice

All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the Providence Veterans Affairs Medical Center and complied with the Public Health Service policy. Male, 8-week-old AKR mice were purchased from the Jackson Laboratory. All mice were housed at the animal facility at the Providence Veterans Affairs Medical Center in standard conditions (12 h:12 h light:dark cycle, 68–72° F, and a humidity of 30–70%) in ventilated racks fitted with automatic watering systems and were fed with standard chow *ad libitum*.

CS Exposure of Mice

Mice designated in the CS exposure group were temporally transported into standard shoebox cages with water and standard chow *ad libitum*. Mice were exposed to CS for 6 hours per day, 4 days per week for up to 6 weeks using a TE-10 mouse smoking machine (Teague Enterprises) and 3R4F reference cigarettes (University of Kentucky, Tobacco Research Institute), as we previously described (10). Mice housed on a shelf near the smoking machine were used as room air control animals.

CSE Exposure of Cultured Cells

CSE was prepared as previously described (9). Briefly, each 3R4F reference cigarette was lit for 5 minutes, and the smoke from five cigarettes was drawn into 30 ml PBS solution by a vacuum. This solution was referred to as 100% CSE. The control solution (sham PBS) was prepared using the same method, except that the cigarettes were unlit. The 100% CSE or PBS were diluted with culture medium and used immediately.

Fluorescence Microscopy

LMVECs were grown to 100% confluency on gelatin-coated cover slides and then

Figure 1. Effects of cigarette smoke extract (CSE) on mitochondrial morphology of primary lung microvascular endothelial cells. Rat lung microvascular endothelial cells were treated with 10% CSE for varying times (30 min to 3 h) (A and B) or with varying concentrations (1–10%) of CSE for 3 hours (C and D). Cells treated with 10% PBS (vehicle) for 3 hours were used as control cells. Mitochondrial morphology was assessed by fluorescence microscopy using mitoTracker Red (A and D) or by immunofluorescence microscopy using antibodies directed against Tom20 (translocase of outer membrane 20) (B) or HSP60 (heat shock protein 60) (C). β -catenin (catenin β 1) was used to indicate the cell border. Representative images from three independent experiments are shown. The images outlined in yellow are shown in middle panels with magnifications. Scale bars, 10 μ m. Ctrl=control.

treated as described in figure legends. The treated cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X-100. The slides were then stained with the primary antibodies of interest followed by species-specific fluorescence probe-conjugated secondary antibody. For mitoTracker and mitoSOX staining, treated cells were incubated with 100 nM of mitoTracker Red or 5 μ M of mitoSOX Red for 10 minutes at 37°C and then washed thoroughly before fixation with 4% paraformaldehyde. DAPI was used in slide-mounting solution to stain nuclei. Fluorescence images were captured by Nikon imaging systems at 100 $\times$ magnification.

Assessment of Mitochondrial Reactive Oxygen Species by Fluorescence Plate Reader

LMVECs were seeded on a black-walled 96-well plate and grown to 100% confluence and then treated as described in figure legends. The treated cells were incubated with 10 nM Hoechst 33342 and 5 μ M mitoSOX Red for 20 minutes at 37°C. The fluorescence signals of Hoechst 33342 and mitoSOX Red were captured by fluorescence plate reader at excitation/emission of 360 nm/460 nm and 510 nm/580 nm, respectively. The signals of mitoSOX Red in each well were adjusted by their Hoechst 33342 signals. The data are presented as the

ratio of mitoSOX signal in treated cells to that of untreated cells.

Assessment of Mitochondrial Reactive Oxygen Species by Flow Cytometry

Treated cells were incubated with 5 μ M mitoSOX Red for 20 minutes at 37°C and then trypsinized into a single-cell suspension, fixed with 4% paraformaldehyde, and stained with antibody directed against Drp1-pS616 followed by species-specific Alexa Fluor 405-conjugated second antibody. The fluorescence signals of mitoSOX Red and Drp1-pS616 were captured and quantified by flow cytometry.

Mitochondria and Cytosol Fractionation

As we previously described (29), treated LMVECs were scraped and harvested by centrifugation at 600 $\times$ g for 10 minutes. Cell pellets were washed and resuspended in buffer A (20 mM HEPES-potassium hydroxide [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonylfluoride) containing 250 mM sucrose and homogenized by 25 strokes. The homogenates were centrifuged twice at 750 $\times$ g for 5 minutes each to remove cell debris and nuclei. The supernatants were centrifuged again at 10,000 $\times$ g for 10 minutes at 4°C. The supernatants were collected as cytosolic fraction, and the pellets were collected as mitochondrial fraction. Mitochondrial fraction was lysed in radioimmunoprecipitation assay buffer and then subjected to IB.

IB

Cells were lysed in radioimmunoprecipitation assay buffer containing phosphatase inhibitor cocktail. To collect lung homogenates, mouse lung lobes were snap-frozen in liquid nitrogen and stored at -80°C until homogenization. The same lung lobes were homogenized in tissue homogenization buffer on ice 3 times for 30 seconds each time, with 15-second breaks. Cell lysates and lung homogenates were dissolved in standard 1 $\times$ Laemmli sample buffer (containing 0.1% β -mercaptoethanol). To detect Drp1 oligomers, cell lysates and lung homogenates were dissolved

Figure 1. (Continued).

in mildly reduced 1 × Laemmli sample buffer (containing 0.05% β-mercaptoethanol). Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The proteins of interest were detected by primary antibodies, which were recognized by horseradish peroxidase-conjugated or fluorophore (IRDye 800CW and IRDye 680RD)-conjugated, species-specific secondary antibodies. The horseradish peroxidase-dependent signals were detected by enhanced chemiluminescence substrate followed by chemiluminescent capture by X-ray and quantified by image J software. The fluorescence signals were captured and quantified by LiCOR Odyssey Imaging Systems.

Assessment of Endothelial Monolayer Permeability

Assessment of endothelial monolayer permeability was as we previously described (30).

Measurement of Cell Viability

Cell viability was assessed by MTT assay according to the recommendations of the manufacture.

Assessment of Mitochondrial Oxygen Consumption Rate

Oxygen consumption rate (OCR) was measured using the XF-96 Extracellular Flux Analyzer (Agilent Technologies) and MitoStress Test kit, as we previously described (31). Briefly, rat LMVECs (3.0×10^4 cells per well) were seeded on a Seahorse 96-well plate and treated as described in figure legends and then subjected to the measurement of OCR by the Seahorse mitochondrial analyzer. The viable cells were stained with the CyQUANT direct cell assay (ThermoFisher Scientific) and the signals were used to adjust for equal numbers of cells. Each experimental condition included three to four wells that were averaged as one experimental data point.

Data Analysis

The number of mice used in each experiment is indicated in the figure legends. All

experiments using cultured LMVECs were performed at least three independent times. Data are presented as mean ± SE. The difference between two means was assessed using unpaired Student's *t* test. The differences among three or more means were assessed using two-way ANOVA and Fisher's least significant difference *post hoc* test using Graphpad Prism program. Differences among means are considered statistically significant when $P < 0.05$.

Results

CSE Altered Mitochondrial Morphology in LMVECs

To evaluate the role of mitochondrial dysfunction in CS-induced endothelial injury, we first characterized effects of CSE on the mitochondrial morphology of primary LMVECs by conducting time-course (30'–3 h) and dose-response (1–10%) experiments. Mitochondrial morphology was visualized by fluorescence microscopy using multiple mitochondrial markers, including mitoTracker Red staining, Tom20 (an outer mitochondrial membrane protein), and HSP60 (a mitochondrial matrix protein). As expected, the control LMVECs, which were incubated with media containing 10% PBS (vehicle) for 3 hours, displayed tubular mitochondrial networks stretching from the perinuclear region to the periphery of cells (Figures 1A–1D). After CSE exposure, the tubular mitochondrial networks of LMVECs were progressively shortened in a time-dependent manner, and only perinuclear mitochondria remained after 3 hours of exposure to 10% CSE (Figures 1A and 1B). Similarly, dismantled mitochondrial networks were observed in LMVECs treated with increased concentrations of CSE in a dose-dependent manner (Figure 1C). Using β-catenin to label cellular plasma membranes, we further demonstrated that CSE exposure altered cell shape from elongated to rounded and disrupted the peripheral mitochondrial networks, leaving only perinuclear mitochondria (Figure 1D). Taken together, our results indicate that CSE exposure shortened mitochondrial networks in LMVECs in a time- and dose-dependent manner.

CS Caused Imbalance of Mitochondrial Fusion and Fission in Primary LMVECs and Lung Tissue

The shortening of mitochondrial networks could be due to increased mitochondrial

Figure 2. Effects of cigarette smoke (CS) on mitochondrial fission and fusion in primary lung microvascular endothelial cells and mouse lungs. (A–F) Rat lung microvascular endothelial cells were treated with 10% CSE for varying times (30 min to 6 h) (A, B, and F) or with varying concentrations (5–15%) of CSE for 3 hours (C–E). Cells treated with 10% PBS for 3 hours were used as controls (Ctrl). The concentrations of Drp1 (dynamin-related protein 1), phosphorylated Drp1 at serine-616 (Drp1-pS616), and phosphorylated Drp1 at serine-637 (Drp1-pS637) in whole cell lysates were assessed by IB. Mitochondrial protein, HSP60, and commonly used cytosol housekeeping protein β -actin were used as protein loading Ctrl (A–D). A and C are representative blots from three independent experiments ($n=3$). The cellular localization of Drp1-pS616, FIS1 (mitochondrial fission 1 protein), and Mfn2 (mitofusin 2) were assessed by immunofluorescence microscopy (E and F). DAPI staining (blue) was used to label nuclei. Arrows point to the positive staining of Drp1-pS616, FIS1, or Mfn2. Representative images from three independent experiments are shown. (G–J) AKR mice were exposed to room air

fission and/or decreased mitochondrial fusion. Thus, we characterized effects of CS on mitochondrial fission and fusion by assessing well-established molecular markers, including FIS1, Drp1-pS616, and Drp1-pS637 for fission and Mfn2 for fusion, by conducting time-course and dose-response experiments. We found that although CSE had no effect on protein concentrations of Drp1, CSE increased Drp1-pS616 and decreased Drp1-pS637 in LMVECs in a time- (Figures 2A and 2B) and dose- (Figures 2C and 2D) dependent manner. It is known that phosphorylation at Drp1-S616 promotes mitochondrial fission, whereas phosphorylation at Drp1-S637 inhibits mitochondrial fission; thereby, the ratio of Drp1-pS616:Drp1-pS637 determines the mitochondrial fission activity (21). We noted that CSE dramatically elevated the ratio of Drp1-pS616:Drp1-pS637 in LMVECs (Figures 2B and 2D). Using immunofluorescence microscopy, we further confirmed that CSE increased Drp1-pS616 in LMVECs in a dose-dependent manner (Figure 2E). The control LMVECs did not have any visible staining for Drp1-pS616 except for in the cells undergoing division (double nuclei) (Figure 2E). The outer mitochondrial membrane protein FIS1 is a receptor for Drp1-pS616. In coordination with the increase in Drp1-pS616, CSE also promoted FIS1 expression in LMVECs in a time-dependent manner (Figure 2F). On the other hand, the fusion marker Mfn2 staining was progressively reduced over time in LMVECs treated with CSE (Figure 2F). These data demonstrate that CSE increases mitochondrial fission and reduces mitochondrial fusion in LMVECs. Consistent with *in vitro* findings, an increased ratio of Drp1-pS616:Drp1-pS637 and decreased Drp1-pS637 were also shown in lung homogenates of mice exposed to CS for 6 hours and 3 weeks

(Figures 2G–2J). We also observed a trend of an increase in FIS1 and a trend of a decrease in Mfn2 in lung homogenates of mice exposed to CS (Figures 2G and 2H). Taken together, our results suggest that CS causes an imbalance of mitochondrial fusion and fission, an event that leads to the shortening of mitochondrial networks.

CS Caused Drp1-pS616 Mitochondrial Translocation and Oligomerization in Primary LMVECs and Lung Tissue

Drp1-pS616 mitochondrial translocation and oligomerization are key steps for the initiation of mitochondrial fission (24). To explore the mechanism underlying CS-induced mitochondrial fission, we first assessed Drp1 mitochondrial translocation by mitochondria and cytosol fractionation assay, using Tom20 and β -actin to confirm the purity of fractionation. We found that CSE significantly increased Drp1-pS616 in the mitochondrial fraction of LMVECs (Figures 3A and 3B). Drp1-pS637 has been shown to localize in both cytoplasm and mitochondria in human embryonic kidney (HEK) 293T cells (32). We found that Drp1-pS637 predominantly localized in mitochondria in normal LMVECs (Figures 3A and 3B), an effect that was associated with tubular elongated mitochondrial networks. CSE exposure of LMVECs slightly decreased the concentrations of Drp1-pS637 in mitochondria (Figures 3A and 3B).

Drp1-pS616 oligomerization is a critical step for the formation of fission rings (24). To detect Drp1 oligomers by IB, we performed mildly reduced SDS-PAGE using 0.05% of β -mercaptoethanol, which did not disassociate bonds among Drp1 oligomers. In this condition, Drp1-pS616 existed in low concentrations as both 75 kD (monomer) and 300 kD (tetramer) in control LMVECs (Figure 3C). CSE dramatically elevated the concentrations of both 75 kD

and 300 kD Drp1-pS616 in a dose- (Figure 3C) and time- (Figure 3D) dependent manner. On the other hand, Drp1-pS637 existed as 75 kD (monomer) in control cells, and the concentrations of this monomer were reduced over time by CSE exposure (Figure 3D). We found that CSE produced a 150 kD (dimer) of Drp1-pS637 in a dose- (data not shown) and time- (Figure 3D) dependent manner. The antibody directed against Drp1 only recognized 75 kD Drp1 (Figures 3C and 3D).

To assess whether oligomerization of phosphorylated Drp1 occurs *in vivo*, we performed the similar mildly reduced SDS-PAGE using lung homogenates from mice exposed to CS or room air for 6 hours. In consistent with *in vitro* findings, CS significantly elevated the concentrations of both 75 kD and 300 kD Drp1-pS616 in lung homogenates (Figures 3E and 3F). In addition, CS promoted 150 kD Drp1-pS637 in mouse lungs (Figures 3E and 3F).

Mitochondrial Fission Mediated CS-induced Endothelial Barrier Dysfunction

Mdivi-1 is a small molecule that inhibits the GTPase activity of Drp1-pS616 and thus prevents mitochondrial fission (33). We next elucidated the role of mitochondrial fission in CS-induced endothelial barrier dysfunction by using Mdivi-1. As expected, 20 μ M Mdivi-1 was able to antagonize CSE-induced shortening of mitochondrial networks (Figure 4A). Interestingly, the inhibition of mitochondrial fission by Mdivi-1 prevented CSE-induced endothelial barrier dysfunction (Figure 4B).

CS Caused Abnormal Mitophagy in Primary LMVECs and Lung Tissue

Mitophagy is the mitochondrial quality-control program that aims to eliminate damaged, fragmented, and unwanted

Figure 2. (Continued). (RA) or CS for 6 hours or 3 weeks. The concentrations of Drp1, Drp1-pS616, Drp1-pS637, FIS1, and Mfn2 in lung homogenates were assessed by IB, using β -actin as protein loading Ctrl. G and I are representative blots. For all proteins in A and C; Drp1, Drp1-pS616, and respective β -actin in G; and Drp1, Drp1-pS637, and respective β -actin in I, the primary antibodies were recognized by horseradish peroxidase-conjugated species-specific secondary antibodies. The horseradish peroxidase-dependent signals were detected by enhanced chemiluminescence substrate followed by chemiluminescent capture by X-ray. Densitometry was performed using the Image J program. The mean $\pm$ SE of densitometry data are shown in B, D, H, and J ($n = 3-5$). Unpaired *t* tests were used to compare each time point or each concentration with the Ctrl. * $P < 0.05$ versus Ctrl. For FIS1, Drp1-pS637, Mfn2, and respective protein loading Ctrl in G and Drp1-pS616 and its protein loading control β -actin in I, the primary antibodies were recognized by fluorophore (IRDye 800CW and IRDye 680RD)-conjugated species-specific secondary antibodies. The fluorescence signals were captured and quantified by LiCOR Odyssey Imaging Systems. The mean $\pm$ SE of the densitometry data are shown in H and J ($n = 4-5$). Unpaired *t* test was used to compare RA with CS. * $P < 0.05$ versus RA. Scale bars, 10 μ m.

Figure 3. Effects of CS on Drp1 mitochondrial translocation and oligomerization in lung microvascular endothelial cells (LMVEC) and mouse lungs. (A and B) Rat LMVEC were treated with 10% CSE or Ctrl (10% PBS) for 3 hours and then mitochondrial and cytosolic fractions were isolated. The protein concentrations of Drp1-pS616 and Drp1-pS637 in each cellular fraction and whole cell lysates were assessed by IB, using Tom20 and β -actin as mitochondrial and cytosolic markers, respectively. (A) Representative blots from three independent experiments. (B) Mean $\pm$ SE of the densitometry data from these independent experiments ($n=3$). The concentrations of Drp1-pS616 and Drp1-pS637 in whole cell lysates and cytosolic fractions were normalized by β -actin, whereas the concentrations of Drp1-pS616 and Drp1-pS637 in mitochondrial fractions were normalized by Tom20. An unpaired t test was used to compare CSE with Ctrl. $*P < 0.05$ CSE versus Ctrl. (C and D) Rat LMVEC were treated with varying concentrations (10–15%) of CSE for 3 hours (C) or treated with 15% CSE for varying times (30 min to 3 h) (D). Cells treated with 15% PBS for 3 hours were used as Ctrl. The concentrations of

mitochondria. We have previously shown that CSE time-dependently increased the conversion of microtubule-associated protein 1 light chain 3 β (LC3B)-I to LC3B-II without impacting Beclin 1 in cultured human pulmonary artery endothelial cells (15), suggesting the activation of autophagy and/or mitophagy. Because CS promoted mitochondrial fission, we anticipated that CS would promote mitophagy. Therefore, we examined mitophagy by assessing PINK1 and Parkin. Using immunofluorescence microscopy, we found that CSE increased Parkin concentrations without altering PINK1 concentrations in LMVECs (Figure 4C). Using immunoblot analysis, we confirmed that CSE promoted Parkin protein concentrations in LMVECs in a time- and dose-dependent manner (Figure 4D). Increased Parkin with no changes in PINK1 was also seen in lung homogenates from mice exposed to CS for 6 hours (Figures 4E and 4F). An increase in PINK1 is required for sufficient mitophagy initiation (34). Taken together, our results suggest that CS may induce aberrant mitophagy.

Mitochondrial Oxidative Stress Mediated CS-induced Endothelial Barrier Dysfunction

Excessive mitochondrial fission generates oxidative stress (24). To assess the effect of CSE on mitochondrial oxidative stress, we assessed mitochondrial-specific superoxide production by mitoSOX staining, which was captured by fluorescence microscopy. We found that LMVECs treated with 10% CSE for 3 hours had dramatic increase in mitoSOX staining compared with control cells (Figure 5A). We also used a fluorescence plate reader and flow cytometry to quantify reactive oxygen species concentrations. Hoechst 33342 was used to counterstain viable cells for the normalization of mitoSOX signals. We

found that 2.5% and 5% CSE significantly increased mitochondrial reactive oxygen species (mtROS) concentrations, whereas low doses (0.625% and 1.25%) of CSE did not increase mitochondrial oxidative stress (Figure 5B). Using flow cytometry, we also demonstrated that exposure to 10% CSE for 3 hours significantly increased the numbers of LMVECs that stained positive for both Drp1-pS616 and mitoSOX (Figure 5C), suggesting that excessive mitochondrial fission may lead to increased mitochondrial oxidative stress. This notion was supported by findings that the mitochondrial fission inhibitor Mdivi-1 significantly attenuated CSE-induced increased mitoSOX signals (Figure 5D). We next assessed the effect of the mtROS scavenger mitoTEMPO on the CSE-induced increase in endothelial monolayer permeability. We found that mitoTEMPO completely prevented CSE-induced increase in endothelial monolayer permeability in LMVECs (Figure 5E).

CSE Caused Mitochondrial Dysfunction and Cell Death via Increased Mitochondrial Fission in LMVECs

Mitochondrial morphological plasticity may reflect altered mitochondrial function. To examine changes in mitochondrial function, we assessed the effect of CSE on the mitochondrial OCR of LMVECs using the Seahorse system. We found that CSE reduced the basal mitochondrial respiration in a dose- and time-dependent manner (Figures 6A–6D). We also evaluated the effect of CSE on ATP production-associated respiration. Oligomycin A is an ATP synthase (complex V) inhibitor that prevents oxidative phosphorylation of ADP to ATP. Thus, we used oligomycin A (1 μ M) to assess ATP production-associated mitochondrial OCR. We noted that

CSE dose- and time-dependently reduced ATP production-linked OCR (Figures 6A–6D). The proton leak-associated OCR is defined as the basal OCR subtracted by the ATP production-linked OCR. We noted that CSE did not alter the proton leak-associated OCR (Figures 6A–6D). FCCP is a protonophore and mitochondrial uncoupler that transports protons into the mitochondrial matrix via a pathway that is independent of ATP synthase, thereby uncoupling oxidation from ATP production. In other words, FCCP collapses the mitochondrial inner membrane gradient, allowing the electron transport chain to function at its maximal rate. Thus, we used FCCP (0.5 μ M) to assess the maximal respiratory capacity of mitochondria. Interestingly, CSE also progressively impaired the mitochondrial maximal respiratory capacity of LMVECs with increasing doses (5%, 10%, and 15%) and exposure times (1 h and 2 h) (Figures 6A–6D). Mitochondrial reserve capacity (spare respiratory capacity) is defined as the maximal respiratory capacity subtracted by the basal mitochondrial respiration. We found that CSE did not alter the spare respiratory capacity (Figures 6A–6D). To assess whether CSE affects nonmitochondrial respiration, we blocked entire mitochondrial respiration by using rotenone (0.5 μ M) and antimycin A (0.5 μ M) to specifically inhibit complex I and III after complex V inhibition by oligomycin A. We found that CSE did not alter nonmitochondrial respiration (Figures 6A and 6C).

Mitochondrial dysfunction could lead to cell apoptosis. Indeed, we have previously shown that CSE caused lung endothelial cell apoptosis (15). Similarly, CSE progressively reduced the viability of LMVECs with increasing doses and exposure times (Figure 6E). Interestingly, the inhibition of mitochondrial fission by Mdivi-1 dose-dependently attenuated CSE-induced caspase-3 cleavage (Figures 6F and 6G),

Figure 3. (Continued). monomers and oligomers of Drp1-pS616, Drp1-pS637, and total Drp1 in whole cell extract were assessed by IB using reduced concentrations (0.05%) of β -mercaptoethanol in Laemmli SDS-PAGE buffer. β -actin was used as a protein loading control. The blots represent three independent experiments for each panel. (E and F) AKR mice were exposed to RA or CS for 6 hours. The concentrations of monomer and oligomers of Drp1-pS616, Drp1-pS637, and total Drp1 in lung homogenates were assessed by IB using reduced concentrations (0.05%) of β -mercaptoethanol in Laemmli SDS-PAGE buffer. Representative blots are shown in E. The mean $\pm$ SE of the densitometry data are shown in F. $n=3$. An unpaired t test was used to compare RA with CS. $*P<0.05$ versus RA. The primary antibodies were recognized by horseradish peroxidase (HRP)-conjugated species-specific secondary antibodies. The HRP-dependent signals were detected by enhanced chemiluminescence substrate followed by chemiluminescent capture by X-ray. Densitometry was performed using the Image J program. WCL=whole cell extract (lysates).

Figure 4. Role of mitochondrial fission in CS-induced endothelial barrier permeability. (A and B) Rat LMVEC were treated with 20 μ M Mdivi-1 or vehicle (media containing 0.1% DMSO) for 1 hour and then treated with Ctrl (10% PBS) or 10% CSE in the absence or presence of 20 μ M Mdivi-1 for indicated times. Mitochondrial fission was evaluated at 3 hours after the addition of CSE by mitochondrial morphology examined by immunofluorescence microscopy of Tom20 (A). Monolayer permeability was assessed by measuring electrical resistance across the monolayer using electric cell-substrate impedance sensing (ECIS) (B). Representative images are shown in A. B is the mean $\pm$ SE of a total of 5–8 biological replicates. ANOVA and Fisher's least significant difference *post hoc* test were used to determine difference among the four groups. * $P < 0.05$ CSE versus Ctrl. (C) Rat LMVEC were treated with 10% CSE or Ctrl (10% PBS) for 6 hours, and the cellular localization of PINK1 (PTEN-induced kinase 1) and Parkin (E3 ubiquitin-protein ligase) were assessed by immunofluorescence microscopy. DAPI staining was used to label nuclei (blue). The images represent three independent experiments. (D) Rat LMVEC were treated with varying concentrations (5–10%) of CSE or Ctrl (10% PBS) for varying times (2–24 h). The protein concentrations of Parkin in whole cell lysates were assessed by IB, using β -actin as protein loading Ctrl. The blots represent three independent experiments. (E and F) AKR mice were

suggesting a role of mitochondrial fission in CSE-induced apoptosis of LMVECs.

Discussion

Mitochondria undergo dynamic morphological change via fusion and fission to maintain homeostasis in response to external and internal insults (21). In this study, we show that CS altered mitochondrial morphology by shortening mitochondrial networks. CS caused an imbalance of mitochondrial fusion and fission, likely by promoting Drp1-S616 phosphorylation, mitochondrial translocation, and tetramerization and by reducing Mfn2, leading to mitochondrial oxidative stress and mitochondrial dysfunction and ultimately resulting in endothelial cell apoptosis and barrier dysfunction. Our data suggest that the inhibition of mitochondrial fission and mitochondria-targeted antioxidants may be useful therapeutic strategies for CS-induced endothelial injury and associated pulmonary diseases.

Lung endothelial cell denudation (35) and increased apoptosis (14) have been reported in patients with chronic obstructive pulmonary disease (COPD). Using TUNEL staining for apoptosis and von Willebrand factor staining for endothelial cells, we have shown that exposure to CS for 3 weeks increases lung endothelial cell apoptosis in mice (15). In addition, CSE increases both pulmonary artery and microvascular endothelial cell apoptosis *in vitro* via oxidative stress (15). CS exposure also promotes alveolar-capillary permeability in human and animal models (8–10, 12, 13). CS increases pulmonary macrovascular and microvascular endothelial cell permeability *in vitro* and mouse lung vascular permeability *in vivo* by a mechanism involving oxidative stress (8–10, 15–17). However, the mechanism underlying CS-induced mitochondrial oxidative stress is not well understood. It has been shown that increased mitochondrial fission promotes mtROS in human bronchial epithelial cells

(36). Mitochondrial fission is increased in bronchial epithelial cells of patients with COPD (36) and in human alveolar type II cells of smokers (37). CSE induces mitochondrial fission and reactive oxygen species production in cultured human bronchial epithelial cells (36), human bronchial epithelial cell line BEAS-2B (26, 36), primary lung epithelial cells (38), and human airway smooth muscle cells (25). In contrast, CSE has been shown to promote mitochondrial fusion in mouse alveolar epithelial cells (39). However, the effect of CS on mitochondrial fission and fusion in endothelial cells is unknown. In this study, we showed that CSE shortened the tubular mitochondrial networks, increased the concentrations of Drp1-pS616 and FIS1, reduced the concentrations of Drp1-pS637 and Mfn2, and enhanced the ratio of Drp1-pS616:Drp1-pS637 in LMVECs. Similar findings were also observed in the lungs of mice exposed to CS. Taken together, our data indicate that CS causes an imbalance of mitochondrial fusion and fission in lung endothelial cells. We further show that the inhibition of mitochondrial fission by Mdivi-1 prevented CS-induced mitochondrial oxidative stress, indicating that CS increases mitochondrial oxidative stress via excessive mitochondrial fission. Importantly, the mitochondria-targeted antioxidant mitoTEMPO completely prevented CSE-induced endothelial barrier dysfunction. Inhibition of mitochondrial fission by Mdivi-1 prevented CSE-induced endothelial barrier dysfunction and apoptosis in LMVECs. Thus, our data support the notion that CS promotes endothelial cell apoptosis and barrier dysfunction via excessive mitochondrial fission and resultant mitochondrial oxidative stress, as depicted in Figure 7.

The mechanisms of CS-induced mitochondrial fission are not well understood. Reduced Drp1-pS637 has been shown to induce Drp1 mitochondrial translocation (40). In this study, we found that Drp1-pS637 is predominantly located at the mitochondria, whereas Drp1-pS616 is located

in both the cytosol and mitochondria in normal LMVECs. CS increased Drp1-pS616 and decreased Drp1-pS637 in the mitochondria; these effects are associated with excessive mitochondrial fission. These data suggest that CS-induced increased ratio of Drp1-pS616:Drp1-pS637 in mitochondria promotes mitochondrial fission. Drp1 tetramer is capable of self-assembly into higher-order ring-like oligomeric structures to execute fission. In this study, we found that CSE increased the concentrations of Drp1-pS616 tetramers and Drp1-pS637 dimers in both LMVECs and mouse lungs. We did not observe Drp1-pS637 tetramers. Thus, we suggest that CS may promote mitochondrial fission by increasing Drp1-pS616 tetramers and Drp1-pS637 dimers. It remains unclear how CS promotes Drp1 phosphorylation, mitochondrial translocation, and oligomerization. Drp1-S616 can be phosphorylated by Ca^{2+} /CaMKII (calmodulin-dependent kinase II) (41) and ERK1/2 (42), whereas Drp1-S637 can be phosphorylated by 5' adenosine monophosphate-activated protein kinase (AMPK) (43). CSE has been shown to activate Ca^{2+} /CaMKII in oral cancer cells (44). CS also activates ERK1/2 in human pulmonary microvascular endothelial cells and in the lungs of patients with COPD (45). In addition, CSE reduces AMPK activity in bronchial epithelial cells (46, 47) and hepatocytes (48). Whether the activation of Ca^{2+} /CaMKII and ERK1/2 and inhibition of AMPK are involved in CS-induced mitochondrial fission remains to be elucidated. Unlike Drp1-pS616 and Drp1-pS637, we were not able to detect polymerized forms of Drp1. It is possible that the Drp1 antibody we used does not recognize the tetrameric and dimeric forms of Drp1 because of the unavailability of epitope upon dimerization. Additional Drp1 antibody shall be tested in future should it become available to us.

How mitochondrial oxidative stress leads to increased endothelial barrier permeability and apoptosis remains unclear. Mitochondrial oxidative stress can induce mtDNA damage, which is essential for oxidants-induced pulmonary endothelial

Figure 4. (Continued). exposed to RA or CS for 6 hours. The protein concentrations of Parkin and PINK1 in lung homogenates were assessed by IB, using β -actin as protein loading Ctrl. The representative blots are shown in E. The samples in all groups were loaded in the same gel (see Figure E3 in the data supplement). The concentrations of Parkin and PINK1 were normalized by β -actin. The mean $\pm$ SE of the relative concentrations from three mice are shown in F. $n = 3$. Unpaired t test was used to compare RA with CS. * $P < 0.05$ CS versus RA. The primary antibodies were recognized by HRP-conjugated species-specific secondary antibodies. The HRP-dependent signals were detected by enhanced chemiluminescence substrate followed by chemiluminescent capture by X-ray. Densitometry was performed using the Image J program. Scale bars, 10 μm . Mdivi-1 = mitochondrial division inhibitor 1.

Figure 5. Role of mitochondrial oxidative stress in CS-induced endothelial barrier dysfunction. (A) Rat LMVEC were treated with 10% of CSE or Ctrl (10% PBS) for 3 hours, and then mitochondrial reactive oxygen species (ROS) concentrations were assessed by mitoSOX Red staining. The ROS signals were captured by fluorescence microscopy. Representative images are shown. (B) Rat LMVEC were treated with varying concentrations (0–5%) of CSE or Ctrl (5% PBS) for varying times (2–24 h). The concentrations of mitochondria ROS were assessed by mitoSOX Red staining. The live cells were stained by Hoechst 33342. Fluorescence signals of Hoechst 33342 and mitoSOX Red were captured by fluorescence spectrometry at excitation/emission of 360 nm/460 nm and 510 nm/580 nm, respectively. The data are presented as mean $\pm$ SE of mitoSOX adjusted by Hoechst 33342 signal. $n = 3$. An unpaired t test was used to compare room air with CS. $*P < 0.05$ versus Ctrl. (C) Rat LMVEC were treated with 10% of CSE or Ctrl (10% PBS) for 3 hours. The number of cells double positively stained by Drp1-pS616 and mitoSOX Red were examined by flow cytometry. Drp1-pS616 was stained by primary antibody directed against Drp1-pS616 followed by species-specific Alexa Fluor 405-conjugated second antibody. Representative flow images are shown. The quantitative data of the percentage of Drp1-pS616/mitoTracker Red double positively stained cells are presented as mean $\pm$ SE of three independent experiments. An unpaired t test was used to compare CSE with Ctrl. $*P < 0.05$ CSE versus Ctrl. (D) Rat LMVEC were treated with 20 μ M Mdivi-1 or vehicle (media containing 0.1% DMSO) for 1 hour and then treated with Ctrl (10% PBS) or 10% CSE in the absence or presence of 20 μ M Mdivi-1 for 3 hours. Mitochondria ROS concentrations were assessed by mitoSOX Red staining and adjusted by Hoechst 33342 signal. The data are presented as the mean $\pm$ SE of three independent experiments. ANOVA and Fisher's least significant difference *post hoc* test were used to determine the differences among the four groups. $*P < 0.05$ CSE versus Ctrl or CSE vs. Mdivi-1 + CSE. (E) Rat LMVEC were pretreated with 1 μ M mitoTEMPO for 1 h and then treated with 10% of CSE or Ctrl (10% PBS) in the absence or presence of 1 μ M mitoTEMPO for indicated times. Monolayer permeability was assessed over time by measuring electrical resistance across the monolayer using ECIS. The data are presented as the mean $\pm$ SE of the normalized electrical resistance at the selected time points relative to their initial resistance from a total of 4–5 biological replicates. ANOVA and Fisher's least significant difference *post hoc* test were used to determine difference among the four groups. $*P < 0.05$ CSE versus Ctrl. Arrows indicate the time of treatment additions. Scale bars, 5 μ m.

Figure 6. Effects of CSE on mitochondrial respiration and cell death of lung microvascular endothelial cells. (A–D) Rat LMVEC were treated with varying concentrations (5–15%) of CSE for 1 hour (A and B) or 10% CSE for varying times (10 min to 2 h) (C and D). Cells treated with 10% PBS for 1 h were used as Ctrl. Mitochondrial oxygen consumption rate was assessed by a XF96 Seahorse analyzer. (A and C) Representative tracing data, in which oxygen consumption rate was averaged from that of 3–4 wells for each data point. (B and D) Presented as mean $\pm$ SE of 3–4 independent experiments for each

Figure 7. The proposed pathways leading to cigarette smoke-induced endothelial barrier dysfunction. The dashed arrow represents suggested pathway, and the solid lines represent demonstrated pathways. Mt = mitochondria.

barrier dysfunction (49). Mitophagy is the mitochondrial quality-control system that recognizes and eliminates damaged, fragmented, and oxidative mitochondria. If damaged mitochondria are not eliminated by mitophagy, cells may release mitochondrial damage-associated molecular patterns, which can increase endothelial permeability *in vitro* (50) and cause systemic inflammation and organ injury *in vivo* (51). Enhanced mitophagy is usually an early response that promotes cell survival. However, overwhelming or prolonged mitochondrial damage can induce excessive mitophagy, thereby promoting cell death and tissue injury. Parkin overexpression is sufficient to induce mitophagy and attenuate CSE-induced mtROS production and cellular senescence in airway epithelial cells,

suggesting that Parkin induction could mitigate CS-induced cellular injury (52). CS reduced Parkin translocation to damaged mitochondria in emphysematous mouse lungs and in the lungs of chronic smokers and patients with COPD, suggesting that CS impairs mitophagy (53). In this study, we found that CS increased the concentrations of Parkin in LMVECs and mouse lungs. We also found that CSE dose- and time-dependently reduced endothelial cell mitochondrial respiration and viability. In addition, the inhibition of mitochondrial fission attenuated CSE-induced endothelial apoptosis (caspase-3 cleavage). Taken together, our data suggest that CS-induced mitochondrial fission and oxidative stress lead to mitochondrial dysfunction and apoptosis likely because of aberrant mitophagy, as depicted in Figure 7. It is also possible that CS-induced excessive mitochondrial fission and oxidative stress cause endothelial barrier dysfunction via mtDNA damage and/or the release of mitochondrial damage-associated molecular patterns.

To characterize the effects of CSE on mitochondrial fusion and fission, mitochondrial oxidative stress, mitochondrial respiration, endothelial barrier function, and cell death, we conducted both time-course and dose-response experiments. Because robust effects in most endpoint measurements were seen in treatment with 10% CSE for 3 hours, this condition was chosen for some experiments. Small variations in time points and doses used in various experiments were necessary to meet specific experimental conditions. For example, LMVECs do not attach well on rigid surfaces (e.g., glass cover slides) and are sensitive to detachment upon CSE treatment. Therefore, for fluorescence microscopy studies, slightly lower

doses and shorter time points were used.

As shown by IB, compared with mice exposed to CS, control mice, which were exposed to room air, had much lower concentrations of FIS1 (Figure 2G) and Parkin (Figure 4E) in lung homogenates. Similarly, the basic concentrations of FIS1 and Parkin (Figure 4D) in control endothelial cells are very low and much lower than those in endothelial cells exposed to CSE, as demonstrated by IB. Therefore, we believe that almost invisible concentrations of FIS1 (Figure 2F) and Parkin (Figure 4C) by immunofluorescence microscopy (IF) in control cells is due to lower sensitivity of immunofluorescence microscopy assay.

This study has some limitations. We characterized mitochondrial fusion and fission and mitophagy *in vivo* relying on lung homogenates from mice exposed to CS. However, this approach does not specifically address effects on lung endothelial cells *in vivo*. *In vivo* imaging or freshly isolated lung endothelial cells from mice exposed to CS will be used to characterize imbalance of mitochondrial fission and fusion and mitophagy in future studies. Although CSE exposure of cultured cells is a widely used and generally accepted *in vitro* system for mechanistic studies, we recognize that the nature and concentrations of smoke components may differ between CS inhalation *in vivo* and CSE exposure of endothelial cells *in vitro*. CSE contains water-soluble smoke components that lung endothelial cells are likely to be exposed *in vivo*. Results found in lung tissues of mice exposed to CS were consistent with results found using cultured LMVECs exposed to CSE.

In summary, we have shown that CS promoted mitochondrial fission and reduced mitochondrial fusion, likely by

Figure 6. (Continued). panel. Unpaired *t* tests were used to compare each time point or each concentration to respective Ctrl. **P* < 0.05 versus Ctrl. (E) Rat LMVEC were treated with varying concentrations (0–10%) of CSE for varying times (2–24 h). Cell viability was assessed by MTT assay (a colorimetric assay for assessing cell metabolic activity). Data are presented as mean ± SE of three independent experiments (*n* = 3). Unpaired *t* tests were used to compare each concentration of CSE at each time point to respective Ctrl. **P* < 0.05 versus Ctrl. (F and G) Rat LMVEC were treated with varying doses (1–100 μM) of Mdivi-1 or vehicle for 1 hour and then treated with vehicle (10% PBS) or 10% CSE in the absence or presence of varying doses of Mdivi-1 for 6 hours. Cell apoptosis was assessed by IB of the cleaved (19 kD and 17 kD) caspase-3. β-actin was used as protein loading Ctrl. Representative blots from three independent experiments are shown in F. Densitometry data (G) are presented as the mean ± SE. ANOVA and Fisher's least significant difference *post hoc* test were used to determine difference among treatment groups for p19 and p17. **P* < 0.05. The primary antibodies were recognized by HRP-conjugated species-specific secondary antibodies. The HRP-dependent signals were detected by enhanced chemiluminescence substrate followed by chemiluminescent capture by X-ray. Densitometry was performed using Image J program. FCCP = carbonyl cyanide-4-phenylhydrazone; OCR = oxygen consumption rate.

enhancing Drp1-S616 phosphorylation, mitochondrial translocation, and tetramerization, leading to mitochondrial oxidative stress and impaired mitochondrial respiration and ultimately resulting in endothelial cell apoptosis and

barrier dysfunction (Figure 7). These results suggest potential new therapeutic strategies to prevent and treat CS-associated pulmonary diseases by inhibiting mitochondrial fission and oxidative stress. ■

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