Cigarette smoke-induced mitochondrial fragmentation and dysfunction in human airway smooth muscle

Bharathi Aravamudan,1 Alexander Kiel,1 Michelle Freeman,1 Philippe Delmotte,2 Michael Thompson,1 Robert Vassallo,3 Gary C. Sieck,1,2 Christina M. Pabelick,1,2 and Y. S. Prakash1,2

1Department of Anesthesiology, Mayo Clinic, Rochester, Minnesota; 2Department of Physiotherapy and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota; and 3Department of Medicine, Mayo Clinic, Rochester, Minnesota

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Cigarette smoke (CS) is thought to contribute to the onset and/or development of respiratory diseases by adversely affecting these processes (20, 31, 46, 48, 77, 87). However, the mechanisms by which CS can induce these effects are still not clear, and are important to understand, in the context of minimizing the detrimental effects of CS in the airway.

There is increasing interest in the nonenergetic roles of mitochondria and the potential role of mitochondrial dysfunction in disease. Mitochondria can act as cytoplasmic Ca2+ buffers (41, 67, 72) and modulate cell fate (proliferation vs. apoptosis) (38, 50, 88). Under normal conditions, mitochondria exhibit tubular, reticular, or networked morphology, which is regulated by dynamic remodeling, represented by a balance between mitochondrial fission vs. fusion, with this balance shifting in response to changes in environment (14, 15, 50, 89). The equilibrium between fission and fusion processes, initially thought of as exclusively morphology oriented, is now widely appreciated for its multiple and far-reaching implications, including content mixing between mitochondria, protecting mitochondrial DNA stability, respiratory functions, cell fate determination, and adaptation to cellular stress (13). Proteins involved in the regulation of mitochondrial morphology and the maintenance of the delicate fission-fusion balance are now known to be essential for overall cellular health (14, 15, 50, 89). Of particular interest are the fission protein dynamin-related protein 1 (Drp1, a master regulator of fission and a member of the dynamin family of GTPases) and the fusion proteins of the mitofusin family. Drp1 translocates from cytoplasm and polymerizes into spirals around specific mitochondrial sites, constricting and breaking mitochondrial membranes at these sites, thus bringing about fission (73). Mitofusins 1 and 2 (Mfn1 and Mfn2, respectively), on the other hand, are evolutionarily conserved GTPases on the mitochondrial outer membrane and are indispensable for mitochondrial fusion (32). As vital determinants of fission-fusion balance, Drp1 and Mfn2 likely share a reciprocal relationship, and factors perturbing this balance are likely to also skew the stoichiometric relationship between these two proteins.

There is currently no information on mitochondrial fission/fusion dynamics in ASM, or its importance in asthma or CS effects. The clinical relevance of these processes lies in their potential contributed to altered airway structure and function that is key to the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). For example, a recent study showed that chronic CS exposure increases mitochondrial fragmentation and expression of fission/fusion proteins in airway epithelial cells of patients with COPD (42). While epithelial cells are likely to be the first to encounter inhaled CS, effects on ASM cannot be ignored, considering that volatile agents and other permeable components can reach the underlying smooth muscle layer.

Another important function of mitochondria, specifically relevant to CS effects, is the production of reactive oxygen species (ROS) and cellular oxidative stress (4, 78, 80), which activate multiple signaling pathways. While cells produce ROS...
at baseline as a result of mitochondrial oxidative phosphorylation (OXPHOS) reactions, the basal level of ROS resulting from residual, unreduced molecular oxygen (~1–3%) during OXPHOS can increase drastically when mitochondria encounter damaging stimuli. CS is a concoction of ~5,000 reactive chemicals, and contains >10^{15} free radicals in the gas phase and 10^{15} free radicals/g in the tar phase (48, 63), and includes ROS such as superoxide, hydrogen peroxide, and hydroxyl anion. Accordingly, given such a high complement of exogenous reactive radicals provided by CS, it becomes important to understand the effect of CS on mitochondrial structure and function in the context of ROS and downstream effects. In this regard, there is currently no information on these relationships in the airway.

In the present study, we hypothesized that CS disrupts mitochondrial fission-fusion balance, affecting mitochondrial morphology, and that this process involves several intracellular signaling mechanisms, including ROS. We tested this hypothesis in nonasthmatic human ASM cells using quantitative imaging and molecular biology techniques. Furthermore, to provide clinical context, we compared ASM cells from patients with moderate asthma. Our results show that even low levels of cigarette smoke extract (CSE) have substantial impact on ASM mitochondria, increased expression of Drp1, and correspondingly reduced expression of Mfn2 (thus affecting mitochondrial morphology), and altered mitochondrial energy production. Furthermore, we demonstrate that CSE-induced changes in ASM mitochondrial morphology are mediated via multiple signaling and transcriptional pathways that are relevant to diseases such as asthma and COPD. Overall, these data offer a novel insight into the mechanisms that regulate mitochondrial morphology, especially in the context of CS exposure, and may thus be relevant in airway diseases linked to CS.

**MATERIALS AND METHODS**

**Materials.** MitoTracker Green and MitoSOX were purchased from Invitrogen/Molecular Probes (Carlsbad, CA). Drp1, NF-κBp50, NF-κBp65, and nuclear erythroid 2-related factor 2 (Nrf2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Mfn2 antibodies were from Abcam (Cambridge, MA), and GAPDH antibodies were from Cell Signaling (Beverly, MA). Primers used for PCR were synthesized by Integrated DNA Technologies (Coralville, IA). Other chemicals were from Sigma Aldrich (St. Louis MO) unless stated otherwise.

**ASM cells.** Human ASM cells were isolated using previously described techniques (60, 62). Briefly, under Institutional Review Board-approved protocols, third- to sixth-level bronchi from human lung specimens, incidental to patient thoracic surgery at St. Mary’s Hospital, Mayo Clinic Rochester, MN, were obtained from Surgical Pathology. Based on medical histories, only ASM cells from nonsmoker patients were used in the current report. Samples from moderate astmatic patients vs. nonastmatic patients were identified from patient histories. Normal-appearing areas of airways were dissected from surgical samples for focal, noninfectious diseases (typically focal nodules of nonbronchovascular carcinomas requiring lobectomies, pneumoctomies for other indications). Bronchial specimens were immersed in ice-cold Hanks’ balanced salt solution (HBSS; 2.5 mM Ca^{2+}), the epithelial layer was removed by blunt dissection, and the ASM layer was excised and finely minced in ice-cold Ca^{2+}-free HBSS. Cells were isolated using collagenase digestion, followed by ovomucoid-albumin separation. Subsequent cell pellets were resuspended in DMEM-F-12 supplemented with 10% FBS (DMEM Complete), centrifuged, resuspended, seeded in culture flasks, and were passaged to a maximum of two subcultures. Cells were serum deprived at least for 24 h before all experiments. Maintenance of ASM phenotype in cells with subculture was frequently verified by the presence of smooth muscle actin and myosin, Ca^{2+} channel regulatory proteins such as TRPC3, CD38, and Orai1, but absence of markers such as E-cadherin and fibroblast surface protein. Asthmatic ASM phenotype was verified by increased expression of TRPC3 or Orai1 that have been found to be relevant in asthma pathophysiology and airway inflammation (9, 19, 58, 76, 79).

**CSE preparation.** Aqueous CSE was prepared using a modification (82) of the Blue and Janoff method (8) via a smoking apparatus (50-ml plastic syringe with a 3-way stopcock) to which a cigarette (Kentucky 1RF4 cigarettes) and a sterile plastic tip were attached. Via the plastic tip, 35 ml of CS was slowly bubbled into 30 ml of sterile RPMI 1640 at 37°C for 30 min. One cigarette per 10 ml medium was used. The CSE solution was then sterile filtered and used immediately. Analysis of nicotine and other metabolite concentrations in CSE have been analyzed and reported previously (82). ASM cells were exposed to CSE diluted in serum-free medium.

**Mitochondrial fission-fusion morphology.** ASM cells in eight-well Labtks were washed with 2 mM Ca^{2+}–HBSS (pH 7.4), loaded with 400 nM MitoTracker Green (room temperature, 5 min), and visualized under a Nikon Eclipse Ti imaging system using a ×100/1.45 NA Nikon Plan Apo λ lens and an LED fluorescence light source and 16-bit high-sensitivity CCD camera.

Using NIH Image J software, masks were created to isolate a single cell within images following correction for background fluorescence. A MATLAB script was used to threshold and identify mitochondria, via edge detection. Area-weighted averages of mitochondrial form factor (an index of mitochondrial branching) and aspect ratio (an index of mitochondrial branch length) in each cell were calculated using the procedures developed by Koopman and colleagues (7, 83).

**ROS measurements.** Cells in eight-well Labtks were loaded with 5 μM MitoSOX Red (37°C, 30 min) and imaged using the Nikon system as above. Cells were treated post hoc with 1 μM DAPI for 5 min to identify nuclei and individual cells.

**Antioxidant treatment.** Superoxide dismutase (SOD)-polyethylene glycol (125 U) or 60 U of catalase were added to 500 μl serum-free DMEM-F-12 medium in each well. For CSE experiments, cells were incubated with antioxidants and 1% CSE concurrently.

**Measurement of mitochondrial respiration.** ASM cells were seeded on 24-well XF-24 plates (SeaHorse Biosciences, Billerica, MA), at a population density of about 100,000/well, in DMEM Complete medium. After 24 h, cells were exposed to serum-free medium alone or to serum-free medium supplemented with artificial epithelial lining fluid (ELF) (39, 47) before exposure to CSE. A further 24 h later, oxygen consumption rate (OCR; an indicator of mitochondrial respiration) was measured in all four groups, in real time, using the XF24 Extracellular Flux Analyzer (SeaHorse Biosciences). OCR measurements were acquired in the presence of 10 mM glucose, before (basal OCR) and after mitochondrial respiration inhibitors were injected in the system. The inhibitors used were as follows: 9 μM oligomycin (ATP uncoupler), 0.3 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (accelerates electron transport chain), 11 μM antimycin A (a complex III inhibitor), and 11 μM rotenone (a complex I inhibitor), allowing for determination of basal respiration, ATP production, maximal respiration, and spare respiratory capacity (59), which were normalized for total protein measured post hoc.

**Q-PCR on cDNAs from ASMs.** ASM cells from nonasthmatic or asthmatic individuals were treated with 1% CSE for 48 h. Total RNA was isolated from the cells, using an RNeasy mini kit (Qiagen, Valencia, CA), and reverse transcribed using the Transcripter reverse transcription kit (Roche, Indianapolis, IN). The resultant cDNA was subjected to Real-Time PCR (optimized for the Roche LC480 Light Cycler), with ribosomal protein S16 as an internal control. The ratio of fold change in expression of the mRNA of interest was calculated by normalization of cycle threshold (Ct) values of the target genes.
(Drp1, Fis1, Mfn1, Mfn2, and Opa1) to the reference gene (S16) using the comparative Ct (ΔΔCt) method. Unexposed control was used as the calibrator for quantification. Primers used for RT-PCR are listed in Table 1.

**Transfection with small-interfering RNAs.** ASM cells, grown to about 80% confluence, were treated with serum and antibiotic-free medium for 24 h before being transfected with 200 pM small-interfering RNA (siRNA) against Drp1 or Mfn2 (Ambion-Applied Biosystems, Austin, TX), using Lipofectamine transfection reagent (Invitrogen). Vehicle-alone treatment and transfection with a scrambled (nontargeting) RNA were performed as controls.

**Preparation of cytoplasmic and nuclear fractions.** ASM cells were transfected, as described above. Posttransfection (48 h), cells were either left untreated or exposed to 1% CSE for 2 h, following which cytoplasmic and nuclear fractions were collected, using the NE-PER kit (Pierce Biotechnology). An equal amount of protein from each sample was used for checking nuclear translocation of transcription factors on Western blots. Cytoplasmic and nuclear fractions from at least three populations were analyzed.

**Immunoblotting.** ASM cells were grown under normal conditions before being serum deprived for either CSE exposure or treatment with inhibitor drugs (see legends for Figs. 1–9 for final inhibitor concentrations).

### Table 1. Oligonucleotide primers used in PCR

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Forward and reverse primers specific for human (h) amplicons targeted in the current study were obtained from Integrated DNA Technologies. Primers were used at a final concentration of 500 nM in the quantitative real-time PCR reactions.

A

**Fig. 1.** Changes in mitochondrial morphology of human airway smooth muscle (ASM) cells caused by cigarette smoke (CS). A: representative images of MitoTracker Green-labeled ASM mitochondria upon exposure to 0 (untreated), 0.5, 1, or 2% cigarette smoke extract (CSE) for 24 or 48 h. B: representative scatter plot showing the range of mitochondrial morphology parameters (form factor representing mitochondrial branching and aspect ratio presenting branch length) at baseline and upon 1% CSE treatment. Each blue or red circle represents individual cells from one patient of control vs. 1% CSE group, respectively. C and D: quantification of changes in mitochondrial morphology in response to different concentrations of CSE showed concentration-dependent decreases in form factor (C) and aspect ratio (D) compared with untreated cells at both 24 and 48 h of CSE exposure. *Significant effect of CSE compared with untreated control. %Significant CSE concentration dependence (P < 0.05).
concentrations). Standard techniques were applied to isolate and separate whole cell lysates on denaturing PAGE. Approximately 15 μg of total protein were loaded onto gels. Protein expression was detected, and densities of bands were quantified on a Li-Cor Odyssey IR scanning system (Lincoln, NE). Band intensities were normalized against GAPDH. Extracts from at least three ASM populations were used for each experiment.

Statistical analysis. All imaging experiments were performed in quadruplicate using different sets of ASM cells isolated from at least three different nonasthmatic or asthmatic individuals. At least 10 cells were tested in each set. For mitochondrial morphology calculations, at least 10 images from each population per treatment were chosen; all mitochondria within a selected cell were analyzed for these parameters, and data from all cells were averaged. Weighted averages were then calculated and used for generating graphs. Controls represent cells not exposed to CSE or those that were not transfected. For PCR, two reverse transcription reactions per RNA sample were performed, and each of the resultant cDNAs was used in duplicate PCR reactions. For immunoblotting, extracts were obtained from three nonasthmatic or asthmatic populations. For all experiments, comparisons were performed across groups with independent Student’s t-test or two-way ANOVA as appropriate. Repeated comparisons required Bonferroni correction. Statistical significance was tested at the \( P < 0.05 \) level. Values are reported as means ± SE. “\( N \)” values representing numbers of individuals are provided in the legends to Figs. 1–9.

RESULTS

CSE effects on mitochondrial morphology. As a first step toward understanding how CSE affects mitochondrial fission-fusion balance in human ASM, we conducted a CSE dose-response study. ASM cells from nonasthmatic subjects were exposed to increasing concentrations of CSE (0.5, 1, and 2%) for 24 or 48 h. Mitochondria were then marked with MitoTracker Green and imaged. Although some levels of mitochondrial fission vs. fusion continuously occur even under normal (baseline) circumstances (resulting in both elongated networks and shorter, fragmented mitochondria within the same cell), in order to keep fission-fusion balance, most cells maintain contiguous mitochondrial tracks and display a complex, networked, morphology. This networked morphology was visibly present

![A](#) (**)  

**A**: in human ASM cells, exposure to 1% CSE increased mRNA expression for the fission protein dynamin-related protein (Drp1) but decreased mRNA levels for the fusion mitofusins (Mfn1, Mfn2). **B**: these mRNA changes were matched by increased expression of Drp1 and decreased expression of Mfn2 protein in human ASM cell lysates. **C** and **D**: quantification of the immunoblots showed that exposure to different concentrations of CSE for either 24 (C) or 48 (D) h resulted in concentration-dependent changes in Drp1 vs. Mfn2. *Significant effect of CSE compared with untreated control. %Significant CSE concentration dependence (\( P < 0.05 \)).
in untreated (control) cells in our study (Fig. 1A). However, with CSE exposure, this morphology was progressively lost, based on CSE concentration and/or time of exposure (Fig. 1A). In other words, mitochondrial fragmentation was enhanced while longer mitochondrial branches and networks were dismantled as CSE concentration and/or time of exposure increased.

Mitochondrial morphology can be quantified in terms of two specific parameters: aspect ratio (length, indicative of mitochondrial fusion) and form factor (degree of branching, indicative of mitochondrial networking) (7, 83). A representative scatter plot exemplifying the distribution in form factor vs. aspect ratio in control (no CSE) cells vs. those exposed to 1% CSE for 48 h is shown in Fig. 1B. With CSE exposure, the mix of cells with longer more branched mitochondrial networks was reduced to short-branched, fragmented mitochondrial patterns. Treatment with higher concentrations of CSE (such as 2%) and longer time of exposure (i.e., 48 h) causes a dramatic reduction in both form factor and aspect ratio (P < 0.05 for each CSE concentration at each time, and across concentrations at 24 or 48 h; Fig. 1, C and D), indicating reduced mitochondrial branching and track lengths.

**CSE effects on mitochondrial fission/fusion proteins.** To determine the mechanisms by which CSE alters mitochondrial morphology in ASM, we first examined expression of proteins known to regulate mitochondrial fission and fusion, namely Drp1 and Mfn2. In human ASM cells from nonasomatic subjects, 24 h of 1% CSE induced significant increases in mRNA (Fig. 2A) and protein (Fig. 2B, summary Fig. 2, C and D) expression for the fission protein Drp1 (P < 0.05 for both mRNA and protein). At the protein level, these effects were concentration- and exposure time-dependent (P < 0.05; Fig. 2, C and D). Conversely, CSE significantly reduced mRNA (Fig. 2A) and protein (Fig. 2B, summary Fig. 2, C and D) levels of the fusion protein Mfn2 (P < 0.05 for both mRNA and protein), again in a concentration- and time-dependent fashion. At the mRNA level, the fission protein Fis1 was also increased, whereas Mfn1 and Opa1, involved in fusion, were decreased following CSE exposure (P < 0.05, Fig. 2).

**Role of Drp1 and Mfn2 in CSE effects on mitochondrial morphology.** ASM cells from nonasomatic patients were transfected with siRNAs targeting Drp1 or Mfn2 (with appropriate nontargeting RNA and vehicle control). Western analysis showed 60 and 40% reduction in expression of Drp1 and Mfn2 respectively (Fig. 3A). Densitometric quantification of immunoblot data showing Mfn2 and Drp1 siRNA efficacy is shown in Fig. 3B. Quantification of mitochondrial morphology, in terms of form factor (C) and aspect ratio (D), showed that both morphological parameters are increased when Drp1 expression is suppressed (consistent with a fission role for Drp1), whereas parameters are reduced when Mfn2 expression is inhibited (consistent with a fusion role for Mfn2). siRNAs against either protein substantially reversed changes in mitochondrial morphology induced by 1% CSE, albeit to different extents. *Significant effect of CSE compared with untreated control. #Significant siRNA effect (P < 0.05).
Mfn2, respectively (P > 0.05 for each protein; Fig. 3, A and B). In unexposed (control) cells, preventing fission (via Drp1 siRNA) significantly increased mitochondrial branching (form factor; P < 0.05, Fig. 3C) and mitochondrial track length (aspect ratio, P < 0.05; Fig. 3D). Nontargeting RNA was without effect. Importantly, Drp1 siRNA reversed mitochondrial fragmentation in 1% CSE-exposed ASM cells, as reflected by substantial recovery of both form factor (P < 0.05, Fig. 3C) and aspect ratio (P < 0.05; Fig. 4C) at 24 h. Conversely, inhibiting mitochondrial fusion via Mfn2 siRNA resulted in a fragmented morphology even in control cells, with substantially reduced form factor and aspect ratio (P < 0.05, Fig. 3, C and D). Under these conditions, CSE effects were significantly exacerbated, particularly for form factor.

Mechanisms regulating CSE-induced changes in mitochondrial morphology and fission/fusion proteins. Given the large number of components in CSE, it is likely that a number of signaling pathways can be potentially activated. We focused on potential mechanisms that are also involved in airway inflammation and diseases such as asthma: extracellular signal-regulated kinase (ERK) 1/2, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and protein kinase C (PKC) (21, 25, 28, 49, 55). Pharmacological inhibition of mitogen-activated protein kinase (MAPK) with PD-98059 (2 μM), Akt using Calbiochem Akt1 Inhibitor XIII (500 nM), PI3K with wortmannin (50 nM), and PKC with bis-indolylmaleimide (10 ng/ml; BIS) resulted in significant blunting of 1% CSE effects on mitochondrial fragmentation at 24 h as represented by recovery of form factor (P < 0.05 for all inhibitors, Fig. 4A) and aspect ratio (P < 0.05 for all inhibitors, Fig. 4B). The extent of blunting of CSE effects was most obvious for form factor and MAPK inhibition. Importantly, all of these inhibitors reversed CSE-induced increases in Drp1 and reductions in Mfn2, albeit to different extents (P < 0.05 for all inhibitors, both Drp1 and Mfn2; Fig. 4, C and D).

Fig. 4. Multiple signaling pathways mediate CSE effects on mitochondrial fission and fusion proteins in human ASM. A and B: quantification of mitochondrial morphology, in terms of form factor (A) and aspect ratio (B), when ASM cells are exposed to 1% CSE, in the presence of inhibitors for mitogen-activated protein (MAP) kinase (PD-98059), protein kinase B (Akt) (Akt inhibitor XIII), phosphatidylinositol 3-kinase (PI3K) (wortmannin), protein kinase C (PKC) (bisindolylmaleimide; BIS), or proteasome (lactacystin). Perturbing these pathways blocked CSE-induced mitochondrial fragmentation, albeit to different extents, with substantial effect of lactacystin and PD-98059, particularly for form factor. C and D: these morphological changes were accompanied by appropriate lack of changes in expression of Drp1 vs. Mfn2 following 1% CSE exposure. *Significant effect of CSE compared with untreated control. $Significant inhibitor effect (P < 0.05).
Because both Mfn2 and Drp1 have been shown as targets for ubiquitination (56, 91), we examined the effect of the proteasome inhibitor lactacystin (5 nM) on CSE-induced changes in mitochondrial morphology, Drp1, and Mfn2. Lactacystin was highly effective in reversing 1% CSE-induced reduction in form factor and the changes in Drp1 and Mfn2 ($P < 0.05$, Fig. 4).

Regulation of CSE-induced mitochondrial fission/fusion by transcription factors. CSE can influence protein expression at the transcription level. Again, with airway inflammation in mind, we focused on NF-κB and Nrf2 (21, 25, 28, 49, 55). To determine if these transcription factors are involved in CSE effects on mitochondrial fission-fusion, we used the following pharmacological inhibitors: SN-50 (NF-κB inhibitor, 20 μM) and trigonelline (Nrf2 inhibitor; 100 nM). Inhibiting activation of either transcription factor partly but significantly reversed 1% CSE-induced changes in form factor ($P < 0.05$, Fig. 5A) and aspect ratio ($P < 0.05$, Fig. 5B) at 24 h. Importantly, these inhibitors also prevented CSE-induced changes in Drp1 or Mfn2 ($P < 0.05$, Fig. 5, C and D).

In human ASM cells, exposure to 1% CSE for 2 h resulted in nuclear translocation of NF-κB subunits P50 and P65, as well as of Nrf2, as indicated by an increase in the nuclear-to-cytoplasmic ratio of their protein content (Fig. 6A, summaries in Fig. 6, B–D). Interestingly, transfection of ASM cells with Drp1 siRNA blunted nuclear translocation of these factors, even in the presence of CSE, whereas reducing Mfn2 expression with siRNA had an exacerbating effect. Even in control cells, Mfn2 siRNA promoted nuclear translocation of NF-κB subunits and Nrf2 ($P < 0.05$, Fig. 7).

CSE-induced ROS and mitochondrial fragmentation. ROS are known to cause mitochondrial damage (3, 70), and CS increases production of ROS (4, 78, 80). Accordingly, we examined whether CSE-initiated ROS generation contributes to mitochondrial fission. Using MitoSOX Red, we examined mitochondrial ROS levels in human ASM cells treated with CSE. Whereas untreated cells exhibited minimal fluorescence, exposure to increasing CSE concentrations resulted in increased MitoSOX fluorescence (Fig. 7A shows representative fluorescence images, Fig. 7B shows summary of measurements...
Mitochondria sense and respond to damage signals such as ROS. A potential confounding factor in the above studies was whether oxidative damage at baseline could occur in ASM cells (leading to injury) because of insufficient endogenous antioxidants. We examined whether an exogenous antioxidant was necessary for proper mitochondrial function in these cells. To this end, we added 10% ELF (39, 47) to the medium. With the use of the Seahorse Bioanalyzer, measurement of mitochondrial respiration showed that all of the relevant parameters (ATP production, maximum and spare respiratory capacity; Fig. 7C) decreased with exposure to CSE but that addition of ELF did not produce any additional protection in this regard (Fig. 7D). Importantly, even in control cells (no CSE), there was no effect of ELF on these parameters (Fig. 7D).

Exposure of ASM cells to SOD or catalase prevented CSE-induced changes in Drp1 or Mfn2 (P < 0.05 for either intervention, Fig. 7E) as well as form factor and aspect ratio (P < 0.05; not shown). Interestingly, inhibition of Drp1 expression using siRNA substantially blunted the CSE-induced increase in ROS levels; conversely, Mfn2 siRNA substantially elevated ROS levels (P < 0.05, Fig. 7F), thus linking these fission/fusion proteins to CSE-induced ROS generation.

Mitochondrial morphology in asmatic patients. To place the significance of mitochondrial fission-fusion machinery and CSE influence in a clinical context, we examined ASM cells isolated from patients with clinically diagnosed moderate asthma (n = 3 patients for asthmatic vs. nonasthmatic each). Mitochondria in ASM cells from asmatic subjects displayed an increased level of fragmentation at baseline compared with cells from nonasmatic subjects, reflected by loss of mitochondrial networking and shortening of mitochondrial tracks, and thus reduced form factor (Fig. 8A) and aspect ratio (P < 0.05, Fig. 8B). Also, at baseline, there was increased expression of Drp1 mRNA and protein, by reduced Mfn2 expression in cells from asmatic subjects (Fig. 8C). Furthermore, fission-related mRNAs (Drp1 and Fis1) are more abundantly expressed in ASM cells isolated from asthmatic humans (Fig. 8C). A concomitant reduction in other fusion gene levels (Mfn1, Opal) was also observed.
DISCUSSION

By quantitatively assessing mitochondrial structure and examining the mechanisms that regulate mitochondrial networks, the present study validates the emerging concept of mitochondrial dysfunction as a potential mechanism underlying CS effects in the airway. Exposure to CSE has direct consequences on mitochondrial morphology of human ASM cells, leading to the loss of the normal, connected, and networked structure typical of undamaged and functional mitochondria. CSE exposure causes not only a general change in the appearance of mitochondria but also a marked reduction in mitochondrial branching (form factor) and mitochondrial track lengths (aspect ratio), mediated by altered expression of the recently identified regulatory proteins Drp1 and Mfn2. Furthermore, mitochondrial fragmentation appears to be linked to mitochondrial function in that increased fragmentation leads to enhanced ROS generation, while in turn can contribute to increased Drp1 and reduced Mfn2, and thus greater mitochondrial fission (Fig. 9). These interactions are likely further perpetuated by enhanced signaling of pathways such as ERK, PI3K/Akt, and PKC and transcriptional regulation by NF-κB and Nrf2 that are relevant not only to CS-induced airway disease but airway...
inflammation and asthma as well. In this regard, asthmatic ASM appear to be more sensitive to CSE, with more fragmented mitochondria at baseline that are increasingly so following CSE exposure. From the perspective of alleviating the effects of CS in the airway, our data showing that interference with mitochondrial fission/fusion balance alters ROS generation and CS effects suggest a potentially novel target that may also be relevant to asthmatic ASM.

Cigarette smoking and secondhand smoke exposure result in respiratory disorders such as asthma and bronchitis, characterized by inflammatory changes of airway and lung parenchyma, and accompanied by exaggerated bronchial narrowing (hyperreactivity). Furthermore, CS has exacerbating effects on individuals with preexisting airway conditions such as asthma (43, 74, 84, 86). A key mechanism that could contribute to all of these parameters in the airway, but is still an emerging field of study, is mitochondrial dysfunction. In addition to being the powerhouse of the cell, mitochondria buffer cytoplasmic Ca\(^{2+}\) (41, 67, 72), modulate cell proliferation/death (38, 50, 88), and, relevant to CS and inflammation, produce ROS (4, 78, 80). In turn, ROS may influence intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and cell proliferation. Whereas the importance of mitochondrial ROS in the pathophysiology of asthma and other airway diseases has been suggested (23, 24, 42), the mechanisms by which CS can alter ASM mitochondrial function to produce airway disease are yet to be established. The potential clinical relevance of such an approach lies in emerging evidence that mitochondria-permeable antioxidants, which protect the electron transport chain from damage, can reduce allergic inflammation and airway hyperreactivity (17, 75), highlighting the importance of mitochondrial integrity and function in the airway.

The field of mitochondrial dynamics is still evolving but is being recognized as a key aspect of mitochondrial and cellular function. Two major themes regarding mitochondrial dynamics are potentially relevant to CS effects in ASM, a cell type in which there is currently no information. One aspect is that Ca\(^{2+}\) buffering by mitochondria is influenced by their proximity to other structures, especially the sarcoplasmic reticulum and plasma membrane (27, 57), where a number of [Ca\(^{2+}\)] regulatory proteins exist. Such spatial relationships are normally maintained by dynamic mitochondrial movement mediated by actin, tubulin, and other cytoskeletal structures (11, 50, 69, 71). On the other hand, the significance of mitochondrial shape and morphology in the context of function has only recently been studied in nonlung tissues (14, 15, 50, 89). Mitochondrial morphology varies between two extremes of a reticular network of fused mitochondria and a highly fragmented distribution. Within a cell, mitochondria dynamically undergo a fission-fusion cycle, breaking apart and joining together continually. When fused, mitochondria appear as tubular, branched, and networked assemblies, whereas fusion makes them look fragmented, with a shorter and less complex morphology. Both aspects are regulated by proteins involved in fusion vs. fission, with mutations in relevant genes being involved in several diseases (including Huntington’s and Alzheimer’s diseases). Regulation of mitochondrial fission/fusion is complex and still under investigation. Mfn1 and -2 and Opa1 have vital roles in mitochondrial fusion, whereas the dynamin-related protein Drp1 and Fis1 seem to mediate fission (14, 15, 50, 89). The GTPases Miro 1 and 2 control mitochondrial trafficking in a Ca\(^{2+}\)-dependent manner (34). The functional relevance of these proteins is suggested by the fact that Bax and Bak, members of the Bcl2 family of proteins that regulate apoptosis (12, 33, 44), are downstream of the mitochondrial fission/fusion proteins. The present study is the first to define the existence of Drp1 and Mfn1 in human ASM and, more importantly, to demonstrate their critical role in maintenance of mitochondrial structural and functional integrity with insults such as CS.

Maintenance of the precise fission-fusion is essential for mitochondrial function and therefore for cell survival and growth. Increased fission or fragmentation can lead to impaired energetics, impaired Ca\(^{2+}\) buffering, increased ROS generation, and perhaps greater apoptosis. Furthermore, elevated [Ca\(^{2+}\)] or ROS themselves can influence mitochondrial fission (as noted in our study using SOD and catalase), thus setting up a potential perpetual and detrimental cycle. Perturbation of mitochondrial fusion (which may or may not be equivalent to fission) results in defects in mitochondrial membrane potential and respiration, poor cell growth, and increased susceptibility to cell death. Excessive fusion can also result in impaired cellular energetics and deregulated cell proliferation. Overall, these emerging data suggest that a balance between mitochondrial fission vs. fusion is a key aspect of appropriate mitochondrial function. Accordingly, disruption of this balance in either direction can result in a multitude of effects on energetics, Ca\(^{2+}\) homeostasis, and cell survival/proliferation. In the current study, we demonstrate that CSE does indeed shift this balance not only toward enhanced fission (reflected by fragmentation) but also impaired fusion, thus blocking a beneficial,
compensatory pathway in ASM cells. More specifically, we show that CSE operates at the level of protein expression to regulate mitochondrial fission and fusion, as evidenced by an increase in Drp1 and a concomitant decrease in Mfn2, these expression patterns reflecting the opposing functions of these two proteins. This “tipping” of balance is a vital phenomenon involved in deciding the fate of a cell. It is generally believed that increased mitochondrial fragmentation signals cellular damage and initiates apoptotic death of the cell and that an unbroken fusion process, on the other hand, may lead to uncontrolled proliferation. Our experiments were designed to accommodate a maximum of 48 h exposure to 1% CSE, since longer durations cause massive cell death (data not shown). Although the correlation between these in vitro parameters and in vivo CS exposure can be debated, the striking amount of mitochondrial fragmentation that CSE induces in ASM cells within a short time period predicts imminent apoptosis, supporting the idea of CS-induced mitochondrial damage with potential long-term consequences for the airway. Taken at face value, this observation, however, appears incongruent in the context of asthma, an airway disease characterized simultaneously by increased ASM proliferation and remodeling. However, it should be noted that some studies suggest that upregulation of mitochondrial fission is a hallmark of cell proliferation and its constituent volatile oxidants and toxins. Whether epithelial mitochondrial morphology is affected to a greater extent is not well studied. Recently, Hoffmann et al. used BEAS-2B epithelial cells and primary bronchial epithelial cells from COPD patients and, interestingly, even though fragmented epithelial mitochondrial morphology was observed following addition of 10% CSE, expression of Drp1, Fis1, or Mfn2 mRNA was unaltered. An increase in Opal1 message was observed. These data are in sharp contrast to the differential influence of CSE on Drp1 vs. Mfn2 (or Opal1) in human ASM and suggest that the nature and extent of CS effects on mitochondrial structure and function may vary between airway cell types. Our data in ASM, which likely experience sublethal levels of inhaled CS compared with epithelium, demonstrate the potency of this insult in terms of mitochondria. Here, it is important to acknowledge that CSE is not an absolute equivalent of CS because of the absence of volatile oxidants and a higher number of toxins. On the other hand, the potent effects of CSE per se on ASM mitochondria only highlight the potential for CS to influence ASM and links mitochondrial fission and fusion to CS effects in the airway.

Our data also provide mechanistic insights into the regulation of structural/network changes in mitochondria, a novel aspect relevant to regulation of ASM structure and function, as well as cell signaling relevant to airway inflammation. Here, it appears that multiple signaling cascades are involved in mitochondrial fission/fusion regulation in ASM, including MAPK, PI3K/Akt, PKC, and ubiquitination. Production of ROS, which is a well-studied effect of CS exposure, has been known to activate these pathways. For example, the MAPK pathway was shown to be activated by ROS, in the context of cytokine signaling, and the Akt pathway was reported to be involved in peroxide-induced autophagy and changes in mitochondrial morphology in glioma cells. Recently, Gan et al. showed that oxidative stress-induced activation of the ERK pathway is a potential regulator of mitochondrial fission/fusion balance in neurons and nowak and Baskajova discerned a link between PKC-α and mitochondrial function in renal proximal tubular cells. However, to our knowledge, our current study is the first to identify these major signaling pathways as modulators of mitochondrial morphology in human ASM cells that have been exposed to CS. The relevance of these pathways also lies in their recognized role in airway inflammation. It is interesting to note here that, although previous studies did not find a reverse correlation, i.e., ERK/MAPK regulation of Mfn2-mediated mitochondrial morphology, our data suggest otherwise, at least in human ASM. A recent report by Qi et al. indicated that phosphorylation of Drp1 at Ser579 by PKC8 increases the mitochondrial localization of this fission protein. The increase in fusion/networked morphology that we observe with PKC inhibitor (BIS) treatment is in agreement with this observation, strengthening the notion that mitochondrial morphology may be controlled by cell proliferation/death-related mechanisms.

A further interesting finding presented here is that protein turnover mechanisms may be involved in controlling mitochondrial morphology. Drp1 has been known to be ubiquitinated by MARCH5 (MITOL) and Parkin (an E3 ubiquitin ligase), and the inhibition of the latter results in an accumulation of Drp1. The increase in Drp1 and Mfn2 is relevant to our finding of a more fragmented mitochondrial network at baseline in asthmatic ASM cells.

Given such a tight connection between the structural integrity of the mitochondria and a cell’s fate, it is not too surprising that signaling pathways that regulate one phenomenon are also involved in the other. Mfn2 has been shown to be an antiproliferation factor that acts by inhibiting the ERK/MAPK pathway. It is interesting to note here that, although previous studies did not find a reverse correlation, i.e., ERK/MAPK regulation of Mfn2-mediated mitochondrial morphology, our data suggest otherwise, at least in human ASM. A recent report by Qi et al. indicated that phosphorylation of Drp1 at Ser579 by PKC8 increases the mitochondrial localization of this fission protein. The increase in fusion/networked morphology that we observe with PKC inhibitor (BIS) treatment is in agreement with this observation, strengthening the notion that mitochondrial morphology may be controlled by cell proliferation/death-related mechanisms.

In addition to cytosolic signaling cascades, CS also seems to activate nuclear factors NF-κB and Nrf2. NF-κB has been known to mediate inflammation responses in the airway, and our data suggest that activation of this potent transcription factor can influence mitochondrial morphology. Interestingly, when Drp1 is downregulated, NF-κB translocation to the nucleus is blocked, indicating that the status of mitochondrial integrity may also influence activation of nuclear factors. Another signaling protein that we examined was Nrf2, an oxidative stress-responsive transcription factor that regulates expression of antioxidant genes by binding to the antioxidant...
response element (ARE) sequence on their promoters (37, 45). Nrf2 has been shown to be activated, in a macrophage cell line, in response to CS exposure (within 6 h) and trigger the expression of heme oxygenase-1 gene via the ERK/MAPK pathway (37). Consistent with this, we also see an increased nuclear translocation of Nrf2 within 2 h of CSE exposure. As a final point, it should be noted that the various signaling pathways that are potential regulators of mitochondrial morphology in CS-exposed ASM cells may not always act directly or in isolation; cross talk between pathways as occurs with Nrf2 and PKC (29, 30, 54) may color the eventual outcome in terms of mitochondrial structure and function, as well as ASM cell proliferation, apoptosis, or other functions.

There is increasing interest in the role of ROS in the pathophysiology of diseases such as asthma, where an oxidant-antioxidant imbalance is thought to occur in the presence of inflammation, resulting in increased epithelial mucus secretion, airway remodeling (e.g., cell proliferation), and increased airway contractility (1, 5, 65, 87). With mitochondria being a major source of ROS, and mitochondrial ROS generation known to be modulated by inflammation, this is an obvious target of CS (also a cellular stress akin to inflammation). Although it was beyond the scope of the current study to examine the complex chemistry of ROS generation per se in ASM, our data show that disruption of mitochondrial dynamics (fission/fusion) is one aspect of CS-induced ROS generation and its downstream effects relevant to asthma. Whereas low levels of ROS generation are part of cellular metabolism, and could promote cellular proliferation, differentiation, or apoptosis depending on cell type and redox potential, the substantially higher ROS induced by CS may induce apoptosis or DNA damage, resulting in abnormal cell proliferation of ASM. Here, a complicating factor may be how CS alters mitochondrial Ca\textsuperscript{2+} concentration, which could indirectly influence mitochondrial ROS, a topic that may be explored in future studies.

The relevance of our findings regarding mitochondrial morphology lies in the potential exacerbation of conditions such as asthma by factors such as CS. Given the focus on CS in this study, we examined asthmatic ASM in a limited fashion, using only moderate astmatic patients where sufficient remodeling and other changes are likely to have occurred to involve mitochondria. Accordingly, the three asthmatic ASM samples may not represent the wide range of asthma patients, or the potential variations in CS effects because of individual genetic and/or environmental factors. Nonetheless, our data do suggest that asthmatic ASM is already “primed” in terms of mitochondrial fragmentation, allowing factors such as CS to cause further degradation. Human ASM cells have been shown to be a significant source of cytokines, growth factors, and other inflammatory agents (60–62, 68). By affecting the mitochondria, and thus the downstream cellular functions, CS can actually activate secretion of these factors by ASM cells. This, in turn, may lead to exacerbation of asthma pathophysiology. In summary, the present study provides novel evidence to suggest that exposure to CSE triggers changes in mitochondrial structure in the airway, by modulating the mitochondrial fission-fusion apparatus via multiple cytosolic and nuclear signaling factors. Direct inhibition of fission protein expression, or inactivating signals that promote fission effectively, reverses the damages inflicted by CSE and disease conditions such as asthma on mitochondria.

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