Mitochondrial fragmentation in cigarette smoke-induced bronchial epithelial cell senescence

Hiromichi Hara,1 Jun Araya,1 Saburo Ito,1 Kenji Kobayashi,1 Naoki Takasaka,1 Yutaka Yoshii,1 Hiroshi Wakui,1 Jun Kojima,1 Kenichiro Shimizu,1 Takanori Numata,1 Makoto Kawaishi,1 Noriki Kamiya,2 Makoto Odaka,2 Toshiaki Morikawa,2 Yumi Kaneko,1 Katsutoshi Nakayama,1 and Kazuyoshi Kuwano1

1Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan; and 2Division of Chest Diseases, Department of Chest Surgery, Jikei University School of Medicine, Tokyo, Japan

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Mitochondrial fragmentation in cigarette smoke-induced bronchial epithelial cell senescence. Am J Physiol Lung Cell Mol Physiol 305: L737–L746, 2013. First published September 20, 2013; doi:10.1152/ajplung.00146.2013.—Mitochondria are dynamic organelles that continuously change their shape through fission and fusion. Disruption of mitochondrial dynamics is involved in disease pathology through excessive reactive oxygen species (ROS) production. Accelerated cellular senescence resulting from cigarette smoke exposure with excessive ROS production has been implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD). Hence, we investigated the involvement of mitochondrial dynamics and ROS production in terms of cigarette smoke extract (CSE)-induced cellular senescence in human bronchial epithelial cells (HBEC). Mitochondrial morphology was examined by electron microscopy and fluorescence microscopy. Senescence-associated β-galactosidase staining and p21 Western blotting of primary HBEC were performed to evaluate cellular senescence. Mitochondrial-specific superoxide production was measured by MitoSOX staining. Mitochondrial fragmentation was induced by knockdown of mitochondrial fusion proteins (OPA1 or Mitofusins) by small-interfering RNA transfection. N-acetylcysteine and Mito-TEMPO were used as antioxidants. Mitochondria in bronchial epithelial cells were prone to be more fragmented in COPD lung tissues. CSE induced mitochondrial fragmentation and mitochondrial ROS production, which were responsible for acceleration of cellular senescence in HBEC. Mitochondrial fragmentation induced by knockdown of fusion proteins also increased mitochondrial ROS production and percentages of senescent cells. HBEC senescence and mitochondria fragmentation in response to CSE treatment were inhibited in the presence of antioxidants. CSE-induced mitochondrial fragmentation is involved in cellular senescence through the mechanism of mitochondrial ROS production. Hence, disruption of mitochondrial dynamics may be a part of the pathogenic sequence of COPD development.

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD), which is characterized by progressive airflow limitation (3), is one of the leading causes of death worldwide (8, 32). Lung function declines with age, and the majority of patients diagnosed with COPD are elderly, suggesting intrinsic association between COPD and aging (1, 21, 22). Increased cellular senescence is a major feature of aging (5), and cellular senescence is accelerated in COPD lung (2, 47), indicating that cellular senescence may be fundamentally involved in the pathogenesis of COPD. Furthermore, cigarette smoke (CS) exposure, a known main cause of COPD (12), has been widely demonstrated to accelerate cellular senescence (15, 19, 48). Although the precise mechanisms of cellular senescence are still obscure, “the free radical theory of aging” has thus far been widely accepted (20). This theory proposes that accumulation of damaged macromolecules caused by reactive oxygen species (ROS) induces functional deterioration, resulting in cellular senescence. Indeed, protein and lipid oxidation increase with age (23, 31, 36), and ROS (11) and oxidative stress-induced lipid and protein modifications (17, 19, 40) have been demonstrated to be increased in COPD lung, suggesting that ROS may play an important role in the pathogenesis of COPD through the acceleration of cellular senescence.

Mitochondria are not only important organelles for ATP production but also the main site for ROS release through respiratory chain reactions (25). Hence, mitochondrial dysfunction has been shown to be responsible for cellular senescence (35, 42). Mitochondria are highly dynamic organelles that constantly fuse and divide (42, 54), and growing evidence suggests that the balance between fusion and fission is critical for determination of not only cellular function but also cell fate through the regulation of mitochondrial ROS production (43, 55). Indeed, mitochondrial dynamics have been demonstrated to play crucial roles in the pathogenesis of neurodegenerative disorders, including Parkinson’s disease (41), diabetes mellitus (43), and heart failure (10). However, the involvement of mitochondrial dynamics in lung diseases has not been studied extensively.

Although several lines of evidence demonstrated that CS exposure induces mitochondrial respiratory chain dysfunction and enhances mitochondrial ROS production in lung epithelial cells (50, 51), the participation of mitochondrial dynamics in CS-induced mitochondrial deterioration in the context of COPD pathogenesis has not been examined. Hence, we explored the involvement of mitochondrial dynamics in mitochondrial integrity, ROS production, and regulation of cellular senescence in the setting of cigarette smoke extract (CSE) exposure.

MATERIALS AND METHODS

Cell culture. Normal airways were obtained from first- through fourth-order bronchi from pneumonectomy and lobectomy specimens for primary lung cancer. Informed consent was obtained from all surgical participants as part of an approved ongoing research protocol.
by the ethical committee of Jikei University School of Medicine. Human bronchial epithelial cells (HBEC) were isolated with protease treatment and characterized as previously described (15, 19). Briefly, airways were treated with protease overnight, and cells derived from the airways were collected and plated on rat tail collagen type I-coated (10 µg/ml) dishes. After incubation overnight, the medium was changed to bronchial epithelial growth medium (Clonetics, San Di- ego, CA). Cultures were characterized immunohistochemically using antivimentin (Sigma-Aldrich, St. Louis, MO) and anticytokeratin (Lu-5; BioCare Medical, Concord, CA) antibodies. HBEC showed >95% positive staining with anticytokeratin and <5% positive staining with the antivimentin antibody.

Antibodies and reagents. Mouse monoclonal antibodies for Mitofusin1 (Abcam, Cambridge, MA), Mitofusin2 (Abcam), Drp1 (Cell Signaling Technology, Beverly, MA), β-actin (Sigma-Aldrich), TOM20 (Santa Cruz Biotechnology, Santa Cruz, CA), SDHA (Abcam), and HSP90 (BD Biosciences, San Jose, CA) and rabbit polyclonal anti-bodies for p21 (Cell Signaling Technology), OPA1 (Novus Biologicals, Littleton, CO), and Fis1 (Santa Cruz) were used. CM-H2DCFDA and bisbenzimide H 33258, and rat tail type-I collagen were purchased from Sigma-Aldrich. MitoTracker Red CMXRos and Mito SOX Red were purchased from Life Technologies (Carlsbad, CA). N-acyetylcycteine (NAC) and hydrogen peroxide (H2O2) were obtained from Wako (Osaka, Japan). Mito-TEMPO-H was purchased from Enzo Life Sciences (Farmingdale, NY).

Mitochondria isolation. Mitochondria fractions are isolated from HBECs by a commercially available kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions.

Preparation of CSE. CSE solution was prepared as described previously with some modifications (15, 19). Briefly, 10 ml of CS were drawn into a syringe and passed through 10 ml of PBS in a 15-ml Falcon tube. The smoke was expelled, and the process was repeated until one cigarette was used up. CSE solution was filtered (0.22 µm). The resulting solution was designated a 100% CSE solution and was used with the indicated dilution in the cell culture medium.

Senescence-associated β-galactosidase staining. Senescence-asso-ciated β-galactosidase (SA-β-gal) staining was performed using HBEC grown on 24-well culture plates according to the manufacturer’s instructions (β-galactosidase staining kit; BioVision Research Products). Cells were incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining was performed using HBEC from Wako (Osaka, Japan). Mitochondria were measured in each case, and average sizes were listed in Table 1. Mitochondria in COPD tended to be shorter in average size of major axis than that of control cases (Table 1).

CSE increases mitochondrial fragmentation in HBEC. To evaluate morphological changes of mitochondria in response to CSE exposure, we performed immunofluorescence staining with anti-TOM 20 to determine the mitochondrial outer mem-brane in HBEC. According to the staining pattern, the morphology of mitochondria was classified into three groups (elongated, fragmented, mitochondrial shorter than 1 µm without fusion to other mitochondria (usually round shape); others, staining pattern other than former two types.

Mitochondrial superoxide generation. Mitochondrial superoxide production was evaluated by Mito SOX Red (a fluorogenic dye for the highly selective detection of superoxide in the mitochondria) according to the manufacturer’s instructions. Mito SOX Red staining was evaluated by fluorescence microscopy and by flow cytometric analysis with a MACSQuant Analyzer (Milenyi Biotec, Bergisch Gladbach, Germany).

Measurement of ROS production in total cells. CM-H2DCFDA (Invitrogen) was used to measure total cellular ROS according to the manufacturer’s instructions. After incubation with CM-H2DCFDA (10 µM) for 10 min at 37°C, cells were harvested and immediately evaluated by MACSQuant Analyzer.

Western blotting. HBEC grown on six-well culture plates were lysed in RIPA buffer (Thermo Fisher Scientific) with protease inhibit-cocktail (Roche Diagnostics, Basel, Switzerland) and 1 mM sodium orthovanadate. Western blotting was performed as previously described (15, 19). For each experiment, equal amounts of total protein were resolved by 10–15% SDS-PAGE. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and incubation with specific primary anti-body was performed for 1 h at room temperature. After being washed several times with PBST, the membrane was incubated in horseradish peroxidase-conjugated secondary antibody (Bethyl Laboratories) fol-lowed by cheniluminescence detection with a kit (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific) with the LAS-4000 Uvmini system (Fujifilm, Tokyo, Japan).

Small-interfering RNA transfection. Small-interfering RNA (siRNA) targeting MFN1, MFN2, OPA1, Fis1, Drp1, and negative control siRNAs were purchased from Life Technologies. Transfections of HBEC were performed using the Neon transfection system (Invitrogen), using matched optimized transfection kits for airway epithelial cells.

The sequences of siRNA are as follows: siRNA against MFN1 was 5′-GCAGAACUCUGAGAUAACAU-3′ (Invitrogen); siRNA against MFN2 was 5′-GCAUUAGACUGCCGA-UAAU-3′ (Invitrogen); siRNA against OPA1 was 5′-GCUCUAGACGAGCCGGA-3′ (Invitrogen); siRNA against Drp was 5′-GGUUAAGACUGCAU- UAAU-3′ (Invitrogen); and siRNA against Fis1 was 5′-GGAUA- UCGAGAGCCCUAA-3′ (Invitrogen).

Statistics. Data are shown as averages (±SD) taken from at least three independent experiments. Student’s t-test was used for comparison of two data sets. Significance was defined as P < 0.05. Statistical software used was Excel (Microsoft).

RESULTS

Mitochondria in bronchial epithelial cells are fragmented in COPD lung tissues. To characterize the mitochondrial morphology in bronchial epithelial cells in COPD lung tissues, we examined mitochondria in surgically resected lung tissues by using electron microscopy. Mitochondria in bronchiolar epithelial cells were prone to be swollen and more fragmented in COPD compared with those in controls (smokers without COPD) (Fig. 1A). The major axes of more than 100 mitochondria were measured in each case, and average sizes were listed in Table 1. Mitochondria in COPD tended to be shorter in average size of major axis than that of control cases (Table 1).

CSE increases mitochondrial fragmentation in HBEC. To evaluate morphological changes of mitochondria in response to CSE exposure, we performed immunofluorescence staining with anti-TOM 20 to determine the mitochondrial outer membrane in HBEC. According to the staining pattern, the morphology of mitochondria was classified into three groups (elongated, fragmented, and others; Fig. 1B), as described in MATERIALS AND METHODS. CSE clearly induced mitochondrial fragmentation (Fig. 1C, top). We observed no significant increase in cell death (necrosis and apoptosis) after CSE exposure, which was examined by means of trypan blue staining and DNA fragmentation assays (data not shown). Quantification analysis of the
mitochondrial morphology shows that CSE exposure increased the percentage of cells with fragmented mitochondria after 48 h (Fig. 1D). CSE treatment also attenuated mitochondrial membrane potential compared with nontreated cells, which was determined by MitoTracker Red staining (Fig. 1C, middle), suggesting that the CSE-induced fragmented mitochondria may be dysfunctional.

Table 1. Mitochondrial size in lung tissue

<table>
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<th>Mitochondria &lt; 5 μm in</th>
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<tr>
<td></td>
<td>Major Axis, μm</td>
<td>Major Axis, %</td>
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<tr>
<td>Control 1 (smoker without COPD)</td>
<td>0.72 ± 0.44</td>
<td>41</td>
</tr>
<tr>
<td>Control 2 (smoker without COPD)</td>
<td>0.77 ± 0.45</td>
<td>32</td>
</tr>
<tr>
<td>Control 3 (smoker without COPD)</td>
<td>0.67 ± 0.41</td>
<td>45</td>
</tr>
<tr>
<td>COPD 1</td>
<td>0.43 ± 0.17</td>
<td>74</td>
</tr>
<tr>
<td>COPD 2</td>
<td>0.51 ± 0.36</td>
<td>68</td>
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<tr>
<td>COPD 3</td>
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Values are means ± SD. COPD, chronic obstructive pulmonary disease.

Mitochondrial ROS production is involved in CSE-induced HBEC senescence. Our previous study has demonstrated that CSE accelerated cellular senescence (15, 19), which was at least partly mediated through protein oxidation (19). Although CSE contains a wide array of toxic components, including ROS, the precise source of ROS involved in progression of cellular senescence in response to CSE exposure remains to be clearly elucidated. Mitochondria are a major source of intrinsic ROS production, and mitochondrial ROS is considered to be associated with aging (24, 44, 52). Accordingly, we hypothesized that mitochondrial ROS production participates in the regulation of CSE-induced cellular senescence. First, we examined the effect of CSE on total intracellular and mitochondrial ROS production. A DCFDA assay showed an increase in total intracellular ROS production in response to CSE (Fig. 2A), and MitoSOX Red staining demonstrated that CSE increased mitochondrial-specific ROS production (Fig. 2, B and C). NAC (an efficient antioxidant for intracellular ROS) and Mito-TEMPO (a specific antioxidant for mitochondrial ROS) inhib-
Mitochondrial fragmentation increases mitochondrial ROS production and HBEC senescence. Next, we investigated the role of mitochondrial fragmentation in ROS production and in cellular senescence. Mitochondrial fragmentation was induced by knockdown of fusion proteins, OPA1 or MFNs (MFN1, MFN2). MFN1 and -2 share high amino acid identity and appear to function coordinately to regulate mitochondrial fusion (29). Thus, to evaluate the role of MFNs, simultaneous knockdown of MFN1 and -2 was performed. Knockdown of OPA1 and MFNs apparently induced mitochondrial fragmentation, which was shown by immunofluorescence detection of TOM20 staining (Fig. 3A). Intriguingly, attenuation of mitochondrial membrane potential accompanied by increased ROS production was also observed by knockdown of either protein (Fig. 3, A and B). Knockdown of OPA1 and MFNs also induced HBEC senescence as measured by SA-β-gal positive staining and p21 expression (Fig. 3, C and D). This senescence was significantly inhibited by concomitant treatment with NAC and Mito-TEMPO. In contrast, mitochondrial fusion induced by knockdown of fission proteins (Fis1, Drp1) demonstrated no increase in HBEC senescence (Fig. 3E).

ROS increases mitochondrial fragmentation in HBEC. Although our findings indicate that increased mitochondrial ROS production is at least partly attributed to mitochondrial fragmentation, prior studies have demonstrated that extrinsic ROS can cause mitochondrial fragmentation (13, 38, 45). Furthermore, ROS within CSE has been proposed as the representative mechanism for toxicity (49). Hence, we examined the involvement of ROS in mitochondrial fragmentation in the setting of CSE exposure. CSE-induced mitochondrial fragmentation was clearly inhibited by the presence of NAC (Fig. 4A), indicating that ROS from CSE play a role in mitochondrial fragmentation.
Mito-TEMPO partially inhibited CSE-induced mitochondrial fragmentation, suggesting that ROS derived from mitochondria at least partly contributes to CSE-induced mitochondrial fragmentation (Fig. 4B). To further confirm the regulatory participation of ROS in mitochondria dynamics, we examined the effects of H₂O₂ on mitochondrial morphology in HBEC. H₂O₂ induced mitochondrial fragmentation, which was partially reversed by the presence of NAC (Fig. 4, B and C). In addition, NAC also inhibited H₂O₂-induced cellular senescence (data not shown).

**Drp1 and Fis1 were recruited to mitochondria by ROS exposure.** Mitochondrial dynamics are orchestrated by the balance of expression levels between mitochondrial fission and fusion proteins (54). To clarify the molecular mechanism of mitochondria fragmentation in response to CSE exposure, we isolated the mitochondrial fraction and examined the changes in expression levels of fusion proteins (MFN1, MFN2, OPA1) and fission proteins (Fis1, Drp1) in the mitochondrial fraction and total cell lysate, respectively. SDHA and HSP90 were mainly detected in the mitochondrial fraction and cytosolic fraction, respectively, indicating the efficient isolation of mitochondria (Fig. 5A). Although no significant changes in protein levels were observed in total cell lysates, significant increase of Drp1 and Fis1 was observed in the mitochondrial fractions (Fig. 5B). Furthermore, consistent with suppression of mitochondrial fragmentation, NAC inhibited the CSE-induced increase in Drp1 in the mitochondrial fraction (Fig. 5C), suggesting the involvement of ROS in CSE-induced Drp1 translocation. To further elucidate the involvement of Drp1 translocation to the mitochondrion in the mechanism of CSE-induced mitochondrial fragmentation, Drp1 siRNA experiments were performed. Drp1 knockdown clearly inhibited CSE-induced mitochondrial fragmentation, suggesting the potential participation of Drp1 in CSE-induced mitochondrial fragmentation (Fig. 5D). To clarify that Drp1-mediated mitochondrial fragmentation is a crucial step for mitochondrial...
ROS production, we examined MitoSOX staining in Drp1 siRNA-transfected HBEC. Drp1 knockdown clearly reduced MitoSOX staining in response to CSE exposure (Fig. 5E). Taken together, CSE-induced Drp1 translocation to the mitochondrial fraction is involved in mitochondrial fragmentation, which is responsible for mitochondrial ROS production.

DISCUSSION

Recent advances in mitochondrial biology have shown the pivotal involvement of mitochondrial dynamics in a wide array of physiological and pathological processes (10, 29, 30, 41, 43). However, a role for mitochondrial dynamics in lung disease pathogenesis has not been intensively investigated. Here, we initially illustrate the participation of mitochondrial dynamics in lung disease pathogenesis in terms of the regulation of HBEC senescence. Only a limited proportion of smokers develop clinical COPD, which may be attributed to the balance between the toxicity of CS exposure and host defensive mechanisms, suggesting that simple smoking exposure is not sufficient for COPD development. Thus, to clarify the clinically relevant causal link between mitochondrial dynamics and COPD development, we compared mitochondrial morphology in lung tissues from smokers without COPD and from COPD patients. In electron microscopic examination of lung tissues, we demonstrated that mitochondria in bronchial epithelial cells tended to be fragmented in COPD but not in smokers without COPD, suggesting the fission process dominance of mitochondrial dynamics in COPD pathogenesis. These results are in accordance with previous reports demonstrating that mitochondria in skeletal muscles from COPD patients tended to be smaller in size accompanied by increased ROS production (16, 37, 39).

Mitochondria fuse and divide with a purpose. In steady-state conditions, continuous changes of their shape through frequent fusion and fission are necessary for the maintenance of functional mitochondria with intact respiratory activity (54). Mitochondrial fusion rescues physiological stress by allowing functional mitochondria to complement dysfunctional mitochondria by diffusion and sharing of components between organelles (53, 55), and mitochondrial fission helps not only the appropriate distribution of mitochondria according to the local energy demand but also helps the removal of damaged mitochondria through mitochondrial-specific autophagic degradation (mitophagy) (33, 34, 53). In the setting of disruption of mitochondrial...
drial dynamics by excessive stress conditions, mitochondrial hyperfusion may be beneficial by increasing ATP production (stress-induced mitochondrial hyperfusion) (46), whereas excessive mitochondrial fragmentation appears to be potentially harmful for cells (9). Indeed, unbalanced mitochondrial fragmentation has been demonstrated to increase in dysfunctional mitochondria with excessive ROS and cytochrome c release, resulting in cellular dysfunction (9, 43). Consistently, we observed that both accumulation of fragmented mitochondria during CSE treatment and knockdown of fusion proteins are associated with increased ROS production and cellular senescence in HBEC (Figs. 1, 2, and 3). However, previous studies demonstrated that excessive mitochondrial fragmentation facilitates apoptosis (55) and elongation promotes senescence (26), possibly through the balance between cytochrome c release and ROS generation in HeLa cells. We speculate our findings of mitochondrial fragmentation-mediated cellular senescence can be attributed to the cell type-specific apoptosis resistance of HBEC. Indeed, compared with alveolar epithelial cells, HBEC have been demonstrated to be apoptosis resistant (7).

Fragmented damaged mitochondria are generally destined to be eliminated by mitophagy (14, 33); therefore, it is not surprising to speculate that not only the disruption of mitochondrial dynamics but also impairment of mitochondrial degradation by mitophagy are both responsible for accumulation of fragmented mitochondria. We have recently reported insufficient autophagic degradation during CSE-induced HBEC senescence (15), and now our recent findings also suggest there is insufficient mitophagy in the setting of CSE exposure (Ito, unpublished observations).

Mitochondrial dynamics are regulated by the balance of expression levels between mitochondrial fission (Fis1 and Drp1) and fusion (MFN1, MFN2, and OPA1) proteins (6, 34). CSE increased expression levels of Drp1 and Fis1 only in the mitochondrial fraction, suggesting that translocation of Drp1 and Fis1 to the mitochondrial fraction is involved in the mechanism of CSE-induced mitochondrial fragmentation. Posttranslational modification of Drp1 is a prerequisite for translocation to mitochondria, which is a fundamental process during mitochondrial fragmentation (4). Actually, translocation of Drp1 to the mitochondria is a proposed mechanism for ROS-induced mito-
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CSE exposure, future studies need to be performed using mainly based on in vitro experimental models using short-term excessive fragmentation. Finally, because our results were other mechanisms for mitochondrial ROS production with CSE-induced mitochondrial ROS release, it is likely that there mitochondrial fragmentation plays an important regulatory role in dimensional structures of mitochondria. Also, although mitochondrial fragmentation or mitochondrial-targeted antioxidants may ameliorate CS-induced acceleration of cellular senescence in HBEC. Therefore, appropriate control of mitochondrial dynamics or mitochondria-targeted antioxidants may ameliorate CS-induced acceleration of cellular senescence. Taken together, mitochondrial dynamics likely play an important regulatory role in CSE-induced cellular senescence in HBEC, which is a promising clue for understanding the pathogenesis of the senescence-associated lung disease COPD.

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DISCLOSURES

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

AUTHOR CONTRIBUTIONS


REFERENCES


