LSD1-S112A exacerbates the pathogenesis of CSE/LPS-induced chronic obstructive pulmonary disease in mice

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INTRODUCTION

Cigarette smoking is a critical risk factor for the development and progression of chronic obstructive pulmonary disease (COPD), characterized by chronic bronchitis and emphysema (1). Cigarette smoke (CS) induces inflammatory responses and oxidative stress by altering the cellular redox status, resulting in epigenetic modifications. Several studies have demonstrated that epigenetic enzymes including DNA methyltransferase, histone (de)acetylase, and histone (de)methylase, act as crucial regulators of COPD pathogenesis (2). For example, suppression of Histone deacetylase 2 (HDAC2) increases pro-inflammatory cytokine production through NF-κB signaling in CS-exposed cells and patients with COPD (3). Histone deacetylase SIRT1-regulated expression of matrix metalloproteinase 9 is associated with inflammation and lung remodeling in patients with COPD (4). Although epigenetic modifiers are widely reported to be involved in the regulation of COPD pathogenesis (5), the role of histone demethylases in this phenomenon is not well studied. Recent studies revealed that the expression of USP38, a potential deubiquitylating enzyme of lysine-specific demethylase 1 (LSD1), also known as KDM1A, is upregulated in the COPD lung tissue. USP38 enhances the stability of LSD1, thereby activating signaling pathways and promoting cell proliferation and colony formation (6, 7), which suggests a role of LSD1 in COPD pathogenesis.

LSD1 is a member of the flavin adenine dinucleotide-dependent amine oxidase family (8). LSD1 was first isolated as an interaction partner of the histone deacetylase HDAC2 and identified as a histone demethylase (9). LSD1 specifically removes methyl groups from histone H3 lysine 4 and H3 lysine 9, and thus, can operate as a transcriptional repressor or activator, respectively (10). Furthermore, LSD1 can demethylate and regulate the function and stability of non-histone proteins such as p53, DNMT1, HIF-1α and p65, which are involved in gene expression (11). Recent evidence showed that several kinases, such as PKCα, PLK1, CK2 and GSK3β, phosphorylate serine residues in LSD1 and thereby, regulate its biological functions (12-16). For example, PKCα-mediated phosphorylation of LSD1 on S112 regulates diverse biological processes including circadian rhythm, presynaptic plasticity, epithelial-mesenchymal transition, and inflammatory responses (12, 15,
17, 18). Furthermore, phosphorylation of LSD1 by PKCα represents a crucial mode of epigenetic regulation in inflammatory diseases, such as sepsis and inflammatory bowel disease in murine models (15, 19). However, little is known about the precise role of LSD1 in CS-induced pulmonary inflammation in the COPD model.

In this study, we explored the effects of Lsd1-S112A on the inflammatory response and oxidative stress in the COPD model using phosphorylation defective mutant Lsd1-S112A (Lsd1SA/SA) mice.

RESULTS

Lsd1SA/SA mice are susceptible to CSE/LPS-induced COPD

To investigate the role of Lsd1-S112A in the onset of COPD in vivo, as an initial approach, WT and Lsd1SA/SA mice were injected intratracheally with CSE-only, LPS-only, or CSE and LPS (CSE/LPS), which are well-established approaches for developing COPD in mice (Fig. 1A). WT and Lsd1SA/SA mice treated with CSE or LPS showed COPD-like lung pathology, characterized by immune cell infiltration and airspace enlargements. Histopathological examinations revealed that CSE-only and LPS-only injections triggered lung injury and airspace enlargements in both WT and Lsd1SA/SA mice (Fig. 1B). This indicated that CSE and LPS can individually induce emphysema in mice. Airspace enlargement was evaluated by comparing the mean linear intercept (MLI). MLI scoring revealed significant airspace enlargements in Lsd1SA/SA mice than in WT littermates, when comparing the vehicle control, CSE, or CSE/LPS groups independently (Fig. 1C). The difference in the MLI scores between the WT and Lsd1SA/SA mice was greater in the CSE-only group than in the LPS-only group (Fig. 1C). In addition, histological images showed that the infiltration of inflammatory cells around the airways and vessels was induced by CSE and LPS treatment (Fig. 1D), and airway inflammation score, which is evaluated by scoring the presence of peribronchial and perivascular inflammation, was higher in Lsd1SA/SA mice compared with WT mice when CSE/LPS was administered (Fig. 1E).

To address whether Lsd1SA/SA mice exhibit increased inflammation, we evaluated the immune cells in the bronchoalveolar lavage fluid (BALF) one day after the last dose of CSE or LPS in WT and Lsd1SA/SA mice (Fig. 2A). Cells in the BALF were stained with Diff-Quik and the total number of cells was evaluated. Representative images of cells in the BALF from each group are shown in Fig. 2B. LPS-injected WT and Lsd1SA/SA mice exhibited a marked increase in immune cell infiltration in BALF (Fig. 2C), consisting mainly of macrophages and neutrophils (20). In the CSE/LPS-injected Lsd1SA/SA mice, the number of infiltrated immune cells increased by 2.7-fold compared with those in WT mice, reaching levels observed in both WT and Lsd1SA/SA mice injected with LPS-only, but the increased level was much smaller in WT mice (Fig. 2C). Consistently, the number of neutrophils infiltration, the major infiltrating cell type, increased by 6.0-fold in Lsd1SA/SA mice than in WT mice when treated with CSE/LPS (Fig. 2D). CSE has immunosuppressive effects leading to impairment of antimicrobial defense mechanisms in the airway epithelium. Neutrophil recruitment factors released by LPS treatment, such as GM-CSF and IL-8, are known to be reduced following CSE exposure (21). Taken together, we found that Lsd1SA/SA mice were more susceptible to CSE/LPS-induced lung damage and inflammatory responses, compared with those seen in WT mice.

Effect of Lsd1SA/SA on Cox-2 and Ho-1 expressions in CSE/LPS-induced COPD

Exposure to CSE promotes the pathogenesis of COPD by increasing oxidative stress and inflammation in the lung (22). To examine whether the phosphorylation status of LSD1 affects airway inflammation and oxidative stress following CSE and LPS exposure, we compared the expression of Cox-2 and

![Fig. 1. Lsd1SA/SA mice are highly susceptible to CSE/LPS-induced COPD.](image-url)
Role of LSD1 phosphorylation in murine COPD model
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Fig. 2. Analysis of CSE/LPS-induced inflammatory and oxidative stress responses in Lsd1<sup>S/A</sup> mice. (A) Schematic diagrams of murine COPD model for analyzing the number of cells in BALF. (B) Photomicrographs of Diff-Quik-stained cells are shown at 200x magnification and (C) total cells and (D) neutrophils count in BALF from the indicated treatment groups. n = 6 per group; data are presented as mean ± SEM; *P < 0.05. (E, F) Immunoblot analysis was performed using the indicated antibodies in lung homogenates. Lane 1-3: control group in Lsd1<sup>+/+</sup> mice, Lane 4-6: control group in Lsd1<sup>S/A</sup> mice, Lane 7-9: CSE/LPS group in Lsd1<sup>+/+</sup> mice, Lane 10-12: CSE/LPS group in Lsd1<sup>S/A</sup> mice. The immunoblots were quantified using ImageJ. n = 6 per group; data are presented as mean ± SEM; *P < 0.05.

Fig. 3. Increased biomarkers of oxidative stress in Lsd1<sup>S/A</sup> MEFs exposed to CSE and LPS. (A, B) Cox-2 and Ho-1 protein levels in WT and Lsd1<sup>S/A</sup> MEFs challenged with CSE at the indicated dose for 24 h or 10% CSE for the indicated time periods, respectively, were assessed by western blotting. mRNA and protein levels of Cox-2 (C, D) and Ho-1 (E, F) in WT and Lsd1<sup>S/A</sup> MEFs were shown after treatment with CSE and LPS for 4 h or 24 h, respectively. All results represent three independent experiments. Data are presented as mean ± SEM; *P < 0.05. (G) 4-HNE-modified protein was used as an oxidative damage marker. The immunoblots were quantified using ImageJ. Graph was obtained from five independent experiments. Data are presented as mean ± SEM; *P < 0.05, ***P < 0.001.

Increased levels of oxidative stress biomarkers in Lsd1<sup>S/A</sup> MEFs exposed to CSE and LPS

Ho-1 in the WT and Lsd1<sup>S/A</sup> lung tissues from each group. We quantified the Cox-2 and Ho-1 protein levels by immunoblotting and densitometry. When treated with CSE/LPS, Cox-2 levels were significantly upregulated in Lsd1<sup>S/A</sup> mice compared with the level in WT mice (Fig. 2E). Ho-1 expression was also upregulated in Lsd1<sup>S/A</sup> mice upon CSE/LPS treatment (Fig. 2F). These results indicate that Lsd1 phosphorylation on S112 is related to the regulation of Cox-2 and Ho-1 expression upon CSE/LPS exposure in vivo. Taken together, our in vivo data suggest that Lsd1-S112A exacerbates inflammation and oxidative stress in CSE/LPS-induced COPD.

Increased levels of oxidative stress biomarkers in Lsd1<sup>S/A</sup> MEFs exposed to CSE and LPS

Although we detected elevated expression of Cox-2 and Ho-1 in the lungs of Lsd1<sup>S/A</sup> mice compared with that in WT mice upon CSE/LPS treatment, the in vivo data is the final outcome of combinatorial signaling. To investigate whether Lsd1-S112A directly affects inflammation and oxidative stress, we compared the pattern of Cox-2 and Ho-1 expression in WT and Lsd1<sup>S/A</sup> MEFs following CSE and LPS exposure. MEFs were treated with CSE at the indicated doses and time points, and the cell lysates were used for immunoblotting. The Cox-2 and Ho-1 protein levels showed a dose-dependent increase upon CSE treatment, which was further enhanced in Lsd1<sup>S/A</sup> MEFs at a high concentration of CSE (i.e., 20%) compared with those in WT MEFs (Fig. 3A). The time-course analysis also exhibited higher and longer expression of Cox-2 and Ho-1 in Lsd1<sup>S/A</sup> MEFs compared with that in WT MEFs (Fig. 3B). Thus, these results confirm in vivo data that Lsd1<sup>S/A</sup> mice are more sensitive to CSE/LPS treatment with increased expression of Cox-2 and Ho-1.

WT and Lsd1<sup>S/A</sup> MEFs were then treated with CSE, LPS or CSE/LPS for 4 h or 24 h to assess the mRNA and protein levels of Cox-2 and Ho-1. The Cox-2 mRNA and protein levels were higher in Lsd1<sup>S/A</sup> MEFs compared with WT MEFs in each group (Fig. 3C, D). Interestingly, elevated expression of Ho-1 in Lsd1<sup>S/A</sup> MEFs, compared with WT MEFs, was specific to the groups containing CSE (i.e., CSE and CSE/LPS group) (Fig. 3E, F). 4-Hydroxy-2,3-trans-nonenal (4-hydroxynonenal, 4-HNE), the aldehydic product of lipid peroxidation, is widely accepted as a reliable marker for oxidative stress (23). WT and Lsd1<sup>S/A</sup> were.
MEFs were treated with CSE, LPS or CSE/LPS for 24 h to assess the cellular levels of 4-HNE-modified proteins as an indicator of oxidative stress. Immunoblot analysis revealed higher levels of 4-HNE-modified proteins in Lsd1<sup>S112A</sup> MEFs compared with WT cells in all groups (Fig. 3G). 4-HNE is a cytotoxic product of lipid peroxidation and acts as a signaling molecule. It has been uncovered as a factor causing pathological changes under oxidative stress and a key mediator of oxidative stress-induced cell death (24). Therefore, it is likely that Lsd1-S112A MEFs are intrinsically more sensitive to oxidative stress.

**Effect of Go6976 on CSE/LPS-induced Cox-2 and Ho-1 expression in MEFs**

To validate the hypothesis that the phosphorylation defect of Lsd1-S112 is critical for the expression of Cox-2 and Ho-1 proteins in MEFs, we used Go6976, a PKCα inhibitor, to block the phosphorylation of Lsd1-S112. Immunoblot analysis was performed on MEF extracts following 24 h CSE and LPS treatment in the absence or presence of Go6976. Interestingly, pre-treatment with Go6976 enhanced Cox-2 and Ho-1 expression (Fig. 4A, B). In a separate experiment, MEFs were treated with Go6976 or vehicle for 1 h and then treated with CSE, LPS, or CSE/LPS for 4 h to evaluate the Cox-2 and Ho-1 mRNA levels. Pre-treatment with Go6976 markedly increased Cox-2 and Ho-1 mRNA expression (Fig. 4C, D). These data indicate that inhibition of Lsd1-S112 phosphorylation up-regulated CSE-induced Cox-2 and Ho-1 transcription. Our data suggest that the Lsd1-S112A mutation and Go6976 treatment indeed strengthen CSE and LPS-induced Cox-2 and Ho-1 protein expression. Taken together, our findings reveal that the phosphorylation defect on Lsd1-S112 enhances inflammatory and oxidative stress responses and exacerbates CSE/LPS-induced COPD (Fig. 4E).

**DISCUSSION**

LSD1 is an epigenetic regulator that controls lysine methylation status of histones and non-histone proteins, and its biological functions are regulated by post-translational modifications including phosphorylation (12, 13, 17, 18). Emerging evidence indicates that phosphorylation of LSD1 at serine 112 (equivalent to serine 111 on the human protein) plays a crucial role in the regulation of inflammatory responses in sepsis and IBD models (15, 19). To the best of our knowledge, this is the first study to demonstrate the effect of Lsd1-S112A on the murine CSE/LPS-induced COPD model.

Chronic inflammation and oxidative stress are important features of the pathogenesis of COPD. CS, a potent inducer of neutrophilic inflammation, alters the inflammatory responses by modulating the production of cytokines and chemokines with consecutive recruitment of macrophages and neutrophils, and thus, causing further damage to the lung tissue (25). CS is a major exogenous source of oxidative stress in the lung. Oxidative stress is implicated in airway inflammation and is one of the major prognostic factors in the pathogenesis of COPD. High levels of ROS are generated by neutrophils following CS exposure and contribute to oxidative stress (21). In the current study, we showed that intratracheal instillation of CSE combined with LPS led to increased immune cell influx and more severe parenchymal destruction in Lsd1<sup>S112A</sup> mice compared with that in WT mice. Lsd1<sup>S112A</sup> mice exhibited higher expression of both Cox-2 and Ho-1 proteins upon CSE/LPS exposure which contributed to airway remodeling and inflammation. Furthermore, several oxidative stress products, such as 4-HNE and Ho-1, were also increased in the Lsd1<sup>S112A</sup> MEFs after CSE/LPS treatment. Lung tissues from patients with COPD show increased lipid peroxidation, as measured by 4-HNE levels (26). Lipid peroxidation products, such as Ox-PLs and 4-HNE are known to mediate cytotoxicity by disrupting membrane integrity and activating cell death pathways including apoptosis and ferroptosis (27). Our results indicate that Lsd1-S112A mice are more susceptible to inflammation and oxidative stress in response to CSE and LPS, compared with WT mice. This suggests that phosphorylation defect of LSD1 on S112 is involved in exacerbating lung damage. Further investigations are required to understand the
underlying mechanism how LSD1 phosphorylation contributes to the restriction of oxidative stress in CSE-induced response. A recent study revealed that the transcriptional activity of LSD1 is modulated by chromatin remodeling, and depends on steady ROS levels and redox signaling (28). It is also reported that the changes in the redox environment increases LSD1 activity in DNA repair after oxidative damage (29). We speculate that phosphorylation-defective Lsd1-S112A is involved in excessive oxidative stress, leading to lung damage due to endogenous ROS.

Taken together, our findings reveal that the phosphorylation defect on Lsd1-S112 is critical for inflammation and oxidative stress upon CSE/LPS exposure and plays a key role in the exacerbation of COPD. This study provides new insights into the correlation between LSD1 phosphorylation and oxidative stress in exacerbating COPD.

MATERIALS AND METHODS

Animals
Generation of Lsd1<sup>S112A</sup> mice of the C57BL/6J strain have been described previously (12). WT and Lsd1<sup>S112A</sup> female mice aged 6-10 weeks were used in this study. All the animal experiments were conducted strictly in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Sookmyung Women’s University (SMWU-IACUC-1711-029).

Preparation of CSE
We used THIS cigarettes (KT&G, Korea), and each cigarette contained 1.1 mg nicotine, 15 mg tar, and 15 mg carbon monoxide. Briefly, the smoke from five cigarettes was bubbled slowly through 100 ml phosphate-buffered saline using a vacuum pump, which was considered as a 100% CSE solution. The soluble CSE was then sterilized and stored at −80°C.

CSE and LPS-induced COPD murine model
Mice were treated with CSE, LPS or CSE/LPS solutions for establishing the COPD murine model (Fig. 1). Briefly, the solutions were administered by intratracheal injection 4 times at 7-day intervals. The mice were divided into four groups as follows; control group (100 μl PBS), CSE group (100 μl CSE), LPS group (10 μg LPS in 100 μl PBS) and CSE/LPS group (10 μg LPS in 100 μl CSE). The mice were euthanized at day 7 or 24 h after the last dose.

Analysis of bronchoalveolar lavage fluid (BALF)
BALF was obtained through the tracheal cannula by washing the airway lumen with 1 ml of sterile saline. Total cell counts in the BALF were determined using a hemocytometer after lysis of red blood cells with a red blood cell lysis buffer (Sigma, USA). Differential cell counts in the BALF were examined by Cytospin preparation followed by staining with the Diff-Quik reagent (Sysmex International Reagents, Kobe, Japan).

Histological analysis
The left lung tissue of each mouse was fixed with an intratracheal injection of 4% paraformaldehyde and immersed in the same fixative to preserve pulmonary architecture. The fixed lungs were then embedded in paraffin using standard procedures. Lung sections (4 μm) were stained with H&E for histological examinations. MLI and inflammation score were determined blindly by two independent observers (30).

Cell culture and treatments
MEFs were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% Penicillin/streptomycin (Gibco). All cells tested negative for the presence of mycoplasma before experiments. The cells were treated with 10% CSE and 1 μg/ml LPS for the indicated time periods.

Immunoblotting
Lung tissue and cells were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (GenDEPOT). Total protein was quantified using the Bradford assay (Bio-Rad) according to the manufacturer’s instructions and subjected to SDS-PAGE. Primary antibodies used were anti-HO-1 (sc-136960), anti-COX-2 (CST, #12282), anti-Tubulin (Sigma, T6074), anti-GAPDH (sc-25778), and anti-4-HNE (Abcam, ab46543). Specific bands were quantified by densitometry using NIH ImageJ.

Real-time RT-PCR
Total RNA was isolated using the TRIzol reagent (Thermo-Fisher Scientific) according to the manufacturer’s protocol. The complementary DNA (cDNA) was synthesized from 1 μg total RNA using the cDNA synthesis kit (Thermo-Fisher Scientific). Quantitative PCR was performed using SYBR Green (Applied Biosystems) and detected on the StepOnePlus™ Real-Time PCR system (Applied Biosystems). Primers used for the mouse genes are as follows. Ho-1: 5′-GGTGTAGTGCTTC-CTTGATCC-3′ (FW), 5′-AGTGAGGCCCATACCAGA AG-3′ (RV), Cox2: 5′-AACCGCATGCTCTGATAG-3′ (FW), 5′-CATGTTCCAGAGGATGGGAG-3′ (RV), Gapdh: 5′-TCCCCACTCTTCCACCTCGA-3′ (FW), 5′-AGITGGGATAGGGCCTCTTTG-3′ (RV).

Statistical analysis
All values are shown as mean ± standard error of mean (SEM). Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) and then determined with the least significant difference test. The criterion for significance was P < 0.05.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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