Cpt1a alleviates cigarette smoke-induced chronic obstructive pulmonary disease

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Abstract. The current study aimed to determine the expression of carnitine palmitoyltransferase 1A (Cpt1a) in the lung tissue of chronic obstructive pulmonary disease (COPD) patients and its correlation with lung function. An increase in Cpt1a expression improved lung function in patients with COPD by inhibiting apoptosis and the inflammatory response of lung endothelial cells. Lung tissues of 20 patients with COPD and 10 control patients were collected, their Cpt1a expression was determined by western blotting and apoptosis and inflammation were assessed by haematoxylin-eosin staining, TUNEL assay and ELISA. Mice with knockout or overexpression of Cpt1a were constructed by lentivirus *in vivo*. A COPD model was induced by cigarette smoke and the role of Cpt1a in COPD was determined *in vivo* and *in vitro*. Cpt1a expression was positively correlated with lung function and negatively correlated with apoptosis and inflammation. Patients with COPD with higher expression of Cpt1a in lung tissues had improved lung function indices and lung tissue morphology with less apoptosis and decreased inflammatory response. Compared with the control group, COPD mice with Cpt1a knockdown had aggravated lung dysfunction and increased lung inflammation and apoptosis. Overexpression of Cpt1a alleviated lung dysfunction and reduced inflammatory response and apoptosis of lung tissues in COPD mice. Pulmonary microvascular endothelial cells of mice were isolated *in vitro* and the results were consistent with the findings obtained *in vivo*. In conclusion, the clinical, *in vivo* and *in vitro* data confirmed for the first time that Cpt1a alleviated lung dysfunction of patients with COPD by inhibiting apoptosis of endothelial cells and inflammatory responses.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common and frequently occurring respiratory disease with high morbidity and mortality. COPD has become an important public health problem due to the economic burden it imposes on society (1). Numerous studies have shown that cigarette smoke exposure is the predominant risk factor for the development of COPD (2). COPD is characterized by an abnormal inflammatory response, airway obstruction, alveolar destruction and apoptosis (3). Pulmonary vascular endothelial cells are one of the main components of pulmonary vessels. Apoptosis and the pulmonary vascular endothelial cell inflammatory response increase in some patients with COPD, which may aggravate morphologic alterations of lung tissues and lead to significant abnormalities in lung function indices and a corresponding decline in lung function (4). However, the mechanism of cigarette-induced COPD causing apoptosis and inflammation of pulmonary vascular endothelial cells has not been established.

Carnitine palmitoyltransferase 1A (Cpt1a) is a key enzyme located to the inner mitochondrial membrane and involved in the regulation of fatty acid oxidation. Cpt1a transports fatty acids from the cytoplasm to the mitochondria for subsequent fatty acid oxidation (5). Fatty acid oxidation protects against endothelial cell apoptosis and lung injury induced hyperoxia in neonatal mice (6). Metabolism of fatty acid in endothelial cells were found to be dysregulated in patients with COPD. Oxidation of fatty acids reduction increases apoptosis in lung endothelial cells treated with cigarette smoke extract (7). In addition, Cpt1a deficiency can inhibit endothelial cell proliferation and angiogenesis (8). Our previous study showed that L-carnitine upregulates Cpt1a, promotes an increase in substrate fatty acids and inhibits cigarette-induced endothelial cell apoptosis (9). However, the role of Cpt1a in pulmonary vascular endothelial cells in COPD remains to be elucidated.

Therefore, the hypothesis of the current study is that Cpt1a alleviates cigarette smoke-induced chronic obstructive pulmonary disease by promoting fatty acid oxidation to inhibit

the inflammatory response and cell apoptosis. The main contents of the present study were as follows: i) clarifying the correlation between Cpt1a and lung apoptosis, inflammation and lung function in patients with COPD at the clinical level; ii) confirming whether Cpt1a can treat cigarette-induced COPD in an animal model; and iii) clarifying the mechanism underlying Cpt1a protection of COPD lungs.

Materials and methods

Subject recruitment and sample preparation. All animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures of the present study were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (CMTT number 2013012) and in accordance with international standards. Prior to the clinical study, the subjects (n=20) were informed of the nature, purpose, possible benefits and risks of the trial and the subjects voluntarily confirmed their consent to participate. The inclusion criteria were as follows: i) Patients who met the Global Initiative for Chronic Obstructive Lung Disease (GOLD) in 2015 with severe COPD, ii) no bronchiectasis, iii) no bronchial asthma, iv) no heart failure and v) patients who underwent pulmonary resection. The exclusion criteria were as follows: patients i) with severe hepatic and renal dysfunction, ii) hematologic diseases, iii) usage of immunosuppressants in recent 3 months, or iv) severe immune system diseases. All patients were detected for lung function which was diagnosed as different degrees of dyspnoea, shortness of breath, cough, chronic cough and other symptoms. In addition, patients (n=10) without COPD who underwent pulmonary resection in The Second Hospital of Shanxi Medical University during the same period were selected as the control group. Postoperatively, the lung tissues of all selected patients were collected and frozen in a -80˚C refrigerator.

Animal model. The tails of healthy adult C57BL/6 mice (5 groups, 10 mice/group, 50% male and 50% female, Age: 8~10 weeks, weight 18~22 g, 12 h light/12 h dark, Temperature is 18~22˚C, humidity, 50-60%, all mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were injected with pGLVU6/GFP-short hairpin (sh)RNA control, pGLVU6/GFP-shRNA Cpt1a, pGLVU6/GFP control and pGLVU6/GFP-Cpt1a lentivirus to establish mouse models with knockdown of Cpt1a or overexpression of Cpt1a. At two weeks after the injection, the above four groups of mice were placed in a self-made tobacco smoke inhalation exposure device (30x40x90 cm plexiglass cuboid with nine evenly distributed circular exhaust holes, 2 cm in diameter, on the cover) and a wooden rectangular box containing cigarettes (Furong brand, China Tobacco Hubei Industrial LLC) was placed in it. The principal combustion products of cigarette were as follows: nicotine content in smoke, 1.2 mg/cigarette; carbon monoxide content in smoke, 14 mg/cigarette; and tar content, 15 mg/cigarette. The control group were exposed to smoke-free air and raised normally. To establish the model of COPD in mice, the mice in the model group were exposed to cigarette smoke four times a day in the device. A total of six cigarettes were lit each time and left burning for 1 h, with the smoke concentration in the closed box reaching 100-120 mg/m^3. The mice were allowed to breathe smoke-free air for 30 min between two times of smoke exposure. The procedures were conducted six days per week and lasted for four weeks. The experiment included five groups (n=10): control group; pGLVU6/GFP-shRNA control + COPD group; pGLVU6/GFP-shRNA Cpt1a + COPD group; pGLVU6/GFP control + COPD group; and pGLVU6/GFP-Cpt1a + COPD group.

Murine lung function testing. The lung function of mice was tested by using Buxco Fine Pointe Series Whole Body Plethysmography (Buxco Research Systems). The test indexes were as follows: Tidal volume (TV), peak expiratory flow (PEF), 50% expiratory flow (EP50), forced expiratory volume in 0.3 seconds (FEV0.3) and forced vital capacity (FVC).

Detection of differences in primary pulmonary microvascular endothelial cells (PMVECs) fatty acid oxidation. PMVECs from different experimental groups were serum-starved in 12-well plates, then washed with warm PBS. The cells were incubated with ^{14}C-labeled FAO (Fatty acid oxidation (FAO) medium consisting of DMEM-low glucose (Invitrogen; Thermo Fisher Scientific, Inc.), 0.25 μCi/ml (1-14C) palmitate, 0.25 μCi/ml (1-14C) oleate, 50 μM palmitate, 50 μM oleate, 0.5% BSA, 1 mM carnitine and 12.5 mM HEPES (pH-7.4) at 37˚C for 3 h. The procedures were thrice repeated for each group. After 3 h, the culture medium was collected from each well and an equal portion of the culture medium was distributed to a sealed trapping device. $^{14}CO_2$ was removed from the medium fraction by adding perchloric acid and captured in NaOH, which was collected and analyzed by liquid scintillation counting to determine the complete FAO ratio of CO_2. The acidified medium was collected, refrigerated and centrifuged 5 min at 16,000 x g and 4˚C. The acid soluble metabolite (ASM) of FAO was determined by liquid scintillation counting analysis of the equal samples. Cells were thrice rinsed with cold Hank's balanced salt solution (HBSS) and lysed with SDS lysis buffer. The protein concentration of the lysate was determined by the BCA assay. The results of FAO were expressed as a percentage of CO_2.

Determination of ceramide. The lung tissue homogenate sample from mice was placed into the centrifuge tube, then the internal standard mix was added. After vortex oscillation blending, the sample was centrifuged (1,000 x g, 4-8˚C, 5 min) and the supernatant was collected for later detection. Ceramide was analysed by LC-MS. Agilent 6530 Q-TOF was used to identify and analyse levels of ceramide, which was confirmed by comparing retention times and tandem mass spectrometry data with standard compounds. The results were corrected for naturally occurring 13C impurity of the tracers. MassHunter Quantitative Analysis software (Agilent Technologies, Inc.) was used to quantify the ceramide content.

Western blotting. Protein was extracted from RIPA lysate (Huaxingbio) containing protease inhibitor (Thermo Fisher Scientific, Inc.). The tissues or cells were ultrasonically disrupted, then centrifuged at 14,000 x g and 4˚C for 15 min. The supernatant was collected and the protein concentration was determined using the BCA assay. Proteins (30 μg) were

separated by SDS-PAGE (10%) together with a pre-stained protein ladder (Thermo Fisher Scientific, Inc.), then transferred to nitrocellulose membranes (MilliporeSigma, blocked with 5% non-fat milk in Tris-Buffered saline and Tween-20 (TBST; 20 mmol/l Tris-Cl, 150 mmol/l NaCl, 0.05% Tween 20, pH 7.4) at 4~8˚C for 2 h and incubated overnight with primary antibody (cat. no. #97361; 1:1,000, CST) at 4˚C. After being washed with buffer, the membranes were incubated with secondary antibodies(Anti-rabbit IgG, HRP-linked Antibody , cat. no. #7074, 1:1,000, CST, USA) at 4˚C for 40 min. The relative protein content was determined by Bio-Rad laser imaging system (Bio-Rad Laboratories, Inc.) and Image Lab software v6.1 (Bio-Rad Laboratories, Inc.) after the DyLight 800-labeled secondary antibody (1:10,000 dilution; KPL, Inc.) was incubated at room temperature for 1 h the next day(Visualization reagent kit, Cat. No. P0020, Beyotime Institute of Biotechnology). Primary antibody Cpt1a (cat. no. 97361, 1:1,000) secondary Antibodies (Cat. No. #7074,1:1,000) and GAPDH (cat. no. 5174, 1:1,000) were purchased from Cell Signaling Technology, Inc.

Reverse transcription-quantitative (RT-q) PCR. Total RNA in tissues(cells number: $2x10^6$) was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). The first-stand cDNA was synthesized using First Strand cDNA Synthesis kit with gDNA Eraser according to the manufacturer's protocol. PCR was performed using cycling conditions: Denaturation 95˚C for 30 sec, annealing 60˚C for 40 sec and extension at 72˚C for 60 sec; 35 cycles) (Takara, Osaka, Japan). RT-qPCR was performed with SYBR Green Master Mix to examine the relative mRNA levels of indicated genes with an AJ qTOWER 2.2 Real-Time PCR system (Analytik Jena AG) by using a quantitative real-time PCR kit (Takara Bio, Inc.). Sequences for RT-qPCR primers were: Mouse Cpt1a, 5'-CTCCGCCTGAGC CATGAAG-3', mouse GAPDH: 5'-AGGTCGGTGTGAACG GATTTG3'. GAPDH was used as an internal control. Relative gene expression level was calculated by $2^{-\Delta\Delta Cq}$ method (10).

Haematoxylin and eosin staining. Lung samples were dissected and fixed at 18~25˚C in 4% paraformaldehyde solution (Sangon Biotech Co., Ltd.) for 72 h. Tissues were embedded in paraffin (Sangon Biotech Co., Ltd.). Sections of lung were cut at 3-4 μm and prepared for haematoxylin and eosin staining by standard procedures. Samples were immersed in xylene and alcohol, stained with hematoxylin for 5 min, stained with eosin for 3 min and re-immersed in alcohol and xylene. The sections were counterstained with Harris haematoxylin (Sangon Biotech Co., Ltd.) and normal IgG (Merck Millipore Sigma Aldrich) as a negative control. Images were captured with a brightfield DM4B microscope (Leica Microsystems GmbH).

Enzyme-linked immunosorbent assay (ELISA). Lung samples were collected at enrolment and immediately stored at -80˚C in a single biologic resource centre. Lung tissues inflammatory factors (TGFβ, IL-6, IL-β and TNF-α) were determined by commercial ELISA kits (cat. nos. 70-EK981-96, 70-EK206/3-96, 70-EK201B/3-96 and 70-EK282/4-96) purchased from Multisciences (Lianke, Hangzhou, China) Biotech Co., Ltd., according to the manufacturer's instructions. The detection threshold was 0.156 and 1.56 ng/ml. Samples, reagents and buffers were prepared strictly in accordance with the manufacturer's guideline.

Mouse PMVEC isolation. Primary PMVECs were isolated by following these steps. C57BL/6J mice (8-10 weeks old, both male and female) were anesthetized by abdominal injection with pentobarbitone sodium (100 mg/kg body weight). and lungs were removed. The lung samples were enzymatically digested by a mouse lung dissociation kit (Miltenyi Biotec GmbH). Following removal of CD^{45+} cells, CD45-cells were collected, washed and incubated with CD31-conjugated beads (Invitrogen; Thermo Fisher Scientific, Inc.). $CD31^+$ cells were enriched using a MACS column and magnetic field by protocol of reagent kits. For magnetic separation, MACS ART MS Columns were placed into a MiniMACS Separator, rinsed once with 1 ml of MACS ART Binding Buffer (discarded after flow-through), and the $CD45^-$ cells suspension was then placed in the column. The PMVECs, which were bound to CD31-conjugated magnetic microbeads, were then retained in the column. That was because the column was placed in MiniMACS Separator, which is basically a magnet forming magnetic field, which causes the retention of magnetically labeled cells. This $CD31^+$ cells was then washed by adding 4 ml of medium and centrifuged 10 min at 1,000 rpm in 4~8˚C. The freshly isolated cells were considered as passage 0, which was cultured in dish coated with human fibronectin (30 μg/ml). Cells within 5 passages were used for experiments.

Haematoxylin and eosin and TUNEL staining. Haematoxylin-eosin staining is a basic method of histology and pathological examination as the haematoxylin produces crisp, intense blue nuclei providing optimal contrast to the eosin-stained cytoplasm. TUNEL staining detects the DNA breaks formed when DNA fragmentation occurs in the last phase of apoptosis. Lung samples were dissected and fixed at 18~25˚C in 4% paraformaldehyde solution (Sangon Biotech Co., Ltd.) for 72 h. Tissue was embedded in paraffin (Sangon Biotech Co., Ltd.). Sections of lung were cut at ~3-4 μm and prepared for haematoxylin and eosin staining by standard procedures. Then the tissue wax was cut into serial 5 μm sections. After being reconstructed by protease K, the sections were stained by haematoxylin and eosin. The cell samples were collected, washed, fixed and then stained by TUNEL and DAPI. Histological sections were observed in six randomly selected fields for analysis) under a Flirorescent microscope (Nikon Eclipse 80i; Nikon Corporation).

Flow cytometric assay for Annexin v-positive cells. Flow cytometry was used to measure Annexin V-positive cells that are considered as apoptotic cells by Flowjo7.6.1 (Treestar Inc., Ashland, OR). Briefly, cells were collected using the TrypLE (Thermo Fisher Scientific, Inc.). After washed with cold phosphate buffered saline, Annexin V binding buffer (300 μl) was added to resuspend cells. A total $1x10^5$ cells were collected and incubated with 100 μl Annexin V binding buffer along with 5 μl of fluorescein isothiocyanate conjugated Annexin V (Thermo Fisher Scientific, Inc.) and 5 μl of propidium iodide (Life Technology) for 20 min at room temperature. Finally, Annexin V binding buffer (500 μl) was added and mixed

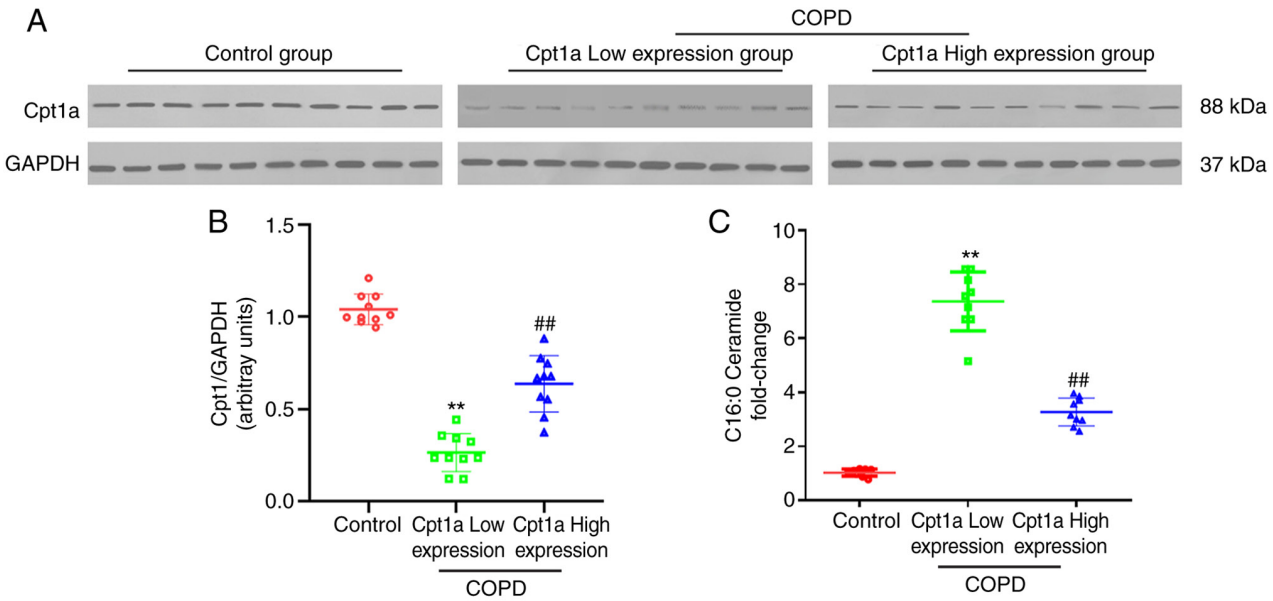

Figure 1. Cpt1a levels and ceramide in lung tissues from patients with COPD and control group. (A) Western blots and (B) quantification of protein expression for Cpt1a in lung tissues from control and patients with COPD. (C) Ceramide were measured in lung tissues from control and patients with COPD by mass spectrometry. Data are expressed as mean ± standard error of the mean; n=10; **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. COPD with low expression of Cpt1a. Cpt1a, carnitine palmitoyltransferase 1A; COPD, chronic obstructive pulmonary disease.

gently. FC-500 (Beckman Coulter, Inc.) was used to detect the Annexin V-positive cells with a total of 20,000 events analyzed. The apoptotic rate was calculated by percentage of early and late apoptotic cells.

Statistical analysis. Experiments of cell cultivation were performed with six biological replicates with a total of five times of technical repetitions for measurements. Data are expressed as mean ± standard error of the mean. One-way analysis of variance with the post Tukey's test was used to determine whether there is any statistical significance between the means of groups. The Student-Newman-Keuls test and Pearson's correlation analysis were used to examine which specific groups of means were statistically different. P<0.05 was considered to indicate a statistically significant difference.

Results

Correlation between Cpt1a levels of lung tissues and lung function in patients with COPD. To determine the correlation between Cpt1a expression and lung function in patients with COPD, clinical data were analysed and lung tissues from patients with COPD were collected for the following tests. In this study, 30 samples were collected. Clinical baselines and data are presented in Table SI. The results of western blotting showed that compared with the control group, the expression of Cpt1a was decreased significantly in lung tissues from patients with COPD. Significant differences in Cpt1a expression were detected between patients with COPD which were then divided into high and low Cpt1a expression groups (Fig. 1A and B). A comparison of lung function data in patients with high and low expression of Cpt1a suggested significant differences in lung function. The lung function indices of patients with high expression of Cpt1a were clearly improved compared with patients with low expression of Cpt1a (Table I). It was also shown that ceramide in the high expression of Cpt1a COPD group was significantly lower than the low expression of Cpt1a COPD group (Fig. 1C). The above findings suggested that Cpt1a may be involved in protecting lung function in patients with COPD.

Cpt1a is critically involved in apoptotic and inflammatory responses in the lung tissues of patients with COPD. Lung inflammation and apoptosis are the most basic pathologic features of patients with COPD. Previous studies have shown that Cpt1a is involved in apoptosis of pulmonary microvascular endothelial cells (6,7,9), but the correlation between Cpt1a expression and apoptosis and inflammation of lung tissues of patients with COPD has not been established. Based on the different levels of Cpt1a expression in lung tissues of patients with COPD, patients were divided into high and low Cpt1a expression groups and the following test results were obtained: Haematoxylin and eosin staining showed that compared with the low Cpt1a expression COPD, the high Cpt1a expression COPD group had less pathologic damage under the microscope, with a smaller amount of inflammatory cell infiltration and more complete lung tissue morphology (Fig. 2A). TUNEL staining showed significantly decreased apoptosis in the high Cpt1a expression COPD group (Fig. 2B). Additionally, compared with the low Cpt1a expression group, the expression of inflammatory factors (TGF-β, TNF-α, IL-6 and IL-1β) in lung tissues of the high Cpt1a expression COPD group was decreased (Figs. 2C and S1). Thus, Cpt1a appeared to be involved in the maintenance of lung morphology and exhibit anti-apoptosis and anti-inflammation properties in patients with COPD.

Cpt1a augmentation ameliorates the COPD-induced apoptosis and inflammation of the lung tissues in COPD mice. To identify the role of Cpt1a in patients with COPD, lentivirus mediated

Table I. Comparison of lung function Indices between patients with COPD with differential expression of Cpt1a and the control group.

Indicator	Control (n=10)	COPD (n=10) Cpt1a low expression	COPD (n=10) Cpt1a high expression	P-value
Tidal volume (ml)	2.73±0.32	1.47±0.25[a]	1.87±0.31[b]	0.001
Peak expiratory flow (ml/s)	37.15±0.18	16.38±2.13[a]	20.48±1.56[b]	0.003
50% expiratory flow (ml/s)	1.79±0.20	1.35±0.22[a]	1.56±1.19[b]	0.021
Forced expiratory volume in 0.3 seconds (ml)	4.42±0.33	2.33±0.32[a]	3.43±0.12[b]	0.013
Forced expiratory volume in 0.3 sec/forced vital capacity (%)	87.61±4.41	64.49±4.30[a]	74.73±5.30[b]	0.001

Results are expressed as mean ± standard deviation. Differences between groups were analyzed by the independent Student t test, χ^2 text, or Wilcoxon test. P-values a vs. b. COPD, chronic obstructive pulmonary disease; Cpt1a, carnitine palmitoyltransferase 1A.

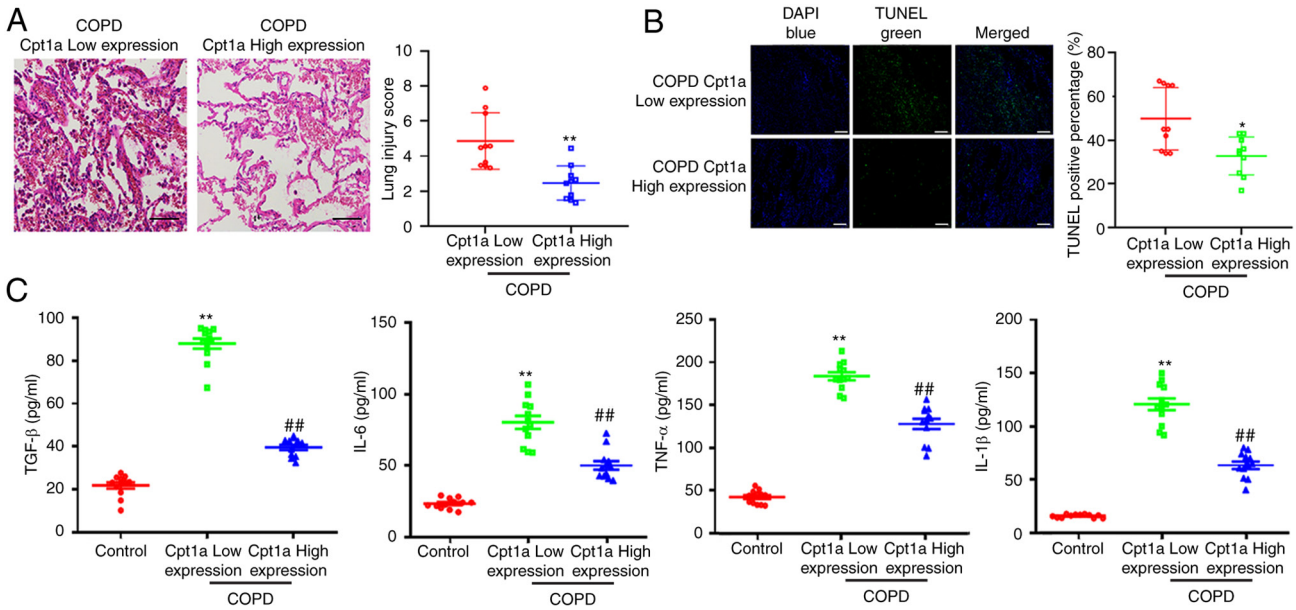

Figure 2. Cpt1a levels are associated with apoptosis and inflammation in lung tissues from patients with COPD. (A) Haematoxylin and eosin-stained sections showing the lung injury in patients with COPD (Bar=100 μm, n=10; **P<0.01 vs. COPD with low expression of Cpt1a). (B) Apoptosis were assessed by TUNEL (Bar=200 μm, n=10; *P<0.05 vs. COPD with low expression of Cpt1a). (C) ELISA demonstrating the levels of TGF-β, IL-6, TNF-α and IL-1β in lung tissues from patients with COPD and control group. Data are expressed as mean ± standard error of the mean; n=10, **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. COPD with low expression of Cpt1a. Cpt1a, Carnitine Palmitoyl Transferase 1A; COPD, Chronic Obstructive Pulmonary Disease.

Cpt1a knockout and overexpression models were established and the following tests were carried out. Transfection of pGLVU6/GFP shRNA Cpt1a and pGLVU6/GFP Cpt1a significantly reduced or elevated the levels of Cpt1a protein and mRNA in lung tissues, respectively (Fig. S2A and B). Haematoxylin and eosin staining showed that compared with COPD model mice, knockdown of Cpt1a significantly aggravated the COPD-induced morphologic disorder of lung tissues, resulted in inflammatory cell infiltration, smooth muscle hyperplasia and partial rupture and fusion of alveolar wall. However, COPD mice with overexpression of Cpt1a had less pathologic damage and inflammatory cell infiltration (Figs. 3A and S2A-D). TUNEL staining showed that Cpt1a knockout aggravated lung cell apoptosis in COPD mice, while Cpt1a overexpression showed the opposite result (Fig. 3B). In addition, *in vivo* results also showed that overexpression of Cpt1a significantly alleviated the lung inflammatory response (Fig. 3C) in COPD mice.

The results of previous studies in our laboratory showed that Cpt1a is mainly expressed in pulmonary microvascular endothelial cells and participates in anti-apoptosis. However, whether overexpression of Cpt1a reduces apoptosis of PMVECs in COPD mice is unknown. Therefore, lung microvascular endothelial cells were isolated from COPD mice. Annexin V-PI flow analysis showed that compared with COPD model mice, knockdown of Cpt1a significantly aggravated the apoptosis of PMVECs induced by COPD, while overexpression of Cpt1a alleviated the apoptosis induced by COPD (Fig. 4A). It was also found that Cpt1a significantly promoted fatty acid oxidation of primary PMVECs in COPD mice

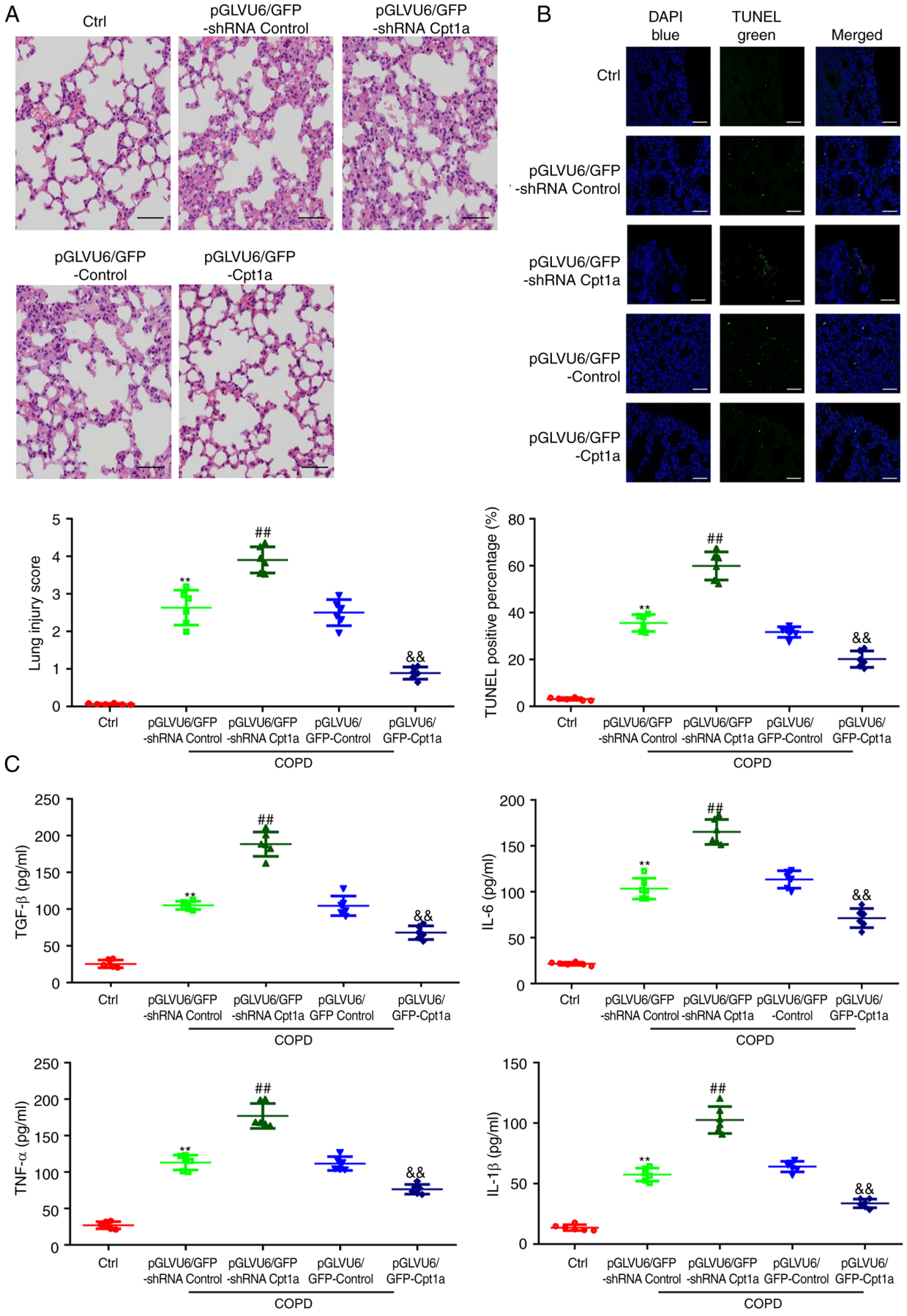

Figure 3. Enhancing Cpt1a attenuating CSE-induced apoptosis and inflammation *in vivo*. (A) Haematoxylin and eosin-stained sections (200x) showing the lung injury *in vivo*. (B) Apoptosis were assessed by TUNEL (Bar=100 μm). (C) ELISA demonstrating the levels of TGF-β, IL-6, TNF-α and IL-1β and in lung tissues. Data are expressed as mean ± standard error of the mean, n=10; **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. pGLVU/GFP-shRNA control; $^{\&\&}$P<0.01 vs. pGLVU/GFP control. Cpt1a, Carnitine Palmitoyl Transferase 1A; CSE, cigarette smoke extract; COPD, chronic obstructive pulmonary disease.

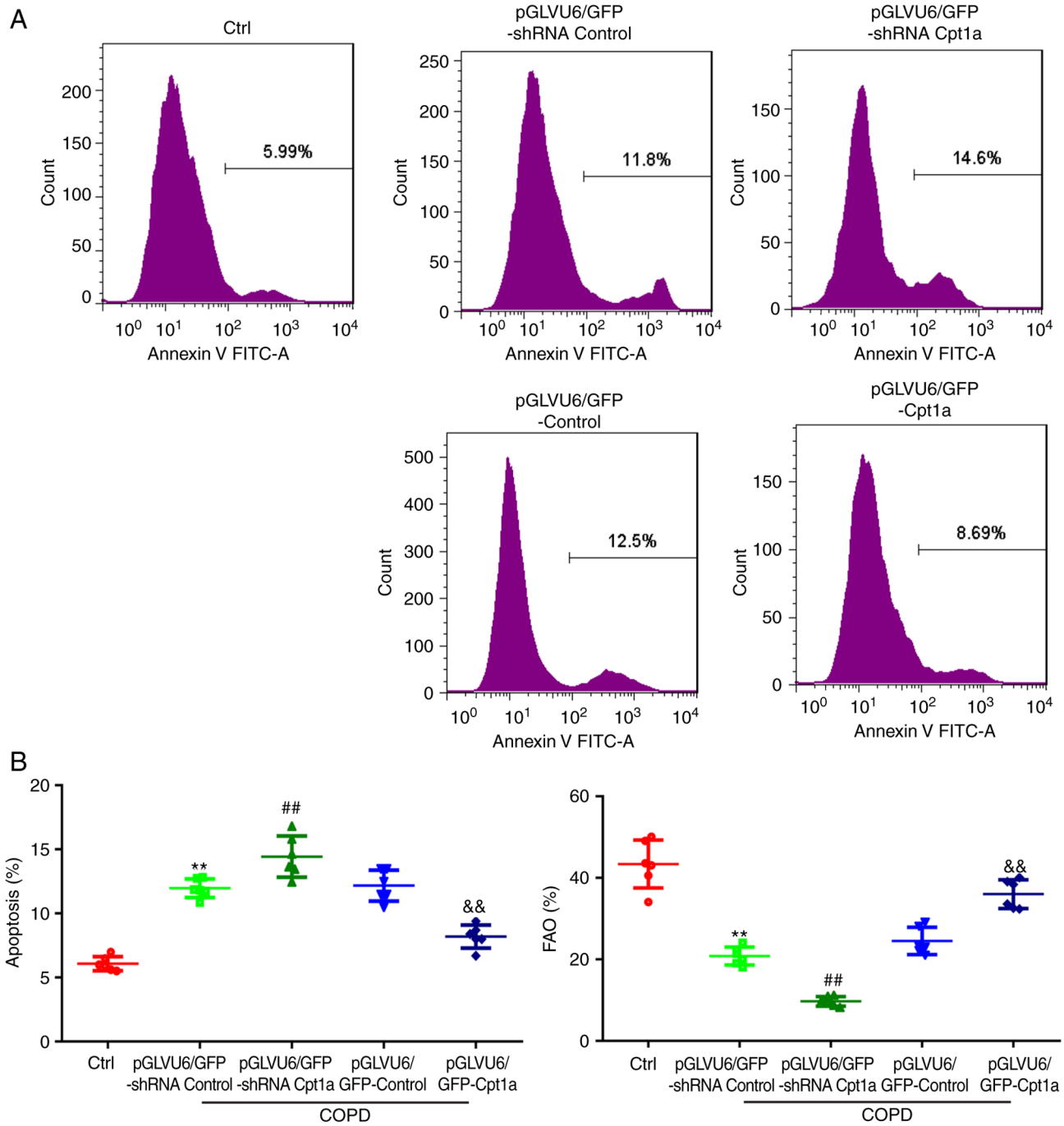

Figure 4. Enhancing Cpt1a increased FAO attenuating CSE-induced apoptosis *in vitro*. (A) Annexin V$^+$ cells were assessed by flow cytometry. (B) FAO was measured by the Seahorse Analyzer. Data are expressed as mean ± standard error of the mean, n=10, **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. pGLVU/GFP-shRNA control; $^{\&\&}$P<0.01 vs. pGLVU/GFP control. Cpt1a, Carnitine Palmitoyl Transferase 1A; FAO, Fatty Acid Oxidation; CSE, Cigarette Smoke Extract; COPD, Chronic Obstructive Pulmonary Disease.

(Fig. 4B). Therefore, Cpt1a alleviated COPD-induced lung tissue disorders in COPD mice.

Cpt1a overexpression significantly improves the lung function in COPD animals. The results of our previous experiments showed that there is a correlation between the difference in Cpt1a expression in clinical patients and the lung function. To identify whether Cpt1a served a role in the treatment of patients with COPD, the present study examined the lung function of the two murine models *in vivo*. Knockdown of Cpt1a significantly decreased lung function indices compared with COPD mice and overexpression of Cpt1a improved the lung function indices of COPD mice (Fig. 5 and Table II). Expression of ceramide in lung tissues of COPD mice with overexpression of Cpt1a was decreased, which is consistent with the clinical results. These results suggest that Cpt1a could induced lung function disorders in patients with COPD.

Discussion

The prevalence of COPD has increased in recent years, especially among young individuals. COPD has become one of the

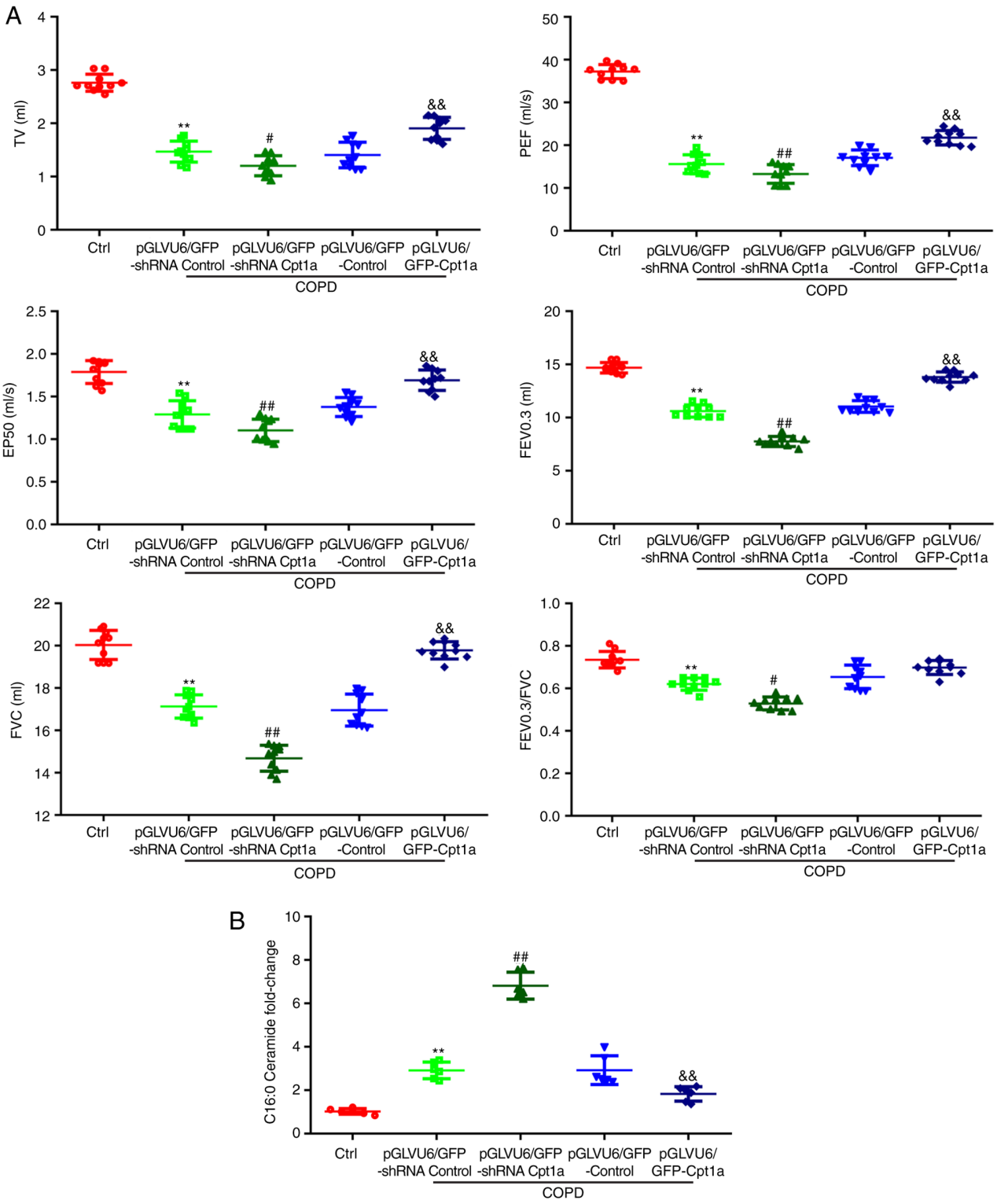

Figure 5. Enhancing Cpt1a inhibited ceramide attenuating CSE-induced lung dysfunction. (A) pulmonary function indicators. (B) Ceramide was measured in lung tissues from model mice by mass spectrometry. Data are expressed as mean ± standard error of the mean; n=10; **P<0.01 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. pGLVU/GFP-shRNA control; $^{\&\&}$P<0.01 vs. pGLVU/GFP control. Cpt1a, carnitine palmitoyltransferase 1A; CSE, cigarette smoke extract; COPD, chronic obstructive pulmonary disease; FAO, fatty acid oxidation.

commonest diseases affecting the health and quality of life of individuals worldwide (2,11). COPD is characterized by apoptosis and inflammation of pulmonary vascular endothelial cells. Increased apoptosis and inflammation of lung tissues result in abnormal lung function and aggravate the course of COPD (12). It has been reported that the expression of Cpt1a is decreased in pulmonary microvascular endothelial cell lines from patients with COPD (5), but whether Cpt1a participates in the protection of lung function in patients with COPD is unclear. The present study showed for the first time that the difference in Cpt1a expression in lung tissues of patients with COPD is related to lung function. The higher the expression of

Table II. Detection of lung function indices of model mice.

Group	Tidal volume (ml)	Peak expiratory flow (ml/s)	50% expiratory flow (ml/s)	Forced expiratory volume in 0.3 sec (ml)	Forced vital capacity (ml)	Forced expiratory volume in 0.3 sec/forced vital capacity (%)
Ctrl	2.76±0.16	37.21±1.66	1.79±0.13	14.69±0.49	20.03±0.69	0.73±0.04
pGLVU6/GFP-shRNA Control	1.47±0.20	15.60±2.17	1.29±0.16	10.62±0.58	17.13±0.55	0.62±0.03
pGLVU6/GFP-shRNA Cpt1a	1.20±0.19	13.28±2.19	1.10±0.13	7.75±0.48	14.68±0.61	0.53±0.03
pGLVU6/GFP-Control	1.41±0.24	17.07±1.85	1.38±0.11	11.04±0.55	16.96±0.75	0.65±0.06
pGLVU6/GFP-Cpt1a	1.91±0.21	21.76±1.68	1.69±0.12	13.80±0.48	19.78±0.41	0.70±0.03
P^a	0.001	0.0123	0.024	0.001	0.0342	0.0453
P^b	0.001	0.001	0.001	0.002	0.001	0.0234

Results are expressed as mean±standard deviation. Differences between groups were analyzed by the independent Student t test, χ^2 text, or Wilcoxon test. P^a, pGLVU6/GFP-shRNA Cpt1a vs. pGLVU6/GFP-shRNA Control; P^b, pGLVU6/GFP-control vs. pGLVU6/GFP-Cpt1a.

Cpt1a, the more improved the lung function indices, the lower the apoptosis rate and the lower the inflammatory response. Overexpression of Cpt1a was shown to alleviate lung damage, inhibit apoptosis and the inflammatory response of pulmonary microvascular endothelial cells in patients with COPD and serve a role in the treatment of COPD by promoting the rate of substrate fatty acid oxidation and inhibiting the production of ceramide (Fig. 6).

The CPT system consists of two separate proteins located in the outer (Cpt1) and inner (Cpt2) mitochondrial membranes (13). Clinical data have shown that Cpt2 deficiency leads to sudden foetal death (14). Cpt1 is the key molecule of mitochondrial fatty acid oxidation, composing of Cpt1a, Cpt1b and Cpt1c (15), which are expressed primarily in the liver and lungs, skeletal muscles and brain, respectively (14,16,17). Our previous studies showed that Cpt1a is expressed in lung endothelial cells, where Cpt1b and Cpt1c are minimally expressed, which is consistent with previous reports (9,18). Therefore, the present study examined the role of Cpt1a in lung tissues of patients with COPD in detail. Basic studies have shown that Cpt1a-mediated fatty acid oxidation is related to the invasion and metastasis of colon cancer and promotes the occurrence and development of cancer (19-21). Cpt1a also inhibits the proliferation and migration of lung cancer cells (20). In view of these findings, available information on the importance of Cpt1a in some tissues is limited and controversial. The role of Cpt1a in pulmonary vascular endothelial cells is not fully understood and it is unclear whether Cpt1a is involved in maintenance of pulmonary homeostasis. The present study showed for the first time that Cpt1a plays an irreplaceable role in regulating lung function. It found differences in the expression of Cpt1a in the lung tissues of clinical patients with COPD and found that lung function indices in the group with high Cpt1a expression were improved compared with the group with low Cpt1a expression. Consistent with the clinical results, knockout and knockdown of Cpt1a *in vivo* showed that Cpt1a reversed pulmonary dysfunction in COPD mice. Cpt1a could be used as a clinical molecular target to improve lung dysfunction in patients with COPD.

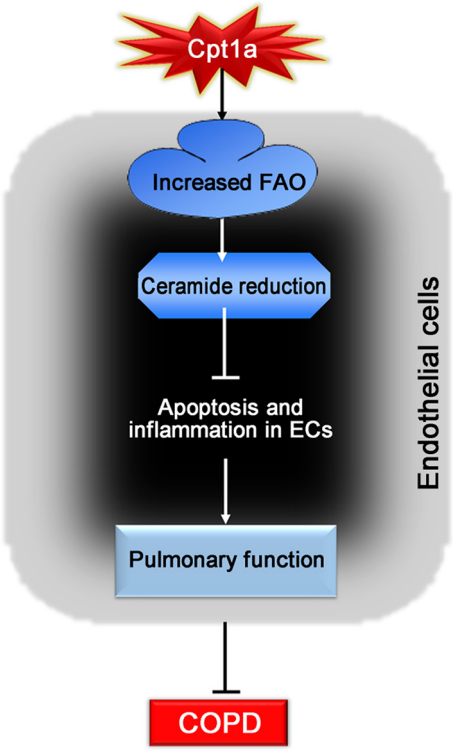

Figure 6. A proposed model showing how Cpt1a suppresses vascular endothelial apoptosis and inflammation induced by COPD. Cpt1a, carnitine palmitoyltransferase 1A; COPD, chronic obstructive pulmonary disease; ECs, endothelial cells.

Tobacco smoke is deadly and has more than 7,000 chemicals, 69 of which are verified as carcinogens (21). Smoking is one important risk factors for COPD and ~80% of patients with COPD were induced by smoking (22). Toxic particles of inhaled smoke induce airway inflammation that is exacerbated in patients with COPD (23). Smoking does great harm

to human beings and is a social problem. Smoking cessation has been confirmed by a large number of studies to be an effective way to prevent COPD, delay airflow restriction and slow down deterioration of lung function (24,25). The current study created a cigarette smoke-induced COPD murine model. Consistent with the previous research results, the present study confirmed the harmful effects of cigarette smoke on lung function through lung function testing. Cigarette smoke increased inflammation and apoptosis in lung tissues, caused morphologic changes of lung tissues and decreased the expression of Cpt1a, the protective factor of lung endothelial cells.

Pulmonary vascular endothelial injury (apoptosis and an inflammatory response) is a common pathologic manifestation in the progression of COPD (7-9). The balance between endothelial injury and anti-injury affects the occurrence and development of COPD (26). Several studies have been conducted to determine how to delay endothelial apoptosis and inhibit the inflammatory response (7-9,26). Our previous study suggests that Cpt1a is the key downstream molecule of L-carnitine, which could inhibit apoptosis of pulmonary vascular endothelial cells in COPD (9), but whether Cpt1a is involved in anti-apoptosis and anti-inflammation of pulmonary vascular endothelial cells in COPD remains to be elucidated. The results of haematoxylin and eosin staining, TUNEL staining and ELISA revealed that the level of Cpt1a expression in patients with COPD was directly associated with infiltration of inflammatory cells, apoptosis and the expression of lung tissue inflammatory factors. Consistent with the clinical results, inflammatory cell infiltration, apoptosis and inflammatory factor expression in lung tissues of the COPD model with Cpt1a knockdown *in vivo* were increased and overexpression of Cpt1a reversed this trend. In addition, primary pulmonary microvascular endothelial cells were isolated from model mice and the results showed that Cpt1a inhibited apoptosis of pulmonary microvascular endothelial cells. These results indicate that Cpt1a plays an important role in pulmonary endothelial cells and subsequent function regulation.

Ceramide is an important lipid molecule that regulates cell differentiation, proliferation, apoptosis, aging and other life activities (27). Studies have shown that ceramide could promote airway inflammation and airway hyperresponsiveness and play an important role in the pathogenesis of asthma (28), COPD (29) and acute lung injury (30). It has been reported that ceramide promotes the expression of MMP-9 in lung epithelial cells by activating the JAK2-STAT3 pathway, thus promoting the development of airway remodelling in lung tissues (31). Upregulation of ceramide is involved in apoptosis of lung epithelial cells (32). Intratracheal instillation of ceramide in mice leads to apoptosis of alveolar epithelium and endothelial cells, resulting in enlargement of air cavity (33). Inhibition of ceramide synthesis by Fumonisin B1, an inhibitor of ceramide synthase, reduces apoptosis of epithelial cells and airway inflammation (34). Nevertheless, it is unclear whether Cpt1a is involved in the production of ceramide in lung tissues of patients with COPD. Ceramide in lung tissues of patients with COPD was detected by LC-MS, which revealed that ceramide production was decreased in the lung tissues of patients with COPD with high expression of Cpt1a, while the opposite trend was shown in the low Cpt1a expression group. Moreover, the results of *in vivo* animal model are consistent with the clinical results. These results suggest that Cpt1a is involved in mediating ceramide production in lung tissues and serves a crucial role in the progression of COPD.

Fatty acid is an important component of blood lipids and the main product of lipid digestion. Fatty acid oxidation plays an essential role in regulating triglyceride metabolism (35). As the main site for fatty acid oxidation, mitochondria provide the ester acyl CoA synthase needed for fatty acid activation, then perform transmembrane transfer, fatty acid β oxidation and acetyl CoA thorough oxidation, thus providing energy (36). The functional state of fatty acid oxidation is closely related to the occurrence and development of a number of pathologic processes, such as lipid accumulation, lipid peroxidation damage and insulin resistance (36). Normally, fatty acids are transported into mitochondria by Cpt1 on the mitochondrial membrane, then undergo the process of fatty acid β-oxidation. The remaining fatty acids can be oxidized under the action of peroxisomes and microsomes to produce reactive oxygen species (37). Abundant clinical data indicate abnormal mitochondrial fatty acid oxidation of lung endothelial cells in patients with COPD. Abnormal expression of Cpt1 leads to abnormal fatty acid oxidation, thus disrupting liver function (38). Our previous results showed that L-carnitine treatment promotes the expression of Cpt1a and oxidation of fatty acids in endothelial cells and alleviates the apoptosis of endothelial cells induced by cigarette smoke (9). In the current study, primary pulmonary microvascular endothelial cells from a murine model were isolated to measure the fatty acid acidification rate. It was found that overexpression of Cpt1a promoted fatty acid oxidation in pulmonary microvascular endothelial cells of the COPD murine model, while the knockdown of Cpt1a led to the opposite results.

In conclusion, the present study showed for the first time that overexpression of Cpt1a could alleviate lung dysfunction and reduce inflammatory response and apoptosis of lung tissues in COPD mice and protect cigarette-induced COPD by promoting the oxidation rate of substrate fatty acids, thus inhibiting the production of ceramide to suppress apoptosis of endothelial cells and inflammatory responses. The data suggested that Cpt1a may be a potential new target for the treatment of patients with COPD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HZ and LL performed the conception and design of the study, and drafted and revised the manuscript. LL, YZ and JG analyzed data and prepared figures. LL, GY, SZ and DR performed the experiments and manuscript review. HZ, GY and LL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (CMTT number 2013012). All patients were informed in detail about the objective and methods of this study and signed a consent form. All procedures of the present study, including animals and patients, were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (CMTT number: 2013012) and in accordance with international standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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