PDK1 inhibition reduces autophagy and cell senescence through the PI3K/AKT signalling pathway in a cigarette smoke mouse emphysema model

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Received March 28, 2022; Accepted November 3, 2022

DOI: 10.3892/etm.2023.11922

Abstract. A number of previous studies have demonstrated the pivotal role of PI3K/AKT signalling in cigarette smoke (CS)-induced emphysema, where phosphoinositide dependent protein kinase 1 (PDK1) is a critical component of this pathway. Therefore, the present study aimed to investigate the effects of a PDK1 inhibitor (GSK-2334470) on the expression levels of PI3K, AKT, cyclin-dependent kinase inhibitor 2A (p16) and LC3B in a CS + CS extract (CSE)-induced mouse emphysema model. CS exposure and intraperitoneal injections of CSE were combined for 4 weeks to establish an emphysema model. Mice (n=35) were randomly divided into the normal control, emphysema (CS), PI3K inhibitor (CS3) and PDK1 inhibitor (CS1) groups. Immunohistochemistry staining of lung tissues was used to measure the expression of the PI3K, PDK1 and AKT proteins in airway epithelial tissues. Immunofluorescence staining was also used to measure the levels of p16 and LC3BII protein expression in the airway epithelial tissues. In addition, PI3K, PDK1, AKT, p16 and LC3B protein expression was semi-quantified using western blotting. The expression of PDK1, PI3K and AKT proteins in the airway epithelial tissues was significantly increased in the CS + CSE group compared with that in the control group. The expression levels of p16 and LC3B were also increased as well in the CS + CSE group compared with those in the control group. The expression levels of PI3K, PDK1, AKT, LC3B and p16 in the airway epithelial tissues of the CS3 group were lower compared with those in the CS + CSE group. A decrease in the expression levels of PDK1, AKT, p16 and LC3B in the airway epithelial tissues of the CS1 group compared with those in the CS + CSE group was also observed. However, there were no significant differences in the expression levels of PI3K between the CS1 and the CS groups. The present study concluded that the inhibition of PDK1 can potentially reduce autophagy and cell senescence by downregulating the expression of PI3K/AKT pathway related proteins in airway epithelial cells, thereby protecting against CS + CSE-induced emphysema in mice.

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Key words: kinase-dependent protein kinase 1, autophagy, cell senescence, emphysema, aging, cigarette smoke, chronic obstructive pulmonary disease, proteostasis, cysteamine

Introduction

Pulmonary emphysema is a major component of chronic obstructive pulmonary disease (COPD) (1-2). Cigarette smoke (CS) exposure and aging are the leading causes of COPD, but the molecular mechanism that mediate the pathogenesis of this disease remains poorly understood (1). It has been previously reported that cell senescence is implicated in the pathogenesis of COPD, which is frequently accompanied with the accumulation of damaged cellular components (1). An age-related increase in neutrophil myeloperoxidase activity and caspase-3/7-related apoptotic cell death was observed that is anticipated to further augment the effect of smoke exposure (1). This data indicates that CS-impaired autophagy serves as a mechanism for severe emphysema progression by inducing aggresome formation that impacts innate and adaptive immune responses, leading to chronic inflammation (1). Previous studies have suggested that CS-induced senescence and autophagy in airway epithelial cells are closely associated with the pathogenesis of COPD (1,3,4). However, the detailed mechanism remains unclear (1).

As a key member of the cAMP-dependent, cGMP-dependent and protein kinase C protein kinase family, phosphoinositide dependent protein kinase 1 (PDK1) serves an important role in a variety of cellular functions, leading to the activation of the PI3K signaling pathway, an event often associated with the onset and progression of several human cancers (5). A previous study has shown that PDK1 is an important component of the PI3K/AKT signalling pathway, which can regulate cell cycle progression, proliferation, metabolism, autophagy and senescence by activating AKT in mammalian cells (6). However, the role of this PDK1-mediated AKT signalling in autophagy and senescence due to emphysema remains unclear (6). It has been previously reported that the PI3K/AKT/mTOR signalling pathway serves a key regulatory

role in autophagy (7), where the inhibition of PI3K contributes to the inhibition of autophagy in mice with COPD (8). AKT can suppress TSC1/2 and activate mTOR to promote protein synthesis and cell growth and regulate cell proliferation by inactivating cell cycle inhibitor (9,10). The PI3K/AKT/mTOR pathway regulates autophagy to induce apoptosis of alveolar epithelial cells in a mouse model of COPD (8,9). This suggests a possible relationship between the PI3K/AKT pathway and cell autophagy associated with emphysema. Since activation of the PI3K/AKT/mTOR signalling pathway is one of the main mechanisms of cell senescence (11,12), it was speculated that activation of PI3K/AKT may result in both cell senescence and autophagy during emphysema (7,8,11,12). LC3B is a structural protein of the autophagosome membrane that is widely used as a biomarker of autophagy (13,14). In addition, cyclin-dependent kinase inhibitor 2A (p16) is a key marker of cellular senescence that has been previously observed to be induced by CS in lung epithelial cells and fibroblasts (15). Since p16 and LC3B proteins are involved in cell aging and autophagy, respectively, it was hypothesized that CS exposure combined with intraperitoneal injections of CS extract (CSE) in mice may induce the expression of LC3B and p16 in airway epithelial cells through the PI3K/PDK1/AKT signalling pathway to promote emphysema-like changes associated with COPD.

In the present study, a mouse model of emphysema was induced with CS + CSE. Expression levels of the PI3K, PDK1, AKT proteins, as well as cell aging marker (p16 protein) and autophagy marker (LC3B protein) were analysed in the normal control group (control), emphysema (CS + CSE), PI3K inhibitor (CS3) and PDK1 inhibitor-treated (CS1) groups. The aim of this was to explore the influence of CS + CSE on the PI3K/AKT signaling pathway and the cell aging and autophagy, as well as their role in the pathogenesis of COPD. In parallel, the impact of PDK inhibitor on cell aging and autophagy in emphysema mice was evaluated.

Materials and methods

Mouse experiments. All experiments were approved by the Animal Ethics Committee of Guizhou Provincial People's Hospital. A total of 35 C57BL/6J mice (4-week-old male; weight, 14-16 g) were purchased from the Sibefu (Beijing) Biotechnology Co., Ltd. All mice were housed in a pathogen-free animal facility, which was maintained at 22-24˚C at 50-60% humidity with 12:12 h light-dark cycle. The mice had free access to food and water before the experiments. As previously described (16), mice were randomly divided into the following four groups: i) Control (n=8); ii) CS + CSE (n=9); iii) CS3 (CS + CSE + PI3K inhibitor; n=9); and iv) CS1 (CS + CSE + PDK1 inhibitor; n=9). During the study, the animals were monitored twice a day by the same researchers. Body condition, posture, coat and grooming, in addition to the visible mucosa, eyes, ears, whiskers and mental status were evaluated. The mental state of the mouse was determined by measuring the autonomic activity, movement, response to the outside world (sluggish or overactive) and gait of the mouse. Animals were individually weighed. Food and water intake were also determined. Criteria used to determine the humane endpoints for the animals were the following: i) Extreme or prolonged weight loss/emaciation (body-weight decrease ≥20%); ii) cramps; iii) paralysis; iv) breathing difficulties; v) diarrhoea (>48 h); vi) prolonged lack of activity; vii) prolonged decreased food and water intake; viii) bleeding from an orifice; ix) repeated or severe self-mutilation; or x) unconsciousness. Any one of these signs would be a criterium for immediate euthanasia (17,18).

CS exposure was combined with intraperitoneal injections of CSE to build an emphysema model (16). The smoke exposure box was 66.3x51.8x36.5 cm. For the first 2 days the mice were exposed to the smoke of 8 cigarettes (China Tobacco Guizhou Industrial Co., Ltd.) smoked in sequence twice a day; then, from day 3 onwards, they were exposed to 10 cigarettes. A single cigarette contained 13 mg tar, 1.2 mg nicotine and 14 mg carbon monoxide. After the last lit cigarette was placed on the burning rack and the lid of the box was closed, timing was started and continued for 40 min each time. After the exposure, the mice were put back into the rearing box and raised normally, before the experiment was performed again after a 4 h interval to administer the second CS exposure of the day. Mice were exposed to CS 40 min/time, twice a day, 6 days per week for 4 weeks. CS exposure was not performed on the day of CSE intraperitoneal injection. During exposure to tobacco smoke, the smoke exhaust equipment was used to divert the tobacco smoke outside the laboratory to avoid poisoning the other laboratory animals and polluting the indoor environment. The normal control group was given fresh air at the same time and frequency.

The CS + CSE, CS3 and CS1 groups received an intraperitoneal injection of 100% CSE on days 1, 12 and 23 of modelling. The dose (PBS; 0.3 ml/20 g) was injected into the abdominal cavity of mice, where the injection was completed within 30 min after the preparation of CSE. Mice in the normal control group were intraperitoneally injected with PBS at the same dose and frequency.

The mice in CS3 group were given the PI3K inhibitor PF-04691502 (10 mg/kg per mouse; APExBio Technology LLC) through oral gavage every day. By contrast, mice in the CS1 group were injected intraperitoneally with a PDK1 inhibitor GSK-2334470 (80 mg/kg per mouse; APExBio Technology LLC) three times per week. The control and the CS + CSE groups were given the same frequency and dose of vehicle intervention.

A total of three mice died during the modelling for unknown reasons. In total, one died on day 21 in the CS + CSE group, one died on day 25 in the CS1 group and one died on day 23 in the CS3 group. The mortality rate (2-7.6%) observed in the present study was similar to previous studies (19,20). Lung biopsies were performed on the dead mice, which suggested that the cause of death may be associated with damage to the lungs caused by smoke. The observed pulmonary histopathological changes of the mice were compromised of alveolar enlargement, alveolar wall thinning, tissue hyperplasia in alveolar septum at different degrees, alveolar sac and alveolar tube enlargement or damage and bronchiole wall injury.

Preparation of CSE. CSE was prepared as previously described (16). Each non-filter Huangguoshu cigarette was first burned and the smoke of one cigarette was bubbled through 10 ml PBS, which was designated as 100% CSE. The solution was utilized within 30 min after preparation.

Tissue processing. Tissue processing was performed as previously described (20). Lung tissue samples were obtained from the right middle lobe of each mouse on day 29 after CS + CSE exposure. Mice were first injected with 3% chloral hydrate into the abdominal cavity at 300 mg/kg to induce anaesthesia, followed by euthanasia through intraperitoneal injections of 100 mg/kg sodium pentobarbital resulting in breathing inhibition (21). Death was confirmed when mice had no breathing or heartbeat and did not respond to gentle stimulation, such as those in the back or abdomen. The mouse was placed on the operating table, before the skin of the operation area was disinfected with alcohol and the superficial skin was cut from the umbilicus along the midline of the abdomen to the neck and mandible. Toothless forceps were then used to bluntly separate the subcutaneous tissue whilst ophthalmic scissors were used to cut the sternum slightly to the right from the xiphoid process. After exposing and severing the femoral artery or internal carotid artery, 5 ml normal saline was slowly injected into the heart for the perfusion of the lung tissue. Straight forceps were used to clamp the right lung and the hilar tissue was cut off by ophthalmology. The stump of the right main bronchus was then ligated using a silk thread. A small incision was cut under the cartilage ring of the trachea and a self-made bronchoalveolar lavage needle was used to insert the needle into the bronchus. A 1-ml syringe was used to take 0.5 ml 4˚C pre-cooled PBS for the pulse lavage of the left lung. Each left lung specimen was washed three times (10-sec rest after each injection of PBS) before the lavage liquid was placed in the same Eppendorf tube. The right middle lobe of each mouse was inflated with 4% paraformaldehyde at a constant pressure of 2.5 KPa and subsequently fixed with 4% paraformaldehyde for 24 h at room temperature, after which it was dehydrated in graded alcohol and embedded in paraffin.

H&E staining of lung tissues and morphological assessment. H&E staining of lung tissues was performed as previously described (16). Paraffin sections (4-μm) were successively added into xylene I (20 min), xylene II (20 min), xylene II (20 min), anhydrous ethanol I (5 min), anhydrous ethanol II (5 min), 95% alcohol (5 min), 90% alcohol (5 min), 80% alcohol (5 min), 70% alcohol (5 min) at room temperature. Then soaked in distilled water for 5 min to dewax. Paraffin-embedded tissues were stained with hematoxylin for 5 min and eosin for 5 min (HE) at room temperature to evaluate the severity of inflammation using a Olympus BX53 biological microscope (magnification, x200). To assess emphysematous changes in mice, H&E-stained sections were collected to measure the mean linear intercept (MLI) and destructive index (DI) (22). The MLI was measured by dividing the length of a line drawn across the lung section by the total number of intercepts counted within this line. The SurePathTM PrepStain automatic liquid-based thin layer cell filmmaking machine was used for preparation and Pap staining. The cell nucleus was observed at 400 times of light microscope for cell classification. The DI was calculated by dividing the defined destructive alveoli (enlarged alveolar space, thinner alveolar septum and destroyed alveolar wall) by the total number of alveoli. ImageJ Software v1.8.0 (National Institutes of Health) was used to analyse MLI.

Bronchoalveolar lavage fluid (BALF), cytokine analysis and measurements of the total and differential cell counts. BALF cell counting was performed as previously described (20). The lavage liquid previously collected was centrifuged at 111 g for 5 min at 4˚C within 2 h and the cell-free supernatants were stored at -80˚C for subsequent cytokine analysis. The pellet was smeared onto slides for cell classification and the counting of BALF. The SurePathTM PrepStain automatic liquid-based thin layer cell filmmaking machine (BD TriPath) was used for preparation and Pap staining. The cell nucleus was observed at 400 times of light microscope for cell classification. Concentrations of IL-6 were measured in the BALF supernatant using the IL-6 ELISA kit (cat. no. E-EL-M0044c; Elabscience Biotechnology, Inc.).

Immunohistochemistry (IHC). The levels of PDK1, PI3K and AKT expression in the airway epithelial tissues were evaluated by IHC staining of paraffin-embedded tissues as previously described (23). Briefly, paraffin-embedded tissues were cut into 4-μm thick sections, dewaxed and rehydrated, as aforementioned. The slices were treated with 3% H_2O_2 in methanol for 15 min at room temperature to inhibit endogenous peroxidase activity. Antigen retrieval was performed in a pressure cooker with 10 mM sodium citrate buffer (pH 6.0) at 100˚C for 15 min. Slices was blocked with normal 5% goat serum (cat. no. AR1009; Wuhan Boster Biological Technology, Ltd.) for 30 min at room temperature. Slices were incubated overnight at 4˚C with anti-PDK1 (1:100; cat. no. 10026-1-Ig; ProteinTech Group, Inc), anti-PI3K (1:200; cat. no. 67121-1-Ig; ProteinTech Group, Inc) and anti-AKT (1:100; cat. no. ab23509; Abcam) primary antibodies. After washing with PBS (cat.no. P1020; Beijing Solarbio Science & Technology Co., Ltd.) and 0.05% Tween-20 (Beijing Solarbio Science & Technology Co., Ltd.), the specimens were incubated with CY3-labeled goat anti-mouse IgG (1:100; cat. no. BA1031; Wuhan Boster Biological Technology) and goat anti-rabbit IgG (1:100; cat. no. BA1032; Wuhan Boster Biological Technology) secondary antibodies at room temperature for 1 h. Subsequently, slides were stained with DAB (cat. no. K5007; Dako; Agilent Technologies, Inc.) for 3 to 10 min at room temperature for color development, and the protein was counterstained with hematoxylin for 2 min at room temperature. Images were captured using a light microscope (magnification, x400). Image J Software v1.8.0 (National Institutes of Health) was used to analyse the average optical density of each protein in the airway epithelial tissue.

Immunofluorescence (IF). The levels of LC3B and p16 protein expression in the airway epithelial tissues were evaluated using IF staining as previously described (21). Paraffin-embedded tissues were cut into 4-μm thick sections, before they were dewaxed and rehydrated as aformentioned. Slices was blocked with normal 5% goat serum (cat. no. AR1009; Wuhan Boster Biological Technology, Ltd.) for 30 min at room temperature. Slices were incubated with anti-LC3B (1:100; cat. no. APG8B; Abcepta) and anti-p16 antibodies (1:100; cat. no. Bs-11652R; BIOSS) overnight at 4˚C. The samples were then washed (PBST; Tween-20 0.05%; cat. no. P1031; Beijing Solarbio Science & Technology Co., Ltd.) extensively and incubated with the appropriate CY3-labeled goat anti-mouse IgG (1:100;

cat. no. BA1031; Wuhan Boster Biological Technology) and goat anti-rabbit IgG (1:100; cat. no. BA1032; Wuhan Boster Biological Technology) HRP-conjugated secondary antibodies for 1 h at room temperature in the dark. Subsequently, the slices were counterstained with DAPI (1:100; cat. no. C1002; Beyotime Institute of Biotechnology) for 5 min at 25˚C in the dark. Immunofluorescence images were captured using a fluorescence microscope (magnification, x400; Leica Microsystems GmbH). The mean fluorescence intensity (MFI) of proteins in the airway epithelial tissues was calculated by the Image J Software v1.8.0 (National Institutes of Health).

Western blotting. PI3K, PDK1, AKT, p16 and LC3B protein expression levels were detected and semi-quantified using western blotting as previously described (21). The lung tissues were lysed at 4˚C in RIPA buffer (CoWin Biosciences) and extracts were clarified at 10,000 x g at 4˚C for 20 min. Protein concentration was determined using a bicinchoninic acid assay kit (CoWin Biosciences). Subsequently, total protein was separated using SDS-PAGE (80-μg protein/well) on an 10% gel. Separated protein was then transferred onto a PVDF membrane. After the PVDF membrane was blocked with 5% BSA (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature, it was incubated overnight at 4˚C with the following primary antibodies: PI3K (1:5,000; cat. no. 60225-1-1g); PDK1 (1:500; cat. no. 18262-1-AP); AKT (1:5,000; cat. no. 60203-1-1g); LC3B (1:500; cat. no. 18725-1-AP) (ProteinTech Group, Inc.) and p16 (1:2,000; cat. no. R22878; Zen BioScience Co., Ltd.). Separated proteins were then transferred onto a PVDF membrane, which was incubated at room temperature for 1 h with the anti-GAPDH (1:10,000; cat. no. ab181602; Abcam). After three washes (TBST; Tween-20 0.05%; cat. no. T1085; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min each, the membrane was incubated with polyclonal anti-rabbit/mouse HRP-conjugated secondary antibodies overnight at 4˚C (1:200; cat. no. CW02029 and CW02030; CoWin Biosciences) and developed with the Pierce ECL Western Blotting substrate (cat. no. CW0049C; CoWin Biosciences). Equal loading of the samples was determined by the semi-quantitation of proteins, as well as by using GAPDH as the loading control. Bands were semi-quantified using optical densities that were analysed using Gel-Pro analyser 4.0 (Media Cybernetics).

Statistical analysis. All data were analysed by SPSS 22.0 statistical software (IBM Corp.). Data from each group are expressed as the mean ± standard deviation. Differences between two groups were compared using the unpaired Student's t-test. Comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. Each experiment was repeated ≥ three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Model evaluation. CS exposure was combined with an intraperitoneal injection of CSE to build an emphysema model. Histomorphological changes in lung tissues from emphysematous mice induced by CS exposure combined with intraperitoneal injection of CSE included enlarged alveolar space, thinner alveolar septum and destroyed alveolar wall. Mouse lung histological results showed that this emphysema model was successfully established in the mice (Fig. 1A and B). The levels of IL-6, number of total cells, the number of neutrophils and the number of macrophages in BALF were significantly increased in the CS + CSE group compared with those in the control ($P<0.05$; Fig. 1E-H). To assess changes in emphysema in mice, H&E-stained sections were analysed to measure the MLI and the DI. The MLI and the DI were significantly greater in the CS + CSE group compared with those in the control group ($P<0.05$; Fig. 1I and J). These results suggest that the combined CS exposure with an intraperitoneal injection of CSE successfully induced lung emphysema. In addition, histomorphology improvements were observed in lung tissues from mice in the CS3 group and the CS1 group, which included improvements in alveolar wall damage (Fig. 1C and D). The levels of IL-6, number of total cells, the number of neutrophils and the number of macrophages in BALF were significantly decreased in the CS3 group and the CS1 group compared with those in the CS + CSE group ($P<0.05$; Fig. 1E-H). The MLI and the DI were significantly decreased in the CS3 group and the CS1 group compared with those in the CS + CSE group ($P<0.05$; Fig. 1I and J).

CS + CSE upregulates PDK1 and the PI3K/AKT signalling pathways in airway epithelial tissues. After IHC staining of the lung tissues, the expression of PI3K, PDK1 and AKT proteins in the airway epithelial tissues presented as brown staining (Fig. 2A-C). Compared with those in the control group, the levels of PI3K, PDK1 and AKT protein expression in the CS + CSE group were significantly increased ($P<0.05$; Fig. 2D-F). Overall, these results suggest that PI3K, PDK1 and AKT protein expression was upregulated in the CS + CSE-induced emphysema mouse airway epithelial tissues.

Effects of CS + CSE on autophagy. IF staining was used to detect the levels of LC3B protein expression in the airway epithelial tissues (Fig. 3A). Under fluorescence microscopy (magnification, x400), the airway epithelial tissues were observed, where red represented the positive expression of the LC3B protein (Fig. 3A). Image J was used to semi-quantitatively analyse the MFI of the LC3B proteins in the airway epithelial tissues. Compared with those in the control group, the expression levels of the LC3B in the CS + CSE group were significantly increased ($P<0.05$; Fig. 3B).

Effects of CS + CSE on cell senescence. IF staining was used to measure the levels of p16 protein expression in airway epithelial tissues (Fig. 4A). The area of positive p16 protein expression was stained in red (Fig. 4A). Compared with those in the control group, the expression levels of p16 in the airway of the CS + CSE group were significantly increased ($P<0.05$; Fig. 4B).

The western blotting were used to detect the levels of PI3K, PDK1, AKT, LC3B and p16 protein expression.The same results were obtained by western blot. Compared with those in the control group, the levels of PI3K, PDK1 and AKT protein expression in the CS + CSE group were significantly increased ($P<0.05$; 5A-C). Compared with those in the control group, the expression levels of the LC3B in the CS + CSE group were

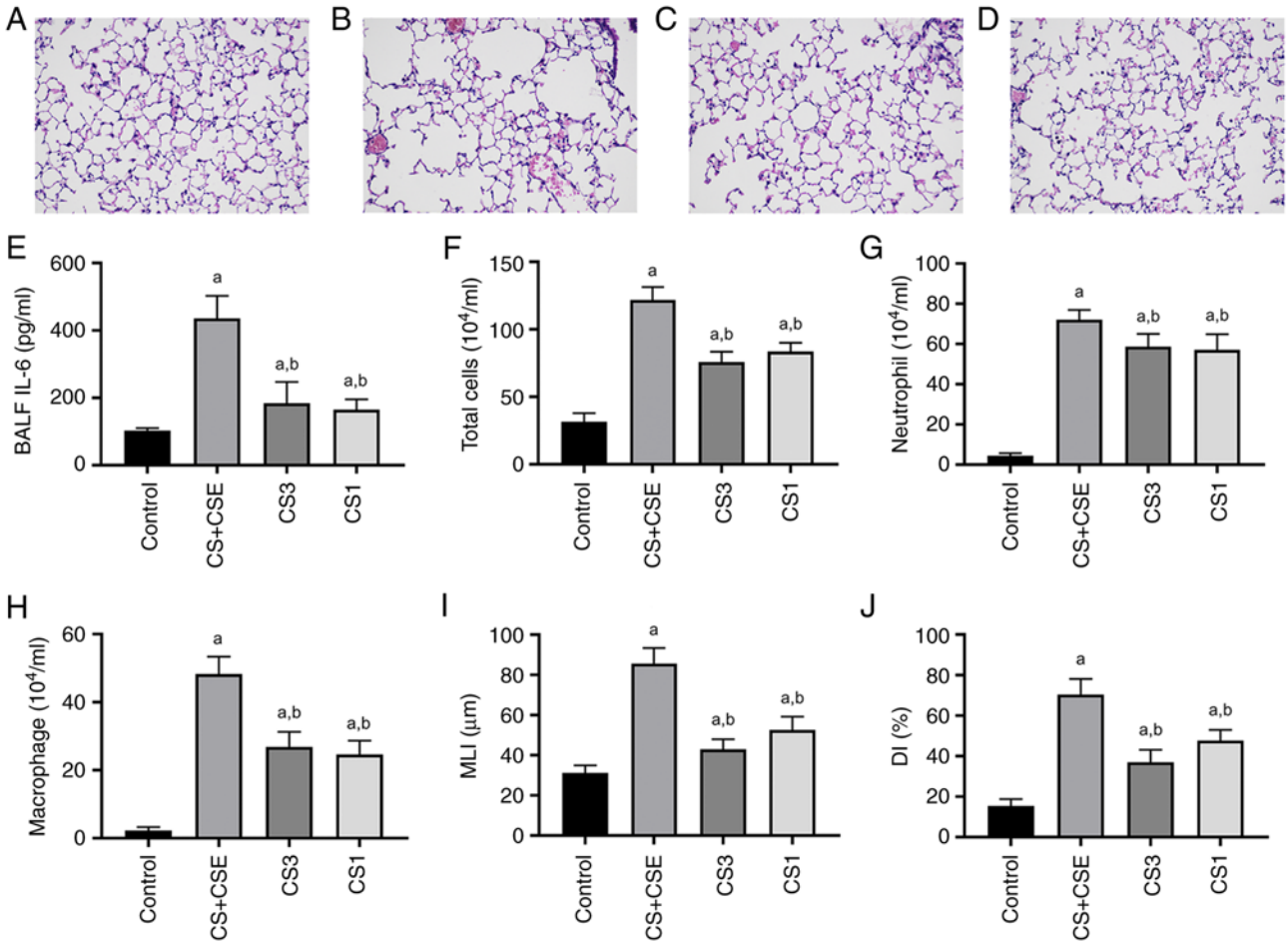

Figure 1. Evaluation of the mouse emphysema model. H&E staining of lung tissues from the (A) control, (B) emphysema, (C) PI3K inhibitor and (D) PDK1 inhibitor groups. Magnification, x400. (E) IL-6 protein levels and (F) total cell count in BALF. Numbers of (G) neutrophils and (H) macrophages in BALF. (I) MLI and (J) DI were measured show the extent of airway remodelling in lung tissues. [a]P<0.05 vs. control. [b]P<0.05 vs. emphysema. BALF, bronchoalveolar lavage fluid; MLI, mean linear intercept; DI, destructive index; CS + CSE, emphysema group; CS3, PI3K inhibitor group; CS1, PDK1 inhibitor group; PI3K, phosphatidylinositol-3-kinase; PDK1, phosphoinositide dependent protein kinase 1.

significantly increased (P<0.05; Fig. 5D). Compared with those in the control group, the expression levels of p16 in the airway of the CS + CSE group were significantly increased (P<0.05; Fig. 5E).

Inhibition of PDK1 reduces the autophagy and cell senescence through the PI3K/AKT signalling pathway in CS + CSE-induced emphysema mice. To explore the role of PDK1 protein expression and PI3K/AKT signalling in the present animal model, small molecule inhibitors were applied. PDK1 inhibitor GSK-2334470 was applied in the CS1 group. PI3K inhibitor PF-04691502 was applied in the CS1 group. Compared with those in the CS + CSE group, the protein expression levels of PI3K, PDK1 and AKT in airway epithelial tissues were significantly decreased in the CS3 group (P<0.05; Figs. 2D-F and 5A-C). In addition, the protein expression levels of LC3B and p16 were also significantly decreased in the CS3 group (P<0.05; Figs. 3B, 4B and 5D-E). The protein expression levels of PDK1, AKT, p16 and LC3B in the airway epithelial tissues of the CS1 group were decreased compared with those in the CS + CSE group (P<0.05; Figs. 2E-F, 3B, 4B and 5B-E). However, there was no significant difference in the expression level of PI3K between the CS1 and the CS + CSE groups (P>0.05; Figs. 2D and 5A). PI3K is the upstream protein of PI3K/PDK1/AKT signalling pathway. PDK1 inhibitor does not inhibit the expression of PI3K, so PI3K does not decrease in CS1 group.

Discussion

CS exposure is the leading cause of emphysema in the world, though the exact mechanism of pathogenesis remain unclear (1). In the present study, CS exposure + short-term CSE intraperitoneal injections were applied to establish an emphysema model in mice to investigate the impact of CS exposure and the pathophysiological changes associated with emphysema progression, in order to explore novel potential therapeutic options for the prevention of this disease. The levels of IL-6, numbers of total cells, neutrophils and macrophages in BALF were found to be significantly higher in the CS + CSE group compared with those in the control group. In addition, the H&E staining of lung tissues revealed histologically advanced emphysema in the CS + CSE group compared with that in the control group, where the MLI and DI were significantly greater, demonstrating the validity of this emphysema model in mice.

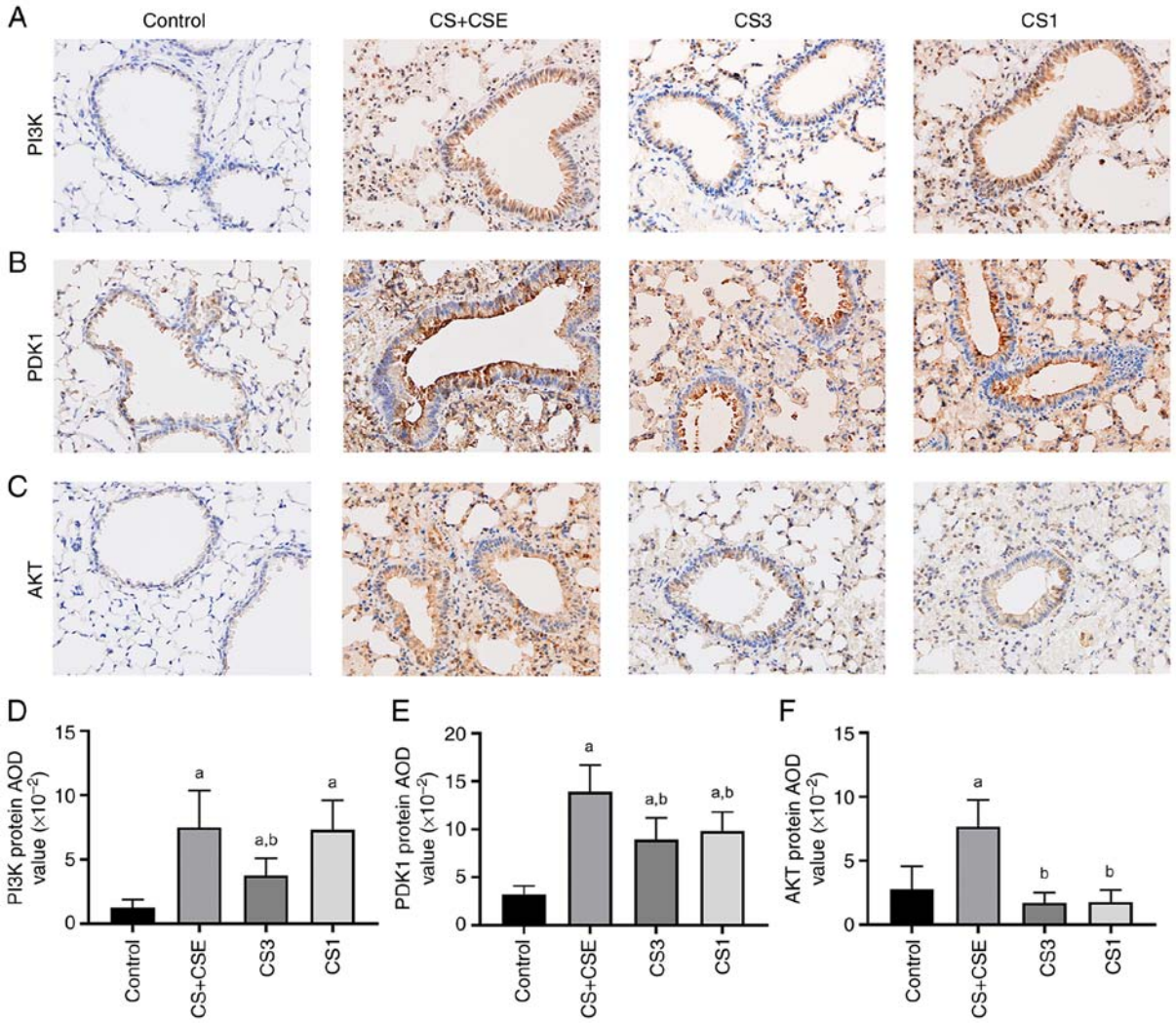

Figure 2. Expression of PI3K, PDK1 and AKT proteins in the airway epithelial tissue. Immunohistochemical staining for (A) PI3K (B) PDK1 (C) AKT expression in the airway epithelial tissues. Magnification, x400. Quantification of (D) PI3K (E) PDK1 (F) AKT protein expression in the airway epithelial tissues in each group. [a]P<0.05 vs. control. [b]P<0.05 vs. emphysema. CS + CSE, emphysema group; CS3, PI3K inhibitor group; CS1, PDK1 inhibitor group; PDK1, phosphoinositide dependent protein kinase 1; AOD, average optical density.

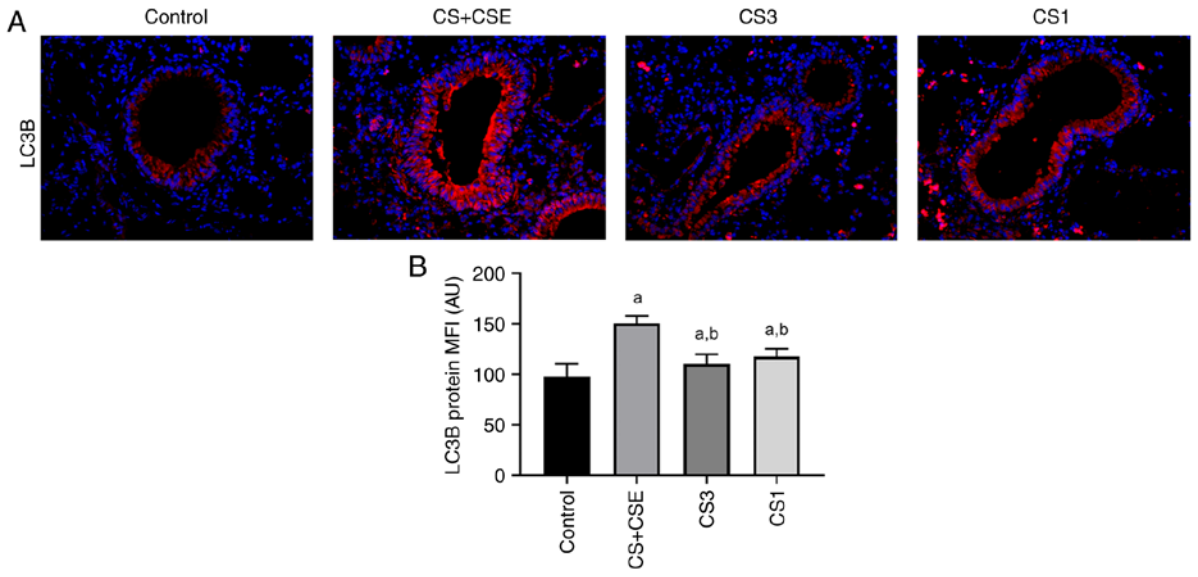

Figure 3. Expression of LC3B protein in airway epithelial tissues. (A) Immunofluorescence staining for LC3BII in airway epithelial tissue. Magnification, x400. (B) Comparison of LC3B protein expression among the experimental groups. [a]P<0.05 vs. control. [b]P<0.05 vs. emphysema. CS + CSE, emphysema group; CS3, PI3K inhibitor group; CS1, PDK1 inhibitor group; PDK1, phosphoinositide dependent protein kinase 1; MFI, mean fluorescence intensity.

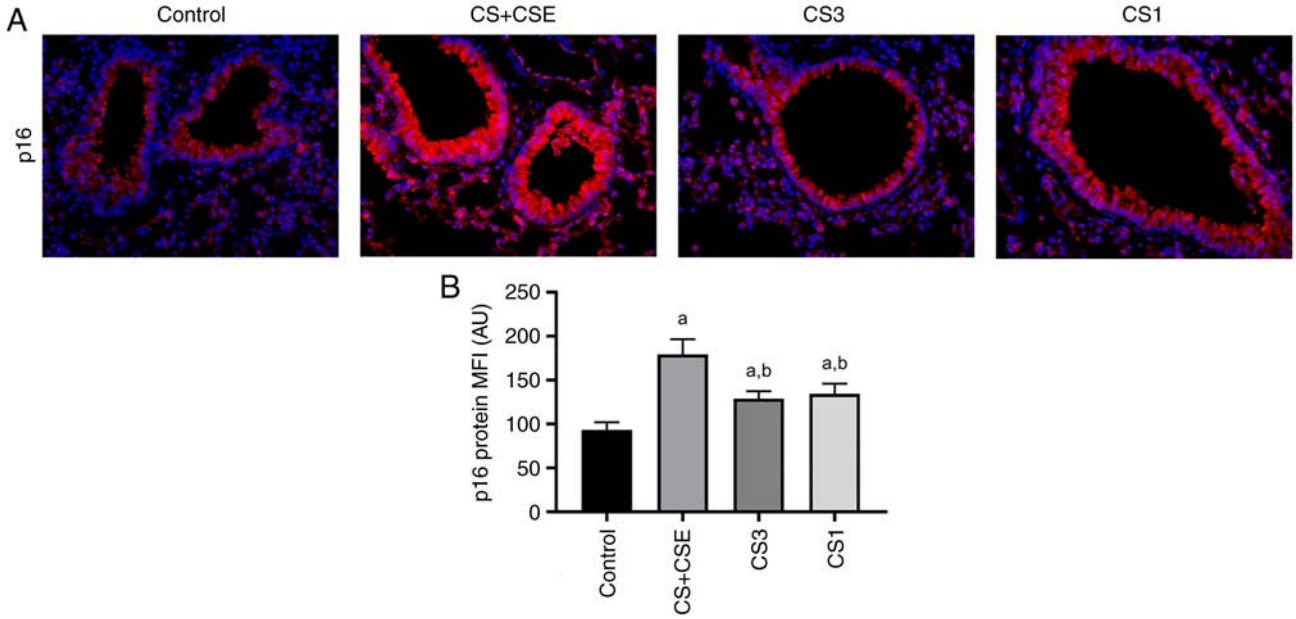

Figure 4. Expression of p16 protein in the airway epithelial tissues. (A) Immunofluorescence staining for the analysis of p16 protein expression in airway epithelial tissues. Magnification, x400. (B) Comparison of p16 protein expression among the experimental groups. [a]P<0.05 vs. control. [b]P<0.05 vs. emphysema. CS + CSE, emphysema group; CS3, PI3K inhibitor group; CS1, PDK1 inhibitor group; PDK1, phosphoinositide dependent protein kinase 1; p16, cyclin-dependent kinase inhibitor 2A.

PDK1 is the major transducer of PI3K downstream (24). The binding of PDK1 to PIP3 activates the PI3K/AKT signalling pathway, which serves an essential role in regulating several important physiological processes, such as cell proliferation, survival and metabolism (25). A previous study demonstrated that activation of the PTEN/PI3K/AKT pathway lead to macrophage M2 polarization in a mouse model of emphysema generated using CS exposure combined with CSE (22). This study combined CS exposure with intraperitoneal injection of CS extract (CSE) to build an emphysema model (22). Activation of the PI3K pathway controls production of transcriptional factors that regulate key inflammatory cytokines (26). The results also showed that there was notably increased p-Akt expression and decreased PTEN expression in the lung tissue of emphysematous mice, and inhibiting PI3K/Akt with LY294002 downregulated the expression of M2 macrophage polarization markers and cytokines (22). Therefore, the present study hypothesized that PDK1 participation in the PI3K/AKT pathway may be involved in the pathogenesis of this type of emphysema. The present results concerning the enhanced expression of PI3K, PDK1 and AKT in the airway epithelial tissues of mice with emphysema were consistent with those observed in a previous study by Lu *et al* (22), which demonstrated the important role of PI3K, PDK1 and AKT in emphysema of mice.

Autophagy is a cellular degradation and recycling process that is highly conserved in all eukaryotes (27). It serves an important role in cell survival and maintenance, the dysfunction of which may contribute to the pathogenesis of a number of diseases, including lung, liver and heart disease, neurodegeneration, myopathies, cancer, ageing and metabolic diseases such as diabetes and COPD (27-29). As previously described by Koukourakis *et al* (13), the most extensively studied endogenous autophagic marker is LC3B, one of the structural proteins of autophagosome membranes, has also been reported to be involved in COPD (14). The present study showed that the expression of LC3B in the airway epithelial tissues of emphysematous mice was significantly increased compared with those in the control group, which is consistent with the previous findings by Chen *et al* (14). This suggests that autophagy is implicated in the pathogenesis of emphysema. In addition, previous studies also showed that the activation of PI3K/AKT signalling can promote cell autophagy and aging (30), whereby CS exposure can increase the expression of senescence marker p16 in the lung tissues of mice (31), consistent with the present results. Therefore, it was hypothesized that activation of the PI3K/AKT signalling pathway in airway epithelial tissues can cause cell autophagy and aging associated with emphysema. Since PDK1 can contribute to the activation of the PI3K/AKT pathway (32), it was speculated that its inhibition may suppress cell autophagy and senescence in emphysematous mice by blocking PI3K/AKT sigalling. However, further studies are needed to clarify the exact mechanisms.

GSK-2334470 is an effective PDK1 inhibitor (32) that can regulate the activity of the PI3K/AKT signalling pathway by inhibiting PDK1, T-loop phosphorylation and AKT activation (33). The PI3K/AKT signaling pathway plays a key regulatory role in autophagy (7). Suppression of PI3K can greatly block the downstream signaling pathways AKT (34). As previously described by Zhang *et al* (8) in mice with COPD, the PI3K inhibitor suppresses protein expression of PI3K, p-AKT and p-mTOR to reduce autophagy. Previous studies demonstrate a pivotal role for the autophagic protein LC3B in CS-induced apoptosis and emphysema (8,14), autophagic protein microtubule-associated protein 1 light chain-3B (LC3B) as a positive regulator of CS-induced lung epithelial cell death (14). In the present study, the levels of PDK1, AKT,

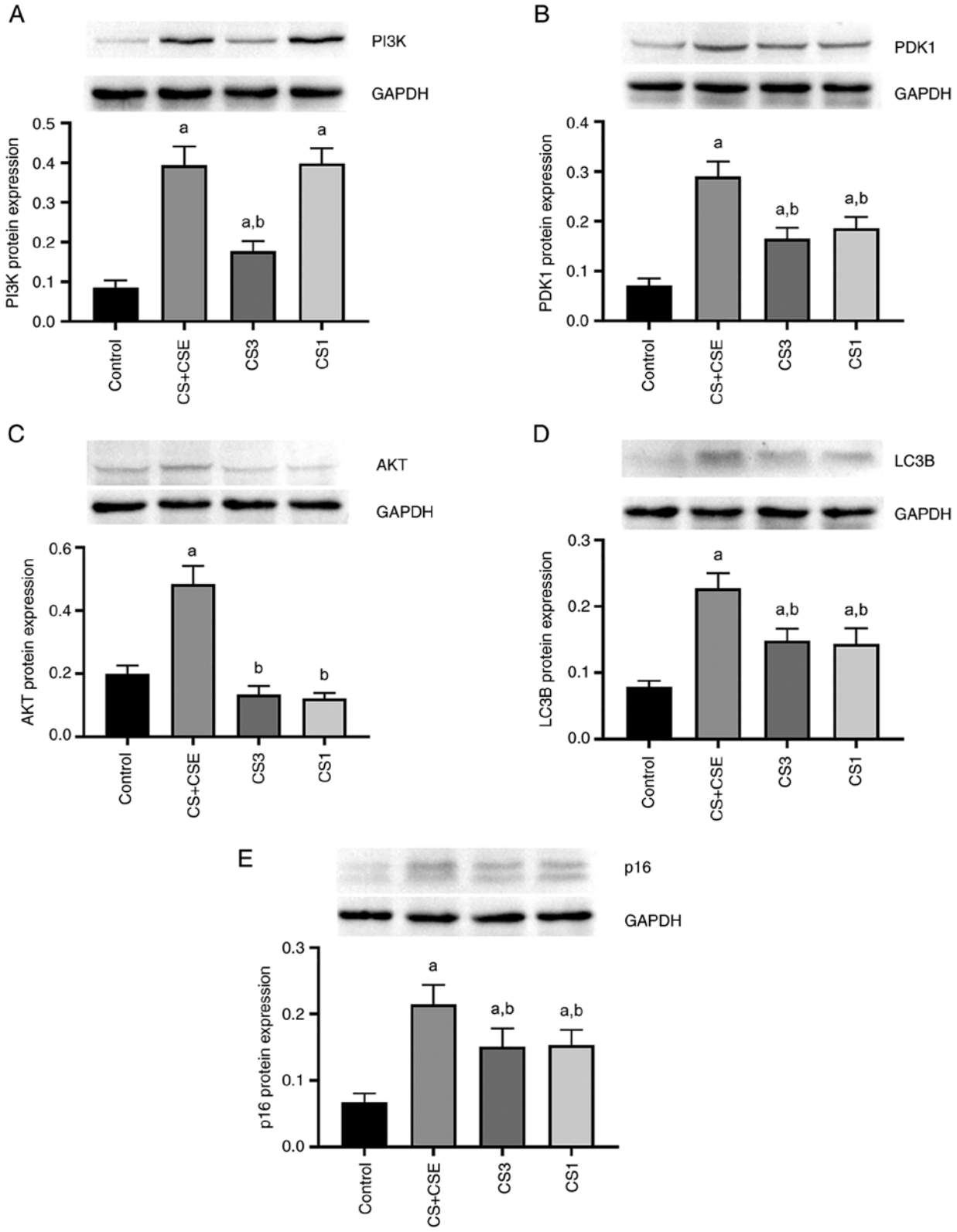

Figure 5. PI3K, PDK1, AKT, LC3B II and p16 protein expression levels. Western blotting for (A) PI3K, (B) PDK1, (C) AKT, (D) LC3B and (E) p16 expression in the airway epithelial tissues. GAPDH as an internal control. Semi-quantification of PI3K, PDK1, AKT, LC3B and p16 protein expression in airway epithelial tissues in each group. [a]P<0.05 vs. control. [b]P<0.05 vs. emphysema. The protein expression levels were expressed as the ratio of band intensity for the target protein relative to that for the internal control GAPDH. Values are expressed as the mean ± standard deviation. CS + CSE, emphysema group; CS3, PI3K inhibitor group; CS1, PDK1 inhibitor group; PDK1, phosphoinositide dependent protein kinase 1; p16, cyclin-dependent kinase inhibitor 2A.

p16 and LC3B expression in the airway epithelial tissues of mice treated with GSK-2334470 were lower compared with those in the CS + CSE group. This suggests that GSK-2334470 may suppress the expression of AKT, p16 and LC3B by partially blocking PDK1. Since PI3K is the upstream activator of PDK1, there was no significant difference in the expression

of PI3K between these two groups, consistent with this theoretical prediction. The present study also showed that PI3K, PDK1, AKT, p16 and LC3B expression were lower in the CS3 group compared with those in CS + CSE group, which further confirmed the important role of PI3K in emphysema and the relationship between PI3K, PDK1 and AKT. It was therefore concluded that PDK1 inhibitors may reduce the expression of p16 and LC3B proteins in airway epithelial cells by regulating the PI3K/AKT signalling pathway, thereby protecting against CS + CSE-induced emphysema in mice. PDK1 inhibitors may represent a potentially novel therapeutic option for the prevention of emphysema in humans.

However, further *in vitro* experiments will be required. The PDK1-specific short hairpin RNA (shRNA) with the highest inhibitory efficiency should be screened out with its interference efficiency verified. Human airway epithelial cells should be treated with different concentrations of CSE (0, 2.5, 5, 10 and 20%) or fixed concentrations of CSE for different times (0, 0.5, 2, 4, 8, 12 and 24 h). Cell proliferation detection kits, such as Cell Counting Kit-8, should be used to measure the proliferation rate of human airway epithelial cells. The optimal concentration and time of CSE should be selected. Therefore, future studies should aim to investigate the effect of PDK1 knockdown on the PI3K/AKT signalling pathway, autophagy and cellular senescence. They will contribute to an in-depth understanding of the molecular regulation mechanism of COPD.

Acknowledgements

Not applicable.

Funding

This study was supported by the National Natural Science Foundation of China (grant no. 1760013; 2018) and the Non-Profit Central Research Institute Fund of Chinese Academy of Medical Sciences (grant no. 2019pt320003).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PZ, YJ and XY designed and performed the experiments, wrote the manuscript, read and approved it. YJ, CZ and YT collected and analysed the data. PZ and YJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The project design was conducted in line with scientific and ethical principles. The institutional review board of Guizhou Provincial People's Hospital (Guiyang, China) approved the present study (approval no. 2017034). The present study was conducted according to the revised Declaration of Helsinki and the experiments were performed following the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, eighth edition (35).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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