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ORIGINAL ARTICLE

Astragaloside IV Inhibits Cigarette Smoke-Induced Pulmonary Inflammation in Mice

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Abstract— The aim of this study was to investigate the effects of Astragaloside IV (AS) on cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD). Our results showed that AS alleviated CS-induced pathological injury in lung tissue. AS also increased superoxide dismutase (SOD) and reduced the level of malondialdehyde (MDA) in serum and lung. AS also reduced cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in serum and lung. More, AS significantly reduced the protein expression of JAK3/STAT3/NF-κB pathway in CS-induced mice. *In vitro*, cigarette smoke extract (CSE) stimulation exposed to normal human bronchial epithelial (HBE) cells. Results further confirmed that AS significantly inhibited the protein levels of JAK3/STAT3/NF-κB pathway in CSE-induced HBE. Our result showed that AS might effectively ameliorate COPD *via* JAK3/STAT3/NF-κB pathway.

KEY WORDS: Astragaloside IV; COPD; inflammation.

INTRODUCTION

The harm of tobacco has become one of the most serious problems in the world. More than 400,000 of people suffer from cardiovascular disease, respiratory disease, and cancer caused by cigarette smoking (CS) [1]. The tobacco production and consumption of our country rank as the top in the world. CS contains thousands of toxic chemicals such as nicotine, tar, and benzopyrene which pose a significant health hazard. The smoke fog may enter the airway and cause the respiratory tract mucosal injury, which leads to increased inflammation in the body. In the lung tissue, CS can induce plenty of disease such as chronic obstructive pulmonary disease (COPD), lung cancer, and interstitial pneumonia [2]. Pulmonary inflammation refers to the terminal airway, pulmonary alveolus, and pulmonary interstitium, which was induced by microorganism, physical and chemical factors, immune injury, irritability, and drugs [3]. Cigarette smoking is one of the most important reasons to cause pulmonary inflammation.

Astragalus mongholicus, a leguminous plant, is widely used in enhancing immunologic function, protecting liver, diuretic, anti-aging, anti-hypertension, and anti-inflammatory [4]. Astragaloside IV (AS) has the best biological activity among *Astragalus* polysaccharide [5]. The aim of this study is to investigate the effects of AS on CS-induced pulmonary inflammation mice.

The Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway is one of the signal transduction pathways which is stimulated by cytokines and participates in proliferation, differentiation, and immunoregulation widely in cells [6]. Besides, it has been proven that it is involved in the development of COPD. Nuclear factor-κB (NF-κB) signal pathway plays an important regulatory role in inflammatory response, immune reactions, and so on [7]. Some research showed that the

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suppression of NF-κB contributes to the inhibition of lung neutrophilia and cytokine production in LPS-induced acute lung injury, asthma, and pulmonary fibrosis. NF-κB signal transduction cascades are also related to COPD. It has been shown that JAK/STAT signal pathway and NF-κB signal pathway have some interaction [8]. NF-κB signal pathway was involved in the neural protection by erythropoietin (EPO), and this was regulated by JAK/STAT signal pathway. Hence, whether the JAK/STAT signal pathway can modulate NF-κB signal pathway to mediate cigarette smoking-induced pulmonary inflammation through JAK/STAT/NF-κB signal pathway needs a further discussion.

MATERIALS AND METHODS

Main Reagents and Kits

AS (purity 97%) was purchased from National Institutes for Food and Drug Control (Beijing, China). Dexamethasone (Dex) was provided by Simcere Drug Store (Nanjing, China). SOD and MDA kits were purchased from the Institute of Jiancheng Bioengineering (Nanjing, China). TNF-α, IL-6, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were supplied from R&D. Cigarettes were purchased from Hongta Tobacco Group Company Limited. P-JAK3 (#3771), JAK3 (#8827), P-STAT3 (#9145), STAT3 (#4904), P-P65 (#3039), P65 (#8242), P-IkBa (#2859), IkBa (#4812), and GAPDH (#5174) were obtained from Cell Signaling Technology (Danvers, USA).

Animals

Sixty male ICR mice (age 8 weeks; weighing 20–22 g) were purchased from Comparative Medicine Centre of Wenzhou Medical University. All animals were housed in a specific pathogen-free (SPF) laboratory in the Animal Center of Wenzhou Medical University at 22 ± 1 °C temperature and 40–50% humidity under a 12-h light/dark cycle with free access to water and standard laboratory chow.

Experimental Protocol

Sixty male ICR mice were randomly assigned to five groups (ten mice in each group): control group, CS group, CS + dexamethasone (Dex, 2 mg/kg) group, CS + AS (10 mg/kg) group and CS + AS (20 mg/kg) group and CS + AS (40 mg/kg) group. All mice (except those in control group) were exposed 5 days a week to the mainstream cigarette smoke of five cigarettes (Reference cigarette3R4F without filter, University of Kentucky, Lexington, KY, USA), four times a day with a 10-min smoke free interval between exposures. A standard smoking apparatus was used with the smoking chamber adapted for a group of mice. A smoke/air ratio of 1/6 was obtained. Control mice were exposed to room air simultaneously. CS exposure started at the weight of 20–22 g and the exposure period was 8 weeks. The mice in group of dexamethasone (Dex) were treated with 2 mg/kg/day dexamethasone by intragastric administration, followed by 4 weeks of smoke exposure. The mice in AS groups were treated with 10, 20, and 40 mg/kg/day AS by intragastric administration, followed by 4 weeks of smoke exposure. The mice in group of control and CS were treated with the same amount of distilled water in the same way.

Tissue Preparation

Blood samples were collected from the orbit and centrifuged at 4500 rpm for 15 min. The supernatant was collected and set aside at − 80 °C. The lungs were harvested subsequently and also stored at − 80 °C.

Cytokine Measurements in Serum, Lung, and Cell Supernatant

Lung samples were homogenized with cold normal saline. After being centrifuged at 12,000 rpm for 10 min at 4 °C, the supernatant of the homogenate was collected into tubes and stored at − 80 °C. The protein contents were determined with a BCA protein assay kit. Serum and cell supernatant IL-1β, IL-6, and TNF-α (R&D, Minneapolis, MN, USA) levels were measured using the ELISA methods according to the manufacturer's instructions with the appropriate commercial kits. Then, the absorbance of each well was read at 450 nm with a microplate spectrophotometer. The contents were calculated according to the standard curves.

Measurement of SOD and MDA Levels in Serum and Lung

Lung samples were homogenized with cold normal saline. After being centrifuged at 12,000 rpm for 10 min at 4 °C, the supernatant of the homogenate was collected into tubes and stored at − 80 °C. The protein contents were determined with a BCA protein assay kit. SOD and MDA levels were measured using the kits according to the manufacturer's instruction.

Histopathology Examination

For the histological analysis, mice were sacrificed after the collection of orbital blood. The left lung was extracted and rapidly fixed in 10% buffered formalin and further fixed for at least 24 h. Sections (4 μm thick) were cut from paraffin-embedded tissues, placed on poly-L-lysine-coated slides, and then incubated overnight at 55–60 °C. Deparaffinized sections were stained with hematoxylin and eosin (H&E). After that, pathological conditions in the lung tissues were visualized under a light microscope. Lung inflammatory cell count based on a 5-point scoring system was performed to estimate the severity of leukocyte infiltration. The scoring system was as follows: 0 no cells; 1 a few cells; 2 a ring of cells with 1 cell layer deep; 3 a ring of cells with 2–4 cell layers deep; and 4 a ring of cells with more than 4 cell layers deep.

Cell Culture

Human bronchial epithelial cells (HBE cells) were purchased from the ATCC (no. 1507); HBE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum and were grown in tissue culture flasks in a humidified gas environment with 95% air and 5% carbon dioxide at 37 °C. The cultures were grouped as follows: (1) normal HBE; (2) cells stimulated with cigarette smoke extract (CSE, 5%, 24 h); (3) cells stimulated with CSE, then pre-incubated with AS (10 μM, 30 min); (4) cells stimulated with CSE, then pre-incubated with AS (20 μM, 30 min); (5) cells stimulated with CSE, then pre-incubated with AS (40 μM, 30 min).

Western Blot

Firstly, the lung and cells were homogenated with RIPA buffer and protease inhibitor for 30 min on ice. Then, the tissue homogenates were centrifuged at 12000 rpm for 15 min, and the supernatant was collected and stored at − 20 °C. The total protein in the lung tissues was determined by the BCA protein assay kit (Beyotime, Nanjing, China). The supernatant was added to the SDS-PAGE loading buffer at the ratio of 4:1 and mercaptoethanol at the ratio of 20:1 and then boiled in boiling water for 5 min. After the protein separation by SDS-PAGE electrophoresis, the

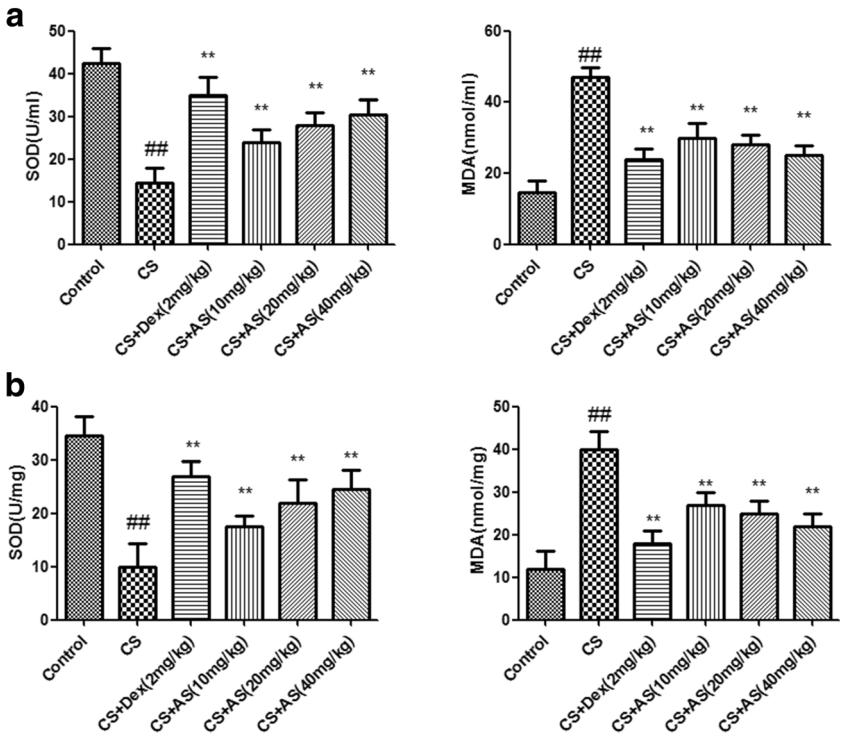

Fig. 1. Effect of Astragaloside IV on SOD and MDA in serum (**a**) and in lung (**b**). Values are expressed as means ± SEM. Compared with control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with cigarette smoke: $^{*}P < 0.05$, $^{**}P < 0.01$.

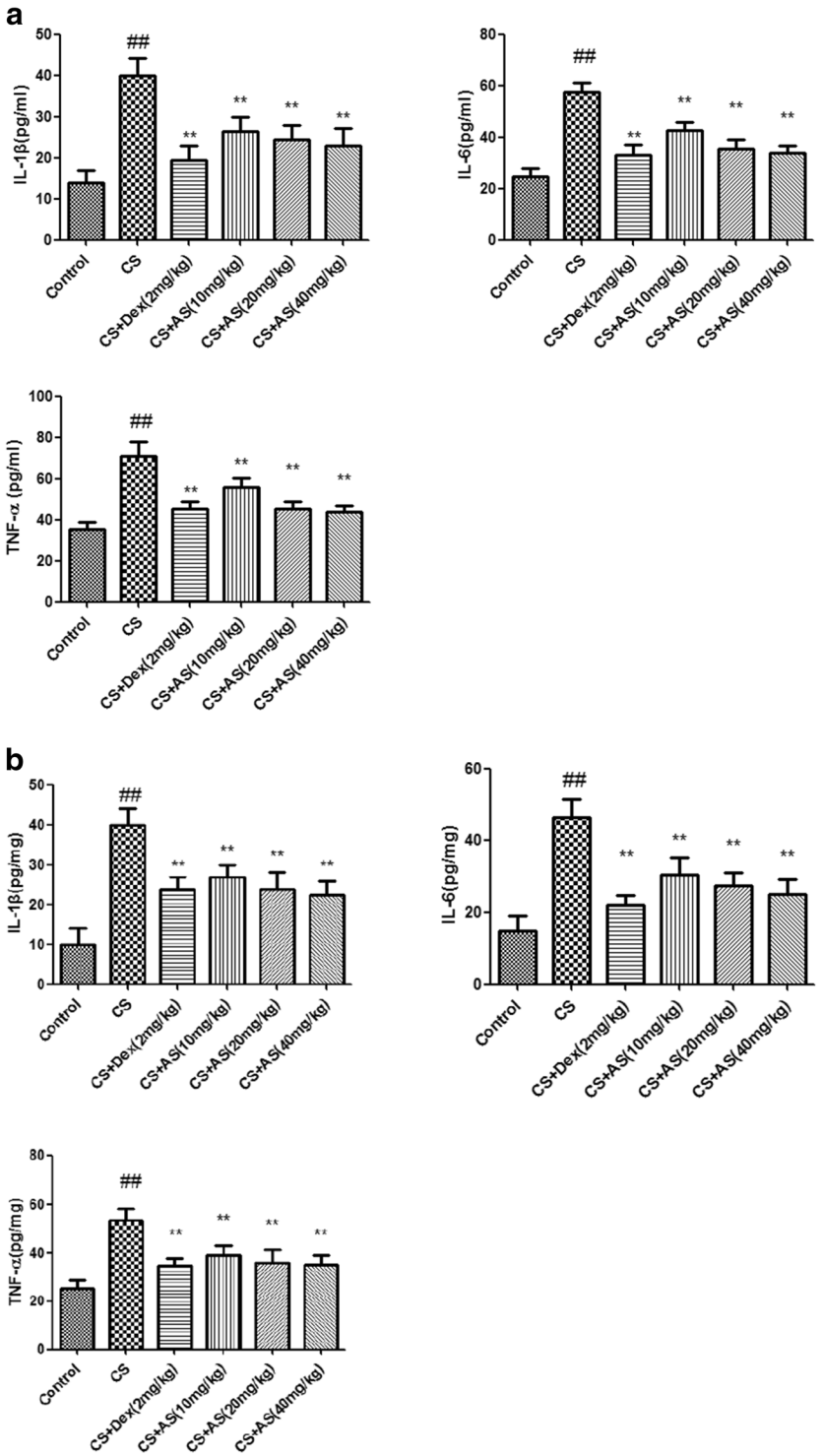

Fig. 2. Effect of Astragaloside IV on pro-inflammatory factors in serum (**a**), lung (**b**), and cell supernatant (**c**). Values are expressed as means ± SEM. Compared with control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with cigarette smoke: $^{*}P < 0.05$, $^{**}P < 0.01$. Values are expressed as means ± SEM. Compared with control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with cigarette smoke extract: $^{*}P < 0.05$, $^{**}P < 0.01$.

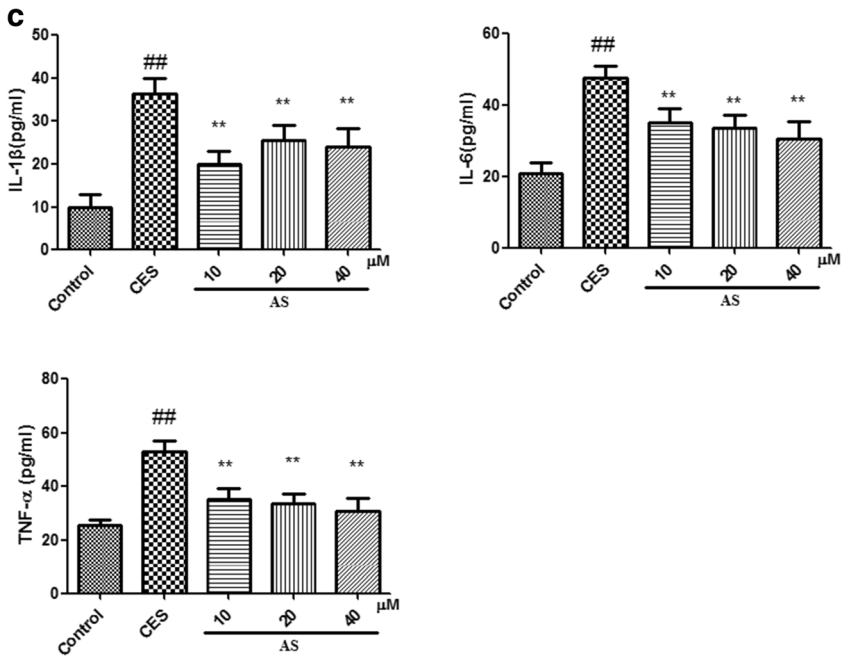

Fig. 2. continued.

protein was transferred to the nitrocellulose membrane (NC). Afterwards, the membranes were block with 5% skim milk in TBST buffer at room temperature for 2 h. Membranes containing target proteins were incubated with primary antibodies diluted in TBST buffer at 4 °C overnight. After washed in TBST buffer for three times, the membranes were incubated with second antibodies for 2 h. The proteins were visualized using an ECL Key-GEN system (KeyGEN Biotechnology, Nanjing, China) and detected with a Clinx ChemiScope chemiluminescence imaging system (Gel Catcher 2850, China).

Statistical Analysis

Experimental data were expressed as means ± standard deviations (SDs). Differences between groups were determined by one-way ANOVA with the Tukey multiple comparison test. P values < 0.05 were considered to be significant. Calculations were made using GraphPad Prism.

RESULTS

Effects of AS on SOD and MDA in Serum and Lung

As depicted in Fig. 1, the CS-induced COPD model group significantly reduced the levels of SOD in serum ($P < 0.01$) and lung ($P < 0.01$), when compared with the control group. However, animals with the treatments of AS (10, 20, 40 mg/kg) and Dex (2 mg/kg) significantly increased the levels of SOD in serum ($P < 0.01$) and lung ($P < 0.01$) compared with those in CS group. The CS-induced COPD model group significantly increased the level of MDA in serum ($P < 0.01$) and in lung ($P < 0.01$) compared with the control group. In contrast, AS (10, 20, 40 mg/kg) ($P < 0.01$) and Dex (2 mg/kg) ($P < 0.01$) both significantly suppressed the elevation of MDA content in serum and in lung.

Effects of AS on Pro-inflammatory Factors

As shown in Fig. 2, the CS-induced COPD mice model group and CSE-induced cells displayed a marked increase in the levels of TNF-α ($P < 0.01$), IL-6 ($P < 0.01$), and IL-1β ($P < 0.01$) in lung tissue and serum cell supernatant. Compared with those in CS group, AS and Dex groups apparently reduced the contents of TNF-α ($P < 0.01$), IL-6 ($P < 0.01$), and IL-1β ($P < 0.01$) in lung tissue and serum cell supernatant, indicating that AS prevented COPD progression *via* the reductions of pro-inflammatory factors.

Histopathological Examination of Lung Tissues

As observed in Fig. 3, in control group, scarce obvious histological alteration was viewed in lung

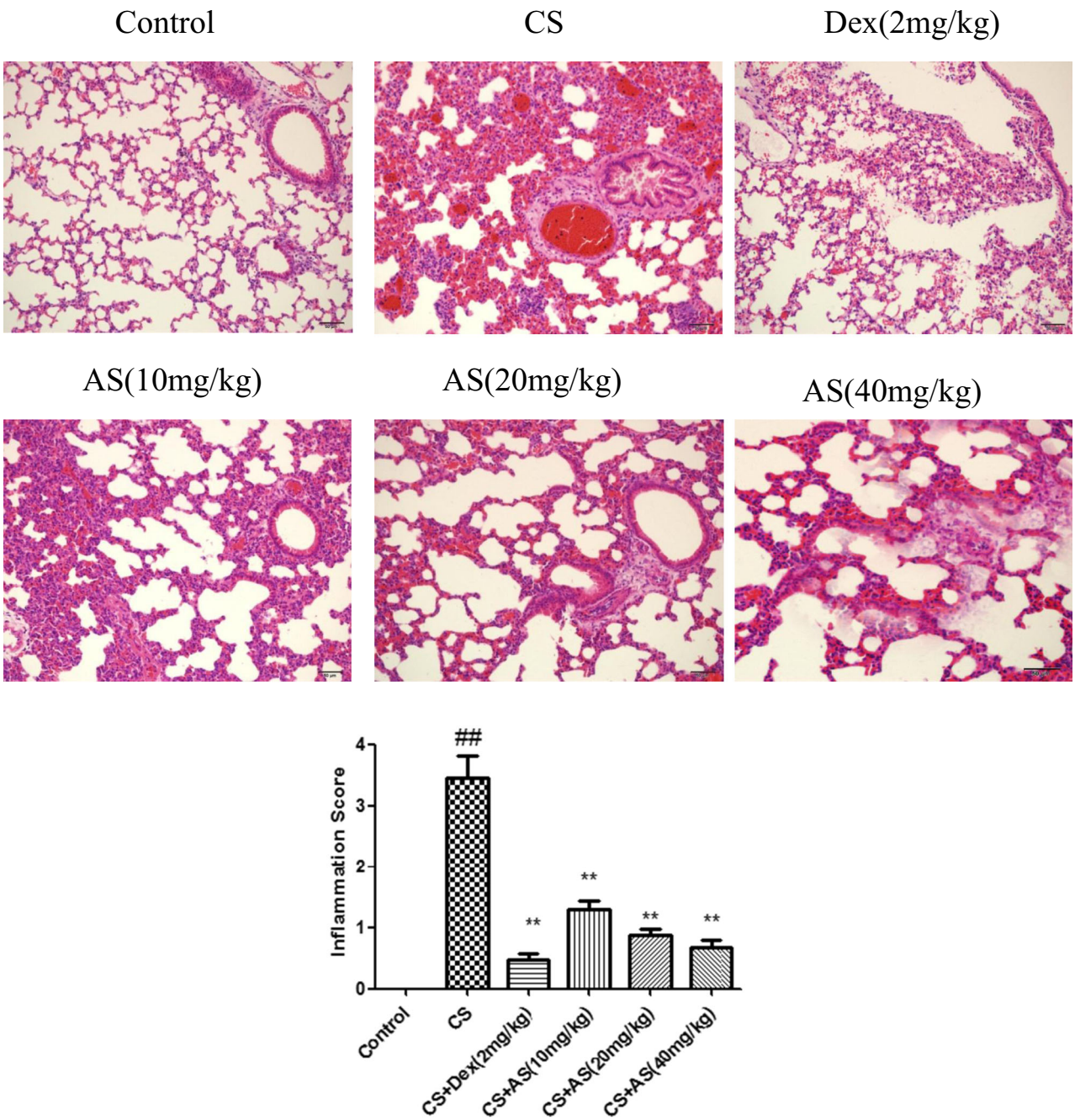

Fig. 3. Histopathological examination of lung tissues. Photomicrographs were taken at × 200. According to the scope and severity of the lung, it was graded on a scale of 0.5–4: 0.5 = minor, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe. Control group showed obvious integrity of lung without pathological injury. Values are expressed as means ± SEM. Compared with control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with cigarette smoke: $^{*}P < 0.05$, $^{**}P < 0.01$.

specimen. By contrast, the pulmonary pathology of CS group showed evident epithelial cell injury of bronchi, goblet epithelium cell metaplasia, inflammatory cell infiltration, mucus secretion, and cell blocking in bronchial lumen. Besides, emphysema, congestion, degeneration, and necrosis of epithelial cells were also relatively clear in CS group. However, the mice treated with AS (10, 20, 40 mg/kg) and Dex (2 mg/kg) notably

reduced the inflammatory infiltration in the alveolar wall and bronchial wall. The results of HE staining indicated the COPD model was successfully established and AS was capable of improving the pathological injury of COPD.

Effects of AS on the Expressions of JAK3/STAT3/NF-κB Signaling-Related Proteins in Mice

As revealed in Fig. 4, Cigarettes caused the up-regulations of the P-JAK3 ($P < 0.01$), P-STAT3 ($P < 0.01$), P-NF-κBp65 ($P < 0.01$), and P-IkBa ($P < 0.01$) protein expressions in mice, suggesting CS might induce COPD through JAK3/STAT3/NF-κB pathway. By contrast, in CS-induced COPD mice, AS (10, 20, 40 mg/kg) and Dex (2 mg/kg) showed inhibitions of expression levels of P-STAT3 ($P < 0.01$), P-NF-κBp65 ($P < 0.01$), and P-IkBa ($P < 0.01$) pathways. Herein, AS exerted the protective effect against COPD possibly *via* the JAK3/STAT3/NF-κB pathway.

Effects of AS on the Expressions of JAK3/STAT3/NF-κB Signaling-Related Proteins in Cells

As revealed in Fig. 5, CSE caused the up-regulations of the P-JAK3 ($P < 0.01$), P-STAT3 ($P < 0.01$), P-NF-κBp65 ($P < 0.01$), and P-IkBa ($P < 0.01$) protein expressions in cells, suggesting CSE might induce cell injury through JAK3/STAT3/NF-κB pathway. By contrast, in CSE-induced cell injury, AS (10, 20, 40 μM) showed inhibitions of expression levels of P-STAT3 ($P < 0.01$), P-NF-κBp65 ($P < 0.01$), and P-IkBa ($P < 0.01$) pathways. Herein, AS exerted the protective effect against COPD possibly *via* the JAK3/STAT3/NF-κB pathway.

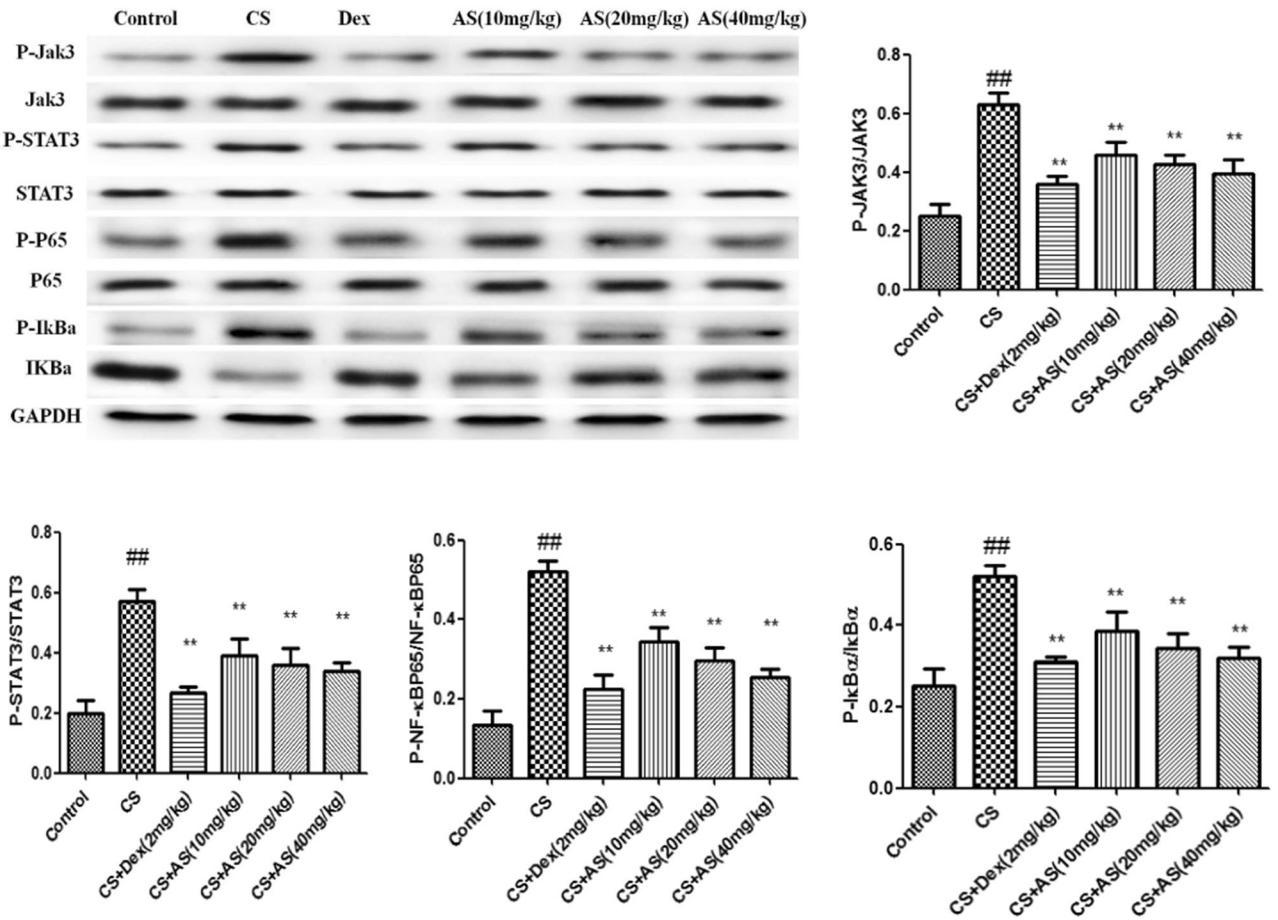

Fig. 4. Effect of Astragaloside IV on the expressions of JAK3/STAT3/NF-κB signaling-related proteins in lung. Values are expressed as means ± SEM. Compared with control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with cigarette smoke: $^{*}P < 0.05$, $^{**}P < 0.01$.

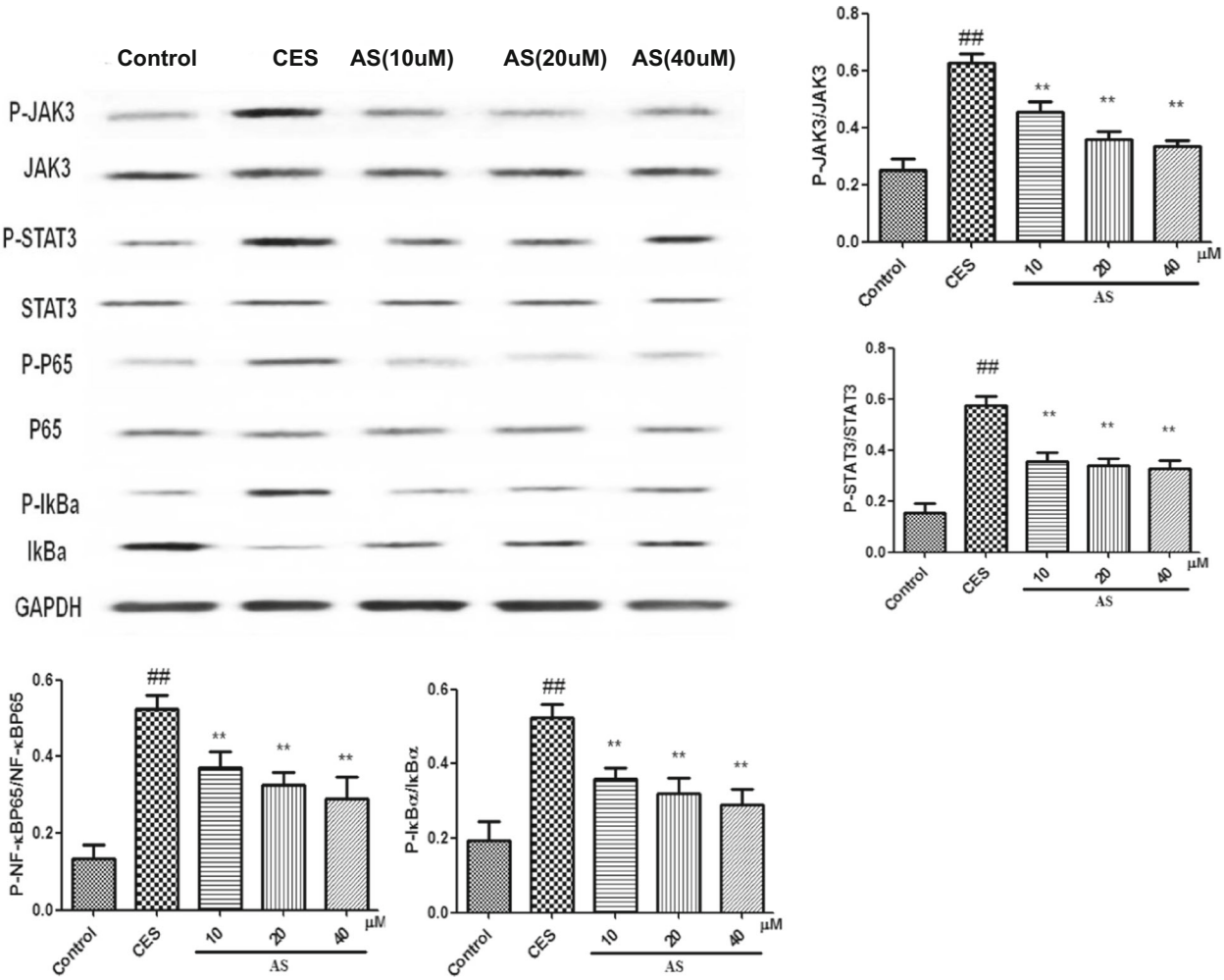

Fig. 5. Effects of Astragaloside IV on the expressions of JAK3/STAT3/NF-κB signaling-related proteins in cells. Values are expressed as means ± SEM. Compared with control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with cigarette smoke extract: $^{*}P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

The harm of cigarette smoking has become one of the most serious problems in the world, which perceived to be a potential injury to self especially the lung tissues. It is known that there are plenty of harmful substances among smoke from cigarette, such as CO, nicotine, phenols, and aldehydes, some of which have strong irritation [9]. The stimulation of smoke will induce the apoptosis of macrophages and reduction of neutrophil infiltration and release plenty of inflammatory mediators, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [10]. TNF-α is produced by mononuclear macrophage, which has the function of immunoregulation and inflammation regulation. IL-1β is regarded as a kind of pro-inflammatory cytokine, which participates in destruction or edema of tissue. IL-6 possesses various immune regulation functions which can enhance the immune function [11–13]. Our study suggested that the CS-induced pulmonary inflammation mouse model group displayed a marked increase in the levels of TNF-α, IL-6, and IL-1β in lung tissue and serum. AS (10, 20, and 40 mg/kg) and AS (10, 20, 40 μM) groups apparently reduced the contents of TNF-α, IL-6, and IL-1β in lung tissue and serum cell supernatant, indicating that AS prevented pulmonary inflammation progression *via* the reductions of pro-inflammatory factors.

When exposed to smoke, the organism will continue to endure the damage of oxidation environment. Reactive oxidative stresses (ROS) such as superoxide anion and hydroxyl are active unpaired electrons which can process a series of biochemical reactions, and it is one of the key factors to induce oxidative damage [14]. ROS can cause the oxidation of protein, DNA, and lipid, which results the lung injury. Besides, ROS can also change the extracellular matrix, remodel the vascular, stimulate the secretion of mucus, regulate cell proliferation, and induce apoptosis [15]. Previous studies have reported that ROS participate in plenty of pulmonary diseases such as pulmonary inflammation [16], pulmonary fibrosis [17], acute respiratory distress syndrome [18], and lung cancer [19]. Superoxide dismutase (SOD) is a kind of active agent which can eliminate the harmful substance through the process of metabolism. What is more, it was regard as the primary substance to eliminate the free radical. Previous studies have reported that lipid peroxidation reaction in organisms will produce molondialdehyde (MDA), which leads to crosslinking and polymerization of protein or nucleic acid, and has plenty of cytotoxicity [20]. Our research indicated that the CS-induced COPD model group significantly reduced the levels of SOD in serum and lung. However, animals with the treatments of AS (10, 20, and 40 mg/kg) and Dex (2 mg/kg) significantly increased the levels of SOD in serum and lung. The CS-induced COPD model group significantly increased the level of MDA in serum and in lung. In contrast, AS (10, 20, and 40 mg/kg) and Dex (2 mg/kg) both significantly suppressed the elevation of MDA content in serum and in lung.

Finally, we chose western blot to discuss the mechanism of Astragaloside IV (AS) on cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD). Janus kinase signal transducer and activator of transcription (JAK/STAT) signal pathway is one of the most important pathways in cell signaling transduction, which can regulate the growth, activation, differentiation, and apoptosis of cell [21]. The present studies have shown that numerous external stimuli signal could lead to the activation of NF-κB signaling pathway including cigarette smoking [22]. The main function of IκB protein is to prevent the NF-κB protein entering the nucleus and combining with the DNA, which keeps the NF-κB protein to stay in the cytoplasm [23]. Therefore, the research of IκB protein seems so important when exploring the mechanism of NF-κB signaling pathway. In our study, cigarettes and CES caused the up-regulations of the JAK3/STAT3/NF-κB signaling-related protein expressions in mice and cells, suggesting CS might induce COPD through JAK3/STAT3/NF-κB. By contrast, in CS-induced mice and CES-induced cells, AS (10, 20, and 40 mg/kg) and AS (10, 20, 40 μM) showed inhibitions of expression levels of JAK3/STAT3/NF-κB pathway. Herein, AS exerted the protective effect against COPD possibly *via* the JAK3/STAT3/NF-κB pathway.

In conclusion, our study indicated that Astragaloside IV (AS) could exert protective effect on CS-induced chronic obstructive pulmonary disease (COPD), which might be attributed to the inhibition of JAK3/STAT3/NF-κB pathway.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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