



Chikusetsusaponin V attenuates lipopolysaccharide-induced acute lung injury in mice by modulation of the NF- κ B and LXR α

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ABSTRACT

Acute lung injury (ALI) is an excessive and uncontrolled inflammatory response in lung, of which remains the leading cause of morbidity and mortality worldwide. Chikusetsusaponin V (CsV), a bioactive compounds derived from *Panax Japonica*, has been reported to have anti-inflammatory effects. However, it is still unclear whether CsV can protect mice against ALI. This study aimed to investigate the protective roles and potential mechanisms of CsV on lipopolysaccharide (LPS)-induced ALI in mice. The mice were pretreated with CsV (5, 10, and 20 mg/kg) four days before LPS treatment. 24 h later LPS administration, the histopathological changes, wet/dry ratio, and MPO activity in lung tissues were detected. The inflammatory cells, including total cells, neutrophils, and macrophages in the bronchoalveolar lavage fluid (BALF) were detected under a light microscope. The levels of pro-inflammatory cytokine TNF- α , IL-1 β , and IL-6 in the BALF were assessed by ELISA. In addition, the expressions of NF- κ B and LXR α in lung tissues were detected by western blot analysis. The results showed that pretreatment of CsV attenuated the lung histopathological damages, lung wet/dry ratio, and MPO activity induced by LPS. In addition, CsV also reduced the LPS-induced increases in the number of inflammatory cells and pro-inflammatory cytokine TNF- α , IL-1 β , and IL-6 in the BALF. Furthermore, western blot analysis showed that CsV significantly inhibited the activation of NF- κ B signaling pathway. CsV dose-dependently increased the expression of LXR α . In vitro, the anti-inflammatory effects of CsV can be reversed by LXR α inhibitor, GGPP. In conclusion, the results showed that CsV protected against LPS-induced ALI due to its ability to activate LXR α .

1. Introduction

Acute lung injury (ALI) is defined as pulmonary dysfunction caused by pathogenic factors [1]. ALI is a serious disease in clinics with persistent high morbidity and mortality due to complicated pathogenesis and a pathological course, and the pathophysiology of this disease is associated with a complex array of molecular, cellular, and physiological mechanisms [2,3]. The factor of ALI is multitudinous, including pneumonia, aspiration, contusion, sepsis, trauma, and pancreatitis. The major characteristics of ALI are loss of the alveolar-capillary barrier, increased permeability of alveolar epithelium, the infiltration of neutrophils, as well as the activation of coagulation [4]. Although a large amount of studies have focus on exploring the potential drugs for ALI, the morbidity and mortality is still high [5].

Panax japonicas, belongs to *Araliaceae Panax family*, is served as a folk medicine in Tujia and the Hmong [6,7]. Chikusetsusaponin V (CsV)

is one of the most abundant and bioactive compounds in the *Panax Japonica* and has been known to have anti-inflammatory and anti-oxidative properties [8,9]. Evidences suggested that CsV inhibited LPS-induced pro-inflammatory cytokines TNF- α , IL-1 β , and iNOS production in RAW264.7 cells. Furthermore, LPS-induced up-regulation of SIRT1 and TLR4/CD14-mediated NF- κ B and MAPKs signaling pathways were inhibited by CsV in a dose-dependent manner [9,10]. Others results showed that CsV inhibited transaminase (ALT) and aspartate aminotransferase (AST) production in LPS-induced liver injury in mice. In addition, CsV also inhibited the levels of pro-inflammatory cytokines by inhibiting the activation of NF- κ B and MAPK signaling pathways. However, the effects of CsV on ALI have not been reported. The aim of this study was to evaluate the protective effects and mechanisms of CsV on LPS-induced ALI in mice.

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2. Materials and methods

2.1. Reagents and chemical

Chikusetsusaponin V (purity > 95%) was purchased from Chengdu Purechem Standard Co., Ltd. (Chengdu, Sichuan, China). Lipopolysaccharide (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). MPO detection kit and Wright-Giemsa stain kit were purchased from Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). Mouse TNF- α , IL-1 β , and IL-6 ELISA kits were purchased from R & D (USA). Antibodies against NF- κ B p65/phosphor-NF- κ B p65, I κ B α /phosphor-I κ B α , and LXR α were purchased from Cell Signal Technology, Inc. (Dancers, MA, USA). Antibodies against β -actin and HRP-conjugated secondary antibodies were purchased from Abcam (Cambridge, UK). All other chemicals used for analysis were reagent grade.

2.2. Animals

Seventy-two C57BL/6 mice (6–8 weeks) were purchased from the center of Experimental Animals of Baiqiu Medical College of Jilin University (Jilin China). All mice were housed at a 12 h light-dark cycle environment, with free access to water and food. Animal experiments comply with the manual the care and use of laboratory animals published by the US National Institutes of Health.

2.3. Animal model of ALI

Mice were randomly divided into six groups ($n = 12$, each group): control group, LPS treated group, LPS + CsV (5 mg/kg, 10 mg/kg, and 20 mg/kg) treated groups (1 h before), and LPS + CsV (20 mg/kg) group (1 h later). A previous study showed that CsV at the dose of 20 mg/kg had no toxicity to mice [8]. In the CsV treatment groups, mice were given CsV for four consecutive days. One hour after the last treatment of CsV, mice were induced ALI by intranasal instillation LPS (5 mg/kg) dissolved in 50 μ L PBS, whereas the control group was treated with equal amount of PBS. 24 h later, the mice were sacrificed and the lung tissues and BALF were harvested and stored at -80°C until used [8,11,12].

2.4. Histopathological analysis

The harvested lung tissue samples were fixed in 10% neutral formalin and embedded in paraffin. After deparaffinization with a series of dehydration, the samples were cut into sections (4 μ m) and stained with hematoxylin and eosin. The lung histopathological changes were detected by microscopy.

2.5. Lung wet/dry ratio

The right lung tissues in each group were collected and the wet weights were recorded. Then the lung tissues were incubated in an incubator at 60°C for 72 h to remove moisture, and the dry weights were recorded. The lung wet/dry ratio was calculated to assess the edema.

2.6. Lung MPO activity

The lung tissue samples were weighted and homogenated with PBS ($w/v = 1:19$). The homogenate was centrifuged at 12000 rpm for 10 min at 4°C , and the supernatant was collected for assessing MPO activity by MPO detection kit in accordance with the manufacturer's instructions. The absorbance was measured at the OD with 460 nm.

2.7. BALF analysis

The BALF was collected by intratracheal injection with 1.2 mL ice-PBS (0.4 mL PBS each time, three times) with 18-G sterile needle with blunt end, and was centrifuged at $3000 \times g$ for 15 min at 4°C . The pellet was resuspended in 0.5 mL PBS and stained with Wright-Giemsa stain. The number of total cells, neutrophils, and macrophages were counted under a microscope (Olympus, Tokyo, Japan). For pro-inflammatory cytokines detection, TNF- α , IL-1 β , and IL-6 levels in BALF were assessed by ELISA kits according to the manufacturer's instruction.

2.8. Western blot assess

The lung tissue samples from each group were harvested and were then subjected to protein extraction with tissue protein extraction reagent (T-PER). The concentrations of proteins were detected by BCA protein assay kit. The proteins were electrophoresed on 10% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked with 3% BSA for 2 h at room temperature. After that, the membranes were incubated with primary antibodies NF- κ B p65, phosphorylation NF- κ B p65, I κ B α , phosphorylation I κ B α and LXR α (1:1000) at 4°C overnight. HRP-conjugated secondary antibody was incubated for 1 h after washing with T-BST for three times. The membranes were subsequently visualized on X-ray films using a chemoluminescence detection system (ECL). The β -actin was served as an internal control.

2.9. In vitro experiment

A549 cells were cultured in DMEM supplemented with 10% FBS. MTT assay was used to detect the effects of CsV on A549 cell viability. The cells were pretreated with CsV 1 h before LPS treatment. The level of IL-8 was measured by ELISA. For LXR α inhibition assay, A549 cells were pretreated with or without 20 μ M GGPP for 2 h. Then, the cells were pretreated with CsV 1 h before LPS treatment.

2.10. Statistical analysis

Data are expressed as the mean \pm SD. Results were analyzed by GraphPad Prism 5 (GraphPad Software Inc., CA, USA) using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. p values < 0.05 were considered statistically significant.

3. Results

3.1. CsV ameliorates LPS-induced lung histopathological changes

Histopathological changes of lung tissues were analyzed by H & E staining. The results showed that CsV (5 mg/kg, 10 mg/kg and 20 mg/kg) treatment could dose-dependently alleviate lung histopathological damages, such as pulmonary edema, hemorrhage and inflammatory cells infiltration into alveoli during ALI induced by LPS (Fig. 1). Treatment of CsV (20 mg/kg) 1 h after LPS could also attenuate LPS-induced lung histopathological damages.

3.2. CsV reduces LPS-induced lung wet/dry ratio

The lung wet/dry ratio was used to assess lung edema. As shown in Fig. 2, 24 h later LPS treatment, the lung wet/dry ratio was higher than that in the control group. However, CsV (5 mg/kg, 10 mg/kg and 20 mg/kg) dose-dependently inhibited the lung wet/dry ratio induced by LPS. Treatment of CsV (20 mg/kg) 1 h after LPS could also attenuate LPS-induced lung wet/dry ratio.

3.3. CsV reduces LPS-induced lung MPO activity

MPO activity was often used to evaluate the activation and

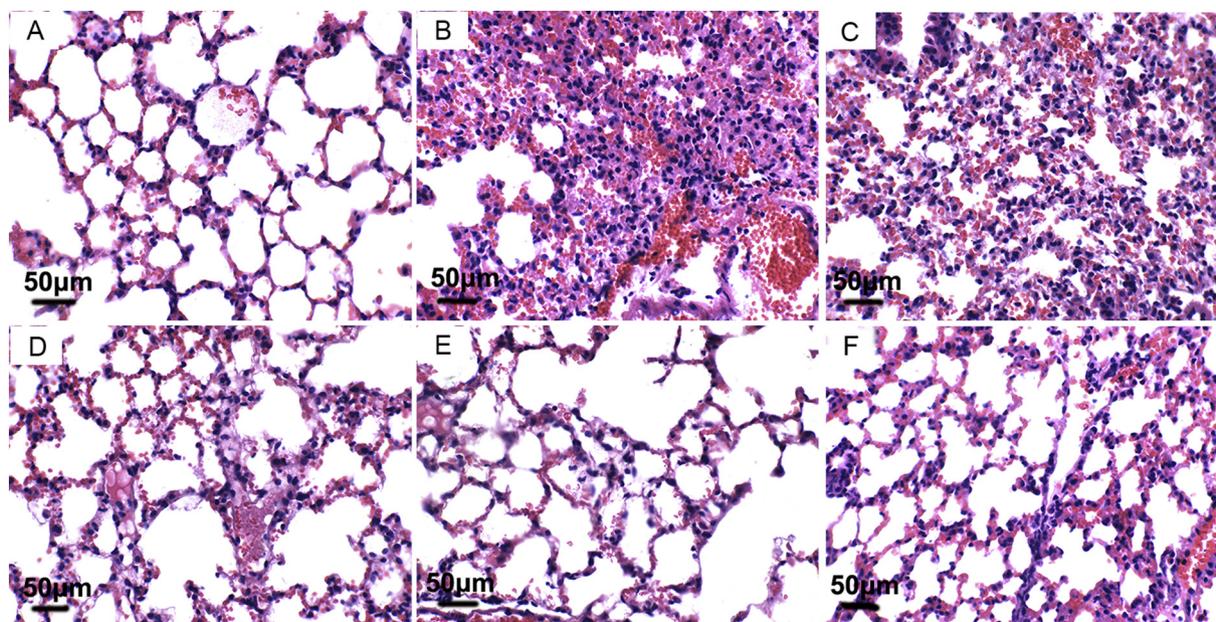


Fig. 1. Effects of CsV on histopathological changes in lung tissues in LPS-induced ALI mice. Representative histological changes of lung obtained from mice of different groups. A: Control group, B: LPS group, C: LPS + CsV (5 mg/kg) group, D: LPS + CsV (10 mg/kg) group, E: LPS + CsV (20 mg/kg) group (Hematoxylin and eosin staining, magnification 200×).

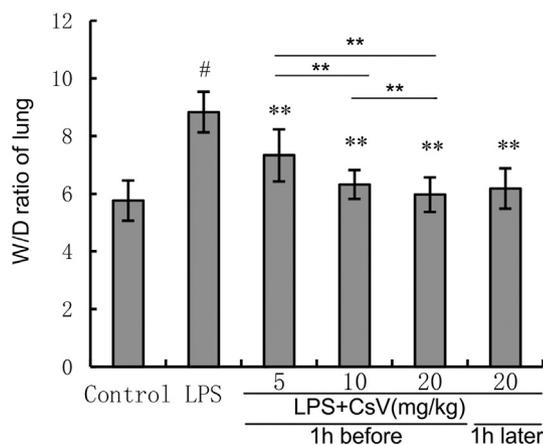


Fig. 2. Effects of CsV on the lung W/D ratio of LPS-induced ALI mice. The values presented are the means ± SEM of three independent experiments. #*p* < 0.01 vs. control group, **p* < 0.05 and ***p* < 0.01 vs. LPS group.

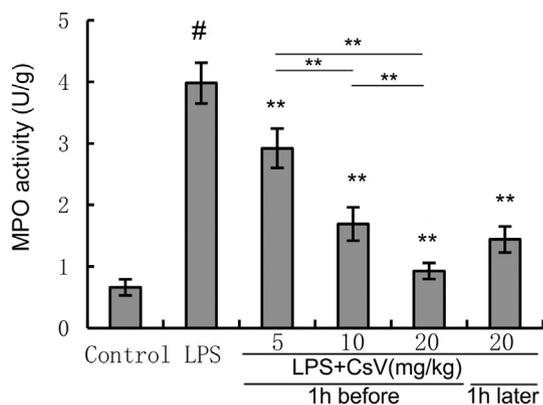


Fig. 3. Effects of CsV on MPO activity and MDA content. The values presented are the mean ± SEM of three independent experiments. #*p* < 0.01 vs. control group, **p* < 0.05, ***p* < 0.01 vs. LPS group.

accumulation of neutrophils in lung tissues. In the present study, we found that MPO activity was markedly enhanced induced by LPS. However, MPO activity was reduced by the administration of CsV in a dose-dependent manner (Fig. 3). Treatment of CsV (20 mg/kg) 1 h after LPS could also inhibit LPS-induced MPO activity.

3.4. CsV reduces LPS-induced inflammatory cells numbers in BALF

In order to explore the anti-inflammatory properties of CsV, the numbers of inflammatory cells in BALF were counted. As shown in Fig. 4, after treatment of LPS, total cells, neutrophils, and macrophages were significantly increased in BALF. However, CsV (5 mg/kg, 10 mg/kg and 20 mg/kg) treatment groups had a dose-dependent effect on decreasing LPS-induced total cells, neutrophils and macrophages in BALF. Treatment of CsV (20 mg/kg) 1 h after LPS could also inhibit LPS-induced inflammatory cell infiltration.

3.5. CsV reduces LPS-induced pro-inflammatory cytokines levels in BALF

The levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in BALF were tested using mouse ELISA kits. As shown in Fig. 5, the levels of TNF-α, IL-1β, and IL-6 were clearly increased in the LPS group compared with that in the control group. However, treatment with CsV resulted in a clear reduce on the levels of TNF-α, IL-1β, and IL-6. Especially, CsV (20 mg/kg) treatment group significantly reduced total cells, neutrophils and macrophages compared with those in the LPS groups. Treatment of CsV (20 mg/kg) 1 h after LPS could also inhibit LPS-induced TNF-α, IL-1β, and IL-6 production.

3.6. CsV inhibits LPS-induced the activation of NF-κB signaling pathway

The activation of NF-κB signaling pathway is associated with the production of pro-inflammatory cytokines, such as TNF-α and IL-1β. After LPS stimulation, the expression of phosphorylation NF-κB p65 and phosphorylation IκBα were significantly increased. However, these increases were dose-dependently inhibited in mice treated with CsV (Fig. 6).

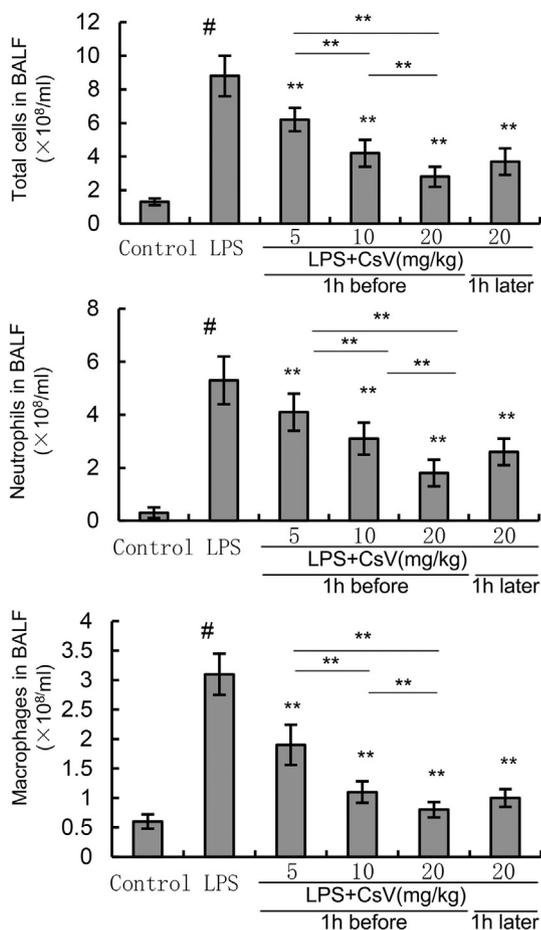


Fig. 4. Effects of CsV on inflammatory cell number in the BALF of LPS-induced ALI mice. The values presented are mean \pm SEM of three independent experiments. # p < 0.01 vs. control group, * p < 0.05, ** p < 0.01 vs. LPS group.

3.7. CsV increases LPS-induced the expression of LXR α

LXR α is recognized as an important anti-inflammatory transcription factor, and it participated in the development of LPS-induced ALI. In the present study, we found that pretreatment with CsV dose-dependently increased the expression of LXR α in LPS-induced ALI in mice (Fig. 7).

3.8. Anti-inflammatory effects of CsV were through activating LXR α

In vitro, we detected the effects of CsV on LPS-induced IL-8 production in A549 cells. The results showed CsV significantly inhibited LPS-induced IL-8 production. However, the inhibitory effect of CsV on IL-8 production was reversed by LXR α inhibitor GGPP (Fig. 8).

4. Discussion

Acute lung injury (ALI) remains a severe disease that threatens human life worldwide and remains a major challenge for clinicians [13,14]. A large amount of studies have shown that CsV, the main component of saponins from *Panax japonicas*, exhibited anti-inflammatory effects [9,10,15]. However, the biological function of CsV in ALI has not been reported. In this study, an LPS-induced ALI mouse model was used to explore the protective effects and mechanisms of CsV on ALI.

Accumulation of inflammatory cells in lungs and the production of pro-inflammatory cytokines is a crucial indicator of ALI. The numbers of total cells, neutrophils and macrophages in BALF were counted in the present study. We found that compared with the control group, the LPS

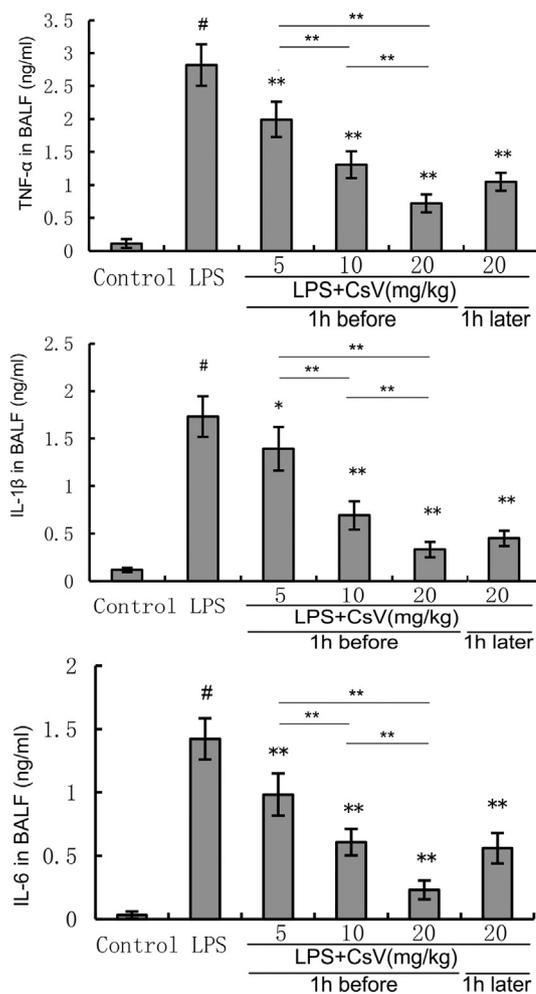


Fig. 5. Effects of CsV on TNF- α , IL-1 β , and IL-6 production in the BALF of LPS-induced ALI mice. The values presented are mean \pm SEM of three independent experiments. # p < 0.01 vs. control group, * p < 0.05, ** p < 0.01 vs. LPS group.

group showed higher levels of total cells, neutrophils and macrophages in BALF. However, CsV dose-dependently reduced the number of those inflammatory cells induced by LPS. MPO, an indicator of the amount of neutrophils and degree of inflammation, is a crucial enzyme released by neutrophils during the inflammatory process [16,17]. In the present study, the lung MPO activity was assessed in each group. MPO activity sharply increased after the administration of LPS. However, a dose-dependently reduction on the level of MPO activity was found in ALI mice pretreated with CsV. TNF- α is particularly worth mentioning, because of it plays an important role in the disruption of endothelial and epithelial barrier [18]. In addition, TNF- α is a crucial factor that induces endothelial and epithelial cells apoptosis [19,20]. IL-1 β is produced in the early stage of inflammation [21], and it is involved in the activation and chemotaxis of neutrophils during the inflammatory responses [22]. Hence, the levels of pro-inflammatory cytokines, TNF- α and IL-1 β , in BALF were detected to assess the anti-inflammatory effects of CsV in the present study. The results showed that the levels of TNF- α , IL-1 β , and IL-6 were increased in the LPS group compared with the control group. However, pretreatment with CsV to mice with LPS-induced ALI, the levels of TNF- α , IL-1 β , and IL-6 were significantly decreased.

The production of pro-inflammatory cytokines, such as TNF- α and IL-1 β , is required for the activation of NF- κ B signaling pathway. Under resting conditions, NF- κ B is in a deactivation state that it is bound by I κ B α in the cytoplasm. Once stimulated by LPS, the I κ B depolymerization

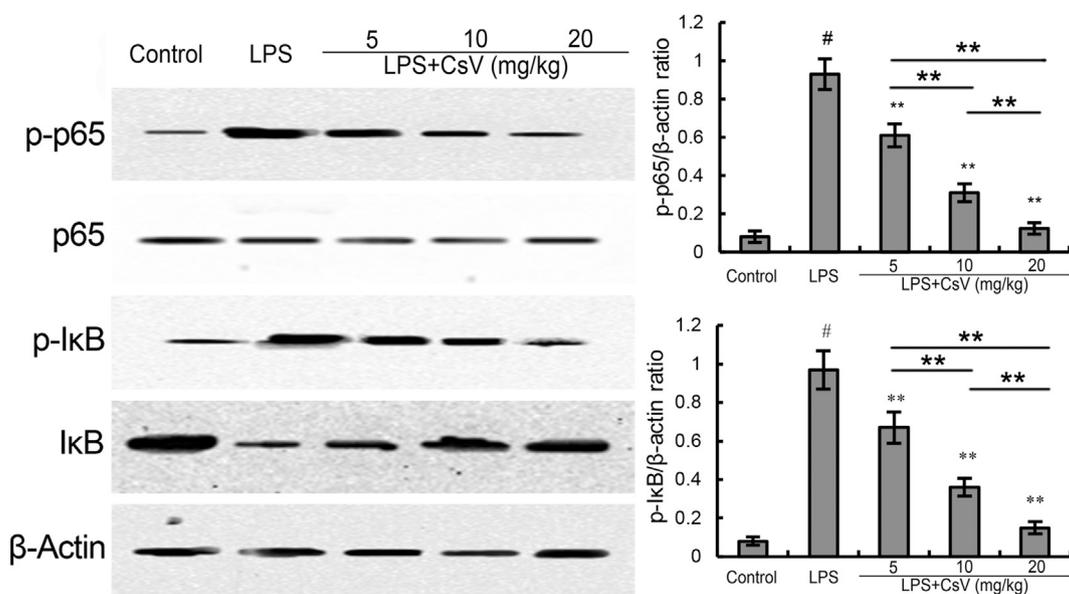


Fig. 6. CsV inhibits LPS-induced NF-κB activation. The values presented are the means ± SEM of three independent experiments. #*p* < 0.01 vs. control group, **p* < 0.05, ***p* < 0.01 vs. LPS group.

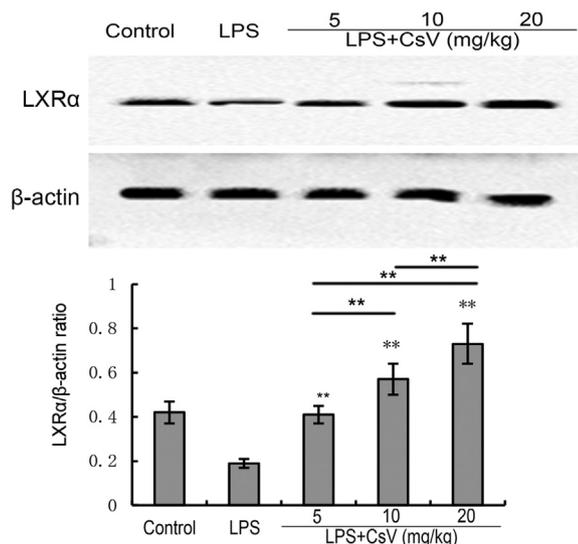


Fig. 7. Effects of CsV on LXRα expression. The values presented are the means ± SEM of three independent experiments. #*p* < 0.01 vs. control group, **p* < 0.05, ***p* < 0.01 vs. LPS group.

and degradation by proteasomes and then induces NF-κB p65 translocates into the nucleus to induce the expression of pro-inflammatory cytokines [23–25]. Studies suggested that activation of NF-κB signaling pathway is closely related to the development of LPS-induced ALI [26,27]. To test the anti-inflammatory mechanisms of CsV, the effect of CsV on the expression of NF-κB was measured in the present study. The results showed that CsV dose-dependently inhibited the activation of NF-κB signaling pathway induced by LPS.

Liver X receptor (LXRs) were recognized as orphan members of the nuclear receptor superfamily of transcription factors that involved in the regulation of lipid and cholesterol metabolism [28]. Recently, many evidences have been reported that LXRα is an important anti-inflammatory transcription factors that participated in the development of inflammatory diseases [29,30]. TO901317, a LXR agonist, inhibited inflammatory responses by inhibition the activation of NF-κB and JNK/p38 MAPK signaling pathway in paraquat-induced ALI [31]. In addition, studies demonstrated that activation of LXRα could inhibit LPS-

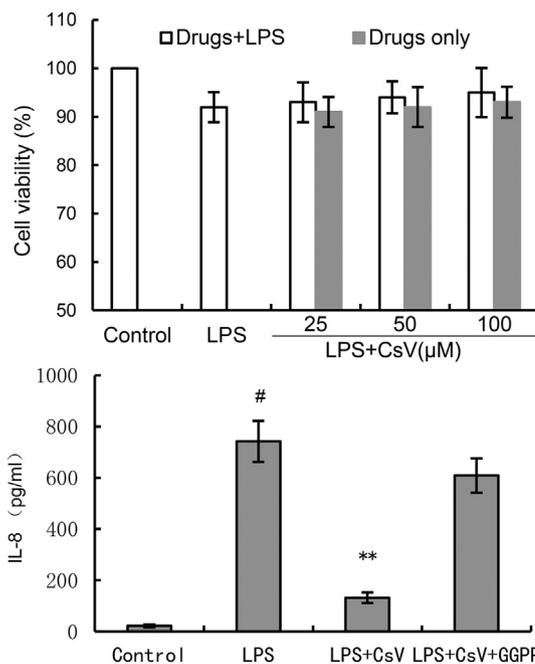


Fig. 8. Anti-inflammatory effects of CsV were through activating LXRα. The values presented are the means ± SEM of three independent experiments. #*p* < 0.01 vs. control group, **p* < 0.05, ***p* < 0.01 vs. LPS group.

induced NF-κB activation [32]. In the present study, we found that CsV significantly increased the expression of LXRα in LPS-induced ALI. Furthermore, our in vitro study showed that the anti-inflammatory effects of CsV can be reversed by LXRα inhibitor GGPP. These results suggested CsV exhibited its anti-inflammatory effects through activating LXRα.

In conclusion, our results clearly indicated that treatment with CsV had an obviously protective effect against LPS-induced ALI in mice. This effect may be due to its anti-inflammatory properties by regulation the expression of NF-κB and LXRα, and CsV may represent a new treatment medicine for treatment of LPS-induced ALI.

Conflict of interest

All authors declare that they have no conflict of interest.

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