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Obeticholic acid alleviate lipopolysaccharide-induced acute lung injury via its anti-inflammatory effects in mice

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ABSTRACT

Acute lung injury (ALI) is a common disease that may result in acute respiratory failure and death. However, there are still no effective treatments for ALI. Several studies have shown that farnesoid X receptor (FXR) has an anti-inflammatory effect. We investigated the effects of obeticholic acid (OCA), an agonist of FXR, on Lipopolysaccharide (LPS)-induced ALI in mice. Sixty male mice were randomly divided into six groups, and orally administered with or without OCA once daily for 3 consecutive days before LPS (1.0 mg/kg). Animals were sacrificed at 0 h, 2 h or 6 h after LPS. As expected, OCA enhanced pulmonary FXR activity. OCA prevented LPS-induced ALI. Additional experiment showed that OCA alleviated LPS-induced up-regulation of pulmonary pro-inflammatory and chemokine genes. Moreover, OCA also repressed LPS-induced the release of TNF-α and KC in serum and bronchoalveolar lavage fluid. In contrast, OCA further up-regulated LPS-induced the expression of *Il-10*, an anti-inflammatory cytokine. Further study showed that OCA inhibited LPS-evoked NF-κB signaling in the lungs. OCA attenuated LPS-induced ERK1/2, JNK, p38 and Akt phosphorylation in the lungs. Overall, these results suggest that OCA prevent LPS-induced ALI may be through enhancing pulmonary FXR activity and then blockading several inflammatory signaling pathways.

1. Introduction

Acute lung injury (ALI) and its more serious form, the acute respiratory distress syndrome (ARDS), are common diseases in worldwide that may result in acute respiratory failure and death. An early cohort study from European countries showed that there were 463 patients (7.1%) with ALI among 6522 admissions. The crude case fatality rates of hospital and intensive care units (ICU) were 32.7% and 22.6% for mild ALI, and 57.9% and 49.4% for ARDS, respectively [1]. Another prospective, multicenter, observational study from Brazil also found that the crude case fatality rates of hospital and 28-day were 49.2% 38.5% for ALI or ARDS, respectively [2]. Severe sepsis was the most common cause that leads to multiple organ failure including ALI and ARDS [3]. Lipopolysaccharide (LPS), also known as endotoxin, is a surrogate for sepsis that often is used to establish experimental models of sepsis-induced ALI in rodents [4–6]. However, there are still no effective treatments for sepsis-induced ALI. Thus, novel therapeutic strategy for sepsis-induced ALI remains to be explored.

LPS is a ligand of toll-like receptor 4 (TLR4). Numerous reports recognized that LPS can induce the production and the release of large amounts of pro-inflammatory cytokines and chemokines through activating TLR4 and its downstream signaling pathway, such as nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and PI3K/Akt signaling pathways [7–9]. The inhibition of TLR4 and its downstream signaling pathway attenuated LPS-induced ALI in rodent animals [10,11]. Obeticholic acid (OCA) is a pharmaceutical currently in clinical trials for the treatment of nonalcoholic steatohepatitis. OCA is also a synthetic agonist of farnesoid X receptor (FXR) that is a ligand-activated transcription factor [12]. FXR has an anti-inflammatory effect through binding to NF-κB subunits and then repressing NF-κB activity [13,14]. Increasing evidences showed that OCA inhibited inflammation via activating FXR activity [15,16]. Indeed, FXR is highly expressed in

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human and rodent animal lungs [17,18]. Nevertheless, it remains an urgent need to explore whether OCA treatment can alleviate LPS-induced ALI.

In this study, we investigated the efficacy of pretreatment with OCA on LPS-induced ALI in mice. The present study found that pretreatment with OCA markedly attenuated LPS-induced ALI. Further study demonstrated that the number of pulmonary nuclear FXR-positive cells was obviously increased in OCA-treated mice. Moreover, pretreatment with OCA significantly inhibited LPS-induced the release of pro-inflammatory cytokines and chemokines via repressing pulmonary NF-κB, MAPK, and PI3K/Akt signaling pathway. Taken together, our results suggest that OCA protect against LPS-induced ALI at least partially through enhancing pulmonary FXR activity and then blockading several inflammatory signaling pathways.

2. Material and methods

2.1. Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and obeticholic acid (OCA) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclear factor-kappa B (NF-κB) p65, FXR, phosphor-p38 (pp38), phosphor-extracellular regulated protein kinases 1/2 (pERK1/2), ERK1/2, phosphor-c-Jun NH2-terminal kinase (p-JNK), JNK, β-actin and Lamin A/C antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). NF-κB p50 phosphor-AKT (p-Akt), and Akt antibody was from Cell Signaling Technology (Beverley, MA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All the other reagents were from Sigma or as indicated in the specified methods.

2.2. Animals and treatments

Male BALBc mice (8–10 week-old) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times. After three days for adaptation, sixty male mice were randomly divided into six groups, and orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. The selection of the dosage of LPS was based on our previous study [19]. The dosage of OCA used in this study was based on previous studies [20,21]. Serum was collected for measurement of TNF-α and KC. Bronchoalveolar lavage fluid (BALF) was collected for measurement of TNF-α and KC and inflammatory cell counts. Lung was collected for histopathology, immunohistochemistry, western blotting and real-time RT-PCR. The Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University approved all the animal experimentations conducted in this study (Permit Number: 14-0017).

2.3. Isolation of total RNA and real-time RT-PCR

Pulmonary RNA was extracted using TRI reagent. RNA was treated with RNase-free DNase and then was reverse-transcribed with AMV (Promega). Real-time RT-PCR was performed to measure mRNAs of inflammatory cytokines and chemokines in the lungs. Specific primers of genes were listed in Table 1. The amplification reactions were carried out on a LightCycler 480 Instrument referred to other study [22].

Table 1
Primers for real-time RT-PCR.

Genes	Forward (5′–3′)	Reverse (5′–3′)
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Tnf-α	CCCTCCTGGCCAACGGCATG	TCGGGGCAGCCTTGTCCCTT
Il-1β	GCCTCGTGCTGTCGGACCCATAT	TCCTTTGAGGCCCAAGGCCACA
Tgf-β	CGGGAAGCAGTGCCCGAACC	GGGGGTCAGCAGCCGGTTAC
Kc	ACTCAAGAATGGTCGCGAGG	GTGCCATCAGAGCAGTCTGT
Mcp-1	GGCTGGAGAGCTACAAGAGG	GGTCAGCACAGACCTCTCTC
Il-10	GCTCCTAGAGCTGCGGACTGC	TGCTTCTCTGCCTGGGGCATCA

2.4. Western blotting

For total protein extraction, pulmonary tissue homogenate was prepared using a lysis buffer containing PMSF. After 20 min on ice, tissue homogenate was centrifuged for 15 min at 15,000 rpm. Supernatant was collected and then protein concentration was measured with Lowry referred to the manufacturer's instructions. The protein sample was denatured for 10 min at 98 °C. Pulmonary nuclear protein was extracted through homogenizing 400 mg lung tissue in 5 mL lysis buffer A (0.6% NP-40, 150 mM NaCl, 10 mM Hepes-KOH pH 7.9, 1 mM EDTA, 0.5 mM PMSF). Tissue homogenate was centrifuged for 30 s at 600 rpm. Supernatant was collected and then was centrifuged for 20 min at 6000 rpm. Precipitate was collected and then was lysed with 100 μL lysis buffer B (20 mM Hepes-KOH pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, Protease Inhbitor Cocktail and 25% glycerol). After 1 h on ice, lysis was centrifuged for 15 min at 15,000 rpm. Supernatant was collected and then protein concentration was measured with Lowry referred to the manufacturer's instructions. For western blotting, same amount of protein (15–30 μg) was electrophoresed by 12.5% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. For total protein, the membranes were incubated for 1–2 h with the following antibodies: p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38, p-Akt, Akt and β-actin. For nuclear protein, the membranes were incubated for 2 h with Lamin A/C, NF-κB p65 or p50 antibodies. The membranes were washed for three times and then were incubated with second antibody for 1–2 h. After washes in DPBS, signal was measured using an ECL luminescence.

2.5. Immunohistochemistry (IHC)

Lungs were fixed with 4.0% formalin and embedded in paraffin based on the standard protocol. Paraffin embedded lung was cut 5 μm thick. After dewaxing and hydration, antigen retrieval was conducted with 0.01 M sodium citrate buffer solution (pH 6.0). Sections were incubated with anti-FXR, anti-NF-κB p65 or p50 monoclonal antibodies (1:200) at 4 °C overnight. The color reaction was developed with HRP-linked polymer detection system and counterstaining with hematoxylin before dehydrating in graded alcohols. Nuclear FXR-, p65- and p50-positive cells were counted in twelve randomly selected fields from each slide at a magnification of ×400.

2.6. Bronchoalveolar lavage fluid collection and cell count

Bronchoalveolar Lavage Fluid (BALF) was collected from each mouse by lavaging the lung with PBS three times and supernatants were then collected for later analysis as previously described [23]. The pellets were resuspended in saline for total cell counts using a hemocytometer. The BALF was centrifuged 1500 rpm for 10 min at 4 °C and the supernatant was collected for ELISA. The remaining cell pellet was resuspended in 200 μL of PBS and centrifuged 700 rpm for 5 min. To determine the differential cell counts, BALF was centrifuged and made into cell smears onto slides. The slides were dried, and the inflammatory cells were fixed and stained using Diff Quik® staining reagent (SBJ-0265-2, Nanjing SenBeiJia Biological Technology Co., Ltd.) according to the manufacturer's instructions. A microscopic cell count was then conducted using an Olympus BX53 microscope at 400× magnification.

2.7. Enzyme-linked immunosorbent assay

Commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, Oxon, UK) kits were used to measure serum TNF-α and KC levels referred to the manufacturer's protocol.

2.8. Statistical analysis

Quantified data were expressed as means ± S.E.M. Differences among different groups were evaluated using one way ANOVA. Data that were not normally distributed were assessed for significance using non-parametric tests techniques. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of OCA on LPS-induced acute lung injury

As shown in Table 2, pulmonary weight was slightly increased at 6 h after LPS injection. In addition, lung coefficient also slightly increased at 6 h after LPS injection. The effects of OCA on the infiltration of inflammatory cells in LPS-induced acute lung injury were analyzed. LPS induced a mild pulmonary edema in mice (data not shown). As expected, an infiltration of inflammatory cells was observed in the lungs in LPS-treated mice (Fig. 1A). OCA treatment significantly alleviated LPS-induced pulmonary edema (data not shown). Moreover, OCA treatment markedly attenuated LPS-induced infiltration of inflammatory cells in mice lungs (Fig 1A and B).

3.2. Effects of OCA on LPS up-regulated the expressions of inflammatory cytokines and chemokines in the lungs

The effects of OCA treatment on LPS-induced the expression of pro-inflammatory and chemokine genes in the lungs were analyzed. As expected, mRNA levels of pulmonary *Tnf-α* and *Il-1β*, two pro-inflammatory genes, were significantly increased both 2 h and 6 h after LPS injection (Fig. 2A, B). Pulmonary *Tgf-β* mRNA, another pro-inflammatory gene, was also markedly up-regulated at 2 h but not 6 h after LPS injection (Fig. 2C). Of interest, OCA treatment significantly alleviated LPS-induced up-regulation of pro-inflammatory cytokines (Fig. 2A–C). Moreover, mRNA levels of pulmonary keratinocyte chemoattractant (*Kc*) and *Mcp-1*, two chemokine genes, were markedly up-regulated both 2 h and 6 h after LPS injection (Fig. 2D, E). Interestingly, OCA treatment significantly repressed LPS-induced up-regulation of pulmonary chemokines both 2 h and 6 h after LPS injection (Fig. 2D, E). The effects of OCA on LPS up-regulated the expressions of anti-inflammatory gene in the lungs were then analyzed. As shown in Fig. 2F, the level of pulmonary *Il-10* mRNA, an anti-inflammatory gene, was significantly increased both 2 h and 6 h after LPS injection. Although OCA alone had little effect on pulmonary *Il-10* mRNA, OCA significantly elevated LPS-induced up-regulation of pulmonary *Il-10* at 6 h but not 2 h after LPS injection (Fig. 2F).

3.3. Effects of OCA on LPS-induced inflammatory cytokines and chemokines in serum

The effects of OCA treatment on LPS-induced pro-inflammatory cytokines and chemokines in serum were analyzed. As expected, the level of serum TNF-α, a pro-inflammatory cytokine, was significantly increased at 2 h after LPS injection remained elevated at 6 h after LPS injection (Fig. 3A). OCA treatment significantly inhibited LPS-induced release of TNF-α in serum (Fig. 3A). Moreover, serum KC, a chemokine, was markedly increased at 2 h after LPS injection remained elevated at 6 h after LPS injection (Fig. 3B). LPS-evoked release of KC in serum was repressed when mice were treated with OCA before LPS (Fig. 3B).

3.4. Effects of OCA on LPS-induced inflammatory cytokine and chemokine in BALF

The effects of OCA treatment on LPS-induced pro-inflammatory cytokine and chemokine in BALF were also analyzed. As expected, the levels of TNF-α and KC in BALF were significantly increased at 2 h after LPS injection remained elevated at 6 h after LPS injection (Fig. 4A, B). OCA treatment significantly inhibited LPS-induced release of TNF-α and KC in BALF (Fig. 4A, B). Moreover, total cell counts were then measured in BALF. As shown in Fig. 4C–E, the number of total cells, neutrophils and macrophages in BALF was significantly increased in LPS-treated mice. LPS-induced the increase of inflammatory cells was repressed when mice were treated with OCA before LPS (Fig. 4C–E).

3.5. Effects of OCA on LPS-activated NF-κB signaling in the lungs

The effects of on LPS-activated pulmonary NF-κB signaling were analyzed. As expected, the protein levels of nuclear NF-κB p65 and p50 subunits in the lungs were increased at 2 h after LPS injection remained markedly increased at 6 h after LPS injection (Fig. 5A–C). Of interest, the protein levels of nuclear NF-κB p65 and p50 subunits in the lungs were significantly reduced in OCA + LPS-treated mice as compared with LPS-treated mice (Fig. 5A). Immunohistochemistry also showed that the number of pulmonary nuclear NF-κB p65- and p50-positive cells was significantly increased in LPS-treated mice (Fig. 5D–G). Interestingly, the number of nuclear NF-κB p65- and p50-positive cells in the lungs was significantly reduced in OCA + LPS-treated mice as compared with LPS-treated mice (Fig. 5D–G).

3.6. Effects of OCA on LPS-activated MAPKs signaling in the lungs

To investigate the effects of OCA on LPS-activated MAPKs signaling in the lungs, pulmonary p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38 and p38 levels were measured. As expected, LPS and OCA had no effect on the protein levels of ERK1/2, JNK and p38 (Fig. 6A–D). Pulmonary p-ERK1/2 level was increased at 2 h after LPS injection remained elevated at 6 h after LPS injection (Fig. 6A, B). Of interest, OCA treatment significantly alleviated LPS-induced pulmonary ERK1/2 phosphorylation at 2 h but not at 6 h after LPS injection (Fig. 6A, B). The level of pulmonary p-JNK was then analyzed. As shown in Fig. 6A and C, Pulmonary p-JNK level was increased at 2 h but not at 6 h after LPS

Table 2
Body weight and lung weight.

	Control	OCA	LPS 2 h	LPS + OCA 2 h	LPS 6 h	LPS + OCA 6 h
Body weight (g)	21.95 ± 0.17	21.18 ± 0.24	21.76 ± 0.31	21.16 ± 0.20	21.50 ± 0.16	21.25 ± 0.26
Absolute lung weight (g)	0.1182 ± 0.005	0.1175 ± 0.004	0.1245 ± 0.003	0.1215 ± 0.002	0.1287 ± 0.004*	0.1262 ± 0.004
Relative lung weight (%)	0.54 ± 0.02	0.55 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.60 ± 0.01*	0.60 ± 0.02

* $P < 0.05$ as compared with Control group.

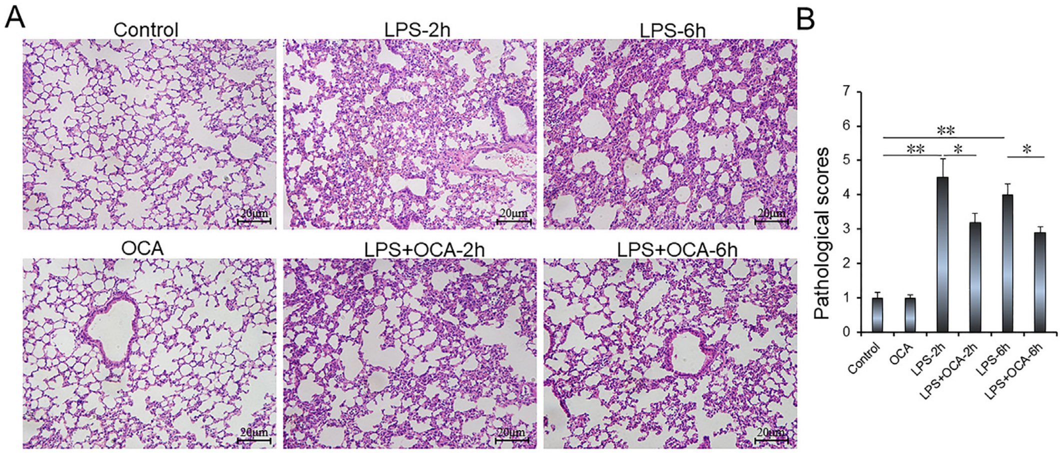

Fig. 1. Effects of OCA on LPS-induced acute lung injury. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. Acute lung injury was evaluated by HE staining. (A) Representative photomicrographs of pulmonary histology were shown. Original magnification: ×200. (B) Pathological scores for pulmonary injury were evaluated in each slide at a magnification of 200×. Data were expressed as means ± SEM of 10 samples from 10 different mice. $^{**}P < 0.01$.

injection. OCA significantly alleviated LPS-induced pulmonary JNK phosphorylation (Fig. 6A, C). Moreover, the level of phosphorylated p38 was also increased at 2 h after LPS injection further elevated at 6 h after LPS injection (Fig. 6A, D). Pulmonary p38 phosphorylation was significantly inhibited when mice were treated with OCA before LPS injection (Fig. 6A, D).

3.7. Effects of OCA on LPS-activated PI3K/Akt signaling in the lungs

The effect of OCA on LPS-activated pulmonary PI3K/Akt signaling was measured. As expected, LPS and OCA had no effect on the Akt protein levels in the lungs (Fig. 7A, B). The level of phosphorylated Akt was significantly increased at 6 h after LPS injection (Fig. 7A, B). Interestingly, OCA alleviated LPS-induced pulmonary Akt phosphorylation (Fig. 7A, B).

3.8. Effects of OCA on pulmonary FXR signaling

The effect of OCA on pulmonary FXR activity was analyzed. Although OCA had no effect on pulmonary *Fxr* mRNA (Fig. 8A), the number of pulmonary nuclear FXR-positive cells was significantly increased in OCA-treated mice (Fig. 8B, C).

4. Discussion

The present study explored the roles of pretreatment with OCA on LPS-induced ALI in male mice. As expected, an infiltration of inflammatory cells was observed in the lungs at 2 h and 6 h after LPS injection. Moreover, OCA significantly alleviated LPS-induced edema and infiltration of inflammatory cells in the lungs. These results suggest that pretreatment with OCA attenuate LPS-induced ALI in mice.

It is increasingly recognized that pro-inflammatory cytokines involve in LPS-induced ALI. Several studies in animal models

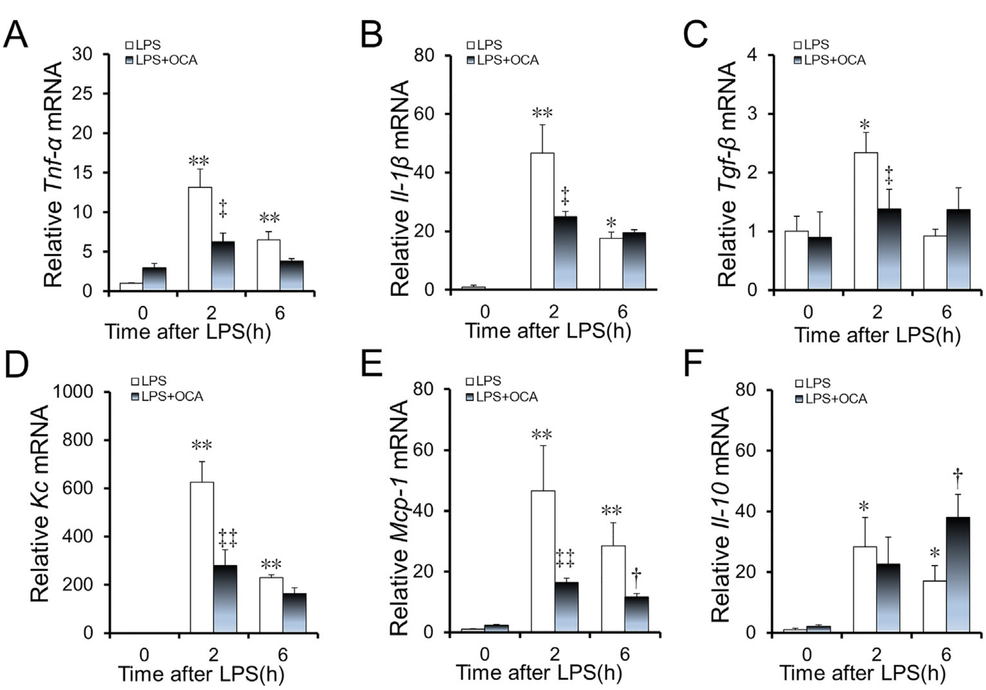

Fig. 2. Effects of OCA on LPS up-regulated the expressions of inflammatory cytokines and chemokines in lung. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. Pulmonary mRNA levels were measured by real-time RT-PCR. (A) *Tnf-α*. (B) *Il-1β*. (C) *Tgf-β*. (D) *Kc*. (E) *Mcp-1*. (F) *Il-10*. All data were expressed as means ± SEM of 10 samples from 10 different mice. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with LPS 0 h group. $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ as compared with LPS 2 h group. $^{†}P < 0.05$ as compared with LPS 6 h group.

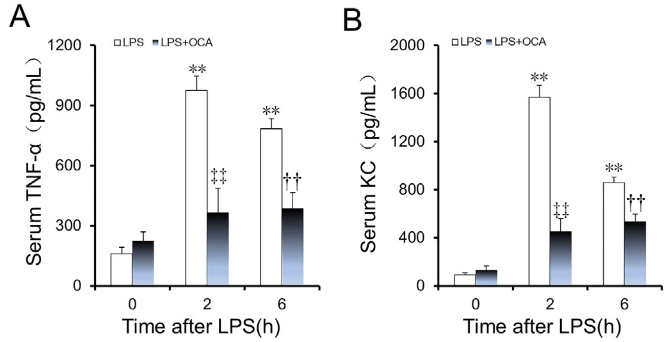

Fig. 3. Effects of OCA on LPS-induced inflammatory cytokines and chemokines in serum. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. Serum TNF-α and KC were measured by ELISA. (A) Serum TNF-α. (B) Serum KC. All data were expressed as means ± SEM of 10 samples from 10 different mice. $^{**}P < 0.01$ as compared with LPS 0 h group. $^{‡‡}P < 0.01$ as compared with LPS 2 h group. $^{††}P < 0.01$ as compared with LPS 6 h group.

demonstrated that TNF-α and IL-1β, two pro-inflammatory cytokines, KC and MCP-1, two chemokines, played key roles in the development of LPS-induced ALI [23–27]. A recent case-control study also found that TNF-α, IL-1β and IL-8 were significantly higher in the epithelial lining fluid and BALF from patients with ALI than controls [28]. Indeed, OCA has an anti-inflammatory activity [15,20]. The present study investigated the roles of OCA on systemic and local inflammatory cytokines in LPS-induced ALI. We found that OCA significantly inhibited LPS-induced the release of TNF-α and KC in serum. Moreover, OCA obviously alleviated LPS-induced up-regulation of pulmonary pro-inflammatory cytokines and chemokines. OCA also repressed LPS-induced the increase of inflammatory cytokines and inflammatory cell counts in BALF. These results indicate that OCA attenuate LPS-induced ALI partially attributed to its anti-inflammatory activity.

IL-10 is a key anti-inflammatory cytokine. A recent case-control study showed that pneumonia patients with high frequency of pulmonary $CD19^{+}$CD24(hi)CD38(hi) regulatory B cells, a well-known immunocyte of secreting IL-10, was associated with a decreased risk of ALI [29]. Several experimental studies also demonstrated that treatment with IL-10 significantly alleviated LPS-induced ALI through its anti-inflammatory activity [30,31]. In contrast, IL-10 knockout mice were more susceptible to inflammation-mediated ALI [32]. The present study analyzed the effect of OCA on pulmonary *Il-10* mRNA. As expected, the level of pulmonary *Il-10* mRNA was obviously elevated in LPS-treated mice. Of interest, OCA further elevated LPS-induced up-regulation of pulmonary *Il-10* gene at 6 h after LPS injection. A recent study also found that OCA further increased serum IL-10 level in LPS-treated mice [16]. Taken together, these results suggest that OCA attenuates LPS-induced ALI not only through repressing the release of pro-inflammatory cytokines and chemokines but also may be partially through up-regulating the expression of anti-inflammatory cytokine in the lungs. Moreover, the present study found that OCA alone had little effect on pulmonary *Il-10* mRNA. Thus, additional study is necessary to investigate the mechanism through which OCA up-regulates the expression of pulmonary *Il-10* gene in LPS-treated mice.

NF-κB signaling plays an important role in regulating TLR4-mediated inflammatory genes. LPS can induce the release of pro-inflammatory cytokines and chemokines through activating NF-κB signaling [33,34]. Indeed, several previous studies showed that OCA can repress placental and hepatic NF-κB activation partially through preventing NF-κB dissociation from inhibitory sub-unit [15,16]. The present study also found that the levels of nuclear NF-κB p65 and p50 subunits in the lungs were obviously increased at 2 h after LPS injection remained markedly increased at 6 h after LPS injection. Moreover, OCA obviously inhibited LPS-induced the increase of nuclear NF-κB p65 and p50 subunits in mice lungs. These results indicate that OCA inhibited LPS-induced the release of pro-inflammatory cytokines and chemokines partially through the blockade of NF-κB signaling pathway. MAPKs and PI3K/Akt are another two downstream signaling that regulate TLR4-mediated inflammatory genes. Numerous reports from in vivo and in vitro experiments showed that the activation of MAPKs and PI3K/Akt pathways played vital roles in LPS-induced inflammatory reaction

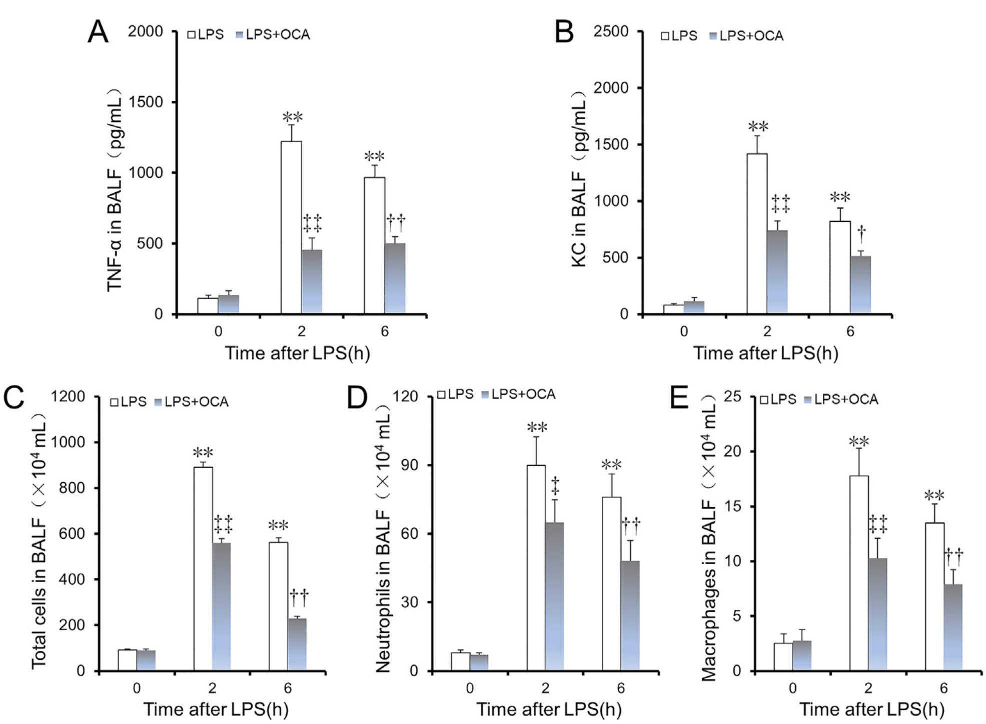

Fig. 4. Effects of OCA on LPS-induced inflammatory cytokines and chemokines in BALF. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. (A and B) TNF-α and KC in BALF were measured by ELISA. (A) TNF-α in BALF. (B) KC in BALF. (D–E) Inflammatory cells were counted in BALF. (C) Total cells in BALF. (D) Neutrophils in BALF. (E) Macrophages in BALF. All data were expressed as means ± SEM of 10 samples from 10 different mice. $^{**}P < 0.01$ as compared with LPS 0 h group. $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ as compared with LPS 2 h group. $^{†}P < 0.05$, $^{††}P < 0.01$ as compared with LPS 6 h group.

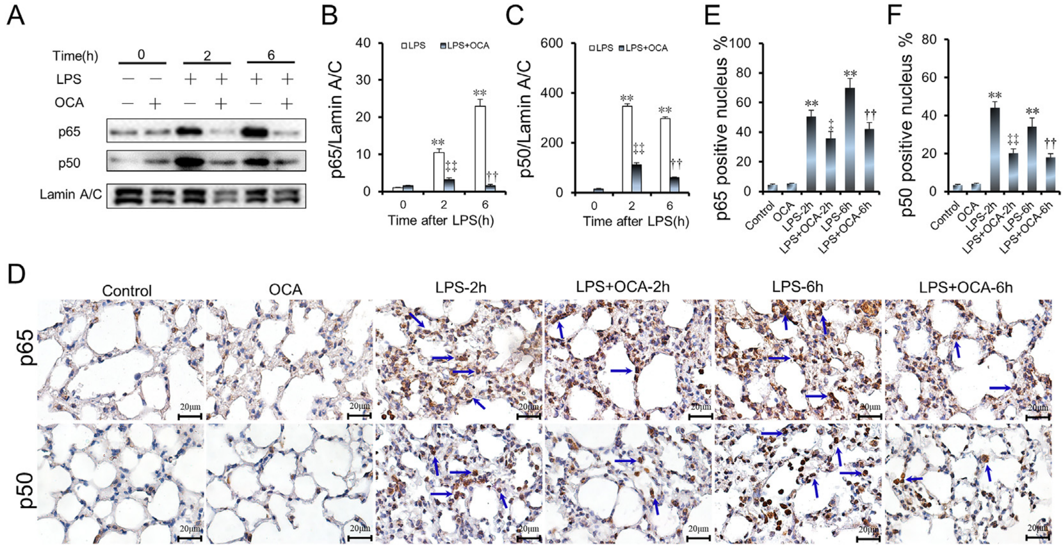

Fig. 5. Effects of OCA on LPS-activated NF-κB signaling in the lungs. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. (A) A representative gels for NF-κB p65 (upper panel), p50 (middle panel) and Lamin A/C (lower panel) were shown. (B) Quantitative analysis of scanning densitometry for p65 was performed. (C) Quantitative analysis of scanning densitometry for p50 was performed. Nuclear NF-κB p65 and p50 subunits in the lungs were measured using IHC. (D) Representative photomicrographs of pulmonary p65 are shown. Original magnification: 400×. Arrowheads indicate nuclear p65-positive cells. (E) Nuclear p65-positive cells were compared among different groups. (F) Representative photomicrographs of pulmonary p50 are shown. Original magnification: 400×. Arrowheads indicate nuclear p50-positive cells. (G) Nuclear p50-positive cells were compared among different groups. All experiments were duplicated for three times. All data were expressed as means ± SEM. $^{**}P < 0.01$ as compared with LPS 0 h group. $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ as compared with LPS 2 h group. $^{††}P < 0.01$ as compared with LPS 6 h group.

[35–37]. The present study explored the roles of OCA on LPS-activated MAPKs and PI3K/Akt pathways in mice. Interestingly, LPS-induced pulmonary ERK1/2, JNK and p38 phosphorylation, three downstream molecules of MAPKs pathway, was alleviated when male mice were treated with OCA before LPS. Moreover, the present study also found that OCA obviously inhibited LPS-induced Akt phosphorylation in the lungs. These results suggest that OCA attenuates LPS-induced pulmonary inflammation and ALI not only through repressing NF-κB signaling pathways but also partially through inhibiting MAPKs and PI3K/Akt signaling pathways.

The mechanism through which OCA represses LPS-activated pulmonary several inflammatory signaling pathways remains unclear. Numerous early reports showed that activated nuclear receptors, such as small heterodimer partner and nuclear steroid and xenobiotic receptor, suppressed NF-κB signaling pathways [38,39]. Two recent studies in mice also found that activated vitamin D receptor (VDR), a ligand-activated nuclear receptor, inhibited LPS-evoked NF-κB signaling pathways in the placenta and kidney through enhancing the interaction between VDR and NF-κB p65 subunit [40,41]. OCA is also a synthetic agonist of farnesoid X receptor (FXR) that is a nuclear receptor [12]. Indeed, FXR has an anti-inflammatory effect through binding to NF-κB subunits and then repressing NF-κB activity [13,14]. The present study investigated the effect of OCA on FXR activation in the lungs. As expected, although OCA had no effect on the expression pulmonary *Fxr* gene, the number of pulmonary nuclear FXR-positive cells was significantly increased in OCA-treated mice. These results suggest that OCA represses LPS-activated pulmonary several inflammatory signaling pathways may be through activating pulmonary FXR activity.

In summary, the present study explored the roles of pretreatment with OCA on LPS-induced ALI in mice. We found that OCA markedly attenuated LPS-induced infiltration of inflammatory cells in the lungs. Further study demonstrated that the number of pulmonary nuclear FXR-positive cells was obviously increased in OCA-treated mice. Moreover, OCA significantly inhibited LPS-induced the release of pro-

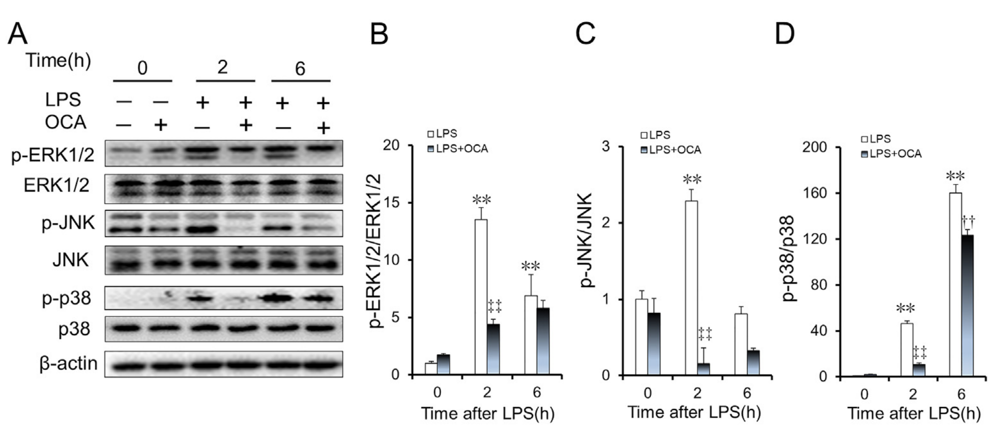

Fig. 6. Effects of OCA on LPS-activated MAPKs signaling in the lungs. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. (A) Representative gels for p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38 and β-actin were shown. (B) Quantitative analysis of scanning densitometry for p-ERK1/2 was performed. (C) Quantitative analysis of scanning densitometry for p-p38 was performed. All experiments were duplicated for three times. All data were expressed as means ± SEM. $^{**}P < 0.01$ as compared with LPS 0 h group. $^{‡‡}P < 0.01$ as compared with LPS 2 h group. $^{††}P < 0.01$ as compared with LPS 6 h group.

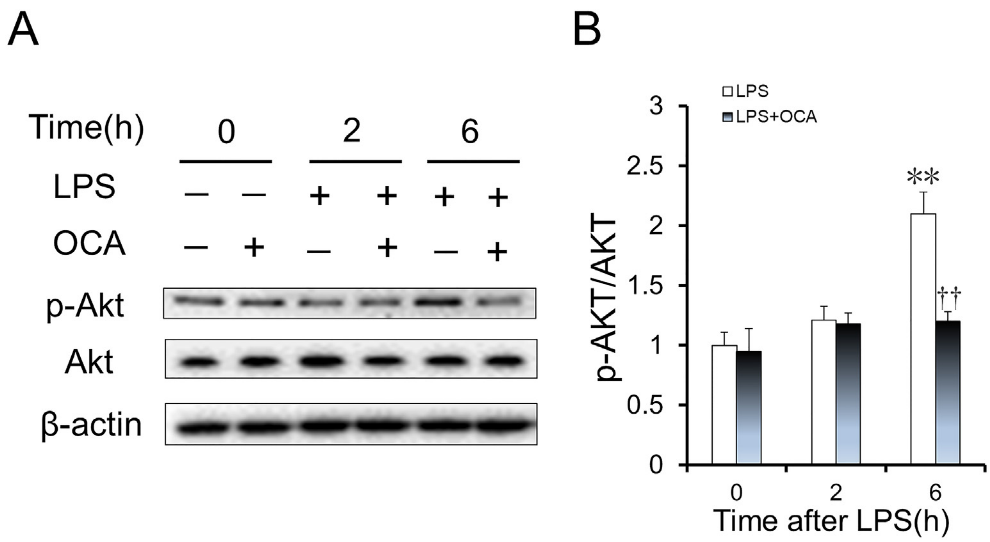

Fig. 7. Effects of OCA on LPS-activated PI3K/Akt signaling in the lungs. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. (A) Representative gels for p-Akt, Akt and β-actin were shown. (B) Quantitative analysis of scanning densitometry was performed. All experiments were duplicated for three times. All data were expressed as means ± SEM. $^{**}P < 0.01$ as compared with LPS 0 h group. $^{††}P < 0.01$ as compared with LPS 6 h group.

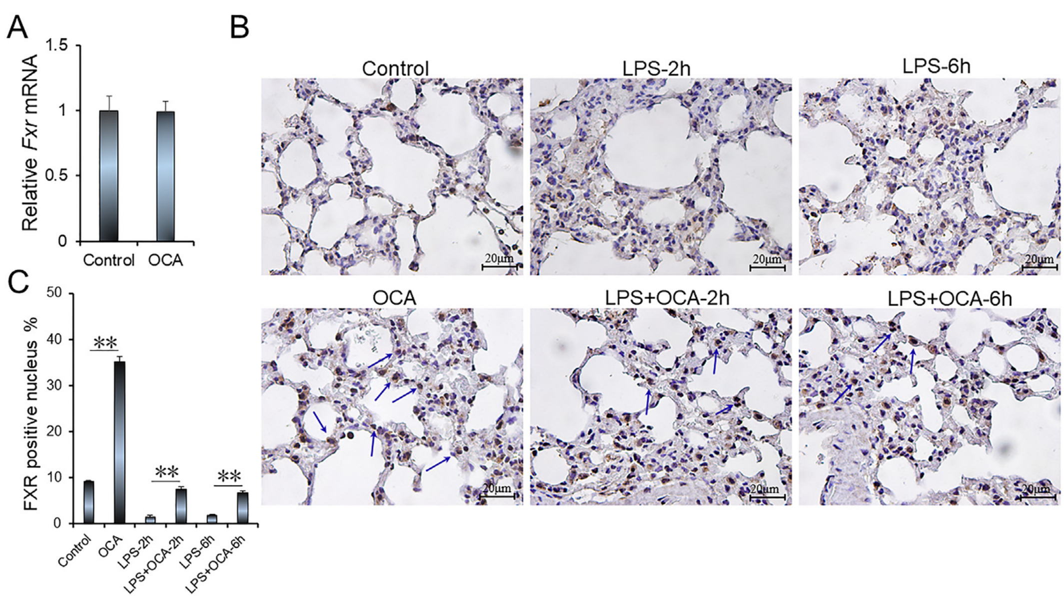

Fig. 8. Effects of OCA on pulmonary FXR signaling. Sixty male mice were orally administered with OCA (5 mg/kg, dissolved in corn oil) or corn oil once daily for 3 consecutive days. All mice were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg) at 1 h after the last OCA or corn oil treatment. In the control and OCA groups, mice were sacrificed at 0 h after LPS injection. In the LPS and OCA + LPS groups, mice were sacrificed either 2 h or 6 h after LPS injection. (A) Pulmonary *Fxr* mRNA was measured by real-time RT-PCR. (B and C) Nuclear FXR was analyzed by IHC. (B) Representative photomicrographs of pulmonary histology are shown. Original magnification: 400×. Arrowheads indicate nuclear FXR-positive cells. (C) Pulmonary nuclear FXR-positive cells were counted in each slide at a magnification of 400×. Data were expressed as means ± SEM of 10 samples from 10 different mice. $^{**}P < 0.01$.

inflammatory cytokines and chemokines via repressing pulmonary NF-κB, MAPK, and PI3K/Akt signaling pathways. Taken together, the present study suggests that OCA protect against LPS-induced ALI may be through activating pulmonary FXR activity and then blockading several inflammatory signaling pathways. Thus, our results indicated that OCA may be used as a potential pharmaceutical to treat inflammation-induced ALI.

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Competing interests

The authors report no conflict of interests.

CRediT authorship contribution statement

Jun Fei: Methodology, Formal analysis. **Lin Fu:** Methodology, Formal analysis. **Biao Hu:** Methodology. **Yuan-Hua Chen:** Methodology. **Hui Zhao:** Methodology. **De-Xiang Xu:** Conceptualization, Writing - original draft. **Jia-Bin Li:** Conceptualization, Resources, Writing - original draft.

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