Research Article

Osthole Protects against Acute Lung Injury by Suppressing NF-κB-Dependent Inflammation

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Inflammation is a key factor in the pathogenesis of ALI. Therefore, suppression of inflammatory response could be a potential strategy to treat LPS-induced lung injury. Osthole, a natural coumarin extract, has been reported to protect against acute kidney injury through an anti-inflammatory mechanism, but its effect on ALI is poorly understood. In this study, we investigated whether osthole ameliorates inflammatory sepsis-related ALI. Results from in vitro studies indicated that osthole treatment inhibited the LPS-induced inflammatory response in mouse peritoneal macrophages through blocking the nuclear translocation of NF-κB. Consistently, the in vivo studies indicated that osthole significantly prolonged the survival of septic mice which was accompanied by inflammation suppression. In the ALI mouse model, osthole effectively inhibited the development of lung tissue injury, leukocytic recruitment, and cytokine productions, which was associated with inhibition of NF-κB nuclear translocation. These findings provide evidence that osthole was a potent inhibitor of NF-κB and inflammatory injury and suggest that it could be a promising anti-inflammatory agent for therapy of septic shock and acute lung injury.

1. Introduction

Sepsis is a systemic and deleterious inflammatory response elicited by microbial infection [1–3]. The number of reported cases of sepsis continues to increase by 5–10% each year [4], making it one of the leading causes of death in intensive care facilities [5]. Among these case reports, approximately 30% of patients progress to multiorgan dysfunction syndrome (MODS) with 18% developing acute lung injury (ALI) [6–8]. ALI is a severe form of diffuse lung disease described as a clinical syndrome of acute respiratory failure with high morbidity and mortality [9]. It is characterized with persistent pulmonary inflammation [10] and increase in microvascular permeability [11]. Even with patients surviving ALI, the quality of life remains poor. Therefore, there is a great need for more effective therapeutic approaches.

Endotoxin, especially lipopolysaccharide (LPS) [12], is well recognized in the pathogenesis of ALI. LPS is a potent activator of toll-like receptor 4 (TLR4) [13–16], triggering the nuclear factor-kappa B (NF-κB) pathway, thereby producing proinflammatory molecules, such as cytokines interleukin 6 (IL-6) [17], IL-1b [18], and tumor necrosis factor α (TNF-α) [19]. Excessive inflammatory responses induced by endotoxin can lead to tissue destruction, fibrosis, and eventual organ failure [4, 18, 20]. Therefore, blocking inflammatory cascades is considered an effective strategy to attenuate lung injury. However, current effective therapeutic agents remain inadequate.

Osthole, a natural coumarin extract from the fruit of Cnidium monnieri (L.) [21], has several beneficial pharmacological properties, such as antiseizure [22], antiosteoporosis [23], and antitumour [24] activities. Recently, studies found
that osthole attenuates chronic kidney failure by inhibition of inflammation [25]. It also suppresses acute inflammatory responses in acute mechanical brain injury and kidney injury [26–28]. However, its potential protective effects on sepsis and ALI are not well characterized. In the present study, we evaluated the protective effects of osthole on LPS-induced ALI and its underlying mechanism. Our findings would provide important insight on potential new therapeutic approaches for inflammation-related lung injury.

2. Materials and Methods

2.1. Reagents. Osthole was purchased from Aladdin (Shanghai, China). The chemical structure is shown in Figure 1(a). Osthole was dissolved in dimethyl sulfoxide (DMSO) as 100 mM stock and diluted before use in assays. The final concentration of DMSO did not exceed 0.1%. All other reagents not mentioned were obtained from Sigma unless otherwise specified.

2.2. Cell Culture and Treatment. Mouse peritoneal macrophages (MPMs) were prepared as follows and also described in our previous paper [29]. Briefly, each ICR mice (8 weeks) was stimulated by intraperitoneal (ip) injection of 3 mL 6% thioglycollate solution (beef extract (0.3 g), tryptone (1 g), sodium chloride (0.5 g), and soluble starch (6 g)) dissolved in 100 mL water and filtered with 0.22 μm filter. Three days later, MPMs were harvested by washing the peritoneal cavity with 8 mL PBS containing 30 mM of EDTA. The suspension was centrifuged at 4°C, 1000 rpm, resuspended in RPMI-1640 (Gibco/BRL life Technologies, Eggenstein, Germany) with 10% (v/v) FBS (HyClone, Logan, UT, USA), 100 U/mL penicillin G, and 100 mg/mL streptomycin. Cells were cultured in a 37°C, 5% CO₂ incubator, washed with medium 3 h after incubation, and used for studies after adherence firmly to culture plates.

GAPDH, P65, IκB-α, and lamin B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TNF-α and CD68 antibodies were obtained from Abcam (Abcam, USA). The secondary antibody was purchased from Santa Cruz Biotechnology.

2.3. Immunofluorescence and Immunoblotting. MPMs were plated (1 × 10⁶) into a 6-well plate, pretreated with osthole for 1 h, and stimulated with LPS at indicated concentrations and time. After treatment, the cells were fixed with 4% paraformaldehyde and permeabilized with 100% methanol at 4°C for 5 min. The cells were washed twice with PBS containing 1% BSA and incubated with primary antibodies for anti-P65 antibody (1:200) overnight at 4°C, followed by a PE-conjugated secondary antibody (1:200). The cells were counterstained with DAPI and viewed by a Nikon fluorescence microscope (200x amplification, Nikon, Japan).

For Western blot analysis, the cells or lung tissue (30–50 mg) was prepared into homogenate samples and lysed (Boster Biological Technology, USA) and the protein concentration determined by a Bio-Rad protein assay kit (Bio-Rad, USA). Nuclear protein fractions were prepared using a kit from Beyotime (Shanghai, China) and were loaded and separated in 10% or 12% SDS-PAGE gels. The separated bands were electroblotted to a nitrocellulose membrane and blocked in Tris-buffered saline, pH7.6, containing 0.05% Tween 20 and 5% nonfat milk. Specific antibodies were incubated to probe for markers, and the immunoreactive bands were detected by incubating with a secondary antibody conjugated with horseradish peroxidase and visualized using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA).

2.4. Determination of TNF-α and IL-6 by ELISA. The TNF-α and IL-6 contents in culture medium or animal samples were determined by ELISA according to the manufacturer’s instructions (Bioscience, San Diego, CA). The amount of TNF-α and IL-6 was normalized to protein concentration of cells, weight of animal tissues, or serum volume.

2.5. Real-Time Quantitative PCR. Total RNA was isolated from 1 × 10⁶ cells or tissues (50–100 mg) using TRIzol (Life Technologies, Carlsbad, CA). Reverse transcription and quantitative PCR (RT-qPCR) were performed using M-MLV Platinum RT-qPCR Kit (Life Technologies). Real-time qPCR was carried out using the Eppendorf RealPlex 4 instrument (Eppendorf, Hamburg, Germany). Primers for genes (i.e., TNF-α, IL-6, and β-actin) were obtained from Life Technologies. The primer sequences used are shown in Table 1. The relative amount of each gene was normalized to β-actin.

2.6. Mouse Models. Male C57BL/6 mice weighing 18–22 g were obtained from the Animal Centre of Wenzhou Medical University (Wenzhou, China). The mice were housed at constant room temperature with a 12:12 h light-dark cycle, fed a standard rodent diet and water, and acclimated to the laboratory for at least 3 days before use for studies. All animal care and experimental procedures were approved by the Wenzhou Medical College Animal Policy and Welfare Committee.

2.6.1. LPS-Induced Sepsis Model. Osthole was dissolved in 30% PEG 400 (Ludwigshafen, Germany), 1% volume of DMSO (Solarbio, Beijing, China), and 69% saline. Mice were pretreated with osthole (20 or 40 mg/kg) by ip injection, and 0.5 h later, 20 mg/kg LPS was injected through the tail vein. The mice were euthanized with chloral hydrate 6 h after LPS injection, and lung and liver tissues were excised aseptically, blotted dry, weighed, and immediately frozen in liquid nitrogen. The samples were stored at −80°C for later analyses. The body weight and mortality were recorded for 7 days.

2.6.2. LPS-Induced ALI. The mice were randomly divided into four groups as follows: control (7 mice received vehicle of 0.9% saline), LPS (7 mice received LPS alone), 20 + LPS (7 mice received 20 mg/kg/day osthole and LPS), and 40 + LPS (7 mice received 40 mg/kg/day osthole and LPS). Osthole was a pretreatment given by ip injection for one week. LPS challenge was made by intratracheal injection of 50 μL of LPS (3 mg/kg, dissolved in 0.9% saline) or 50 μL 0.9% saline as vehicle control. Mice were euthanized with chloral hydrate 6 h after LPS injection, and bronchoalveolar lavage fluid (BALF) and blood samples were collected. Lung and liver
Osthole inhibits LPS-induced inflammatory cytokines in mouse peritoneal macrophages (MPMs). (a) The chemical structure of osthole. (b) MPMs were treated with various concentrations of osthole for 24 h. The viability of cells was measured by MTT assay and values reported as mean ± SEM; n = 3. (c) MPMs were treated with osthole (20, 50, or 100 μM) for 24 h, and light micrographs were recorded using a microscope. Representative of three evaluations, n = 3, micron bar = 20 μm. (d–e) MPMs were pretreated with osthole (20, 50, or 100 μM) for 0.5 h and stimulated with LPS (0.5 μg/mL) for 24 h, and secreted IL-6 (d) and TNF-α (e) were measured from conditioned media by ELISA (Materials and Methods). Values normalized to total protein of cells and reported as mean ± SEM relative to LPS alone; n = 3. (f–g) MPMs were pretreated with osthole (20, 50, or 100 μM) for 0.5 h and stimulated with LPS (0.5 μg/mL) for 6 h, and total RNA was extracted for real-time qPCR for IL-6 (f) and TNF-α (g). Values normalized to β-actin mRNA and reported as mean ± SE M; n = 3. (d–g) ***p < 0.001 versus control; ### p < 0.001 versus LPS alone.
tissues were excised aseptically, frozen in liquid nitrogen, and stored at −80°C before later analyses.

2.7. BALF Analysis. The collected BALF was centrifuged at 1000 rpm for 10 min at 4°C. The supernatant was used for protein concentration and cytokine determination. The cell pellet was resuspended using 50 μL physiological saline for total cell count determination using a cell counting instrument (Count Star, Shanghai, China). The number of neutrophils in BALF was determined using Wright Giemsa staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and microscopic fields were counted under a Nikon fluorescence microscope (200x amplification; Nikon, Japan).

2.8. Lung Wet/Dry Ratio. For determining the ratio of wet to dry weight of lung tissue, the middle lobe of the right lung was collected and the wet weight was recorded. The tissue dry weight of lung tissue, the middle lobe of the right lung was reported as index of pulmonary edema.

2.9. Immunohistochemical Determination. Lung tissues were routinely fixed in 4% formalin, processed in graded alcohol and xylene, and embedded in paraffin. Paraffin blocks were sectioned into 5 μm thick sections. After rehydration, the sections were stained with hematoxylin and eosin (H&E assay kit, Beyotime, Shanghai, China).

For immunohistochemistry, paraffin was removed from the sections with xylene, rehydrated in graded alcohol series, subjected to antigen retrieval in 0.01 mol/L citrate buffer (pH 6.0) by microwaving, and then placed in 3% hydrogen peroxide in methanol for 30 min at room temperature. After blocking with 5% BSA (Sigma, USA), the sections were incubated with anti-TNF-α antibody (1:500, Abcam, USA) or anti-CD68 (1:500, Abcam, USA) overnight at 4°C, followed by the secondary antibody (1:200; Santa Cruz, USA). The reaction was visualized with DAB (ZSGB-Bio, Beijing, China), counterstained with hematoxylin, dehydrated, and viewed under a Nikon fluorescence microscope (200x amplification, Nikon, Japan).

2.10. Statistical Analysis. All data represent three independent experiments and are expressed as means ± SEM. Statistical analyses were performed using GraphPad Pro Prism 5.0 (GraphPad, San Diego, CA). One-way ANOVA followed by multiple comparisons test with Bonferroni correction was used to analyze the differences between sets of data.

3. Results

3.1. Osthole Inhibits LPS-Induced Production of IL-6 and TNF-α in MPMs. The MTT assay was used to investigate the effect of osthole on cell viability. For study, MPMs were treated with several doses for 24 h. The results indicated that osthole did not impair cell viability at the range of concentrations used (Figure 1(b)) or alter cell morphology (Figure 1(c)). Therefore, we selected 20–100 μM of osthole to investigate its anti-inflammatory activity. We first evaluated the effects of osthole on LPS-stimulated production of proinflammatory cytokines, IL-6 and TNF-α, in MPMs. Results indicated that LPS (0.5 μg/mL) stimulation for 24 h of MPMs robustly increased the secretion of both IL-6 and TNF-α into medium (Figures 1(d) and 1(e), resp.). Pretreatment of MPMs with osthole for 0.5 h suppressed the LPS-induced cytokine secretion in a dose-dependent manner (Figures 1(d) and 1(e)). The osthole-induced inhibition of cytokine secretion was associated with inhibition of the transcription of IL-6 and TNF-α (Figures 1(f) and 1(g)). The results indicated that osthole can inhibit LPS-induced inflammatory response in MPMs.

3.2. Osthole Blocks LPS-Induced Activation of NF-κB. The production of proinflammatory cytokines is predominantly regulated by NF-κB and/or AP-1 at the transcriptional level. MPMs were pretreated with osthole for 1 h and stimulated with LPS (0.5 μg/mL) for 1 h, and cell lysates were immunobotted for IkB-α or the p65 subunit of NF-κB. Results indicated that LPS significantly stimulated the degradation of IkB-α, which was effectively prevented by osthole pretreatment in a dose-dependent manner (Figure 2(a)). The inhibition of the LPS-stimulated IkB-α degradation was accompanied by inhibition of nuclear translocation of the p65 subunit of NF-κB as detected by Western blot analysis of nuclear and cytoplasmic cell fractions (Figure 2(b)). Additionally, immunofluorescence staining of the p65 subunit similarly showed that osthole pretreatment prevented the LPS-induced nuclear translocation (Figure 2(c)). These data indicated that osthole suppressed LPS-induced activation of NF-κB signaling.

3.3. Osthole Ameliorates Sepsis In Vivo. We used the LPS-induced sepsis mouse model to evaluate the protective effects of osthole. Mice given an iv injection of LPS died within 60 h (Figure 3(a)), as well as sharp loss of body weight (about 20%) (Figure 3(b)). However, mice pretreated with osthole, 20 or 40 mg/kg, improved survival beyond 72 h (p < 0.05) (Figure 3(a)), as well as body weight gain (Figure 3(b)). Uncontrollably sustained and vigorous inflammatory responses are characteristic of sepsis. In septic mice, both IL-6 and TNF-α were significantly elevated in serum and lung tissue (Figures 3(c) and 3(d)). Following osthole pretreatment, the increases in IL-6 and TNF-α content in serum and lung tissue were prevented (Figures 3(c) and 3(d)).
and 3(d)). The findings indicated that osthole was a potent inhibitor of inflammatory cytokine production, which likely protected against sepsis.

3.4. Protective Effect of Osthole on LPS-Induced Acute Lung Injury (ALI). We next investigated the protective effects of osthole in the LPS ALI mouse model. Histological examination of the lung morphology stained with H&E indicated that LPS induced the expected pathologic changes, including areas of inflammatory infiltration, hemorrhage, interstitial edema, thickening of the alveolar wall, and lung tissue destruction (Figure 4(a), top panel). Moreover, LPS induced significant macrophage infiltration, as indicated by increased tissue localization of marker CD68 (Figures 4(a), middle panel and 4(b)), as well as increased tissue content of TNF-α (Figures 4(a), lower panel and 4(c)). However, osthole pretreatment effectively prevented the LPS-induced morphological derangements, as well as the induced increases in CD68 and TNF-α in lung tissue (Figures 4(a)–4(c)).

Additionally, osthole pretreatment inhibited the LPS-induced extravasation and tissue recruitment of leukocytes in ALI. Results indicated that osthole inhibited the LPS-induced increase in total cell number and neutrophils in BALF (Figures 5(a) and 5(b)). This was further corroborated by the finding that the LPS-induced increase in lung tissue MPO activity, an index of neutrophil, was significantly inhibited by osthole pretreatment (Figure 5(c)). An important characteristic of ALI is pulmonary edema, which can be assessed by the wet/dry lung ratio. LPS induced ~30% increase in the wet/dry ratio, indicating the presence of pulmonary edema, which was prevented by osthole pretreatment (Figure 5(d)). Further, LPS induced increases of
proinflammatory cytokines IL-6 and TNF-α, in both BALF and serum (Figures 5(e) and 5(f)). As expected, osthole pre-treatment inhibited these increases in BALF and serum (Figures 5(e) and 5(f)). These results provide strong evidence that osthole was a potent anti-inflammatory agent with therapeutic potential for treatment of ALI.

3.5. Osthole Suppresses the NF-κB Nuclear Translocation in ALI Mouse Model. We investigated the signaling mechanism by which osthole protected against inflammatory injury responses observed in our in vivo and in vitro studies. The involvement of NF-κB was first evaluated by determining IκB-α degradation in the mouse lung tissues of ALI mice. Western blot results indicated that LPS induced significant IκB-α degradation, which was effectively prevented by osthole pretreatment (Figures 6(a) and 6(b)). Moreover, LPS stimulated the nuclear translocation of the p65 subunit of NF-κB, but osthole pretreatment prevented the translocation (Figures 6(c)–6(d)). The data illustrated that osthole inhibited NF-κB signaling in lung tissue of ALI in vivo.

4. Discussion

ALI is characterized by persistent pulmonary inflammation [10] and increase in microvascular permeability. Enhanced inflammatory responses [30, 31], manifesting as elevated inflammatory cytokine production, and macrophage infiltration in lung are the most important pathological mechanisms [32].
Therefore, anti-inflammatory therapy could be an attractive option to improve the quality of life in ALI patients. However, the progress in the development of novel effective therapeutic drugs for treatment of ALI is still disappointing. Discovery of active compounds from natural products would speed up the pace presently. Interestingly, several natural compounds with anti-inflammatory activities have been demonstrated to prevent inflammatory responses in experimental animal models of ALI.

Osthole has been shown to be effective in acute kidney injury via suppressing inflammatory response. However, the underlying mechanism is poorly understood. Here, we show that osthole alleviated ALI by inhibiting LPS-induced productions of IL-6 and TNF-α, likely through a mechanism in modulating NF-κB activity. It was closely related to the efficacy of osthole against ALI, such as lung tissue derangements, pulmonary edema, tissue recruitment of leukocytes, and prolonging of the survival time of septic mice. Although the dose of osthole

Figure 4: Osthole protects against inflammatory tissue injury in LPS-induced ALI. C57BL/6 mice were pretreated for 0.5 h with 20 mg/kg or 40 mg/kg osthole via ip injection, followed by intratracheal injection of 5 mg/kg LPS, and lungs were sampled 6 h later for analyses. (a, top panel) Representative light micrograph of lung histological changes stained with H&E, (a, middle panel) of immunolocalization of CD68, marker for macrophages (brown), and (a, bottom panel) of tissue localization of TNF-α (brown), micron bar = 50 μm. Quantification of positive lung tissue staining for (b) CD68 and (c) TNF-α in (a); values are reported as % mean ± SEM; n = 4. ***p < 0.001 versus control (Ctrl). ###p < 0.001 versus vehicle.

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Figure 5: Osthole inhibits inflammatory cell infiltration and cytokine secretion in ALI. ALI was induced as described (Materials and Methods). Briefly, C57BL/6 mice were pretreated for 30 min with 20 mg/kg or 40 mg/kg osthole via ip injection, followed by intratracheal injection of 5 mg/kg LPS, and lungs were sampled 6 h later for analyses. (a) Number of total cells and (b) neutrophils in BALF was determined by counting microscopic fields; \( n = 7 \). (c) Lung tissue myeloperoxidase (MPO) activity assay was determined as an index of neutrophil activity; values are reported as mean ± SEM in U/g tissue; \( n = 7 \). (d) Wet and dry weights of lung tissue were measured and reported as the ratio of wet to dry as index of pulmonary edema; values are reported as mean ± SEM; \( n = 7 \). ELISA detection of (e) IL-6 and (f) TNF-α in BALF (upper graph) and serum (lower graph) from the experimental mice was determined, and values are reported as mean ± SEM; \( n = 7 \). ns = not significant; * \( p < 0.05 \) and *** \( p < 0.001 \), versus control (Ctrl). # \( p < 0.05 \), ## \( p < 0.01 \), and ### \( p < 0.001 \), versus vehicle.
Figure 6: Osthole suppresses NF-κB signaling in vivo. ALI was induced as described (Materials and Methods). Briefly, C57BL/6 mice were pretreated for 30 min with 20 mg/kg or 40 mg/kg osthole via ip injection, followed by intratracheal injection of 5 mg/kg LPS, and lungs were sampled 6 h later for Western blot analyses. (a) Representative Western blot detecting for IκB-α; GAPDH as loading control. (b) Densitometric quantification of blot in (a); values are reported as mean ± SEM relative to control (Ctrl); n = 4. The bands were quantified by ImageJ and normalized to loading controls. (c) Representative Western blot analysis of the p65 subunit of NF-κB from cytoplasmic and nuclear cell fractions (C-p65 and N-p65, resp.); GAPDH and lamin B as respective loading controls. Densitometric quantification of (d) N-p65 and (e) C-p65 from (c); values are reported as mean ± SEM; n = 4. The bands were quantified by ImageJ and normalized to loading controls. ns = not significant; *p < 0.05, **p < 0.001 versus control (Ctrl). #p < 0.05, ##p < 0.01 versus vehicle.
was relatively high, no toxic effects on body weight and cell proliferation were observed in our study. In addition, the effective dose of osthole we used was consistent with literatures reported previously [33, 34]. Furthermore, osthole is a natural coumarin derivative. Coumarin has been approved for some medical uses as pharmaceuticals in the treatment of lymphedema and anticoagulation via its anti-inflammatory properties. Thus, the present study would also provide a basis for expanding the indications of coumarin.

Macrophages are important participants in immune response [35–37] by providing an immediate defense against foreign agents or organisms. Besides, neutrophil infiltration into inflamed and infected tissues is a fundamental process of the innate immune response [38–41], which increased recruitment by the stimulation of LPS as the data shows. Two transcription factors, NF-κB [42–44] and AP-1 [45], are well-characterized regulators in the expression of proinflammatory cytokines [46], including TNF-α, IL-6, IL-1β, and IL-18 [47]. Our findings indicated that the inhibition of inflammatory response by osthole was through suppressing IκB-α degradation, resulting in NF-κB inhibition and thereby inhibition of its downstream cytokine gene expression. However, osthole showed no significant effect on JNK and p38 MAPK signaling pathways (data not shown). Overall, our results indicated that osthole potently inhibited LPS-activated NF-κB signaling of macrophages in the lung tissue of ALI mice, leading to significant suppression of inflammation and tissue injury. Even though nonsteroidal or steroidal anti-inflammatory drugs have potent anti-inflammation efficacy, the underlying mechanisms and side effects limit their applications in clinic. So, the present study would provide a potential strategy for treating ALI.

In summary, in this study we examined the potential pharmacological effect of osthole on sepsis and ALI and its underlying mechanism. Our work highlights osthole as a potential new candidate for treatment of injurious inflammatory responses in sepsis and ALI. However, the direct target of osthole upstream NF-κB remains unknown. The family of toll-like receptors (TLRs) are known as initiator of innate immune response. TLR4 is a typical member of TLRs which responsible for chronic and acute inflammatory disorders. Moreover, TLR4 is the primary receptor for LPS. Future studies are needed to investigate the relationship between osthole and TLR4.

Conflicts of Interest

The authors declare no competing financial interests.

Authors’ Contributions

Yiyi Jin, Li Li, Zhongxiang Xiao, Xuemei Chen, and Weiwei Zhu performed the research. Guang Liang designed the research study. Suqing Zheng, Xiong Chen, and Weixin Li contributed essential reagents or tools. Guang Liang, Jianchang Qian, Yiyi Jin, Xin Ju, and Xiaodong Bao analysed the data. Guang Liang and Wencan Wu wrote the paper. Yiyi Jin and Jianchang Qian contributed equally to the work.

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