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Shikonin exerts anti-inflammatory effects in a murine model of lipopolysaccharide-induced acute lung injury by inhibiting the nuclear factor-kappaB signaling pathway

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ABSTRACT

Shikonin, an analog of naphthoquinone pigments isolated from the root of *Lithospermum erythrorhyzon*, was recently reported to exert beneficial anti-inflammatory effects both *in vivo* and *in vitro*. The present study aimed to investigate the potential therapeutic effect of shikonin in a murine model of lipopolysaccharide (LPS)-induced acute lung injury (ALI). Dexamethasone was used as a positive control to evaluate the anti-inflammatory effect of shikonin in the study. Pretreatment with shikonin (intraperitoneal injection) significantly inhibited LPS-induced increases in the macrophage and neutrophil infiltration of lung tissues and markedly attenuated myeloperoxidase activity. Furthermore, shikonin significantly reduced the concentrations of TNF-α, IL-6 and IL-1β in bronchoalveolar lavage fluid induced by LPS. Compared with the LPS group, lung histopathologic changes were less pronounced in the shikonin-pretreated mice. Additionally, Western blotting results showed that shikonin efficiently decreased nuclear factor-kappaB (NF-κB) activation by inhibiting the degradation and phosphorylation of IκBα. These results suggest that shikonin exerts anti-inflammatory properties in LPS-mediated ALI, possibly through inhibition of the NF-κB signaling pathway, which mediates the expression of pro-inflammatory cytokines. Shikonin may be a potential agent for the prophylaxis of ALI.

1. Introduction

Acute lung injury (ALI) is a critical illness syndrome characterized by noncardiogenic edema, pulmonary inflammation, severe systemic hypoxemia, decreased neutrophil apoptosis in the lung and multiple organ failure [1,2].The incidence and mortality of ALI in humans are notably higher than previously reported, despite the implementation of proactive therapeutics [3]. Thus, animal models with relevant characteristics of ALI are needed to elucidate possible mechanisms and determine effective therapeutics. Lipopolysaccharide (LPS) is an endotoxin of gram-negative bacteria. A murine model with LPS-induced ALI has become a common ALI model because the experiment results are reproducible. LPS-induced ALI mainly causes alveolar epithelial permeability, a neutrophilic inflammatory response and an increase in intrapulmonary cytokines regulated by the TLR4 pathway [4–6]. In some cases, novel treatments for ALI are assessed for their ability to attenuate the inflammatory response and relevant symptoms.

Shikonin, an analog of naphthoquinone pigments extracted from the root of *Lithospermum erythrorhyzon* [7–9], has been reported to produce multiple pharmacological effects, including antibacterial, wound-healing, analgesic, anti-inflammatory, antithrombotic and antitumour effects [10–17]. The results of recent studies have shown that shikonin exerts anti-inflammatory properties in LPS-activated macrophages via downregulating MAPK and nuclear factor-kappaB (NF-κB) signaling [18] and reducing tumor necrosis factor-α (TNF-α) production through the selective blockade of pre-mRNA splicing of TNF-α [19,20]. Recently, there have been reports of shikonin modifying inflammatory responses and leading to partial edema in mice by interfering with IκBα degradation [21]. However, there have been a limited number of published reports on the anti-inflammatory effect of shikonin on LPS-induced ALI. In this study, the effect and possible mechanism of shikonin on LPS-induced ALI in mice were investigated. This study may provide a new potential treatment for ALI from natural products.

2. Materials and methods

2.1. Animals

Male BALB/C mice weighing 20–22 g were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China). All animals were housed in microisolator cages and fed

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standard laboratory chow and water ad libitum. The mice were kept at a temperature of 24 ± 1 °C and a relative humidity of 50%–60% for at least 3 days prior to the experiments throughout the study. This study was approved by the Jilin University Animal Care and Use Committee. The protocols were reviewed and approved by the committee.

2.2. Reagents

Shikonin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and suspended in phosphate-buffered saline (PBS) with 0.1% dimethyl sulfoxide (DMSO). LPS (*Escherichia coli* 055:B5) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The myeloperoxidase (MPO) kit was provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Mouse TNF-α, IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (CA, USA). Mouse mAb phospho-NF-κB p65, mouse mAb phospho-IκBα and rabbit mAb IκBα were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). HRP-conjugated goat anti-rabbit and goat-mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). The Nuclear and Cytoplasmic Protein Extraction Kit was provided by Beyotime Institute of Biotechnology (Jiangsu, China). All other chemicals were of reagent grade and endotoxin free.

2.3. LPS-induced ALI in mice

To evaluate the effect of shikonin, mice were randomly assigned into six groups: control group, LPS and shikonin (0.5, 2, and 4 mg/kg) groups, LPS and dexamethasone (DEX) group, and an LPS group. Each group contained six mice. The mice were treated as previously reported [22,23] and intraperitoneally injected with shikonin (0.5, 2, or 4 mg/kg) 1 h before the intranasal (i.n.) administration of LPS. DEX 0.5 mg/kg was administered intragastrically as a positive control. The control and LPS mice were given an equal volume of PBS instead of shikonin. After 1 h, these mice were slightly anesthetized through inhalation of diethyl ether, and 10 μg of LPS was instilled i.n. in 50 μl PBS to induce lung injury. The control group mice were given 50 μl PBS with 0.1% DMSO i.n. without LPS [22]. The doses of these drugs and LPS were based on our previous studies and preliminary experiments (data not provided).

2.4. Lung wet/dry ratio and inflammatory cell count

The lungs were removed at 7 h after LPS challenge, and the wet weight was recorded. The lungs were then placed in an incubator at 65 °C for 48 h, and the dry weight was determined. Bronchoalveolar lavage fluid (BALF) was collected from the left lung through the intratracheal injection of 1 ml PBS followed by gentle aspiration. The recovered fluid was pooled and centrifuged at 250 ×g for 10 min at 4 °C. The cell pellet was resuspended in PBS, and the total cells recovered in the BALF were counted. The remaining cell samples were smeared on a slide, and the percentage of neutrophils was calculated under light microscopy.

2.5. Measurement of cytokines in BALF

After the mice were killed under diethyl ether anesthesia and their tracheas were exposed, BALF was collected at 7 h following LPS challenge. BALF was collected by injecting 1 ml PBS (pH 7.4) through a silastic catheter, and fluid was collected by gentle suction. The levels of the inflammatory cytokines TNF-α, IL-6 and IL-1β in BALF were measured using specific ELISA kits according to the manufacturer's instructions.

2.6. MPO activity assay

Neutrophil accumulation in the lung tissue was assessed by MPO activity. Briefly, at 7 h after LPS administration, lung tissue samples were frozen and homogenized in cool normal saline (lung tissue to normal saline, 1:10). Then, the homogenates were processed according to the manufacturer's instructions. MPO activity was measured with a spectrophotometer at 450 nm.

2.7. Histopathologic evaluation

To evaluate the changes of lung issues, the lungs were obtained after the optimal time of LPS injection determined via the preliminary experiments from mice selected randomly from each group. The lung tissue was fixed in 10% neutral buffered formalin overnight and then dehydrated, paraffin embedded and sliced. After hematoxylin and eosin staining, pathological changes in the lung tissues were observed under a light microscope.

2.8. Western blotting analysis

Tissues were harvested at 7 h after LPS administration and immediately frozen in liquid nitrogen until homogenization. Proteins were extracted from the lungs using the Nuclear and Cytoplasmic Protein Extraction Kit. Whole cell lysates were prepared using cell lysis buffer for Western blotting according to the manufacturer's protocol. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Next, 60 μg of protein from lung tissue homogenates was separated on a 10% acrylamide gel, the proteins were transferred onto a polyvinylidene difluoride membrane, and the membrane was blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) supplemented with 5% skim milk (Sigma) at room temperature for 2 h. Phosphorylation-specific antibodies against IκBα and p65 were used as the primary antibody. They were diluted 1:1000 in TBS-T and incubated with the membrane at 4 °C overnight. Subsequently, the membrane was incubated with peroxidase-conjugated secondary antibody at room temperature for 1 h. The immunoreactive proteins were detected using an enhanced chemiluminescence Western blotting detection kit. The β-actin protein served as an internal control.

2.9. Statistical analysis

All values were expressed as the mean ± SEM. Differences between the mean values of normally distributed data were assessed with a one-way ANOVA (Dunnett's *t* test) and the two-tailed Student's *t* test. P-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effects of shikonin on LPS-induced lung wet/dry weight ratio and inflammatory cell count in the BALF of mice with ALI

Intranasal LPS administration induced airway and lung inflammation characterized by capillary leakage, large increases in neutrophils and macrophages and edema. The lung wet-to-dry weight ratio was used to quantify the magnitude of pulmonary edema in the control group, LPS group, shikonin (0.5, 2, or 4 mg/kg) + LPS groups and DEX group. As illustrated in Fig. 1, the lung wet-to-dry weight ratio was significantly increased after LPS (0.5 mg/kg) challenge compared with the control group ($p < 0.01$). Intraperitoneal shikonin injection at 0.5 mg/kg did not noticeably reduce the lung wet-to-dry weight ratio, while 2 and 4 mg/kg shikonin and DEX (0.5 mg/kg) caused the lung wet-to-dry weight ratio to decline significantly compared with the LPS group ($p < 0.05$). The total cells, neutrophils and macrophages in the BALF were evaluated at 7 h after LPS challenge. As shown in Fig. 2, the number of total cells, neutrophils and macrophages was significantly increased