Dioscin alleviates lipopolysaccharide-induced acute lung injury through suppression of TLR4 signaling pathways

Jun Zhu1,2, Qingnian Li3 and Tianyu Li1*

1Department of Traumatic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
2Department of Anesthesiology, Pu Ai Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
3Department of Emergency Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Abstract

Acute lung injury (ALI) is a life-threatening inflammatory syndrome that lacks an effective therapy. Dioscin, a natural steroid saponin isolated from a variety of herbs, could serve as an anti-inflammatory agent, as suggested in previous reports. The purpose of this study was to explore the effects of dioscin on lipopolysaccharide (LPS)-induced ALI and validate the potential mechanisms. An ALI model was induced by intratracheal administration of LPS. Dioscin (20, 40, and 80 mg/kg) was administered intragastrically once daily for seven consecutive days prior to LPS challenge. Our data revealed that dioscin significantly suppressed LPS-induced lung pathological changes, pulmonary capillary permeability, pulmonary edema, inflammatory cell infiltration, myeloperoxidase (MPO) activity, and cytokine production, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and keratinocyte chemoattractant (KC). Moreover, dioscin inhibited LPS-induced nuclear factor-kappaB (NF-κB) activation as well as Toll-like receptor 4 (TLR4) expression. In brief, the results indicated that dioscin alleviates LPS-induced ALI through suppression of TLR4 signaling pathways.

Introduction

Acute lung injury (ALI) and its more severe complication, acute respiratory distress syndrome (ARDS), are life-threatening inflammatory syndromes with high morbidity and mortality [1]. The canonical pathogenesis of ALI/ARDS is characterized by disruption of the alveolar-capillary barrier, progressive pulmonary edema and infiltration of inflammatory cells into the alveolar space, especially polymorphonuclear neutrophils (PMNs) [2]. A principal cause of ALI is sepsis mainly due to gram-negative bacteria. Specifically, the shedding of LPS from gram-negative bacteria into the circulation is a major factor leading to inappropriate neutrophil infiltration, which is the primary indicator of ALI development. Consequently, intratracheal administration of LPS has been extensively utilized to simulate human Gram-negative ALI [3-5]. However, despite several studies, the mechanisms governing increased lung epithelial and endothelial permeability remain undefined, and few specific pharmacological therapies for clinical ALI/ARDS have been discovered [6-8]. Considering the high morbidity and mortality of ALI, the exploration of novel treatments for ALI is urgently needed.

Dioscin, a typical steroid saponin, is derived from various medicinal herbs [9]. Pharmacological research has corroborated that dioscin has a broad range of effects...
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such as anti-viral [10], anti-fungal [11], anti-tumor [12-16], anti-hyperuricemic [17], anti-obesity [18], anti-ischemia–reperfusion injury [19-22], and hepatic protective activity [23-27]. Additionally, dioscin exerts therapeutic effects on bone and arthritis diseases [28-30]. It is noteworthy that dioscin also has anti-inflammatory activity [31], which has been identified as a crucial component in the treatment of inflammatory injuries including LPS-induced acute liver injury [32]. However, few studies regarding the effects and molecular mechanisms of dioscin in the inhibition of LPS-induced ALI have been reported thus far. In this study, we aimed to identify the protective effects of dioscin on LPS-induced ALI in a mouse model and elucidate the potential anti-inflammatory mechanisms.

Materials and Methods

Reagents

Dioscin (purity >99%) was provided by Shanghai Winherb Medical S&T Development Co. Ltd. (Shanghai, China). The dioscin was dissolved with 0.1% dimethyl sulfoxide (DMSO) for in vitro experiments and suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) before in vivo experiments. LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The myeloperoxidase (MPO) measurement kit was provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits for mouse tumor necrosis factor (TNF)-α, interleukin (IL)-6, and keratinocyte chemoattractant (KC) were purchased from BioLegend (San Diego, CA, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Anti-TLR4, anti-COX-2, anti-pNF-κB p65, anti-NF-κB p65, anti-pIκBα, anti-IκBα, and anti-GAPDH monoclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals were of analytical grade (AR, ≥99.9%).

Cell culture

Human alveolar cells (A549) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin, and streptomycin in a suitable environment containing 95% O₂ and 5% CO₂ at 37.

Toxicity assay

Before treatment, A549 cells were grown in 24-well plates at a density of 6×10⁴ cells per well for 24 h and then incubated in the presence of different concentrations of dioscin (75, 150, 300, 600, 1,200, and 2,400 ng/ml) for another 24 h. The control group was treated with DMSO. Cell proliferation was measured using the MTT method.

Cell proliferation assay

A549 cells were seeded into 24-well plates at a density of 6×10⁴ cells per well for 24 h and then incubated in the presence of different concentrations of dioscin (150, 300, and 600 ng/ml) caused by LPS (100 ng/ml) for 6, 12, and 24 h. Cell proliferation was calculated based on the MTT method.

Animal model of ALI

All of the procedures involving animals conformed to the Declaration of Helsinki of the World Medical Association and were approved by the Animal Care and Use Committee of Tongji Hospital of Huazhong University of Science and Technology. Male BALB/c mice (8–10 weeks, 23–27 g) were purchased from Beijing HFK Biotechnology Co. Ltd. (Beijing, China). Before the development of animal models, mice were acclimatized to laboratory conditions for one week.
Mice were randomly divided into six groups containing 12 mice each, including the control group, LPS group, LPS + dioscin (20, 40, and 80 mg/kg) group (22, 23,30,32), and LPS + dexamethasone (DEX, 5 mg/kg, served as the positive control) group. Dioscin and DEX were administered intragastrically once daily for seven consecutive days prior to LPS administration according to a previous study [32]. Twenty micrograms of LPS in 50 μl phosphate-buffered saline (PBS) was administered intratracheally to induce ALI after general anesthesia (2% isoflurane inhalation) in mice at a dosage of 20 mg/kg. Mice in the control group were administered 50 μl of PBS plus 0.5% CMC-Na solution at a volume equal to that administered to the LPS + dioscin group. The LPS group mice were treated with LPS and 0.5% CMC-Na at the same volume administered to the control group. Briefly, anesthetized mice hanging on the vertical plane were subjected to trachea intubation, prepared LPS was slowly dripped into the tracheal tube with a Micro-sprayer (Penn-Century, Wyndmoor, PA, USA), from the bronchial to the pulmonary alveoli. Mice without bucking were send to an incubator until sacrifice 6 hours later. Animal euthanasia and tissue collection were performed 6 h after intratracheal instillation.

**Blood gas analysis**

PaO2, PaCO2, and PH were detected by IL1302-type blood gas analysis instrument (Instrumentation Laboratory Inc.) through collecting the arterial blood before euthanasia. Simply, once being anesthetized with sodium pentobarbital (50 mg/kg), mice were fixing on the operating table. And the abdominal aorta was exposed through blunt dissection after median laparotomy. With a heparinized syringe 0.6-ml was collected to measure.

**Preparation and evaluation of bronchoalveolar lavage fluid (BALF)**

At the end of the experiment, the lungs were lavaged three times by intratracheal instillation with 1 ml of autoclaved and precooled PBS. The collected BALF was centrifuged at 1,200×g for 5 min at 4°C. Afterwards, the supernatant from the first 1 ml was aliquoted and stored at -80°C.

Total protein concentration of BALF was determined using a BCA kit (23225; Pierce) according to the manufacturer’s instructions. The concentration of TNF-α, IL-6, and KC was measured with ELISA kits (BioLegend) based on the manufacturer’s protocol. After the supernatant of BALF was aliquoted, red blood cells (RBCs) in cell pellets from BALF were subjected to lysis by Ammonium-Chloride-Potassium Lysing Buffer (C3702; Beyotime, Shanghai, China). A standard hemocytometer was employed to measure the total number of white blood cells (WBCs). Wright–Giemsa staining was adopted for the quantification of neutrophils (three counts per slide, 300 cells per count).

**MPO Assay**

The level of neutrophil infiltration of the alveolar space was assessed by MPO activity. The MPO activity in homogenates of lung tissue was determined through test kits (Jiancheng Bioengineering Institute) in accordance with the provided protocols.

**Lung wet-to-dry weight (W/D) ratio**

As an important reference for lung edema evaluation, the weight ratio of the wet lung to the dry lung was calculated. Firstly, the whole lung was excised, blotted dry, and weighed to acquire the “wet” weight. Then, the lung was placed in an oven maintained at 65°C for 48 h to obtain the “dry” weight. Lastly, the W/D ratios were computed (wet weight / dry weight).

**Analysis of pulmonary capillary permeability**

Under general anesthesia (2% isoflurane inhalation), Evans blue dye (20 mg/kg; E2129; Sigma–Aldrich) was injected into the tail vein of mice 30 min in advance of
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Euthanasia. After sacrifice, tracheotomy was performed, and the lungs were perfused with 4–5 ml of PBS through the right ventricle to evacuate blood. The lungs were then excised from the trachea and thoracic cavity, the surrounding mediastinal structures were removed, and the main stem bronchi were separated, followed by imaging of the lungs. The homogenized lungs were incubated in 500 μl formamide for 48 h at 60°C and centrifuged at 7,000×g for 30 min at room temperature. Supernatants containing Evans blue dye were collected and quantitated in a spectrophotometer at 620-nm absorbance. The Evans blue dye concentration was calculated against a standard curve.

**Histopathological evaluation**

Histopathological evaluation was performed on mice that were not subjected to BALF measurement. The left lungs were inflated with 4% paraformaldehyde and dehydrated in graded alcohol before embedding in paraffin. Then, hematoxylin and eosin (H&E) staining of sections (5 μm thickness) was performed.

**Western blot analysis**

Frozen lung tissues were defrosted and homogenized with radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor. Thereafter, the homogenate samples were centrifuged at 12,000×g for 30 min at 4°C. Protein concentrations were measured with the BCA protein assay kit. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking of the nonspecific sites with 5% non-fat dry milk, the membranes were incubated with specific primary antibodies at 4°C overnight. Next, the membranes were washed with PBS with Tween (PBS-T) followed by incubation with the secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. Ultimately, the immune-active protein expression was detected with an enhanced chemiluminescence western blotting detection kit.

**Statistical analysis**

All values are expressed as means ± standard error of the mean (SEM). Statistically significant differences between groups were determined by analysis of variance (ANOVA) followed by Student’s t test. Statistical significance was accepted at p<0.05.

**Results**

Dioscin alleviates proliferation of A549 cells treated with LPS *in vitro*

As shown in the Supplemental Figure 1B and 1C, in the cell viability dioscin with the different concentrations of 75, 150, 300, and 600 ng/ml for A549 cells presented no statistically significant difference. However, the concentrations of 1200, and 2400 ng/ml for A549 cells under 24 h treatment significantly reduce the cell viability. In contrast to the LPS group, at the concentrations of 150, 300, and 600 ng/ml under 6, 12, and 24 h treatment, the dioscin effectively inhibited cell proliferation.

**LPS-mediated lung histopathological changes**

In this study, we primarily evaluated lung histological changes. Normal lung structures showed no edema, few inflammatory cells, and integral alveolar structure (Figure 1A). Nevertheless, lung histological sections from LPS-induced mice revealed inflammatory cell infiltration, hemorrhage in stroma, edema, and alveolar collapse (Figure 1B). Challenge with dioscin (20, 40, and 80 mg/kg) effectively ameliorated LPS-induced lung injury in a dose-dependent manner compared with that in the control group (Figure 1C–E). In addition, as a positive control, pretreatment with DEX (5 mg/kg) notably attenuated LPS-induced lung injury (Figure 1F).

**Change in lung permeability**

To evaluate LPS-induced disruption of the alveolar–capillary barrier, we performed
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...the Evans blue dye exudation assay to examine lung permeability. As shown in Fig. 2A, dioscin (20, 40, and 80 mg/kg) markedly inhibited LPS-induced augmentation of lung permeability, as indicated by lung color variation compared with that in the control. Statistically, dioscin (40 and 80 but not 20 mg/kg) suppressed Evans blue dye extravasation (Figure 2B).

LPS-induced lung edema

To validate the effect of dioscin on LPS-induced lung edema, the lung W/D ratio was assessed. As illustrated in figure, the W/D ratio was remarkably increased after LPS administration compared with that in the control group. However, pretreatment with dioscin (20, 40, and 80 mg/kg) strikingly reduced the W/D ratio.

Inflammatory cell counts in BALF and MPO activity in lung tissues

The infiltration of total inflammatory cells and assembly of neutrophils into BALF are the hallmarks of LPS-induced ALI. As presented in figure 4A and B, the accumulation of total cells and neutrophils in BALF after LPS stimulation significantly increased compared with that in the control group. Notwithstanding, pretreatment with dioscin (40 and 80 mg/kg) or DEX significantly decreased the number of total cells and neutrophils. Additionally, the MPO activity of the lung tissue, reflecting PMN accumulation, was increased in the LPS group compared with the control group. Similarly, dioscin (20, 40, and 80 mg/kg) or DEX reduced MPO activity compared with that in the LPS group (Figure 4C).

![Figure 1](image1.png)

![Figure 2](image2.png)
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Total protein and cytokine concentration in BALF

Both protein leakage and cytokine production are important biological characteristics of ALI [33,34]. The concentrations of total protein and cytokines were respectively measured by BCA protein assay and ELISA. As shown in figure 5, after LPS stimulation, the concentrations of total protein and cytokines were significantly increased compared with those in the control group. Nonetheless, pretreatment with dioscin (40 and 80 mg/kg) or DEX distinctively attenuated protein leakage (Figure 5A). As demonstrated in figure 5B, challenge with dioscin (20, 40, and 80 mg/kg) or DEX notably decreased production of the cytokine TNF-α; similarly, dioscin (40 and 80 mg/kg) or DEX significantly reduced the production of IL-6 and KC compared with that in the LPS group (Figure 5C and D).

LPS-induced TLR4 expression and NF-κB activation

As shown in figure 6A, in the LPS group, the expression of TLR4 and NF-κB was significantly higher than in the control group. However, pretreatment with dioscin (20, 40, and 80 mg/kg) or DEX effectively inhibited the expression of TLR4 and the phosphorylation of inhibitory κB (IκB) α and NF-κB p65 (Figure B–D). Interestingly, challenge with dioscin at 20 mg/kg, unlike at 40 and 80 mg/kg, did not significantly suppress the expression of COX-2 compared with that in the LPS group (p>0.05; Figure 6E).
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Discussion

Previous reports revealed the anti-inflammatory activity of dioscin \[31,32\]. Our present study aimed to investigate its potent effect on LPS-induced ALI. Indeed, the data demonstrated that dioscin can attenuate LPS-induced respiratory failure and respiratory acidosis, lung histopathological changes, lung permeability, pulmonary edema, inflammatory cell infiltration, protein leakage, and cytokine production. Furthermore, our results revealed that dioscin can significantly inhibit the expression of TLR4 and the activity of NF-κB and its downstream proteins, such as COX-2. These results verified that dioscin could be a potential therapeutic candidate for ALI.

ALI is characterized by a massive inflammatory cascade within the lungs in addition to severely impaired gas exchange, resulting from alveolar–capillary barrier disruption and pulmonary edema \[1,3\]. In evidence, mice suffering to ALI shows respiratory failure and respiratory acidosis with decreased pH, oxygen deficit, and, carbon dioxide retention similar to ALI’s patients. In our study, Supplementary Table 1 shown dioscin significantly improved respiratory failure via blood gas analysis. In this study, the Evans blue dye extravasation assay (a marker for pulmonary capillary permeability) was employed to quantify the extent of alveolar–capillary barrier disruption. Compared with other treatments recommended for ALI, our study not only provided statistical data of Evans blue dye but also images of the Evans blue dye extravasation assay; obviously, the images were more convincing. Pretreatment with
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Dioscin significantly diminished the pulmonary capillary permeability (Figure 2). To evaluate the magnitude of pulmonary edema, the lung W/D ratio was calculated. The results illustrated that pretreatment with dioscin decreased the lung W/D ratio (Figure 3), which indicates that dioscin could prevent the progression of pulmonary edema.

Extensive infiltration and accumulation of leukocytes, particularly PMNs, in both the alveolar and interstitial spaces of the lung are principal pathological hallmarks of ALI [35,36]. Our study confirmed that pretreatment with dioscin considerably impeded the enhancement of total leukocytes as well as PMNs after stimulation with LPS (Figure 4A and 3B). MPO activity, a marker of neutrophils, is estimated to quantify neutrophil activation and accumulation in tissues (37). Similarly, our study demonstrated that pretreatment with dioscin markedly restrained MPO activity in the lung tissues (Figure 4C).

Cytokines play a pivotal role in the initiation and development of inflammatory cascade response [38]. During the cascade of ALI, proinflammatory modulators including TNF-α, IL-6, and KC are critical. Our results revealed that dioscin dose-dependently suppressed LPS-induced inflammatory cytokine production including of TNF-α, IL-6, and KC (Figure 5). Moreover, COX-2 is increased by pro-inflammatory mediators and further exacerbates the inflammatory immune response in lung injury [39]. In this study, pretreatment with dioscin notably inhibited the expression of COX-2 (Figure 6). It is well known that NF-κB plays a critical role in the pathogenesis of ALI [40]. Furthermore, the production of inflammatory cytokines depends on NF-κB, and these cytokines in turn activate NF-κB [41]. Thus, suppression of NF-κB activation is necessary to lessen its downstream cytokine levels. In our experiment, pretreatment with dioscin significantly downregulated activated NF-κB. A previous study suggested that dioscin can suppress phosphorylation of IκBα [24], a pivotal inhibitory protein for NF-κB activation. In the current research, the phosphorylation of IκBα, which can limit the nuclear translocation of NF-κB, was also markedly suppressed. From another aspect, TLR4, a pattern recognition receptor (PRR), can trigger both damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) as well as recognize LPS from gram-negative bacteria. Accumulating evidence has confirmed that it plays a dominant role in innate and adaptive immune response to ALI [42,43]. LPS-induced activation of TLR4 induces activation of NF-κB, which plays an essential role in regulating cytokine gene transcription. Our results proved that dioscin dose-dependently inhibits LPS-induced TLR4 expression and NF-κB activation.

In the recent years, considerable advances have been made in understanding the pathogenesis of ALI, but approaches to develop drug therapies have not been productive. Specifically, targeting single inflammatory factors has failed to improve ALI outcomes because of its complex pathogenesis and nature. Alternatively, several natural products hold promising therapeutic capacity to inhibit multiple inflammatory pathways associated with ALI in experimental models [44]. For instance, curcumin, alpinetin, and licochalcone A inhibit LPS-induced lung injury by inhibiting inflammatory and/or apoptotic response [45,46], while shikonin significantly suppressed LPS-induced total proteins in BALF and decreased MPO and nitric oxide in lung tissues [47]. Our data strongly suggested that preventing and/or treating ALI with dioscin is an attractive approach that simultaneously limits inflammation, protein leakage, and oxidative stress. In our study, dioscin was administered for seven days prior to ALI model establishment to maintain a stable circulating and tissue concentration of dioscin and its metabolic derivatives, which corresponds to a preventive effect on ALI occurrence and progression in the clinic. Until now, no pharmacological agents have demonstrated efficacy in treating ALI or improving its symptoms, and thus preventing ALI is critical. The seven-day pretreatment approach was also supported by previous studies [19,32,48].
In conclusion, we found that dioscin could alleviate LPS-induced ALI via inhibiting the production of COX-2, TNF-α, IL-6, and KC. The mechanism of ALI amelioration mainly involves blocking of the TLR4 signaling pathway. Overall, these data demonstrated that dioscin is a potential therapeutic candidate for ALI.

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Authors’ contributions

Tianyu Li conceived and designed all the experiments. Jun Zhu performed the experiments. Qingnian Li analyzed the data and made the interpretation. Tianyu Li, Jun Zhu, and Qingnian Li drafted and revised the article. All authors read and approved the final version.

Ethics approval and consent to participate

All of the procedures involving animals were approved by the Animal Care and Use Committee of Tongji Hospital of Huazhong University of Science and Technology.

Supplementary Tables & Figure

References


