Original Article

Effects and mechanisms of oridonin in the treatment of acute respiratory distress syndrome mice

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Abstract: Objective: To investigate the protective effects and possible mechanisms of oridonin (ORI) in the treatment of acute respiratory distress syndrome (ARDS) in mice. Methods: ARDS mice models were established by tracheal instillation of lipopolysaccharide (LPS). Male C57BL/6 mice aged from 8 to 10 weeks were randomized into three groups: a sham operation group (5 mice), in which only sterile phosphate buffered saline (PBS) was instilled into the trachea; an experimental group, in which after the establishment of ARDS mice models, different doses of ORI (30 mg/kg, 90 mg/kg, 150 mg/kg, 300 mg/kg respectively; 5 mice in each group) were given by intraperitoneal injection; and a control group, in which the same doses of PBS were given (5 mice). The lung injuries were observed on the base of the detection of protein quantification in alveolar lavage fluid, the lung wet-to-dry ratio (W/D), and the pathological sections of lung tissues stained with hematoxylin and eosin (HE). The expression of inflammatory factors in lung tissues of the mice in these two groups was detected by fluorescent quantification polymerase chain reaction (PCR) and the release of inflammatory factors was detected by enzyme-linked immuno sorbent assay (ELISA); western blot was employed to analyze the activation of the NF-κB's pathway. Results: Compared with the control group, protein quantification in bronchoalveolar lavage fluid and the lung W/D ratio were significantly reduced after the injection of ORI (P<0.05). The pathological sections showed that the inflammation in lung tissues was relieved and the injuries were significantly reduced. The expression of TNF-α, IL-6 in lung tissues and serum greatly decreased (P<0.05). The results of western blot showed that the expression of NF-κB p65 in the experimental group decreased and the expression of Iκ-Bα increased. Conclusion: ORI could alleviate ARDS lung injuries by inhibiting the activation of signaling pathway of NF-κB and the expression of inflammatory factors.

Keywords: Oridonin (ORI), ARDS, lung injuries, NF-κB, Iκ-Bα

Introduction

Acute respiratory distress syndrome (ARDS), is a commonly seen critical disease in clinical diagnosis, which currently has no effective treatment methods and the mortality rate is extremely high, up to 30%-60% [1]. With the advance of pathogenesis study and diagnosis and treatment methods, the rehabilitation rates in young patients have slightly improved, but pulmonary functions cannot be completely recovered, which brings severe physical and emotional traumas to patients with ARDS. Lung is the first target organ of multiple organ dysfunction syndrome (MODS), and it has abundant of alveolar epithelium and vascular endothelium, which makes it easily invaded by external injury factors. ARDS can be initiated by both direct injuries caused by all kinds of pulmonary infections and indirect injuries caused by severe traumas, and essentially, it is a serious adverse consequence induced by the imbalance between inflammatory responses and anti-inflammatory responses [2]. In the course of disease, the overactivity of neutrophils can lead to the imbalance between pro-inflammatory and anti-inflammatory responses, which is a key process for ARDS [3]. The release of various kinds of cell factors and inflammatory mediators can cause alveolar epithelial injuries and vascular endothelial injuries, leading to the occurrence of ARDS.

ORI is a kind of 7, 20-epoxy-enantiomer-kaurane diterpenoid compounds and a major active ingredient extracted from traditional Chinese
Effects and mechanisms of oridonin

ORI is a widely used medicine, and according to the studies, it had obvious inhibiting and killing effects on tumor cells [5, 6]. Furthermore, ORI has the ability of enhancing immunity and has efficacies as neuroprotection, antibacterial, anti-inflammatory functions and so on [7, 8]. The recent studies showed that ORI also had protective effects on mice with septic diseases [9]. NF-κB signaling pathway can activate various inflammatory factors, which is of great use in the treatment of ARDS lung injuries [10]. Besides, according to studies, it also could play an anti-inflammatory role by inhibiting the activation of NF-κB [11]. Therefore, it is assumed that ORI is able to decrease the inflammatory responses by inhibiting the activation of NF-κB, so as to protect human beings from ARDS lung injuries. This research tries to determine the effects and possible mechanisms of ORI in the treatment of ARDS by studying ARDS mice models.

Materials and methods

Experimental animals and major reagents

The healthy male C57BL/6 mice reaching cleaning level, 8-10 weeks old, weighing 20-25 g, were purchased from Shanghai Slac Laboratory Animal Company. Lipopolysaccharide (LPS, 055:B5) was purchased from Sigma Company in USA, ORI from American Sigma Company, Trizol reagent from Ambion Life Technologies in USA, and reverse transcription kits, PrimeScript 1st Strand cDNA Synthesis Kit from TaKaRa in Japan. Protein quantification of bicinchoninic acid (BCA) kits were purchased from Pierce, IL in USA, and enzyme-linked immunosorbent assay test kits of TNF-α and IL-6 were purchased from Wuhan Boster Company.

LPS-induced ARDS mice models

Mice were anesthetized with 2.5% sodium pentobarbital (40 mg/kg) by intraperitoneal injection, and the limbs and heads were fixed in an operation table. Their tongues were pulled open with tweezers to expose the glottis, inhalation syringes with LPS (2 mg/ml, 50 µL) were placed in the glottis, and LPS was sprayed into the trachea swiftly. If small blisters sound was heard, it meant that the liquid was correctly sprayed into the lung and the model establishment succeeded. Equal doses of sterile PBS were given to the sham operation group with the same approach. Different doses of ORI were injected into the mice in the treatment group by intraperitoneal injection after model establishments, while the control group was assigned with equal doses of PBS. After 12 hours, the mice were sacrificed by broken neck execution after certain amount of blood extracted from their eye balls, then bronchoalveolar lavage (BAL) was conducted and lung tissues were collected for further detection.

Bronchoalveolar lavage and measurement of protein

After the mice were executed, their necks and chests were cut open, and the tracheas and the left lobes were ligated. Then puncture needle was inserted into the top of the trachea, which was rinsed with 0.4 mL PBS for 3-5 times. The lavage obtained was centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was collected and placed at -20°C for further test. The protein concentration was determined by BCA, and the operational steps were carried out in strict accordance with the instructions of BCA Protein Assay Kit.

Measurement of the lung W/D ratio

After the mice were executed, their chests were cut open immediately and its lungs were collected. Filter paper was used to dry the lung surface, and the wet lung tissues were weighed. Then the lung was placed to an 80°C oven, dried to a constant weight, and the dry weight was weighed. At last, the lung W/D ratio was calculated to assess the severity of pulmonary edema.

Fluorescent quantitative PCR assay

The total RNA was extracted from fresh lung tissues by Trizol and the cDNA was obtained by Takara reverse transcription kit. Fluorescent quantitative PCR assay was performed by ABI PRISM 7500 FQ-PCR fluorescent quantitative instrument (Applied Biosystems, USA) with glyceraldehyde triphosphate dehydrogenase (GAPDH) as the internal reference. Primers were synthesized by Shanghai Sangon Company. The specific primers are shown in Table 1. The conditions of amplification were as follows: Pre-denaturation at 95°C for 20 s, followed by 95°C for 3 s, 60°C for 30 s for 40
Effects and mechanisms of oridonin

**Table 1.** Specific primers (5'-3')

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<thead>
<tr>
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<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>IL-6</td>
<td>CTCTGGGAAATCGTGGAAAT</td>
<td>CCAGTTTGGTAGCATCCATC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TCTCTTCAAGGGACAAGGCT</td>
<td>GGCAGAAGAGGTTGACTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AACTTTGCGATGTGGAAGG</td>
<td>ACACATTGGGGGTAGGAACA</td>
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**Pathological observation of lung tissues**

After the mice were executed, the lung tissues were collected, and fixed at 10% paraformaldehyde for 48 hours. The pathological changes were observed in each lung tissue under optical microscope after regular dehydration, embedding, and HE staining.

**Protein expression of NF-κB p65/p-p65, Iκ-Bα/p-Iκ-Bα by western blot**

After the lung tissues of mice in each group were obtained, they were split respectively, the supernatant was collected, and the protein concentration was detected by BCA. A total of 60 µg mixed protein sample was added in each electrophoresis channel to conduct polyacrylamide gel electrophoresis and transfer membrane. Primary antibodies (NF-κB p65/p-p65, Iκ-Bα/p-Iκ-Bα, β-actin, rabbit polyclonal antibody, dilution: 1:500) and secondary antibody (goat anti-rabbit monoclonal antibody, dilution: 1:5000) were added successively to conduct hybridization. Gel imaging system (UVP Company) was applied to scan and observe the results.

**Statistical methods**

The measurement data was expressed as mean and standard deviation, and analyzed by SPSS17.0. The data of the two groups were compared with by one-way ANOVA. P<0.05 was considered as statistically significant.

**Results**

**Lung W/D ratio of mice in different groups**

After the establishment of mice models with LPS, the lung W/D ratio increased significantly and showed marked difference, which is shown in Figure 1 (P<0.001). ORI could tremendously decrease the lung W/D ratio of ARDS mice, and the decrease became more distinct as the dose increased. When the dose reached 150 mg/kg, the difference had statistical significance (P<0.05), but the lung W/D ratio did not keep decreasing when the dose increased continuously.

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*Figure 1. Detection of lung wet-to-dry W/D ratio in mice of different groups, *P<0.05, ***P<0.001.

*Figure 2. Quantitative detection of protein in BALF in each group, *P<0.05, ***P<0.001.*
Effects and mechanisms of oridonin

Quantitative detection of the protein in bronchoalveolar lavage fluid (BALF) of mice in different groups

Compared with the control group, protein in BALF significantly increased after the establishment of ARDS mice models, the difference was statistically significant (Figure 2, P<0.001). ORI could reduce the protein content in lung tissues of ARDS mice. Similarly, the reduction became more distinct as the dose increased. When the dose reached 150 mg/kg, the difference was marked and statistically significant (P<0.05), which showed that ORI could remarkably relieve lung injuries caused by LPS. However, the protein content did not show further reduction when the dose increased to 300 mg/kg, which indicated that 150 mg/kg ORI was the appropriate dose for the treatment of LPS-induced ARDS mice (Figure 2).

Pathological changes of lung tissues

In the control group, the lung tissues (Figure 3A) had a clear and complete structure, with no infiltration into inflammatory cells or interstitial congestion, the alveolar wall was thin and no edema was presented. The lung tissues in ARDS modeling group were infiltrated into a lot of inflammatory cells (Figure 3B), the alveolar septum was widened greatly, alveolar wall was destroyed, and the infiltration of red blood cells and mononuclear macrophages was visible in alveolar cavity. After the treatment of lung tissues with ORI (Figure 3C-F), the pathological changes gradually relieved with the increasing dose, the edema alleviated, and the infiltration of inflammatory cells gradually reduced. When the dose of ORI arrived at 150 mg/kg, the injuries in lung tissues improved significantly, which indicated that 150 mg/kg was the appropriate dose for the treatment of ARDS mice.

Q-PCR detection of changes in inflammatory factors’ expression

To determine the influence of ORI in the expression of inflammatory factors on lung tissues, the appropriate dose (150 mg/kg) of ORI was applied. We found that ORI could reduce the expression of IL-6 and TNF-α in lung tissues by q-PCR; the difference had statistical significance (P<0.05). The result is shown in Figure 4.

Detection of changes of inflammatory factors in serum by ELISA

The production of a large number of inflammatory factors is crucial in the occurrence and
Effects and mechanisms of oridonin

development of ARDS. To study the influence of ORI on the production of inflammatory factors, we used ELISA to detect the levels of inflammatory factors in the serum of ARDS mice, and found that IL-6 and TNF-α in the ORI-treated group were markedly reduced; the difference was statistically significant (Figure 5, P<0.05).

Detection of different levels of expression of NF-κB p65/p-p65 and Iκ-Bα/p-Iκ-Bα by western blot

In a further study of mechanisms, we used western blot to analyze the production of NF-κB-related proteins. As shown in Figure 6, ORI could reduce the production of NF-κB p65/p-p65, increase Iκ-Bα greatly, and also slightly increase p-Iκ-Bα, which indicated that ORI could decrease the production of inflammatory factors by inhibiting the signaling pathway of NF-κB, thereby protecting the LPS-induced ARDS.

Discussion

ARDS is a kind of acute and progressive respiratory failure caused by risk factors inside and outside the lung which is not cardiogenic, and it's a pulmonary inflammatory responses characterized by the increasing permeability of alveolar-capillaries. As a common clinical severe disease, it brings great harms to patients, and it is difficult to be cured completely, especially for the aged patients; the mortality is as high as 60% [12]. Therefore, further study of its pathogenesis and searching for better medications and treatments are of great clinical significance. In recent years, increasing Chinese medicines were applied to various kinds of clinical diseases. ORI, an active ingredient extracted from traditional Chinese medicine, has many pharmacological activities such as anti-inflammatory, anti-tumor functions and neuroprotection [8, 9, 13]; thus it is of great significance in clinical applications. The previous researches indicated that ORI showed great effects in vari-
Effects and mechanisms of oridonin

LPS is an important risk factor that induced sepsis and ARDS. This research used a classical model, LPS-induced ARDS mice. After being administered with LPS, not only direct injuries of pulmonary microvascular were initiated in mice, but also the imbalance between anti-inflammatory and inflammatory responses was induced, leading to the accumulation of various kinds of inflammatory cells and factors [15, 16], which further induced lung injuries and caused liquid leak and the increase of permeability in pulmonary capillary endothelial cells [17]. In this study, ORI could reduce the lung W/D ratio of ARDS mice and the protein content in bronchoalveolar lavage fluid, which indicated that the injuries of pulmonary vessels and the liquid leak were alleviated, and the edema was reduced greatly. In the course of ARDS, the production of a large number of inflammatory factors in lung played a crucial role. In the occurrence and development of ARDS, TNF-α and IL-6, as vital biomarkers, could predict the morbidity and mortality of mice [18]. TNF-α is one of the earliest inflammatory factors that have been released on the onset of ARDS. It could not only cause direct injuries in pulmonary vascular endothelial cells, but also regulate the chemotaxis of polymorphonuclear cells (PMN) and promote the generation of reactive oxygen species in PMN [19]. As an important inflammatory factor, IL-6 significantly increases in the burn, major surgery, sepsis and other acute injuries [20]. IL-6 signaling pathway is involved in the occurrence and development of lung injuries, and can be used as an indicator of the severity of ARDS patients [20, 21]. In this study, the expression of IL-6 and TNF-α significantly decreased and the release of inflammatory factors in serum detected by ELISA also reduced after the application of ORI. Besides, it was observed from pathological sections that the edema got relieved, the infiltration of inflammatory reduced, and the lung injuries of ARDS alleviated obviously. Some researchers found that ORI could inhibit the inhibition of NF-κB in liver cancer cells and macrophages [22]. Besides, this research also found that ORI could inhibit the production and activation of NF-κB when acute lung injuries occurred, and significantly increase the amount of the inhibitor, IκB. It showed that ORI could reduce the production of inflammatory factors and relieve the inflammatory responses in lung tissues possibly by blocking the signaling pathway of NF-κB, so as to play a protective effect on mice with ARDS.

In conclusion, this study found that ORI could relieve inflammatory responses by inhibiting the signaling pathway of NF-κB and had obvious protective effects on ARDS lung. Therefore, there is positive clinical significance and practical value in the development and utilization of ORI.

Disclosure of conflict of interest

None.

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Effects and mechanisms of oridonin


