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Usnic acid protects LPS-induced acute lung injury in mice through attenuating inflammatory responses and oxidative stress

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ABSTRACT

Usnic acid is a dibenzofuran derivative found in several lichen species, which has been shown to possess several activities, including antiviral, antibiotic, antitumoral, antipyretic, analgesic, antioxidative and anti-inflammatory activities. However, there were few reports on the effects of usnic acid on LPS-induced acute lung injury (ALI). The aim of our study was to explore the effect and possible mechanism of usnic acid on LPS-induced lung injury. In the present study, we found that pretreatment with usnic acid significantly improved survival rate, pulmonary edema. In the meantime, protein content and the number of inflammatory cells in bronchoalveolar lavage fluid (BALF) significantly decreased, and the levels of MPO, MDA, and H_2O_2 in lung tissue were markedly suppressed after treatment with usnic acid. Meanwhile, the activities of SOD and GSH in lung tissue significantly increased after treatment with usnic acid. Additionally, to evaluate the anti-inflammatory activity of usnic acid, the expression of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and anti-inflammatory cytokine IL-10, and chemokines interleukin-8 (IL-8) and macrophage inflammatory protein-2 (MIP-2) in BALF were studied. The results in the present study indicated that usnic acid attenuated the expression of TNF-α, IL-6, IL-8 and MIP-2. Meanwhile, the improved level of IL-10 in BALF was observed. In conclusion, these data showed that the protective effect of usnic acid on LPS-induced ALI in mice might relate to the suppression of excessive inflammatory responses and oxidative stress in lung tissue. Thus, it was suggested that usnic acid might be a potential therapeutic agent for ALI.

1. Introduction

Acute lung injury (ALI), especially its more severe form, acute respiratory distress syndrome (ARDS), is characterized by severe hypoxemia, pulmonary edema, and neutrophil accumulation in the lung. Many factors could contribute to ALI, such as severe sepsis, trauma, shock, burns, and inhaling harmful gas [1,2]. Despite technical development and advanced supportive treatment in intensive care units, ALI/ARDS takes responsibility for a high mortality rate of 30% to 40% [3]. Lipopolysaccharide (LPS) is the primary component of outer membrane of Gram-negative bacteria and the predominant inducer of inflammatory responses to these bacteria [4]. Intratracheal administration of LPS into lung has been extensively accepted as an ideal pharmacological research model of ALI characterized by increased release of reactive oxygen species, neutrophil infiltration, protein content, inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and interleukin-10 (IL-10), in the bronchoalveolar lavage fluid (BALF) [5].

Usnic acid (D-2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3 (2H, 9bH)-dibenzofurandione, UA) is a naturally occurring dibenzofuran derivative found in several lichen species, including *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae), *Lecanora* (Lecanoraceae), *Ramalina* (Ramalinaceae), *Evernia*, *Parmelia* (Parmeliaceae) and other lichen genera. It was first isolated by German scientist W. Knop in 1844 and first synthesized by Curd and Robertson in 1933 [6,7]. Growing evidences have shown that UA possessed significant antiviral, antibiotic, antiprotozoal, antitumoral, antipyretic, analgesic, antioxidative and anti-inflammatory activities [8,9]. In indomethacin-induced gastric ulcers model, usnic acid was found to significantly increase the activities of superoxide dismutase (SOD) and glutathione (GSH), and also decrease the levels of myeloperoxidase (MPO) and malondialdehyde (MDA) in gastric tissues [9]. Nuclear factor kβ (NF-κB) is a nuclear transcription factor that regulates the expression of many genes encoding inflammatory cytokines and chemokines including TNF-α, IL-6, IL-8,

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IL-10 and MIP-2, and the balance between pro-inflammatory cytokines TNF-α, IL-6 and anti-inflammatory cytokine IL-10 is crucial to the outcome of inflammatory response [10,11]. Previous studies demonstrated that UA significantly reduced TNF-α and IL-6 expression in LPS-challenged RAW 264.7 cells. Meanwhile, UA could significantly increase the expression of anti-inflammatory cytokines, such as IL-10 and HO-1 [12]. These studies concluded that UA was a potentially effective antioxidative and anti-inflammatory drug. In the clinical cases, despite great progress in the treatment of ALI was observed in the past forty years, there are still few effective medicines applied for the treatment of ALI. ALI still maintains a significantly high mortality rate. Therefore the development of novel therapies for ALI is urgently needed. In this study, we explored whether UA could exhibit protective effects in ALI stimulated by intratracheal instillation of LPS.

2. Materials and methods

2.1. Animals and materials

Male Kunming (KM) mice, weighing approximately 18 to 22 g, were obtained from the Medical Laboratory Animal Center of Guangdong Province, China (Certificate No. SCXK2008-0002). The animals were kept on a 12-hour light/12-hour dark cycle under laboratory temperature (24 ± 1 °C) and relative humidity (50 ± 10%) and received food and water ad libitum. All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine.

2.2. Chemicals

Usnic acid (HPLC, purity: >98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China) (Fig. 1). Dexamethasone (DEX; purity: >99.6%) was purchased from the Guangdong Huanan Pharmaceutical Group Co., Ltd (Dongguan, Guangdong, China). The myeloperoxidase (MPO), glutathione (GSH), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), mouse interleukin-8 (IL-8) and macrophage inflammatory protein-2 (MIP-2) enzyme-linked immunosorbent assay kits and BCA protein assay kits were provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Mouse TNF-α, IL-6 and IL-10 enzyme-linked immunosorbent assay kits were obtained from eBioscience (CA, USA). LPS (*Escherichia coli* O111:B4) was provided by the Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China).

2.3. Experimental design

To evaluate mortality rate, mice were randomly divided into five groups: control group; LPS + UA group (25, 50 or 100 mg/kg); and LPS group. In the LPS + UA group, UA (25, 50 or 100 mg/kg body weight, p.o.) was given once a day for 5 consecutive days, while the control and LPS group were given an equal volume of 0.5% Tween-80. One hour after the last administration, all animals were anesthetized by 3% chloral hydrate. And mice from the LPS group and LPS + UA groups received intratracheal administration of LPS at 20 mg/kg, while mice from the control group were given an equal volume of phosphate buffered saline (PBS). The survival rate of mice was checked twice every day for 6 days after LPS stimulation.

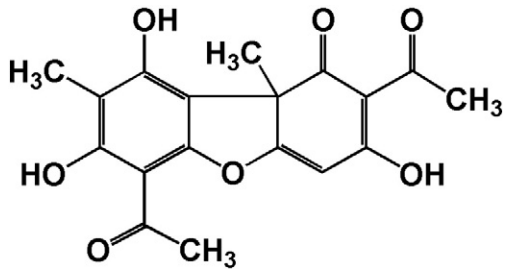

Fig. 1. Structure of usnic acid.

In the other experiments, mice were also randomly divided into six groups: control group; LPS group; LPS + UA group (50 or 100 mg/kg); and LPS + DEX (5 mg/kg) group. In the UA + LPS and DEX + LPS groups, mice were administrated with UA (50 or 100 mg/kg, p.o.) or DEX (5 mg/kg, p.o.) once a day for 5 consecutive days, 1 h after the last administration. Meanwhile mice from the control and LPS groups were given an equal volume of 0.5% Tween-80 instead of UA and DEX. After 1 h, mice from LPS, LPS + UA and LPS + DEX groups were anesthetized by 3% chloral hydrate before being received intratracheal administration of 5 mg/kg LPS to cause ALI. And mice from the control group received an equal volume of PBS (20 μL/10 g body weight). The mice were humanely sacrificed 24 h after LPS stimulation. Lung tissues were collected for the analysis of the W/D ratio and oxidation index. And the lungs from other mice were lavaged with 1.5 mL ice-cold PBS three times, the recovery ratio of the fluid was about 90%. The bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 800 ×*g* for 10 min at 4 °C, and the supernatant was collected and frozen at −80 °C for protein concentration analyses, cytokine and chemokines analyses [1].

2.4. Lung wet-to-dry weight ratio

After the mice were killed humanely, lung tissues were excised and immediately weighed to record the "wet" weight and then were heated at 80 °C for 48 h to obtain the "dry" weight. The ratio of wet to dry weight is important for the assessment of the lung edema [13].

2.5. Histological study

Histopathological evaluation of lung tissues was performed on mice which were not subject to BALF collection. The lung tissues were removed and fixed with 4% paraformaldehyde and imbedded in paraffin. After deparaffinization and dehydration, the lung tissues were cut into 5 μm sections and stained with hematoxylin and eosin subsequently. The severity of lung injury was scored by a blinded observer according to lung pathological changes, including infiltration or aggregation of neutrophils in air spaces or vessel walls; alveolar congestion; hemorrhage and thickness of alveolar wall/hyaline membrane formation. Each indicator was graded according to a five-point scale: 0 = minimal damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage and 4 = maximal damage [14,15]. Thus, the severity of lung injury was evaluated according to the sum of the four criteria.

2.6. Inflammatory cell counts of in BALF

The mice were humanely sacrificed 24 h after LPS stimulation. BALF was collected and centrifuged (800 ×*g*, 10 min, 4 °C) to pellet the cells. The resulting pellet were resuspended in PBS for the total cell counts via hemacytometry, and cytospins were prepared for differential cell counts by a Wright-Giemsa stained kit (Jiancheng Company, Nanjing, China) [5].

2.7. MPO activity assay

The activity of MPO enzyme as an important index of neutrophil infiltration would increase in the lung tissue after LPS challenge. In the present study, the lung tissue samples were homogenized for further measurement according to the manufacturer's instructions (Jiancheng