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Protective effects of Isofraxidin against lipopolysaccharide-induced acute lung injury in mice

Xiaofeng Niu, Yu Wang, Weifeng Li *, Qingli Mu, Huani Li, Huan Yao, Hailin Zhang

School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, PR China

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ABSTRACT

Acute lung injury (ALI) is a life-threatening disease characterized by serious lung inflammation and increased capillary permeability, which presents a high mortality worldwide. Isofraxidin (IF), a Coumarin compound isolated from the natural medicinal plants such as *Sarcandra glabra* and *Acanthopanax senticosus*, has been reported to have definite anti-bacterial, anti-oxidant, and anti-inflammatory activities. However, the effects of IF against lipopolysaccharide-induced ALI have not been clarified. The aim of the present study is to explore the protective effects and potential mechanism of IF against LPS-induced ALI in mice. In this study, We found that pretreatment with IF significantly lowered LPS-induced mortality and lung wet-to-dry weight (W/D) ratio and reduced the levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and prostaglandin E_2 (PGE_2) in serum and bronchoalveolar lavage fluid (BALF). We also found that total cells, neutrophils and macrophages in BALF, MPO activity in lung tissues were markedly decreased. Besides, IF obviously inhibited lung histopathological changes and cyclooxygenase-2 (COX-2) protein expression. These results suggest that IF has a protective effect against LPS-induced ALI, and the protective effect of IF seems to result from the inhibition of COX-2 protein expression in the lung, which regulates the production of PGE_2.

1. Introduction

Inflammation is a self-defensive immune process in which the body reacts to external stimuli, infection, or injury [1]. The development of inflammation is associated with the production of inflammatory cytokines by neutrophils and macrophages, tissue edema caused by the leakage of fluid and proteins, and the infiltration of leucocytes at the site of inflammation [2,3]. Acute lung injury (ALI), a common inflammatory disease in the clinic, shows the characteristics of interstitial edema, neutrophil recruitment, disruption of epithelial integrity, and lung parenchymal injury [4]. Despite significant advances in clinical research and therapeutic trials made in the past several decades, ALI still presents a high mortality in the diseases of shock, sepsis, ischemia reperfusion and viral pneumonia [5]. Therefore, it is extremely urgent to discover effective drugs and clinical therapies for ALI.

Lipopolysaccharide (LPS) is widely used for induction of animal models of ALI for its similar characteristics of human ALI [6]. Ingestion of LPS stimulates vascular permeability, promotes inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) from blood into lung tissues and activates numerous inflammatory cells such as neutrophils and macrophages [7]. In macrophages, LPS challenge induces the transcription of gene encoding pro-inflammatory protein, which leads to cytokine release and synthesis of enzymes, such as cyclooxygenase-2 (COX-2) [8]. COX-2 usually can't be found in normal tissues, but widely induced by pro-inflammatory stimuli, such as cytokines, endotoxins, and growth factors [9]. COX-2 plays a vital role in the regulation of inflammatory process by modulating the production of prostaglandin E_2 (PGE_2). PGE_2, induced by cytokines and other initiator, is an inflammatory mediator which is produced in the regulation of COX-2. Previous researches demonstrated that inhibition of COX-2 produced a dramatically anti-inflammatory effect with little gastrointestinal toxicity [10]. Therefore, inhibition of COX-2 protein expression has far-reaching significance in the treatment of ALI.

Isofraxidin (IF, 7-hydroxy-6,8-dimethoxycoumarin, shown in Fig. 1) is a Coumarin compound that widely exists in natural plants such as *Sarcandra glabra* and *Acanthopanax senticosus*, both of which are traditional Chinese herbs usually used for anti-tumor, anti-bacterial, anti-oxidant, and anti-inflammatory treatments [11]. IF is a bioactive component with definite anti-bacterial and

* Corresponding author at: School of Pharmacy, Xi'an Jiaotong University, No. 76 Western Yanta Road, Xi'an City, Shaanxi Province 710061, PR China. Tel.: +86 29 82655139; fax: +86 29 82655138.
E-mail addresses: niuxf@mail.xjtu.edu.cn (X. Niu), liwf@mail.xjtu.edu.cn (W. Li).

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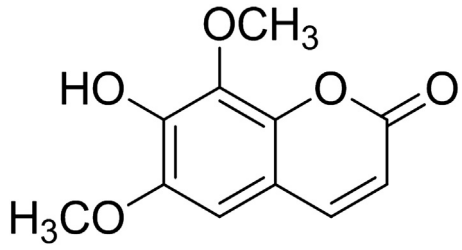

Fig. 1. Chemical structure of Isofraxidin.

anti-oxidant activities [12]. The previous studies have also shown that IF possesses anti-inflammatory activity [13,14]. However, there is no report about the protective effects of IF on LPS-induced ALI. The purpose of the present study is to explore the protective effects of IF against LPS-induced ALI in mice.

2. Materials and methods

2.1. Reagents

IF (purity ≥ 98%) was purchased from Xi'an Honson Biotechnology Co., Ltd. (Xi'an, China) and confirmed by the Pharmacognosy Laboratory, School of Medicine, Xi'an Jiaotong University (Xi'an, China). Dexamethasone (DEX), as a positive control, was supplied by Xi'an Lijun Pharmaceutical Company Limited (Shanxi, China). LPS (*Escherichia coli* serotype O55:B5) was obtained from Sigma (St. Louis, MO). The enzyme linked immunosorbent assay (ELISA) kit for mouse TNF-α, IL-6, and PGE_2 was purchased from R&D Systems (Minneapolis, MN, USA). The kit for biochemical analysis of myeloperoxidase (MPO) was provided by Jiancheng Bioengineering Institute (Nanjing, China). Histostain-Plus kits, DAB (3, 3′-diaminobenzidine) staining kit and rabbit anti-cyclooxygenase-2 were supplied by Beijing Biosynthesis Biotechnology Co., Ltd. RPMI-1640 was purchased from Gibco (Gibco-BRL, Gaithersburg, MD, USA). Antibody against COX-2 (UniProt: P35354) were supplied by Epitomics Inc. (an Abcam Company, U.S.A.). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membranes were provided by Pall Gelman Laboratory (Ann Arbor, MI, USA). All other reagents used in the study were of analytical grade.

2.2. Animals

All male Kunming mice (22–25 g) were supplied by the Experimental Animal Center, Xi'an Jiaotong University (Xi'an, China). Animals were raised under standard conditions with a 12 h day/night cycle and acclimatized to their environment for at least one week before the beginning of animal experiments. All animal experiments were performed in line with the National Institute of Health guidelines.

2.3. Mouse model of LPS-induced ALI

The mice were divided into six groups randomly (n = 12/group): Control group; LPS group (LPS, 5 mg/kg, i.p.); LPS + DEX group (5 mg/kg, i.p.); LPS + IF groups (5, 10, 15 mg/kg, i.p.). The doses and administration route of IF and DEX were confirmed based on preliminary tests. IF or DEX were administrated intraperitoneally 1 h prior to LPS challenge. The control and LPS groups were given an equal volume of vehicle according to experimental design. The severity of LPS-induced pulmonary injury was assessed at 6 h after LPS administration. Blood samples were collected from the retro-orbital plexus, and then all mice were euthanized. Bronchoalveolar lavage fluid (BALF) was performed through the left lungs. The superior lobe of the right lung was removed for histopathological analysis and immunohistochemical study. The middle lobe of the right lung was removed for the evaluation of lung wet-to-dry weight (W/D) ratio. The lower lobe of the right lung was removed and rapidly cut into two parts. One part of the lower lobe was used for MPO analysis, and the other part was used for Western blot analysis.

2.4. LPS-induced mortality in ALI mice

The normal control group and the negative control group were given an equal volume of vehicle according to experimental design. All other groups received intraperitoneal injection of IF (5, 10, 15 mg/kg) or DEX (5 mg/kg). An hour later, mice received intraperitoneal injection of LPS (20 mg/kg). Within 72 h after LPS injection, the mortality of mice was observed every 12 h in each group (n = 12/group).

2.5. Lung W/D ratio

After the mice were euthanized by cervical dislocation, the middle lobe of the right lungs were excised and weighed to record the "wet" weight. Then, the lung tissues were arranged in an oven at 80 °C for 48 h to obtain the stable "dry" weight. The lung W/D ratios were computed to assess the severity of pulmonary edema.

2.6. Collection of serum and BALF

Six hours after LPS administration, blood samples were collected from the retro-orbital plexus and immediately centrifuged at 3000 rpm for 10 min at 4 °C to obtained the serum. The left lung was lavaged through a tracheal cannula with 1.5 mL autoclaved phosphate buffered saline (PBS) three times in each group. The recovery rate of BALF was better than 90%. BALF was immediately centrifuged at 1500 rpm for 10 min for pelleting the cells.

2.7. Measurement of the levels of TNF-α, IL-6, and PGE_2 in serum and BALF

The BALF was centrifuged at 1500 rpm for 10 min at 4 °C, and the supernatant of BALF was used for determining the concentrations of TNF-α, IL-6, and PGE_2. The levels of TNF-α, IL-6, and PGE_2 in serum and BALF were evaluated by mouse ELISA kits according to the manufacturer's directions. The absorbance was read at 450 nm and the samples were detected three times.

2.8. Inflammatory cell counts of BALF

The BALF were centrifuged at 1500 rpm for 10 min at 4 °C to pellet the cells and the cell pellets were resuspended in PBS. A hemacytometer was used for the total cell counts and cytospins were essential for different cell counts via staining with the Wright–Giemsa staining method.

2.9. MPO activity in the lung tissues

MPO activity is an important index of neutrophils accumulation in inflammatory tissues. After the lungs were removed, one part of the lower lobe were homogenized in PBS by a homogenizer. The mixtures were centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatants were used for MPO analysis. MPO activity were measured with an MPO activity kit based on the manufacturer's instructions. MPO activities were expressed as units per gram of protein.

2.10. Histopathological study of the lung tissues

LPS-induced ALI mice possess the characteristics of congestion, edema, and the infiltration of inflammatory cells. To assess the lung