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Osthole protects lipopolysaccharide-induced acute lung injury in mice by preventing down-regulation of angiotensin-converting enzyme 2

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ABSTRACT

The renin–angiotensin–aldosterone system (RAAS) plays an important role in the pathogenesis of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Angiotensin converting enzyme 2 (ACE2) plays a protective role in acute lung injury. Osthole, a natural coumarin derivative extracted from traditional Chinese medicines, is known to have anti-inflammatory effect, but the effect of osthole on the ALI is largely unknown. The aim of this study is to explore whether and by what mechanisms osthole protects lipopolysaccharide(LPS)-induced acute lung injury. Herein, we found that osthole had a beneficial effect on LPS-induced ALI in mice. As revealed by survival study, pretreatment with high doses of osthole reduced the mortality of mice from ALI. Osthole pretreatment significantly improved LPS-induced lung pathological changes, reduced lung wet/dry weight ratios and total protein in BALF. Osthole also inhibited the release of inflammatory mediators TNF-α and IL-6. Meanwhile, osthole markedly prevented the loss of ACE2 and Ang1-7 in lung tissue of ALI mice. ACE2 inhibitor blocked the protective effect of osthole in NR 8383 cell lines. Taken together, our study showed that osthole improved survival rate and attenuated LPS-induced ALI and ACE2 may play a role in it.

1. Introduction

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), despite intensive care, take responsibility for significant morbidity and is associated with an up to 35% mortality rate (Meade et al., 2008; Rubenfeld et al., 2005). Lipopolysaccharide (LPS), as a component of the cell walls of gram-negative bacteria, has been recognized as a main component in the pulmonary inflammation and sepsis leading to ALI or ARDS (Brigham and Meyrick, 1986; Rubenfeld et al., 2005), the interactions among cellular and soluble mediators responsible for ALI/ARDS are complex and incompletely understood.

Osthole (7-methoxy-8-isopentenoxycoumarin) is a natural coumarin isolated from the fruit of *Cnidium monnieri* (L.) Cusson, a Chinese herb which has a variety of pharmacological, biological, and therapeutic uses (Hoult and Paya, 1996; Teng et al., 1994), has been showed a inhibitory effect on the release of inflammatory mediators (Nakamura et al., 2009). However, whether osthole exhibited a protective effect on ALI is unknown. Studies have shown that the renin–angiotensin system (RAS) has a crucial role in severe acute lung injury. The angiotensin converting enzyme 2 (ACE2) counterbalances the function of angiotensin converting enzyme (ACE) and negatively regulates angiotensin II (AngII) production (Imai et al., 2005), and it is also a key negative regulatory factor for severity of lung edema and acute lung failure (Kuba et al., 2005). Therefore, we hypothesized that osthole might also protect lung injury, and the effect is associated with ACE2. In the present study, the anti-inflammatory effect of osthole was investigated in a mice acute lung injury (ALI) model. This study provided new insights into the mechanisms underlying the osthole's lung protective effects.

2. Materials and methods

2.1. Animals and materials

Adult male BALB/c mice (18–22 g) were obtained from the Animal Center of the Fourth Military Medical University (Xi'an, China). All studies were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Osthole was obtained from YiLe Bio-Tech laboratory (Xi'an, Shanxi, China) with

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HPLC purity >98%. LPS (*Escherichia* coli lipopolysaccharide, 055:B5) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits of TNF-a, IL-6 and angiotensin1-7 (Ang(1-7)) were purchased from R&D Corporation (R&D Systems Inc., MN, USA). DX-600 (ACE2 inhibitor) was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). WST-1 reagent was purchased from Roche Diagnostics (Indianapolis, IN, USA). Anti-ACE2 polyclonal antibody was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA) and anti-β-actin monoclonal antibody was purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Animal grouping

Mice were randomly divided into four groups, i.e., control (saline)-treated ($n = 12$), osthole-treated ($n = 12$), LPS-treated ($n = 12$) and osthole + LPS treated ($n = 12$) groups. In the LPS-treated group, mice received an intratracheal instillation of LPS (5 mg/kg). In the control or osthole-treated group, animals received a gavage of saline or osthole. In the osthole + LPS treaded group, osthole (40 mg/kg) was given to mice by gavage for four times in four consecutive days. LPS was administered 1 h after the last dose of osthole. Mice were sacrificed 6 h after LPS administration.

2.3. Survival studies

For the assessment of mortality rates, mice were given 50 mg/kg LPS with or without different doses of osthole (20 or 40 mg/kg body weight) pretreatment by gavage for four times in four consecutive days. The mortality of mice was recorded every 6 h for 72 h after the LPS injection in each treated group. Experiments were performed with littermate mice and each group contains 20 animals.

2.4. Preparation of BALF and measurements

At the 6 h after LPS administration, mice were anesthetized with intraperitoneal pentobarbital. The lungs were lavaged with 1 ml ice-cold phosphate buffered saline five times in all groups. The recovery ratio of the fluid was about 90%. The collected BAL fluid was centrifuged at 520 g for 20 min at 4 °C and the supernatant was frozen at −80 °C for subsequent protein study. BAL protein concentration was measured by Lowry's method with bovine serum albumin as a standard.

2.5. Lung histology and immunohistochemistry

At the end of the experiments, the lung tissues in all groups were removed and fixed with 4% paraformaldehyde for 24 h. The tissues were embedded in paraffin and cut into 5 μm sections. Hematoxylin-eosin stains were performed using standard protocol. For immunostainings, sections were deparaffinized, rehydrated in graded alcohols, and blocked by incubating in 0.3% H_2O_2 for 30 min. Antigen retrieval was performed by treating the slides in citrate buffer in a microwave oven for 10 min. The slides were incubated for 1 h with normal goat serum, and then incubated in a moist chamber with anti-ACE2 polyclonal antibody at 4 °C overnight. After a complete wash in phosphate buffered saline (PBS), the tissues were incubated in biotin-labeled goat anti-mouse anti-body for 30 min at 37 °C, rinsed with PBS, and incubated with avidin-biotin peroxidase complex for 30 min at 37 °C. The signal was detected using diaminobenzidine (DAB).

2.6. Lung wet/dry ratios

To quantify the magnitude of pulmonary edema, we evaluated the wet to dry ratios. At the end of the experiments, the body of all groups was weighed. The pulmo sinister were excised and the wet weight was recorded. Then the dry weight was obtained after the lobus were dried in a drying oven at 70 °C until a stable dry weight was achieved after 72 h. The wet/dry ratios were then calculated.

2.7. Quantitative real-time PCR

Total RNA was isolated and real-time PCR was performed as previously (Nosotti et al., 2005). The primers for ACE2 (189 bp) were (forward) 5′-AGGATGTCTCGGGGCCGCAT-3′ and (reverse) 5′-ACCTTTGATGCCAGTGACAATCAGG-3′, and for β-actin (266 bp) were (forward) 5′-GTCCCTCACCCTCCCAAAAG-3′ and (reverse) 5′-GCTGCCTCAACACCTCAACCC-3′, respectively.

2.8. Western blotting

Tissue samples were homogenized and the proteins were extracted according to instructions of Total Protein Extraction Kit. Protein concentrations were determined by BCA protein assay kit. Samples were separated on a denaturing 10%SDS – polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with Tris buffered saline (TBS) containing 5% non-fat dry milk at room temperature for 2 h, followed by incubation with mouse polyclonal antibodies against ACE2 (1:200) and β-actin (1:5000) overnight. The secondary antibody (anti-rabbit or anti-mouse IgG peroxidase conjugated, 1:5000) was incubated. Detection was performed using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA).

2.9. Cell viability assay

The rat lung-derived macrophages, NR 8383 were maintained in a 96-well plate (Ham's F12 medium with 15% fetal calf serum). Cells were then incubated in serum-free medium with osthole (50 μg/ml) in the presence or absence of ACE2 inhibitor DX-600 (100 nM) for 4 h, followed by incubation with LPS (1 μg/ml) for 8 h. After 8 h, 10 μl/well WST-1 was added to the 96-well plate, which was then incubated for 4 h. The plate was agitated thoroughly for 1 min on a shaker prior to measurement. The absorbance was measured at 450 nm wavelength (reference wavelength 630 nm) by using a spectrophotometer (Power Wave XS, BioTek Inc, Vermont, USA).

2.10. Elisa

The concentrations of Ang(1-7) in lung tissue and the levels of TNF-α and IL-6 in BALF and supernatant of cultured cells were measured using ELISA kits according to the manufacturer's instructions.

2.11. Statistical analysis

Data are expressed as mean ± S.E.M. and statistical analysis was performed with analysis of variance (ANOVA). Survival data were presented by the Kaplan Meier method and comparisons were made by the log rank test. A statistical difference was accepted as significant if $P < 0.05$.

3. Results

3.1. Effect of osthole on LPS-induced mortality in mice

To evaluate the protective effect of osthole on mice with endotoxemia, osthole (20 or 40 mg/kg body weight) was administrated