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Daidzein attenuates lipopolysaccharide-induced acute lung injury via toll-like receptor 4/NF-kappaB pathway

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ABSTRACT

Daidzein, a diphenolic isoflavone from many plants and herbs, has been reported to have anti-inflammatory properties. However, the effects of daidzein on lipopolysaccharide (LPS)-induced acute lung injury have not been determined. The aim of this study was to detect the effects of daidzein on LPS-induced acute lung injury and investigate the molecular mechanisms. Daidzein was intraperitoneally injected (2, 4, 8 mg/kg) 30 min after intratracheal instillation of LPS (5 mg/kg) in rats. The results showed that daidzein treatment remarkably improved the pulmonary histology and decreased the lung wet/dry weight ratios. We also found that daidzein significantly inhibited LPS-induced increases of macrophages and neutrophils infiltration of lung tissues, as well as markedly attenuated MPO activity. Moreover, daidzein effectively reduced the inflammatory cytokines release and total protein in bronchoalveolar lavage fluids (BALF). Furthermore, daidzein significantly inhibited LPS-induced toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88) protein up-expressions and NF-κB activation in lung tissues. In vitro, daidzein obviously inhibited the expressions of TLR4 and MyD88 and the activation of NF-κB in LPS-stimulated A549 alveolar epithelial cells. In conclusion, these data indicate that the anti-inflammatory effects of daidzein against LPS-induced ALI may be due to its ability to inhibit TLR4-MyD88-NF-κB pathway and daidzein may be a potential therapeutic agent for LPS-induced ALI.

1. Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), which is the severest form of injury, are the leading causes of morbidity and mortality in critically ill patients [1,2]. ALI often occurs in various acute severe illnesses complicated by systemic inflammation. A variety of clinical syndromes, such as pneumonia, aspiration of gastric contents, sepsis, major trauma and acute pancreatitis, can induce the occurrence of ALI [3]. Among these, the most common pathological condition of ALI/ARDS is gram-negative bacteria-induced sepsis [4].

Lipopolysaccharide (LPS), the primary component of outer membrane of gram-negative bacteria, is regarded as the predominant microbial initiators of inflammatory responses and is responsible for the overwhelming innate immune responses in ALI patients [4,5]. Toll-like receptor 4 (TLR4) is a transmembrane protein and act as signal transduction molecule [6]. TLR4 signaling pathway plays a crucial role in the innate immune system as the first line of defense against pathogens [7]. TLR4 has been regarded as the main sensors for recognition of LPS and transmits its associated downstream regulators. In LPS-relevant ALI, LPS binds TLR4 on the surface of epithelial cells and activates NF-κB through a MyD88-dependent pathway that ultimately trigger an inflammatory response, resulting in acute lung injury [8]. Alveolar epithelial cells (AECs), which are typically the first cells challenged by pathogenic microorganisms [2], play important roles against bacterial infection and participate in the initiation and progression of acute lung injury [9]. LPS stimulation of AECs is a widely used model to simulate the LPS-induced ALI in vitro. Despite marked efforts, little therapeutic progress has been made, and the mortality rate of ALI/ARDS still remains high [3,10]. Therefore, the development of novel therapies for ALI is urgently needed.

Daidzein is a plant-derived diphenolic isoflavone found in a number of plants and herbs like *Trifolium pratense* and *Pueraria mirifica*, as well as in food sources such as soybeans [11,12]. In recent years, daidzein has been shown to exert various pharmacological properties such as anti-inflammation, anti-oxidant and anti-cancer. Previous research indicated that daidzein could inhibit LPS-induced NF-κB transcriptional activity in mouse macrophages and fibroblasts [13]. In another study, daidzein could significantly down-regulate LPS-induced NO and IL-6 production by inhibiting NF-κB and STAT1 pathway in RAW264.7 murine macrophages [14]. Recently, it has been reported that daidzein exerts anti-inflammatory effect by enhancing the efferocytic capability of

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macrophage cells [15]. Moreover, daidzein reduced myocardial injury in a rat ischemia/reperfusion model by inhibiting NF-κB activation [16] (Fig. 1).

Although previous studies have showed the anti-inflammatory potential of daidzein, its ability to protect against LPS-induced acute lung injury and its anti-inflammatory mechanism remains poorly understood. In the present study, we investigated the protective effects of daidzein on LPS-induced acute lung injury and elucidated the potential molecular mechanism.

2. Materials and methods

2.1. Materials

Daidzein and LPS (*Escherichia coli* 055:B5) were obtained from Sigma (St. Louis, MO, USA). The myeloperoxidase (MPO) determination kit was provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kit were obtained from American R&D Corporation (R&D Systems Inc. Minneapolis, MN, USA). Anti-TLR4, anti-p-NF-κB p65 and anti-NF-κB p65 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MyD88, anti-IκB-α and anti-p-IκB-α antibodies were obtained from Cell Signaling Technology Inc (Beverly, MA). All other reagents were of analytical grade.

2.2. Animals

Adult male Sprague–Dawley rats (weighing 250 to 300 g) were provided by the Experimental Animal Center of Xuzhou Medical College, kept in a 12 h dark/12 h light cycle in a temperature- and humidity-controlled room and fed on standard laboratory diet and water. All procedures were performed in accordance with the Declaration of Helsinki of the World Medical Association.

2.3. Animal experimental design

Animals were randomly divided into six groups and each group contained six rats: (1) control group (saline); (2) daidzein (8 mg/kg) group; (3) LPS group (received LPS intratracheal instillation 5 mg/kg); (4) LPS + daidzein (2 mg/kg) group; (5) LPS + daidzein (4 mg/kg) group; (6) LPS + daidzein (8 mg/kg) group. LPS (5 mg/kg) or vehicle (saline) was intratracheally administered to induce acute lung injury [17]. Daidzein (2, 4 or 8 mg/kg) was intraperitoneally injected 30 min after LPS injection. The doses of these drugs were on the basis of previous studies and our preliminary experiments [16,18]. At 7 h after LPS administration, the rats were sacrificed, and samples were collected.

2.4. Cell culture and treatment

The human alveolar epithelial cells A549 were obtained from Dr. Huang (Department of Oncology, Beijing Shijitan Hospital, Beijing, China). A549 cells were seeded into six-well plates and were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown until 70% confluence before drug treatments. Cells were divided into four groups ($n = 6$ in each group): (1) control group (saline); (2) daidzein (100 μM) group; (3) LPS group (received LPS 10 μg/ml stimulation); (4) LPS + daidzein group. A549 cells were treated with daidzein (100 μM) 15 min after LPS (10 μg/ml) stimulation in LPS + daidzein group. The cell samples were harvested at 6 h after the addition of LPS to analyze the expressions of TLR4, MyD88, p-NF-κB p65 and p-IκB-α.

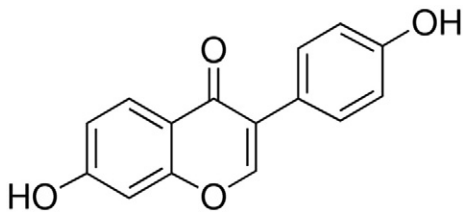

Fig. 1. Chemical structure of daidzein.

2.5. Lung wet/dry weight ratio

The water content of lungs was determined by calculating the wet/dry weight ratio of lung tissues. The inferior lobe of right lung was excised, rinsed briefly in PBS, blotted and then weighed to obtain the 'wet' weight. The lung was then dried at 80 °C for 72 h to obtain the 'dry' weight. The wet/dry ratio was calculated by dividing the wet weight by the dry weight.

2.6. Determination of bronchoalveolar lavage proteins and cell counts

Bronchoalveolar lavage (BAL) was performed by intratracheal injection of 5 mL ice-cold phosphate-buffered saline (PBS) followed by gentle aspiration. The recovery ratio of the fluid was about 90%. Then the recovered fluid was pooled and centrifuged at 1200×*g* for 10 min at 4 °C. Supernatants were preserved for the measurement of total protein concentration by the Bradford method with bovine serum albumin as a standard. The cell pellet was re-suspended in 50 μl PBS, and total cells recovered in BALF were counted. The cell differentiation was determined for 200 cells by examination of the HE-stained smears.

2.7. Cytokine measurements

The levels of TNF-α and IL-6 in the supernatants of BALF were measured with a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.8. MPO activity assay

Lung tissues were homogenized in hydroxyethyl piperazine ethanesulfonic acid (HEPES) (pH8.0) containing 0.5 % cetyltrimethyl ammonium bromide (CTAB) and subjected to three freeze–thaw cycles. The homogenate was centrifuged (4 °C, 12,000×*g*, 30 min). The MPO activity was assayed using a test kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Samples were diluted in phosphate citrate buffer (pH 5.0) and the absorbance of the sample was measured at 460 nm using a microplate reader. The specific activity of MPO in the lung is expressed as U/g of the tissue.

2.9. Histological examination

The right lobes were excised and fixed with 10% neutral phosphate-buffered formalin, imbedded in paraffin and sliced. After hematoxylin and eosin (H&E) staining, pathological changes of lung tissues were observed under a light microscope.

2.10. Western blot analysis

Protein concentrations were determined by BCA protein assay kit, and 20 μg protein was loaded per well on a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred onto polyvinylidene difluoride membrane. After being blocked for 3 h in