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Original article

Digitoflavone (DG) attenuates LPS-induced acute lung injury through reducing oxidative stress and inflammatory response dependent on the suppression of TXNIP/NLRP3 and NF-κB

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ABSTRACT

Acute lung injury is a severe disease with a high rate of mortality. Digitoflavone (DG) was suggested to possess bioactivities to reduce oxidative stress, inflammation and to regulate apoptosis. In our study, the normal saline, a low dose of DG (12.5 mg/kg), a medium dose of DG (25 mg/kg) and a high dose of DG (50 mg/kg) were administered to male C57BL/6 mice by gavage. And then, the mice were intratracheally injected with either normal saline or lipopolysaccharide (LPS). We found that DG ameliorated LPS-induced lung injury and platelets activation, accompanied with reduced CD41 expression and neutrophil platelet aggregates (NPAs). Further, pulmonary myeloperoxidase (MPO) activity and neutrophil infiltration in the lung tissues induced by LPS were abolished by DG dose-dependently. Additionally, LPS-triggered oxidative stress and secretion of pro-inflammatory cytokines were reduced by DG administration through suppressing thioredoxin-interacting protein (TXNIP) and nuclear factor-κB (NF-κB) signaling pathways, and their down-streaming and up-streaming signals, including xanthine oxidase (XO), NLR family, pyrin domain-containing 3 (NLRP3), ASC, Caspase-1, as well as IκB kinase-α (IKK-α), and IκBα. Moreover, mitogen-activated protein kinases (MAPKs) pathway was also inactivated by DG in LPS-induced mice. The in vitro study further confirmed that DG ameliorated LPS-induced inflammation and oxidative stress, which was associated with reduction of ROS. In conclusion, our data suggested that DG treatment could be considered as a promising therapy for treating acute lung injury.

1. Introduction

Acute lung injury and acute respiratory distress syndrome are reported as life-threatening diseases that significantly contribute to more critical illness [1]. Presently, although advances have been made in understanding the pathophysiology of acute lung injury and acute respiratory distress syndrome, there is still no effective therapeutic strategy for cute lung injury and acute respiratory distress syndrome [2,3]. Additionally, despite various molecular mechanisms are included in the pathogenesis and progression of acute lung injury, oxidative stress and inflammatory response are two major causes [4]. Acute lung injury is an extreme inflammatory process, which is characterized by excessive neutrophil infiltration into the lung tissues, the release of pro-inflammatory cytokines, and lung endothelium and epithelium injuries, which lead to the lung oedema and gas exchange deterioration [5,6].

Reactive oxygen species (ROS)-induced oxidative stress regulated the progression of acute lung injury [7]. The production of ROS is one of the most important factors to stimulate various signaling pathways, including TXNIP/NLRP3, NF-κB as well as MAPKs [8]. TXNIP/NLRP3 activation has been illustrated to link with acute lung injury. TXNIP could block anti-oxidant function, and its over-expression has been observed in many types of diseases [9,10]. Accumulating evidences have indicated that the NLRP3 inflammasome initiates the innate immunity and promotes inflammatory responses [11]. Importantly, TXNIP links the oxidative stress to the activation of NLRP3 in a ROS-sensitive manner [12]. The process accelerates the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, which is related to NF-κB activity, and contributes to lung injury [13]. And MAPKs are implicated

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in cellular inflammatory processes responding to oxidative stress [14].

Digitoflavone (3,0,4,5,7-tetrahydroxyflavone, DG) is a member of the flavone subclass of flavonoids, which are abundant in various vegetables and fruits [15]. Plants containing a large amount of digitoflavone have been applied in Chinese traditional medicine for a long time to treat inflammatory diseases, hypertension, and cancer [16,17]. Recently, it was also indicated that digitoflavone displayed anti-oxidant role, which was associated with enhancement of nuclear factor-erythroid 2-related factor 2 (Nrf2). Digitoflavone induced Nrf2 activation and reduced ROS generation, suppressing inflammatory response in rodents [18]. Considering the effects of digitoflavone on suppressing ROS and inflammation, it was first time that digitoflavone was used in treating acute lung injury.

In our study, we demonstrated that LPS induced acute lung injury, along with platelets activation, inflammation and oxidative stress, which were associated with the activity of NF-κB and TXNIP/NLRP3. Digitoflavone was found to effectively alleviate LPS-triggered lung injury, which might be the results of inhibition of NF-κB and TXNIP/NLRP3 and potentiation of Nrf2. Thus, digitoflavone treatment could be considered as a promising therapy against acute lung injury.

2. Materials and methods

2.1. Animals and treatment

Male C57BL/6 mice aged 6 to 8 weeks, weighed 18–20 g, were purchased from Shanghai Experimental Animal Center (Shanghai, China), and provided with food and water ad libitum and kept in climate-controlled quarters with a 12 h light/dark cycle with food and water in cages under the germ-free conditions at a temperature of 25 °C. The mice were housed for a minimum of one week for environmental adaptation prior to experimentation. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China, and before the animal experiments were performed, the procedures were approved by the Research Ethical Committee of Huai'an First People's Hospital, Nanjing Medical University (Nanjing, China).

C57BL/6 mice were randomly divided into five groups (n = 20/group): (i) Control (Con); (ii) LPS treatment (LPS); (iii) Low dose of DG treatment (LPS treatment + oral gavage of 12.5 mg/kg DG for 3 days, LD-DG); and (iv) Medium dose of DG treatment (LPS treatment + oral gavage of 25 mg/kg DG for 3 days, MD-DG); and (v) High dose of DG treatment (LPS treatment + oral gavage of 50 mg/kg DG for 3 days, HD-DG). The mice from the control and LPS groups received an equal volume of normal saline instead of DG. DG was purchased from Xian Shunyi Bio-Chemical Technology Co., Ltd. (HPLC ≥ 98%, Shanxi, China). Three hours after drug administration on the third day, the mice were slightly anesthetized with inhalation of diethyl ether, and 10 μg of LPS in 50 μl of PBS was instilled intranasally to induce lung injury. The mice in the control group were given 50 μl of normal saline without LPS. Twenty-four hours after the LPS treatment, the mice were sacrificed by an intraperitoneal injection of pentobarbital (50 mg/kg; Sigma-Aldrich, USA) and bronchoalveolar lavage fluid (BALF) and lung tissue samples were harvested for further researches. The ratios of lung wet/dry weight were calculated by dividing the wet weight by the dry weight. The middle lobe of the right lung was excised and the wet weight was recorded, and the lung was then placed in an incubator at 80 °C for 24 h to obtain the dry weight.

2.2. Cells and culture

Human lung epithelial cells, BEAS-2B, were obtained from Shanghai Haoran Biological Technology Co., LTD (Shanghai, China) and cultured in DMEM/F12 supplemented with 1% penicillin/streptomycin and 10% FBS (Hyclone). RAW264.7 murine macrophage-like cells, purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, China), were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS (Hyclone). All cells were cultured in a humidified atmosphere with 5% CO_2 and 95% humidity at 37 °C in an incubator.

2.3. Immunohistochemical analysis

Histopathological evaluation was performed on mice that were not subjected to BAL. Lungs were inflated and fixed with 10% buffered formalin for 48 h and embedded in paraffin (Beyotime Institute of Biotechnology). Tissue sections at 4 μm thickness were cut and stained with hematoxylin and eosin (H&E) following the regular staining method for histological analysis. The severity of injury was judged based on the following criteria: 0: No injury; 1: injury to 25% of the field; 2: injury to 50% of the field; 3: injury to 75% of the field; and 4: diffuse injury. The ultimate score was obtained by adding the aforementioned scores. Immunohistochemistry was performed using the paraffin-embedded tissue sections at 4 μm thickness mounted on glass slides. The slides were then deparaffinized and rehydrated. Then, the lung sections were incubated with primary antibodies against mouse CD41, TXNIP and XO, which were all purchased from Abcam (UK), and then with biotin secondary antibodies (Sigma Aldrich) an Vectastain ABC Kit, Vector laboratories (USA). Numbers of CD41-, XO-, and TXNIP-positive cells in lung sections were counted using Pax-it software (Paxcam, Villa Park, IL).

2.4. Biochemical assays

Levels of superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx) and malondialdehyde (MDA) in lung tissue sample were determined by commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions. H_2O_2 levels in lung tissue were measured through a hydrogen peroxide assay kit, obtained from Beyotime Institute of Biotechnology, following the manufacturer's instructions. O_2^- in lung tissue samples was measured through lucigenin chemiluminescence method. Briefly, lung tissues of mice under different conditions were weighed and homogenized in a homogenization buffer using 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium (Hepes) and ethylenediaamine tetra-acetic acid (EDTA). After centrifugation, an aliquot of the supernatant was incubated with 5 μM lucigenin in Krebs–Hepes buffer. Light emission was measured with a Tecan Infinite 200. Specificity for O_2^- was evaluated by adding SOD (350 U/ml) to the incubation medium. Protein concentration was assessed with BCA Protein Quantitative Analysis Kit (Thermo Fisher Scientific, USA).

2.5. Toxicity analysis of DG

30 male C57BL/6 mice aged 6–8 weeks, weighed 18–20 g were divided into two groups: (1) the Control group; and (2) DG group (50 mg/kg). All mice were provided with food and water ad libitum and kept in climate-controlled quarters with a 12 h light/dark cycle with food and water in cages under the germ-free conditions at a temperature of 25 °C. The mice were housed for a minimum of one week for environmental adaptation prior to experimentation. The mice in the control group were given 50 μl of normal saline, while