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Withaferin A attenuates lipopolysaccharide-induced acute lung injury in neonatal rats

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Abstract

Withaferin A (WFA) is an active compound from *Withania somnifera* and has been reported to exhibit a variety of pharmacological activities such as anti-inflammatory, immunomodulatory and anti-tumor properties. In the present study, we investigated the potential protective role of WFA on acute lung injury in neonatal rats induced by lipopolysaccharide (LPS). We found that WFA significantly attenuated the pathological changes of lungs induced by LPS injection. Administration with WFA obviously decreased pulmonary neutrophil infiltration accompanied with decreased MPO concentrations. WFA also reduced the expression of pro-inflammatory cytokines including MIP-2, TNF-α, IL-1β and IL-6. Meanwhile, the expression levels of anti-inflammatory mediators such as TGF-β1 and IL-10 were significantly increased following WFA administration. Moreover, WFA protected LPS-treated rats from oxidative damage via up-regulation of TBARS and H_2O_2 concentrations and down-regulation of ROS contents. Taken together, the present study demonstrated that WFA administration attenuated LPS-induced lung injury through inhibition of inflammatory responses and oxidative stress.

Key words: Withaferin A, acute lung injury, lipopolysaccharide.

Introduction

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome (ARDS), are characterized by increased capillary leakage and microvascular permeability due to the epithelial and endothelial injury (1,2). Up to now, ALI and ARDS are still the leading causes of death in pediatric and adult intensive care units. Accumulating evidences indicate that the pathophysiological mechanism of ARDS/ALI is associated with the uncontrolled inflammatory response in lungs (3). Thus, attenuation of inflammation can reduce the morbidity and mortality of ALI and it is critical to develop new potential anti-inflammatory drugs.

Many natural products exhibit immunomodulatory and anti-inflammatory activities and possess a wide prospect of clinical applications. *Withania somnifera*, a well known medicinal plant, has long been used to prevent dermatological disorders and infectious wounds (4). Withaferin A (WFA) is a steroidal lactone derived from *Withania somnifera* and considered to be a bioactive compound. It has drawn much attention from researchers due to its anti-carcinogenic and anti-inflammatory properties (5-7). Accumulating evidences supported the regulatory effects of WFA on multiple molecules including heat shock proteins [14] Akt [15], estrogen receptor [16] and NFkappaB (8,9). However, the biological role of WFA on LPS-induced lung injury has never been investigated. Therefore, our present study aimed to investigate the possible protective effects of WFA on LPS-induced ALI in a rat model.

Materials and methods

Animals

This study was approved by the animal care and use committee. Animal procedures were carried out in compliance with Institutional Standards for Use of Animal Laboratory Animals. Wistar rats with dated pregnancies were purchased from Chinese academy of sciences (Shanghai, China). They were bred in house and have free access to water and standard laboratory chow. Forty neonatal Wistar rats were randomly divided into 5 groups (n=8): control group, ALI group and WFA group with different concentrations. Neonatal rats were intraperitoneally injected with 2, 4 and 6 mg/kg of WFA (Sigma, St. Louis, MO, USA) 1 hour before LPS (Sigma, St. Louis, MO, USA) injection. Animals in control group were injected with an equal volume of sterile saline. ALI was induced by LPS via intratracheal injection according to the previous published report (10).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the contents of IL-6, IL-1β, TNF-α, TGF-β1 and IL-10 in the serum according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Absorbance was measured at 450 nm by microplate assay.

Histology

Animals were killed using sodium pentobarbital and the lungs were immediately harvested, post-fixed in 4% paraformaldehyde for 24 hours. Paraffin-embedded sections with the thickness of 5 mm were stained with hematoxylin and eosin (HE) for visualization under a

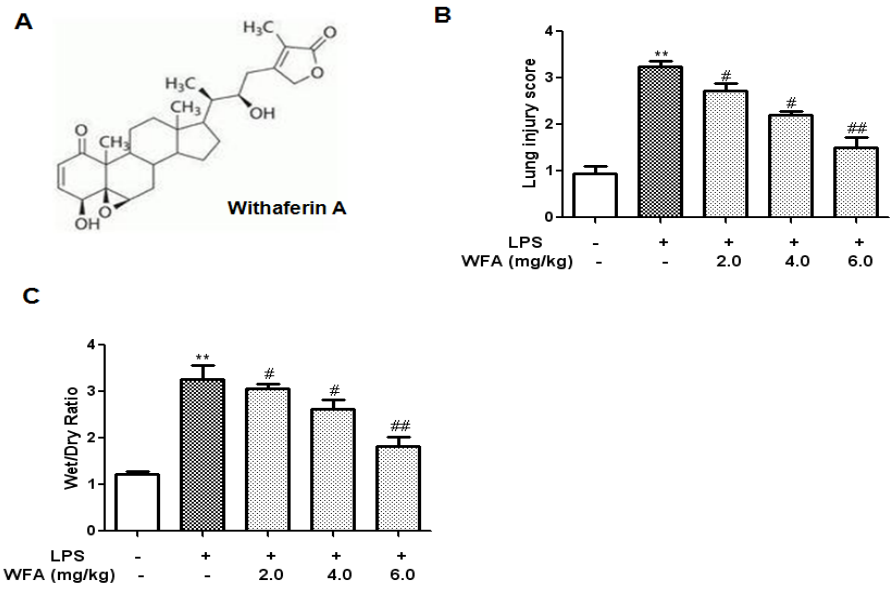

Figure 1. Effects of the WFA on the lung pathological changes. (A) Structure of WFA. After LPS injection with or without WFA treatments, rats were sacrificed by an anaesthetic overdose. Determination of the lung injury degree (B) and wet/dry weight ratio (C). The data were expressed as means ± SD, n=8 in each group. * P<0.05 versus the control group. #P<0.05 versus the LPS group.

light microscope (Leica Microsystems, Wetzlar, Germany) at 200 x magnification. Lung injury was scored using an average score of the following items: alveolar congestion, hemorrhage, infiltration of neutrophils into airspace or the vessel wall, and thickness of the alveolar wall. Lung injury scores were scaled from 1, mild; 2, moderate; 3, severe; and 4, very severe (2). The lung injury scores were evaluated by two pathologists who were blinded to the experimental conditions.

Measurement of lung wet/dry weight ratio

The severity of pulmonary edema was assessed by the wet to dry weight ratio (W/D ratio). The left lower lungs weighed and then dehydrated at 60 °C for 72 h in an oven.

Myeloperoxidase and thiobarbituric acid reactive substances (TBARS) assay

Concentration of myeloperoxidase (MPO), an index of neutrophil sequestration in the lungs, was measured as previously described (7). TBARS level in serum was measured using OxiSelect TBARS assay kit (Geneteks Biosciences, Inc.).

Statistical analysis

Data were presented as means ± SD and treated for statistics analysis by SPSS 16.0. Comparison between groups was made using ANOVA and statistically significant difference was defined as P<0.05.

Results

Effects of the WFA on the lung pathological changes of rats

Compared with the control group, the lungs in LPS group showed significant lung injury, including hemorrhage, edema, and infiltration of neutrophils in lung parenchyma. In contrast, pretreatment with WFA (Fig. 1A) markedly decreased the lung injury scores in a dose-dependent manner, which represented histopathological lung injury (Fig. 1B). Then, lung wet/dry weight ratio (W/D) was employed to assess the pulmonary edema in the lungs. Higher W/D was observed in the neonatal rats received LPS injection compare with the control group. However, WFA administrations significantly reduced the W/D (Fig. 1C). These results suggested that WFA treatment attenuated the lung pathological changes induced by LPS in rats.

Effects of WFA on inflammatory responses

MPO reflects the effect of WFA on pulmonary neutrophil infiltration. We found that WFA treatment reduced the levels of MPO compared with the LPS group (Fig. 2A). In order to evaluated the effects of WFA on cytokine production, ELISA assay was performed to detect the expression of inflammatory mediators in different groups. We found that WFA dose-dependently decreased the contents of several pro-inflammatory cytokines such as MIP-2 (Fig. 2B), TNF-α (Fig. 2C), IL-1β (Fig. 2D) and IL-6 (Fig. 2E). Meanwhile, the anti-inflammatory mediator TGF-β1 and IL-10 were reduced in LPS-injected rats, which were reversed by WFA treatment (Fig. 2F and G). These data suggested that WFA treatment modulated LPS-induced cytokine production.

WFA attenuated oxidative stress in ALI model

Oxidative stress has been demonstrated to be associated with LPS-induced lung injury. Therefore, we evaluated the oxidative status by assessment of TBARS and H_2O_2 in BALF. Results showed that the contents of TBARS and H_2O_2 in ALI group were obviously elevated compared with the control group. However, pretreatment with WFA remarkably suppressed the levels of TBARS and H_2O_2 in a dose-dependent fashion (Fig. 5A and B). In addition, the concentration of ROS was significantly decreased in LPS-treated rats, which was reversed after WFA administration (Fig. 5C). Collectively, these results indicated that WFA protected against

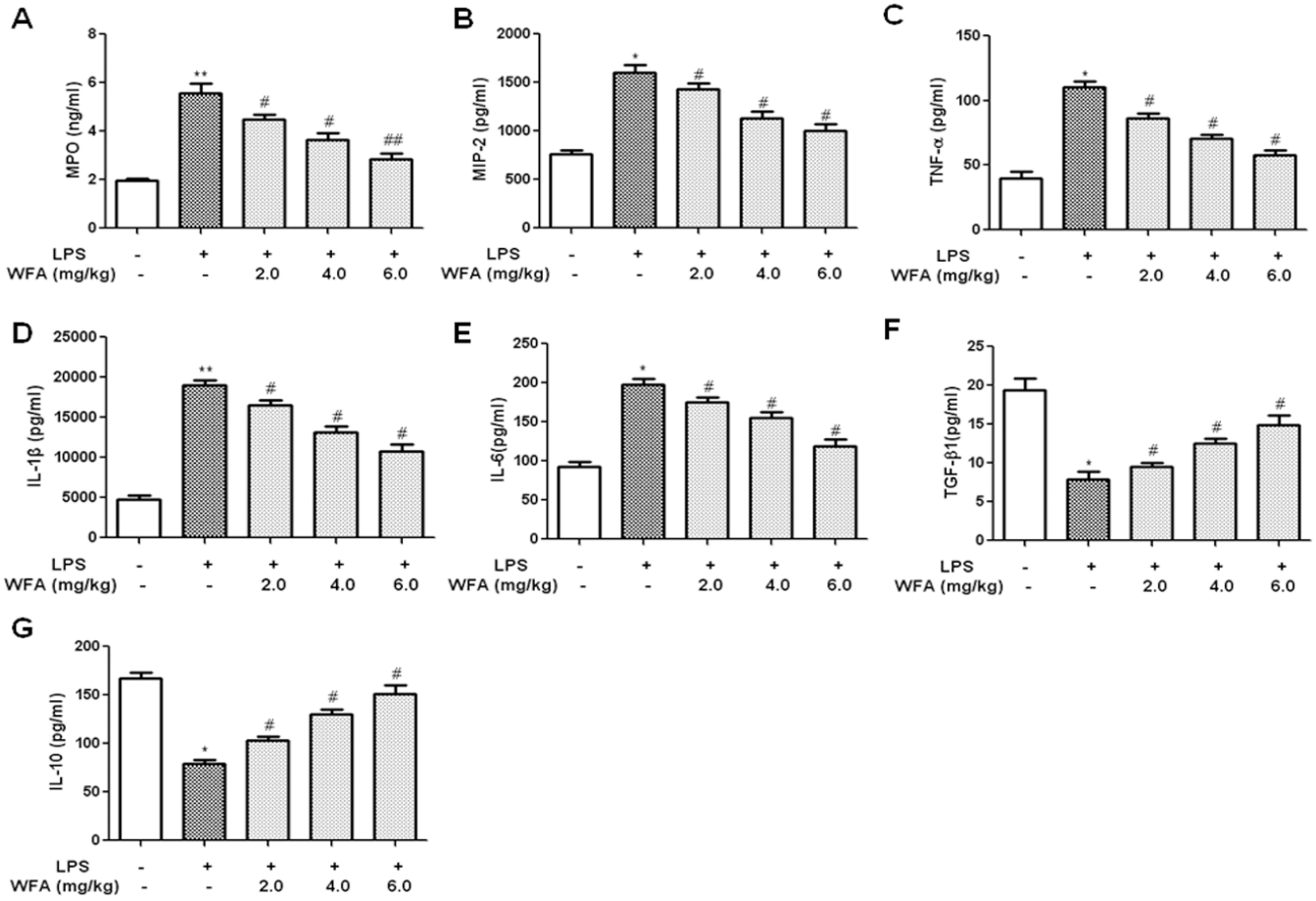

Figure 2. Effects of the WFA on inflammatory response in ALI. Measurement of MPO (A) and MIP-2 (B) activity, TNF-α (C), IL-1β (D), IL-6 (E), TGF-β1 (F) and IL-10 (G) in LPS-induced ALI rats with or without WFA treatment. The data were expressed as means ± SD, n=8 in each group. * P<0.05 versus the control group. #P<0.05 versus the LPS group.

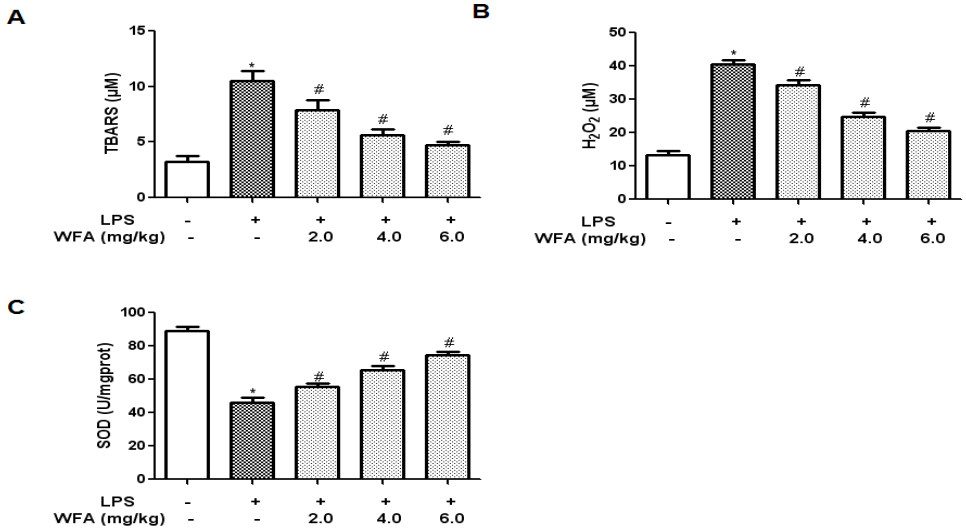

Figure 3. WFA treatment attenuated LPS-induced oxidative stress. The concentrations of TBARS (A), H_2O_2 (B) and SOD (C) were measured in LPS-induced ALI rats in the present or absence of WFA. The data were expressed as means ± SD, n=8 in each group. * P<0.05 versus the control group. #P<0.05 versus the LPS group.

oxidative damage induced by LPS.

Discussion

ALI and ARDS are severe clinical disorders of acute respiratory failure which results in high morbidity and mortality rates, and currently, there are no effective drugs in the clinic (11). Thus, it is important to develop effective therapeutics for ALI. Currently, a large number of herbal medicines exhibit pharmacological activities which have received considerable attention around the world (12-14). In the present study, we evaluated the possible protective effects of WFA on ALI induced by LPS in neonatal rats.

LPS is a main component of the outer membrane of Gram-negative bacteria, which can enter the blood stream and induce inflammatory responses (15). ALI model is well established by intraperitoneal administration of LPS. In this study, we demonstrated LPS injection elicited typical lung pathological changes. However, WFA administration remarkable attenuated such pathological changes. In addition, analysis of lung wet/dry weight ratio suggested that WFA decreased the LPS-induced pulmonary edema. These results revealed that WFA treatment protected neonatal rats against LPS-induced lung injury.

Release of various cytokines/chemokines plays an important role in the pathogenesis of ALI, leading to an increase in the pulmonary permeability of the alveolar-capillary barrier and a consequent impairment in arterial oxygenation (16). Therefore, inhibition of lung inflammation has been considered as a critical target in pharmacologic treatment of ALI patients. It has been shown that the predominant inflammatory cells are the neutrophils. MPO is an enzyme mainly located in the primary granules of neutrophils and is considered as a marker of neutrophil influx (17). We found that the LPS injection elevated the MPO activity, but WFA pretreatment reduced such changes. The MIP-2 belongs to the family of chemotactic cytokines and contributes to production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (18). Furthermore, IL-10 and TGF-β exhibit an anti-inflammatory activity in immunity and autoimmunity (19). In the present study, we found that WFA reduced the levels of MIP-2, TNF-α, IL-1β and IL-6 compared to that from the ALI group. On the contrary, WFA treatment elevated the production of anti-inflammatory cytokines TGF-β and IL-10, which were down-regulated by LPS. These results demonstrated that WFA treatment suppressed inflammatory response in the lungs induced by LPS injection.

Oxidative damage is another stimulator of ALI due to overproduction of reactive oxygen species. It has been suggested that oxidative stress is implicated in LPS-induced lung injury and serves a significant therapeutic target in the context of ALI (20). Neutrophil recruitment into the lung is a hallmark of ALI. Activated neutrophils produce vast quantities of ROS and nitrogen species, which cause cell injury through direct damage to DNA, lipid peroxidation with formation of vasoactive molecules, and oxidation of proteins (21). Thus, both inhibition of inflammatory response and elevated ROS better contributes to control ALI. We further assessed the oxidative status in rats treated with LPS or WFA. We observed that LPS injection significantly enhanced the concentrations of TBARS and H_2O_2. However, WFA treatment dramatically reduced the TBARS and H_2O_2 production. SOD was an antioxidant enzyme with scavenging the superoxide free radical, and reduction in SOD activity suggests the enhancement of oxidative stress (22,23). We found that the SOD activity was dramatically reduced LPS. However, this LPS-induced SOD activity decrease was obviously elevated by WFA treatment, indicating that WFA could attenuate the oxidative damage induced by LPS.

In conclusion, the present study for the first time shows that application of WFA can effectively attenuate LPS-induced lung injury in neonatal rats. The protective effects of WFA on ALI are partly due to inhibition of inflammatory responses and oxidative stress. Therefore, our findings suggest that WFA may be considered as an effective candidate drug for the potential treatment of LPS-induced lung injury.

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