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Astilbin alleviates LPS-induced ARDS by suppressing MAPK signaling pathway and protecting pulmonary endothelial glycocalyx

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a devastating disorder that is characterized by increased vascular endothelial permeability and inflammation. Unfortunately, no effective treatment beyond supportive care is available for ARDS. Astilbin, a flavonoid compound isolated from Rhizoma Smilacis Glabrae, has been used for anti-hepatic, anti-arthritic, and anti-renal injury treatments. This study examined the effects of Astilbin on pulmonary inflammatory activation and endothelial cell barrier dysfunction caused by Gram-negative bacterial endotoxin lipopolysaccharide (LPS). Endothelial cells from human umbilical veins or male Kunming mice were pretreated with Astilbin 24 h before LPS stimulation. Results showed that Astilbin significantly attenuated the pulmonary histopathological changes and neutrophil infiltration 6 h after the LPS challenge. Astilbin suppressed the activities of myeloperoxidase and malondialdehyde, as well as the expression of tumor necrosis factor-α and interleukin-6 in vivo and in vitro. As indices of pulmonary edema, lung wet-to-dry weight ratios, were markedly decreased by Astilbin pretreatment. Western blot analysis also showed that Astilbin inhibited LPS-induced activation of mitogen-activated protein kinase (MAPK) pathways in lung tissues. Furthermore, Astilbin significantly inhibited the activity of heparanase and reduced the production of heparan sulfate in the blood serum as determined by ELISA. These findings indicated that Astilbin can alleviate LPS-induced ARDS, which potentially contributed to the suppression of MAPK pathway activation and the degradation of endothelial glycocalyx.

1. Introduction

Acute lung injury (ALI) and its severe manifestation, acute respiratory distress syndrome (ARDS), are well-known fatal conditions with extremely high morbidity rates in critically ill patients. The distinguishing features of ARDS are neutrophil recruitment, interstitial edema, disruption of endothelial and epithelial integrity, and parenchymal lung disease [1].

Lipopolysaccharide (LPS) administration disturbs immune and inflammatory responses and may stimulate the production of proinflammatory cytokines and reactive oxygen species, resulting in increased infiltration of inflammatory cells [2,3].

Inflammatory responses, oxidative stress, and activation of mitogen-activated protein kinase (MAPK) pathways play an important role in the pathogenesis of ARDS [4]. The glycocalyx is a complex gel between the flowing blood and endothelial cells. Specifically, the endothelial glycocalyx is a heparan sulfate (HS)-rich layer of glycosaminoglycan and associated proteoglycans. Sepsis is associated with the activation of pulmonary heparanase (HPA), a HS-specific glucuronidase that causes the degradation of pulmonary endothelial glycocalyx and consequently, with endothelial dysfunction and inflammatory lung injury [5]. HPA is also a pro-inflammatory enzyme that upregulates TNF or IL-1β signals and induces transcription and translation in various cells, including epithelial cells, endothelial cells, and leukocytes [6]. HPA activation leads to the degradation of pulmonary endothelial glycocalyx with consequent endothelial dysfunction and inflammatory lung injury [7].

Astilbin (Fig. 1) is an active flavonoid compound isolated from the rhizome of *Smilax china* L. (Smilacaceae), which is widely used in traditional Chinese medical treatment. *S. china* reportedly demonstrated antioxidative [8], anti-arthritic [9], insecticidal [10], hepatoprotective [11], anti-inflammatory [12], anti-edematogenic [13], and immunosuppressive effects. The current study investigated the protective effects and

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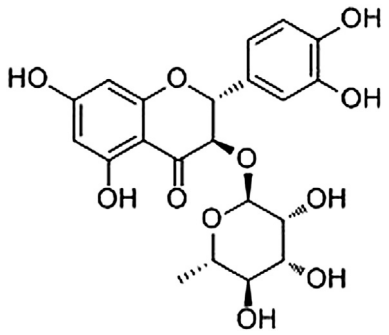

Fig. 1. The chemical structure of Astilbin.

important mechanisms of Astilbin in LPS-induced injury both in vivo and in vitro. Overall, the results may provide a pharmacological basis for the use of Astilbin in the treatment of ALI.

2. Material and methods

2.1. Animals

Adult male Kunming mice weighing 18–20 g were purchased from Lu Kang Animal Feed Sales Center (Shandong, China). All animals were housed in plastic cages at 21 ± 1 °C and kept on a 12 h light/dark cycle with 40%–80% humidity. They were supplied with food and water ad libitum. The animals were used in complete compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23; revised in 1996).

2.2. Reagents

Astilbin was purchased from Dalian Meilun Biology Technology Co., Ltd. (Liaoning, China) with purity of >98%. LPS, dimethyl sulfoxide (DMSO), 3-(4.5-dimethylthiazol-2-y1)-2.5-diphenyltetrazolium bromide (MTT), and streptomycin/penicillin were purchased from Sigma Aldrich Co. TNF-α, IL-6, myeloperoxidase (MPO), malondialdehyde (MDA), and enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Lengton Bioscience Co., Ltd. (Shanghai, China). Rabbit monoclonal antibody, ERK, JNK, p38, β-actin, *p*-ERK, p-JNK, p-p38 were purchased from Cell Signaling Technology, Inc. HRP-conjugated goat anti-rabbit antibodies were purchased from Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd.

2.3. In vitro study

2.3.1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from China Cell Line Bank (Beijing, China). Cells were cultured in DMEM supplemented with 10% heated-inactivated fetal bovine serum (Invitrogen/Gibco Life Technologies, Carlsbad, CA), penicillin (100 U/mL), and streptomycin (100 g/mL) at 37 °C under a humidified atmosphere of 5% CO_2. For all the experiments, cells were subcultured at confluence and used between the third and eighth passages. Astilbin was consistently dissolved in DMSO and used immediately. The final concentration of DMSO in the culture medium upon different treatments was <0.1%. All endothelial cells were incubated in the presence or absence of various concentrations of Astilbin, which was always added 24 h prior to LPS (1 μg/mL) stimulation.

2.3.2. Cell viability assay

Cell viability was evaluated by MTT formation. HUVECs were plated at a density of 4×10^5 cells/mL onto 96-well plates in a 37 °C, 5% CO_2 incubator for 1 h. Cells were then treated with different concentrations of Astilbin (0–100 μg/mL, 100 μL/well) for 24 h, followed by LPS stimulation (1 μg/mL, 100 μL/well). After 18 h, 20 μL of MTT (5 mg/mL) was added to each well, and incubation was continued for 4 h. After 4 h, supernatant was removed and the formation of formazan was resolved with 150 μL/well of DMSO. The optical density was measured at 570 nm on a microplate reader. Concentrations were determined for three wells of each sample, and each experiment was performed in triplicate.

2.3.3. Enzyme-linked immunosorbent assay

Cells were plated onto 96-well plates and added with different concentrations of Astilbin (12.5, 25, and 50 μg/mL). After 24 h, cells were incubated with or without LPS (1 μg/mL) in a 37 °C, 5% CO_2 incubator for 6 h. An equal volume of DMEM was added to the control group. TNF-α, IL-6, MPO, MDA, and HPA activities in the supernatant were all measured by ELISA. All procedures were conducted in accordance with the manufacturer's instructions.

2.4. In vivo study

2.4.1. Mouse model building

All experimental animals were randomly allocated into five groups: control group; LPS group (20 mg/kg); and LPS plus Astilbin (12.5, 25, or 50 mg/kg) groups. The doses and administration form of Astilbin were prepared on the basis of our preliminary experiments. The LPS plus Astilbin groups received Astilbin via intraperitoneal administration. The control and LPS groups were pretreated with an equal volume of vehicle. After 24 h, the LPS group and the LPS plus Astilbin groups were intraperitoneally injected with LPS (20 mg/kg) to induce lung injury. The control group received an equal volume of normal saline without LPS. All mice were alive after 6 h of LPS stimulation. Animals were euthanized 6 h after LPS administration. Then, bronchoalveolar lavage fluid, serum samples and lung tissue samples were harvested.

2.4.2. Lung wet-to-dry weight (W/D) ratio

After the mice were euthanized, a median sternotomy allowed exposure of both lungs of the mice. The left lungs were blotted dry and weighed to obtain the "wet" weight. The lungs were then placed in an oven at 80 °C for 48 h to obtain the "dry" weight. Lung W/D weight ratio was used to assess pulmonary edema.

2.4.3. Pulmonary histopathology

Histopathologic examination was performed on mice but not subjected to bronchoalveolar lavage fluid (BALF) collection. Part of each pulmonary tissue sample was fixed in 4% normal buffered formalin for 48 h, followed by dehydration in graded alcohol and embedding in paraffin wax. After staining with hematoxylin and eosin (H&E), pathological changes of the lung tissues were observed under a light microscope. The lung injury score (LIS) assessment was using the method described by Nishina et al. [14]. The character of lung injury was alveolar congestion, hemorrhage, infiltration, or aggregation of neutrophils in the airspace or vessel wall and thickness of the alveolar wall or hyaline membrane. The severity of lung injury was scored as follows: 0, minimum; 1, mild; 2, moderate; 3, severe; and 4, maximum. Each stained sample selected Six high-magnification fields randomly, then graded for the average LIS.

2.4.4. Collection of BALF and cytokine assays with ELISA

At 6 h after LPS stimulation, BALF collection was achieved thrice through a tracheal cannula with an autoclaved PBS instilled up to 1.5 mL. The BALF of each sample was centrifuged (4 °C, 3000 rpm, 20 min) immediately. Levels of the inflammatory cytokines TNF-α and IL-6 in BALF were measured by ELISA. All procedures were conducted in accordance with the manufacturer's instructions.