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Salidroside attenuates LPS-induced pro-inflammatory cytokine responses and improves survival in murine endotoxemia

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ABSTRACT

Salidroside is a major component isolated from the *Rhodiola rosea*. In the present study, we investigated the anti-inflammatory effects of salidroside on cytokine production by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages in vitro, and the results showed that salidroside reduced tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) secretions. This inspired us to further study the effects of salidroside in vivo. Salidroside significantly attenuated TNF-α, IL-1β and IL-6 productions in serum from mice challenged with LPS, and consistent with the results in vitro. In the murine model of endotoxemia, mice were treated with salidroside prior to or after LPS challenge. The results showed that salidroside significantly increased mouse survival. Further studies revealed that salidroside could downregulate LPS-induced nuclear transcription factor- B (NF- B) DNA-binding activation and ERK/MAPKs signal transduction pathways production in RAW 264.7 macrophages. These observations indicated that salidroside modulated early cytokine responses by blocking NF- B and ERK/MAPKs activation, and thus, increased mouse survival. These effects of salidroside may be of potential usefulness in the treatment of inflammation-mediated endotoxemia.

1. Introduction

Lipopolysaccharide (LPS/endotoxin), which is the major component of outer membranes of Gram-negative bacteria, can induce the release of pro-inflammatory cytokines and other inflammatory mediators, including TNF-α, IL-1β and IL-6. The progressive production of these inflammatory mediators may result in clinical syndrome of sepsis, including septic shock and multiple organ dysfunction syndrome [1,2]. During the past several decades, the incidence of sepsis and sepsis-related deaths appears to be increasing [3]. The mortality rate from severe sepsis remains over 30% [4]. Unfortunately, there are currently few effective adjuvant therapies in clinical use except activated protein C (APC) which targets the coagulation system [5]. However, APC is only recommended in patients at high risk of death. Therefore, it is very important to search for new anti-sepsis drugs.

In recently years, there was a resurgence of interest in developing natural anti-sepsis drugs from medicinal plants, such as hesperidin [6] and Schisantherin A [7]. They could reduce bacterial numbers during infection, decrease the level of serum LPS, inhibit LPS-stimulated cytokines release, and suppress infection-induced endotoxin shock in mice. *Rhodiola rosea* has been widely used in traditional Chinese medicine for a long time [8]. Previous studies prove that it has various pharmacological functions, such as improving exercise endurance and fatigue, antimicrobial, anti-cancer, preventing high altitude sickness, promoting blood circulation, eliminating toxins from the body, and treating various endemic diseases [9–12]. Salidroside (p-hydroxyphenethyl-β-D-glucoside, $C_{14}H_{20}O_7$) is a phenylpropanoid glycoside extracted from *R. rosea* and regarded as the most important bioactive component [13]. It has been reported to have various pharmacological properties including anti-aging, anti-cancer, hepatoprotective, antivirus, antioxidative effect, and suppressing the release of prostaglandin E_2 in vitro [14–17]. However, no available study has evaluated the effects of salidroside treatment on LPS-induced endotoxemia in a mouse model. Thus, the aim of the present study was to investigate the anti-inflammatory effects of salidroside on LPS-activated pro-inflammatory cytokine production in RAW 264.7 macrophages and mortality rate in mice with endotoxic shock that were treated with salidroside prior to or after LPS challenge. Our results showed that salidroside significantly inhibited the production of pro-inflammatory cytokines induced by LPS in vitro and in vivo, increasing mouse survival. The related-mechanism is by blocking LPS-induced NF- B and ERK/MAPKs signalling pathways.

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2. Materials and methods

2.1. Materials

Salidroside (purity >98%) was ordered from the National Institute for the Control of Pharmaceutical and Biological Products (China). Dimethyl sulfoxide (DMSO), LPS (*Escherichia coli* 055:B5), 3-(4,5-dimethylthiazol-2-y1)-2,5-dipheny-ltetrazolium bromide (MTT), and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α, IL-6 and IL-1β ELISA kits were purchased from Biolegend. Dulbeccos modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). The rabbit polyclonal anti-ERK and mouse monoclonal anti-phospho-ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H + L) were purchased from PTG (Chicago, IL, USA).

2.2. Experimental animals

C57BL/6 male mice weighing 18–22 g were purchased from Jilin University Experimental Animal Center and acclimatized for 1 week before use. Rodent laboratory chow and tap water were provided ad libitum and maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of 40–80%, and a 12-h light/12-h dark cycle. All of the procedures were in strict accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS at 37 °C under a humidified atmosphere of 5% CO_2.

2.4. Cell viability assay

Cytotoxicity studies induced by salidroside were performed by MTT assay. RAW 264.7 cells were mechanically scraped, plated at a density of 4×10^5 cells/ml onto 96-well plates (Costar USA) containing 100 μl of DMEM medium, and incubated overnight. After overnight incubation, the cells were treated with various concentrations of salidroside (0–200 μg/ml) in the presence or absence of LPS (1 μg/ml) according to the experimental design. After 24 h, 50 μl of MTT was added to each well and the cells were further incubated for 4 h at 37 °C with 5% CO_2. MTT was removed and cells were lysed with 100 μl/well DMSO. The optical density (OD) values were measured at 570 nm on a microplate reader (TECAN, Austria).

2.5. Cytokine assays in vitro

To determine the effects of salidroside on cytokine responses from LPS-induced cells, RAW 264.7 cells were plated onto 24-well plates (10^5 cells/well), and incubated in the presence of either 1 μg/ml LPS alone, or LPS plus salidroside 30 μg/ml, 60 μg/ml, and 120 μg/ml for 12 h at 37 °C with 5% CO_2. Cell-free supernatants were collected and stored at −20 °C until assayed for cytokines. The concentrations of cytokine TNF-α, IL-1β and IL-6 in the supernatants of RAW 264.7 cells culture were measured by ELISA using commercially available reagents according to the manufacturer's instructions (BioLegend, Inc. Camino Santa Fe, Suite E San Diego, CA, USA).

2.6. Cytokine assays in vivo

Cytokine concentrations in vivo were measured in serum. Salidroside (120 mg/kg) was given with an intraperitoneal injection (ip). Control mice received an equal volume of vehicle instead of salidroside. One hour later, all mice received LPS (30 mg/kg) by intraperitoneal injection (ip). Serum was separated from clotted blood at 0, 1, 3, 6 and 12 h following administration of intraperitoneal LPS. Serum was stored at −70 °C and concentrations of cytokine TNF-α, IL-1β and IL-6 were measured by ELISA using commercially available reagents according to the manufacturer's instructions.

2.7. Murine model of LPS-induced endotoxemia

C57BL/6 mice were challenged in groups of four with LPS (dose range: 10–40 mg/kg) by ip. Mice were observed on mortality for 6 days and twice a day. The items include feeding, activity and grooming (smooth and shiny coats vs dull and ruffled coats). LPS concentration that induced 80–90% lethal as working solution in the next step was used. In drug testing, using our established dose of LPS that was 80–90% lethal, the effect of salidroside (30, 60 and 120 mg/kg) on LPS-induced mortality was assessed by dosing intraperitoneal salidroside 1 h before LPS challenge. To further observe the effects of salidroside on endotoxemia, mice were administered salidroside (120 mg/kg) at 0, 1 or 4 h after LPS challenge, respectively. Mice in control and LPS groups were only given vehicle or LPS.

2.8. Measuring NF- B (p65) DNA-binding activation

RAW264.7 cells were plated at a density of 4×10^5 cells/ml onto 6-well plates (Costar USA). After overnight incubation, cells pretreated with 30, 60 or 120 mg/L of salidroside 1 h prior to treatment with 1 μg/ml of LPS were incubated for another 0.5 h and then collected on ice. Nuclear protein was extracted using commercially available reagents according to the manufacturer's instructions.

NF- B (p65) DNA-binding activity was examined using the TransAM™ ELISA kit according to the manufacturer's protocol. In brief, 0.5 μg of nuclear extract was subjected to the binding of NF- B to an immobilized consensus sequence in a 96-well plate, and the primary and secondary antibodies were added. After the colorimetric reaction, OD value of samples was measured in an ELISA reader at 450 nm. Recombinant NF- B p65 was used as a protein standard.

2.9. Western blot analysis

In brief, RAW 264.7 cells (4×10^5 cells/ml), cultured in 6-well plates for 24 h, were pretreated with 30, 60 and 120 mg/L of salidroside 1 h prior to treatment with 1 μg/ml LPS for 30 min (for ERK/MAPKs) in a 37 °C, 5% CO_2 incubator. Then, the cells were collected on ice, washed twice with ice-cold PBS, and suspended in 40 μL of the lysis buffer. After incubating the lysates on ice for 30 min, they were subjected to centrifugation (12,000 g) at 4 °C for 5 min to obtain the cytosolic fractions. Protein concentration was determined by the Bradford assay (Bio-Rad, Munich, Germany) before storage at −70 °C. For phosphorylated p42/p44 ERK, 40 μg of total cellular protein was separated by 10% acrylamide SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were then washed in Tris-Tween-buffered saline (TTTS), blocked overnight with 5% (wt/vol) nonfat dry milk, and probed with monoclonal phospho-specific antibodies to p42/p44 ERK in 5% (wt/vol) BSA dissolved in TTBS. With the use of peroxidase-conjugated secondary anti-mouse antibody, bound antibodies were detected by plus ECL. To confirm equal loading of proteins between samples, the membranes were probed with rabbit polyclonal p42/p44 ERK antibody.