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DGAEE, a newly synthesized derivative of glycyrrhetinic acid, potently attenuates mouse septic shock *via* its main metabolite DGA in an IL-10-dependent manner

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ABSTRACT

Endotoxin can stimulate inflammatory cytokine release from monocytes/macrophages and result in septic shock. Glycyrrhetinic acid (GA), the main bioactive component of licorice, possesses substantial anti-inflammatory activity. Here, we explored effect of 11-deoxy-18α-glycyrrhetinic acid-30-ethyl ester (DGAEE), a newly synthesized derivative of GA, on septic shock. DGAEE and its main metabolite 11-deoxy-18α-glycyrrhetinic acid (DGA) significantly alleviated septic shock as evidenced by improvements of survival rates, lung histopathological changes and wet/dry ratio in lipopolysaccharide (LPS)/D-galactosamine-stimulated mice, and decreased blood pressure in LPS/D-galactosamine-stimulated rats. The two compounds decreased serum levels of NO, TNF-α, IL-6, IL-1β, and increased the level of IL-10 more potently in mice. In LPS-stimulated RAW 264.7 cells, DGA but not DGAEE showed marked regulation of NO, TNF-α, IL-6 and IL-10 levels, suggesting that DGAEE display anti-shock effect by DGA rather than itself. Moreover, the neutralizing antibody against IL-10 markedly prohibited the inhibitory effect of DGA on the production of cytokines from RAW 264.7 cells, and AS101 (an inhibitor of IL-10 biosynthesis) almost completely reversed the anti-shock effect of DGA in mice. In addition, DGA did not affect activation of NF-κB-p65 and p38 MAPK as well as IκBα degradation, but moderately reduced activation of ERK and JNK, and markedly increased phosphorylation of GSK3β in LPS-stimulated RAW 264.7 cells. LY294002 (an inhibitor of GSK3β phosphorylation) and LiCl (an inhibitor of GSK3β activity) diminished and potentiated increase of IL-10 levels by DGA, respectively. In conclusion, DGAEE alleviates septic shock through DGA in an IL-10-dependent manner, and the mechanism is related to inactivation of GSK3β.

1. Introduction

Sepsis, a systemic inflammatory response syndrome, is the fourth leading cause of long-stay intensive care admissions, and the death of patients may occur if the host response to infection is either excessive or insufficient. Septic shock is codified as sepsis complicated by either hypotension that is refractory to fluid resuscitation or by hyperlactatemia. The mortality has been reported to be as high as 45% during five months after the onset of septic shock [1–3]. Bacterial components are the main inducers of the occurrence of septic shock such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid and exotoxins. Following initial infection occurs, macrophages, monocytes and other host cells will activate and release large amount of inflammatory mediators, and these mediators act on the relevant receptors on neutrophils and endothelial cells. Concurrently, other effector molecules are released, and cause organ damage and further recruit activated neutrophils to the site of injury [4–6].

Data have shown that inflammatory mediators occupy capital position in the occurrence and development of septic shock, and they can be roughly classified into pro-inflammatory and anti-inflammatory ones, such as TNF-α, IL-6, IL-1β and IL-10. Pro-inflammatory mediators are able to destroy pathogens, however their overproduction will induce cytotoxicity and inflammatory responses. In contrast, anti-inflammatory mediators are important for avoiding the excessive inflammation of tissues and for improving host survival. The deficiencies of both IL-1 receptor and TNF-α additively prevent LPS-induced mortality of septic shock mice, while IL-10 gene-deficient mice demonstrate an enhanced endotoxin-induced mortality and multiple organ failure [7,8]. Therefore, rebalance of interrelated inflammatory mediators is identified as the key aim of therapeutic intervention for septic shock.

Licorice, a well-known herb plant with pleiotropic bioactivities, is frequently used in food additives and traditional Chinese medicine for

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a long time. Glycyrrhetinic acid (GA) is the most important bioactive component of licorice, and exists in two stereoisomers the *trans* form 18α-glycyrrhetinic acid (18α-GA) and the *cis* form 18β-glycyrrhetinic acid (18β-GA). Both of them belong to 11-carbonyl group of GA and have multiple biological effects such as anti-inflammation and anti-cancer [9–10]. However, clinical data indicated that 11-carbonyl group of GA could directly induce side effect of pseudoaldosteronism, though the side effect of 18α-GA was relatively lower. 11-deoxy-18α-glycyrrhetinic acid-30-ethyl ester (DGAEE) is a newly synthesized derivative of 18α-GA by changing 11-carbonyl to 11-hydroxyl with reduced side effects, and 11-deoxy-18α-glycyrrhetinic acid (DGA) is its main *in vivo* metabolite. Herein, we comparatively investigated the effects of DGAEE and DGA on septic shock in mice, and explored the underlying mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

11-deoxy-18α-glycyrrhetinic acid-30-ethyl ester (DGAEE, purity: 96.5%) and 11-deoxy-18α-glycyrrhetinic acid (DGA, purity: 97.9%) were obtained from Jiangsu Chia-Tai Tianqing Pharmaceutical Co., Ltd. (Nanjing, China) (Fig. 1); Dexamethasone (DEX) was purchased from Zhejiang XianJu Pharmaceutical Co., Ltd. (Taizhou, China); LY294002 (an inhibitor of GSK3β phosphorylation) was purchased from Abmole Bioscience (Kowloon, Hong Kong); AS101 (a white crystalline synthetic organic tellurium compound, an inhibitor of IL-10 biosynthesis) was purchased from Santa Cruz Biotechnology (CA, USA); LiCl (an inhibitor of GSK3β activity) and lipopolysaccharide (*Escherichia coli* O55: B5, LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); D-galactosamine (D-gal) was purchased from Nanjing Dingsi Technology, Inc. (Nanjing, China); Neutralizing antibody against IL-10 (clone JES5-2A5, rat IgG1) and isotype control antibody (clone eBRG1, rat IgG1) were purchased from eBioscience (San Diego, CA); TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA); HiScript QRT SuperMix and SYBR Green Master Mix were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China); Antibodies against p-NF-κB-p65, p-IκBα, p-p38 MAPK, p-ERK, p-JNK, p-GSK3β, IκBα, p38 MAPK, ERK, JNK and GSK3β were purchased from Bioworld Technology, Inc. (Georgia, USA); Antibody against NF-κB-p65 was purchased from Cell Signaling Technology, Inc. (Boston, American). The other chemicals and reagents used were of analytical grade available.

2.2. Animals

Female C57/BL6 mice, 6–8 week-old, were purchased from the Comparative Medicine Center of Yangzhou University (Yangzhou, China). All experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and the related ethical regulations of China Pharmaceutical University. All efforts were made to minimize animals' suffering and reduce the number of animals used.

2.3. LPS/D-gal-induced septic shock in mice

Septic shock was induced in mice by an intraperitoneal injection of LPS (4 mg/kg)/D-gal (500 mg/kg) [11–13]. To examine the anti-septic shock effects of DGAEE and DGA, mice were randomly divided into the following groups: normal group, model group, DGAEE (40, 80 mg/kg) groups, DGA (40, 80 mg/kg) groups, DEX (0.5 mg/kg) group. To verify the participation of IL-10 in the anti-septic shock effect of DGA, mice were randomly divided into the following groups: normal group, model group, DGA (40, 80 mg/kg) groups, AS101 (10 μg/mouse) group, AS101 (10 μg/mouse) + DGA (40, 80 mg/kg) group and DEX (0.5 mg/kg) group.

DGAEE, DGA and DEX were intraperitoneally injected 2 h before LPS/D-gal challenge; AS101 was intraperitoneally injected 12 h before LPS/D-gal challenge. Mice in the normal and model groups were administered an equal volume of vehicle in the same schedule. In addition, the general condition and mortality of mice were observed and recorded for up to 72 h.

2.4. Histopathologic analysis

Right lungs of mice were excised at 4 h after LPS/D-gal challenge, and immediately fixed in 10% formalin. The fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E).

2.5. Lung wet/dry weight ratio

Left lungs of mice were obtained 4 h after LPS/D-gal challenge. The wet weights were obtained immediately. Then, lungs were drying in an oven at 80°C for 48 h until achieving stable dry weights. The lung wet/dry weight ratio was calculated to assess lung edema.

2.6. Cell culture and viability assay

RAW 264.7 cells, a mouse macrophage cell line, were obtained from American Type Culture Collection (ATCC). They were cultured in DMEM medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and maintained at 37 °C in 5% CO_2 humidified air.

Cell viability was evaluated by MTT assay. Briefly, RAW 264.7 cells (1×10^6 cells/mL) were seeded in 96-well plates and cultured in a 37 °C, 5% CO_2 incubator for 24 h. Then, they were incubated with DGAEE or DGA at different concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80 μM) in the absence or presence of LPS (1 μg/mL) for 20 h [14]. Whereafter, 20 μL of MTT solution (5 mg/mL in PBS) was added into each well, and cells were continuously cultured for 4 h. The supernatants were removed and the formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm using a Model 1500 Multiskan spectrum microplate Reader (Thermo, Waltham, MA, USA).

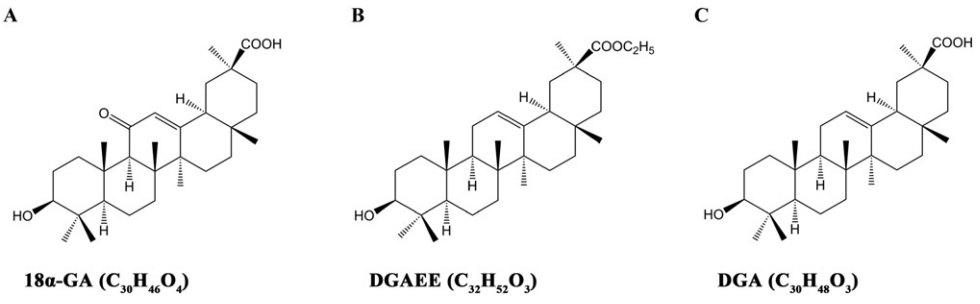

Fig. 1. Chemical structures of 18α-GA, DGAEE and DGA.