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Geniposide, from *Gardenia jasminoides Ellis*, inhibits the inflammatory response in the primary mouse macrophages and mouse models

Yunhe Fu [a,1], Bo Liu [a,1], Jinhua Liu [b], Zhicheng Liu [a], Dejie Liang [a], Fengyang Li [a], Depeng Li [a], Yongguo Cao [a], Xichen Zhang [a], Naisheng Zhang [a], Zhengtao Yang [a,*]

[a] Department of Clinical Veterinary Medicine, College of Animal Science and Veterinary Medicine, Jilin University, Changchun, Jilin Province 130062, People's Republic of China
[b] Jilin Entry-exit Inspection and Quarantine Bureau, Changchun, Jilin Province 130062, People's Republic of China

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ABSTRACT

Geniposide, a main iridoid glucoside component of gardenia fruit, has been known to exhibit antibacterial, anti-inflammatory and other important therapeutic activities. The objective of this study was to investigate the protective effects of geniposide on inflammation in lipopolysaccharide (LPS) stimulated primary mouse macrophages in vitro and LPS induced lung injury model in vivo. The expression of pro-inflammatory cytokines was determined by enzyme-linked immunosorbent assay (ELISA). Nuclear factor-kappa B (NF-κB), inhibitory kappa B (IκBα) protein, p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Toll-like receptor 4 (TLR4) were determined by Western blot. Further analysis was carried out in mTLR4 and mMD-2 co-transfected HEK293 cells. The results showed that geniposide markedly inhibited the LPS-induced TNF-α, IL-6 and IL-1β production both in vitro and in vivo. Geniposide blocked the phosphorylation of IκBα, p65, p38, ERK and JNK in LPS stimulated primary mouse macrophages. Furthermore, geniposide inhibited the expression of TLR4 in LPS stimulated primary mouse macrophages and inhibited the LPS-induced IL-8 production in HEK293-mTLR4/MD-2 cells. In vivo study, it was also observed that geniposide attenuated lung histopathologic changes in the mouse models. These results suggest that geniposide exerts an anti-inflammatory property by down-regulating the expression of TLR4 up-regulated by LPS. Geniposide is highly effective in inhibiting acute lung injury and may be a promising potential therapeutic reagent for acute lung injury treatment.

1. Introduction

Acute lung injury (ALI) is characterized by an acute inflammation process in the airspaces and lung parenchyma involving the inflammatory damage of the alveolar–capillary membrane, polymorphonuclear neutrophil (PMN) adhesion, activation and sequestration [1]. Its severe form, acute respiratory distress syndrome (ARDS), often results in multi-organ failure with a mortality of approximately 30–50% [2]. Lipopolysaccharide (LPS), a main component of outer membrane of Gram-negative bacteria, has been referred to be an important risk factor of ALI [3,4]. LPS activates macrophages and monocytes to trigger the pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), metalloproteinases cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and prostaglandin E2 (PGE2), which lead to inflammation and various other clinical manifestations [5–7].

So far, 13 members of Toll-like receptors (TLRs) have been reported and identified. TLR4 as one of the best characterized TLRs is associated with CD14 and MD-2 to recognize LPS from Gram-negative bacteria [7–9]. After the complex of CD14-LPS–LBP was presented to TLR4-MD-2 complex, it will activate the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways to secrete pro-inflammatory cytokines [8,9]. Therefore, therapeutic approaches aimed at modulating TLR4 signaling may have potential therapeutic advantages for inflammatory diseases.

Geniposide, a traditional Chinese medicine, is the major iridoid glycoside constituent of gardenia herbs and is responsible for the plant's pharmacological activities. It has been shown that geniposide exhibited a broad spectrum anti-inflammatory effect. Geniposide was found to inhibit IL-6 and IL-8 production in LPS-activated human umbilical vein endothelial cells by blocking p38 and ERK1/2 signaling pathways and to block high glucose-induced cell adhesion through the NF-κB signaling pathway in human umbilical vein endothelial cells [10]. In addition, some reports have demonstrated that geniposide exhibited anti-inflammatory activity in the carrageenan-induced rat paw edema model, displayed inhibitory effects on acetic acid-induced vascular permeability changes, and exerted protective effects against nonalcoholic steatohepatitis [11]. However, the anti-inflammatory effect and its mechanism of geniposide on LPS-induced acute lung injury remain unclear. In this study, we sought to assess the anti-inflammatory effects

*Corresponding author. Tel.: +86 431 87981688; fax: +86 431 87981688.
E-mail address: yzt@jluhp.edu.cn (Z. Yang).
[1] These authors contributed equally to this work.

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of geniposide in a LPS-induced mouse acute lung injury model and elucidated the potential anti-inflammatory mechanism.

2. Materials and methods

2.1. Reagents

Geniposide (Purity: >98%, Fig. 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China), and free of endotoxin. Dimethyl sulfoxide (DMSO), LPS (*Escherichia coli* 055:B5), and 3-(4,5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, Fetal bovine serum (FBS), penicillin and streptomycin for cell culture were obtained from Invitrogen-Gibco (Grand Island, NY). Mouse TNF-α, IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (CA, USA). Mouse monoclonal phospho-specific p46–p54 JNK antibodies, mouse monoclonal phospho-specific p42–p44 ERK antibodies, mouse monoclonal phospho-specific p38 antibodies, mouse mAb phospho-NF-κB p65, mouse mAb phospho-IκBα and rabbit mAb IκBα were purchased from Cell Signaling Technology Inc. (Beverly, MA). Mouse mAb TLR4 were purchased from GeneTex. HRP-conjugated goat anti-rabbit and goat-mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

2.2. Animals

Male BALB/c mice (8–12 weeks), which weighed 18–20 g, were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China). All animals were housed in microisolator cages, and received food and water ad libitum. Laboratory temperature was 24 ± 1 °C, and relative humidity was 40–80%. Before experimentation, mice were housed for a minimum of 2–3 days to adapt them to the environment. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

2.3. In-vitro study

2.3.1. Cells

Female C57 mice were injected i.p. with 2 ml of 4% thioglycollate broth (Difco Laboratories, Detroit, MI). Four days later, peritoneal cells were harvested with phosphate-buffered saline (PBS). The cells were cultured in RPMI 1640 medium supplemented with 10% FCS. HEK293 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS.

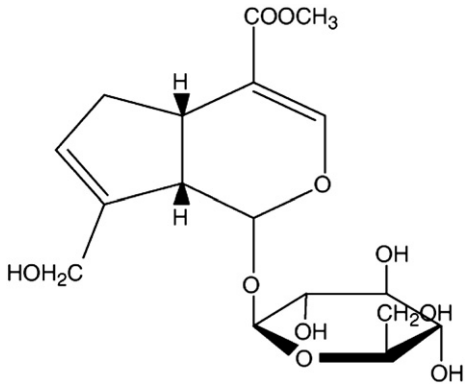

Fig. 1. Chemical structure of geniposide.

2.3.2. Expression vectors and transient transfection

pEGFP-N1-TLR4 and pDsRED-N1-MD2 were maintained in laboratory. All constructions involving PCR were verified by sequencing.

HEK293 cells were transfected with the expression plasmids using FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions.

2.3.3. Cell viability assay

Cell viability was evaluated by MTT assay. Briefly, the cells were plated at a density of 4×10^5 cells/ml in 96-well plates in a 37 °C, 5% CO_2 incubator for 1 h, then the cells were treated with 50 μl of geniposide at different concentrations (0–300 μg/ml) for 1 h, followed by stimulation with 50 μl LPS (4 μg/ml). After 18 h of LPS stimulation, 20 μl MTT (5 mg/ml) was added to each well, and the cells were further incubated for an additional 4 h. The 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 (TECAN, Austria).

2.3.4. ELISA assay

Primary mouse macrophages were plated onto 24-well plates (1.0×10^5 cells/well), and treated with various concentrations of geniposide for 1 h followed by incubation with or without 1 μg/ml LPS for 18 h. Cell-free supernatants were subsequently employed for the pro-inflammatory cytokine assays using a mouse ELISA kit, according to the manufacturer's instructions.

2.3.5. Western blot analysis

The total proteins from the treated cells were extracted by M-PER Mammalian Protein Extraction Reagent (Thermo). The protein concentration was determined by the BCA method. Individual samples, each containing 15 μl protein and 5 μl LDS Sample Buffer, were separated by SDS-PAGE using Tris–HCl Precast Gels. The PVDF membrane had been soaked in anhydrous methanol before it was used for protein transfer. The resulting membrane was blocked with phosphate buffer solution containing 0.05% Tween-20 (PBS-T), supplemented with 5% skim milk (Sigma) at room temperature for 2 h on a rotary shaker, and followed by PBS-T washing. The specific primary antibody, diluted in PBS-T containing skim milk, was incubated with the membrane at 4 °C overnight. Subsequently, the membrane was washed with PBS-T followed by incubation with the peroxidase-conjugated secondary antibody at room temperature for 1 h. The blots were washed twice with PBS-T and then developed with an ECL Plus Western Blotting Detection System.

2.4. In-vivo study

2.4.1. LPS-induced ALI model in mice

All mice were randomly divided into 4 groups: control group, LPS group, geniposide + LPS group and dexamethasone (DEX) + LPS group. Geniposide, 50 mg/kg, and DEX, 0.5 mg/kg, as a positive control, were given intraperitoneally. Control and LPS mice were given an equal volume of distilled water instead of geniposide or DEX. 1 h later, mice were slightly anesthetized with an inhalation of diethyl ether, 10 μg of LPS was instilled intranasally (i.n.) in 50 μl PBS to induce lung injury. The control group mice were given a 50 ml PBS i.n. instillation without LPS. All the mice were alive after 7 h LPS treatment. Collection of bronchoalveolar lavage fluid (BALF) was performed three times through a tracheal cannula with autoclaved PBS, instilled up to a total volume of 1.3 ml.