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Glycyrrhizin inhibits lipopolysaccharide-induced inflammatory response by reducing TLR4 recruitment into lipid rafts in RAW264.7 cells

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ABSTRACT

Background: The aim of this study was to investigate the effect of glycyrrhizin on LPS-induced endotoxemia in mice and clarify the possible mechanism.
Methods: An LPS-induced endotoxemia mouse model was used to confirm the anti-inflammatory activity of glycyrrhizin in vivo. In vitro, RAW264.7 cells were stimulated with LPS in the presence or absence of glycyrrhizin. The expression of cytokines was determined by ELISA. Toll-like receptor 4 (TLR4) was determined by Western blot analysis. Nuclear factor-kB (NF-κB) and Interferon regulatory factor 3 (IRF3) activation were detected by Western blotting and luciferase assay. Lipid raft staining was detected by immunocytochemistry.
Results: In vivo, the results showed that glycyrrhizin can improve survival during lethal endotoxemia. In vitro, glycyrrhizin dose-dependently inhibited the expression of TNF-α, IL-6, IL-1β and RANTES in LPS-stimulated RAW264.7 cells. Western blot analysis showed that glycyrrhizin suppressed LPS-induced NF-κB and IRF3 activation. However, glycyrrhizin did not inhibit NF-κB and IRF3 activation induced by MyD88-dependent (MyD88, IKKβ) or TRIF-dependent (TRIF, TBK1) downstream signaling components. Moreover, glycyrrhizin did not affect the expression of TLR4 and CD14 induced by LPS. Significantly, we found that glycyrrhizin decreased the levels of cholesterol of lipid rafts and inhibited translocation of TLR4 to lipid rafts. Moreover, glycyrrhizin activated ABCA1, which could induce cholesterol efflux from lipid rafts.
Conclusion: Glycyrrhizin exerts an anti-inflammatory property by disrupting lipid rafts and inhibiting translocation of TLR4 to lipid rafts, thereby attenuating LPS-mediated inflammatory response.
General significance: Learning the anti-inflammatory mechanism of glycyrrhizin is crucial for the anti-inflammatory drug development.

1. Introduction

Inflammation is a reaction of tissue to irritation, injury or infection, typically caused by various bacterial infections [1]. Bacterial LPS, a potent immune system activator, is an important risk factor for inflammation. Macrophages play an important role in various inflammatory responses [2]. LPS activates macrophages, triggering inflammatory mediators, including 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) [3–5]. These inflammatory mediators lead to inflammation and various other clinical manifestations.

Toll-like receptors (TLRs) are a large family of Type I transmembrane receptors that play an integral role in the innate immune system. A total of 13 Toll-like receptors (TLRs) have been recently identified [6–8]. Located on the cell plasma membrane or within endosomes, TLRs recognize conserved, characteristic molecular structures on infectious agents called "pathogen associated molecular patterns" (PAMPs). LPS, the integral molecules within the outer membrane of Gram-negative bacteria, activates the expression of TLR4 [9–11]. LPS primarily signals via TLR4 receptors. TLR4 associates with CD14 and MD-2 to recognize LPS. First, LPS binds to CD14 to form the CD14–LPS–LBP complex. Then, the complex is presented to the TLR4–MD-2 complex [12]. Finally, the intact plasma membrane microdomains facilitate the formation of the receptor complex to initiate TLR4 signaling.

The activation of TLR4 by LPS induces MyD88-dependent and MyD88-independent signaling pathways. The MyD88-dependent pathway requires signal transduction intermediates such as IL-1RI-associated protein kinases (IRAKs), and transforming growth factor-activated kinase (TAK1), for the activation of NF-κB and the production of pro-inflammatory cytokines [13]. The MyD88-independent pathway requires

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signal transduction intermediates, such as TICAM1 and TICAM2, to ultimately activate the transcription factor IRF3 and to induce the production of IFNβ and IFN-inducible genes [14,15]. The activation of TLR4 by LPS induces NF-κB and IRF3 activation, resulting in the release of cytokines [13]. Therefore, treatments aimed at modulating TLR4 signaling might have potential therapeutic advantages for inflammatory diseases.

Glycyrrhizin, a triterpene glycoside isolated from licorice root (Fig. 1), is responsible for the pharmacological activities of this plant. It has been shown that glycyrrhizin exhibits a broad spectrum of anti-inflammatory effect. Glycyrrhizin inhibits nitric oxide (NO) activity, prostaglandin E_2 (PEG_2) activity and inflammatory cytokine production in LPS-activated macrophages [16] and LPS-induced acute lung injury in mice [17]. Recently, it has been reported that the anti-inflammatory effects of glycyrrhizin are mediated by blocking the activation of NF-κB signaling [18,19]. However, the molecular targets of the anti-inflammatory actions of glycyrrhizin in LPS-stimulated macrophages remain unclear.

The aim of this work was to examine the anti-inflammatory effects of glycyrrhizin in LPS-stimulated macrophage and to identify the molecular targets of glycyrrhizin in the TLR4 signaling pathway. The results showed that glycyrrhizin inhibited LPS-induced NF-κB and IRF3 activation and cytokine production through the inhibition of TLR4 translocation and the disruption of lipid rafts.

2. Materials and methods

2.1. Materials

Glycyrrhizin (99.8%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 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) and fetal bovine serum (FBS) were obtained from Hyclone. Mouse TNF-α, IL-6 and IL-1β ELISA kits were purchased from BioLegend (CA, USA). Mouse RANTES ELISA kits were purchased from R&D Systems (Minneapolis, MN). Mouse mAb Phospho-NF-κB, mouse mAb NF-κB, mouse mAb Phospho-IRF3 and rabbit mAb IRF3 were purchased from Cell Signaling Technology Inc. (Beverly, MA). HRP-conjugated goat anti-rabbit antibodies were provided by GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

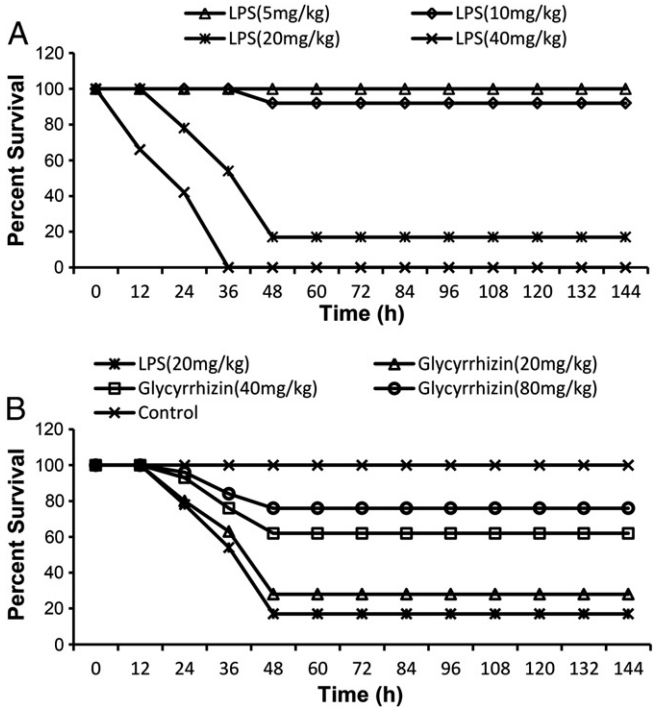

Fig. 1. Effects of glycyrrhizin on LPS-induced lethality in mice. Mice were given an intraperitoneal injection of glycyrrhizin (20, 40 and 80 mg/kg) 1 h prior to LPS challenged. A, the survival rate of mice challenged with LPS of different doses. B, effect of glycyrrhizin (20, 40 and 80 mg/kg) treatment on LPS-induced lethality. The survival was monitored every 12 h for 7 days. $^{\#}p < 0.01$ vs. control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS group.

2.2. Cell culture and treatment

The RAW264.7 mouse macrophage cell line was purchased from the China Cell Line Bank (Beijing, China) and cultured in DMEM containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. HEK293 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Media was changed once every 48 h. In all experiments, macrophages were incubated in the presence or absence of various concentrations of glycyrrhizin that was always added 1 h prior to LPS (1 μg/ml) treatment.

2.3. Plasmids

Recombinant vectors encoding TLR4 (NM_021297), MD2 (NM_001159711), MyD88 (NM_010851), TRIF (BC094338), and IKKβ (AF026524; GenBank accession numbers in parentheses) were generated by the PCR-based amplification of RAW264.7 cDNA, followed by subcloning into the pcDNA3.1 eukaryotic expression vector (Invitrogen) as previously described. The TBK1 and IFNβ PRDIII-I luciferase plasmids were obtained from Kate Fitzgerald (University of Massachusetts Medical School) via Addgene. The NF-κB-luciferase reporter plasmid was purchased from Stratagene (La Jolla, CA, USA).

2.4. Animals

Male BALB/c mice, 6–8 weeks, weighing approximately 18 to 20 g, were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China). And this study was approved by the Jilin University Animal Care and Use Committee. The protocols were reviewed and approved by the committee. The mice were housed in microisolator cages and received food and water. The laboratory temperature was 24 ± 1 °C, and relative humidity was 40–80%. Mice were housed for 4–6 days to adapt the environment before experimentation. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.5. LPS-induced endotoxemia in mice

The 48 healthy male BALB/c mice were randomly classified into four groups and challenged with LPS (5–40 mg/kg) by i.p. The mortality of mice was observed twice a day for 7 days. In drug testing, the effect of glycyrrhizin (20, 40 and 80 mg/kg) on LPS-induced mortality was assessed by given glycyrrhizin 1 h before LPS challenge. Survival in each group was assessed every 12 h for 7 days.

2.6. Cell transfection and luciferase assay

HEK293 cells were co-transfected with a luciferase plasmid and various expression plasmids or the corresponding empty plasmid vectors using FuGENE HP transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The β-galactosidase plasmid was co-transfected as an internal control. Luciferase activity was measured using the Luciferase Reporter-Gene Assay (Promega). β-Galactosidase enzyme activity was determined