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Magnolol inhibits lipopolysaccharide-induced inflammatory response by interfering with TLR4 mediated NF-κB and MAPKs signaling pathways

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ABSTRACT

Ethnopharmacological relevance: *Magnolia officinalis* as a traditional Chinese herb has long been used for the treatment of anxiety, cough, headache and allergic diseases, and also have been used in traditional Chinese medicine to treat a variety of mental disorders including depression.
Aim of the study: Magnolol, a hydroxylated biphenyl compound isolated from *Magnolia officinalis*, has been reported to have anti-inflammatory properties. However, the underlying molecular mechanisms are not well understood. The aim of this study was to investigate the molecular mechanism of magnolol in modifying lipopolysaccharide (LPS)-induced signal pathways in RAW264.7 cells.
Material and methods: The purity of magnolol was determined by high performance liquid chromatography. RAW264.7 cells were stimulated with LPS in the presence or absence of magnolol. The expression of proinflammartory cytokines were determined by ELISA and reverse transcription-PCR. Nuclear factor-κB (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 analyses were performed on mTLR4 and mMD2 co-transfected HEK293 cells.
Results: The result showed that the purity of magnolol used in this study was 100%. Magnolol inhibited the expression of TNF-α, IL-6 and IL-1β in LPS-stimulated RAW264.7 cells in a dose-dependent manner. Western blot analysis showed that magnolol suppressed LPS-induced NF-κB activation, IκBα degradation, phosphorylation of ERK, JNK and P38. Magnolol could significantly down-regulated the expression of TLR4 stimulating by LPS. Furthermore, magnolol suppressed LPS-induced IL-8 production in HEK293-mTLR4/MD-2 cells.
Conclusions: Our results suggest that magnolol exerts an anti-inflammatory property by down-regulated the expression of TLR4 up-regulated by LPS, thereby attenuating TLR4 mediated the activation of NF-κB and MAPK signaling and the release of pro-inflammatory cytokines. These findings suggest that magnolol may be a therapeutic agent against inflammatory diseases.

1. Introduction

Inflammation is a reaction of tissue to irritation, injury or infection which is usually caused by various bacteria (Conese and Assael, 2001). Bacterial LPS, a potent immune system activator, has been referred to be an important risk factor of inflammation. Macrophages play an important role in various inflammatory responses (Mayeux, 1997). LPS activates macrophages triggering the pro-inflammatory mediators which include tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), matrix metalloproteinases (MMP), cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and prostaglandin E2 (PGE2) (Boje, 2004; Hartlage-Rubsamen et al., 1999; Takeuchi et al., 2006). These pro-inflammatory mediators lead to inflammation and various other clinical manifestations.

Toll-like receptors (TLRs) located on the cell plasma membrane or within endosomes, are a large family of type I transmembrane receptors that play an integral role in the innate immune system. Recently, 13 kinds of Toll-like receptors (TLRs) have been reported

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(Akira et al., 2006; Alexopoulou et al., 2001; Takeda and Akira, 2005). TLR4 as one of the best characterized TLRs can associate with CD14 and MD-2 to recognize LPS from Gram-negative bacteria (Akashi et al., 2001; da Silva Correia et al., 2001; Nagai et al., 2002). LPS binds to CD14 to form the CD14-LPS-LBP complex. Then the complex is presented to the TLR4-MD-2 complex (Triantafilou and Triantafilou, 2002). LPS signals mainly via TLR4 receptors. Activation of TLR4 by LPS induces the activation of NF-κB and MAPK pathways and finally results in the release of pro-inflammatory cytokines (Medzhitov and Kagan, 2006). Therefore, treatments aimed at modulating TLR4 signaling pathways may have potential therapeutic advantages for inflammatory diseases.

Magnolia officinalis as a traditional Chinese herb has long been used for the treatment of anxiety, cough, headache and allergic diseases (Chen et al., 2011; Matsuda et al., 2001; Wang et al., 1995; Wu et al., 2011), and also have been used in traditional Chinese medicine to treat a variety of mental disorders including depression (Maruyama et al., 1998; Nakazawa et al., 2003; Watanabe et al., 1983). Magnolol, a compound purified from *Magnolia officinalis,* is responsible for the plant's pharmacological activities. It has been shown that magnolol exhibited a board spectrum anti-inflammatory effect. Magnolol was found to inhibit nitricoxide (NO) production in LPS-activated macrophages (Matsuda et al., 2001), to suppress inflammatory cytokines production in THP-1 cells (Park et al., 2004) and to inhibit TPA-induced skin inflammation in mice (Kuo et al., 2010). Recently, it has been reported that the anti-inflammatory effects of magnolol are mediated by blocking the activation of NF-κB and MAPKs signaling pathways (Lai et al., 2011; Li et al., 2010). However, the detailed mechanisms underlying the anti-inflammatory effect of magnolol in LPS-stimulated macrophage remain unclear. In this study, we sought to examine the anti-inflammatory effects of magnolol in LPS-stimulated macrophage and elucidate the potential anti-inflammatory mechanism. The results showed that magnolol inhibits LPS-induced pro-inflammatory cytokines by interfering with TLR4 mediated NF-κB and MAPK signaling pathways.

2. Materials and methods

2.1. Chemicals and reagents

Magnolol was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China) (Fig. 1A). 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), fetal bovine serum (FBS) 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 and mouse mAb NF-κB, 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. High-performance liquid chromatography (HPLC)

The purity of magnolol was determined by high performance liquid chromatography. The test was performed on an Agilent 1100 series (Agilent Technologies, Palo Alto, CA). Chromatography was performed through an ODS-3 analytical HPLC column (5 μm, 150 × 4.6 mm, Phenomenex, Torrance, CA). Elution was carried out with acetonitrile/ultrapure water (v/v, 70:30), operating at a flow rate of 1 mL min^{-1} with UV detection at 294 nm. The column temperature was controlled at 35 °C.

2.3. Cell culture and treatment

The RAW 264.7 mouse macrophage cell line was purchased from the China Cell Line Bank (Beijing, China) and cultured in DMEM containing 10% FBS at 37 °C with 5% CO_2. HEK293 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in DMEM containing 10% FBS at 37 °C with 5% CO_2. The culture media was changed once every 48 h. In all experiments, macrophages were incubated in the presence or absence of various concentrations of magnolol that was always added 1 h prior to LPS (1 μg/ml) treatment.

2.4. Cell transfection

HEK293 cells were co-transfected with pEGFP-N1-m*TLR*4 and pDsRED-N1-m*MD*2 plasmids using FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions.

2.5. 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 treated with 50 μl of magnolol at different concentrations (0–60 μg/ml) for 1 h, followed by stimulation with 50 μl LPS. 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).

2.6. Total RNA extraction and RT-PCR

Total RNA from RAW264.7 cells was extracted using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions after the cells were stimulated with 1.0 μg/ml LPS for 3 h. The RNA was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo). The gene expressions of TNF-α, IL-1β and IL-6 were amplified from the synthesized cDNA. The primers used were listed in Table 1. The PCR reactions were carried out as the following: 94 °C for 3 min; 30 cycles of 94 °C for 45 s, 58 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products were verified on a 1.5% agarose gel.

2.7. ELISA assay

RAW 264.7 cells or HEK293-mTLR4/MD-2 cells were seeded in 24-well plates (10^5 cells/well), and incubated in the presence of either LPS alone 1 μg/ml, or LPS plus magnolol 15, 30, 60 μg/ml for 18 h. Cell-free supernatants were subsequently employed for the pro-inflammatory cytokine assays using a mouse enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (BioLegend, Inc, Camino Santa Fe, Suite E, San Diego, CA, USA).