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Inhibitory effects of a spinasterol glycoside on lipopolysaccharide-induced production of nitric oxide and proinflammatory cytokines via down-regulating MAP kinase pathways and NF-κB activation in RAW264.7 macrophage cells

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ABSTRACT

Extracts from the leaves of *Stewartia koreana* are known to exhibit strong anti-inflammatory activity. Investigation of bioactive compounds from *S. koreana* has led to the isolation of 3-*O*-β-D-glucopyanosylspinasterol (spinasterol-Glc), a spinasterol glycoside. In the present study, we examined the effects of spinasterol-Glc on production of nitric oxide (NO) and proinflammatory cytokines in LPS-treated RAW264.7 macrophage cells and in mouse models. Our results showed that spinasterol-Glc inhibited the production of NO and proinflammatory cytokines such as TNF-α, IL-6 and IL-1β in dose-dependent manners in LPS-treated RAW264.7 cells. Spinasterol-Glc inhibited the expression of iNOS and the proinflammatory cytokine genes. Spinasterol-Glc also inhibited phosphorylation of IκB-α and IKKα/β as well as translocation of NF-κB to the nucleus. We demonstrated that spinasterol-Glc reduced transcription of the NF-κB minimal promoter and NF-κB DNA binding activity. Administration of the spinasterol-Glc significantly decreased the plasma levels of these inflammatory mediators including TNF-α, IL-6 and IL-1β in LPS-injected mice and improved survival of septic mice with lethal endotoxemia. These results suggest that spinasterol-Glc has effective inhibitory effects on production of inflammatory mediators via inhibition of MAP kinases/NF-κB activities, and can be used as a potential anti-inflammatory agent for the prevention and treatment of inflammatory diseases.

1. Introduction

Inflammation is a physiological response to injury associated with physical, chemical stimuli or biological toxins, and is a protective process of the body that functions to destroy invading organisms or to repair tissues after injury. However, excessive inflammation causes chronic inflammatory diseases including arthritis, asthma, multiple sclerosis and atherosclerosis [1–4]. One of the most potent stimuli for macrophage is bacterial endotoxin (lipopolysaccharide, LPS), which activates cells via Toll-like receptor 4 (TLR4) and induces the production of large amounts of pro-inflammatory cytokines such as TNF-α and IL-1β [5–8]. The inflammatory response is accompanied by the upregulation of inflammatory cytokines and the release of various inflammatory mediators including nitric oxide (NO) and prostaglandins. NO is generated via the oxidative deamination of L-arginine by a family of nitric oxide synthases [9]. In particular, inducible NOS (iNOS), which is expressed in macrophages in response to pro-inflammatory cytokines and LPS, plays important roles in inflammatory processes [10,11].

Stimulation of cells with inflammatory stimuli initiates a cascade of signaling events, including nuclear factor kappa B (NF-κB) and MAP kinase pathways such as extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 kinase pathway [12,13]. NF-κB, which induces expression of cytokines, factors and enzymes including tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β), interleukin-6 (IL-6), and iNOS, has a key role in the regulation of inflammatory responses [14]. The major form of NF-κB is a heterodimer composed of p50 and p65, both of which belong to the Rel family. In unstimulated cells, NF-κB resides in cytoplasm through the association with inhibitor proteins of the IκB family, such as IκB-α and IκB-β. Activated IKKs phosphorlyate IκBs, leading to the ubiquitination and proteasomal degradation of IκBs [15–17]. NF-κB factors are released, translocate into the nucleus where they activate the transcription of target genes, including inflammatory cytokines and iNOS.

A variety of herbs and plants have been traditionally used in oriental folk medicine for the treatment of inflammatory diseases. *Stewartia koreana* (family Theaceae), a deciduous tree, is native to, and grows throughout Korea. Several studies reported that extracts of *S. koreana* leaves exhibited various biological activities, such as antioxidant activity,

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inhibition of NO production in LPS-stimulated macrophage cells, and inhibition of HIV protease activity [18–20]. A number of phytosterols such as α-amyrin, isoquercitrin, and 3-*O*-β-D-glucopyanosylspinasterol (spinasterol-Glc) were isolated from methanol extracts of *S. koreana* leaves. Recently, spinasterol-Glc was shown to promote procollagen production and to inhibit matrix metalloproteinase-1 expression in UVB-irradiated human dermal fibroblast cells [21]. In the present study, we demonstrate that spinasterol-Glc strongly inhibits the production of NO and proinflammatory cytokines such as TNF-α, IL-6 and IL-1β in LPS-stimulated RAW264.7 macrophage cells. Our results showed that spinasterol-Glc suppresses expression of the cytokine genes and iNOS via inhibition of NF-κB and MAPKs signaling pathways. Moreover, we evaluated its anti-inflammatory activity in vivo using LPS-injected mouse models.

2. Materials and methods

2.1. Materials and cell culture

The leaves of *S. koreana* were harvested in 80% aqueous MeOH and spinasterol-Glc was isolated from the leaves of *S. koreana* as previously described [21]. Cell culture medium, reagents and other chemicals were obtained commercially as indicated. Murine macrophage RAW264.7 (ATCC) cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM glutamate, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin in humidified incubator of 5% CO_2. Cells were stimulated with LPS (Sigma, St Louis, MO, USA) at 1 μg/ml concentration along with various concentrations of spinasterol-Glc for 12 h to 24 h as indicated.

2.2. Animal experiment

Animal experiments were approved by the Institutional Review Board (IRB), Kyung Hee University (KHUASP-10-009). Male C57BL/6NCrSlc black mice (8 weeks, 25–30 g, Central Lab. Animal Inc. Korea) were housed in an environmentally controlled room with a 12 h light/dark cycle and allowed free access to food and water. Mice were divided into four groups and treated with saline, LPS (4 mg/kg), LPS with spinasterol-Glc (10 mg/kg) and LPS with spinasterol-Glc (50 mg/kg). Mice were peritoneally injected with spinasterol-Glc or saline twice at 24 h intervals. After 12 h of the second injection, LPS or saline was intraperitoneally injected. Twelve hours later, a whole blood sample was collected by infraorbital plexus and plasma was prepared by centrifugation at 12,000 *g* for 20 min at 4 °C. Animals were sacrificed under light anesthesia with diethyl ether. For evaluation of spinasterol-Glc in the LPS model of endotoxemia, 8-weeks old C57BL6 mice were used. Spinasterol-Glc was intraperitoneally injected at a dose of 10 mg/kg 24 h before LPS challenge, which was injected intraperitoneally at a dose of 30 mg/kg. The death of an animal was monitored by vigorous physical examination. The survival rate was studied in $n = 11$ in each group, and recorded for over the next 7 days.

2.3. Measurement of NO and cell viability assay

Nitric oxide was determined by measuring the amount of nitrite, a stable oxidized product in cell culture supernatant as previously described [19]. To test the effect of the spinasterol-Glc on the iNOS enzymatic activity, RAW264.7 cells (1×10^4 cells/well) were grown in culture media in 96-culture well plate for 24 h. Cells were washed with PBS twice and pretreated with spinasterol-Glc in serum-free medium for 6 h. Cells were then stimulated with 1 μg/ml of LPS (Sigma, St. Louis, MO, USA) in the presence of spinasterol-Glc for 18 h. One hundred microliters of cell culture supernatant was mixed with 100 μl of Griess reagent (Sigma, St. Louis, MO, USA) in a 96-well plate, followed by spectrophotometric measurement at 550 nm according to the manufacturer's instructions. Nitrite concentrations in the supernatant were determined by comparison with a sodium nitrite standard curve. The cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-thetazolium bromide (MTT) assay as previously described [19].

2.4. Reverse transcriptase-polymerase chain reaction analysis

Total RNA was prepared from RAW264.7 cells using a Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA). Total RNA (2 μg) was reverse transcribed using M-MuLV reverse transcriptase (Fermentas Life Science). The following primers were used for PCR amplification: iNOS, 5′-TCTTCGAAATCCCACCTGAC-3′ (sense) and 5′-CCATGATGGTCACATTCT-GC-3′ (antisense), TNF-α, 5′-TTGACCTCAGCGCTGAGTTG-3′ (sense) and 5′-CCTGTAGCCCACGTCGTAGC-3′ (antisense); IL-6, 5′-GAGGATACCACT-CCCAACAG-3′ (sense) and 5′-TTCACAGAGGATACCACTCC-3′ (antisense); IL-1β, 5′-GAAGCTGTGGCAGCTACCTATGTCT-3′ (sense) and 5′-CTCTGCTT-GTGAGGTGCTGATGTAC-3′ (antisense). β-actin mRNA levels were used as internal controls.

2.5. Western blot analysis

RAW264.7 cells were pretreated with various concentrations of spinasterol-Glc for 6 h and then stimulated with 1 μg/ml of LPS for 18 h. The cells were lysed in RIPA buffer containing proteinase inhibitors. Protein concentration was quantified with a protein assay kit (Bio-Rad Laboratories). Proteins (50 μg/lane for analysis of IκB; 20 μg/lane for other samples) were resolved with SDS-polyacrylamide gel electrophoresis, and Western blot analysis was performed as described previously [19]. Rabbit anti-iNOS and anti-p65 (Research Santa Cruz, Santa Cruz, CA), mouse anti-phospho IκB-α, rabbit anti-IκB-α, rabbit anti-phospho IKKα/β, mouse anti-IKKα/β, phospho ERK1/2, ERK1/2, phospho JNK, JNK, phospho p38 and p38 (Cell signaling, Denvers, MA) were utilized as primary antibodies and peroxidase-conjugated antibody was used as a secondary antibody. The membranes were developed with an enhanced chemiluminescence system from Amersham and exposed for 30 s to X-ray film (FUJI Photo Film Co., Ltd).

2.6. ELISA assay

Levels of pro inflammatory cytokine such as TNF-α, IL-1β and IL-6 were measured in cell culture media and in mouse plasma using ELISA kits (Bender MedSystems, Inc., USA). Murine RAW264.7 peritoneal macrophage cells were cultured in 48-well plates for 24 h, washed with PBS, and grown in fresh medium containing various concentrations of spinasterol-Glc. Six hours after pretreatment with the compound, macrophage cells were stimulated with 1 μg/ml of LPS for 18 h and supernatants of the cultures were collected. The levels of TNF-α, IL-1β and IL-6 released into the culture supernatants were measured using enzyme-linked immunosorbent assay kits according to the manufacturers' recommendations.

2.7. Electrophoretic mobility shift assay (EMSA)

RAW264.7 cells were pretreated with various concentrations of spinasterol-Glc for 6 h and then stimulated with LPS for 2 h. Preparation of nuclear extract and EMSA was performed as described previously [19]. A double-stranded NF-κB-specific deoxyoligomer (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and a non-specific deoxyoligomer (5′-CGTGGGAAAATCCAGT-3′) were used as a probe and competitors. Oligomer probe was radiolabeled by kination using [γ-^{32}P] ATP (Amersham, USA) and T4 polynucleotide kinase. The reaction products were separated on 6% polyacrylamide gel. The gel was dried and subjected to autoradiography.