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Research article

Molecular mechanism of protopanaxadiol saponin fraction-mediated anti-inflammatory actions

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A B S T R A C T

Background: Korean Red Ginseng (KRG) is a representative traditional herbal medicine with many different pharmacological properties including anticancer, anti-atherosclerosis, anti-diabetes, and anti-inflammatory activities. Only a few studies have explored the molecular mechanism of KRG-mediated anti-inflammatory activity.
Methods: We investigated the anti-inflammatory mechanisms of the protopanaxadiol saponin fraction (PPD-SF) of KRG using *in vitro* and *in vivo* inflammatory models.
Results: PPD-SF dose-dependently diminished the release of inflammatory mediators [nitric oxide (NO), tumor necrosis factor-α, and prostaglandin E_2], and downregulated the mRNA expression of their corresponding genes (inducible NO synthase, tumor necrosis factor-α, and cyclooxygenase-2), without altering cell viability. The PPD-SF-mediated suppression of these events appeared to be regulated by a blockade of p38, c-Jun N-terminal kinase (JNK), and TANK (TRAF family member-associated NF-kappa-B activator)-binding kinase 1 (TBK1), which are linked to the activation of activating transcription factor 2 (ATF2) and interferon regulatory transcription factor 3 (IRF3). Moreover, this fraction also ameliorated HCl/ethanol/-induced gastritis via suppression of phospho-JNK2 levels.
Conclusion: These results strongly suggest that the anti-inflammatory action of PPD-SF could be mediated by a reduction in the activation of p38-, JNK2-, and TANK-binding-kinase-1-linked pathways and their corresponding transcription factors (ATF2 and IRF3).
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1. Introduction

Inflammation is an important process because it is one of the natural defense mechanisms caused by the release of inflammatory mediators [e.g., (nitric oxide) NO and prostaglandin (PG)E_2], cytokines [e.g., tumor necrosis factor (TNF)-α], and chemokines [1,2]. This event requires the activation of inflammatory cells such as macrophages via the ligation of their surface receptors (e.g., Toll-like receptors) [3]. The activation of Toll-like receptors in macrophages by ligands derived from pathogens triggers various cellular signaling cascades to activate transcription factors including nuclear factor (NF)-κB (p50 and p65), activator protein (AP)-1 [c-Fos, c-Jun, and activating transcription factor (ATF)-2], and interferon regulatory transcription factor (IRF)-3 to trigger the new expression of inflammatory genes [4–6]. Although inflammation is a normal response, acutely, excessive induced, or chronically sustained

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inflammatory responses are known to cause serious diseases including cancer, stroke, and diabetes. Therefore, it must be stressed that normalization of upregulated inflammation is crucial in prevention of such diseases [7–9].

Korean Red Ginseng (KRG, steamed root of *Panax ginseng* Meyer, Araliaceae) is a well-known herbal medicine traditionally used in Korea [10]. It has been used for a long time without displaying any toxic properties, thus, developing some anti-inflammatory preparation with KRG could be considered beneficial. Unlike acid polysaccharides that are known as major components contributing to upregulation of the body's immune responses [11], red ginseng saponin fractions enriched with protopanaxadiol (PPD)-type ginsenosides have been reported as strong anti-inflammatory preparations [12]. Some PPD-type ginsenosides such as ginsenoside (G)-Rb1, G-Rb2, and G-Rd display strong anti-inflammatory properties under various conditions [13]. This notion led us to establish a hypothesis that PPD-type saponins could be used as an anti-inflammatory remedy. In this study, therefore, we investigated the anti-inflammatory activity and molecular mechanism of the protopanaxadiol saponin fraction (PPD-SF).

2. Materials and methods

2.1. Materials

PPD-SFs, prepared by previously established methods [14], from KRG with higher amounts of protopanaxadiol-type ginsenosides (G-Rb1, G-Rc, G-Re, and G-Rb2) were kindly supplied by the Korea Ginseng Cooperation (Daejeon, Korea). N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BX795 and SP600125 were obtained from Calbiochem (La Jolla, CA, USA). Luciferase constructs containing promoters with binding sites for NF-κB, AP-1, and IRF-3 were used, as reported previously [15]. RAW264.7 cells, a BALB/c-derived murine macrophage cell line (ATCC No. TIB-71), and HEK293 cells, a human embryonic kidney cell line (ATCC No. CRL-1573), were obtained from American Tissue Culture Collection (Rockville, MD, USA). TANK (TRAF family member-associated NF-kappa-B activator)-binding kinase (TBK)1 and adaptor molecule [TIR-domain-containing adapter-inducing interferon-β (TRIF) or myeloid differentiation primary response gene 88 (MyD88)] were used as reported previously [16]. Fetal bovine serum and RPMI 1640 were purchased from Gibco (Grand Island, NY, USA), and phospho-specific or total antibodies to c-Jun, c-Fos, ATF-2, IRF-3, extracellular signal-regulated kinase (ERK), p38, C-Jun N-terminal kinase (JNK), mitogen-activated protein kinase kinase 4 (MKK4), MKK3/6, transforming growth factor-β-activated kinase 1 (TAK1), TBK1, lamin A/C, and β-actin were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals were purchased from Sigma Chemical Co.

2.2. Treatment of PPD-SF

A stock solution (8 mg/mL) of PPD-SF was prepared with culture medium and diluted to 0–400 μg/mL: with media for *in vitro*, cellular assays, or suspended in 1% sodium carboxymethylcellulose for *in vivo* experiments.

2.3. Animal experiments

Male imprinting control region (ICR) mice (6–8 weeks old, 17–21 g) were obtained from Daehan Biolink (Chungbuk, Korea) and maintained in plastic cages under standard conditions. Water and pelleted food (Samyang, Daejeon, Korea) were supplied *ad libitum*. Studies (approval ID: SKKUBBI 13-6-2) were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee at Sungkyunkwan University, Suwon, Korea.

2.4. Cell culture

RAW 264.7 and HEK293 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine, and antibiotics (penicillin and streptomycin) at 37°C under 5% CO_2. For experiments, cells were detached with a cell scraper. Under our experimental cell density (2×10^6 cells/mL), the proportion of dead cells was < 1% according to Trypan blue dye exclusion tests.

2.5. NO, PGE_2, and TNF-α production

After preincubation for 18 hours, RAW264.7 cells (1×10^6 cells/mL) were pretreated with PPD-SF (0–400 μg/mL) or the standard compounds (L-NAME, SP600125, or BX795), and incubated with LPS (1 μg/mL) for 24 hours. The inhibitory effects of PPD-SF or standard compounds on NO, TNF-α, or PGE_2 production were determined by analyzing the NO, PGE_2, or TNF-α levels quantified with Griess reagent, enzyme immunoassay, or enzyme-linked immunosorbent assay, respectively, as described previously [17,18].

2.6. Cell viability test

After preincubation for 18 hours, PPD-SF (0–400 μg/mL) was added to RAW264.7 cells (1×10^6 cells/mL) followed by incubation for 24 hours. The cytotoxic effects of PPD-SF were evaluated by MTT assay, as reported previously [19,20].

2.7. HPLC of PPD-SF

Phytochemical characteristics of PPD-SF with standard ginsenosides were identified by high performance liquid chromatography (HPLC) as reported previously [21,22]. The HPLC system was equipped with a Knauer (Wellchrom, Berlin, Germany) HPLC-pump K-1001, a Wellchrom fast scanning spectrophotometer K-2600, a WellChrom UV Detector K-2600, and a four-channel degasser K-500. Elution solvent (acetonitrile), step gradients (0, 20%, 32%, 50%, 65%, or 90% for 0 minutes, 10 minutes, 40 minutes, 55 minutes, 70 minutes, or 80 minutes, 1.6 mL/minute, 203 nm), and a phenomenex gemini C_{18} ODS (250 mm × 4.6 mm, 5 μm) column were used. Based on these conditions, the contents of ginsenosides from PPD-SF were calculated with the peak area curve of standard ginsenosides.

2.8. mRNA analysis by quantitative reverse transcriptase-polymerase chain reaction

To evaluate cytokine mRNA expression levels, RAW264.7 cells pretreated with PPD-SF (0–400 μg/mL) for 30 minutes were incubated with LPS (1 μg/mL) for 6 hours. Total RNA was isolated with TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions and stored at −70°C until use. The mRNA was quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR) with SYBR Premix Ex Taq, according to the manufacturer's instructions (Takara, Shiga, Japan), using a real-time thermal cycler (Bio-Rad, Hercules, CA, USA), as reported previously [23,24]. Results were expressed as the ratio of the optical density relative to glyceraldehyde 3-phosphate dehydrogenase. The primers used (Bioneer, Seoul, Korea) are described in Table 1.

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