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

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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)E2], 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...
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^2-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 O111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tissue Culture Collection (Rockville, MD, USA). TANK (TRAF family and/or-associated NF-kappa-B activator)-binding kinase (TBK)1 and adaptor molecule (TIR-domain-containing-adapter-interferon-β (TRIF) or myeloid differentiation primary response gene 88 (MyD88)) 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-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% CO2. 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, PGE2, 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 PGE2 production were determined by analyzing the NO, PGE2, 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, 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 phenom-exnemex Gemini C18 3 μm, 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 (Bio- neer, Seoul, Korea) are described in Table 1.
2.9. Plasmid transfection and luciferase reporter gene activity assay

HEK293 cells (1 x 10^6 cells/mL) were transfected with 1 μg of plasmid containing β-galactosidase and NF-κB-Luc, AP-1-Luc, or IRF-3-Luc in the presence or absence of PMA, or overexpressed adaptor molecules (TRIF or MyD88) using the polyethylenimine (PEI) method in 12-well plates. The cells were treated with PPD-SF for 12 hours prior to termination. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison, WI, USA), as previously reported [24,25].

2.10. Preparation of total lysates and nuclear extracts, and immunoblotting analysis

Stomach tissues or RAW264.7 cells (5 x 10^6 cells/mL) were washed three times in cold phosphate-buffered saline with 1mM sodium orthovanadate, and then lysed using a sonicator (Thermo Fisher Scientific, Waltham, MA, USA) or a Tissuemizer (Qiagen, Germantown, MD, USA) in lysis buffer [26] for 30 minutes with rotation at 4°C. Lysates were clarified by centrifugation at 16,000 x g for 10 minutes at 4°C and stored at -20°C until use. Nuclear fractions were prepared with RAW264.7 cell-derived lysates in a three-step procedure [27]. After treatment, cells were collected with a rubber policeman, washed with 1/2 phosphate-buffered saline, and lysed in 500 μL lysis buffer [28] on ice for 4 minutes. Lysates were centrifuged at 19,326 x g for 1 minute in a microcentrifuge. The pellet (nuclear fraction) was washed once in washing buffer (lysis buffer without Nonidet P-40) and then treated with extraction buffer (lysis buffer containing 500mM KCl and 10% glycerol). The nuclei/extraction buffer mixture was frozen at -80°C, thawed on ice, and centrifuged at 19,326 x g for 5 minutes. The supernatant was collected as a nuclear extract. Soluble cell lysates (30 μg/lane) were immunoblotted. Either the phosphorylated or total levels of c-Jun, c-Fos, ATF2, FRA (Fos-related antigen), IRF3, ERK, p38, JNK, MKK4, MKK3/6, TAK1, TBK1, lamin A/C, and β-actin were visualized as previously described [29].

### Table 1

| Gene name | Sequence (5’→3’) |
|-----------|------------------|
| iNOS      | F CTCCTCCGAAGTTTCTGGCAGCAG R GGCTGTCAGAGCCTCGTGGCTTTGG |
| COX-2     | F CACTACATCTGAGCCTCCTT R ATGCTCCTGCTTGAGTATGT |
| TNF-α     | F TGGCTATGGCTCCATGCTT R GAGGCATTTGGGAACTT |
| GAPDH     | F GACCTACGGCAATTCACACGGCA R TACCCACGACATACTCAGCAC |

**COX** = cyclo-oxygenase; **GAPDH** = glyceraldehyde 3-phosphate dehydrogenase; **iNOS** = inducible NO synthase; **TNF-α** = tumor necrosis factor; **PDD-SF** = protopanaxadiol saponin fraction; **L-NAME** = Nethylene glycol; **MTT** = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PGE2** = prostaglandin E2; **PPD-SF** = protopanaxadiol saponin fraction; **TNF** = tumor necrosis factor.

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Fig. 1. In vitro anti-inflammatory activity of PPD-SF in RAW264.7 cells and HPLC analysis of PPD-SF. (A) Levels of NO, PGE2, and TNF-α were determined from culture supernatants of RAW264.7 cells treated with LPS (1 μg/mL) in the presence or absence of PPD-SF (left panel) or L-NAME (right panel) for 24 hours. (B) Viability of RAW264.7 cells under PPD-SF exposure in the absence of LPS was determined by MTT assay. (C) Phytocchemical characteristics of ginsenosides in PPD-SF were analyzed using high performance liquid chromatography. **p < 0.05 compared to the control. **p < 0.01 compared to the control. **i-NAME** = N'-Nitro-l-arginine methyl ester hydrochloride; **LPS** = lipopolysaccharide; **MTT** = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PGE2** = prostaglandin E2; **PPD-SF** = protopanaxadiol saponin fraction; **TNF** = tumor necrosis factor.
A

inOS, TNF-α, and COX-2 Inhibition (Gene expression)

LPS (1 μg/mL)  -  +  +  +  +
PPD-SF (μg/mL)  -  50  100  200

B

AP-1-mediated luciferase activity (Fold increase)

PMA (100 nM)  -  +  +  +  +
AP-1-Luc  +  +  +  +  +
PPD-SF (μg/mL)  -  100  200  400

C

AP-1-mediated luciferase activity (Fold increase)

TRIF  -  +  +  +  +
AP-1-Luc  +  +  +  +  +
PPD-SF (μg/mL)  -  100  200  400

D

AP-1-mediated luciferase activity (Fold increase)

MyD88  -  +  +  +  +
AP-1-Luc  +  +  +  +  +
PPD-SF (μg/mL)  -  100  200  400

E

NF-κB-mediated luciferase activity (Fold increase)

PMA (100 nM)  -  +  +  +  +
NF-κB-Luc  +  +  +  +  +
PPD-SF (μg/mL)  -  100  200  400

F

IFN-β-mediated luciferase activity (Fold increase)

TRIF  -  +  +  +  +
IRF-3-Luc  +  +  +  +  +
PPD-SF (μg/mL)  -  100  200  400

G

|                | 30 min | 60 min | LPS (1 μg/mL) | PPD-SF (μg/mL) |
|----------------|--------|--------|---------------|----------------|
| c-Fos          |        |        | -  +  +  +    | -  200         |
| c-Jun          |        |        | -  +  +  +    | -  200         |
| p-ATF2         |        |        | -  +  +  +    | -  200         |
| ATF2           |        |        | -  +  +  +    | -  200         |
| p-FRA          |        |        | -  +  +  +    | -  200         |
| p-IRF3         |        |        | -  +  +  +    | -  200         |
| IRF3           |        |        | -  +  +  +    | -  200         |
| Lamin A/C      |        |        | -  +  +  +    | -  200         |
2.11. Enzyme assay

To evaluate the inhibition of MKK4, MKK6, and MKK7 kinase activities using purified enzymes, we used the kinase profiler service from Millipore (Billerica, MA, USA).

2.12. Ethanol/HCl-induced gastritis

Stomach inflammation was induced in mice using HCl/ethanol according to a published method [30,31]. Fasted ICR mice (7 mice/group) were orally treated with PPD-SF (200 mg/kg) or ranitidine (40 mg/kg) twice daily for 3 days. At 30 minutes after the final injection, 400 µL of 60% ethanol in 150mM HCl was administered orally. Animals were anaesthetized and sacrificed with urethane 1 hour after the administration of necrotizing agents. Stomachs were excised and gently rinsed under running tap water. After opening the stomachs along the greater curvature and spreading them out on a board, the area (mm²) of mucosal erosive lesions was measured using a pixel counter by a technician blinded to the treatment conditions. Experimental groups included a normal group (sham-operated/treated with vehicle), control group (HCl/ethanol injected/treated with vehicle), and drug-treated groups [HCl/ethanol injected/treated with PPD-SF (200 mg/kg) or ranitidine (40 mg/kg)]. Immunoblotting analysis was used to detect the phosphorylated and total levels of JNK from stomach tissue lysates.

2.13. Statistical analysis

The data in this paper are presented as the mean ± standard error of the mean of three different experiments performed using four samples for the in vitro experiments, or as the mean ± standard deviation for the six mice used in the in vivo tests and the kinase assay for three samples. For statistical comparisons, these results were analyzed using analysis of variance/Scheffe’s post hoc and Kruskal–Wallis/Mann–Whitney tests. A p value < 0.05 was considered statistically significant. All statistical tests were performed using the SPSS 16.0 computer program (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

To test the anti-inflammatory activity of PPD-SF, we first used in vitro inflammatory models established with LPS-treated RAW264.7 cells. Under these conditions, we could achieve optimal levels of NO, PGE2, and TNF-α after 24 hours incubation with LPS, as reported previously [15]. The levels of these inflammatory mediators during LPS exposure were 45µM (NO), 21.6 ng/mL (PGE2), and 6.8 ng/mL (TNF-α), whereas normal levels of these mediators were below 0.6µM (NO), 0.01 ng/mL (PGE2), and 0.3 ng/mL (TNF-α). As we expected, PPD-SF (0–400 µg/mL) dose-dependently suppressed the production of these molecules (Fig. 1A left panel), which shows a higher activity than those of KRG water extract [32]. In particular, this fraction more strongly inhibited the release of PGE2, indicating that this fraction is able to ameliorate more effectively PGE2-derived pain and inflammatory responses. The fact that i-NAME, a standard inducible NO synthase inhibitor, diminished NO production (Fig. 1A right panel) validates our in vitro inflammatory models. There was no cytotoxicity seen at up to 400 µg/mL PPD-SF (Fig. 1B), therefore, the inhibitory activity of PPD-SF in in vitro models could not have been due to its nonspecific cytotoxicity. Meanwhile, HPLC analysis showed that this fraction (PPD-SF) mostly contained G-Rb1 (33.2%), G–Rc (29.4%), G-Rb2 (31.7%), and G-Rb3 (5.4%) (Fig. 1C), implying that these specific ginsenosides could contribute to the mediation of the anti-inflammatory activity of PPD-SF.

To understand the molecular mechanism of PPD-SF-induced anti-inflammatory activity, we next examined whether this fraction inhibited the secretion of inflammatory mediators at the transcriptional level. We measured the mRNA levels of iNOS, TNF-α, and cyclo-oxygenase-2 by real-time PCR. Like the upregulation of inflammatory mediators, the mRNA levels of their corresponding genes were also markedly upregulated by LPS, up to 200–1,400-fold (Fig. 2A), similar to findings that have been reported previously [15]. Similarly, PPD-SF strongly decreased the mRNA levels of the genes in a dose-dependent manner (Fig. 2A). Moreover, the promoter-binding activities of AP-1 and IRF3, but not NF-κB, triggered by PMA (Fig. 2B, 2E) and adaptor molecules (TRIF and MyD88) (Fig. 2C, 2D, 2F) were also dose-dependently inhibited by PPD-SF, indicating that this red ginseng fraction could modulate the transcriptional activation of AP-1 and IRF-3. In agreement with these results, this fraction suppressed the nuclear translocation of c-Jun and the phosphorylation of ATF-2 and IRF-3 (Fig. 2G), implying that the nuclear translocation and phosphorylation events of these transcription factors could be targeted by PPD-SF. Considering that red ginseng marc oil was able to block the expression of inflammatory genes in LPS-treated RAW264.7 cells by suppression of NF-κB [33], and that Panax notoginseng saponins were also found to block the NF-κB pathway [34], the pharmacological features of PPD-SF from KRG seem to be distinctive from those of marc oil and P. notoginseng sapoinos. However, because there is still a possibility that PPD-SF can suppress the activation of NF-κB, we will further evaluate its potential inhibitory activity under LPS-stimulated conditions.

Therefore, we further investigated PPD-SF-targeted molecular events regulating the activation and translocation of AP-1 and IRF-3 in LPS-treated RAW264.7 cells. Previously, it has been reported that ERK, p38, and JNK are major proteins involved in the regulation of AP-1 family activation [35]. TBK1 is also regarded as an important upstream enzyme regulating IRF-3 phosphorylation [4]. PPD-SF clearly suppressed the phosphorylation of p38 from 5 minutes to 30 minutes after treatment, and the phosphorylation of JNK at 15–30 minutes after treatment (Fig. 3A), suggesting that these two enzymes could be directly or indirectly inhibited by PPD-SF. However, the upstream enzymes for p38 and JNK phosphorylation were not inhibited by PPD-SF between 2 minutes and 10 minutes after treatment (Fig. 3B), whereas the phosphorylation of TBK1, the phosphorylating enzyme of IRF-3 [4], was suppressed (Fig. 3C). These results seem to imply that MKK4, MKK6, MKK7, and TBK1 upstream kinase could be directly targeted by this fraction. However, we did not observe any inhibitory effect of PPD-SF in a direct enzyme assay performed with purified MKK4, MKK7, and MKK6,

![Fig. 2](image-url) Effect of PPD-SF on the transcriptional regulation of inflammatory genes in LPS-treated RAW264.7 cells. (A) RAW264.7 cells (5 × 10⁶ cells/mL) were incubated with LPS (1 µg/ml) in the presence or absence of PPD-SF for 6 hours. The mRNA levels of iNOS, TNF-α, and COX-2 were determined by real-time PCR. (B–F) HXK293 cells transfected with plasmid constructs (NF-κB-Luc, AP-1-Luc, and IRF-3-Luc) and β-gal (as a transfection control) were treated with PMA (100nM) or cotransfected with adaptor molecules (MyD88 or TRIF). Then, the cells were further incubated with PPD-SF for 12 hours. Luciferase activity was measured using a luminometer. (G) The phospho- or total protein levels of transcription factors were detected by immunoblotting analysis of the lysates of LPS-treated RAW264.7 cells. "p < 0.05 compared to the control. "*p < 0.01 compared to the control. AP – activator protein; COX – cyclooxygenase; iNOS – inducible NO synthase; IRF – interferon regulatory factor; LPS – lipopolysaccharide; MyD88 – myeloid differentiation primary response gene 88; NF – nuclear factor; P38 – phospholipase serine; PPD-SF – protopanaxadiol saponin fraction; TNF – tumor necrosis factor; TRIF – TR-Domain-containing adapter-inducing interferon-β.
indicating that these enzymes are not targets of PPD-SF. Moreover, we could not test the upstream TBK1-phosphorylating enzyme, because the TBK1-phosphorylating enzymes have not yet been identified [36]. Therefore, we will continue to identify targets specifically inhibited by PPD-SF for the suppression of AP-1 and IRF-3 pathways. Meanwhile, the inhibitory activities of SP600125, a JNK inhibitor, and BIX795 were determined by Griess assay and EIA. *p < 0.05 compared to the control. **p < 0.01 compared to the control. AP = activator protein; COX = cyclooxygenase; ERK = extracellular signal-regulated kinase; iNOS = inducible NO synthase; IRF = interferon regulatory factor; JNK = C-Jun N-terminal kinase; LPS = lipopolysaccharide; MKK = mitogen-activated protein kinase kinase; PGE2 = prostaglandin E2; PPD-SF = protopanaxadiol saponin fraction; TAK = transforming growth factor-β-activated kinase 1; TBK = TANK-binding kinase.

To develop a strong and safe anti-inflammatory remedy, determining whether the preparation is orally active in an in vivo model is critical. Although orally administered KRG-water extract is reported to have anti-inflammatory activity in a mouse inflammation model with allergic rhinitis [40], whether PPD-SF is able to ameliorate in vivo inflammatory symptoms was examined using a HCl/ethanol-induced mouse gastritis model. As Fig. 4A shows, PPD-SF strongly suppressed the formation of gastric ulcer triggered by HCl/ethanol. In particular, it was also revealed that the level of phospho-JNK2 was markedly decreased by PPD-SF, according to immunoblotting analysis with stomach lysates (Fig. 4B). Therefore, these results also strongly suggest that PPD-SF can be an orally active anti-inflammatory compound.
effective anti-inflammatory preparation with JNK inhibitory properties.

In summary, we found that PPD-SF is capable of diminishing in vitro inflammatory responses mediated by macrophage-like RAW264.7 cells treated with LPS and suppressing in vivo gastritis symptoms induced by HCl/ethanol in mice. Through the analysis of transcription factors and their upstream signaling enzymes, it was demonstrated that c-Jun, ATF-2, and IRF-3 and their upstream activation pathways including p38, JNK, and TBK1 could be targeted by PPD-SF, as summarized in Fig. 5. Therefore, our results strongly suggest that PPD-SF can be developed as a KRG-derived anti-inflammatory remedy.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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