Research article

In vitro and in vivo anti-inflammatory activities of Korean Red Ginseng-derived components

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Abstract

Background: Although Korean Red Ginseng (KRG) has been traditionally used for a long time, its anti-inflammatory role and underlying molecular and cellular mechanisms have been poorly understood. In this study, the anti-inflammatory roles of KRG-derived components, namely, water extract (KRG-WE), saponin fraction (KRG-SF), and nonsaponin fraction (KRG-NSF), were investigated.

Methods: To check saponin levels in the test fractions, KRG-WE, KRG-NSF, and KRG-SF were analyzed using high-performance liquid chromatography. The anti-inflammatory roles and underlying cellular and molecular mechanisms of these components were investigated using a macrophage-like cell line (RAW264.7 cells) and an acute gastritis model in mice.

Results: Of the tested fractions, KGR-SF (but not KRG-NSF and KRG-WE) markedly inhibited the viability of RAW264.7 cells and splenocytes at more than 500 μg/mL, significantly suppressed NO production at 100 μg/mL, diminished mRNA expression of inflammatory genes such as inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, and interferon-β at 200 μg/mL, and completely blocked phagocytic uptake by RAW264.7 cells. All three fractions suppressed luciferase activity triggered by interferon regulatory factor 3 (IRF3), but not that triggered by activator protein-1 and nuclear factor-kappa B. Phospho-IRF3 and phospho-TBK1 were simultaneously decreased in KRG-SF. Interestingly, all these fractions, when orally administered, clearly ameliorated the symptoms of gastric ulcer in HCl/ethanol-induced gastritis mice.

Conclusion: These results suggest that KRG-WE, KRG-NSF, and KRG-SF might have anti-inflammatory properties, mostly because of the suppression of the IRF3 pathway.

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1. Introduction

Inflammation is an innate immune response consisting of a series of complex biological processes to protect the body from infection by pathogens, including bacteria, viruses, and fungi [1,2]. Inflammation is characterized by key symptoms, including recruitment of white blood cells, pain, redness, swelling, heat, tissue damage, and organ dysfunction. During the inflammatory response, different types of immune cells are actively recruited to the inflamed lesions to remove the invading pathogens. Among these immune cells, the macrophage is one of the major effector cells governing inflammatory responses by producing various inflammatory mediators, including nitric oxide (NO), reactive oxygen/nitrogen species (ROS/RNS), prostaglandin E2 (PGE2), and different types of proinflammatory cytokines. The latter include tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 [under the control of the activator protein (AP)-1], nuclear factor kappa B (NF-κB), and interferon (IFN) regulatory factor 3 (IRF3) [2–7]. Although inflammation is a host defense mechanism to protect the body from invading pathogens, chronic inflammation, which is a prolonged
Korean ginseng (Panax ginseng) is a perennial plant that has been traditionally used as an herbal medicine to ameliorate the symptoms of various diseases in eastern Asia. The ginseng root has been used as a common ethnopharmacological remedy to support vitality [11,12]. Because fresh ginseng is easily degraded at room temperature, it needs to be processed to red ginseng by steaming and drying, and accumulating evidence has revealed that red ginseng has higher biological activity and lower side effects compared to fresh or white ginseng [13]. Korean Red Ginseng (KRG) has been known to have various biological activities, including immune enhancement, antioxidant effects, memory enhancement, improvement of menopausal disorder, and induction of metabolic energy [14–17]. However, although KRG has been studied in human health and immunity, the therapeutic potential of each component derived from KRG extract in modulating inflammatory responses and in preventing inflammatory diseases has been poorly understood.

Therefore, in this study, we prepared three fractions of KRG—water extract (KRG-WE), nonsaponin fraction (KRG-NSF), and saponin fraction (KRG-SF) —and investigated their therapeutic potency in inflammatory responses and diseases using lipopolysaccharide (LPS)-stimulated macrophages and an acute inflammatory gastritis mouse model.

2. Materials and methods

2.1. Materials

KRG-WE, KRG-NSF, and KRG-SF were kindly supplied by the Korea Ginseng Corporation (Daejeon, Korea). Male imprinting control region (ICR) mice (6–8 wk old, 17–21 g) were purchased from Orient Bio (Gyeonggi, Korea). RAW264.7 and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco’s modified Eagle’s medium, Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal bovine serum (FBS), streptomycin, penicillin, and L-glutamine were purchased from the American Type Culture Collection (Manassas, VA, USA). RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine, and antibiotics (penicillin and streptomycin) at 37°C in a 5% CO2 humidified incubator.

2.2. Mice

Male ICR mice (6–8 wk old, 17–21 g) were obtained from Orient Bio (Gyeonggi, Korea) and maintained in plastic cages under standard conditions. Water and pelleted food (Samyang, Daejeon, Korea) were supplied ad libitum. Studies were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Sungkyunkwan University, Suwon, Korea.

2.3. Preparation of splenocytes from mice

Splenocytes from ICR mice were prepared as previously described [20].

2.4. Cell culture

RAW264.7, HEK293 cells and splenocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine, and antibiotics (penicillin and streptomycin) at 37°C in a 5% CO2 humidified incubator.

2.5. High-performance liquid chromatography analysis

For determination of ginsenosides from KRG-WE, KRG-SF, and KRG-NSF, high-performance liquid chromatography (HPLC) was conducted as described previously [21,22].

2.6. Cell viability assay

The cytotoxic effects of KRG-WE, KRG-SF, and KRG-NSF were determined using the MTT assay, as reported previously [23].

2.7. Phagocytosis assay

RAW264.7 cells treated with KRG-WE, KRG-SF, or KRG-NSF were resuspended in 100 μL phosphate-buffered saline (PBS) containing 1% human AB serum and incubated with FITC-dextran (1 mg/mL) at 37°C for 30 min. The incubations were stopped by adding 2 mL ice-cold PBS containing 1% human serum and 0.02% sodium azide. The cells were then washed three times with cold PBS-azide and analyzed on a FACScan flow cytometer, as reported previously [24].

2.8. NO production assay

RAW264.7 cells were pretreated with either KRG-WE, KRG-NSF, or KRG-SF and incubated with LPS (1 μg/mL) for 24 h. NO production level was determined using Griess reagent as described previously [25].

2.9. mRNA analysis by semiquantitative RT-PCR

RAW264.7 cells pretreated with KRG-WE, KRG-NSF, or KRG-SF for 1 h were incubated with LPS (1 μg/mL) for 6 h. Total RNA was isolated with TRI reagent according to the manufacturer’s instructions and stored at −70°C until use. cDNA from 1 μg of total RNA was synthesized using MuLV reverse transcriptase according to the manufacturer’s instructions, and semiquantitative RT-PCR

| Name  | Sequence (5’ to 3’) |
|-------|---------------------|
| INOS  | F CACCTGCCAGTTTTCTGGCACG  |
|       | R CTCGTCTACAGGGTGCCCTTTCCG |
| COX-2 | F CACCATCATCTGTACCCACCTT  |
|       | R ATGCTCTGCGTGAGTAGATG    |
| TNF-α | F TTAGCCTCAGGCGCTCAGTTG  |
|       | R CTGTAGGCCACCTCTGAGCC    |
| IFN-β | F CAGGATGGACACATGGACCC    |
|       | R CTCGGACAGATCTACCCAAC    |
| GAPDH | F CAATGAGGGAAATTCACACC    |
|       | R GACTTTCAGCACATCTACCA    |

COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN-β, interferon beta; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor alpha
reactions were conducted using the primers specific for inducible NO synthase (iNOS), cyclooxygenase (COX)-2, TNF-α, IFN-β, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as previously reported [26]. The primer sequences used in this study are listed in Table 1.

2.10. Luciferase reporter gene assay

HEK293 cells were transfected with β-galactosidase and NF-κB-Luc, AP-1-Luc, IRF3-Luc, MyD88, or TBK1 using PEI. The cells were treated with KRG-WE, KRG-NSF, or KRG-SF for 12 h prior to termination. Luciferase assays were performed using the Luciferase Assay System according to the manufacturer’s instructions (Promega, Madison, WI, USA).

2.11. Preparation of whole cell and nuclear lysates and Western blot analysis

RAW264.7 cells were treated with KRG-WE, KRG-NSF, or KRG-SF for the indicated time, and whole cell and nuclear lysates were prepared as described previously [23]. Western blot analysis was performed as previously reported [23] using the indicated antibodies.

2.12. HCl/ethanol-induced gastritis in mice

Fasted ICR mice (7 mice/group) were orally treated with KRG-WE, KRG-NSF, KRG-SF (200 mg/kg), or ranitidine (40 mg/kg) twice daily for 3 d. At 30 min after the final administration, stomach inflammation was induced by oral administration of HCl/ethanol (EtOH), and the degree of inflammation was analyzed according to a published method [27].

2.13. Statistical analysis

All data in this paper are presented as the mean ± standard deviation of an experiment performed with six or three replicates. For statistical comparisons, these results were analyzed using Kruskal–Wallis/Mann–Whitney U tests. A p value < 0.05 was considered statistically significant. All statistical tests were carried out using SPSS version 16.0.

Table 2
HPLC profile of ginseng components from KRG-WE, KRG-NSF, and KRG-SF

| Component | KRG-WE | KRG-SF | KRG-NSF |
|-----------|--------|--------|---------|
| Rg1       | 1.03   | 13.33  | 0.00    |
| Re        | 1.21   | 15.68  | 0.00    |
| Rf        | 1.04   | 13.39  | 0.00    |
| Rh1       | 0.96   | 11.54  | 0.00    |
| Rg2s      | 1.43   | 15.75  | 0.00    |
| Rb1       | 5.19   | 64.20  | 0.00    |
| Rb2       | 2.02   | 25.98  | 0.00    |
| Rd        | 1.88   | 22.96  | 0.00    |
| Rg3s      | 2.67   | 8.29   | 0.00    |
| Rg3r      | 0.96   | 21.69  | 0.00    |

HPLC, high-performance liquid chromatography; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract.

Fig. 1. HPLC profiles. (A) KRG-WE. (B) KRG-SF. (C) KRG-NSF. HPLC, high-performance liquid chromatography; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract.
out using the computer program SPSS (Version 22.0, 2013; IBM Corp., Armonk, NY, USA).

3. Results and discussion

In this study, the regulatory roles of KRG-WE, KRG-NSF, and KRG-SF in the inflammatory response were investigated using macrophages and an inflammatory disease animal model. First, the major active saponin components of KRG-WE, KRG-NSF, and KRG-SF were identified and analyzed by HPLC analysis. A variety of active ginseng components, including ginsenoside (G)-Rg1, G-Re, G-Rf, G-Rh1, G-Rg2s, G-Rb1, G-Rc, G-Rb2, G-Rd, G-Rg3s, and G-Rg3r were identified in both KRG-WE and KRG-SF, in varying amounts (Table 2), whereas none of them was found in KRG-NSF (Figs. 1A—1C). Along with ginseng components, amino acids and sugars were also identified from KRG-WE, KRG-NSF, and KRG-SF in varying amounts (data not shown). These HPLC analysis results indicate that ginsenosides and their metabolites, known as the major active pharmaceutical components of ginseng [28], are contained only in KRG-WE and KRG-NSF, but not in KRG-NSF.

To examine the cytotoxicity of KRG-WE, KRG-NSF, and KRG-SF in macrophages prior to exploring their anti-inflammatory activities, RAW264.7 cells were treated with KRG-WE, KRG-NSF, and KRG-SF, and the cell viability was determined. As shown in Fig. 2A, KRG-WE and KRG-NSF decreased the cell viability of RAW264.7 cells by 10—20% with doses up to 800 μg/mL, whereas SF exerted significant cytotoxicity from 400 μg/mL (~80% decrease in viability) to 800 μg/mL (~90% decrease in viability). Similarly, KRG-WE and KRG-NSF did not show cytotoxicity in splenocyte culture, whereas SF significantly reduced splenocyte viability from 250 μg/mL (~40%) up to 1,000 μg/mL (60%) (Fig. 2B). These results suggest that, unlike KRG-NSF, KRG-SF has a severe cytotoxic effect at higher doses, and ginsenosides that are contained only in KRG-SF are regarded as the main contributors for this cytotoxicity. These data highlight the importance of considering the optimal dose of KRG-SF or ginsenosides for their pharmaceutical use.

Inflammatory responses are characterized by the release of inflammatory mediators such as NO and the expression of proinflammatory cytokines such as TNF-α and IFN-β. Therefore, we examined whether KRG-WE, KRG-NSF, and KRG-SF exert anti-inflammatory effects in LPS-treated macrophages. First, NO levels were evaluated in the LPS-stimulated RAW264.7 cells after treating them with KRG-WE, KRG-NSF, or KRG-SF. As shown in Fig. 3A, NO production induced by LPS in RAW264.7 cells was markedly decreased by KRG-SF in a dose-dependent manner (left panel), consistent with the use of L-NAME, an iNOS inhibitor (right panel). The effects of KRG-WE, KRG-NSF, and KRG-SF on the expression of inflammatory genes were further examined in macrophages. The mRNA expression levels of inflammatory genes such as iNOS, COX-2, TNF-α, and IFN-β were determined by semiquantitative RT-PCR in LPS-stimulated RAW264.7 cells after treating them with KRG-WE, KRG-NSF, or KRG-SF. Induced expression of these genes in LPS-stimulated RAW264.7 cells was significantly decreased by KRG-SF, whereas both KRG-WE and KRG-NSF did not exert suppressive effects on the expression of these genes (Fig. 3B). These results strongly indicate that KRG-SF, but not KRG-WE and KRG-NSF, plays an anti-inflammatory role in macrophages through suppressing the release and mRNA expression of inflammatory mediators. The effects of KRG-WE, KRG-NSF, and KRG-SF on the generation of ROS, which are representative inflammatory mediators produced in macrophages, were also examined; however, none of them suppressed ROS generation in LPS-stimulated RAW264.7 cells (data not shown). These results indicate that, although both NO and ROS are inflammatory mediators produced in macrophages, the molecular mechanism by which SF modulates the production of NO and ROS in macrophages is different, and this needs to be further investigated. The phagocytic activity of macrophages is another indicator of macrophage activation, and the effect of KRG-WE, KRG-NSF and KRG-SF on the phagocytic activity of macrophages was examined. As shown in Fig. 3C, only SF markedly suppressed the phagocytic activity of RAW264.7 cells, whereas KRG-WE and KRG-NSF only slightly decreased the phagocytic activity. This suggests that KRG-SF may exhibit its anti-inflammatory effects by suppressing the activation of macrophages.

The molecular mechanism by which SF exerts anti-inflammatory responses was next examined in macrophages. It is well known that inflammatory responses in macrophages are dominantly mediated by the activation of several signal transduction pathways, such as NF-κB, AP-1, and IRF3 signaling pathways [3,4,29—31]. To examine whether KRG-WE, KRG-NSF, and KRG-SF play a role in modulating the transcriptional activities of NF-κB, AP-1 and IRF3, a luciferase reporter gene assay was used using NF-κB-Luc, AP-1-Luc, and IRF3-
Luc constructs in HEK293 cells. KRG-WE, KRG-NSF, and KRG-SF markedly suppressed the luciferase activities of IRF3 induced by TBK1 in HEK293 cells (Fig. 4A), whereas KRG-WE, KRG-NSF, and KRG-SF failed to significantly modulate the luciferase activities of both NF-κB and AP-1 induced by MyD88 (Figs. 4B and 4C). Because KRG-WE, KRG-NSF, and KRG-SF suppressed the luciferase activity of IRF3, we further examined whether they could decrease the nuclear translocation of IRF3 in RAW264.7 cells. As expected, KRG-WE, KRG-NSF, and KRG-SF decreased the nuclear translocation of phospho-IRF3 in RAW264.7 cells (Fig. 4D). Interestingly, and in accordance with the results of the luciferase gene assay, only SF dramatically decreased the nuclear translocation of phospho-IRF3 induced by LPS in RAW264.7 cells (Fig. 4D). To investigate which intracellular signaling molecules are involved in the IRF3 signaling pathways suppressed by KRG-WE, KRG-NSF, and KRG-SF, LPS-stimulated RAW264.7 cells were treated with KRG-WE, KRG-NSF, or KRG-SF, and changes in the activity of TBK1 (located upstream of IRF3) were determined. The phosphorylation of TBK1 induced by LPS was not detectable in the RAW264.7 cells treated with KRG-SF, whereas the phosphorylation levels of TBK1 in the RAW264.7 cells treated with KRG-WE or KRG-NSF were comparable (Fig. 4E). KRG-WE and KRG-NSF suppressed the nuclear translocation of IRF3 (Fig. 4D), but they did not decrease the phosphorylation levels of TBK1 (Fig. 4E), suggesting that the molecular mechanisms of the SF-mediated anti-inflammatory effects differ from those of KRG-WE and KRG-NSF. KRG-SF exerts its anti-inflammatory effects by inhibiting the activity of TBK1 and suppressing the nuclear translocation and transcriptional activity of its downstream molecule, IRF3. In contrast, KRG-WE and KRG-NSF exert their anti-inflammatory effects by suppressing the nuclear translocation and transcriptional activity of IRF3, and by inhibiting molecules upstream of IRF3 (rather than TBK1) in macrophages.

Although in vitro and ex vivo studies support the anti-inflammatory effects of KRG-WE, KRG-NSF, and KRG-SF, and provide the molecular and cellular mechanisms of their anti-

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**Fig. 3.** Effects of KRG-WE, KRG-NSF, and KRG-SF on NO production, mRNA expression, and phagocytic activity. (A) RAW264.7 cells were pretreated with the indicated concentration of KRG-WE, KRG-NSF, KRG-SF, or l-NAME and incubated with LPS (1 μg/mL) for 24 h. NO production was determined by Griess assay using culture supernatants of the cells. (B) RAW264.7 cells were pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) and incubated with LPS (1 μg/mL) for 6 h. mRNA levels of iNOS, COX-2, TNF-α, and IFN-β were determined by semiquantitative PCR. (C) RAW264.7 cells were pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) and incubated with FITC-dextran (1 mg/mL) for 30 min. The uptake levels of FITC-dextran were measured by flow cytometry. *p < 0.05 and **p < 0.01 compared to controls. COX-2, cyclooxygenase-2; FITC, fluorescein isothiocyanate; IFN, interferon; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract; l-NAME, N*-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PCR, polymerase chain reaction; TNF, tumor necrosis factor.
inflammatory roles in macrophages, a study regarding their in vivo anti-inflammatory roles in animal models of inflammatory diseases is also critical for the development of efficacious and safe drugs to treat inflammatory diseases. The in vivo anti-inflammatory effects of KRG-WE, KRG-NSF, and KRG-SF were explored during acute experimental gastritis induced in mice by injecting HCl/EtOH. Because previous studies have revealed that oral administration of ginseng or its metabolites/derivatives is a reasonable route to test their in vivo anti-inflammatory effects in animal models [19,32,33], KRG-WE, KRG-NSF, and KRG-SF were orally administered in the HCl/EtOH-induced gastritis mice, and their anti-inflammatory activities were examined according to the time schedule described in Fig. 5A. KRG-WE, KRG-NSF, and KRG-SF ameliorated the symptoms of gastric ulcer (Fig. 5B) and significantly reduced the area of the disease lesions (Fig. 5C) in the HCl/EtOH-induced experimental gastritis mice. These results strongly suggest that oral administration of KRG-WE, KRG-NSF, and KRG-SF could be a potential and effective anti-inflammatory remedy to treat gastritis.

In summary, we found that KRG-WE, KRG-NSF, and KRG-SF exhibited in vivo and in vitro anti-inflammatory effects by ameliorating the symptoms of gastric ulcer in the gastritis mouse model and by suppressing the IRF3 signaling pathway in macrophages. Interestingly, although KRG-WE, KRG-NSF, and KRG-SF exerted in vitro anti-inflammatory effects by suppressing the IRF3 signaling pathway in macrophages, the activity of TBK1 was reduced only by KRG-SF, but not by KRG-WE and KRG-NSF. In addition, although KRG-SF decreased phagocytic activity, NO production, and mRNA expression of inflammatory genes in inflammatory macrophages, KRG-NSF may exert an in vivo anti-inflammatory activity by a mechanism other than the modulation of macrophage-mediated inflammatory

![Fig. 4. Effects of KRG-WE, KRG-NSF, and KRG-SF on activation of IRF3, NF-κB, and AP-1 pathways. HEK293 cells transfected with the following constructs for 36 h were treated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) for 12 h. (A) IRF3-Luc and TBK1 expression constructs. (B) NF-κB-Luc and MyD88 expression constructs. (C) AP-1-Luc and MyD88 expression constructs. Luciferase activities were measured using a luminometer. (D) RAW264.7 cells pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) were further incubated with LPS (1 μg/mL) for 30 min, after which the level of phosphorylated IRF3 was determined by Western blot analysis. (E) RAW264.7 cells pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) were further incubated with LPS (1 μg/mL) for 5 min, after which the level of phosphorylated TBK1 was determined by Western blot analysis. *p < 0.05, **p < 0.01 compared with control cells. AP-1, activator protein-1; IRF3, interferon regulatory factor 3; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NO, nitric oxide; PCR, polymerase chain reaction; TNF, tumor necrosis factor.](image)
responses. Exactly how KRG-NSF plays an anti-inflammatory role in macrophages needs to be further investigated. Our data strongly suggest that KRG-WE, KRG-NSF, and KRG-SF have anti-inflammatory activities with different molecular and cellular mechanisms, and could therefore be developed as potential anti-inflammatory remedies to prevent and treat inflammatory diseases.

Conflicts of interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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