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Research Article

Korean Red Ginseng saponin fraction exerts anti-inflammatory effects by targeting the NF-κB and AP-1 pathways

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A R T I C L E I N F O

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Background: Although ginsenosides and saponins in Korea red ginseng (KRG) shows various pharmacological roles, their roles in the inflammatory response are little known. This study investigated the anti-inflammatory role of ginsenosides identified from KRG saponin fraction (RGSF) and the potential mechanism in macrophages.
Methods: The ginsenoside composition of RGSF was identified by high-performance liquid chromatography (HPLC) analysis. An anti-inflammatory effect of RGSF and its mechanisms were studied using nitric oxide (NO) and prostaglandin E_2 (PGE_2) production assays, mRNA expression analyses of inflammatory genes and cytokines, luciferase reporter gene assays of transcription factors, and Western blot analyses of inflammatory signaling pathways using the lipopolysaccharide (LPS)-treated RAW264.7 cells.
Results: HPLC analysis identified the types and amounts of various panaxadiol ginsenosides in RGSF. RGSF reduced the generation of inflammatory molecules and mRNA levels of inflammatory enzymes and cytokines in LPS-treated RAW264.7 cells. Additionally, RGSF inhibited the signaling pathways of NF-κB and AP-1 by suppressing both transcriptional factors and signaling molecules in LPS-treated RAW264.7 cells.
Conclusion: RGSF contains ginsenosides that have anti-inflammatory action via restraining the NF-κB and AP-1 signaling pathways in macrophages during inflammatory responses.
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1. Introduction

Inflammation is a protective immune response from infection with harmful pathogens and is a response to danger signals derived from cellular stress [1–3]. However, chronic inflammation, which is repeated inflammation and lasts for months to even years, has been considered a major risk factor of numerous human diseases [4–6]. An inflammatory response is initiated by the interaction of pattern-recognition receptors (PRRs) with the various molecular patterns associated with pathogens and danger signals [7–9]. The initiation of inflammatory response activates signal transduction pathways, such as nuclear factor-kappa B (NF-κB), activated protein-1 (AP-1), and interferon regulatory factors (IRFs) by stimulating the signaling cascades of various intracellular inflammatory molecules. These events result in the generation of inflammatory molecules and transcriptional up-regulation of pro-inflammatory enzymes and cytokines [10–13].

Korean ginseng (*Panax ginseng* Meyer), cultivated in far-east Asia, is traditional herbal medicine and has been reported to play an ameliorative role in numerous human diseases [14–18]. Fresh ginseng has high moisture content and decays easily; therefore, it is necessary to produce red ginseng by repeating steaming and drying several times. Interestingly, compared to fresh ginger, Korean red

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ginseng (KRG) shows higher chemical content and biological activity with fewer adverse effects [19]. KRG improves essential biological functions, such as immune response, energy induction, sexual functions, memory, cognitive functions, and offers antioxidant activity [20–25]. KRG was also reported to have anti-inflammatory activities by alleviating the inflammatory response [16,26–31]; however, the KRG components that show anti-inflammatory activities and the potential mechanism that manifests these activities are still unclear.

Therefore, this study prepared the ginsenoside composition of the KRG saponin fraction (RGSF) and investigated the anti-inflammatory role of RGSF as well as the potential mechanism in lipopolysaccharide (LPS)-activated macrophage, RAW264.7 cells.

2. Materials and methods

2.1. Materials

RGSF was kindly supplied from the Korea Ginseng Cooperation (Daejeon, Korea). RAW264.7 and HEK293 cells were purchased at the American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), streptomycin, and penicillin were purchased at Gibco (Grand Island, NY, USA). Lipopolysaccharide (LPS), crystal violet, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), luciferin, and polyethyleneimine (PEI) were purchased at Sigma Aldrich (St Louis, MO, USA). PCR primers were synthesized at Bioneer Inc. (Daejeon, Korea). Real-time PCR dye was purchased at PCR Biosystems (London, United Kingdom). TRI reagent® was purchased at Molecular Research Center Inc. (Cincinnati, OH, USA). MuLV reverse transcriptase and Lipofectamine® 2000 reagent were purchased at Thermo Fisher Scientific (Waltham, MA, USA). NF-κB-Luc, AP-1-Luc, CREB-Luc, and TRIF-expressing constructs were purchased at Addgene (Cambridge, MA, USA). Antibodies for Western blot analysis were purchased at Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Dallas, Texas, USA). An enhanced chemiluminescence system was purchased at AbFrontier (Seoul, Korea).

2.2. Cell culture

RAW264.7 and HEK293 cells were incubated in RPMI 1640 medium that contains 10% heat-inactivated FBS and penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. The cells were freshly maintained by splitting them three times per week.

2.3. Cell viability assay

The cytotoxicity of RGSF was quantified by an MTT method as previously described [32]. In brief, RAW264.7 cells were incubated with various doses of RGSF for 24 h, and the numbers of live cells were quantified and compared by an MTT assay.

2.4. NO production assay

RAW264.7 cells incubated with various doses of RGSF or prednisolone (Pred) for 30 min were activated with LPS (1 μg/mL) for 24 h, after which NO amount in culture medium was quantified by a Griess assay as previously described [33].

2.5. PGE_2 production assay

RAW264.7 cells incubated with various doses of RGSF for 30 min were activated with LPS (1 μg/mL) for 24 h, after which PGE_2 amount in culture medium was quantified by an enzyme immunoassay as described previously [34].

2.6. Quantitative real-time polymerase chain reaction (PCR)

RAW264.7 cells incubated with various doses of RGSF for 30 min were activated with LPS (1 μg/mL) for 6 h, after which total RNA was extracted using TRI reagent®. cDNA was immediately synthesized from the extracted RNA using a MuLV reverse transcriptase, and mRNA of iNOS, TNF-α, COX-2, and IL-1β were quantified by a quantitative real-time polymerase chain reaction (PCR) using primers specific for each target. The information of primers is summarized in Table 1.

2.7. Luciferase reporter gene assay

RAW264.7 cells incubated with various doses of RGSF and LPS (1 μg/mL) were transfected with either an NF-κB-Luc, AP-1-Luc, or CREB-Luc construct along with a β-gal construct using Lipofectamine® 2000 reagent 48 h. HEK293 cells transfected with an AP-1-Luc construct along with a β-gal construct using PEI for 24 h were treated with various doses of RGSF for another 24 h. AP-1-Luc reporter activity was quantified by incubating luciferin with cell lysates.

2.8. Western blot analysis

RAW264.7 cells incubated with RGSF (100 μg/mL) for 30 min were activated with LPS (1 μg/mL), and nuclear as well as whole-cell lysates were prepared as previously described [33]. Western blot analysis was conducted as previously described [33] with the antibodies specific for each target.

2.9. High-performance liquid chromatography analysis

Types and amounts of ginsenosides in RGSF were analyzed by high-performance liquid chromatography (HPLC) as previously described [35].

2.10. Statistical analysis

All data were described as the mean ± standard error of the mean (SEM) of independent experiments performed more than three times. Statistical significance between the control versus experimental groups was evaluated by either a Mann-Whitney test or one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

Table 1
The information of primers used in this study for quantitative real-time PCR.

Target		Sequence (5′–3′)
iNOS	F	CCCTTCCGAAGTTTCTGGCAGCAG
	R	GGCTGTCAGAGCCTCGTGGCTTTGG
TNF-α	F	TTGACCTCAGCGCTGAGTTG
	R	CCTGTAGCCCACGTCGTAGC
COX2	F	CACTACATCCTGACCCACTT
	R	ATGCTCCTGCTTGAGTATGT
IL-1β	F	TAGAGCTGCTGGCCTTGTTA
	R	ACCTGTAAAGGCTTCTCGGA
GAPDH	F	CAATGAATACGGCTACAGCAAC
	R	AGGGAGATGCTCAGTGTTGG

3. Results and discussion

In this study, ginsenoside components were identified in RGSF, and anti-inflammatory role of RGSF was evaluated in LPS-activated macrophage, RAW264.7 cells. The types and amounts of panaxdiol ginsenosides in RGSF were first determined by HPLC analysis; the panaxdiol ginsenosides (Gs) G-Rg1, G-Re, G-Rf, G-Rb1, G-Rc, G-Rb2, G-Rb3, G-Rd, G-F2, and G-Rg3 were identified (data not shown), as reported previously [36,37]. The total amount of these ginsenosides was 520.6 mg/g, and the amount of each ginsenoside is summarized in Supplementary Table 1. Among the identified ginsenosides, the amount of G-Rb1 was the highest (158.0 mg/g), followed by G-Rc (107.6 mg/g) and G-Rb2 (80.0 mg/g). Numerous previous studies have demonstrated an anti-inflammatory role of G-Rb1 [15,38,39], G-Rc [37,40], and G-Rb2 [15,37,40], strongly indicating that RGSF may also have anti-inflammatory effect.

Therefore, the anti-inflammatory effects of RGSF and its underlying molecular mechanism were examined in LPS-activated RAW264.7 cells. Pharmacological agents are useless if they exhibit cytotoxicity or adverse effects. Therefore, RGSF cytotoxicity was tested in macrophages, and RGSF exerted no cytotoxicity in RAW264.7 at any of the test doses (Fig. 1A), indicating that it confers no cytotoxicity at the doses tested in this study. Anti-inflammatory effect of RGSF was nest investigated in LPS-activated RAW264.7 cells. RGSF decreased NO and PGE_2 production (Fig. 1B) and also down-regulated mRNA levels of pro-inflammatory enzymes, such as iNOS and COX-2 as well as cytokines, such as TNF-α and IL-1β in LPS-activated RAW264.7 cells (Fig. 1C). Meanwhile, prednisolone showed significant suppression of NO production as previously reported [41], implying that the experimental condition of this study is properly established. Given the results, ginsenosides in RGSF exert a strong anti-inflammatory role by reducing inflammatory mediator production and the mRNA levels of pro-inflammatory enzymes and cytokines in macrophages.

An inflammatory response is induced by activating intracellular signal transduction pathways of NF-κB, AP-1, and CREB in macrophages, therefore, inhibitory role of RGSF in the activation of these inflammatory signal transduction pathways was evaluated in the LPS-activated RAW264.7 cells. Inhibitory effect of RGSF on the luciferase reporter gene activity induced by NF-κB, AP-1, and CREB transcription factors was evaluated in LPS-activated RAW264.7 cells as well as TRIF-transfected HEK293 cells. RGSF markedly reduced AP-1-Luc reporter activity at 50 and 100 μg/mL and marginally reduced NF-κB-Luc reporter activity at 100 μg/mL in the LPS-activated RAW264.7 cells (Fig. 2A). However, RGSF showed no suppressive effect on CREB-Luc reporter activity at all doses (25,

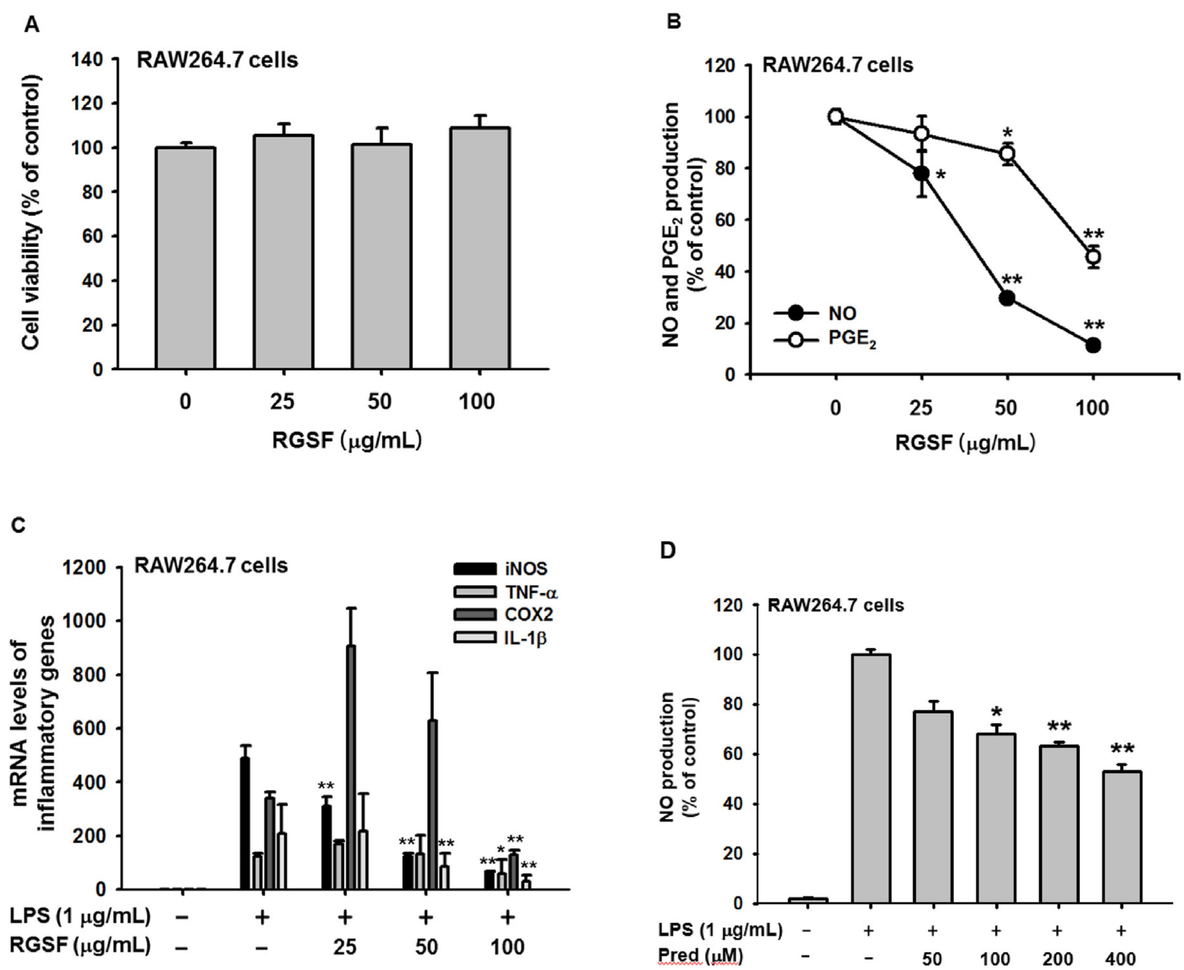

Fig. 1. Suppressive role of RGSF on inflammatory mediator production and mRNA levels of pro-inflammatory enzymes and cytokines (A) RAW264.7 cells were incubated with RGSF (0–100 μg/mL) for 24 h, and viable cells were quantified by an MTT assay. (B and D) RAW264.7 cells incubated with RGSF (0–100 μg/mL) or Pred (0–400 μM) for 30 min were stimulated with LPS (1 μg/mL) for 24 h, and NO and PGE_2 in culture medium were quantified by a Griess assay and enzyme immunoassay, respectively. (C) RAW264.7 cells incubated with RGSF (0–100 μg/mL) for 30 min were stimulated with LPS (1 μg/mL) for 6 h, and mRNA levels of iNOS, TNF-α, COX-2, and IL-1β were quantified by quantitative real-time PCR. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the control.

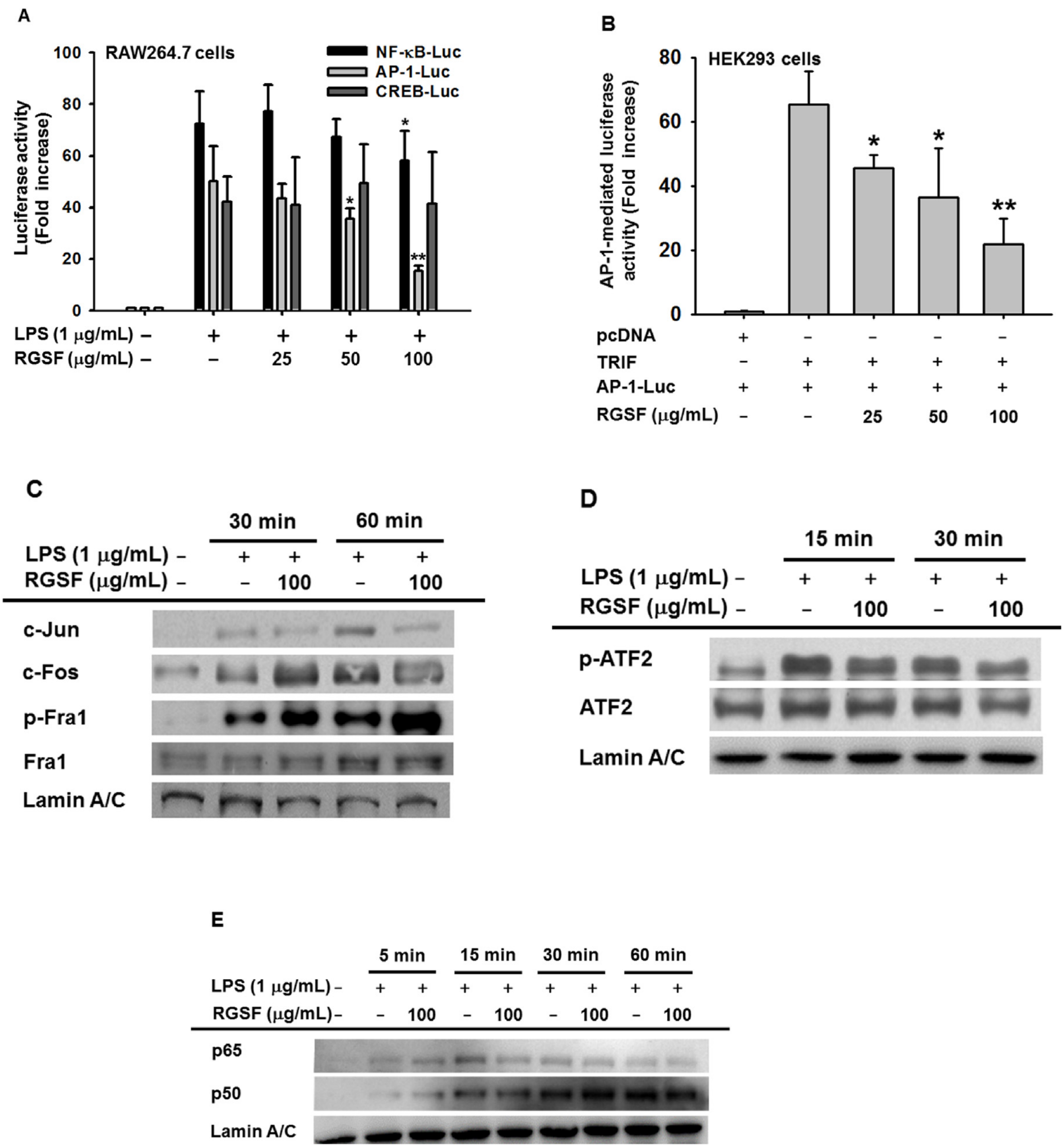

Fig. 2. Suppressive role of RGSF on NF-κB, CREB, and AP-1 transcriptional activities (A) RAW264.7 cells co-transfected with NF-κB-Luc, AP-1-Luc, or CREB-Luc along with a β-gal were incubated with RGSF (0, 25, 50, and 100 μg/mL) and LPS (1 μg/mL), and luciferase reporter activity was quantified. (B) HEK293 cells co-transfected with TRIF (1 μg/mL) and AP-1-Luc along with a β-gal were incubated with RGSF (0–100 μg/mL), and AP-1-Luc reporter activity was quantified. (C–E) RAW264.7 cells 0–100 μg/mL with RGSF (100 μg/mL) for 30 min were stimulated with LPS (1 μg/mL). Western blot analysis of phosphorylated and total forms of c-Jun, c-Fos, Fra1, Lamin A/C (C), ATF2 (D), and p65 (E) and p50. *$P < 0.05$ and **$P < 0.01$ compared to the controls.

50 and 100 μg/mL) (Fig. 2A). Inhibitory effect of RGSF on AP-1-Luc reporter activity was further confirmed in the TRIF-transfected HEK293 cells, since TRIF is an intracellular adaptor that leads to activation of the AP-1 signaling pathway [42]. RGSF significantly suppressed AP-1 luciferase reporter gene activity at all doses in TRIF-transfected HEK293 cells (Fig. 2B). Inflammatory signaling is activated by the translocation of transcription factors into the nucleus of macrophages [10,12,13], illustrating the suppressive effect of RGSF on nuclear translocation of these transcription factors in LPS-activated RAW264.7 cells. RGSF (100 μg/mL) markedly suppressed AP-1 nuclear translocation, such as c-Jun (30 and 60 min), c-Fos (60 min) (Fig. 2C), and p-ATF2 (15 and 30 min) (Fig. 2D), but not p-Fra1 (Fig. 2C), in LPS-activated RAW264.7 cells. Additionally, RGSF (100 μg/mL) also suppressed NF-κB nuclear translocation, such as p65 (15 min), but not that of p50, in LPS-activated RAW264.7 cells (Fig. 2E). Given the results, RGSF exerts an anti-inflammatory effect via suppressing NF-κB and AP-1 transcription factors during macrophage-mediated inflammatory response.

Many cytosolic molecules, such as kinases, mitogen-activated protein kinases (MAPKs), and MAPK kinases (MAPKKs) in NF-κB and AP-1 pathways are activated through phosphorylation in macrophages during inflammatory response [10,12,13]. Since RGSF suppressed NF-κB and AP-1 activation in macrophages during inflammatory response, the suppressive effect of RGSF extends to

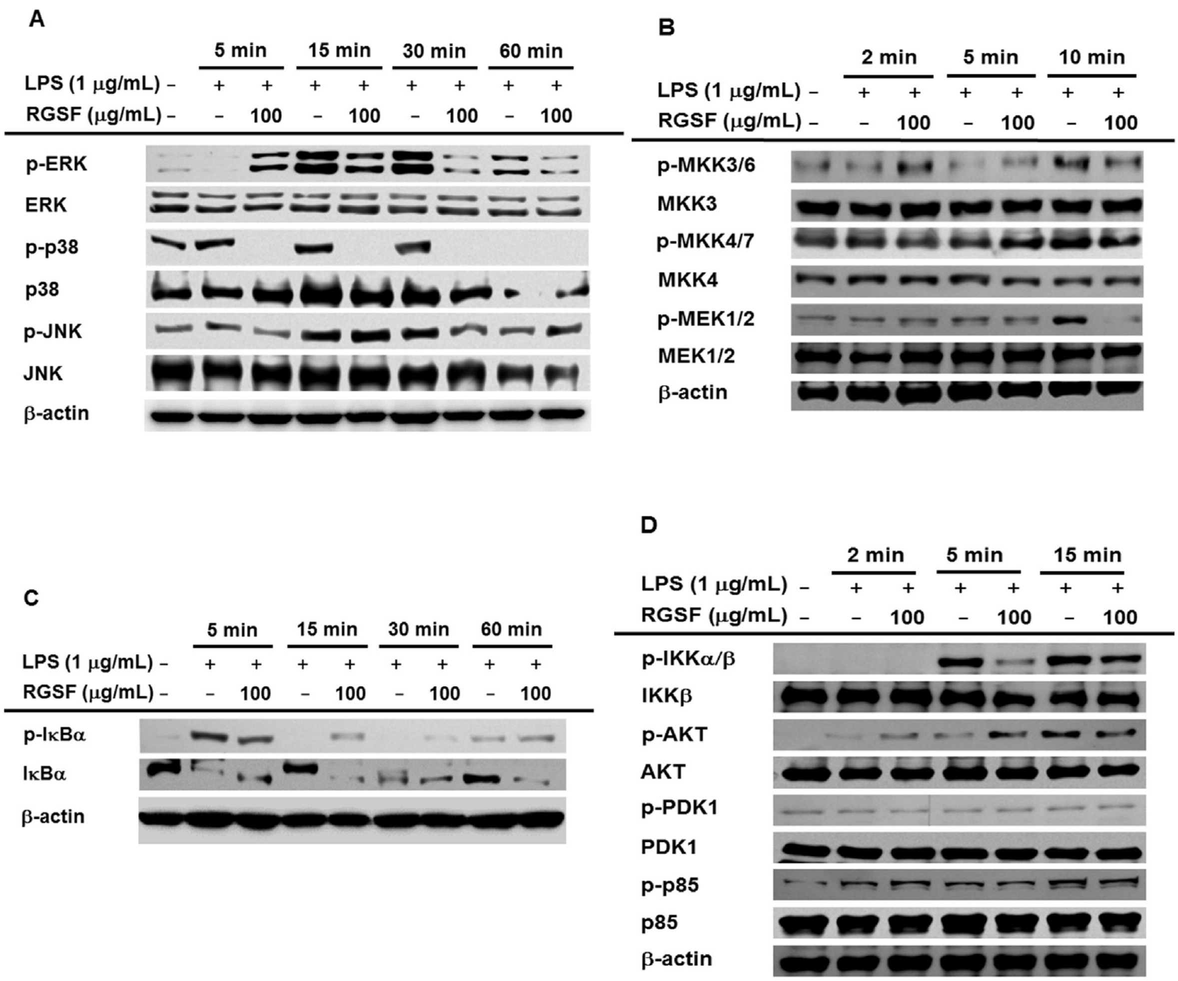

Fig. 3. Suppressive effect of RGSF on NF-κB and AP-1 signaling pathways. (A, B, C, and D) RAW264.7 cells incubated with RGSF (100 μg/mL) for 30 min were stimulated with LPS (1 μg/mL). Western blot analysis of phosphorylated and total forms of ERK, p38, and JNK (A), MKK3/6, MKK4/7, MEK1/2 (B), and β-actin, IκBα (C), and IKKα/β, AKT, PDK1, p85 (D), and β-actin.

activation of inflammatory molecules stimulating NF-κB and AP-1 transcription factors in LPS-activated RAW264.7 cells. RGSF (100 μg/mL) inhibited phosphorylation of MAPKs, such as ERK (15, 30, and 60 min) and p38 (5, 15, and 30 min), but not JNK in LPS-activated RAW264.7 cells (Fig. 3A). Inhibitory effect of RGSF on MAPKKs activation was further evaluated in LPS-activated RAW264.7 cells, and RGSF (100 μg/mL) was found to inhibit the phosphorylation of MKK3/6 (10 min) and MEK1/2 (10 min), but not MKK4/7 (Fig. 3B). In addition, the inhibitory effect of RGSF on activation of inflammatory molecules in NF-κB signaling pathway was evaluated in LPS-activated RAW264.7 cells, and RGSF (100 μg/mL) increased phosphorylation-induced breakdown of IκBα (5, 15, 30, and 60 min) (Fig. 3C). Furthermore, suppressive effect of RGSF on activation of IκBα-upstream inflammatory molecules was also evaluated in LPS-activated RAW264.7 cells, and RGSF (100 μg/mL) inhibited the phosphorylation of IKKα/β (5 and 15 min), but not that of PDK1 or p85 (Fig. 3D), as seen in the previous papers [36,43]. Given the results, RGSF exhibits its anti-inflammatory activity by restraining activation of intracellular inflammatory molecules in AP-1 and NF-κB pathways, such as ERK, p38, MKK3/6, MEK1/2, IκBα, and IKKα/β in macrophages.

In conclusion, this study identified ginsenosides in RGSF and demonstrated the anti-inflammatory role of RGSF in LPS-activated

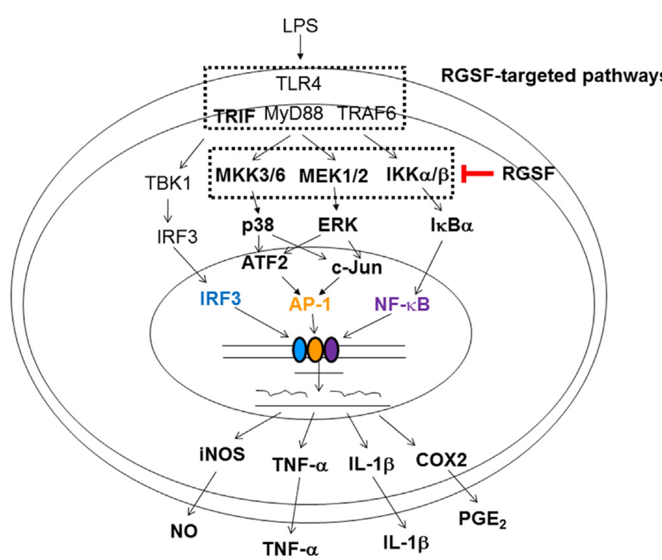

Fig. 4. Schematic summary of the RGSF-mediated anti-inflammatory effect in macrophages stimulated by LPS.

macrophages. RGSF contained various panaxadiol ginsenosides, including Rg1, Re, Rf, Rb1, Rc, Rb2, Rb3, Rd, F2, and Rg3 that have anti-inflammatory effects. RGSF exerted anti-inflammatory effects without any cytotoxicity by reducing inflammatory mediator production and mRNA levels of pro-inflammatory enzymes and cytokines, which was accomplished by suppressing activation of AP-1 and NF-κB inflammatory signaling pathways during macrophage-mediated inflammatory responses (Fig. 4). Taken together, the findings of this study could increase the knowledge of the anti-inflammatory effects mediated by KRG at a molecular level and also provide insight into the use of KRG when developing anti-inflammatory treatments that prevent and treat human inflammatory diseases.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.02.004.

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