Research article

In vitro antioxidative and anti-inflammatory effects of the compound K-rich fraction BIOGF1K, prepared from Panax ginseng

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Background: BIOGF1K, a compound K-rich fraction prepared from the root of Panax ginseng, is widely used for cosmetic purposes in Korea. We investigated the functional mechanisms of the anti-inflammatory and antioxidative activities of BIOGF1K by discovering target enzymes through various molecular studies.

Methods: We explored the inhibitory mechanisms of BIOGF1K using lipopolysaccharide-mediated inflammatory responses, reporter gene assays involving overexpression of toll-like receptor adaptor molecules, and immunoblotting analysis. We used the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to measure the antioxidative activity. We cotransfected adaptor molecules, including the myeloid differentiation primary response gene 88 (MyD88) and Toll/interleukin-receptor domain containing adaptor molecule-inducing interferon-β (TRIF), to measure the activation of nuclear factor (NF)-κB and interferon regulatory factor 3 (IRF3).

Results: BIOGF1K suppressed lipopolysaccharide-triggered NO release in macrophages as well as DPPH-induced electron-donating activity. It also blocked lipopolysaccharide-induced mRNA levels of interferon-β and inducible nitric oxide synthase. Moreover, BIOGF1K diminished the translocation and activation of IRF3 and NF-κB (p50 and p65). This extract inhibited the upregulation of NF-κB-linked luciferase activity provoked by phorbol-12-myristate-13 acetate as well as MyD88, TRIF, and inhibitor of κB (IkB)α, and IRF3-mediated luciferase activity induced by TRIF and TANK-binding kinase 1 (TBK1). Finally, BIOGF1K downregulated the NF-κB pathway by blocking IKKα and the IRF3 pathway by inhibiting TBK1, according to reporter gene assays, immunoblotting analysis, and an Akt-IKKβ-TBK1 overexpression strategy.

Conclusion: Overall, our data suggest that the suppression of IKKβ and TBK1, which mediate transcriptional regulation of NF-κB and IRF3, respectively, may contribute to the broad-spectrum inhibitory activity of BIOGF1K.

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1. Introduction

Inflammation is defined as the complex biological response of the immune system against harmful pathogens [1]. To maintain the immune homeostasis system in the body, both acute and chronic inflammatory responses play an important role in the natural defense mechanisms of the body's innate immune system. Phagocytic uptake of infected materials via receptors, likely toll-like receptors (TLRs), that interact with molecular patterns of pathogen-derived materials including lipopolysaccharide (LPS) and peptidoglycan triggers upregulation of macrophage functions that mediate inflammatory responses [2,3]. To activate inflammatory cells,
intracellular signaling cascades linked to tyrosine kinases (e.g., Src), serine-threonine kinases, and inhibitor of κB (IκB) kinase (IKK)-activating nuclear factor-κB (NF-κB) are required. Subsequent transcriptional activation of various inflammatory genes such as inducible nitric oxide (NO) synthase (iNOS) occurs [4,5]. Consequently, several inflammatory mediators such as NO and proinflammatory cytokines such as interleukin (IL)-1, interferon (IFN)-β, and tumor necrosis factor-α are secreted to further stimulate other inflammatory cells [6]. These responses help protect the body against pathogenic infections. Nevertheless, prolonged inflammation causes severe diseases, including cancer, arthritis, diabetes, and atherosclerosis [7–9]. Thus, in recent decades, immunologists have focused on the development of safe and effective anti-inflammatory and antioxidative therapy to prevent chronic inflammatory diseases.

Numerous studies have expanded our perception on the TLR-signaling pathway [10]. After LPS stimulation, TRlr4 is activated and delivers outside signals into the cytoplasm by recruiting two adaptor molecules, myeloid differentiation primary response protein-88 (MyD88) and Toll/IL-1 receptor-domain containing adaptor molecule-inducing interferon-β (TRIF), through an interaction between their Toll/interleukin-1 receptor (TIR) domains [11]. MyD88, but not TRIF, interacts directly with Toll-like receptor (TLR) signaling proteins such as IL-1 receptor-associated kinases (IRAK1 and IRAK4), protein kinase B (AKT), and TANK-binding kinase 1 (TBK1) [12]. When these proteins are activated, complex signaling cascades composed of Src, phosphoinositide 3-kinase, phosphoinositide-dependent kinase-1, and AKT trigger the NF-κB activation pathway linked to IKK, IκBα [13,14], and the interferon regulatory factor 3 (IRF3) activation pathway [15]. These two major pathways participate in the release of proinflammatory cytokines and inflammatory mediators. Free radicals such as reactive oxygen species (ROS) are highly reactive molecules that are generated in micro- or macrolevel inflammatory responses [16,17]. Oxidative stress can damage proteins, DNA, and small molecules other than lipids. Biologists and clinicians are interested in the capacity of antioxidants to defend the human body against damage by reactive free radicals found in immunological diseases such as cancer, atherosclerosis, and aging [18,19].

The root of Korean ginseng (Panax ginseng) Meyer) has been prescribed as a herbal medicine in Korea, China, and Japan, and its pharmacologically active compounds display antioxidant, anti-inflammatory, and anticancer effects [20,21]. Recently, our group has developed an interesting fraction (BIOGF1K) with a higher amount of compound K, an active metabolite displaying anti-inflammatory, anticancer, and skin-protective activities [22,23]. In the present study, we benchmark the molecular mechanisms of anti-inflammatory and antioxidative activities of BIOGF1K by exploring target proteins through molecular studies.

2. Materials and methods

2.1. Materials

LPS (Escherichia coli O111:B4), ascorbic acid, phorbol-12-myristate-13 acetate (PMA), and (3-4-5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The luciferase construct with the 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma myristate-13 acetate (PMA), and (3-4-5-dimethylthiazol-2-yl)-2-

2.2. Preparation of BioGF1K

BIOGF1K, a compound K-rich fraction, was gifted by Amorepacific R&D unit (Yongin, Korea). Briefly, P. ginseng root (2 kg) was pulverized into powder using a mechanical grinder. After weighing, P. ginseng roots were transferred to a flask, treated with 70% ethanol (4 L) for 2 kg of powdered P. ginseng root, refluxed to extract three times, and deposited for 6 h at 15°C. After that the extracts were suspended in water and subsequently fractionated using diethyl ether (1 L) and 1-butanol (500 mL). Dried 1-butanol extract (10 g) was continuously suspended into a citrate buffer solution (pH 4.0, 1 L) and added with pectinase (15 g, Multifect Pectinase FE, originating from Aspergillus niger) during agitation in a water bath for 48 h at 30°C. After the reaction, the incubation mixture was extracted using the same amount of ethylacetate three times and concentrated by speed vac. Finally, BIOGF1K (compound K, 3.2 g, and ginsenoside F1, 1.5 g) was obtained by separation using column chromatography (choleform:methanol = 9:1); yield = 87.8%.
and stored at /C0 24-well plates) were transfected with 1 TRIzol Reagent (Gibco) according to the manufacturer
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IFN-β immunoblotting visualized as described previously[39]. The cells were incubated with BIOGF1K for 24 h before
assay System (Promega, Madison, WI, USA), as described previ-
method. The cells were incubated with BIOGF1K for 24 h before
(A1), while 200 μg/mL BIOGF1K suppressed up to 67% of this increase (Fig. 1A, left panel). Mean-
while, the standard NO inhibitory compound L-NAME displayed a
strong inhibitory activity (Fig. 1A, right panel), as expected[40]. To
prove the radical-scavenging activity of BIOGF1K, we employed the
DPPH assay (for electron-donating activities). BIOGF1K showed
significant electron-donating activities (p < 0.01), which were
comparable with that of the standard compound ascorbic acid
(100μM, Fig. 1B). Cell viability was intact in RAW264.7 and HEK293
cells treated with BIOGF1K (Fig. 1C), implying that the NO inhibi-
tory activity of this extract was not due to any distracted toxicity.
Finally, the level of compound K in BIOGF1K was analyzed using
HPLC. As Fig. 1D shows, a peak with a similar retention time to
standard compound K was observed at 9.5 min in this extract.

2.2. Statistical analysis
All data presented in this paper are the mean ± standard devi-
ation of an experiment performed with six (Fig. 1) or three (Figs. 2–
4) replicates. For statistical comparisons, these results were
analyzed using analysis of variance/Scheffe's post hoc test and
Kruskal–Wallis/Mann–Whitney U tests. A p value < 0.05 was
considered statistically significant. All statistical tests were carried
out using the computer program SPSS (version 22.0, 2013; IBM
Corp., Armonk, NY, USA).

3. Results
3.1. Effects of BIOGF1K on NO production and radical scavenging activity
We first investigated BIOGF1K-induced suppression of NO pro-
duction in LPS-treated RAW264.7 cells to determine the ability of
this extract to modulate inflammatory responses. LPS upregulated
NO levels in RAW264.7 cells (Fig. 1A), while 200 μg/mL BIOGF1K
suppressed up to 67% of this increase (Fig. 1A, left panel). Mean-
while, the standard NO inhibitory compound L-NAME displayed a
strong inhibitory activity (Fig. 1A, right panel), as expected[40]. To
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Corp., Armonk, NY, USA).

2.1. Preparation of total lysates and nuclear extracts, and
immunoblotting
Total lysates prepared from HEK293 or RAW264.7 cells (5 × 10⁶ cells/ mL) were subjected to western blot analysis of
various total or phospho-proteins. Nuclear lysates were extracted in
a three-step procedure described previously [38]. Total or phos-
phorylated protein levels of transcription factors (p65 and p50),
Akt, IκBα, IKKα/β, TBK1, IRF3, Src, HA, and β-actin (as a control) were
visualized as described previously [39].

2.12. Statistical analysis
All data presented in this paper are the mean ± standard devi-
ation of an experiment performed with six (Fig. 1) or three (Figs. 2–
4) replicates. For statistical comparisons, these results were
analyzed using analysis of variance/Scheffe’s post hoc test and
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out using the computer program SPSS (version 22.0, 2013; IBM
Corp., Armonk, NY, USA).

| Table 1  |
|----------|
| Name     | Sequence (5’–3’) |
| INOS     | F                |
|          | CCCCTGGAAGTCTCTGACGACAG |
|          | R                |
|          | GGCCTGACAGCTCTGGCTGTTTCG |
| IFN-β    | F                |
|          | CAAGTGAGAGACAGGGAGCTATCATC |
|          | R                |
|          | TGGACATCTTCACCAGCTAA |
| GAPDH    | F                |
|          | CACCTGCCCAATTACACGCCCA |
|          | R                |
|          | GACCTCAGCACTACATCAGCAC |

GAPDH, Glycerinaldehyde 3-phosphate dehydrogenase; IFN-β, interferon beta; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction.
Fig. 1. In vitro anti-inflammatory and antioxidative effects of BIOGF1K. (A) The NO level in the culture supernatant of RAW264.7 cells treated with LPS (1 μg/mL) in the presence or absence of BIOGF1K (left panel) or L-NAME (right panel) for 24 h was analyzed with a Griess assay. (B) The antioxidative activity (electron-donating activity) of BIOGF1K and ascorbic acid was measured by DPPH assay. After adding an ethanolic DPPH solution, the absorbance was monitored at 517 nm and enzyme activity was calculated as described in the "Materials and methods" section. (C) Cell viability of RAW264.7 and HEK293 cells under BIOGF1K treatment conditions was determined using the MTT assay. (D) The phytochemical analysis of the level of compound K in BIOGF1K was analyzed by HPLC. *p < 0.05, compared with the control. **p < 0.01, compared with the control. MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl.
Fig. 2. Effect of BIOGF1K on the expression of proinflammatory genes and their transcriptional activation. (A) The mRNA levels of iNOS and IFN-β were determined by semi-quantitative RT-PCR. (B–E) The promoter binding activity of the transcription factor NF-κB was analyzed using a reporter gene assay in HEK293 cells transfected with plasmid constructs NF-κB-Luc (1 μg/mL), IRF3-Luc (1 μg/mL), β-gal (as a transfection control), PMA (100nM), MyD88 (1 μg/mL), or TRIF (1 μg/mL) in the presence or absence of BIOGF1K. Luciferase activity was measured using a luminometer. *p < 0.05, compared with the control. **p < 0.01, compared with the control. iNOS, inducible nitric oxide synthase; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-κB; PMA, phorbol-12-myristate-13 acetate; RT-PCR, reverse transcriptase-polymerase chain reaction; TRIF, Toll/interleukin-receptor domain containing adaptor molecule-inducing interferon-β; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
under IKKβ cotransfection. As we expected, BIOGF1K dose-dependently blocked NF-κB-mediated luciferase activity induced by IKKβ (Fig. 3E) in a dose-dependent manner.

3.4. Effect of BIOGF1K on activation of the IRF3 pathway

As Fig. 2E depicts, TRIF alone induced luciferase activity up to 234-fold, and 200 μg/mL of this extract dose-dependently and significantly (p < 0.01) downregulated luciferase activity up to 63%. BIOGF1K (200 μg/mL) also suppressed phosphorylated IRF3 in both nuclear fractions and whole lysates at 30 min (Figs. 4A, 4B). BIOGF1K also inhibited the phosphorylation of TBK1 (Fig. 4C), which is a critical event for IRF3 phosphorylation[15], indicating that TBK1 could be targeted in BIOGF1K-mediated inhibition of IRF3 activation. Moreover, we investigated the effect of BIOGF1K on upstream signaling events for IRF3 phosphorylation. LPS-induced phosphorylation of TBK1 at 2 min and 3 min was strongly diminished. Fig. 4D shows that BIOGF1K was able to dose-dependently (50 μg/mL and 200 μg/mL) inhibit both events triggered by LPS at 5 min. Validation work with overexpressed TBK1 (Fig. 4E) showed upregulation of TBK1 phosphorylation and IRF3-mediated luciferase activity, confirming that TBK1 might be a target of BIOGF1K-mediated anti-inflammatory action.

4. Discussion

Here, we benchmarked in vitro anti-inflammatory activities of BIOGF1K with LPS-treated macrophages and investigated its electron-donating activity induced by DPPH assay, based on its use as a skin care product and moisturizer in the cosmetic industry. Additionally, to elucidate the prospect of developing this extract as a source of potential anti-inflammatory and antioxidative remedies, we identified the molecular and pharmacological mechanisms of this extract.

As shown in Figs. 1 and 2, BIOGF1K inhibited production of the inflammatory mediator NO and the expression of the inflammatory genes iNOS and IFN-γ in LPS-stimulated RAW264.7 cells. As iNOS expression is increased in various skin diseases such as atopic eczema and psoriasis [43,44], the inhibitory effect of BIOGF1K on NO release can explain its known efficacy in cosmetic skin care formulations as well as in curing various skin inflammatory
symptoms and diseases. In addition, the antioxidative activity of BIOGF1K (Fig. 1B), as assessed by a DPPH assay, can also protect the skin from stress or UV radiation-induced free radical generation [45]. In fact, strong antioxidants are popularly used in many skin care formulations and as a functional food source [46,47]. Although BIOGF1K was prepared to increase the level of compound K from ginseng roots, as shown in Fig. 1D, these effects on NO production and antioxidative activity were similar to those reported from Korean Red Ginseng water extract and ginseng saponin fraction [20,48]. Therefore, the anti-inflammatory and antioxidative components of Korean ginseng could also be extracted in BIOGF1K.

To elucidate the molecular mechanisms of BIOGF1K-mediated anti-inflammatory activity, we explored its effect on the activation of NF-κB, a key transcription factor modulating inflammation [49]. Overall, the data imply that the NF-κB pathway might be a target transcription factor in the BIOGF1K-mediated anti-inflammatory effect. BIOGF1K blocked nuclear translocation of NF-κB subunits (p50 and p65; Fig. 3A) and diminished PMA-induced and MyD88- or TRIF-mediated NF-κB activation (Figs. 2B–2D). BIOGF1K also suppressed IκBα phosphorylation, which is an essential step for NF-κB translocation [50] (Fig. 3B). Phosphorylation of IκBα/β was strongly diminished by this extract at 2–5 min under LPS-stimulated conditions (Fig. 3C). Overexpression of IκBβ increased NF-κB-mediated luciferase activities up to more than 4,000-fold, which was dose-dependently diminished by BIOGF1K (Fig. 3E). Moreover, molecular validation work with AKT and IκBα overexpression in a reporter gene assay (Fig. 3E) and in immunoblotting analysis (Fig. 3D) clearly confirmed that IκBα could directly be

Fig. 4. Effect of BIOGF1K on activation of IRF3 and its upstream signaling. (A) Levels of phospho-IRF3, IRF3, and lamin A/C in nuclear fractions were determined by nuclear fractionation and immunoblotting analysis. (B–D) Phospho-protein or total protein levels of IRF3, TBK1, and β-actin in whole cell lysates were determined by immunoblotting analysis. (E) Inhibition of TBK1-induced IRF3 activation by BIOGF1K was analyzed using a reporter gene assay in HEK293 cells transfected with IRF3-Luc (1 μg/mL) and TBK1 in the presence or absence of BIOGF1K. Luciferase activity was measured using a luminometer. **p < 0.01, compared with the control. IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; TBK1, TANK-binding kinase 1.
In summary, BIOGF1K possesses strong in vitro antioxidative and anti-inflammatory activities through blockade of both IKK and TBK1, and suppression of the downstream activation of NF-κB and IRF3, as described in Fig. 5. Further studies will focus on validating BIOGF1K as a new anti-inflammatory candidate in preclinical studies.

Conflicts of interest

The authors report no conflicts of interest.

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