BIOGF1K, a compound K-rich fraction of ginseng, plays an antiinflammatory role by targeting an activator protein-1 signaling pathway in RAW264.7 macrophage-like cells

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1. Main text

Inflammation is a host defense mechanism that protects the body from invading pathogens and has ﬁve hallmarks: redness, swelling, heat, pain, and loss of function [1–3]. Inflammatory responses are initiated by macrophages recognizing pathogen-associated molecular patterns by pattern recognition receptors expressed on their surfaces and activating inﬂammatory signaling pathways, such as nuclear factor-kB (NF-kB), activator protein-1 (AP-1), and interferon-regulatory factors (IRFs) [4–8]. Toll-like receptors (TLRs) are the main pattern recognition receptors in macrophages, and TLR4 is a molecular receptor of lipopolysaccharide (LPS), the most powerful agonist derived from gram-negative bacteria able to activate inﬂammatory responses. LPS binding with TLR4 transduces inﬂammatory signaling cascades by activating various intracellular signaling kinases in macrophages, resulting in the overexpression of inﬂammatory genes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and the production of inﬂammatory mediators, including tumor necrosis factor-alpha, interleukin-1β (IL-1β), IL-6, nitric oxide (NO), and prostaglandin E2 [4–6,8,9].

Ginsenosides are the main active ingredients found in ginsengs and were reported to have many functions, including antiinflammatory, anticancer, antiviral, and antioxidative activities [10–14]. Recently, we prepared a new fraction of Korean ginseng containing a high amount of compound K, named BIOGF1K, and demonstrated its antiinflammatory activity [15]. Despite this study reporting an antiinflammatory role of BIOGF1K, mechanisms by which BIOGF1K plays a protective role in inﬂammatory responses remains unclear. Therefore, in this study, the antiinflammatory activity of BIOGF1K and the underlying mechanism present during inﬂammatory responses were investigated using an in vitro inﬂammatory cell model, specifically LPS-stimulated RAW264.7 cells. RAW264.7 and HEK293 cells (ATCC, Rockville, MD, USA) were cultured in Roswell Park Memorial Institute 1640 medium and Dulbecco’s modiﬁed Eagle’s medium (Gibco, Grand Island, NY, USA), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Gibco), streptomycin, penicillin, and l-glutamine at

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cellular signal-regulated kinase (ERK), mitogen-activated protein (ATF2), fos-related antigen 1, c-Jun N-terminal kinase, p38, extra-phorylated forms of c-Jun, c-Fos, activating transcription factor 2

...polyvinylidene fluoride membranes. Total and phosphorylated forms of c-Jun, c-Fos, activating transcription factor 2 (ATF2), fos-related antigen 1, c-Jun N-terminal kinase, p38, extra-cellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK)/ERK kinase 1/2 (MEK1/2), MAPK kinase 3/6 (MKK3/6), TAK1, HA, lamin A/C, and β-actin were detected by the antibodies specific for the targets and were visualized with an enhanced chemiluminescence solution (AbFrontier, Seoul, Korea). The data are presented as a mean ± standard deviation. Statistical analyses were performed by analysis of variance/Scheffe post hoc test and Kruskal–Wallis/Mann–Whitney U test. A p value < 0.05 was regarded statistically significant. All data were analyzed using an SPSS program (SPSS Inc., Chicago, IL, USA).

We first examined the effect of BIOGF1K on NO production, one of the critical inflammatory mediators in macrophage-mediated inflammatory responses. Prednisolone was used as a standard compound because it has been known to downregulate COX-2 and inflammatory cytokines [20]. Like prednisolone, BIOGF1K dose-dependently suppressed NO production in LPS-treated RAW264.7 cells (Fig. 1A) without serious cytotoxicity (Fig. 1B), indicating the antiinflammatory activity of BIOGF1K by the reduction of inflammatory mediators without cytotoxicity at the doses studied herein.

To examine the effect of BIOGF1K on mRNA expression of inflammatory genes, RAW264.7 cells were treated with BIOGF1K and LPS, and mRNA expression of iNOS, COX-2, and tumor necrosis factor-alpha was measured by semiquantitative and real-time reverse transcriptase polymerase chain reaction. BIOGF1K dose-dependently inhibited mRNA expression of iNOS and COX-2 in LPS-treated RAW264.7 cells (Fig. 2A). Inflammatory gene expression is governed by inflammatory signaling pathways, such as AP-1, NFκB, and IRF-3 [4–8], and the regulatory activity of BIOGF1K in NFκB and IRF-3 signaling pathways have been demonstrated in a previous study by Hossen et al [15]. Therefore, the effect of BIOGF1K on an AP-1 signaling pathway was examined by a luciferase assay. HEK293 cells transfected with plasmids expressing AP-1-Luc and Flag-MyD88, an adaptor molecule able to activate AP-1 signaling, were treated with BIOGF1K. BIOGF1K markedly suppressed the AP-1–mediated luciferase activity (Fig. 2B), suggesting that BIOGF1K downregulates mRNA expression of inflammatory genes by suppressing an AP-1 signaling pathway in macrophages.

Mechanisms by which BIOGF1K suppresses an AP-1 pathway in macrophage during inflammatory responses was further investigated by use of immunoblotting analysis. The hallmark of transcription factor activation is their nuclear translocation. Therefore, nuclear translocation of AP-1 transcription factors was examined in macrophages. BIOGF1K inhibited nuclear translocation of c-Jun (at 90 minutes), ATF2 (at 90 minutes), and phospho (p)-fos-related antigen 1 (at 60 minutes and 90 minutes) in LPS-treated RAW264.7 cells (Fig. 3A). The effect of BIOGF1K on the activities of MAPKs in AP-1 signaling was examined. BIOGF1K inhibited the activation of p38 (60 minutes) and ERK (90 minutes) in LPS-treated RAW264.7 cells (Fig. 3B). Phosphorylation of MAPK kinases (MAPKks), upstream signaling molecules of MAPKs were further investigated and found that BIOGF1K inhibited the activation of MEK1/2 (30 minutes) and MKK3/6 (30 minutes) in LPS-treated RAW264.7 cells (Fig. 3C). This result strongly supports the hypothesis of this study, since MEK1/2 and MKK3/6 are known as upstream MAPKks of ERK and p38, respectively [21,22]; moreover, BIOGF1K inhibited the activation of MAPKks earlier (30 minutes) than that of MAPKs (60 minutes) (Fig. 3B, C). These results suggest that BIOGF1K inhibits the AP-1 signaling pathway by inhibiting ERK and p38 MAPKs and MEK1/2 and MKK3/6, the upstream MAPKks of ERK and p38, in macrophages during inflammatory responses. However, the common upstream molecule of MEK1/2 and MKK3/6, TAK1, was not affected by BIOGF1K. We further confirmed the suppressive effect of BIOGF1K on the activation of these MAPKs and MAPKks in HEK293 cells by transfecting HA-TAK1, an activator of AP-1 signaling was examined. BIOGF1K inhibited the activation of MEK1/2 and ERK (Fig. 3D) and MKK3/6

### Table 1

| Name | Sequence (5' to 3') |
|------|---------------------|
| iNOS | CCCCCTGGAAGGTTCGTGGCAAGCAG | F |
| R    | GGGTGACAGGCGCTGGTGTGTTG |
| COX-2| CACATACCTGACCCACCTT | F |
|       | ATGGCTGGCCTGAGTATG | R |
| TNF-α| TTGGACCTCGACGCTG ACGTG | F |
| GAPDH| CACTACCGGAATTCACACAGCAA | F |
| R    | GACCTGACGCTGGTACGACCA |

COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcriptase polymerase chain reaction; TAK1, tumor necrosis factor-alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Fig. 1. LPS, lipopolysaccharide; NO, nitric oxide; PRED, prednisolone.

Fig. 2. COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide.
and p38 (Fig. 3E), respectively, in HA-TAK1-transfected HEK293 cells.

In this study, we investigated an antiinflammatory activity of BIOGF1K in macrophage-mediated inflammatory responses. We found that BIOGF1K suppressed the activation of an AP-1 pathway by targeting MAPKs, such as ERK and p38, and MAPKKs, such as MEK1/2 and MKK3/6, as summarized in Fig. 4, thereby suppressing inflammatory gene expression, such as iNOS and COX-2, as well as inflammatory mediator production, such as NO in macrophages during inflammatory response. For further validating inhibitory mechanism of BIOGF1K, whether TAK1 can be directly inhibited by BIOGF1K will be evaluated by employing a kinase assay. Collectively, these results strongly suggest that BIOGF1K, an active ingredient in ginseng, plays a protective role in macrophage-mediated inflammatory responses and provides evidence that BIOGF1K should be further examined as a promising antiinflammatory agent to prevent and treat inflammatory diseases.

Conflicts of interest

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2018.02.001.
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