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Research article

Panax ginseng–derived fraction BIOGF1K reduces atopic dermatitis responses via suppression of mitogen-activated protein kinase signaling pathway

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ABSTRACT

Background: BIOGF1K, a fraction of *Panax ginseng*, has desirable antimelanogenic, anti-inflammatory, and antiphotoaging properties that could be useful for treating skin conditions. Because its potential positive effects on allergic reactions in skin have not yet been described in detail, this study's main objective was to determine its efficacy in the treatment of atopic dermatitis (AD).
Methods: High-performance liquid chromatography was used to verify the compounds in BIOGF1K, and we used the (3-4-5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide method to determine its cytotoxicity in RBL-2H3 and HMC-1 cell lines. RBL-2H3 cells were induced using both anti–DNP-IgE/DNP-BSA and calcium ionophore (A2187) treatments, whereas HMC-1 cells were induced using A2187 alone. To measure mast cell degranulation, we performed histamine (enzyme-linked immunosorbent assay) and β-hexosaminidase assays. To quantify interleukin (IL)-4, IL-5, and IL-13 levels in RBL-2H3 cells, we performed quantitative polymerase chain reaction (PCR); to quantify expression levels of IL-4 and IL-13 in HMC-1 cells, we used semiquantitative reverse transcription polymerase chain reaction (RT-PCR). Finally, we detected the total and phosphorylated forms of extracellular signal-regulated kinase, p-38, and c-Jun N-terminal kinase proteins by immunoblotting.
Results: BIOGF1K decreased the AD response by reducing both histamine and β-hexosaminidase release as well as reducing the secretion levels of IL-4, IL-5, and IL-13 in RBL-2H3 cells and IL-4 and IL-13 in HMC-1 cells. In addition, BIOGF1K decreased MAPK pathway activation in RBL-2H3 and HMC-1 cells.
Conclusions: BIOGF1K attenuated the AD response, hence supporting its use as a promising and natural approach for treating AD.

1. Introduction

Atopic dermatitis (AD) is a skin condition with a relapsing and inflammatory nature that affects many people around the world [1] and is usually accompanied by itchy, scaly skin, and swollen eczematous flares [2]. Risks of AD include a strong genetic background (a strong family history of type I allergies) [3], impaired immunity, weakened skin barrier function, and environmental influences [4]. AD is also a direct consequence of an imbalance between T helper cells 1 and 2. In AD, the predominant cell type is T helper 2 [4]; hence, cytokines produced by this cell type, including interleukin (IL)-4, IL-5, and IL-13, are often found in high quantities in AD acute lesions and greatly influence allergic response development [5]. In general, IL-5 is associated with eosinophil recruitment [6], whereas IL-4 and IL-13 are related to the inhibition of antimicrobial peptides; the increase in both marks the probability of *Staphylococcus aureus* infection and the exacerbation of skin inflammation [7].

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In addition to this role, IL-4 provides positive feedback on T helper 2 cells (which, as a consequence, release more IL-4) and induces B isotype cell differentiation into IgE antibody–producing cells [8]. Cross-linking between IgE antibodies and FCεRI, a mast cell–specific receptor, triggers a signaling cascade, in which the mitogen-activated protein kinase (MAPK) pathway is activated. MAPK pathway activation, which includes the stimulation of proteins such as p38, extracellular signal-regulated kinase, and c-Jun N-terminal kinases, increases intracellular calcium, triggering mast cell degranulation as well as histamine and β-hexosaminidase secretion [9]. Histamine, one of the main mediators of allergic reaction, is released along with the granule-stored enzyme β-hexosaminidase [10]. For this reason, both of them are useful biomarkers for mast cell degranulation [11]. Intracellular calcium–increasing compounds such as calcium ionophore (e.g., A2187) are also effective activators of the MAPK signaling pathway and mast cell degranulation [12].

Topical corticosteroids and topical calcineurin inhibitors have been commonly used for managing AD-associated inflammation [13]. Although effective against the release of proinflammatory cytokines, both corticosteroids [14] and calcineurin inhibitors [15] can lead to serious secondary effects, therefore increasing the importance of identifying less toxic and effective treatments for AD. According to traditional Chinese medicine, *P. ginseng* has diverse benefits for treating skin conditions including wound healing [16], psoriasis, skin inflammation, and AD [17]. Currently, many researchers are focusing their studies on individual ginsenosides (steroid-like components abundant in ginseng species) [18] and their mechanism of action, in hopes of optimizing treatments for diverse diseases [17].

BIOGF1K, a novel fraction of *P. ginseng* whose previous analysis revealed high contents of compound K and compound Y [19], has shown significant antioxidant, anti-inflammatory [20], photoprotective, and antimelanogenic activities [19]. However, its effect on allergic reactions has not yet been described in detail. Therefore, in this study, we aimed to determine if BIOGF1K has the potential to treat AD and its related symptoms.

2. Materials and methods

2.1. Materials

RBL-2H3, a rat basophil cell line, and HMC-1, a human mast cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS), (3-4-5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT), streptomycin–penicillin solution, Dulbecco's modified Eagle's medium, and Roswell Park Memorial Institute medium were purchased from Gibco (Grand Island, NY, USA). Calcium ionophore (A2187), 4-nitrophenyl n-acetyl-β-d-glucosaminide, and anti–DNP-IgE antibody (D8406) were obtained from Sigma Aldrich (St. Louis, MO, USA). 2,4-Dinitrophenyl albumin from bovine serum (DNP-BSA) was purchased from Invitrogen (Waltham, MA, USA). The enzyme-linked immunosorbent assay (ELISA) kit for histamine detection (BA E−1000) was acquired from Labor Diagnostika Nord (Nordhorn, Germany). TRIzol was purchased from Thermo Fisher Scientific (Waltham, MA, USA). RT-PCR premix was purchased from Bio-D Inc. (Seoul, Korea), and SyGreen mix for quantitative PCR was purchased from PCRBIO (London, UK). The primer sets used in both polymerase chain reactions were synthetized by Macrogen (Seoul, Korea). Antibodies acquired from Cell Signaling Technology (Beverly, MA, USA) were used to detect the total and phosphorylated forms of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase, and β-actin.

2.2. Cell culture

RBL-2H3 cells were sustained in Dulbecco's modified Eagle's medium supplemented with 1% penicillin–streptomycin and 10% FBS, whereas HMC-1 cells were sustained in Roswell Park Memorial Institute media supplemented with 1% penicillin–streptomycin and 10% FBS. Both cell lines were kept in a 5% humidified incubator at 37°C.

2.3. Analysis and preparation of BIOGF1K

In accordance with previous reports [21], high-performance liquid chromatography (HPLC) was performed to analyze the phytochemical compounds existing in BIOGF1K. BIOGF1K was prepared following previously reported methods [22]. The HPLC profile of BIOGF1K is included in Supplementary Figure 1. To prepare a 100-mg/mL BIOGF1K stock solution, the compound was dissolved using dimethyl sulfoxide. To prepare the BIOGF1K target concentrations used in this study (12.5, 25 and 50 μg/mL), we used a serial dilution method, in which an initial concentration was diluted continuously until obtaining each of the working concentrations [20,23].

2.4. Cell viability assay

A density of 5×10^5 cells per mL was used to seed RBL-2H3 cells in a 96-well plate. After being cultivated for 24 h, cells were treated with concentrations from 0 to 50 μg/mL of BIOGF1K for 24 h. HMC-1 cells were seeded in a 96-well plate to a cell density of 1×10^6 cells per mL. After being cultivated for 24 h, cells were also treated using the same conditions. Cell viability for both cell lines was determined using the MTT method following previously reported procedures [19,24].

2.5. β-Hexosaminidase assay

RBL-2H3 cells were seeded at a density of 5×10^5 cells per mL in a 12-well plate. After being harvested for 24 h, cell sensitization was achieved by treating the cells overnight with 0.1 μg/mL anti-DNP IgE. Sensitized cells were washed with phosphate-buffered saline, pretreated with BIOGF1K in concentrations from 0 to 50 μg/mL for 30 min, and induced for 2 h with 1 μg/mL of DNP-BSA. In the case of A2187-mediated induction, RBL-2H3 cells were pretreated with BIOGF1K in concentrations from 0 to 50 μg/mL for 30 min and then treated with 1 μg/mL of A2187 for 24 h [25]. A β-hexosaminidase release assay was performed as previously described [26], in which 60 μL of the supernatant was combined with 60 μL of 7.5-mM 4-nitrophenyl n-acetyl-β-d-glucosaminide (in 80 mM of citric acid buffer, pH 4.5) in a 96-well plate. The solution was maintained at 37°C for 2 h. Then, 120 μL of 0.2-M concentrated glycine buffer (pH 10.7) was added to stop the reaction, and the absorbance was determined at 405 nm. Finally, β-hexosaminidase release was calculated as a percentage of control according to the normal values (absorbance sample-absorbance media/absorbance normal-absorbance media*100).

2.6. Histamine production assay (ELISA)

A density of 5×10^5 cells per mL was used for seeding RBL-2H3 cells in a 12-well plate. After being maintained for 24 h, RBL-2H3 cells were pretreated for 30 min with BIOGF1K in concentrations from 0 to 50 μg/mL and then induced with A2187 (1 μg/mL) for 24 h. For inducing cross-linking with IgE/DNP-BSA in RBL-2H3 cells, sensitization was achieved by treating the cells overnight with anti-DNP IgE (0.1 μg/mL), followed by a rinse with phosphate-buffered

saline, pretreatment for 30 min with BIOGF1K (0-50 μg/mL), and induction with DNP-BSA (1 μg/mL) for 2 h. For the histamine ELISA assay, 50 μL of supernatant was collected and the assay was performed as instructed by the manufacturer [Labor Diagnostika Nord (LDN)].

2.7. Quantitative real-time and semiquantitative PCR analysis of mRNA levels

A density of 5×10^5 per mL was used for seeding RBL-2H3 cells in a 12-well plate. After being maintained for 24 h, BIOGF1K pretreatment and both calcium ionophore (A2187)– and IgE/DNP-BSA–mediated inductions were performed as mentioned previously (section 2.6). Real-time and semiquantitative PCR were carried out according to previously published reports [27]. The sequences of the primers used for performing this experiment can be found in Table 1.

2.8. Immunoblotting

RBL-2H3 cells were seeded at a density of 5×10^5 cells per mL in a 6-well plate. After 24 h, calcium ionophore (A2187)–mediated induction, IgE/DNP-BSA–mediated induction, and BIOGF1K pretreatment were performed as formerly mentioned in section 2.6. HMC-1 cells were seeded at a density of 1×10^6 cells per mL in a 6-well plate. After 24 h, HMC-1 cells were induced with calcium ionophore (A2187) and pretreated with BIOGF1K as formerly mentioned in section 2.6. Cell lysates were prepared in accordance with the previously described procedures used by Park et al [28,29], in which the lysates were separated based on size using sodium dodecyl sulfate–polyacrylamide gel electrophoresisand later transferred to a polyvinylidene fluoride membrane. Finally, through the use of specific antibodies and chemiluminescence reagents, target proteins (total and phosphorylated forms) were detected.

2.9. Statistical analysis

The values of are presented as mean ± standard deviation of a minimum of three independent experiments in this article. The Mann–Whitney test was performed to evaluate differences between the experimental and control groups; a p value < 0.05 was considered to be statistically significant. Analyses were performed using SPSS (SPSS, Chicago, IL, USA).

Table 1
Primer sequences (human) used in real-time and semiquantitative PCR

Name	Direction	Primer
Real-time		
IL-4	Forward	TGTACCGGGAACGGTATCCA
	Reverse	ACATCTCGGTGCATGGAGTC
IL-5	Forward	AGAATCAAACTGTCCGAGGGG
	Reverse	ACTCATCACGCCAAGGAACTC
IL-13	Forward	GCTCTCGCTTGCCTTGGTGG
	Reverse	CATCCGAGGCCTTTTGGTTA
GAPDH	Forward	GTTACCAGGGCTGCCTTCTC
	Reverse	GATGGTGATGGGTTTCCCGT
Semiquantitative		
IL-4	Forward	ATCTTTGCTGCCTCCAAGAACA
	Reverse	CTCTGGTTGGCTTCCTTCACA
IL-13	Forward	AGAATCCGCTCAGCAATCCTC
	Reverse	ATTGCTCTCACTTGCCTTGG
GADPH	Forward	CACTCACGGCAAATTCAACGGCAC
	Reverse	GACTCCACGACATACTCAGCAC

IL, interleukin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3. Results

3.1. BIOGF1K analysis and effects on cell viability

According to the cell viability (MTT) assay, cell viability in RBL-2H3 cells was not affected by BIOGF1K up to a concentration of 50 μg/mL, but appeared to be slightly toxic at the same concentration in HMC-1 cells (Fig. 1A and B). HPLC was used to verify the main compounds present in the BIOGF1K fraction, which corresponded to compound K and compound Y (Supplementary Fig. 1).

3.2. BIOGF1K decreased mast cell degranulation

BIOGF1K decreased the release of β-hexosaminidase in concentrations up to 50 μg/mL in calcium ionophore (A23187) (Fig. 2A) while decreasing it dose dependently from 12.5 to 50 μg/mL in IgE/DNP-BSA–induced RBL-2H3 cells (Fig. 2B). The ELISA assay showed there was a decrease in histamine release while using a BIOGF1K concentration of 50 μg/mL in both calcium ionophore (A23187) (Fig. 2C) and IgE/DNP-BSA (Fig. 2D) treatments.

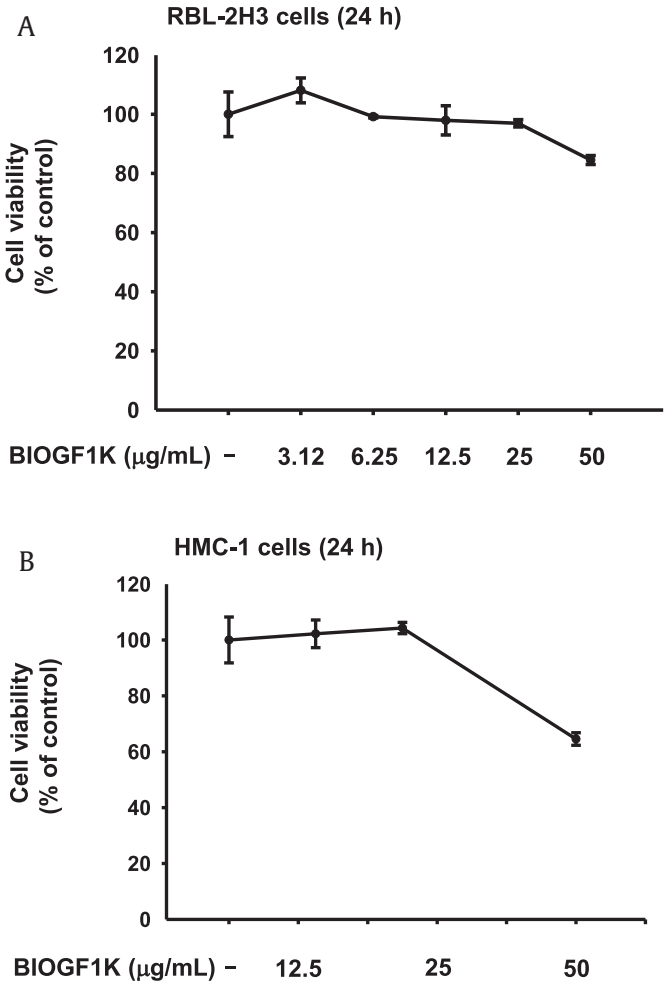

Fig. 1. Cell viability of RBL-2H3 and HMC-1 cells after BIOGF1K treatment. (A and B) RBL-2H3 and HMC-1 cells were incubated with BIOGF1K in concentrations from 0 to 50 μg/mL for 24 h. MTT assay was used to determine their cell viability. (C) HPLC analysis and profile of the phytochemical compounds present in BIOGF1K. MTT, (3-4-5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; HPLC, high-performance liquid chromatography.

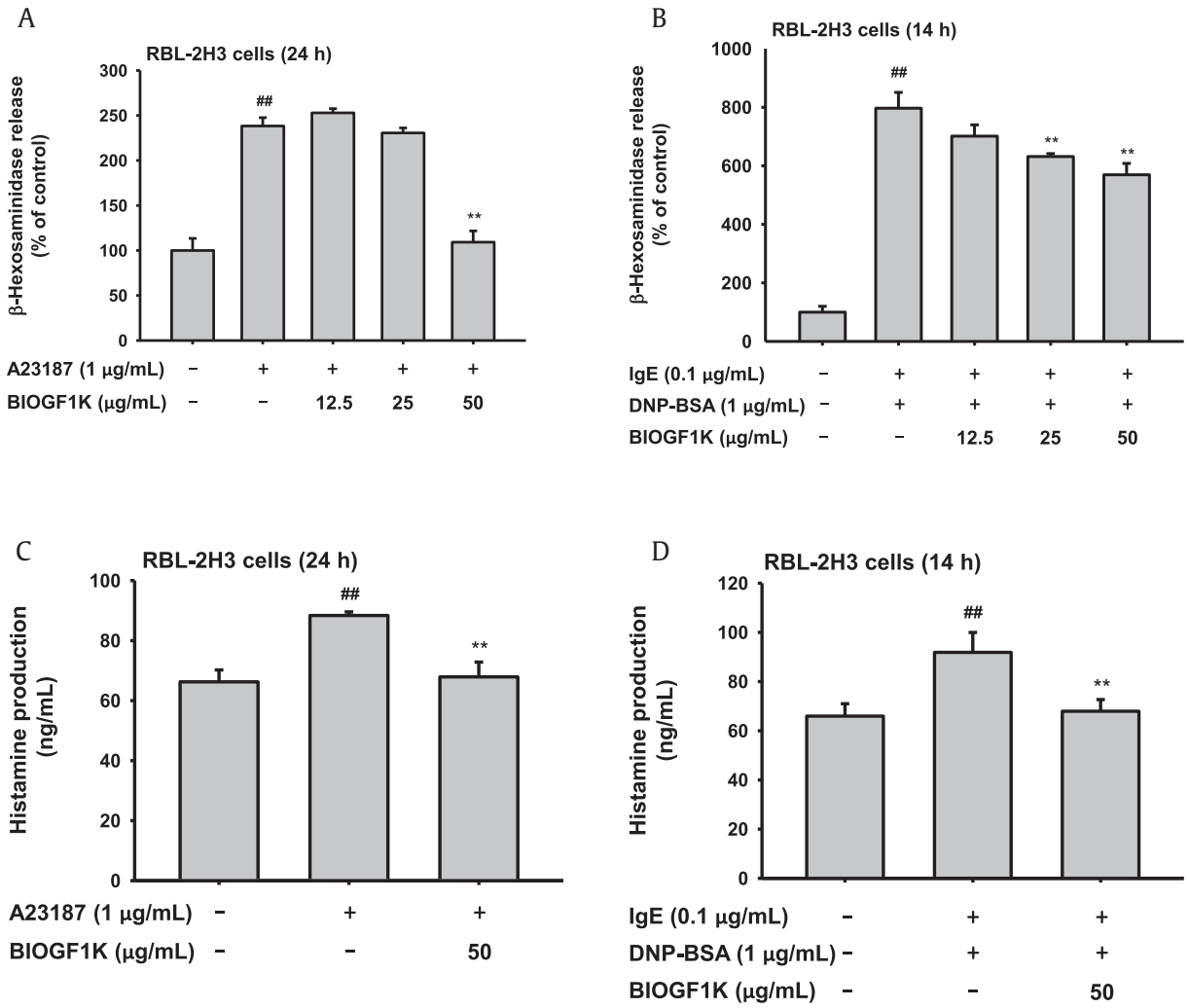

Fig. 2. BIOGF1K decreased the effect of mast cell degranulation. A density of 5×10^5 cells per mL was used to seed RBL-2H3 cells in a 12-well plate. (A, C) After 24 h of incubation time, RBL-2H3 cells were pretreated for 30 min with BIOGF1K (0-50 μg/mL), then induced with 1 μg/mL of A2187 for 24 h. (B, D) RBL-2H3 cell sensitization was performed overnight with IgE (0.1 μg/mL), washed with PBS, pretreated for 30 min with BIOGF1K (0-50 μg/mL), and treated with 1 μg/mL of DNP-BSA for 2 h. (A, B) For the β-hexosaminidase secretion assay, 60 μL of the supernatant was mixed with 60 μL of p-NAG and kept for 2 h in a 96-well plate at 37°C. Glycine buffer (120 μL) was added, and the absorbance was measured at 405 nm. (C, D) 50 μL of supernatant was collected and a histamine release assay (ELISA) was performed using the manufacturer's specifications. $^{\#\#}p < 0.01$ versus a normal (untreated) group, $^{**}p < 0.01$ versus a control (induced) group. DNP-BSA, 2,4-dinitrophenyl albumin from bovine serum; p-NAG, 4-nitrophenyl n-acetyl-β-d-glucosaminide; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

3.3. BIOGF1K diminished the expression of AD-related cytokines

BIOGF1K decreased the expression of IL-4, IL-5, and IL-13 in a dose-dependent manner in RBL-2H3 cells previously induced using calcium ionophore (A23187) (Fig. 3A, C, and E). However, in the IgE/DNP-BSA–induced RBL-2H3 cells, BIOGF1K reduced the secretion of IL-4, IL-5, and IL-13 in concentrations up to 50 μg/mL (Fig. 3B, D, and F). BIOGF1K also decreased the expression of both IL-4 and IL-13 cytokines in HMC-1 cells previously induced with calcium ionophore (A23187) (Fig. 3G).

3.4. BIOGF1K lessened MAPK pathway activation

BIOGF1K diminished the activation levels of MAPK signaling pathway proteins in RBL-2H3 cells previously induced with A23187 (Fig. 4A) or anti–DNP-IgE/DNP-BSA cross-linking (Fig. 4B). BIOGF1K also reduced MAPK signaling pathway expression in HMC-1 cells induced with A23187 (Fig. 4C) .

4. Discussion

Because of its healing properties, *P. ginseng* has been used for centuries in Asian medicine for treating many different skin conditions, including skin inflammation, psoriasis, and AD [17]. Nonetheless, relatively few studies investigated *P. ginseng*'s use in the treatment of AD. Recent studies with Korean Red Ginseng extract showed an improvement in AD-related symptoms in patients [30], whereas *in vivo* studies carried out with AD-induced Balb/c mice (compound 40/80) showed a decrease in allergy-related cytokine expression, IgE content in serum, and MAPK pathway activation in phorbol myristate acetate–induced and A23187-induced HMC-1 cells [31].

The main component present in the BIOGF1K fraction was previously found to be compound K or 20-*O*-β-d-glucopyranosyl-20(*S*)-protopanaxadiol (Fig. 1C, Supplementary Fig.1) [19]. GDP has previously shown important antiallergic activity: a GDP-fortified ginseng extract ameliorated the AD response in NC/Nga mice induced with *Dermatophagoides farinae* body extract [32]. Because

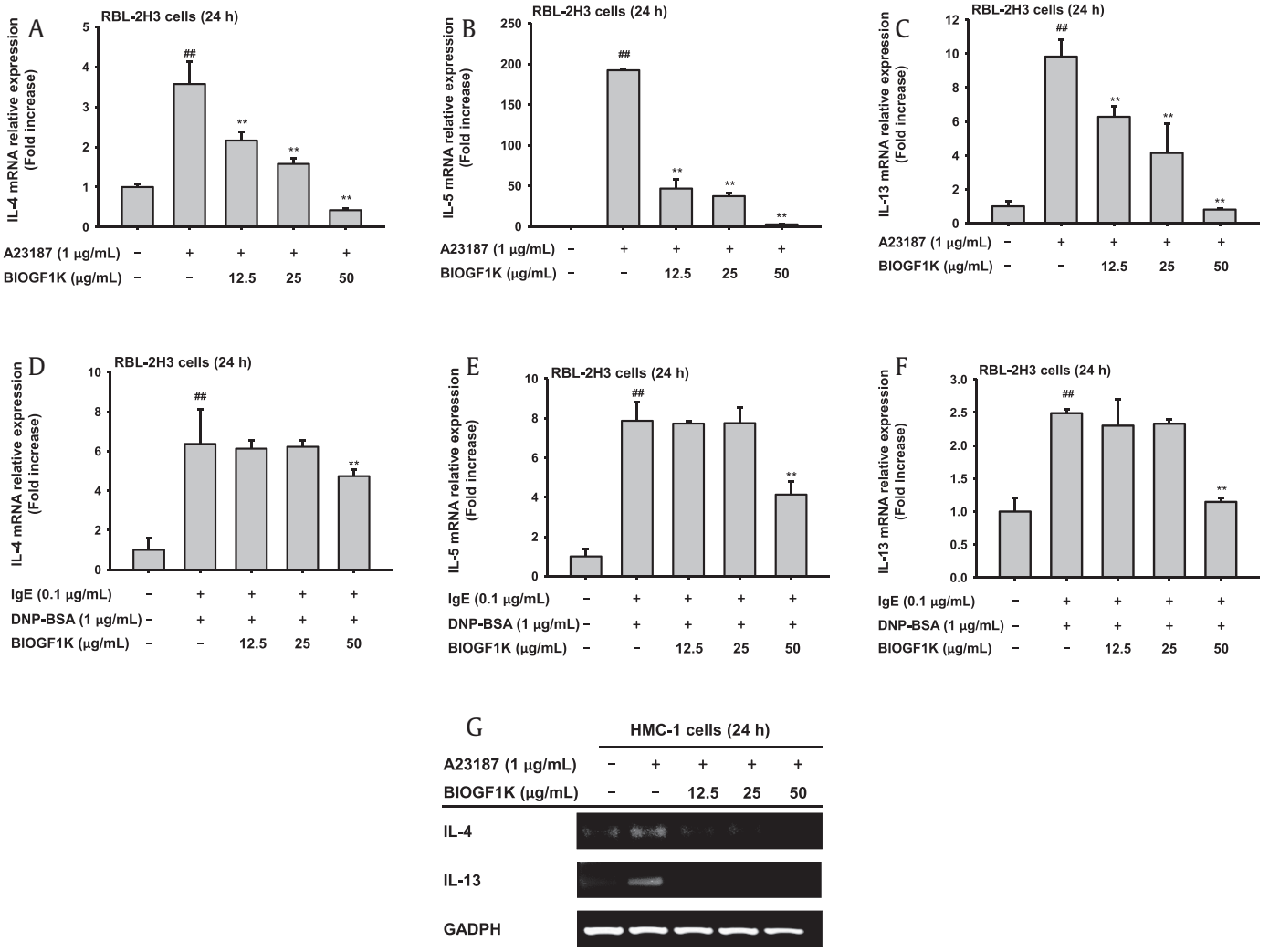

Fig. 3. Downregulating effect of BIOGF1K on atopic dermatitis–related cytokines expression. A density of 5×10^5 cells per mL was used for seeding RBL-2H3 cells in a 12-well plate. (A, C, and E) After 24 h, RBL-2H3 cells were pretreated for 30 min with BIOGF1K (0-50 μg/mL), then treated with 1 μg/mL of A2187 for 24 h. (B, D, and F) RBL-2H3 cell sensitization was performed overnight with IgE (0.1 μg/mL), then the cells were washed with PBS, pretreated for 30 min with BIOGF1K (0-50 μg/mL), and finally induced using 1 μg/mL of DNP-BSA for 2 h. The secretion level of IL-4, IL-5, IL-13, and GADPH were determined by quantitative PCR. (G) A density of 1×10^6 cells per mL was used for seeding HMC-1 cells in a 6-well plate. After 24 h, cells were pretreated for 30 min with BIOGF1K (0-50 μg/mL), then treated with 1 μg/mL of A2187 for 24 h. RT-PCR was used to quantify the expression of IL-4 and IL-13 and GADPH. $^{\#\#}p < 0.01$ versus a normal (untreated) group, $^{**}p < 0.01$ versus a control (induced) group. DNP-BSA, 2,4-dinitrophenyl albumin from bovine serum; IL, interleukin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

of its high compound K content as well as its promising antioxidant, anti-inflammatory [19], antiphotoaging, and antimelanogenic properties [20], we predict that BIOGF1K can be useful in the treatment of AD. Concentrations up to 50 μg/mL of BIOGF1K exhibited low cytotoxicity in RBL-2H3 cells (Fig. 1A) but reduced cell viability slightly in HMC-1 cells (Fig. 1B). This observation might be due to HMC-1 being a suspended, more sensitive cell line [33]. Research to determine toxicity in each cell line as well as trials using *in vivo* models are needed.

As previously mentioned, the MAPK pathway can activate transcription factors involved in the secretion of AD cytokines, including IL-4, IL-5, IL-13, and an increase in intracellular Ca^{2+} that triggers mast cell degranulation [12]. Therefore, many studies have focused on compounds that downregulate the MAPK pathway [34]. The fact that BIOGF1K decreased the activation level of MAPK proteins in RBL-2H3 cells induced using calcium ionophore (A23187) (Fig. 4A) and anti–DNP-IgE/DNP-BSA (Fig. 4B) as well as the HMC-1 cells induced using the calcium ionophore (A23187) (Fig. 4C) suggests BIOGF1K may have beneficial properties for AD treatment.

As previously mentioned, IL-4, IL-5, and IL-13 cytokines are known to take part in AD pathogenesis [5]. The downregulating effect of BIOGF1K on AD responses was further confirmed by dose-dependent (up to a concentration of 50 μg/mL) lessened secretion of IL-4, IL-5, and IL-13 cytokines in calcium ionophore (A23187)–induced RBL-2H3 cells (Fig. 3A, C and E) and IL-4, IL-5, and IL-13 levels in anti–DNP-IgE/DNP-BSA–induced RBL-2H3 cells (Fig. 3B, D and F). In the case of calcium ionophore (A23187)–induced HMC-1 cells, IL-4 and IL-13 secretion was also reduced dose dependently (Fig. 3G).

It has been well documented that histamine, serotonin, β-hexosaminidase, and other granule components released upon mast cell degranulation play a central role in the progression of allergic reactions [11]; thus, efforts have been made to try to reduce their secretion. Previous reports suggested that ginsenosides derived

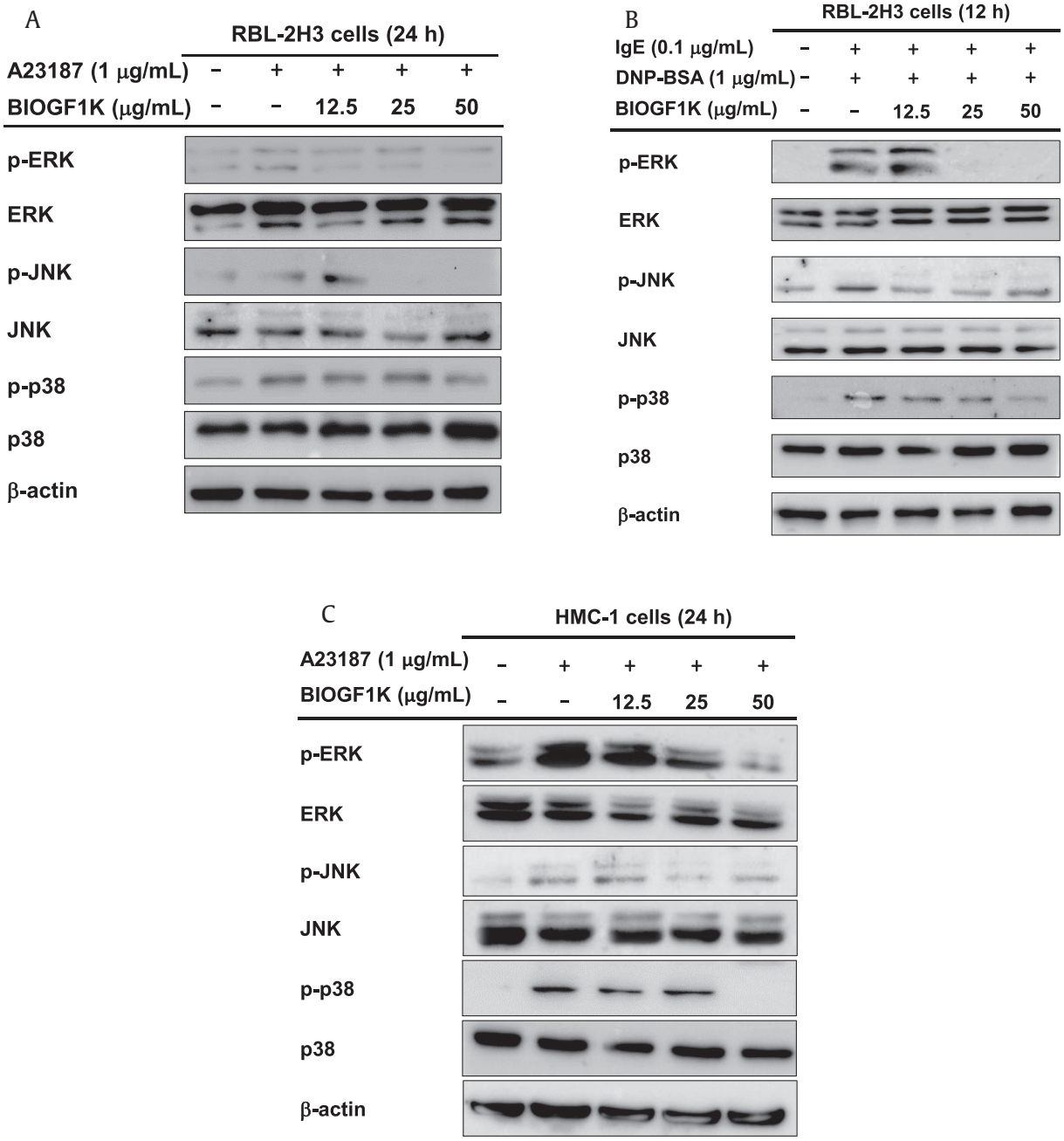

Fig. 4. Downregulation effect of BIOGF1K on the atopic dermatitis–activated MAPK pathway. A density of 5×10^5 cells per mL was used to seed RBL-2H3 cells in a 12-well plate. (A) After 24 h of harvesting time, RBL-2H3 cells were pretreated for 30 min with BIOGF1K (0–50 μg/mL) and induced with A2187 (1 μg/mL) for 24 h. (B) RBL-2H3 cell sensitization was performed overnight with IgE (0.1 μg/mL), and then the cells were washed with PBS, pretreated for 30 min with BIOGF1K (0-50 μg/mL), and treated with DNP-BSA (1 μg/mL) for 2 h. (C) A density of 1×10^6 cells per mL was used for seeding HMC-1 cells in a 6-well plate. Cells were incubated for 24 h, pretreated for 30 min with BIOGF1K (0-50 μg/mL) and then treated with A2187 (1 μg/mL) for 24 h. Phosphorylated and total forms of ERK, p-38, and JNK proteins were determined using immunoblotting. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; DNP-BSA, 2,4-dinitrophenyl albumin from bovine serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase.

from *P. ginseng* reduced mast cell degranulation. Among them, gintonin lessened histamine's secretion in DNFB-induced NC/Nga mice [35], whereas other ginsenosides, including (G)-Rf, (G)-Rh2, and (G)-Rg3, diminished β-hexosaminidase's release in previously IgE-sensitized RBL-2H3 cells [36].

Because BIOGF1K lessened the MAPK pathway's activation, a reduction in mast cell degranulation was also expected. In accordance with these expectations, we detected a decrease in histamine secretion at a concentration of 50 μg/mL in both calcium ionophore (A23187)– (Fig. 2C) and anti–DNP-IgE/DNP-BSA–induced RBL-2H3 cells (Fig. 2 D). A reduction in β-hexosaminidase secretion was also detected in both calcium ionophore (A23187)–induced (Fig. 2A) and anti–DNP-IgE/DNP-BSA–induced RBL-2H3 cells (Fig. 2 B). This suggests BIOGF1K may have a positive effect on AD.

Conclusively, because of its effect on MAPK pathway downregulation, the lessened secretion of AD-associated cytokines (IL-4, IL-5, and IL-13) and the decrease in mast cell degranulation confirmed by a decrease in histamine and β-hexosaminidase secretion as

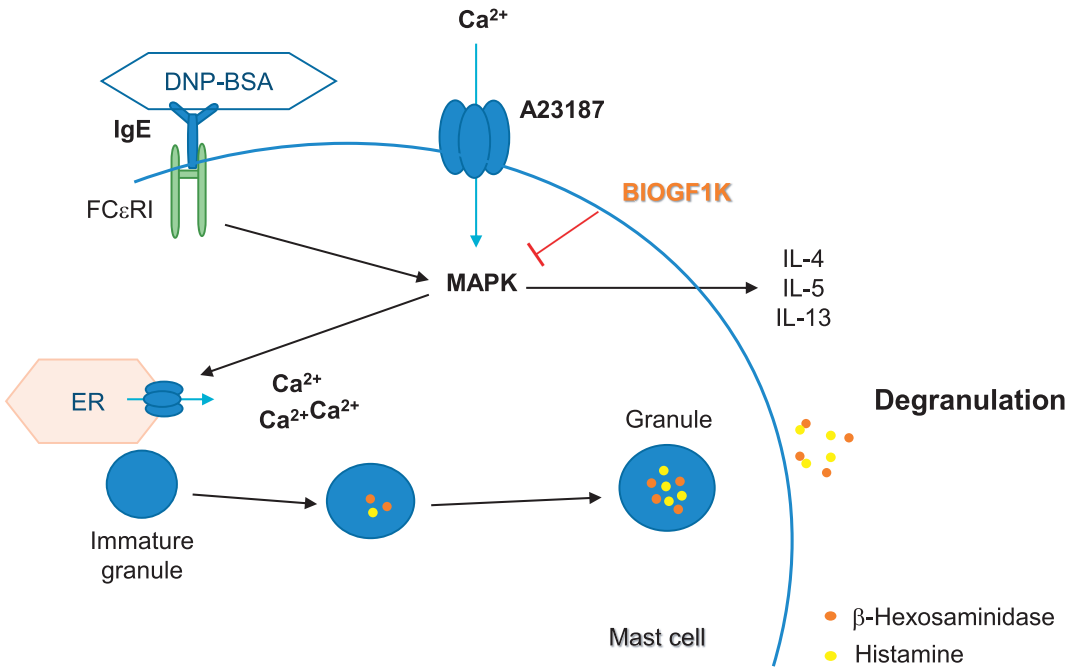

Fig. 5. Schematic pathway summarizing the effect of BIOGF1K on atopic dermatitis induction. Antigen/IgE cross-linking with the mast cell–specific receptor FCεRI and calcium ionophore (A23187) induction activates the MAPK pathway. BIOGF1K decreases MAPK pathway protein activation, decreasing intracellular calcium signaling and therefore diminishing mast cell degranulation, histamine release, and interleukin production. DNP-BSA, 2,4-dinitrophenyl albumin from bovine serum; IL, interleukin; MAPK, mitogen-activated protein kinase.

summarized in Fig. 5, we predict that BIOGF1K has the potential to be used in the treatment of AD. However, more research regarding its toxicity in other cell lines and *in vivo* models and more details on its mechanisms of action are needed to optimize its utility in the pharmacological industry.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of interest

The authors declare to have no conflicts of interest.

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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.2019.02.003.

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