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Fermentation-Mediated Enhancement of Ginseng's Anti-Allergic Activity against IgE-Mediated Passive Cutaneous Anaphylaxis In Vivo and In Vitro

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Ginseng (the root of *Panax ginseng* Meyer) fermented by *Lactobacillus plantarum* has been found to attenuate allergic responses in in vitro and in vivo experimental models. Ginseng has been reported to also possess various biological functions including anti-inflammatory activity. The present study was aimed at comparing the anti-allergic effect of ginseng and fermented ginseng extracts on IgE-mediated passive cutaneous anaphylaxis in vitro in a murine cell line and in vivo in mice. Fermented ginseng extract (FPG) showed higher inhibitory effect against in vitro and in vivo allergic responses when compared with ginseng extract (PG). The secretion of β-hexosaminidase and interleukin (IL)-4 from the IgE-DNP-stimulated RBH-2H3 mast cells were significantly ($p < 0.05$) inhibited by FPG treatment, and this effect was concentration-dependent. Further, MKK4 activation and subsequent JNK phosphorylation were attenuated by FPG treatment. The inhibitory effect of FPG on the in vitro allergic response was verified in vivo against IgE-DNP–induced passive cutaneous anaphylaxis in a mouse model. These data indicated that the fermentation of ginseng with *L. plantarum* enhanced its anti-allergic effects both in vitro and in vivo. We predict that compositional changes in the ginsenosides caused by the fermentation may contribute to the change in the anti-allergic effects of ginseng. The results of our study highlight the potential of the use of FPG as a potential anti-allergic agent.

Keywords: Ginseng, fermentation, anti-allergic, anaphylaxis, *Lactobacillus plantarum*

Introduction

Allergies are hypersensitivity disorders mediated by immunological mechanisms that result in tissue damage and are involved in many allergic diseases such as atopic dermatitis, rhinitis, asthma, and anaphylaxis. Hence, the regulation of allergic reactions is an important and attractive research area [1, 2]. Mast cells and basophils are critical for various allergic disorders [3]. These cells express surface membrane receptors with a high affinity and specificity for IgE (FcεRI) [4]. IL-4 is a key cytokine in IgE-dependent mast cell activation and allergic inflammation. Activated mast cells secrete granules containing various allergic mediators such as β-hexosaminidase, histamines, eicosanoids, and pro-inflammatory cytokines [5–7]. Mechanistically, the interaction of FcεRI with antigen-bound IgE causes the activation of a signaling cascade involving phospholipase C and mitogen-activated protein kinases (MAPKs). The IgE

receptor activates the JNK pathway through the MEKK2-MKK4/7 cascade [8, 9]; this pathway is essential for the induced expression of some cytokine genes such as IL-4, IL-6, or TNF-α and is activated following antigenic mast cell activation [10, 11]. These cytokine-induced reactions then cause tissue inflammation and anaphylaxis, both of which are becoming chronic health problems worldwide [12].

Ginseng (root of *Panax ginseng* C.A. Meyer) from the family Araliaceae is an herbaceous plant cultivated mainly in countries in East Asia, such as Korea, China, and Japan [13]. Ginseng is considered one of the most renowned medicinal agents extensively used to treat various diseases in traditional Chinese and Oriental systems of medicine [14−16].

It is well documented that the fermentation of foods and plants enhances their biological functions [17]. *Lactobacillus* strains occur naturally in the human intestine, and for this reason, they are also preferentially developed for a variety of industrial food fermentations and for commercial use as probiotics [18]. Although the anti-allergic effects of ginseng and red ginseng have been previously reported [19, 20], there is no comparative study between the anti-allergic effects of ginseng and its fermented extract (FPG). In vivo, IgE-mediated passive cutaneous anaphylaxis (PCA) is a well-characterized model for studying immediate-type hypersensitivity [21]. Therefore, in the present study, we aimed to evaluate the effect of FPG on allergic reactions in RBL-2H3 cells in vitro as well as on the prevention of IgE-induced PCA in vivo and compared it with that of ginseng extract (PG). Our results highlight the potential for use of FPG as an anti-allergic agent.

Materials and Methods

Reagents and Antibodies

Minimum essential medium (MEM), fetal bovine serum (FBS), opti-MEM, and penicillin-streptomycin were purchased from Gibco (USA). De Man, Rogosa and Sharpe (MRS) media, Rat IL-4 ELISA kit, and TMB substrate reagent were purchased from BD Biosciences (USA). Lactate dehydrogenase (LDH) assay kit (CytoTox 96) was purchased from Promega (USA). Antibodies against MAPKs and p-MKK4 were purchased from Cell Signaling Technology Inc. (USA). β-actin antibody was purchased from Santa Cruz Biotechnology (USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Evans blue, formamide, anti-dinitrophenyl (DNP)-IgE, dinitrophenyl-conjugated human serum albumin (DNP-HSA), 4-nitrophenyl N-acetyl-β-D-glucosaminide, JNK inhibitor SP600125, and all other chemicals were purchased from Sigma Aldrich Co. (USA).

Preparation of Ginseng

Panax ginseng was cultivated at Jiri Mountain in South Gyeongsang Province, Korea. The roots of *P. ginseng* were collected from 2-year-old plants, and then, washed and dried. The dried roots were crushed and extracted with 70% ethanol for 4 h at 65°C. The obtained extract was then evaporated under reduced pressure at 50°C (BUCHI Rotavapor R-210) to remove ethanol, resulting in the final *P. ginseng* extract (PG) with a concentration of 7%–9% (w/w), which was used for fermentation.

Fermentation of Ginseng

The protocol for the fermentation of ginseng was as reported previously [22] with slight modifications. *L. plantarum* (HSW-01) was cultured in MRS broth at 30°C, and the ginseng extract was inoculated with *L. plantarum* (1 portion of extract: 10 portions of *L. plantarum* cultured media, v/v) and incubated for 100 days at 4°C. After 100 days, the fermented ginseng extract was filtered (Advantec filter paper, 5 μm) and concentrated to achieve 10 brix in a rotary evaporator at 50°C. The concentrates were further mixed with 95% ethanol to elute saponins by agitating for 2 h at 40°C. The eluates were then filtered using a filter paper, concentrated in a rotary evaporator, and dissolved in 40% butylene glycol solution (5x; v/v) for 1 h. Next, butylene glycol was removed under reduced pressure in a rotary evaporator, and the concentrates were finally mixed with 95% ethanol and agitated. The ethanol was then removed using a rotary evaporator. The final yield of fermented ginseng extract (FPG) was approximately 5% of the starting dried ginseng material. The extract from the incubation of *L. plantarum* in MRS broth without ginseng extract under the same conditions (BL) was used as the control.

Cell Culture and Animal Treatment

RBL-2H3 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in MEM with 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator (5% CO_2, 95% air). For the in vivo studies, female BALB/c mice (6 weeks old) were obtained from ORIENT Co., Korea and housed for 1 week on a 12-h light-dark cycle at 24 ± 1°C with access to food and water *ad libitum*. All experiments were performed in accordance with the institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University, Korea (Permit number KU18001).

Degranulation Assay of β-Hexosaminidase

Degranulation from the RBL-2H3 cells was evaluated by measuring the activity of β-hexosaminidase enzyme secreted in the extracellular medium. Cells were pre-cultured in 24-well plates (1.5×10^5 cells/well) overnight and then sensitized with anti-DNP IgE (110 ng/ml) antibodies for 2 h. After sensitization, the medium was replaced with opti-MEM, and cells were incubated for 1 h with or without BL, FPG, PG, or SP600125. Subsequently, surface IgE was cross-linked with DNP-HSA

(25 ng/ml) for 15 min.

After sensitizing the cells, the culture supernatant (30 μl) was transferred to 96-well plates and incubated with 30 μl of substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in citrate 0.05 M, pH 4.5) for 60 min at 37°C. The cells were then lysed with 0.1% Triton X-100 before removing the supernatant to measure the total β-hexosaminidase activity. The reaction was stopped by adding 200 μl of 0.1 M Na_2CO_3-NaHCO$_3$ buffer at pH 10. The color generated was measured at 405 nm using a spectrophotometric microplate reader (Molecular Devices Corp., USA). The percentage of specific β-hexosaminidase release was calculated as follows: percentage release = 100 × supernatant activity/(supernatant activity + cell lysate activity) [23]).

Assessment of Cytotoxicity Via Cell Viability (MTT) and Cell Cytotoxicity (LDH) Assays

The protocol for MTT and LDH assay was followed as described previously [24]. Briefly, cells were cultured in 96-well plates (1 × 10⁴ cells/well) overnight and then treated with diluted BL, FPG, or PG and incubated for 24 h. Cell cytotoxicity was measured with a LDH assay method using the cytotox 96 kit (G1780) according to the manufacturer's instructions. Percent cytotoxicity = 100 × (leaked LDH OD value of experimental group - LDH OD value of non-treated cells group)/(LDH OD value of positive control group – LDH OD value of non-treated cells group). For MTT assay, the medium was discarded completely and 100 μl of MEM medium containing MTT (500 μg/ml) was added to each well, followed by incubation for 2 h at 37°C. After incubation, medium was discarded and DMSO was added to each well for the solubilization of formazan. Optical density was measured at 550 nm, and value was determined in comparison to control cells. Percent viability = 100 × experimental cells OD/Non-treated cells OD.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-4 Secretion

Supernatants of the cultured RBL-2H3 cells were collected 6 h after DNP-HSA stimulation, and the concentration of IL-4 was measured with a commercial sandwich ELISA method using the BD OptEIATM Set (555198) according to the manufacturer's instructions. The color generated was measured at 450 nm using a spectrophotometric microplate reader.

SDS-PAGE and Immunoblotting

The conditions used for the immunoblot analysis were as described previously [24]. The membrane was developed for visualization using an ECL detection kit developed by Chemiluminescence imaging systems (Davinch-Chemi; Davinch-K, Korea).

IgE-Mediated PCA in Mice

BALB/c mice were passively sensitized with IgE by intradermal injection in the ears, using 500 ng monoclonal mouse dinitrophenol-specific (anti-DNP) IgE in 20 μl of PBS. After 24 h, the mice were intravenously injected with 100 μg of DNP-HSA in 200 μl of PBS containing 2% Evans blue. FPG (25 or 50 mg/kg) and PG (100 mg/kg) were administered intraperitoneally 1 h before DNP-HSA injection. The mice were sacrificed after 20 min, and the Evans blue was extracted by incubating ear samples of equal size in 200 μl of formamide for 12 h at 64°C. The optical absorbance of the Evans blue was measured at 620 nm using a spectrophotometric microplate reader.

LC-MS/MS Analysis

A Waters UPLC system and tandem quadrupole mass spectrometer system (USA) were used for LC-MS/MS analysis. Chromatographic separation was performed using a UPLC BEH C18 column (2.1 × 100 mm²) with 1.7 μM packing. The gradient and running time were as shown in Table 1. The electrospray ionization interface (ESI) was operated in both negative modes (desolvation temperature 300°C, source temperature 120°C, desolvation gas flow 600 l/h, cone gas flow 50 l/h). The mass spectrometer was operated in MS–MS modes with collision cell gas pressure (argon) set at 1.2×10^{-3} T.

Statistical Analyses

Data are represented as mean ± standard error of mean (S.E.M.). The statistical analysis of the results was performed by one-way ANOVA, followed by Tukey's multiple comparison tests. Data were analyzed using statistical Prism Software (GraphPad Software Inc., USA) and values of $p < 0.05$ were considered statistically significant.

Results

FPG Inhibits FcεRI-Mediated Allergic Reactions In Vitro

RBL-2H3 cells were used as the in vitro model to evaluate the anti-allergic effect of FPG and PG. The cytotoxicity of BL, FPG, and PG on RBL-2H3 cells was examined using the

Table 1. Column gradients and sample running time[†].

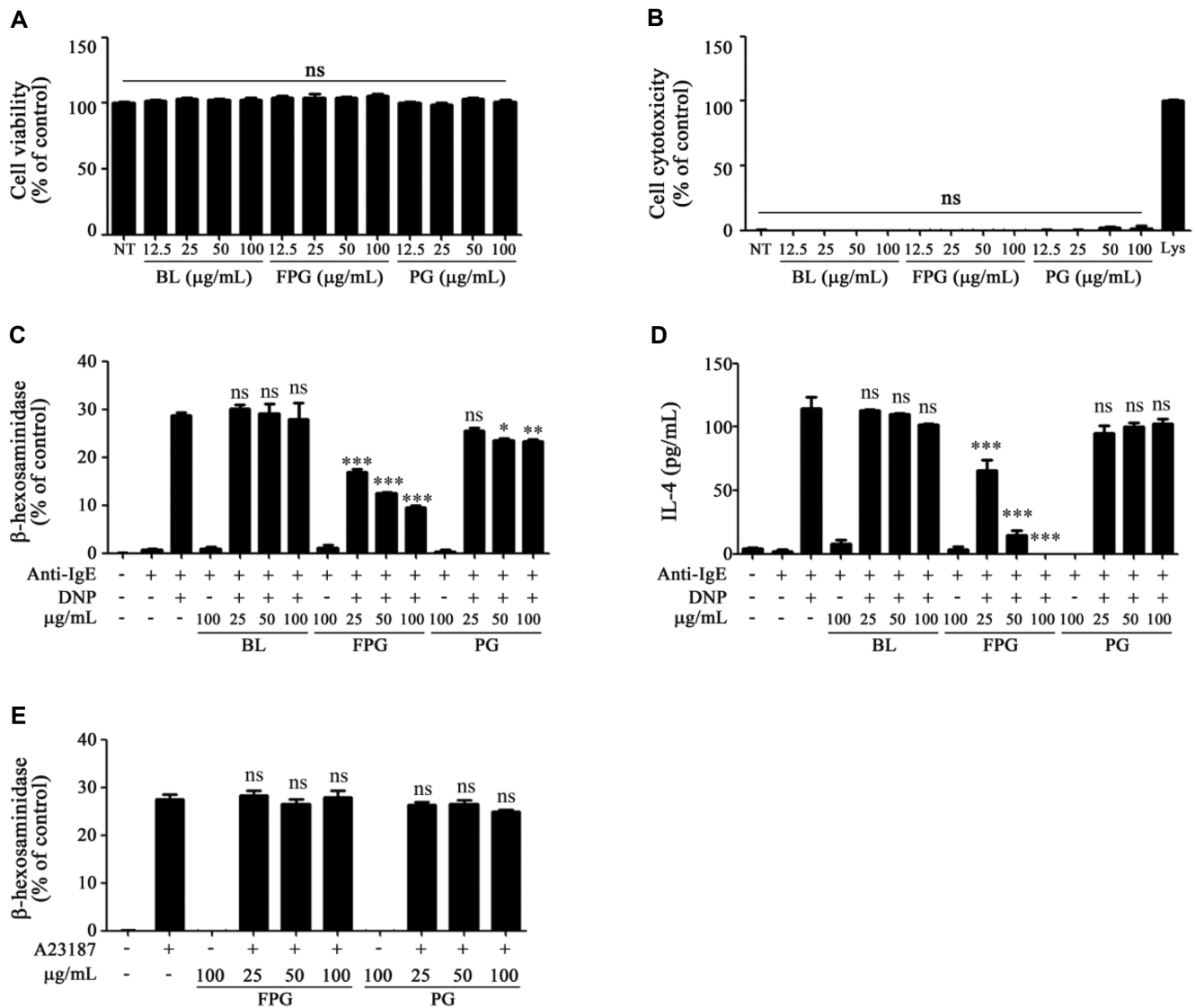| Time (min)	Flow (ml/min)	% A (0.1% Formic acid)	% B (Acetonitrile)
Initial	0.3	80	20
0.10	0.3	80	20
2.00	0.3	68	32
7.00	0.3	67	33
20.00	0.3	48	52
23.00	0.3	47	53
26.00	0.3	20	80
26.90	0.3	0	100
27.00	0.3	80	20
30.00	0.3	80	20

Fig. 1. Inhibitory effects of FPG on allergic responses in vitro and in vivo.

RBL-2H3 cells were treated with various concentrations of BL, FPG, and PG for 24 h. Cell viability was measured using the MTT assay (**A**), and cytotoxicity was measured using the LDH assay (**B**). Non-treated cells (NT) were used as the negative control, and whole cell lysate (Lys) was used as the positive control. (**C**) RBL-2H3 cells were incubated with anti-DNP IgE (110 ng/ml) and then treated with various concentrations of BL, FPG, or PG; subsequently, surface IgE was cross-linked with or without DNP-HSA (25 ng/ml) for 15 min. The percentage of specific β-hexosaminidase release was determined as described in the methods. (**D**) FcεRI-induced RBL-2H3 cells were stimulated with or without DNP-HSA for 6 h. IL-4 production was determined using ELISA. (**E**) RBL-2H3 cells were treated with various concentrations of FPG and PG and then stimulated with or without A23187. Data represent the mean ± SEM of three independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: non-significant.

MTT and LDH assays. As shown in Figs. 1A and 1B, treatment with BL, FPG, and PG at concentrations up to 100 μg/ml did not result in cytotoxicity. Therefore, we chose 100 μg/ml as the maximum concentration for subsequent experiments in RBL-2H3 cells.

Mast cells mediate the early phase of type I hypersensitivity reactions by releasing granule contents and cytokines after cross-linking with FcεRI [25]. Therefore, we measure the inhibitory effects of FPG and PG on FcεRI-mediated degranulation in RBL-2H3 cells with the release of β-hexosaminidase as the degranulation biomarker [26]. As shown in Fig. 1C, the IgE-DNP–induced β-hexosaminidase release was significantly inhibited by FPG treatment, and this effect was concentration dependent. However, BL did

not show a significant inhibitory effect on β-hexosaminidase release. PG showed marginal inhibition in β-hexosaminidase release; however, the decrease in β-hexosaminidase release with PG was not as marked as that with FPG.

Further, IL-4 plays an important role in facilitating mast cell activation as well as in inducing the secretion of other Th2 type cytokines [27, 28]. In this study, IgE-sensitized RBL-2H3 cells were treated with BL, FPG, or PG and challenged with DNP-HSA. The results showed that FPG treatment, but not BL or PG treatment, significantly ($p <$ 0.05) decreased the secretion of IL-4, and this effect was concentration dependent (Fig. 1D). Since BL did not show any significant inhibitory effect on allergic responses in vitro, we excluded them in subsequent experiments and only examined the inhibitory activities of FPG and PG on allergic responses in vitro and in vivo.

The calcium ionophore A23187 can increase intracellular concentrations of Ca^{2+}; this influx of Ca^{2+} subsequently triggers the degranulation of mast cells by bypassing receptor activation [29]. Therefore, to explore the effects of FPG/PG on receptor bypassing degranulation from RBL-2H3 cells, we pre-treated the cells with FPG or PG and subsequently induced them with A23187. We found that neither FPG nor PG had any effect on the release of β-hexosaminidase (Fig. 1E), indicating that FPG effectively attenuates FcεRI-mediated hypersensitivity reaction in mast cells.

Together these results from our in vitro experiments indicated that the fermentation of ginseng with *L. plantarum* enhances its anti-allergic properties.

FPG Attenuates PCA In Vivo

To verify the inhibitory effects of FPG on allergic responses in vivo, we used a mouse model of IgE-DNP–
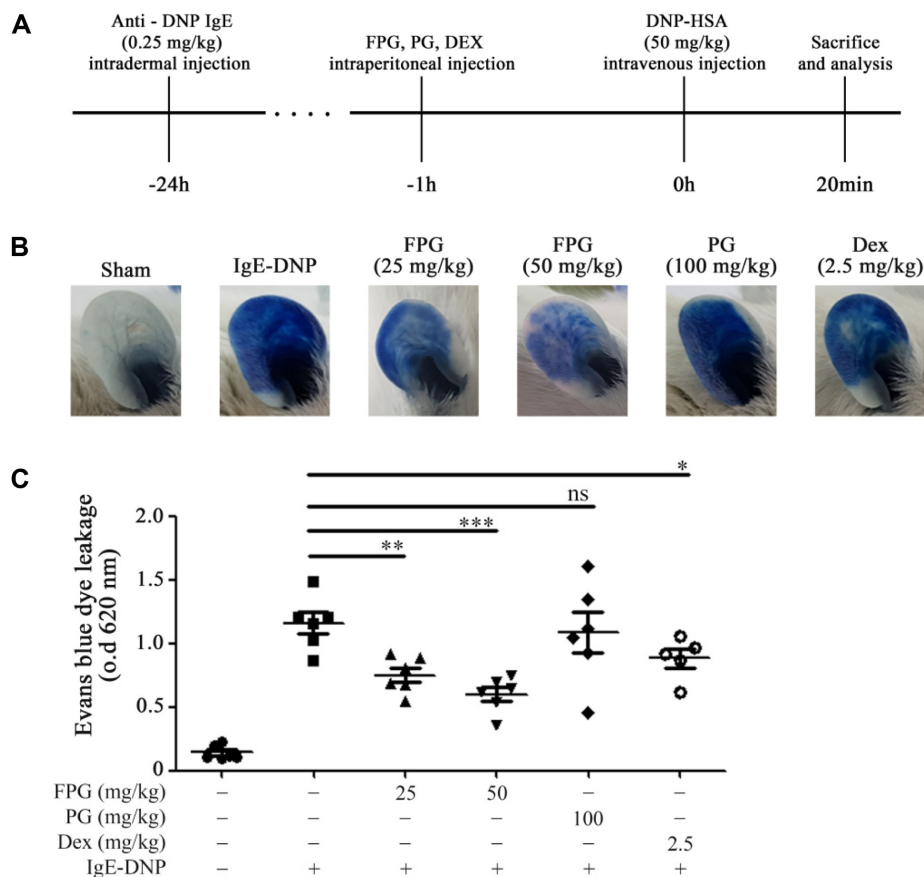

Fig. 2. In vivo efficacy of PG and FPG on PCA.

(**A**) The experimental scheme for PCA and treatment with FPG (25, 50 mg/kg), PG (50 mg/kg), or DEX (2.5 mg/kg) (*n* = 6). (**B**) Upon DNP-HSA challenge, vascular leakage was assayed by measuring Evans blue extravasation into ear tissues. (**C**) Ear samples of equal size were excised 20 min after the intravenous challenge with antigen and Evans blue, and subsequently extracted in formamide. Each point showed the mean optical density at 620 nm. Data represent the mean ± SEM of three independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: non-significant.

induced PCA (Fig. 2A). The ears of BALB/c mouse were injected intradermally with monoclonal DNP-specific IgE antibody and FPG or PG was subsequently injected intraperitoneally. As shown in Figs. 2B and 2C, the DNP-antigen induced strong PCA concomitant with rapidly occurring capillary dilatation and leakage of Evans blue dye into the reaction site of the mouse ears. Leakage of the injected Evans blue dye was significantly and dose-dependently suppressed by pre-treatment with either FPG or dexamethasone, a known anti-allergic positive control [6]. PG, even with at a higher concentration (100 mg/kg) than that used for FPG (50 mg/kg) could not attenuate the anaphylactic responses.

FPG Attenuates FcεRI-Mediated JNK Activation

FcεRI aggregation activates diacylglycerol (DAG), inducing the activation of protein kinase C (PKC) and the downstream MAPK pathway, including the phosphorylation of JNK, p38, and ERK; these in turn lead to the transcription of cytokine genes [9, 30]. Therefore, we investigated the potential involvement of MAPKs in FPG-induced regulation to elucidate the mechanism underlying the role of FPG in the FcεRI-triggered signaling pathway, which is stimulated by the binding of FcεRI to IgE cross-linked to DNP. As shown in Fig. 3A, FPG decreased IgE-DNP–induced phosphorylation of JNK, but not that of ERK or p38. Mitogen-activated protein kinase kinase 4 (MKK4), a member of the MAP kinase kinase family, directly phosphorylates and activates the JNK pathway [31]. We therefore aimed to determine the role of FPG in the phosphorylation of MKK4 at 7 and 15 min after the cross-linking of FcεRI with IgE-DNP. FPG also decreased the IgE-DNP-induced phosphorylation of MKK4 in a concentration-dependent manner (Fig. 3B). Next, to elucidate the role of FPG in the correlation between MKK-mediated JNK activation and degranulation, RBL-2H3 cells were pre-incubated with SP600125, a JNK inhibitor, and then subjected to IgE-DNP treatment. As shown in Fig. 3C, both SP600125 and FPG dose-dependently and significantly ($p < 0.05$) inhibited the release of β-hexosaminidase from RBL-2H3 cells, indicating that the MKK-JNK pathway is at least partly involved in the FPG-mediated regulation of degranulation from RBL-2H3 cells.

Analysis of Ginsenosides in FPG and PG

Extensive studies on the role of ginsenosides have led to a partial understanding of the physiological functions of ginseng. In our experiments, to analyze the prominent anti-allergic function of FPG, we analyzed 30 kinds of ginsenosides from PG and FPG. Table 2A shows the ginsenosides that exhibited similar levels in FPG and PG or those whose levels were decreased due to fermentation. The levels of many other ginsenosides were also increased upon fermentation of ginseng (Fig. 2B).

Discussion

In the present study, we found that the fermentation of *P. ginseng* extract enhanced its anti-allergic effects in vitro and in vivo. We also found that the improvement in the anti-allergic effects upon fermentation was due to the attenuation of the FcεRI-induced phosphorylation of the MKK4-JNK pathway, which resulted in reduced secretion of FcεRI-mediated β-hexosaminidase and IL-4 in mast cells. Further, our study revealed that the levels of several constituent ginsenosides were altered by the fermentation of ginseng. FPG significantly and more strongly inhibited IgE-induced PCA compared with PG, suggesting that the increase in the anti-allergic effect of ginseng upon fermentation may be due to the altered active constituents. Our experiments also showed that the levels of some of the ginsenosides were increased, while those of some others were decreased upon fermentation.

In a previous study, the ginsenosides Rb1, Rd, F2, and compound K (C-K) were reported to induce β-hexosaminidase release from RBL-2H3 cells [3]. In contrast, the ginsenosides Rh1, Rh2, Rg1, and Rg3 have been reported to inhibit allergic responses [32−35]. Furthermore, Rg3 has been reported to reduce inflammatory responses in human asthmatic airway epithelial cells and tissues [35]. Consistent with these reports, we found that FPG showed lower levels of Rb1, Rb2, and C-K and had enhanced anti-allergic effect compared to PG and that the levels of Rh1, Rh2, and Rg3 were higher in FPG than in PG. Although the levels of Rg1 were decreased and those of Rd and F2 were increased in FPG, which is contrary to previous reports [3, 36], FPG showed more intense anti-allergic activity. One limitation of our study is that we were unable to identify the ginsenosides actively involved in the improvements in the anti-allergic effect of PG upon fermentation. Future studies are needed to determine whether the anti-allergic effects of FPG are controlled by a single ginsenoside or if these effects are a coordinated action of multiple ginsenosides.

However, we could not identify the exact active constituent present in ginsenosides that helped in the enhanced anti-allergic effect of PG upon fermentation. Further studies are needed to determine whether the anti-allergic effects of
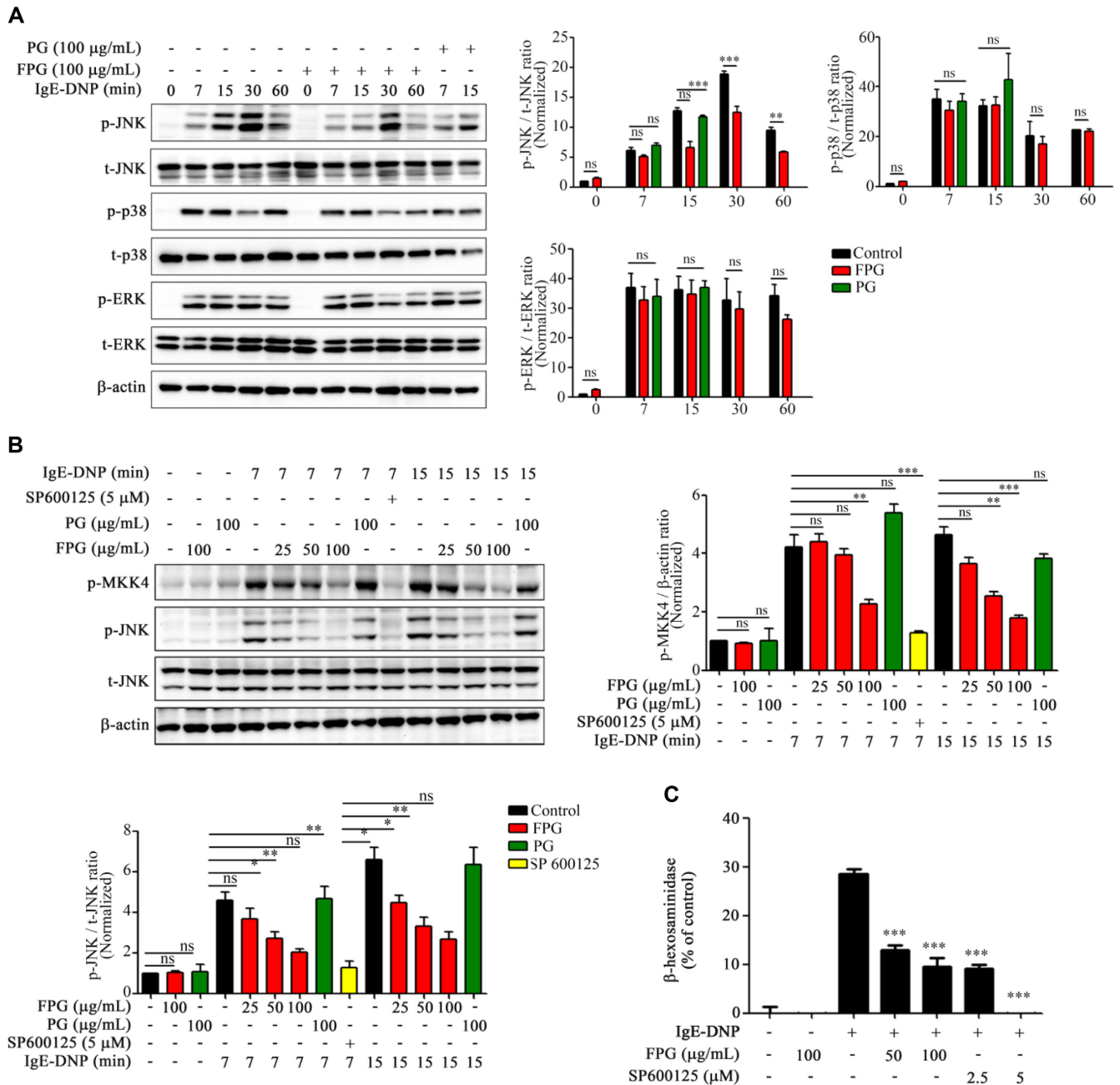
Fig. 3. Involvement of FcεRI-mediated MKK4-JNK pathway in the anti-allergic effect of FPG.

(**A**, **B**) RBL-2H3 cells were incubated with anti-DNP IgE (110 ng/ml) for 2 h, treated with the indicated amount of FPG, PG, or SP600125 for 1 h, and then cross-linked with or without DNP-HSA for the indicated times. Cell lysates were analyzed by western blot analyses using specific antibodies. β-actin was used as the internal control. The statistical band intensities are indicated with the histogram. (**C**) RBL-2H3 cells were incubated with anti-DNP IgE (110 ng/ml), treated with various concentrations of FPG or SP600125, and subsequently stimulated with or without DNP-HSA (25 ng/ml) for 15 min. The percentage of specific β-hexosaminidase release was determined as described in the methods. The bar graphs present the mean values ± SEM of three independent experiments; *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$, ns: non-significant.

FPG are controlled by a single ginsenoside constituent or a combined effect of all ginsenosides present in FPG.

In conclusion, we observed that the fermentation of ginseng is a beneficial technique to increase the anti-allergic effect of ginseng. This study provides scientific evidence for the therapeutic efficacy of FPG in the prevention of allergic

Table 2. Altered constituents of ginsenosides in FPG and PG.

A

Ginsenosides (mg/kg)	PG	FPG	PG - FPG (⇩)
Rb1	4415.65	246.7	4168.95
Re	2699.66	2465.4	234.26
Rg1	2071.3	793	1278.3
Rc	1921.93	494.6	1427.33
Rb2	1598.29	788.9	809.38
Rf	945.63	34.2	911.43
Ro	483.97	10.47	473.5
C-K	39.83	8.45	31.38

B

Ginsenosides (mg/kg)	PG	FPG	FPG - PG (⇧)
Rd	1156.94	2000.8	843.86
Rb3	180.64	1115.4	934.76
Rg2(S)	180.24	452.6	272.36
F2	13.84	436.1	422.26
F1	10.66	383.3	372.64
C-O	4.78	312.8	308.02
Rg6	3.88	285.4	281.52
C-Mx1	-	276	276
Rg2(R)	3.49	261.8	258.31
Rh1(S)	37.04	241.83	204.79
Rh4	5.88	208.33	202.45
Rg3(S)	20.51	200.1	179.59
C-Mc1	10.26	195.8	185.54
Rg4	3.58	142.62	139.04
Rg5	3.98	141.7	137.72
Rg3(R)	0.1	107.78	107.68
Rk1	30.87	87.85	56.98
Rh1(R)	14.44	82.07	67.63
PPTol	0.6	73.2	72.6
Gyp17	23.5	68.5	45
Rk3	-	40.9	40.9
Rh2(S)	-	25.72	25.72
Rh2(R)	-	15.74	15.74
Rh3	-	13.81	13.81
Rk2	-	11.22	11.22
PPDol	3.88	8.06	4.18
C-Y	2.79	5.61	2.82
C-Mx	0.32	5.2	4.88
C-Mc	-	3.94	3.94

Representative chromatograms from LC-MS/MS are shown. (A) Ginsenosides with reduced levels (⇩) in FPG compared with PG. (B) Ginsenosides with increased levels (⇧) in FPG compared with PG. Data represent the mean ± SEM of three independent experiments; ***$p < 0.001$, ns: non-significant.

reactions and highlights the potential of the development of FPG as a promising agent in the treatment of anaphylaxis, pruritus, and other inflammatory diseases.

Acknowledgements

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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