Research article

20(S)-Protopanaxatriol inhibits release of inflammatory mediators in immunoglobulin E-mediated mast cell activation

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ABSTRACT

Background: Antiallergic effect of 20(S)-protopanaxatriol (PPT), an intestinal metabolite of ginseng saponins, was investigated in guinea pig lung mast cells and mouse bone marrow-derived mast cells activated by a specific antigen/antibody reaction.

Methods: Increasing concentrations of PPT were pretreated 5 min prior to antigen stimulation, and various inflammatory mediator releases and their relevant cellular signaling events were measured in those cells.

Results: PPT dose-dependently reduced the release of histamine and leukotrienes in both types of mast cells. Especially, in activated bone marrow-derived mast cells, PPT inhibited the expression of Syk protein, cytokine mRNA, cyclooxygenase-1/2, and phospholipase A2 (PLA2), as well as the activities of various protein kinase C isoforms, mitogen-activated protein kinases, PLA2, and transcription factors (nuclear factor-κB and activator protein-1).

Conclusion: PPT reduces the release of inflammatory mediators via inhibiting multiple cellular signaling pathways comprising the Ca2+ influx, protein kinase C, and PLA2, which are propagated by Syk activation upon allergic stimulation of mast cells.

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1. Introduction

It has been reported that Panax ginseng Meyer exerts a variety of pharmacological effects on the immune system [1]. In the studies with human and rodents, ginseng extracts or various ginseng components exhibited mitogenic activity on T and B lymphocytes [2,3], and immunomodulatory effects in a variety of diseases [4,5]. Ginseng saponin enhances the phagocytic activity of macrophage by increasing the intracellular Ca2+ level ([Ca2+]i) and the protein kinase C activity [6], and ginsenoside Rb1 inhibits cyclooxygenase-2 (COX-2) expression and nuclear factor (NF)-κB activation [7]. However, a person engaged in Korean ginseng wholesale was recently reported to be suffering from occupational asthma [8].

In the inflammatory and allergic responses, protopanaxadiol Rb1 inhibits histamine release and leukotrienes (LTs) in guinea pig lung mast cells (GPMCs) activated by specific antigen/antibody reaction [9]. Ginseng ameliorates chronic histopathologic changes in murine asthma [10]. Ginsan, a polysaccharide derived from P. ginseng, also has tumoricidal activities [11], antiseptic activity [12], and antiasthmatic effects in a mouse OVA (ovalbumin)-induced asthma model [13].

A variety of ginseng saponin metabolites are formed by intestinal bacteria when total ginseng extracts or total ginseng saponins is administered orally to a human or rat, respectively, and absorbed into the systemic circulation [14,15], and then their metabolites including nonsugar moieties of ginsenosides (aglycones) are detected in blood and in urine. Compound K [16] and Rh2 [17] among various ginseng metabolites have potent inhibitory activity on β-hexosaminidase release from RBL-2H3 cells.

Mast cells are well known as major effector cells for immunoglobulin (Ig) E-mediated immediate hypersensitivity and chronic allergic reactions such as asthma. Antigen-specific IgE bound to the...
high-affinity receptor (FcεRI) on the mast cell membrane encounters the multivalent antigen, and then the cells are activated. Activated mast cells secrete preformed mediators [histamine, tryptase, chymase, tumor necrosis factor (TNF)α, and other proteins] as well as newly synthesized proinflammatory mediators such as prostaglandin D2, LTs, cytokines, and chemokines [18–21]. These mediators have been postulated to be responsible for airway inflammation and remodeling in allergic asthma [19,22]. Although 20(S)-dihydroprotopanaxadiol and 20(S)-dihydroprotopanaxatriol have distinctive immunological responses [23,24], the effects of 20(S)-protopanaxatriol (PPT, Fig. 1A), one of the ginseng saponin metabolites formed by human intestinal bacteria, on the inflammatory mediator release during mast cell activation, which is a well-known event in allergic asthma, has not been reported yet. Therefore, we examined the effects of PPT on the release of inflammatory mediators from the guinea pig lung mast cells (GPMCs) and mouse bone marrow-derived mast cells (BMMCs) activated by a specific antigen/antibody reaction to determine the role of ginseng intestinal metabolites of orally administered ginseng extract.

2. Materials and methods

2.1. Animals

Female Hartley guinea pigs weighing 200–250 g and female BALB/c mice weighing 20 g (age 8 weeks) were purchased from Samtako BioKorea (Osan, Korea) and maintained in specific...
pathogen-free conditions prior to being sacrificed. All animals were housed in accordance with the guideline from the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all protocols were approved by the Institutional Review Board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University, Suwon, Korea.

2.2. Reagents

OVA (fraction V), collagenase (type I), elastase (type I, porcine pancreatic), human serum albumin—dithionithrophenol (HSA-DNP), Fluo-3 AM, and percoll were purchased from Sigma-Aldrich Chemical Co (St Louis, MO, USA); leukaotiene immunoassay kit and [3H]arachidonic acid were purchased from PerkinElmer (Waltham, MA, USA); and gelatin was obtained from Difco Laboratories (Detroit, MI, USA). PPT (molecular weight, 476.7) was prepared by the chemical modification of ginseng saponin with periodic acid as described previously [25]. Its structure is shown in Fig. 1A. Other chemicals and reagents used in this experiment were of the best grade.

The PPT was dissolved in 50 mM stock solution in dimethyl sulfoxide (DMSO), and diluted prior to use. The percentage (%) of DMSO in experimental solution or media was 0.01%, 0.02%, and 0.04% for 10 μM, 50 μM, or 100 μM PPT, respectively. These percentages of DMSO did not affect release of mediators (data not shown). The concentration of PPT was chosen in preliminary experiments.

2.3. Immunization and preparation of anti-OVA antibody

Ten outbred female guinea pigs were immunized by injecting a mixture of 50 μg/200 μL OVA and complete Freund’s adjuvant into a foot pad. After 1 week, 100 μg/200 μL OVA was injected intradermally at one side of the back of the animals and 200 μg/200 μL of OVA at the other side of the back of the animals. One week later, mice were sacrificed for getting sera, and the sera were kept in aliquots at −70°C until use [26]. To separate IgG1 antibody from sera, guinea pig serum was applied to anti-IgG2 affinity column and the column was washed with 0.1 M citric acid (pH 2.1). IgG1 passed through and it was concentrated under pressure for the experiments. The titers of anti-OVA antibody were in the range 1,600/70 to 10,000/70. The amounts of LTs in each supernatant obtained from GPMCs or BMMCs were determined in preliminary experiments, and both types of mast cells were treated with PPT (10 μM, 50 μM, or 100 μM) 5 min prior to the antigen challenge.

2.7. Histamine assay

Histamine release in each supernatant obtained after activation of GPMCs or BMMCs were quantified by the automated fluorometric analyzer (with dialysis; Series 300 Analyzer; Astoria Clackamas, OR, USA). The detection limit of this assay is approximately 5 ng/mL of histamine. Amounts of histamine release were expressed as the percentage of the total histamine in non-stimulated cells [26].

2.8. LT immunoassay

The amounts of LTs in each supernatant obtained from GPMCs or BMMCs were determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Briefly, 50 μL samples were incubated with anti-LT antiserum (diluted 1:120) and acetylcholinesterase-linked LTs (diluted 1:120) in wells that were coated with mouse monoclonal antibodies for 18 h at room temperature. After rinsing with washing buffer, color was developed using Ellman’s reagent, and the plates were read at 412 nm with a spectrophotometer. The concentrations of LTs were then calculated using standard curves.
generated with specific Lts standards and using analysis tools on the Cayman Chemical website (http://www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z).

2.9. Reverse transcription—polymerase chain reaction

Total cellular RNA was isolated from BMMCs (1 × 10^6 cells) using Trizol reagent. Reverse transcription—polymerase chain reaction (RT-PCR) was performed in a final volume of 50 μL using an amfi-ivert one-step RT-PCR kit (GenDEPOT, Barker, TX, USA) in an automated thermal cycler (BIORO Technology, Hangzhou, China). PCR assays were performed for 35 cycles. Each cycle consisted of the following steps: denaturation at 94 ºC for 30 s, annealing at 56 ºC for 45 s, and extension at 72 ºC for 1 min. PCR products were analyzed using 1.0% agarose gel containing ethidium bromide.

The primer sequences used were as follows: IL-1β sense, 5′-TGA AGG GCT GCT TCC AAA CCT TGG ACC-3′; IL-1β antisense, 5′-TGT CGG TGAG TGA GCT TTC GGC-3′; IL-4 sense, 5′-TGG CAA GTT TGA AGC AGG TC-3′; IL-6 antisense, 5′-GAT GCA GGG ATG ATG TTC-3′; IL-10 sense, 5′-CAT GGG TCT TGG GAA GAA AA-3′; IL-10 antisense, 5′-CAT CCC AGT AGG AAT TTC GGC-3′; IL-13 sense, 5′-CAG CTC CCT GCT GTC TCT CTC AC-3′; IL-13 antisense, 5′-CCA CAC TCC ATC ATA TCA GCC TGG-3′; TNFα sense, 5′-TAA ATG GGT GTC GGT AAC GG-3′; TNFα antisense, 5′-TGG CCA CTG AAA CCA TGA TCA-3′; interferon-γ sense, 5′-GCT CGT AGA CAA TGG CTG CT-3′; interferon-γ antisense, 5′-AAA GAG ATA ATC TGG CTG TGC-3′; GAPDH sense, 5′-GAT GCA GGG ATG ATG TTC TGG-3′; and GAPDH antisense, 5′-GTC AGG GCT GCT TCC AAC AC-3′ [28].

2.10. Measurements of the intracellular Ca^{2+} level in the activated mast cells

The sensitized GPMCs (4 × 10^6 cells) or BMMCs (4 × 10^6 cells) were incubated for 30 min after adding Fluoro-3 AM (5μM) and placed on a glass slide treated with poly-L-lysine, and then OVA (1.0 μg/mL) for GPMCs or HSA-DNP for BMMCs was flowed out on a glass slide for stimulation. The cells were treated with PPT (10μM, 50μM, or 100μM) 5 min prior to each antigen challenge (OVA or HSA-DNP). The [Ca^{2+}]_i was measured with LSM510 software (Mitutoyo America Corporation, Aurora, IL, USA). Relative intensity (RI) indicated the ratio of optical fluorescence density versus the control (RI = 1).

2.11. Immunoprecipitation for Syk

Immunoprecipitation of Syk protein was performed according to method provided previously [29]. Briefly, agaroar conjugate (50 μL) was washed twice with washing buffer (PBS, pH 7.4), centrifuged for 10 s at 12,000 × g at room temperature and then resuspended in washing buffer. Agaroar conjugate was added to 10 μL anti-Syk antibody (Upstate, Lake Placid, NY, USA), incubated for 60 min at room temperature with gentle mixing, and then centrifuged at 3,000 × g for 2 min at 4 ºC. Samples were washed with 1 mL washing buffer, centrifuged at 3,000 × g for 2 min at 4 ºC, and this step was repeated at least twice. Cell lysates (200 μg protein) were added to agaroar conjugate-bound antibody, and incubated overnight at 4 ºC with gentle mixing. Immunoprecipitated complexes were washed with washing buffer and centrifuged at 3,000 × g for 2 min at 4 ºC. Pellets were washed with 1 mL washing buffer and centrifuged at 3,000 × g for 2 min at 4 ºC. This step was repeated at least three times. The pellet was resuspended with 25–100 μL Laminll sample buffer [0.125M Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue]. Samples were heated at 95 ºC for 5 min, centrifuged for 30 s at 12,000 × g at room temperature, and then the supernatants collected (immunoprecipitation sample).

Immunoprecipitation samples were stored in sample buffer at −70 ºC until assay. Samples and molecular weight standards were run with known concentrations on sodium dodecyl sulfate—polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotting performed.

2.12. Measurements of protein kinase C activity in the activated GPMCs

The sensitized GPMCs (1 × 10^6 cells) were preincubated in the presence of histone (0.2 mg/mL), phosphatidylserine (40 μg/mL), and [γ-32P] ATP (1 μM) at 30 ºC for 5 min. The cells were treated with 100μM PPT 5 min prior to OVA stimulation. The changes in the protein kinase C (PKC) activity in mast cells were measured at 10 min after 1.0 g/mL OVA stimulation. The reaction was stopped by adding 1 mL of 10% OVA antibody, followed by inactivation of the reaction by filtering through a glass fiber disk (GF/B; Whatman, Maidstone, Kent, UK) to remove unreacted [γ-32P] ATP, and then were washed four times with 20mM tetrasodium pyrophosphate and once with absolute ethanol. After drying the glass fiber disk, radioactivity was measured with a liquid scintillation counter [26].

2.13. Measurements of phospholipase A2 activity in the activated GPMCs

GPMCs (1 × 10^6 cells) were preincubated with [3H] arachidonic acid (1 μCi) at 37 ºC for 1 h. The labeled mast cells were washed twice and resuspended in TGCM buffer. The cells were sensitized by anti-OVA antibody (1 × 10^6 cells/mL antibody) at 37 ºC for 45 min and then stimulated with 1.0 μg/mL OVA at 37 ºC for 10 min, and the reaction were stopped by the addition of 1N formic acid. The cells were treated with 100μM PPT 5 min prior to the OVA challenge. The mast cells were then centrifuged at 800 × g for 10 min. Reactivities released in supernatant were counted by adding Aquasol (PerkinElmer, Boston, MA, USA) for liquid scintillation spectrometry [30].

2.14. Immunoblotting for signaling molecules in the activated mast cells

The activated GPMCs (1 × 10^6 cells) or BMMCs (1 × 10^6 cells) were homogenized in lysis buffer [10mM HEPES (pH 7.9), 10mM KCI, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, 2.0 μg/mL aprotinin, 2.0 μg/mL leupeptin], and allowed to swell on ice for 10 min. Cell lysates (μg) were subjected to 8% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Bioscience, Amersham, Buckinghamshire, UK). Membranes were washed with PBS containing 0.1% Tween 20 (PBST), and then blocked for 1 h in PBST containing 5% skim milk. After the membranes were washed with PBST, they were incubated for 60 min at room temperature with antibodies against Syk, PKC isotypes (α, β, η, ι), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, COX-1/2, secreted phospholipase A2 (PLA2), actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), as well as p-PKC, p-ERK, p-JNK, p-p38, diluted with PBST (1:1,000). Membranes were washed with PBST and treated with horseradish peroxidase-conjugated rabbit anti-goat IgG (diluted to 1:5,000–1:10,000; Zymed Laboratory Inc., San Francisco, CA, USA) in PBST for 60 min. After washing, the
protein bands were visualized using enhanced chemiluminescent solution (Amersham Biosciences) [28].

2.15. Electrophoretic mobility shift assay

To prepare nuclear extracts, BMMCs (1 × 10⁶ cells) were washed twice with ice-cold PBS and resuspended in 1 mL ice-cold buffer A (10mM Hepes/KOH pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.5mM DTT, 0.2mM PMSF, 1µg/mL leupeptin, and 1µg/mL aprotinin. After incubation on ice for 15 min, the cells were lysed by adding Nonidet P40 (10 µL 10% Nonidet P40, to a final concentration of 0.625%, v/v) and immediately vortexed for 10 s. Nuclei were harvested by centrifugation at 20,000 × g for 1 min and resuspended in 40 µL ice-cold buffer C (20mM Hepes/KOH pH 7.9, 0.42M NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 25% glycerol, 0.2mM PMSF, 1µg/mL leupeptin, and 1µg/mL aprotinin). After incubation at 4°C for 20 min on a shaking platform, the nuclei were centrifuged by precipitation at 15,000 × g for 10 min. The supernatant (nuclear extract) was then transferred to a new tube and quantified using Bradford’s method. Nuclear extracts were stored at −80°C until required [28].

Ten microliters of a mixture of NF-κB (5’-AGT TCA GTC GGC CTT-3’) or activator protein-1 (AP-1: 5’-GCC TTC ATG ACT CAG CCG GAA-3’) oligonucleotide were added and incubated at 0°C for 10 min. The reaction was stopped by adding 1 µL EDTA (10mM Tris-EDTA buffer pH 8.0, 1mM EDTA), unincorporated nucleotides were separated from the DNA probe by G-25 spin column chromatography. The nuclear extract and gel shift binding 5 × buffer [20% glycerol, 5mM MgCl₂, 2.5mM EDTA, 2.5mM DTT, 250 mM NaCl, 50mM Tris-HCl pH 7.5, and 0.25 mg/mL poly(dI-dC)] were incubated at room temperature for 10 min, and then 20–30 fmol of 32P-labeled NF-κB or AP-1 oligonucleotide was added and incubated at room temperature for 20 min. After stopping the reaction, 1 µL of 10 × gel loading buffer was added to each reaction. Reaction mixtures were electrophoresed on 6% polyacrylamide gels, and the gels were analyzed using FLA-2000 (Fujifilm, Tokyo, Japan).

2.16. Statistical analysis

Experimental data were expressed as mean ± standard error of the mean. An analysis of variance (ANOVA) was used for statistical analysis. Significance between control and experimental groups was determined by Scheffe posthoc test using SPSS version 21 (SPSS Inc., Chicago, IL, USA). The bands for RT-PCR, western blot and electrophoretic mobility shift assay are representative of four independent experiment (n = 4). A p value of 0.05 was regarded as statistically significant.

3. Results

3.1. Effect of PPT on the release of histamine and LTs in the activated GPMCs and BMMCs

It is well known that histamine and LTs are released from mast cells when activated by antigen/antibody reaction. Two types of mast cells, GPMCs and mouse bone marrow-derived mast cells BMMCs were used for this study since commercially available antibodies for guinea pigs have certain limitations for investigating signaling molecules related to allergic responses. In addition, it is known that mast cells are different in their function, depending on their location in the types of organs (lung, bone marrow, etc.) and species (guinea pigs, mice, etc.).

In order to determine whether PPT causes some effects on the release of inflammatory mediators (histamine and LTs) in quiescent GPMCs or BMMCs, unsensitized each types of mast cells pretreated with various concentrations of PPT (5µM, 10µM, 50µM, 100µM, or 300µM) for 5 min were challenged by OVA (10 µg/mL), and both types of mast cells sensitized with anti-OVA antibody were challenged with various concentrations of PPT (5µM, 10µM, 50µM, 100µM, or 300µM) for 30 min or 1 h. It was confirmed that PPT itself did not cause any effects on the mediator releases if not antigen/antibody stimulation (data not shown).

Effects of PPT on the mediator release were examined in GPMCs or BMMCs activated by each specific antigen/antibody reaction. In the activated GPMCs, PPT pretreatment (10µM, 50µM, or 100µM) dose-dependently inhibited histamine release by 5.4%, 41.4%, and 60.5%, respectively (28.6 ± 0.05% for 10µM; 17.7 ± 0.34% for 50µM; and 11.9 ± 0.25% for 100µM PPT), compared with that of OVA stimulation (30.2 ± 0.72%; Fig. 1B, left panel). In the activated BMMCs, histamine release was inhibited by PPT pretreatment by 7.9%, 37.6%, and 70.1% (30.6 ± 0.07% for 10µM; 20.7 ± 0.35% for 50µM; and 9.9 ± 0.26% for 100µM PPT), compared with that of OVA challenge (33.2 ± 0.76%; Fig. 1B, right panel).

Patterns of LT secretion in the activated GPMCs or BMMCs were similar to those of histamine release in both types of mast cells. PPT (10µM, 50µM, or 100µM) inhibited LTs release by 3.4%, 27.6% and 55.4%, respectively, in activated GPMCs (6.5 ± 0.04 × 10² pg/mL for 10µM PPT; 4.9 ± 0.39 × 10² pg/mL for 50µM PPT; 3.0 ± 0.09 × 10² pg/mL for 100µM PPT), compared with that of OVA challenge (6.8 ± 0.13 × 10² pg/mL; Fig. 1B, left panel). In activated BMMCs, PPT inhibited LTs release by 4.7%, 21.9%, and 45.7% (4.1 ± 0.06 × 10² pg/mL for 10µM PPT; 3.3 ± 0.11 × 10² pg/mL for 50µM PPT; and 2.3 ± 0.12 × 10² pg/mL for 100µM PPT), compared with that of OVA challenge (4.3 ± 0.07 × 10² pg/mL; Fig. 1B, right panel).

Histamine and LTs release in the unstimulated GPMCs or BMMCs were 1.3 ± 0.27% and 4.3 ± 0.50%, and 1.3 ± 0.09 × 10² pg/mL and 0.2 ± 0.00 × 10² pg/mL, respectively. Total amounts of histamine in the unstimulated GPMCs or BMMCs were 2,356 ± 70.7 ng/4 × 10⁵ cells and 758 ± 31.3 ng/L × 10⁶ cells, respectively.

3.2. Effect of PPT on the expressions of cytokine mRNA in activated BMMCs

In addition to the release of histamine and LTs, mast cells secrete various types of cytokines, particularly inflammatory cytokines (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TNF-α, and interferon-γ) [20]. Thus, whether the mRNA levels of each cytokines were altered by PPT was examined in the activated BMMCs. PPT pretreatment (10µM, 50µM, or 100µM) inhibited the expressions of all cytokines’ mRNA in dose-dependent manner, except IL-10 mRNA expression, which was increased in the activated BMMCs (Fig. 1C).

3.3. Effect of PPT on [Ca²⁺⁺] in activated GPMCs and BMMCs

Increments of [Ca²⁺⁺] in, crucial for degranulation in activated mast cells [20,26,31,32]. In activated GPMCs, changes in the [Ca²⁺⁺] in were monitored for 10 min after OVA challenge (data shown only for 5 min). [Ca²⁺⁺] reached the maximum level (approximately 1.9 folds of basal level) at 3 min and was maintained until 10 min. PPT pretreatment (100µM) strongly prevented the OVA-induced rise in [Ca²⁺⁺] and kept it at the basal level (Fig. 2A). In the activated BMMCs, [Ca²⁺⁺] increased steadily, reached the maximum at 5 min (approximately 1.9 folds of basal level), and was maintained until 10 min (data shown only for 5 min). PPT (10µM, 50µM, or 100µM) pretreatment significantly suppressed [Ca²⁺⁺] (Fig. 2B). Especially, PPT inhibited [Ca²⁺⁺] in, activated GPMCs more prominently than in
versus OVA or HSA-DNP challenge. C, Syk protein. Numbers below the bands indicate the ratio of band intensity of each band versus that of control and actin. The data are representative of four independent experiments.

![Graph](image)

Fig. 2. Effects of 20(S)-protopanaxatriol (PPT) on the intracellular \([\text{Ca}^{2+}]_i\) level (\([\text{Ca}^{2+}]_j\)) and expression of Syk kinase protein in activated mast cells. The sensitized guinea pig mast cells (GPMCs) or mouse bone marrow-derived mast cells (BMMCs; 4 \times 10^6) were incubated for 30 min after adding Fluo-3 AM (5\(\mu\)M) and fixed on a glass slide treated with poly-L-lysine, and then ovalbumin (OVA; 1.0 \(\mu\)g/mL) or human serum albumin–dinitrophenyl (HSA-DNP), respectively, was flowed out on a glass slide for stimulation. The \([\text{Ca}^{2+}]_i\) was monitored for 10 min using confocal microscopy, and Syk protein expression after immunoprecipitation was determined with western blot, as described in Materials and methods. PPT (10\(\mu\)M, 50\(\mu\)M, or 100\(\mu\)M) reduced the augmented phosphorylation of MAP kinases in the activated BMMCs. PPT (10\(\mu\)M, 50\(\mu\)M, or 100\(\mu\)M) dose-dependently reduced the augmented phosphorylation of MAP kinases in activated BMMCs (Fig. 3C).

3.5. Effect of PPT on the PKC activity in the activated GPMCs and BMMCs

It has been reported that PKC is activated by an elevation of \([\text{Ca}^{2+}]_i\) level in the stimulated B cells and mast cells [26,31]. The changes in the PKC activity in both types of mast cells were measured after stimulation. The activity of PKC was enhanced in OVA-challenged GPMCs by 59.3%. The OVA-induced increment in PKC activity was remarkably counteracted by 100\(\mu\)M PPT (Fig. 3A).

The \([\text{Ca}^{2+}]_i\)-dependent PKC isoforms (PKC\(\alpha\), \(\beta\), and \(\gamma\)) are activated in mast cells, and then the activated PKC isoforms regulate mitogen-activated protein (MAP) kinases [18,33]. Fig. 3B showed that PKC isoforms (\(\alpha\), \(\beta\), \(\gamma\)) are phosphorylated by antigen/antibody reaction in BMMCs, and PPT (10\(\mu\)M, 50\(\mu\)M, or 100\(\mu\)M) inhibited the phosphorylation of PKC isoforms in the activated BMMCs. In addition, we observed the phosphorations of ERK, JNK, and p38 MAP kinases in activated BMMCs. PPT (10\(\mu\)M, 50\(\mu\)M, or 100\(\mu\)M) reduced the augmented phosphorylation of MAP kinases in activated BMMCs (Fig. 3C).

3.6. Effect of PPT on the PLA2 activity in activated GPMCs and BMMCs

The changes in the PLA2 activity in GPMCs were assayed after OVA stimulation. OVA increased the PLA2 activity by 38% of basal activity in stimulated GPMCs. The OVA-induced increment in the enzyme activity was decreased by 30.2% when the cells were pretreated with 100\(\mu\)M PPT (Fig. 4A).

In GPMCs and BMMCs, the level of protein expression of PLA2 was enhanced upon stimulation with OVA challenge (Fig. 4B). PPT pretreatment (10\(\mu\)M, 50\(\mu\)M, or 100\(\mu\)M) dose-dependently reduced the PLA2 expression in each type of activated mast cells.

3.7. Effect of PPT on the activity of COX-1/2 in the activated BMMCs

It is well known that prostaglandins (PGs) are produced by the actions of both constitutive enzyme COX-1 and inducible enzyme COX-2. In mast cells, certain amount of COX-2 is expressed and certain amount of COX-1 is expressed and one amount of COX-2 is induced by the antigen/antibody stimulation [34,35]. It was also observed that expression of COX-2 was enhanced in activated BMMCs, but COX-1 was not affected (Fig. 4C). PPT decreased dose-dependently only the induced level of COX-2 expression by antigen stimulation, but not that of COX-1 expression (Fig. 4C).

3.8. Effect of PPT on the activity of transcription factors in the activated BMMCs

NF-\(\kappa\)B and AP-1 are transcription factors controlling the expression of inflammatory cytokines in mast cells. These transcription factors were upregulated in the activated BMMCs. PPT (10\(\mu\)M, 50\(\mu\)M, or 100\(\mu\)M) inhibited the DNA binding activities of transcription factors in a dose-dependent manner (Fig. 4D).

4. Discussion

Mast cells participate in the induction of innate and adaptive immune responses [20,36]. Mast cells influence both health and diseases, such as allergy, arthritis, autoimmunity, and neoplasia, by releasing and producing a variety of mediators [20,26].
Ginsenosides and ginsan, the major active saponin and polysaccharide components of ginseng, respectively, have many biological activities including inflammatory responses in mast cells as well as allergic inflammation [7,16,37]. Herein, we demonstrated that PPT, one of the ginseng saponin metabolites formed by human intestinal bacteria and of nonsugar moiety of ginsenoside (aglycone), reduced the release of mediators by \( [\text{Ca}^{2+}]_i \) level, PLA2, PKC, and NF-\( \kappa \)B/AP-1 activities which are downstream signals regulated by Syk kinase in Fc\( \varepsilon \)RI-mediated mast cell activation.

The antigen-mediated activation of mast cells via engagement of the IgE bound to Fc\( \varepsilon \)RI receptor represents an initial event in the development of type I hypersensitivity reaction, and results in degranulation, with the release of various inflammatory mediators, such as histamine, LTs, and cytokines [20,26,32]. That is, IgE-mediated signaling pathways lead to the release of preformed mediators (histamine, tryptase, etc.) by PKC activity that is accompanied by \( [\text{Ca}^{2+}]_i \) influx, which is critical for the downstream propagation signals by Syk-mediated phosphorylation and the activation of PLC\( \gamma \) or PLD enzyme [18,20,38,39]. Therefore, we examined the effects of PPT on the well-known signaling pathways in mediator release from IgE-mediated mast cell activation. Our data (Figs. 1B, 2, 3A, 3B) suggest that PPT reduces histamine release via the inhibition of PKC activity caused by blocking of the rise in \( [\text{Ca}^{2+}]_i \), through regulating Syk in Fc\( \varepsilon \)RI-mediated mast cell activations. Similarly, it was reported that ginsenoside Rh1, which has a sugar moiety in its structure, inhibited mediator release in activated GPMCs [9]. However, there has been a report that ginseng saponin enhanced \( [\text{Ca}^{2+}]_i \) and PKC activity in macrophage activation [6]. This may be due to the cell types or the sort of ginsenoside components used in each separate experiment.

Rapid hydrolysis of phospholipids in mast cell membrane also occurs through the activation of cytosolic phospholipase A\( _2 \) (cPLA\( _2 \)) in IgE-mediated signaling pathways. Hydrolyzed phospholipids were converted into arachidonic acids. Then, enzymes such as COX-1/2 or 5-lipoxigenase (LOX) convert them into PGs and LTs, respectively, which affects the early phase of allergic responses in mast cells [34,35,38,40,41]. A rise in \( [\text{Ca}^{2+}]_i \) is necessary for translocation of cPLA\( _2 \) to the cell membrane in Fc\( \varepsilon \)RI-mediated mast cell activation [18,42]. It has been reported that ginsenoside Rh1 inhibited COX-2 [7]. Our data (Figs. 1B, 2A, 2B, 4A–C) suggest that PPT-mediated reduction in \( [\text{Ca}^{2+}]_i \), leads to the decrements in production of LTs via inhibiting the activities of cPLA\( _2 \) and most likely LOX during mast cell activations. In addition, we observed that PPT
causes reduction of COX-1/2 expression, which may result in PG production in combination with the inhibition of PLA2 activity and the reduction of [Ca2+]i. This indicates that PPT may concurrently inhibit the COX-2 and LOX activities, thereby reducing early phase of allergic responses.

IgE-mediated signaling pathways induce production of inflammatory cytokines via activation of MAP kinases during activation of mast cells [32,43,44]. NF-κB and AP-1 are pleiotropic transcription factors that play an important role in regulating the expression of multiple genes including Th2 cytokines [20], and allergic inflammation is associated with increased NF-κB activity in animal lung tissues and BAL cells [28,45]. A rise of [Ca2+]i, level also leads to the activation of MAP kinases and NF-κB, which play important roles in the control of IgE-mediated cytokine synthesis in mast cells [46–49]. Our results (Figs. 1C, 2, 3C, 4D) suggest that PPT inhibits production of inflammatory cytokines via inhibiting an increase of cellular Ca2+ level, the activities of MAP kinases and NF-κB/AP-1 in the IgE-mediated activated mast cells. Similarly, ginsenosides have been reported to inhibit NF-κB activation as well as histamine release in certain cell lines [7,37]. However, IL-10, which is known as an anti-inflammatory cytokine, was not inhibited in the PPT pretreatment.

PPT pretreatment exerted antiallergic effects in the same manner in both types of mast cells when activated by specific antigen/antibody reaction. It can be inferred that PPT acts via regulating expression of Syk kinase, which is critical for the downstream propagation of signals in FcεRI-mediated mast cell activation, although further upstream signaling molecules of Syk and [Ca2+]i were not defined in this experiment. This can be supported by the previous reports that a rise of [Ca2+]i, triggered the induction of activities of cellular signaling molecules such as PKCs, MAP kinases, PLA2, and NF-κB/AP-1 [18,20,38,39,42,46–49].

Fig. 4. Effects of 20(S)-protopanaxatriol (PPT) on the phospholipase A2 (PLA2) activity or expression of cyclooxygenase (COX)-1/2, and the DNA binding activities of transcription factors, nuclear factor (NF)-κB and activator protein (AP)-1 in activated mast cells. GPMCs (1 x 10⁶) pre-incubated with [³²P] arachidonic acid (1 μCi) and BMMCs (1 x 10⁶ cells) were sensitized and challenged by each antigen/antibody reaction. The experimental details and PPT treatment were as described in Materials and methods. (A) PLA2 activity in GPMCs. The data are expressed as mean ± standard error of the mean (n = 8). **p < 0.01 versus control (Con). ++, p < 0.01 versus ovalbumin (OVA) challenge. (B) The expression of PLA2 protein in guinea pig mast cells (GPMCs) or mouse bone marrow-derived mast cells (BMMCs). (C) Expression of COX-1/2 in BMMCs. (D) Activities of transcription factors, NF-κB and AP-1 in BMMCs. Numbers below the bands indicate the ratio of band intensity of each band versus those of control and actin protein or total protein. (a) Negative control; (b) competition. The data are representative of four independent experiments (n = 4). HSA-DNP, human serum albumin–dinitrophenyl.
A variety of ginsenosides affect allergic and inflammatory responses with their characteristic effects. Ginsenosides Rb1, Rg1, Rg2, and Re exert antiallergic effects by inhibiting histamine release from mast cells [7,9,16,17] and anti-inflammatory effects in murine model [10,13]. In addition, PPT, an intestinal metabolite of ginsenosides (an aglycone moiety) inhibited the release and production of inflammatory mediators (histamine, LTE4, and cytokines, respectively) in the GPMCs as well as in BMMCs, which is compatible with the observations by other laboratories that ginseng metabolites such as compound K [16] and Rh2 [17] inhibit release of mediators such as β-hexosaminidase from RBL-2H3 cells. Thus, the sugar moiety attached at their triterpenoid structures is not supposed to affect the mediator release during mast cell activation.

Ginsenoside Re, which induces allergic responses (unpublished data), exists in very low amounts in ginseng extracts. An allergic asthma observed in personnel exposed to ginseng for long periods, such as the workers engaged in Korean ginseng wholesale may be due to ginsenoside Re [8].

The blood concentration of PPT after oral intake of ginseng extract would be much lower than that of PPT used in this experiment. Therefore, it can be supposed that the cumulative blood concentration of ginseng metabolites when taken for longer periods will be much more close to the concentration of PPT used in this experiment. Thus, it is necessary to measure the blood concentrations of ginseng metabolites after long-term use of ginseng extract for more appropriately evaluating the dose-dependent effects of intestinal ginseng metabolites in vivo.

In conclusion, we suggest that PPT, one of the intestinal ginseng metabolites, inhibits the release and production of inflammatory mediators by affecting the cellular signaling pathways initiated by Syk activation in the FcεRI receptor stimulation and it may be used as an auxiliary substance for treating allergy-induced asthma.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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