

Spiraeoside inhibits mast cells activation and IgE-mediated allergic responses by suppressing phospholipase C- γ -mediated signaling

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Abstract: Mast cells are responsible for IgE-mediated allergic responses through the secretion of various inflammatory cytokines and mediators. Therefore, the pharmacological regulation of mast cell activation is an important goal in the development of novel anti-allergic drugs. In this study, we found that spiraeoside (SP) inhibits mast cell activation and allergic responses *in vivo*. SP dose-dependently inhibited the degranulation induced by IgE-antigen (Ag) stimulation in RBL-2H3 mast cells without cytotoxic effects. At the molecular level, SP reduced the Ag-induced phosphorylation and subsequent activation of phospholipase C- γ 2 (PLC- γ 2). Moreover, SP inhibited the phosphorylation of spleen tyrosine kinase (Syk), linker for activation of T cells (LAT), and downstream MAPKs, such as ERK1/2, p38, and JNK, eventually attenuating expression of TNF- α and IL-4. Finally, we found that SP significantly inhibited IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. Taken together, our results strongly suggest that SP suppresses IgE-mediated mast cell activation and allergic responses by inhibiting Lyn-induced PLC- γ 2/MAPK signaling in mast cells.

Key words: Spiraeoside, mast cells, degranulation, PLC- γ , Lyn/Syk kinase, MAPK, allergic response.

Résumé : Les mastocytes sont responsables des réponses allergiques à IgE par l'intermédiaire de la sécrétion de diverses cytokines et médiateurs inflammatoires. Conséquemment, la régulation pharmacologique de l'activation des mastocytes constitue un objectif important du développement de nouveaux médicaments antiallergiques. Dans cette étude, les auteurs ont trouvé que le spiraeoside (SP) inhibe l'activation des mastocytes et les réponses allergiques *in vivo*. Le SP inhibait la dégranulation des mastocytes RBL-2H3 stimulée par le complexe IgE-antigène (Ag) en fonction de sa concentration et ce, sans effets cytotoxiques. Au niveau moléculaire, le SP induisait la phosphorylation et l'activation subséquente de la phospholipase C- γ 2 (PLC- γ 2) induites par l'Ag. De plus, le SP inhibait la phosphorylation de la tyrosine kinase Syk (*spleen tyrosine kinase*), LAT (*linker for activation of T cells*), les MAPK agissant en aval telles ERK1/2, p38 et JNK, atténuant éventuellement l'expression du TNF- α et de l'IL-4. Finalement, les auteurs ont trouvé que SP inhibait de manière significative l'anaphylaxie cutanée passive provoquée par les IgE chez la souris. Dans l'ensemble, les résultats suggèrent fortement que le SP supprime l'activation des mastocytes par les IgE et les réponses allergiques, en inhibant la signalisation de PLC- γ 2/MAPK dans les mastocytes. [Traduit par la Rédaction]

Mots-clés : spiraeoside, mastocytes, dégranulation, PLC- γ , Lyn/Syk kinase, MAPK, réponse allergique.

Introduction

Type I allergy is an immune disorder that is mediated by the expansion of T helper 2 cells (T_H2 cells), together with the isotype switching of B cells to generate IgE antibodies specific for common environmental antigens (Ag), called allergens (Georas et al. 2005). Because the high-affinity receptor for an IgE antibody (Fc ϵ RI) is expressed primarily on the surface of mast cells, exposure to the cognate Ag triggers mast cell activation within minutes, resulting in allergic inflammation. These inflammatory responses lead to many types of allergic diseases, such as allergic rhinitis, asthma, atopic dermatitis, and eczema (Holgate and Polosa 2008). Over the

past several decades, allergic diseases have reached epidemic proportions worldwide, and the incidence of such diseases is continuously increasing. Thus, an understanding of the mechanisms underlying allergic disorders is a prerequisite for the development of novel anti-allergic drugs.

Mast cells have long been considered key players in type I allergic disorders. When activated, mast cells initiate and propagate allergic inflammation by secreting diverse cytokines and mediators, such as tumor necrosis factor (TNF)- α , interleukin-4 (IL4), histamine, and arachidonic acid metabolites. These reactions are primarily mediated by the degranulation process, whereby preformed or newly synthesized cytokines and mediators are se-

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creted from activated mast cells (Theoharides and Kalogeromitros 2006). Because the degranulation of mast cell is tightly regulated by FcεRI-mediated signaling, an understanding of this signaling pathway is unequivocally required for the pharmacological regulation of allergic diseases. When an Ag induces the crosslinking of the IgE-FcεRI complex on the surface of a mast cell, Lyn kinase is activated, which results in the subsequent phosphorylation and activation of spleen tyrosine kinase (Syk). The activated Lyn kinase and Syk then phosphorylate diverse target molecules, including LAT and Gab2, to propagate the FcεRI-mediated signaling such as activation of GTPase Ras, PI3K, and phospholipase C-γ (PLC-γ) (Gilfillan and Tkaczyk 2006); among these molecules, PLC-γ is the most essential for mast cell activation. Upon activation, PLC-γ generates two important second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphates (IP₃), from phosphatidylinositol 4,5-bisphosphates (PIP₂), and these two second messengers are implicated in PKC activation and intracellular Ca²⁺ mobilization, respectively (Rhee 2001). It has been well demonstrated that the elevation of the intracellular Ca²⁺ concentration ([Ca²⁺]_i) initiated by PLC activation is a key step for the degranulation of mast cells (Choi et al. 1993; Wang et al. 2000). In addition, PKC is known to trigger the activation of downstream MAPKs, including ERK1/2 and JNK (Kalesnikoff and Galli 2008). Because MAPK activation is required for the expression of cytokines such as TNF-α and IL-4, PLC-γ plays a pivotal role in allergic responses by regulating activation of mast cells. Therefore, the inhibition of PLC-γ itself or the PLC-γ-mediated signaling pathway is a good strategy to regulate mast cells activation and ameliorate allergic disorders.

Quercetin, 3,3',4',5,7-pentahydroxyflavone, is a typical flavonoid that is widely distributed in many fruits and vegetables (Skibola and Smith 2000), and it is now generally accepted that quercetin has beneficial effects on human health due to its strong antioxidant effects (Boots et al. 2008). However, most dietary quercetin is present in its glycoside forms, in which one or more sugars are bound to the phenolic groups via β-glycosidic linkages (Sesink et al. 2003). The bioactive properties of quercetin glycosides may be more significant in dietary terms than those of quercetin aglycones, because quercetin glycosides are preferentially absorbed in humans over quercetin aglycone (Hollman et al. 1995). Moreover, quercetin glycosides exert diverse cellular effects, and these effects are influenced by the nature and location of the sugar moiety (Williamson et al. 1996; Arts et al. 2004). Therefore, determining the biological effects of each quercetin glycoside is quite an interesting area of study. Spiraeoside (SP; Fig. 1A), also known as quercetin-4'-glucoside, is found in certain vegetables, particularly in onions. Previous studies have demonstrated that SP has several biological effects, such as antioxidant activity (Maksimovic et al. 2007; Murota et al. 2004), inhibitory effects on cancer cell proliferation (Browning et al. 2005), and inhibitory effects on glucose uptake (Cermak et al. 2004). However, the potential effects of SP on FcεRI-mediated signaling in mast cells and on allergic responses are largely unknown.

In this study, to identify novel molecules that regulate allergic responses, we screened a natural product library by monitoring the degranulation of RBL-2H3 mast cells. As a result, we found that SP significantly inhibited the Ag-induced degranulation of RBL-2H3 cells by suppressing Lyn-induced PLC-γ2 activation. Moreover, the activation of MAPK downstream of PLC-γ2, was also attenuated by SP treatment, which subsequently inhibited the expression of TNF-α and IL-4 in RBL-2H3 mast cells upon Ag stimulation. Finally, we showed that the oral administration of SP to mice suppressed the passive cutaneous anaphylaxis (PCA) reaction. Therefore, our results strongly suggest that SP can be utilized as an anti-allergic agent.

Materials and methods

Materials

Spiraeoside (3,5,7-trihydroxy-2-[3-hydroxy-4-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]chromen-4-one) was purchased from INDOFINE Chemical Company (Hillsborough, New Jersey, USA). Evans blue, dinitrophenol-conjugated human serum albumin (DNP-HSA), DNP-specific monoclonal IgE, MTT, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). U73122, U73433, and PP2 were obtained from Calbiochem (La Jolla, California, USA). Mouse monoclonal antibody against phosphotyrosine and rabbit polyclonal antibody against PLC-γ2 were generated. Rabbit polyclonal antibodies against phosphorylated forms of ERK1/2, p38, c-Jun N-terminal kinase (JNK), Syk, linker for activation of T cells (LAT), Gab2, and Syk were obtained from Cell signaling Technologies (Beverly, Massachusetts, USA). A rabbit polyclonal antibody against LAT and a mouse monoclonal antibody against actin were obtained from Upstate Biotechnology (Lake Placid, New York, USA) and ICN Biomedicals, Inc. (Aurora, Ohio, USA), respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, Maryland, USA).

Cell culture

The rat basophilic leukemia cell line RBL-2H3 was kindly provided by Dr. Cho JY from Kangwon National University, Korea. The RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, Maryland, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BioWhittaker), 100 μg/mL streptomycin, and 100 units/mL penicillin under a humidified atmosphere of 5% CO₂ in air at 37 °C.

Animal

Specific pathogen-free Balb/c mice were maintained and housed in the animal facilities at Pohang University of Science and Technology (POSTECH). The mice were kept at a temperature of 22 ± 1 °C and a relative humidity of 55 ± 10% with a 12 h light/dark cycle. This study was done in accordance with the institutional guidelines. The protocol had received prior approval from the Institutional Animal Care and Use Committee (IACUC) at POSTECH.

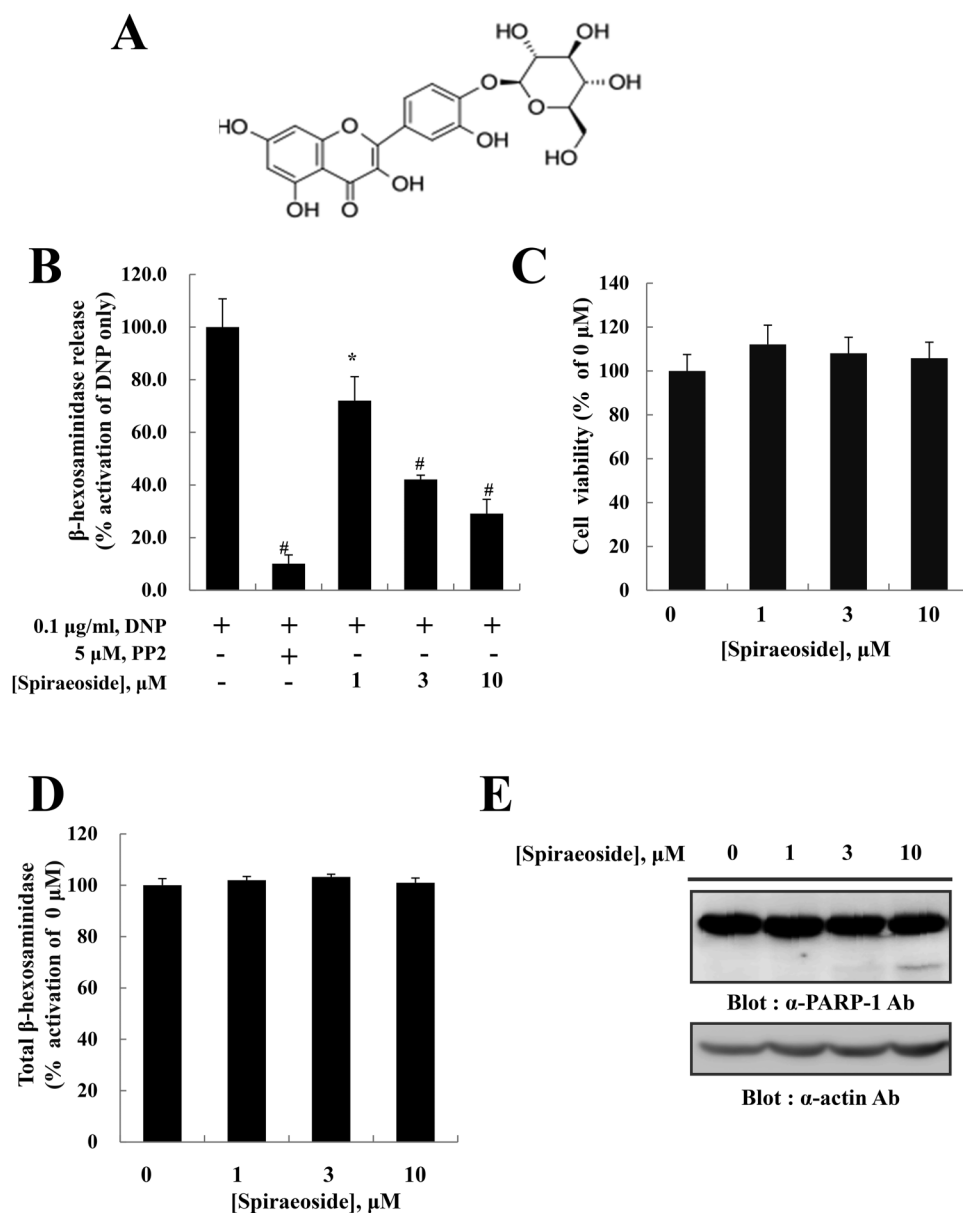
Stimulation and measurement of degranulation in RBL-2H3 cells

The RBL-2H3 cells were plated at 2 × 10⁵ cells/well in a 24-well plate. After 24 h, the cells were incubated with 100 ng/mL of DNP-specific IgE in complete medium for 12 h. The IgE-sensitized cells were washed twice and incubated in Tyrode buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 12 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, 5.6 mmol/L glucose, 25 mmol/L HEPES, 0.1 g/100 mL BSA, pH 7.4) for 1 h with or without SP and then stimulated with 100 ng/mL of DNP-HSA for 30 min. Degranulation was determined by measuring the activity of β-hexosaminidase, as described previously (Lim et al. 2008). Briefly, 40 μL of supernatant and 40 μL of 5 mmol/L *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (in 0.1 mol/L citrate buffer, pH 4.5) were mixed in each well of a 96-well plate for 2 h at 37 °C. The reaction was terminated by adding 100 μL of carbonate buffer (0.1 mol/L Na₂CO₃, 0.1 mol/L NaHCO₃, pH 10.0). The absorbance was measured with a colorimetric microplate reader at 405 nm.

Cell viability and granule content analysis

The RBL-2H3 cells were plated at 2 × 10⁴ cells/well in a 96-well plate. After 24 h, the indicated concentrations of SP were added for another 24 h. MTT was dissolved in PBS at a concentration of 5 mg/mL. This stock solution was added to the culture medium at a final concentration of 0.5 mg/mL, and the plates were incubated

Fig. 1. Effect of SP on Ag-induced degranulation in RBL-2H3 cells. (A) Molecular structure of SP. (B) DNP-specific IgE-sensitized RBL-2H3 cells were stimulated with 100 ng/mL of DNP-HSA for 30 min. Degranulation was determined by measuring the activity of β -hexosaminidase in the supernatant. (C and D) RBL-2H3 cells were incubated with the indicated concentration of SP for 24 h and then subjected to an MTT assay (C) or measurement of the total granule contents (D), as described in Materials and methods. (E) 24 h exposure of SP had little effect on PARP-1 cleavage in the cells. The results shown are representative of 3 independent experiments. The data are the mean $\pm$ SD. * $p < 0.05$ and # $p < 0.01$. PP2 is a general Src-family kinase inhibitor and was used as a negative control.



at 37 °C for 2 h. The formazan crystals, which were formed by the reduction of the tetrazolium ring by the mitochondria of viable cells, were dissolved in dimethylsulfoxide (DMSO), and the absorbance was measured with a colorimetric microplate reader at 540 nm. To measure the total granule content of the cells, the culture medium was removed, and 0.5% Triton X-100 solution was added to each well for 10 min. The β -hexosaminidase activity of each extract was then measured.

Measurement of PLC activity

To monitor PLC activation, we measured the total amounts of inositol phosphates (IPs) in RBL-2H3 cells. Briefly, the cells were plated at 1×10^6 cells/well in a 6-well plate. After 24 h, the cells were incubated with 100 ng/mL of DNP-specific IgE and 1 μ Ci of [^{3} H]-inositol (NEN Life Science Products, Boston, Massachusetts,

USA) per well in 1 mL of inositol-free DMEM for 12 h at 37 °C. The cells were washed twice and incubated in Tyrode buffer for 1 h, with or without SP. After incubation with 10 mmol/L LiCl for 10 min, the IgE-sensitized cells were stimulated with 100 ng/mL of DNP-HSA for 30 min. The reaction was terminated by adding ice-cold 5% perchloric acid. The accumulated [^{3} H]-inositol phosphates were measured as described previously (Kim et al. 2011). Briefly, after 30 min in an ice bath, the extracts were centrifuged and applied to AG 1-X8 anion exchange resin (Bio-Rad Laboratories, Hercules, California, USA). The column was then washed with 2 mL of distilled water followed by 6 mL of 0.06 mol/L ammonium formate containing 5 mmol/L sodium tetraborate. The total inositol phosphates were eluted with a solution containing 1 mol/L ammonium formate and 0.1 mol/L formic acid. The radioactivity in the eluate was measured using a liquid scintillation counter.

RNA extraction and real-time quantitative PCR

Total RNA was isolated from the RBL-2H3 cells using TRIzol agent (Invitrogen, Carlsbad, California, USA) and then reverse transcribed according to the manufacturer's instructions using an oligo (dT) primer and murine leukemia virus reverse transcriptase (Promega, Madison, Wisconsin, USA). cDNA was amplified using the following forward and reverse primers: TNF- α 5'-CCAGACCCCTCACACTCAGAT-3' and 5'-CACCAGTTGGTTGCTTTGA-3' and, IL-4 5'-GGTCTTCGCAA-ATTTTACT-3' and 5'-GCTTCCAGGAAGTCTTTCA-3'.

The real-time quantitative PCR, PCR amplification mixtures (20 μ L) contained 10 μ L of 2 $\times$ SYBR green qPCR Master Mix (USB, Cleveland, Ohio, USA), 2 μ L of a 1 μ M/L forward and reverse primer mix, and 8 μ L of diluted cDNA. The real-time quantitative PCR was performed using the Bio-Rad CFX96 Realtime PCR detection system. The cycling parameters were 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 30 s. After amplification, a melting-curve analysis was performed according to the manufacturer's instructions (Bio-Rad).

Western blot analysis and immunoprecipitation

RBL-2H3 cells were treated under the indicated conditions, washed 2 times with ice-cold PBS, and lysed with lysis buffer (40 mmol/L HEPES, 1 mmol/L EDTA, 120 mmol/L NaCl, 1 mmol/L PMSF, 1.5 mmol/L Na₃VO₄, 50 mmol/L NaF, 10 mmol/L β -glycerol phosphate, and 1% Triton X-100, pH 7.4). The lysates were centrifuged at 14 000 rpm for 15 min at 4 °C. For the western blot analysis, supernatant fractions containing equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Triton X-100 and then incubated with the indicated primary Abs overnight at 4 °C. A secondary antibody linked to horseradish peroxidase was used at 1:5000, and the specific signals were visualized using enhanced chemiluminescence (Amersham Biosciences, Amersham, UK). For immunoprecipitation, supernatant fractions containing equal amounts of protein were used. PLC- γ 2 was immunoprecipitated overnight by incubation with specific Abs and then with protein A-agarose for 2 h at 4 °C. After washing 3 times with lysis buffer, the resulting precipitates were subjected to Western blot analysis.

Passive cutaneous anaphylaxis reactions

An anti-DNP IgE (1 μ g) was intradermally injected into an ear of male Balb/c mice (aged 4 weeks). After 24 h, the mice were challenged with an intravenous injection of 200 μ g of DNP-HSA in 200 μ L PBS containing 1% Evans blue. The indicated doses of SP were administered orally 1 h prior to the challenge. The mice were then sacrificed 30 min after the challenge, followed by the removal of the ear for the measurement of the amount of extravasated dye. The dye was extracted overnight from the ear in 700 μ L of formamide at 63 °C, and the absorbance was measured at 620 nm.

Statistical analysis

Student's *t*-test was used to calculate *P*-values based on comparisons with the appropriate control samples tested at the same time. *P*-values < 0.05 and < 0.01 were considered statistically significant.

Results

SP inhibits the Ag-induced degranulation of RBL-2H3 cells without cytotoxic effects

To identify novel molecules that inhibit mast cells activation, we utilized RBL-2H3 cells and a natural product library. RBL-2H3 cells, the tumor analog of mast cells, are considered to be a well-established model for investigating mast cell activation because IgE-sensitized RBL-2H3 cells release β -hexosaminidase, a marker of mast cells degranulation after cognate Ag exposure (Ortega Soto and Pecht 1988; Granberg et al. 2001). Thus, we screened a natural product library by monitoring the Ag (DNP-HSA; 0.1 μ g/mL)-

induced degranulation of DNP IgE-sensitized RBL-2H3 cells. As a result, we found that SP suppressed mast cells degranulation. As shown in Fig. 1B, SP significantly inhibited the Ag-induced degranulation of RBL-2H3 cells in a dose-dependent manner. We also observed that PP2, a Src-family kinase inhibitor, abolished Ag-induced degranulation, which is consistent with the results of a previous study demonstrating that the Src kinase family plays pivotal roles in mast cell activation (Kim et al. 2009). To ascertain whether SP has possible cytotoxic effects, we performed an MTT assay and found that SP did not affect cell viability (Fig. 1C). In addition, 24 h exposure of SP had little effect on the total granule content and PARP-1 cleavage in the cells (Figs. 1D and 1E). Therefore, these results indicate that SP inhibits the Ag-induced degranulation of RBL-2H3 cells without cytotoxicity.

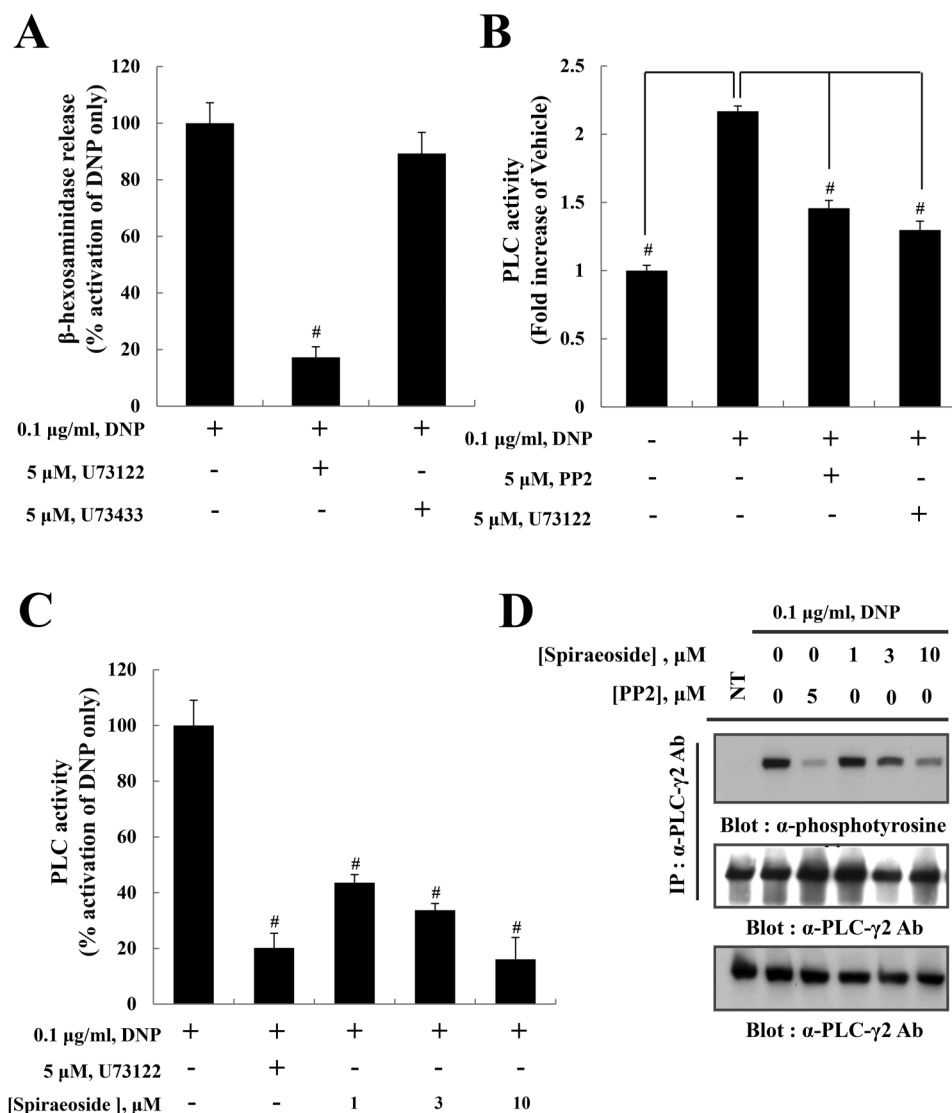
SP inhibits Ag-induced PLC- γ 2 phosphorylation and activation

We next investigated the molecular mechanisms underlying the inhibitory effect of SP on mast cell degranulation. Previous studies have demonstrated that the elevation of [Ca²⁺]_i is a key signal for mast cells degranulation (Aketani et al. 2001). Because PLC can generate a Ca²⁺ signal, we first examined the possible roles of PLC in Ag-induced mast cells degranulation. For this purpose, we utilized U73122, a PLC inhibitor, and found that Ag-induced mast cells degranulation was significantly inhibited by U73122; in contrast, U73433, an inactive analog of the PLC inhibitor, had little effect, demonstrating that the activation of PLC is required for Ag-induced mast cell degranulation (Fig. 2A). In agreement with this observation, the overall PLC activity was increased in the IgE-sensitized RBL-2H3 cells upon Ag stimulation (Fig. 2B). Because PP2 also significantly inhibited Ag-induced PLC activation, Src-family kinases might act as an upstream signal for PLC activation. Based on these results, we next examined whether SP can inhibit Ag-induced PLC activation in RBL-2H3 cells. Surprisingly, we found that SP significantly inhibited Ag-induced PLC activation in a dose-dependent manner (Fig. 2C). Because the activation of Fc ϵ RI induces PLC- γ 2 activation via tyrosine phosphorylation (Han et al. 2009), we then examined whether SP affected the tyrosine phosphorylation of PLC- γ 2. As shown in Fig. 2D, SP attenuated the tyrosine phosphorylation of PLC- γ 2 induced by Ag stimulation. Therefore, these results clearly indicate that SP inhibits the tyrosine phosphorylation and subsequent activation of PLC- γ 2 to suppress the degranulation of RBL-2H3 mast cells during Ag stimulation.

SP inhibits the Ag-induced activation of Src-family kinases in RBL-2H3 cells

Our results indicated that SP inhibited the Ag-induced activation of PLC- γ 2 by negatively affecting the status of tyrosine phosphorylation of PLC- γ 2 (Figs. 2C and 2D). Thus, SP might act upstream of PLC- γ 2 to regulate its tyrosine phosphorylation and subsequent activation. Previously, it has been demonstrated that the aggregation of the IgE-Fc ϵ RI complex triggers the activation of Src-family kinases, such as Lyn kinase and Syk, to phosphorylate their target molecules including PLC- γ 2 (Rivera and Gilfillan 2006). We also observed that Ag-induced PLC activation was significantly inhibited by treatment with PP2, a Src-family kinase inhibitor (Fig. 2B). Thus, we next examined whether SP affected Src kinase family activation during Ag stimulation. As shown in Fig. 3A, we found that the phosphorylation of Syk was drastically reduced by SP treatment in a dose-dependent manner. In addition, the phosphorylation of LAT and Gab2, the direct targets of Syk, was also attenuated by the SP treatment. Because the phosphorylation and subsequent activation of Syk are directly mediated by activated Lyn kinase (Gilfillan and Tkaczyk 2006), these results collectively suggest that SP negatively regulates Fc ϵ RI-mediated signaling in RBL-2H3 mast cells by acting at the level of Lyn kinase.

Fig. 2. Effect of SP on the Ag-induced tyrosine phosphorylation and activation of PLC- γ 2 in RBL-2H3 cells. (A) DNP-specific IgE-sensitized RBL-2H3 cells were incubated with 5 μ M of U73122 or U73433 for 1 h and then stimulated with 100 ng/mL of DNP-HSA for 30 min. The β -hexosaminidase activity in the supernatant was measured. (B) RBL-2H3 cells were incubated with 100 ng/mL of DNP-specific IgE and 1 μ Ci of [^{3} H]-inositol in inositol-free DMEM for 12 h at 37 $^{\circ}$ C and then stimulated with 100 ng/mL of DNP-HSA for 30 min. PP2 and U73122 were used as negative controls. The reactions were quenched with 5% perchloric acid, and the production of total [^{3} H]-inositol phosphates was determined, as described in Materials and methods. (C) The indicated concentrations of SP were added 1 h prior to DNP-HSA stimulation, and the production of total [^{3} H]-inositol phosphates was determined. (D) DNP-specific IgE-sensitized RBL-2H3 cells were stimulated with 100 ng/mL of DNP-HSA for 5 min. The cell lysates were immunoprecipitated with anti-PLC- γ 2 Ab, and the precipitates were analyzed with anti-PLC- γ 2 and anti-phosphotyrosine antibodies. The results shown are representative of 3 independent experiments. The data are the mean $\pm$ SD. * p < 0.05 and # p < 0.01. PP2 is a general Src-family kinase inhibitor. U73122 is a general PLC inhibitor, and U73433 is its inactive analog.



SP inhibits Ag-induced MAPK activation and inflammatory cytokine expression in RBL-2H3 cells

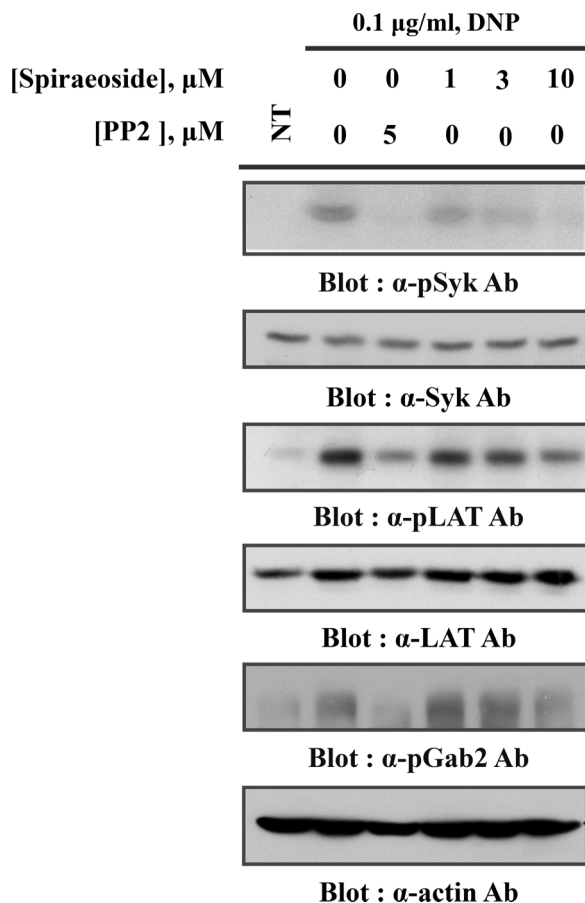
Previous studies have demonstrated that PLC transmits the activation signals of upstream receptors to downstream MAPKs to regulate diverse physiological responses, including inflammation (Yang et al. 2001; Dommisch et al. 2010; Lee et al. 2004). We also observed that the Ag-induced phosphorylation of JNK, P38, and ERK1/2 was dramatically inhibited by U73122 but not by U73433 (Fig. 4A), a result that consistently confirms the essential roles of PLC in Ag-induced MAPK activation in RBL-2H3 cells. Because SP negatively regulated the activation of PLC- γ 2 (Fig. 2), SP might also suppress downstream MAPK activation. As expected, SP inhibited the Ag-induced phosphorylation of JNK, P38, and ERK1/2 in a dose-dependent manner (Fig. 4B). Because the activation of MAPKs is

required for the expression of inflammatory cytokines, particularly TNF- α and IL-4, in mast cells (Theoharides and Kalogeromitros 2006), we next examined the effects of SP on the expression of these cytokines. Consistent with the inhibitory effect on MAPK activation, SP also attenuated the Ag-induced expression of TNF- α and IL-4 in RBL-2H3 cells (Fig. 4C). Therefore, these results strongly suggest that SP simultaneously inhibits degranulation and cytokine expression by suppressing PLC- γ 2/MAPK signaling in RBL-2H3 mast cells.

SP suppresses the mast cells-mediated passive cutaneous anaphylaxis reaction in mice

Based on our finding that SP inhibited degranulation and cytokines expression in RBL-2H3 cells (Figs. 1 and 4), we hypothesized

Fig. 3. Effect of SP on FcεRI-mediated signaling in RBL-2H3 cells. (A) DNP-specific IgE-sensitized RBL-2H3 cells were incubated with the indicated concentrations of SP for 1 h and then stimulated with 100 ng/mL of DNP-HSA for 5 min. The cells were lysed and lysates were then subjected to SDS-PAGE. The levels of activation of Syk by Lyn kinase, LAT, and Gab2 were determined by western blotting analysis. Actin was blotted as a loading control. The results shown are representative of 3 independent experiments.



that SP has an anti-allergic effect. Thus, to determine the potential anti-allergic effect of SP *in vivo*, we used the passive cutaneous anaphylaxis (PCA) reaction in mice, a well-established animal model of IgE-mediated type I allergic reactions (Kabu et al. 2006). PCA was strongly induced by the local injection of a DNP-specific IgE into the ears of mice, followed by the injection of Ag (DNP-HSA) (Fig. 5A). However, the oral administration of SP significantly suppressed the PCA reaction (ED₅₀ of 2.48 mg/kg) (Figs. 5A and B). Therefore, these results strongly suggest that SP has an anti-allergic effect *in vivo*.

Discussion

Mast cells participate in allergic disorders by secreting diverse cytokines and mediators upon activation (Holgate and Polosa 2008). Given that mast cells are key players in allergic inflammation, the pharmacological regulation of mast cell activation is a prerequisite for reducing the severity of the symptoms of allergic diseases. When Ag induce the cross-linking of FcεRI in an allergic conditions, the tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) of the FcεRI β and γ subunits are phosphorylated by Lyn kinase, which results in the recruitment and activation of Syk (Rivera and Gilfillan 2006). Activated Syk, in turn, induces various signaling cascades, including the activation of PI3K, PLC-γ, and MAPKs. These FcεRI-mediated signaling cas-

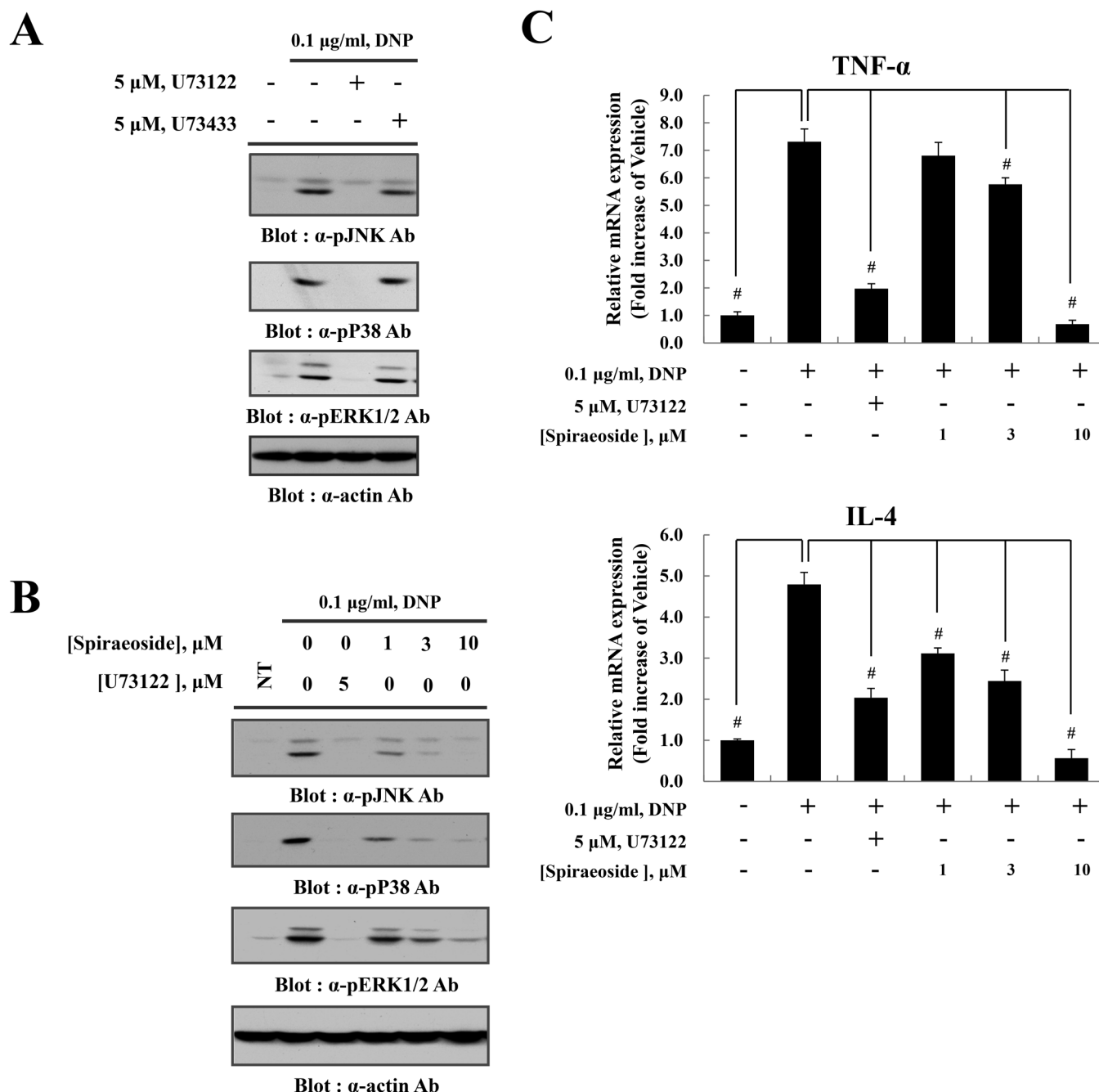
cades eventually regulate and coordinate the mast cell activation and allergic inflammation (Kalesnikoff and Galli 2008). Thus, targeting these signaling cascades is a good strategy for the development of novel anti-allergic drugs. In practice, many studies have been performed to develop regulators for mast cell activation, and several synthetic compounds inhibiting FcεRI-mediated signaling are under clinical trials (Brown et al. 2008). Interestingly, as potential candidates, natural products have recently been used as a good starting point for discovering anti-allergic drugs because of their relatively low cytotoxicity. To date, several natural products, such as saponin and curcumin, have been shown to have anti-allergic effects (Han et al. 2009; Lee et al. 2008). Therefore, the identification of novel natural products that can regulate mast cell activation is important.

In this study we found that SP, a major flavonoid in onions, inhibited mast cell degranulation and suppressed the PCA reaction in mice, highlighting the potential value of SP as an allergy therapeutic. With regard to the underlying molecular mechanism, we showed that SP dramatically reduced the phosphorylation and subsequent activation of PLC-γ2, a key regulator of mast cell activation (Fig. 2). Moreover, the activation of MAPKs such as JNK, P38, and ERK1/2, which are downstream of PLC-γ2, was also attenuated by SP treatment (Fig. 4). Collectively, these results clearly indicate that SP suppresses mast cell activation and allergic responses by blocking PLC-γ2-mediated signaling. Because there are two PLC-γ subtypes, PLC-γ1 and PLC-γ2, we also examined whether Ag induced the phosphorylation of PLC-γ1. However, we barely detected the tyrosine phosphorylation of PLC-γ1 (data not shown), which suggests that PLC-γ2 is the primary subtype involved in FcεRI-mediated signaling in RBL-2H3 mast cells. Thus, targeting PLC-γ2 or PLC-γ2-mediated signaling using a small compound such as SP may be a reasonable approach for the treatment of allergic disorders.

To obtain more insight into how SP suppresses PLC-γ2 activation, we examined the effect of SP on early FcεRI-mediated signaling events, such as the phosphorylation of Syk by Lyn kinase and the phosphorylation of LAT and Gab2 by Syk (Kalesnikoff and Galli 2008). According to our results, SP exhibits an inhibitory effect on the phosphorylation of Syk and its direct target proteins, including LAT and Gab2 (Fig. 3A). These results demonstrate that SP acts primarily at the level of Lyn kinase to suppress PLC-γ2-mediated signaling, although the detailed mode of action was not clearly defined in this study. There are two possible scenarios. First, it is possible that SP directly regulates the kinase activity of Lyn. Previously, several small compounds, including natural products, were found to exhibit an inhibitory effect on Src-family kinases. For example, morin, one of the flavonoids, specifically inhibits the kinase activity of Fyn and improves type I allergic responses in mice (Kim et al. 2009). In addition, the Syk inhibitor R112 has been clinically evaluated for the treatment of symptoms of allergic airway inflammation and allergic rhinitis (Guyer et al. 2006; Meltzer et al. 2005). Thus, SP may directly inhibit the kinase activity of Lyn. Second, SP could interfere with the association between FcεRI and Lyn. It has been demonstrated that when Ag induces the crosslinking of FcεRI, FcεRI aggregation brings FcεRI toward acylated membrane-bound Lyn kinase, leading to the phosphorylation of FcεRI's ITAM and the subsequent activation of Syk after ITAM binding (Hazenbos et al. 2011). Therefore, it is possible that SP suppresses FcεRI-mediated signaling by blocking the association between FcεRI and Lyn. Future studies regarding this issue should provide insight into the exact molecular target of SP.

Traditionally, food-derived flavonoids exhibit various potential pharmacological effects, such as anti-inflammatory and anti-cancer activities (Tunon et al. 2009), and these products have been given much attention (Kobayashi and Tanabe 2006). It is interesting to note that the daily US diet contains approximately 210 mg of flavonoids (Chun et al. 2010). Because quercetin and its glycosides, typical members of the flavonol subclass, are the most common

Fig. 4. Effect of SP on FcεRI-mediated MAPK activation and the subsequent expression of TNF- α and IL-4 in RBL-2H3 cells. DNP-specific IgE-sensitized RBL-2H3 cells were incubated with 5 μ M of U73122 or U73433 (A) or the indicated concentrations of SP (B) for 1 h and then stimulated with 100 ng/mL of DNP-HSA for 5 min. The cells were lysed, and the lysates were then subjected to SDS-PAGE. The levels of phosphorylation of JNK, p38, and ERK1/2 were determined by western blotting. (C) DNP-specific IgE-sensitized RBL-2H3 cells were incubated with the indicated concentrations of SP for 1 h and then stimulated with 100 ng/mL of DNP-HSA. The mRNA levels of TNF- α and IL-4 were determined by qRT-PCR analysis. For quantification, the target was normalized to GAPDH as an internal standard. The results shown are representative of 3 independent experiments. The data are the mean $\pm$ SD. * p < 0.05 and # p < 0.01.

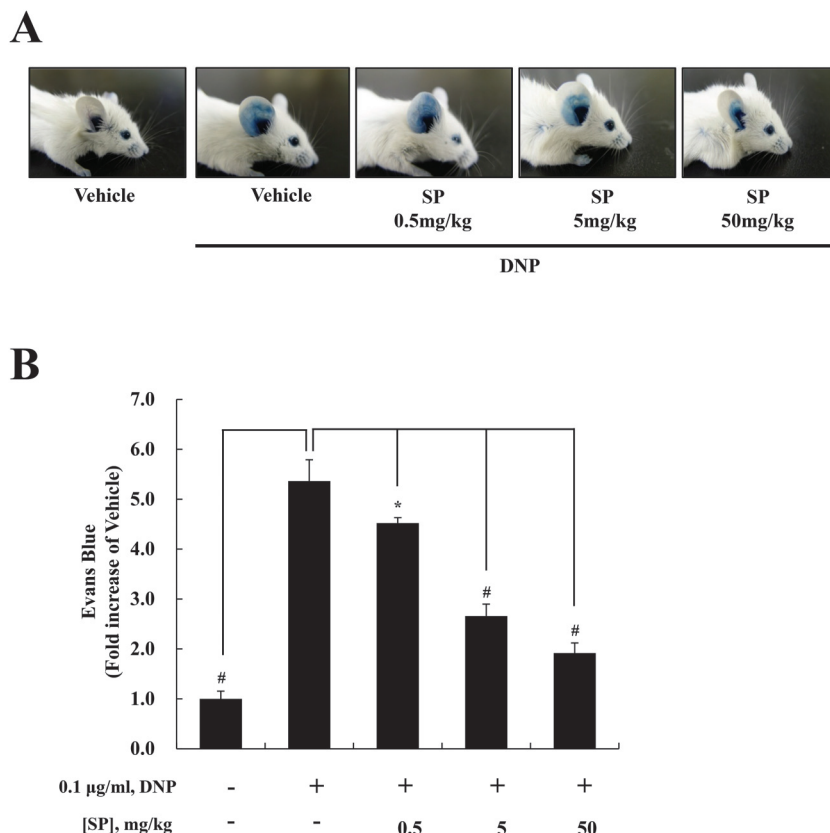


flavonoids in the diet, the biological effects of quercetin and its glycosides need to be clarified in terms of any beneficial effects on human health. Several studies have demonstrated that quercetin has anti-microbial, anti-hypertensive, and anti-allergic effects (Bischoff 2008; Lee et al. 2010); however, the potential effects of its glycosides remain relatively elusive. Given that the biological effects of quercetin glycosides are determined primarily by the nature of the sugar moiety, elucidating the biological effects of quercetin glycosides is necessary to determine the usefulness of these com-

pounds as therapeutic agents. In this regard, our finding that SP (quercetin-4'-glucoside) suppresses mast cells activation is noteworthy. Because we observed that isoquercitrin, quercetin-3'-glucoside, had little effect on mast cell degranulation in our screening system (data not shown), the location of the sugar moiety may be the critical factor determining the inhibitory action of SP in mast cell activation.

In summary, this study is, to our knowledge, the first to demonstrate that SP inhibits the degranulation of and cytokine

Fig. 5. Effect of SP on IgE-mediated PCA in mice. DNP-specific IgE (1 μ g) was intradermally injected into the ears of mice. After 24 h, 200 μ g DNP-HSA in 200 μ L of PBS containing 1% Evans blue was injected i.v. SP was administered orally 1 h prior to antigen (DNP-HSA) stimulation. The mice were then euthanized 30 min after Ag challenges, and the ears were excised. (A) Representative pictures of the ears examined are shown. (B) The extravasated dye was extracted from each excised ear excised, and the amount was measure by absorbance at 620 nm. The data are the mean $\pm$ SE ($n = 5$). * $p < 0.05$ and # $p < 0.01$.



expression in Ag-stimulated RBL-2H3 mast cells and suppresses the mast cell-mediated PCA reaction in mice. These results strongly suggest that SP possesses anti-allergic activity, and that the dietary intake of SP-containing vegetables, such as onions, will have beneficial effects on allergic disorders.

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