

Resveratrol Inhibits IgE-Mediated Basophilic Mast Cell Degranulation and Passive Cutaneous Anaphylaxis in Mice^{1,2}

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Abstract

Resveratrol is a phytoalexin abundantly found in red grape skin and is effective in antitumor and antiinflammation associated with immune responses. This study investigated whether resveratrol suppressed immunoglobulin (Ig)E-mediated allergic responses and passive cutaneous anaphylaxis (PCA) in rat RBL-2H3 mast cells and in BALB/c mice. The release of β -hexosaminidase and histamine was enhanced in mast cells sensitized with anti-dinitrophenyl (DNP)-IgE and subsequently stimulated by DNP-human serum albumin (HSA), indicative of mast cell degranulation. When mast cells were pretreated with nontoxic resveratrol at 1–25 μ mol/L, such induction was dose dependently diminished. Spleen tyrosine kinase (Syk) and phospholipase C γ (PLC γ) of sensitized mast cells were activated by stimulation with DNP-HSA antigen, which was dampened by ≥ 5 μ mol/L resveratrol. The phosphorylation of protein kinase C (PKC) μ and PKC θ was attenuated by administering resveratrol to DNP-HSA-exposed mast cells, whereas quiescent PKC ζ/λ in sensitized cells was dose-dependently activated by resveratrol. Male BALB/c mice were sensitized for 24 h with DNP-IgE and orally administered with resveratrol 1 h before the DNP-HSA challenge. The histamine concentration was enhanced in sensitized mice challenged to DNP-HSA, which was reversed by administration of 10 mg/kg resveratrol. Additionally, it encumbered the tissue activation of Syk, PLC γ , and PKC μ in antigen-exposed mice. Resveratrol decreased IgE-mediated PCA and alleviated allergic edema of mouse ear and dorsal skin. Mast cell degranulation and allergic inflammation, accompanying the induction of monocyte chemotactic protein-1 and macrophage inflammatory protein-2, were inhibited by supplementing resveratrol to antigen-challenged mice. Resveratrol inhibited mast cell-derived, immediate-type allergic reactions, and these responses of resveratrol suggest possible therapeutic strategies in preventing allergic inflammatory diseases. J. Nutr. doi: 10.3945/jn.112.173302.

Introduction

An allergy is a hypersensitivity disorder mediated by immunological mechanisms and can cause tissue damage and common reactions, including eczema, atopic dermatitis, asthma attacks, and anaphylactic shock (1,2). Allergic reactions occur due to innocuous antigens that are normally harmless environmental substances known as allergens, such as certain foods, dust, mites, and pollen (3). Allergy is characterized by the excessive activation of mast cells and basophils by a specific IgE antibody, resulting in extreme inflammatory responses (1,3,4). Antigens called allergens induce the production of antigen-specific IgE antibodies that bind to high affinity-IgE receptors (Fc ϵ RI) on the surface of mast cells or basophils (4).

Mast cells play important roles in both immediate allergic reactions and late-phase reactions (5,6). Mast cell activation due to antigens in the early phase is regulated by the aggregation of Fc ϵ RI, which immediately releases chemical mediators, including histamine, β -hexosaminidase, and prostaglandins through initiating signaling cascades (7,8). In addition, mast cells produce various cytokines, chemokines, and leukotrienes that all play important roles in the infiltration of inflammatory cells and the induction of the late-phase reactions (4,9). However, the regulatory mechanisms for mast cell degranulation are not clearly defined.

Aggregation of Fc ϵ RI sequentially activates antigen-mediated, nonreceptor-type, protein-tyrosine kinases such as spleen tyrosine kinase (Syk)³ and subsequent tyrosine phosphorylation of cellular

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³ Abbreviations used: DNP, dinitrophenyl; HSA, human serum albumin; IgE, immunoglobulin E; MCP-1, monocyte chemotactic protein-1; MIP-2, macrophage inflammatory protein-2; MTT, 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazolium bromide; PCA, passive cutaneous anaphylaxis; PIPES, piperazine-N,N'-bis (2-ethanesulfonic acid); PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; Syk, spleen tyrosine kinase.

proteins such as phospholipase C γ (PLC γ), which leads to mast cell degranulation (6,10). Syk may play an essential role in regulating the trafficking and retention of Fc ϵ RI (11). Tyrosine-phosphorylated PLC γ 1 and PLC γ 2 catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in the generation of inositol-1,4,5-trisphosphate and 1,2-diacylglycerol, which are second messengers that release Ca²⁺ from internal stores and activate protein kinase C (PKC) isomers. The tyrosine phosphorylation and activation of PLC γ are essential to generate signals for a sustained Ca²⁺ influx (6). The sequential cascade of events suggests that mast cells are ideal targets for controlling various allergic responses (12).

Resveratrol is a polyphenol and phytoalexin rich in the skin of red grapes and is a constituent of red wine. It has been reported that resveratrol exerts anticancer, antiinflammatory, blood sugar-lowering activity and other beneficial cardiovascular effects (13). The antiinflammatory effects of resveratrol appear to be associated with its immune responses (14). Resveratrol inhibits the IgE-mediated release of histamine, leukotrienes, and prostaglandin D from bone marrow-derived mouse mast cells (15). Oral administration of fermented grape marc suppresses passive cutaneous anaphylaxis (PCA) reactions in IgE-sensitized, ovalbumin-challenged BALB/c mice (16). However, the molecular mechanism(s) by which resveratrol inhibits mast cell degranulation and allergic responses are not well defined, but it has been shown that resveratrol inhibits mast cell degranulation through reducing Fc ϵ RI-mediated tyrosine phosphorylation of extracellular signal-regulated kinase and PLC γ 1 (17).

This study investigated whether resveratrol (Fig. 1A) modulated allergic responses in rat basophilic leukemic RBL-2H3 cells IgE-sensitized and inflamed by dinitrophenyl (DNP)-human serum albumin (HSA) antigen. For the measurements of antigen-induced mast cell degranulation, the release of β -hexosaminidase and histamine from mast cells was determined in the presence of 1–25 μ mol/L resveratrol. Additionally, the suppressive effects of resveratrol on signaling components of Syk-PLC-PKC were elucidated in antigen-exposed mast cells. Furthermore, this study examined whether oral administration of resveratrol alleviated IgE-mediated PCA in DNP-HSA-challenged BALB/c mice through blocking Syk-responsive signaling. Histamine secretion, allergic ear/dorsal edema, mast cell degranulation, and allergic skin inflammation were determined in sensitized and resveratrol-treated mice.

Materials and Methods

Materials. Rat basophilic leukemic RBL-2H3 mast cells were obtained from American Type Culture Collection. Eagle's minimum essential medium (EMEM) was provided by Gibco Life Technologies and culture reagents were purchased from Sigma-Aldrich Chemicals. FBS, penicillin-streptomycin, and trypsin-EDTA were provided from Lonza. 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was obtained from DUCHEFA Biochemie. Mouse monoclonal anti-2,4-DNP and DNP-DNP-HSA were purchased from Sigma-Aldrich Chemicals. Antibodies against phospho-Syk, phospho-PLC γ , and phospho-PKC family were all provided by Cell Signaling Technology. HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories.

Cell culture. Rat basophilic leukemic RBL-2H3 mast cells were cultured in EMEM containing 10% heat-inactivated FBS, 2 mmol/L glutamine, 100 mg/L streptomycin, and 100,000 U/L penicillin at 37°C in a humidified atmosphere of 5% CO₂ in air. RBL-2H3 cells were sensitized with 0.5 mg/L anti-DNP IgE overnight to induce IgE-mediated allergic responses (18). Sensitized cells were washed twice in piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.4 mmol/L MgCl₂,

40 mmol/L NaOH, 119 mmol/L NaCl, 1 mmol/L CaCl₂, 5 mmol/L KCl, 25 mmol/L PIPES, 5.6 mmol/L glucose, and 1% BSA (pH 7.4) and pretreated with 1–25 μ mol/L resveratrol for 2 h. Subsequently, cells were exposed to 200 μ g/L DNP-HSA for the various times indicated.

The cytotoxicity of resveratrol was determined using an MTT assay. Briefly, cells were cultured in a fresh, 1 g/L MTT medium at 37°C for 3 h. Gentle shaking was performed to dissolve purple formazan product in 0.5 mL isopropanol and the absorbance of formazan dye was determined at λ = 570 nm using a microplate reader (Bio-Rad Model 550). When rat RBL-2H3 basophilic cells were incubated with $\leq$ 25 μ mol/L resveratrol for 24 h, the cell viability was not influenced (data now shown).

Induction of IgE-mediated PCA in a murine model. Five-week-old male BALB/c mice (Hallym University Breeding Center for Laboratory Animals) were kept on a 12-h-light/dark cycle at 23 $\pm$ 1°C with 50 $\pm$ 10% relative humidity under specific pathogen-free conditions. Mice were fed a commercial chow diet containing 20.5% protein, 3.5% fat, 8% fiber, 8% ash, 0.5% calcium, and 0.5% phosphorus (RodFeed, DBL) and consumed water ad libitum at the Animal Facility of Hallym University. Mice were allowed to acclimatize for 1 wk before beginning the allergic experiments. Mice were divided into 4 subgroups (9 mice/subgroup) as follows: untreated control group (not sensitized), anti-DNP (IgE)-sensitized group, DNP-HSA antigen-treated IgE group, and resveratrol/DNP-HSA-treated IgE group. All experiments were approved by the Committee on Animal Experimentation of Hallym University and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals. No mice were dead and no apparent signs of exhaustion were observed during the experimental period.

Experimental protocols for the IgE sensitization and antigen challenge of mice are shown in Figure 1B. Mice were passively sensitized for 24 h with intradermal administration (0.3-cc syringe, 31G needle) of DNP-specific IgE antibody dissolved in PBS into the left or right ear (0.5 μ g in 10 μ L) or dorsal skin (5 μ g in 100 μ L). The resveratrol solution (10 mg/kg in 100 μ L) was orally administered to IgE-sensitized mice 1 h before the DNP-HSA antigen challenge. For the challenge with DNP-HSA, mice were i.v. injected with 250 μ g DNP-HSA in 200 μ L PBS into the tail vein (0.3-cc syringe, 31G needle). When Evan's blue was used, 250 μ g DNP-HSA in 1% Evan's blue (in saline) was i.v. injected into the tail of sensitized mice. Ear swelling was observed for 1 h after DNP-HSA challenge. Control mice were sensitized and challenged with saline as a DNP-HSA vehicle.

β -Hexosaminidase release assay. After challenge with resveratrol and DNP-HSP to sensitized cells, 20 μ L of culture medium was mixed with an equal volume of substrate solution (1 mmol/L p-nitrophenyl-N-acetyl- β -D-glucosaminide in 0.1 mol/L citrate buffer, pH 4.5) for 1 h at 37°C. The reaction was terminated by adding 200 μ L of stop solution (0.1 mol/L Na₂CO₃/NaHCO₃). The absorbance was measured with a microplate reader at λ = 405 nm.

Histamine release assay. After washing in PIPES buffer, anti-DNP-sensitized cells were pretreated with 1–25 μ mol/L resveratrol for 2 h and exposed to DNP-HSA. Culture media were collected and assayed for histamine release by using a histamine ELISA kit (IBL International) according to the manufacturer's instructions.

Plasma histamine concentrations of IgE-sensitized antigen-challenged mice were measured by a fluorescent method with the highly specific fluorescent reagent O-phthalaldehyde (Sigma-Aldrich Chemicals), as previously described (19). Tissue proteins (50 μ L plasma) were precipitated by an addition of 60% perchloric acid and the resulting precipitate was removed by centrifugation. After plasma samples experienced alkalization with 3 N NaOH, a fluorometric reaction with 1% O-phthalaldehyde solution for 30 min and acidification with phosphoric acid were followed. Fluorescence intensity was determined by using a Fluoroscan Ascent FL (LabSystems) at λ = 350 nm excitation and λ = 450 nm emission.

Protein isolation and Western-blot analysis. Western-blot analysis was performed using whole-cell lysates and skin tissue extracts were

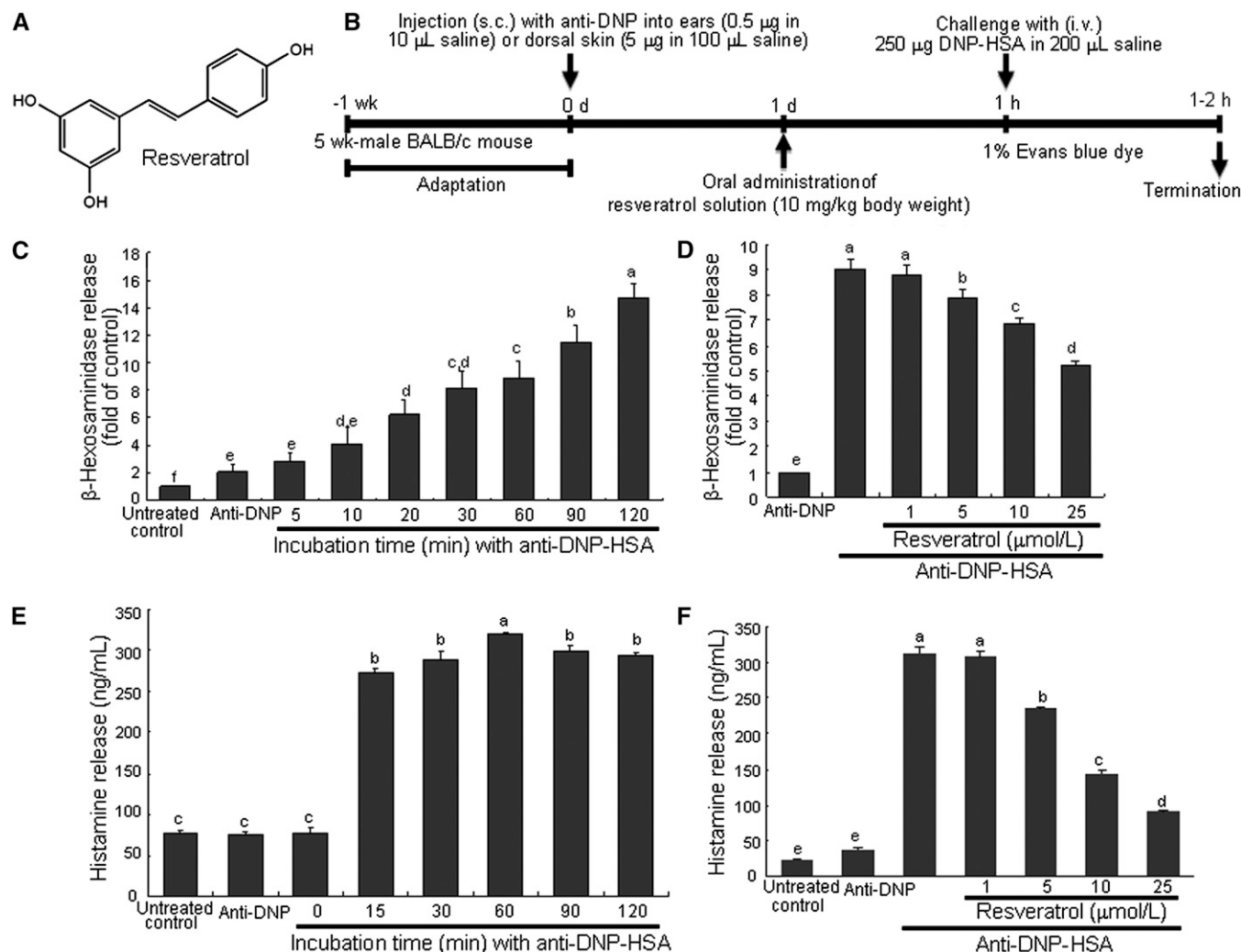


FIGURE 1 Chemical structure of phytoalexin resveratrol (A) and experimental protocols for sensitization and antigen challenge (B). Time course responses of release of β -hexosaminidase and histamine (C,E) and inhibition of release of β -hexosaminidase and histamine by resveratrol (D,F) in IgE-sensitized rat basophilic leukemic RBL-2H3 cells. Data are means $\pm$ SEMs, $n = 3$ (means of duplicates). Means without a common letter differ, $P < 0.05$. DNP, dinitrophenyl; HSA, human serum albumin.

prepared from RBL-2H3 cells and BALB/c mice experienced experimental protocols. Whole RBL-2H3 cell lysates and mouse skin tissue extracts were prepared in lysis buffer containing 1 mol/L Tris-HCl (pH 6.8) lysis buffer containing 10% SDS, 1% β -mercaptoethanol, 1% β -glycerophosphate, 0.1 mol/L Na_3VO_4 , 0.5 mol/L NaF, and protease inhibitor cocktail. Equal volumes of cell culture supernatants and equal amounts of cell lysates or tissue extract proteins were electrophoresed on 8–15% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in TBS-T buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 3 h. The membrane was incubated with a primary rat antibody against phospho-Syk, phospho-PKC family, phospho-PLC γ , and monocyte chemotactic protein-1 (MCP-1). After 3 washes with TBS-T buffer, the membrane was incubated with a goat anti-rabbit IgG conjugated to HRP. The proteins were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology) and AGFA medical X-ray film (Gevaert). Incubation with anti- β -actin was performed for the comparative control.

Immunohistochemistry of dorsal skin. Dorsal skin tissue sections were subjected to a series of immunohistochemical procedures, including the Ag retrieval followed by quenching of endogenous peroxidase activity. For the measurement of tissue concentration of phospho-Syk, an immunohistochemical analysis of the integuments was conducted using a 3,3'-diaminobenzidine chromogenic substrate detection kit (Dako).

Counter staining was conducted with hematoxylin. Each slide was mounted in VectaMount mounting medium (Vector Laboratories). Images of each slide were taken using an optical microscope system (Axiomager).

Histology of ears and dorsal skin and dye extravasation. All mice were killed with an anesthetic (2 $\mu\text{L/kg}$ rompun and 8 $\mu\text{L/kg}$ zoletil, intraperitoneal injection) after a 1-h challenge. The skin specimens from ears and dorsal skin were collected and kept at -80°C for the histological staining with hematoxylin and eosin and toluidine blue (paraffin-embedded tissue sections, 5 μm). For the Evans blue extravasation, the ears were cut into 4–8 pieces and placed in 1 mL dimethylformamide. The pieces of ears and dorsal skin containing extravasated dye were extracted overnight in 700 μL formamide at 63°C . The tube was shaken vigorously for a few seconds and centrifuged at $480 \times g$ for 15 min and the absorbance intensity was measured at $\lambda = 620 \text{ nm}$.

ELISA. The tissue concentration of macrophage inflammatory protein-2 (MIP-2) was examined in BALB/c dorsal skin tissue extracts by using each ELISA kit (microplate reader, Molecular Devices).

Data analysis. The data are presented as means $\pm$ SEMs. Statistical analyses were conducted using a SAS program (SAS Institute). Data were analyzed by 1-factor ANOVA and posthoc Duncan's multiple-range test. Differences were considered to be significant at $P < 0.05$.

Results

Inhibition of β -hexosaminidase and histamine release by resveratrol. Sensitized RBL-2H3 basophilic cells were stimulated with antigen DNP-HSA and release of β -hexosaminidase and histamine was determined for up to 2 h. As expected, the β -hexosaminidase release was gradually and significantly enhanced over the incubation time with antigen (Fig. 1C). β -Hexosaminidase release was very minimal in IgE-sensitized but quiescent RBL-2H3 cells. However, a significant increase in β -hexosaminidase release was observed in cells exposed to DNP-HSA for 2 h compared with that of sensitized cells. Accordingly, sensitized RBL-2H3 cells were incubated for 2 h to determine the effect of resveratrol on β -hexosaminidase release. Resveratrol suppressed the β -hexosaminidase release in a dose-dependent manner (Fig. 1D). On the other hand, histamine was also rapidly released on stimulation of the sensitized cells with the antigen (Fig. 1E). Similar results were observed in inhibiting histamine release by resveratrol (Fig. 1F). These results showed that anti-DNP IgE-sensitized cells caused allergic responses fully within 2 h postexposure to DNP-HSA. These responses were attenuated in RBL-2H3 cells treated with resveratrol at nontoxic doses of $\geq 5 \mu\text{mol/L}$.

Resveratrol attenuation of antigen-triggered Syk and PLC γ activation. This study investigated that resveratrol disturbed Syk-responsive mechanism(s) possibly involved in the antigen-triggered allergic responses. The antigen stimulation instigated Syk activation very rapidly within 5 min poststimulation (Fig. 2A). In addition, PLC γ , a downstream effector of Syk, was similarly activated by treating sensitized RBL-2H3 cells with antigen. When 1–25 $\mu\text{mol/L}$ resveratrol was added to DNP-HSA-stimulated mast cells, the activation of Syk and PLC γ was diminished in a dose-dependent manner (Fig. 2B). Accordingly, the inhibition of release of allergic mediators by resveratrol may be mediated via blocking Syk-responsive mechanism(s).

Blockade of antigen-triggered PKC family activation by resveratrol. Allergic responses are mediated via the activation of the PKC-associated signal-transduction pathway (20). This study elucidated the phosphorylation of PKC isoforms in the DNP-HSA antigen-triggered allergic responses and modulatory effects of resveratrol on its specific isoforms. The stimulation with DNP-HSA promoted PKD (PKC μ) phosphorylation rapidly within 20 min (Fig. 3A).

When DNP-HSA stimulated sensitized mast cells, there was a differential activation of PKC isoforms (Fig. 3B). In addition to PKC μ , PKC δ/θ and PKC θ were rapidly phosphorylated after a 30 min-stimulation of sensitized cells, whereas PKC α/β II and PKC (pan, β II ser660) remained active even in quiescent RBL-2H3 cells. In contrast, PKC ζ/λ was not activated by antigen stimulation. When 1–25 $\mu\text{mol/L}$ resveratrol was administered to sensitized RBL-2H3 cells exposed to DNP-HSA, the activation of these PKC isoforms was disparately manipulated (Fig. 3B). The antigen-enhanced activation of PKC μ , PKC δ/θ , and PKC θ was diminished in a dose-dependent fashion. Strikingly, PKC ζ/λ was activated by increasing the resveratrol doses in the range of 1–25 $\mu\text{mol/L}$. These results showed functional divergence of PKC family isoforms competent to mediate antigen-responsive allergic responses that were influenced by resveratrol.

Resveratrol inhibition of IgE-mediated anaphylactic histamine release. This study examined mast cell-dependent allergic responses in a mouse model. The plasma histamine concentra-

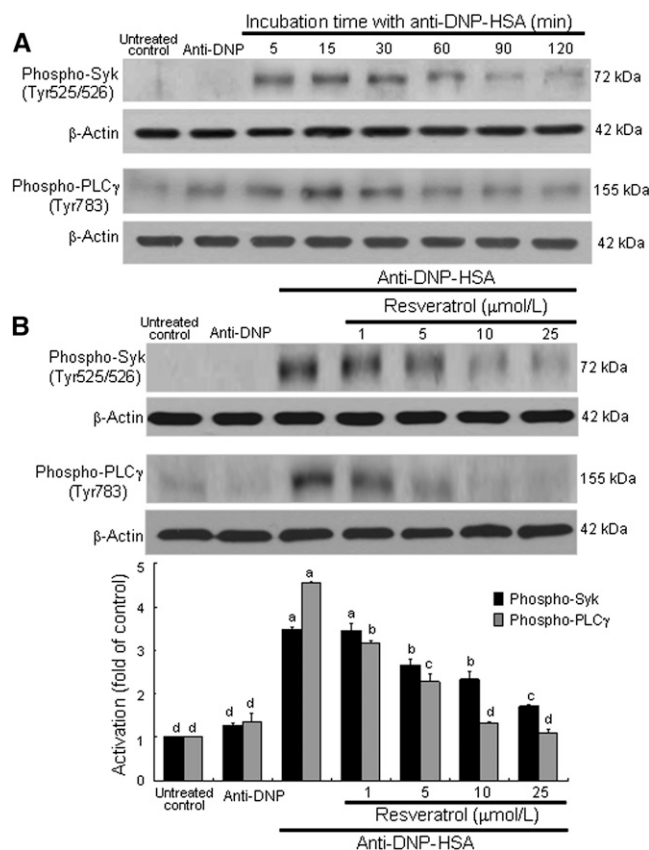


FIGURE 2 Western-blot data showing time course responses (A) and inhibition (B) of Syk and PLC γ activation by resveratrol. RBL-2H3 cells were sensitized with 0.5 mg/L anti-DNP and stimulated with 200 $\mu\text{g/L}$ of DNP-HSA in the absence and presence of 1–25 $\mu\text{mol/L}$ resveratrol. Immunoblot analysis was conducted with a primary antibody against of phospho-Syk and phospho-PLC γ (3 separate experiments). β -Actin protein was used as an internal control for cellular activation of phospho-Syk and phospho-PLC γ . Densitometric data in the bottom panel are means $\pm$ SEMs, $n = 3$. Means for a variable without a common letter differ, $P < 0.05$. DNP, dinitrophenyl; HSA, human serum albumin; PLC, phospholipase C; Syk, spleen tyrosine kinase.

tion was highly elevated by ~ 12 -fold in mice sensitized with DNP-specific IgE and i.v. challenged with the antigen DNP-HSA (Fig. 4A). When 10 mg/kg resveratrol was orally administered to those sensitized mice, the elevated plasma histamine concentration decreased significantly to $\sim 50\%$ (Fig. 4A). These results showed that resveratrol alleviated IgE-mediated anaphylaxis due to antigen in mice.

This study attempted to confirm that resveratrol disturbed the signaling pathway of Syk-PLC γ -PKC μ in sensitized mice exposed to antigen. The stimulation of sensitized mice with DNP-HSA highly activated Syk, PLC γ , and PKC μ in dorsal skin tissues (Fig. 4B). Resveratrol administration to DNP-HSA-challenged mice significantly lowered tissue concentrations of phospho-Syk, phospho-PLC γ , and phospho-PKC μ relative to those of mice challenged only with antigen (Fig. 4B). This indicates that resveratrol ameliorated IgE-mediated anaphylactic histamine release via disturbing the Syk-PLC γ -PKC μ signaling axis.

This study attempted to further investigate Syk activation in the dermis during antigen stimulation (Fig. 4C). The DNP-HSA challenge enhanced Syk phosphorylation (brown staining) in mouse skin integuments, which was diminished by oral administration with 10 mg/kg resveratrol.

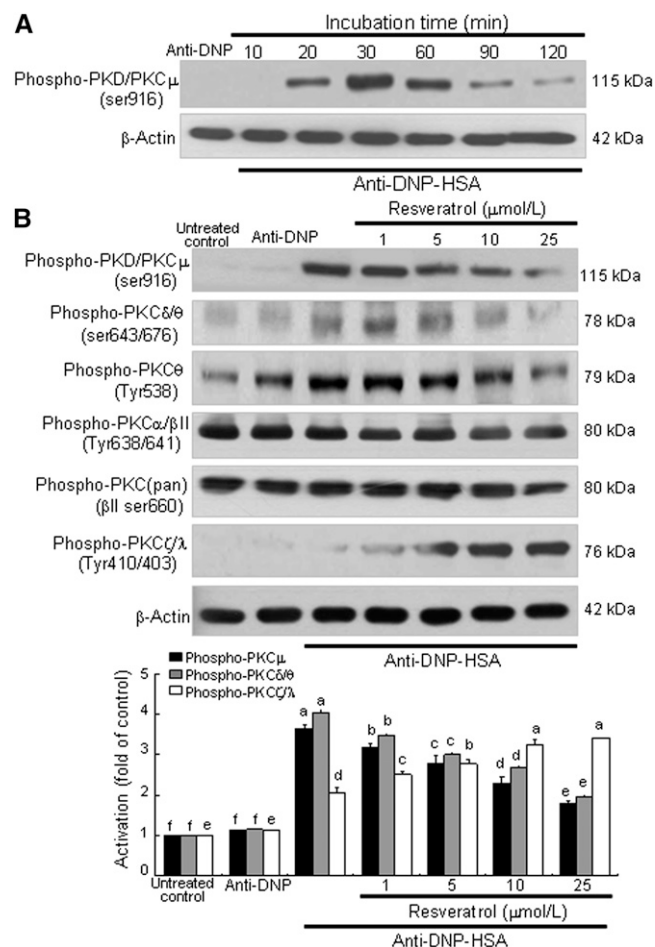


FIGURE 3 Temporal responses of PKC μ activation (A) and modulation of PKC family activation by resveratrol (B) in IgE-sensitized RBL-2H3 cells. Equal amounts of cell lysate proteins were subject to 6–8% SDS-PAGE and Western-blot analysis with a primary antibody against phospho-PKC μ and phospho-PKC family (3 separate experiments). β -Actin protein was used as an internal control. Densitometric data in the bottom panel are means $\pm$ SEMs, $n = 3$. Means for a variable without a common letter differ, $P < 0.05$. DNP, dinitrophenyl; HSA, human serum albumin; PKC, protein kinase C.

Suppression of IgE-mediated PCA reaction by resveratrol. Mice sensitized with DNP-specific IgE and i.v. challenged with the antigen DNP-HSA developed strong PCA concomitant with rapidly occurring capillary dilatation and increased vascular permeability of ears, as visibly manifested by leakage of the Evans blue dye into the reaction site of ears (Supplemental Fig. 1). When resveratrol was orally administrated to those mice, the vascular permeability of the ears was attenuated, as evaluated by the extent of ear blue staining and intensity of Evans blue extraction of the ears (Fig. 5A). Thus, resveratrol suppressed the IgE-dependent PCA reaction in antigen-challenged mice.

Hematoxylin and eosin stains of mouse ears and dorsal skin at 1 h after DNP-HSA challenge revealed increased edema formation, epidermal hyper-thickening, and inflammatory cell infiltration in the anti-DNP IgE-sensitized mice compared with uninfamed control mice (Supplemental Fig. 2). In contrast, the oral treatment of resveratrol diminished ear thickness compared with that of DNP-HSA antigen-exposed mice. This indicates that resveratrol suppressed ear swelling and epidermal thickening in the ear skin (Fig. 5B). Thus, resveratrol administration may alleviate the IgE-mediated immediate hypersensitivity.

Inhibition of mast cell degranulation by resveratrol. This study tested whether resveratrol blocked the release of MCP-1 (chemokine C-C motif ligand 2) and MIP-2 (C-X-C chemokine) at the dorsal dermis of mice. Western-blot data demonstrated that a noticeable increase in the MCP-1 concentration was observed in DNP-HSA-challenged mouse dorsal skin sections compared with mice challenged with only anti-DNP (Fig. 5C). In marked contrast, the tissue concentration of MCP-1 in antigen-exposed mice supplemented with 10 mg/kg resveratrol decreased, almost similar to that of untreated control mice (Fig. 5C). ELISA data revealed that IgE sensitization enhanced the cutaneous secretion of MIP-2 in mice, which was further promoted by the antigen DNP-HSA challenge (Fig. 5D). Conversely, the MIP-2 secretion was suppressed in mice supplemented with 10 mg/kg resveratrol, indicating a possible blockade of macrophage infiltration. These observations suggest that resveratrol lessened the local tissue degranulation associated with PCA reactions in mice sensitized with antigen-specific IgE.

This study further examined mast cells resident in the dorsal skin of mice during the IgE-mediated antigen-dependent PCA. Ear tissue sections of sensitized mice with DNP-HSA challenge were stained with toluidine blue in order to identify mast cell degranulation by light microscopy. In the absence of DNP-HSA challenge (untreated control and anti-DNP sensitized mice), the dorsal skin of mice contained intact toluidine blue-positive mast cells (arrows, Fig. 5E). After challenge with DNP-HSA for 1 h, a marked reduction in the numbers of dermal mast cells was identified in histological tissue sections. This was an effect which most likely reflected extensive mast cell degranulation at the dorsal dermis of mice. When resveratrol was orally administrated to antigen-challenged mice, intact mast cells were detected, indicative of less degranulation (Fig. 5E).

Discussion

This study observed 7 major findings: 1) the release of β -hexosaminidase and histamine was markedly enhanced in IgE-sensitized RBL-2H3 cells stimulated with antigen DNP-HSA, which was attenuated by treatment of nontoxic resveratrol in a dose-dependent manner at $\leq 25 \mu\text{mol/L}$; 2) the phosphorylation of Syk and PLC γ took place rapidly after the antigen stimulation of sensitized mast cells and such phosphorylation was diminished by adding $\geq 5 \mu\text{mol/L}$ resveratrol; 3) PKC μ and PKC θ were rapidly phosphorylated within 30 min in IgE-sensitized and DNP-HSA-exposed RBL-2H3 cells, which was disturbed by treatment with $\geq 10 \mu\text{mol/L}$ resveratrol. In contrast, the PKC ζ/λ phosphorylation was enhanced with doses of resveratrol in IgE-sensitized and DNP-HSA-exposed RBL-2H3 cells; 4) resveratrol lowered the plasma histamine concentration concomitant with the blockade of activation of Syk, PLC γ , and PKC μ in mice sensitized with antigen-specific IgE and exposed to antigen; 5) oral administration of resveratrol suppressed PCA resulting in vascular permeability and the local tissue swelling in mice challenged with antigen-specific IgE; 6) resveratrol administration alleviated macrophage infiltration and mast cell degranulation in DNP-specific IgE-sensitized and antigen-challenged mice. Accordingly, resveratrol dampened the release of allergic mediators by disturbing Syk-PLC γ -PKC-signaling pathways. Resveratrol may be a potential therapeutic agent in ameliorating PCA and allergic inflammation induced by DNP-HSA.

Allergy is called type I (or immediate) hypersensitivity and is distinct due to excessive activation of mast cells and basophils by IgE, resulting in inflammatory responses (1,3,4). Antigens induce the production of antigen-specific IgE that bind to Fc ϵ RI

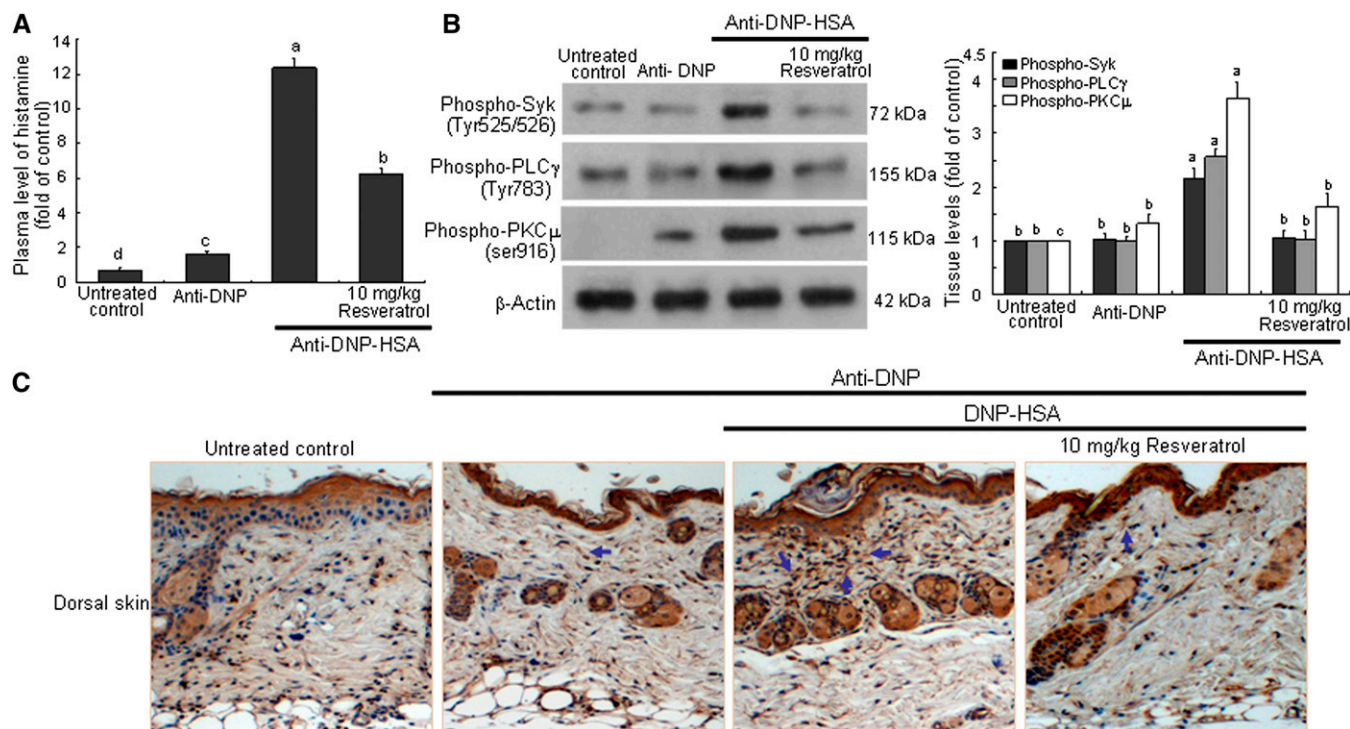


FIGURE 4 Inhibition of histamine secretion (A) and of Syk, PLC γ , and PKC μ activation (B) by resveratrol in IgE-sensitized antigen-challenged BALB/c mice. Histamine secretion was means $\pm$ SEMs, $n = 5$. For the measurement of Syk, PLC γ , and PKC μ activation (B), equal amounts of tissue extract proteins were subject to 6–8% SDS-PAGE and Western-blot analysis with a primary antibody against phospho-Syk, phospho-PLC γ , and phospho-PKC μ . β -Actin protein was used as an internal control. Densitometric data in the right panel are means $\pm$ SEMs, $n = 3$. Means for a variable without a common letter differ, $P < 0.05$. Syk phosphorylation in the dorsal skin tissues of mice (C). Histological sections of mouse dorsal skins were stained using an anti-mouse phospho-Syk and counterstained with hematoxylin. Arrows indicate phosphorylated Syk in the dorsal skin dermis. Magnification: $\times 200$. PKC, protein kinase C; PLC, phospholipase C; Syk, spleen tyrosine kinase.

with high affinity on the surface of mast cells or basophils (4,21). Fc ϵ RI stimulation of basophils and mast cells induces the immediate release of preformed, proinflammatory mediators such as histamine and proteases and the later production and secretion of leukotrienes, cytokines, and chemokines involved in the symptoms of both acute and chronic allergic inflammation (4,9). In the current study, DNP-HSA antigen immediately induced the release of β -hexosaminidase and histamine from IgE-sensitized basophilic mast cells, possibly via eliciting cellular induction of Fc ϵ RI α . In addition, the plasma histamine concentration was markedly enhanced in the DNP-HSA-challenged sensitized mice. Accordingly, the mediators secreted due to antigen-evoked mast cell degranulation may be responsible for PCA, inflammatory cell infiltration, and induction of both early- and late-phase allergic reactions. Accordingly, basophils and mast cells are thought to be ideal targets for controlling various allergic responses (4,7,22).

Resveratrol is a phytoalexin and stilbenstilbenestilbene-type polyphenol abundant in the skin of red grapes and possesses anticancer, antiinflammatory, blood-sugar-lowering, and cardioprotective properties (13). Resveratrol inhibits the IgE-mediated release of histamine, leukotrienes, and prostaglandin D from bone marrow-derived mouse mast cells (15). This study found that resveratrol diminished the release of β -hexosaminidase and histamine from IgE-sensitized and antigen-exposed mast cells as markers of antigen-induced degranulation. These results suggest that this compound inhibited mast cell degranulation. Consistently, oral administration of resveratrol lowered the plasma histamine concentration and PCA in IgE-sensitized HSA-challenged BALB/c mice. Similarly, nonapoptotic trans-resveratrol is effective at inhibiting human eosinophil activation

and degranulation at $<100 \mu\text{mol/L}$ concentrations (23). Another stilbene-type polyphenol piceatannol inhibits mast cell-mediated mediator secretion, cellular signaling, and effector function (24,25). However, the molecular regulatory mechanism(s) for the resveratrol inhibition of cell degranulation and PCA are not well defined. Nevertheless, PKC family isomers have been considered as potential targets to mediate antigen-induced release of chemical mediators and cytokines in the type I allergic reactions (26,27).

Mast cell activation is regulated by antigen-triggered Fc ϵ RI aggregation that sequentially activates receptor-associated protein-tyrosine kinases such as Syk and Lyn (27). Fc ϵ RI-crosslinking of antigens further causes the tyrosine phosphorylation of other proteins, including PLC γ isoforms, phosphatidylinositol-3 kinase isoforms, and PKC isoforms. However, little is known about the trafficking of the internalized Fc ϵ RI. It can be speculated that while not influencing the cellular level of Fc ϵ RI due to an antigen, resveratrol may influence the aggregation and trafficking of Fc ϵ RI. This study also revealed that oral administration of resveratrol suppressed Syk activation and subsequent tyrosine phosphorylation of PLC γ induced by DNP-HSA in IgE-sensitized mast cells, concomitant with the suppression of the serum concentration of histamine. Additionally, resveratrol inhibited the activation of PKC μ and PKC θ , whereas it enhanced PKC ζ/λ phosphorylation with its increasing doses. Consequently, the blockade of the sequential cascade of events by resveratrol may suppress the release of β -hexosaminidase and histamine in mast cells due to mast cell degranulation. It has been reported that Fc ϵ RI-mediated tyrosine phosphorylation of PLC γ 1 and extracellular signal-regulated kinase could be potential cellular targets of resveratrol for the inhibition of

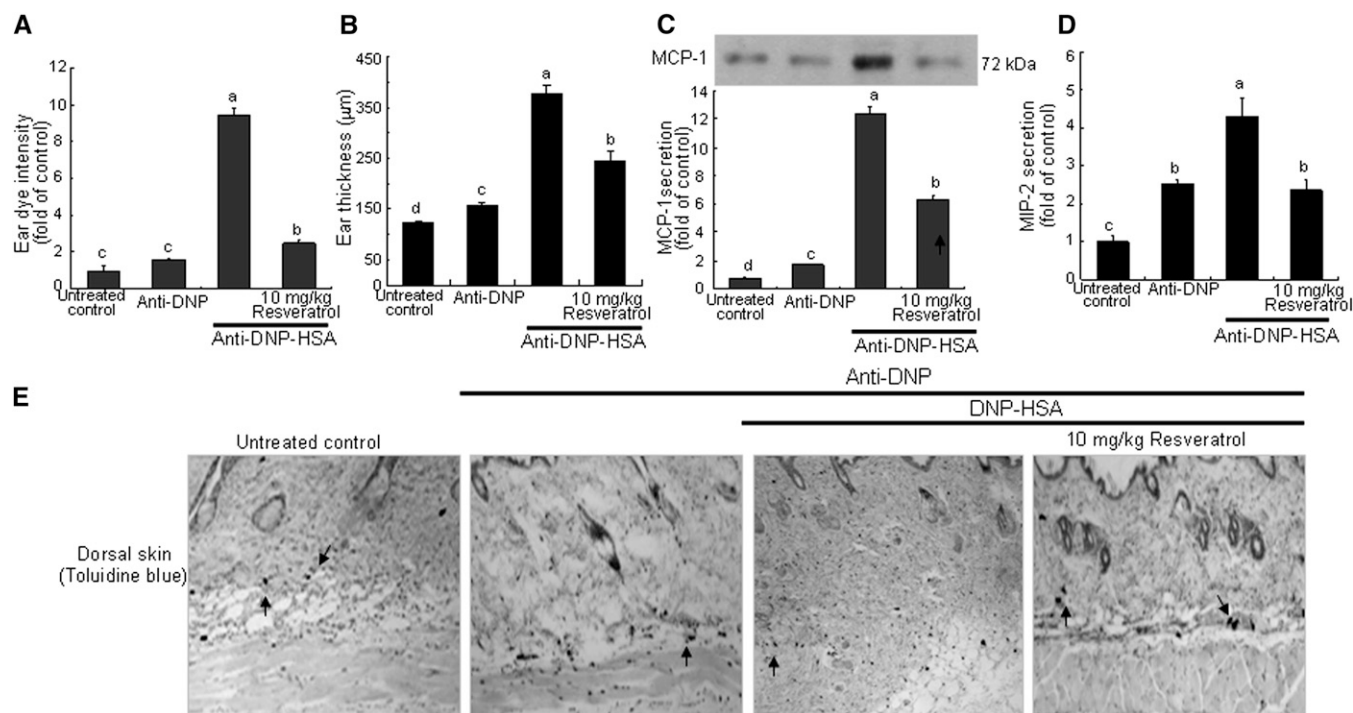


FIGURE 5 Inhibitory effects of resveratrol on IgE-mediated extravasation (A), edema (B), secretion of MCP-1 (C), and MIP-2 (D) and mast cell degranulation (E) in antigen-challenged BALB/c mice. After antigen challenge (Fig. 1B), ears were excised to quantify the extravasated dye (A). The absorbance intensity of Evans blue dye extracted was measured. The amount of Evans blue dye leakage is means $\pm$ SEMs, $n = 3$. The thickness of ear sections was measured to analyze edema (B). For the measurements of the tissue concentration of MCP-1 (C), tissue extracts were subject to Western-blot analysis with a primary antibody against MCP-1 (3 separate experiments). β -Actin protein was used as an internal control for cellular expression of MCP-1. Densitometric data in the bottom panel are means $\pm$ SEMs, $n = 3$. Means without a common letter differ, $P < 0.05$. Dorsal skin tissue sections were stained with toluidine blue for mast cell degranulation (E). Arrows indicate mast cell degranulation into the dorsal skin dermis. Magnification: $\times 400$. MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2.

mast cell degranulation (17). Likewise, procyanidin suppresses Fc ϵ RI-mediated mast cell activation by inhibiting tyrosine phosphorylation of Syk and linker for the activation of T cells, suggesting the antiallergenic effects of the procyanidin-enriched apple extract (28). However, piceatannol inhibits mediator secretion from human basophils independent of inhibiting the activity of Syk kinase (25).

PCA is an evanescent cutaneous reaction, rapidly resulting in capillary dilatation and increased vascular permeability readily visible by leakage into the reaction sites. Oral administration of resveratrol to DNP-HSA-challenged mice suppressed PCA by disturbing the IgE-mediated mechanisms of immediate hypersensitivity. Specific allergic responses of PCA typical of enhanced tissue swelling and vascular permeability in IgE-sensitized mice were most likely mediated by Syk-dependent, PKC μ -responsive signaling of dermal mast cells. Chlorogenic acid, a naturally occurring polyphenol compound, inhibited a mast cell-dependent anaphylactic reaction by decreased calcium uptake and an increased cAMP level (29). Flavonol inhibits IgE-mediated proinflammatory mediator release from human umbilical cord blood-derived mast cells, possibly due to inhibition of intracellular Ca^{2+} influx and PKC θ signaling (30). The increasing concentration of intracellular Ca^{2+} in mast cells is an important factor for histamine release. It is deemed that calcium influx followed by activation of PLC γ and PKC would be attenuated in resveratrol-treated sensitized RBL-2H3 and mice. Furthermore, dietary flavonoid morin inhibits Fyn kinase but not Lyn and Syk in mast cells and IgE-mediated type I hypersensitivity response in mice (31). Unfortunately, this study did not examine the

upstream kinases of Syk. Procyanidin inhibits the generation of intracellular reactive oxygen species (ROS) of mast cells stimulated by Fc ϵ RI cross-linking (28). Thus, one can speculate the involvement of intracellular ROS in the inhibitory mechanisms of resveratrol underlying mast cell degranulation. Because resveratrol is known to be an antioxidant, its antioxidant activity may inhibit tyrosine phosphorylation of Syk and subsequent tyrosine phosphorylation of ROS-sensitive proteins in stimulated mast cells (32). Unfortunately, this study did not investigate the ROS production in sensitized mast cells and mice.

In summary, this study investigated the beneficial effects of the dietary compound resveratrol on allergic responses and PCA. Resveratrol inhibited the antigen-stimulated release of β -hexosaminidase and histamine in IgE-sensitized mast cells, which appeared to be associated with the downregulation of the Fc ϵ RI α -Syk-PLC γ -signaling cascade. The resveratrol inhibition of mast cell degranulation may be mediated via blocking of the PKC isomer-responsive mechanism(s). In addition, the resveratrol administration alleviated the tissue swelling and PCA in sensitized mice challenged with antigen. Antigen-induced inflammatory cell infiltration by MCP-1 and MIP-2 was also attenuated by treating sensitized mice with resveratrol. Therefore, resveratrol is effective in ameliorating allergic and inflammatory diseases.

Acknowledgments

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had primary responsibility for final content. All authors read and approved the final manuscript.

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