

Resveratrol Suppresses Cytokine Production Linked to FcεRI-MAPK Activation in IgE-Antigen Complex-Exposed Basophilic Mast Cells and Mice

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Abstract: A complicated interplay between resident mast cells and other recruited inflammatory cells contributes to the development and progression of allergic inflammation entailing the promotion of T helper 2 (Th2) cytokine responses. The current study examined whether resveratrol suppressed the production of inflammatory Th2 cytokines in cultured rat basophilic leukemia RBL-2H3 cells. Cells pre-treated with resveratrol nontoxic at 1–25 μM were sensitized with anti-dinitrophenyl (anti-DNP), and subsequently stimulated by dinitrophenyl-human serum albumin (DNP-HSA) antigen. Resveratrol dose-dependently diminished the secretion of interleukin (IL)-3, IL-4, IL-13 as well as tumor necrosis factor (TNF)-α by the antigen stimulation from sensitized cells. It was found that resveratrol mitigated the phosphorylation of p38 MAPK, ERK, and JNK elevated in mast cells exposed to Fc epsilon receptor I (FcεRI)-mediated immunoglobulin E (IgE)-antigen complex. The FcεRI aggregation was highly enhanced on the surface of mast cells following the HSA stimulation, which was retarded by treatment with 1–25 μM resveratrol. The IgE-receptor engagement rapidly induced tyrosine phosphorylation of c-Src-related focal adhesion protein paxillin involved in the cytoskeleton rearrangement. The FcεRI-mediated rapid activation of c-Src and paxillin was attenuated in a dose-dependent manner. In addition, the paxillin activation entailed p38 MAPK and ERK-responsive signaling, but the JNK activation was less involved. Consistently, oral administration of resveratrol reduced the tissue level of phosphorylated paxillin in the dorsal skin of DNP-HSA-challenged mice. The other tyrosine kinase Tyk2-STAT1 signaling was activated in the dorsal epidermis of antigen-exposed

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mice, which was associated with allergic inflammation. These results showed that resveratrol inhibited Th2 cytokines- and paxillin-linked allergic responses dependent upon MAPK signaling. Therefore, resveratrol may possess the therapeutic potential of targeting mast cells in preventing the development of allergic inflammation.

Keywords: Allergic Inflammation; Cytokine Secretion; Mast Cells; FcεRI; MAPK; Resveratrol.

Introduction

Allergic inflammation is characterized by pathophysiological dysfunctions and abnormalities leading to allergic disorders including asthma, atopic dermatitis, and rhinitis (Leung, 1998; Fireman, 2003; Hansen *et al.*, 2004). Allergic inflammation is often classified into three temporal phases, early-phase reaction, late-phase reaction, and chronic inflammation (Galli *et al.*, 2008). Persistent or repetitive exposure to allergens, which are typically innocuous substances common in the environment, causes chronic allergic inflammation and in turn generates substantial long-term changes in the organ structure and in their function (Schroeder *et al.*, 1995; Galli *et al.*, 2008). Allergic inflammation entails a complicated interplay between resident and recruited inflammatory cells such as mast cells, basophils, lymphocytes, dendritic cells, eosinophils, T helper 2 (Th2) cells, and neutrophils (Barnes, 2011). These cells produce inflammatory mediators of lipids, purines, cytokines, chemokines, and reactive oxygen species (White, 1999; Barnes, 2011). There are several endogenous anti-inflammatory mechanisms by the potent actions of lipoxins, resolvins, and protectins, which may be defective in allergic disease potentiating the inflammation (Serhan, 2007; Ariel and Timor, 2013). However, better understanding of the pathophysiology of allergic inflammation has identified therapeutic targets, while developing effective novel therapies has been challenging (Harvima *et al.*, 2014).

Mast cells can contribute to multiple features of chronic as well as acute, allergic inflammation. Allergic disorders are known to involve allergen-specific immunoglobulin E (IgE) and Th2 cells that recognize allergen-derived antigens (von Bubnoff *et al.*, 2003; Sokol *et al.*, 2008; Barnes, 2011). In early-phase hypersensitivity reactions that can occur within minutes of allergen exposure, IgE bound to Fc epsilon receptor I (FcεRI) on mast cells and basophils is cross-linked by the allergen, resulting in the cellular release of diverse preformed and newly synthesized mediators (White, 1999; Barnes, 2011). During the early-phase reaction, the allergen exposure triggers the release of cytokines of interleukin (IL)-3, IL-4, IL-9, and IL-13 that cause immediate hypersensitivity (Amin, 2012). Some of the released mediators can promote the local recruitment and activation of leukocytes (White, 1999). The mediators include histamine, serine esterases and prostaglandins, and mast cells produce various cytokines, chemokines and leukotrienes (Siraganian, 2003; Moon *et al.*, 2014). These cytokines play important roles in the infiltration of inflammatory cells and induction of the late-phase reaction (Wynn, 2003; Maezawa *et al.*, 2004). Th2- and mast cell-derived cytokines of IL-3, IL-5, and granulocyte

macrophage colony-stimulating factor stimulate eosinophil activation and leukocyte recruitment to the site of allergen exposure (Amin, 2012).

Suitable activation and fine tuning of mast cell functions are mediated by a complex array of factors including IgE receptors, tyrosine kinases, phosphatases, adaptors, and co-stimulatory molecules (Gilfillan and Tkaczyk, 2006). These molecules may be responsible for the outcome of mast cell-associated pathologies of degranulation, cytokine release, and cytoskeleton rearrangement (Liu *et al.*, 2007). However, the regulatory mechanisms for cytokine genes and mast cells in inducing allergic inflammation are not clearly defined. There is strong evidence that mast cells can substantially influence features of chronic allergic inflammation and tissue remodeling by manipulating several effector cell mechanisms that are dependent or independent of IgE (Galli *et al.*, 2008; Stone *et al.*, 2010). Many new pharmacological agents that target the various steps in the effector cell and mediator pathways implicated in allergic inflammation are being investigated (Barnes, 2006; Holgate and Polosa, 2008). Some of these compounds are designed to exploit molecular mechanisms to suppress effector cell activation during allergic inflammation or to take advantage of other mechanisms that can negatively regulate FcεRI-dependent signaling, one of which activates phospholipase Cγ (PLCγ) and the other of which activates phosphatidylinositol 3-kinase (PI3K) (Gilfillan and Tkaczyk, 2006; Kraft and Kinet, 2007).

There are various approaches employing natural compounds potentially effective as anti-inflammatory agents to manage disorders associated with allergic inflammation (Huang *et al.*, 2008; Naik and Wala, 2013). For instance, the administration of *Gynostemma pentaphyllum* extracts inhibited airway inflammation responses in ovalbumin (OVA)-sensitive mice through the suppression of Th2 activity (Huang *et al.*, 2008). Luteolin exerts a regulatory effect on mast cell activation leading to inflammatory diseases and allergy (Kritas *et al.*, 2013). Resveratrol (Fig. 1A) is a polyphenol that is richly found in the skin of red grapes and is a constituent of red wine. This compound possesses cardioprotective actions through stimulation of nitric oxide production as well as anti-oxidative effects (Nabavi *et al.*, 2014). Recently, our previous investigation revealed that resveratrol inhibited IgE-mediated mast cell degranulation and passive cutaneous anaphylaxis in mice (Han *et al.*, 2013). This study tested whether resveratrol modulated IgE-mediated cytokine (tumor necrosis factor (TNF)-α, IL-3, IL-4, and IL-13) production and allergic responses following the FcεRI stimulation of IgE-sensitized rat basophilic mast cells. Furthermore, the inhibition of allergic inflammation by resveratrol was examined in dinitrophenyl-human serum albumin (DNP-HSA) antigen-exposed mice.

Materials and Methods

Materials

Eagle's minimum essential medium (EMEM) was provided by Gibco Life Technologies (Carlsbad, CA) and culture reagents were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA

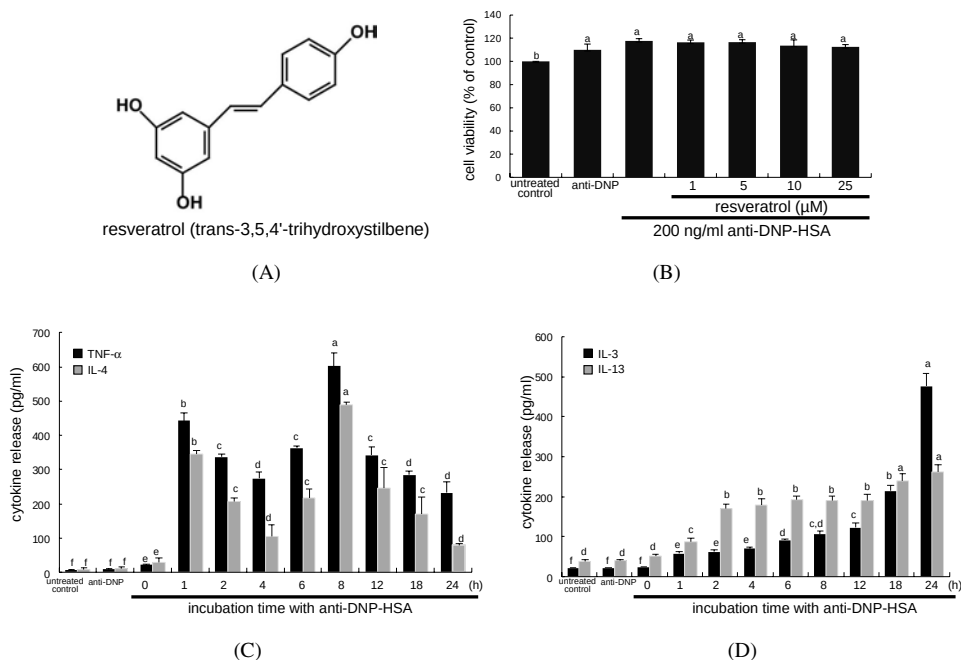


Figure 1. Chemical structure of resveratrol (A), and DNP-HSA cytotoxicity (B) and temporal responses of cytokines by DNP-HSA (C) and (D) in rat basophilic leukemic RBL-2H3 cells sensitized with anti-DNP. After the treatment with 200 ng/ml DNP-HSA for up to 24 h in 0.5 μg/ml anti-DNP-sensitized RBL-2H3 cells, the cell viability was determined using a MTT assay (B). Cell viability is presented as mean ± SEM ($n = 4$) with multiple estimations. The release of TNF-α and Th2 cytokines was measured using commercial cytokine kits (C) and (D). The ELISA data represent mean ± SEM from 3 independent experiments with multiple estimations. Values not sharing a small alphabetical letter refer to significant different at $p < 0.05$.

were provided from Lonza (Walkersville, MD). Rat basophilic leukemic RBL-2H3 mast cells were provided by American Type Culture Collection (Manassas, VA). Mouse monoclonal anti-DNP and DNP-HSA were purchased from Sigma-Aldrich Chemicals. Antibodies against phospho-p38 mitogen-activated protein kinase (p38 MAPK, Thr180/Tyr182), phospho-extracellular signal-regulated kinase (ERK, phospho-p44/42 MAPK, Thr202/Tyr204), and phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK, Thr183/Tyr185) were all provided by Cell Signaling Technology (Beverly, MA). Anti-FcεRIγ was purchased from Abcam Company (Cambridge, MA) and anti-FcεRIα obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat antirabbit IgG, goat antimouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). p38 MAPK inhibitor SB203580, JNK inhibitor SP600125 and ERK inhibitor PD 98059 were all purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

Cell Culture

RBL-2H3 mast cells were cultured in EMEM containing 10% heat inactivated FBS, 2 mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C humidified atmospheres of 5% CO₂ in air. RBL-2H3 cells were sensitized with 0.5 µg/ml anti-DNP overnight to induce allergic responses. RBL-2H3 cells were sensitized with 0.5 µg/ml anti-DNP overnight, washed twice in piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (0.4 mM MgCl₂, 40 mM NaOH, 119 mM NaCl, 1 mM CaCl₂, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1% BSA, pH 7.4) and pretreated with 1–25 µM resveratrol for 2 h. Subsequently, cells were exposed to 200 ng/ml DNP–HSA for various times indicated. Resveratrol at 1–25 µM was not cytotoxic in mast cells sensitized with anti-DNP-IgE and exposed to DNP–HSA antigen (Fig. 1B).

Enzyme-Linked Immunosorbent Assay (ELISA)

After washing in PIPES buffer, cells were pre-treated with 1–25 µM resveratrol for 2 h and exposed to DNP–HSA. At the end of the experiments, culture media were collected, centrifuged at 800× *g* for 10 min and resulting supernatants were collected. Supernatants were assayed for secretion of TNF-α, IL-3, IL-4, and IL-13 was measured using ELISA kits of TNF-α and IL-4 (R&D Systems, Minneapolis, MN), IL-3 (Abcam Company), and IL-13 (EIAab, Wuhan, China) according to the manufacturer's instructions. The absorbance was measured with a microplate reader.

Protein Isolation and Western Blot Analysis

Western blot analysis was performed using whole cell lysates were prepared from RBL-2H3 cells at a density of 5.0×10^5 cells. Whole cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with 1 M Tris-HCl (pH 6.8) lysis buffer containing 10% SDS, 1% β-mercaptoethanol, 1% β-glycerophosphate, 0.1 M Na₃VO₄, 0.5 M NaF, and protease inhibitor cocktail. Cell lysates containing equal amounts of total proteins were electrophoresed on 8–10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in TBS-T buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 3 h. The membrane was incubated overnight at 4°C with a primary antibody against phospho-p38 MAPK, phospho-ERK, phospho-JNK, Src, phospho-Src, paxillin, phospho-paxillin, phospho-tyrosine kinase 2 (Tyk2), or phospho-signal transducers and activators of transcription 1 (STAT1). After three washes with TBS-T buffer, the membrane was incubated with a goat anti-rabbit IgG-conjugated to horseradish peroxidase. The proteins were determined using Immobilon Western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA) and AGFA medical X-ray film (Gevaert, Belgium). Incubation with β-actin was performed for the comparative control.

Immunoprecipitation

At the end of the culture experiments with RBL-2H3 cells, collected cells were assayed for the FcεRI induction using an immunoprecipitation kit (Thermo Scientific, Waltham, MA), according to the manufacturer's instruction.

Immunocytochemical Staining of FcεRIγ

RBL-2H3 cells grown on glass coverslips were fixed with 20% formaldehyde for 20 min and permeated with 0.1% triton-X 100 and 0.1% sodium citrate for 2 min on ice. After blocking non-specific binding with 4% FBS for 1 h, FcεRIγ antibody was added and incubated overnight at 4°C. Subsequently, the cells were incubated with antirabbit IgG-conjugated Cy3. Nuclear counter-staining was also conducted by using 4 mg/ml 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were taken with an Axiomager Optical fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Murine Animal Model

Five week-old male BALB/c mice (Hallym University Breeding Center for Laboratory Animals) were kept on a 12 h light/12 h dark cycle at $23 \pm 1^\circ\text{C}$ with $50 \pm 10\%$ relative humidity under specific pathogen-free conditions. Mice were fed a chow diet that include 20.5% protein, 3.5% fat, 8% fiber, 8% ash, 0.5% calcium, 0.5% phosphorus (RodFeed™, DBL, Umsung, Korea) and were provided with water *ad libitum* at the Animal Facility of Hallym University. Mice were allowed to acclimatize for 1 week before beginning allergic experiments. Mice were divided into four subgroups (9 mice for each subgroup) as follows: Untreated control group (not sensitized), anti-DNP-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 were previously described (Han *et al.*, 2013). 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 dorsal skin (5 μg in 100 μl). Resveratrol solution (10 mg/kg in 100 μl) was orally administered to IgE-sensitized mice 1 h before DNP-HSA antigen challenge. For the challenge with DNP-HSA, mice were intravenously injected with a 250 μg DNP-HSA in 200 μl PBS into the mouse tail vein (0.3 cc syringe, 31G needle). Control mice were sensitized and challenged with saline as a DNP-HSA vehicle.

Plasma levels of TNF-α and IL-4 were measured using ELISA kits of TNF-α and IL-4, according to the manufacturer's instructions. The absorbance was measured with a microplate reader.

Immunohistochemical Staining of Dorsal Skin

For the immunofluorohistochemical analysis, paraffin-embedded tissue blocks were cut into 5 μ M sections in thickness. The sections were placed on glass slides, deparaffinated, hydrated with xylene and alcohol, and antigen was retrieved with boiling sodium citrate buffer. Tissue sections were incubated with a primary phospho-paxillin antibody or CD11b antibody overnight at 4°C. The sections were further incubated with antirabbit IgG-conjugated Cy3 and with DAPI for nuclear staining, or with antirabbit IgG with 3,3'-diaminobenzidine (DAB). The images of stained tissue sections on slide were taken using a fluorescence microscope system (Carl Zeiss).

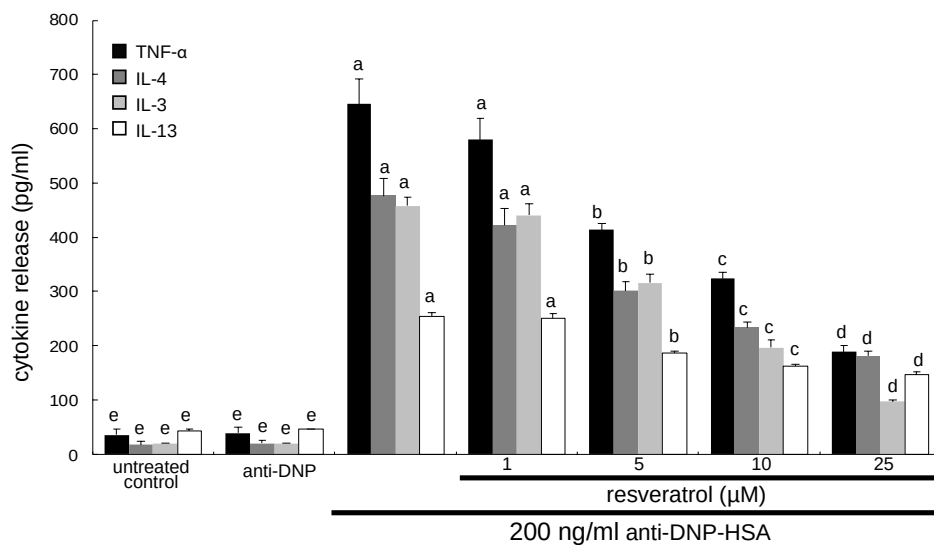
Data Analysis

The results are presented as mean $\pm$ SEM for each treatment group. Statistical analyses were conducted using Statistical Analysis Systems (SAS Institute, Cary, NC). Significance was determined by one-way ANOVA followed by Duncan range test for multiple comparisons and were considered significant at $p < 0.05$.

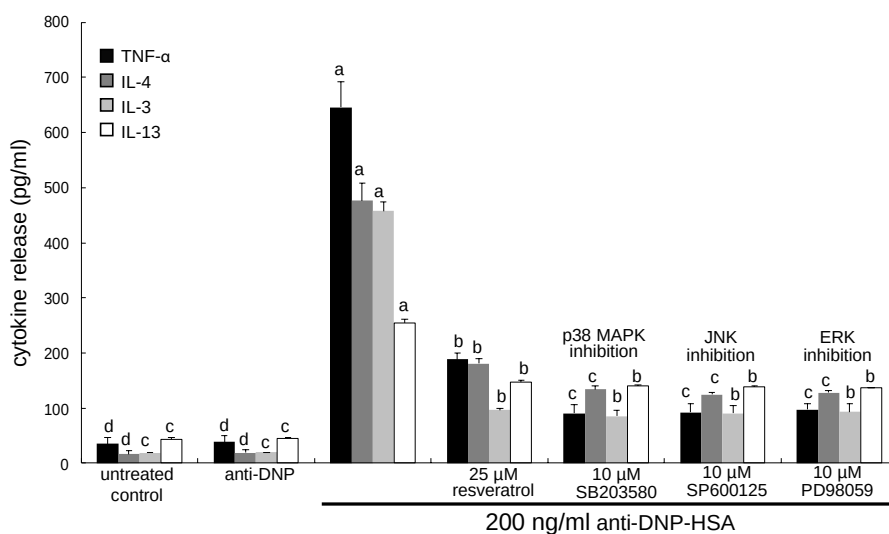
Results

Inhibition of Cytokine Production by Resveratrol

Cytokines secreted from immune cells including mast cells, basophils, T cells, and dendritic cells play an essential role in the late-phase allergic responses (Harvima *et al.*, 2014; Moon *et al.*, 2014). When basophilic mast cells were sensitized with anti-DNP, the release of TNF- α and IL-4 was rapidly enhanced 1 h after the stimulation of DNP-HSA antigen, evidenced by ELISA (Fig. 1C). The release of these two cytokines was repeatedly enhanced at 8 h-exposure to DNP-HSA and thereafter gradually diminished. On the other hand, the secretion of the Th2 cytokines of IL-3 and IL-13 continuously increased up to 24 h after DNP-HSA treatment (Fig. 1D). The present study aimed to test whether resveratrol suppressed the secretion of these cytokines in the allergic model using rat basophilic leukemic RBL-2H3 cells. Resveratrol nontoxic at 1–25 μ M dose-dependently reduced the secretion of all the cytokines of TNF- α , IL-4, IL-3, and IL-13 from anti-DNP-sensitized and DNP-HSA-stimulated cells (Fig. 2A). It should be noted that anti-DNP *per se* did not stimulate the release of these cytokines (Fig. 2A). This study also attempted to explore the involvement of MAPK activation in the secretion of TNF- α and Th2 cytokines induced by DNP-HSA antigen. As with 25 μ M resveratrol, the selective MAPK inhibitors of SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), PD98059 (ERK inhibitor) highly suppressed the release of these cytokines with similar extents of inhibition. Accordingly, the antigen-triggered release of these cytokines may entail the MAPK activation of p38 MAPK, JNK, and ERK1/2, which are possibly responsible for allergic responses.



(A)



(B)

Figure 2. Inhibition of release of TNF- α and Th2 cytokines by resveratrol (A) and MAPK inhibitors (B) in anti-DNP-sensitized rat basophilic leukemic RBL-2H3 cells stimulated with DNP-HSA. Cells were sensitized with 0.5 μ g/ml anti DNP and stimulated with 200 ng/ml of DNP-HSA for 8 h (for TNF- α and IL-4) or 24 h (for IL-3 and IL-13) in the absence and presence of 1–25 μ M resveratrol or 10 μ M of each inhibitor. The release of these cytokine was measured by ELISA analyses. The ELISA data represent mean $\pm$ SEM from 3 independent experiments with multiple estimations. Values in bar graphs not sharing a small alphabetical letter refer to significant different at $p < 0.05$.

Inhibitory Effect of Resveratrol on MAPK Activation

Resveratrol may limit release or production of allergic mediators via disturbing specific MAPK-associated mechanisms. The present study investigated whether resveratrol inhibited the phosphorylation of p38 MAPK, ERK1/2, and JNK in sensitized RBL-2H3 basophilic cells stimulated with DNP-HSA, as evidenced by Western blot analysis. The antigen stimulation instigated the activation of p38 MAPK and ERK1/2 very rapidly within 5 min and the JNK phosphorylation was markedly increased at 30 min post-stimulation (Fig. 3A). When 1–25 μ M resveratrol was added to sensitized and DNP-HSA-stimulated RBL-2H3 cells, the marked activation of three MAPK of p38 MAPK, ERK1/2, and JNK was all diminished in a dose-dependent manner (Fig. 3B). Thus, it is assumed that resveratrol dampened the antigen-triggered release of TNF- α and Th2 cytokines by interrupting MAPK activation.

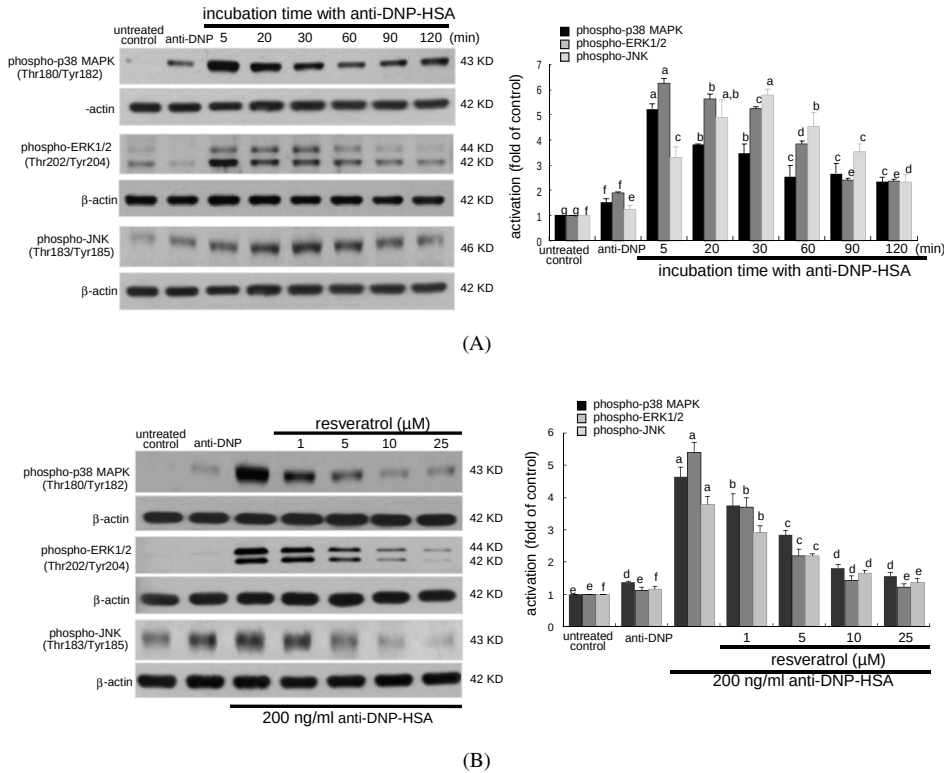


Figure 3. Western blot data showing time course response of MAPK phosphorylation (A) and inhibition of their activation by resveratrol (B) in IgE-sensitized rat basophilic leukemic RBL-2H3 cells. Cells were sensitized with 0.5 μ g/ml anti-DNP, and stimulated with 200 ng/ml of DNP-HSA up to 120 min with and without 1–25 μ M resveratrol for 120 min. Equal amounts of cell lysate proteins were subject to 12% SDS-PAGE and Western blot analysis with a primary antibody against phospho-p38 MAPK, phospho-ERK1/2, and phospho-JNK (3 separate experiments). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM) represent quantitative densitometric results of left bands. Means without a common small alphabetical letter differ, $p < 0.05$.

Blockade of FcεRI Induction by Resveratrol

The high-affinity IgE receptor of FcεRI that is expressed on the surface of mast cells and basophils has a central role in immediate allergic responses. Cross-linking of the high-affinity IgE receptor of FcεRI via IgE-antigen complexes evokes the degranulation of mast cells or basophils and the release of inflammatory mediators (Siraganian, 2003; Prussin and Metcalfe, 2006). FcεRI is a tetrameric receptor complex consisting of the antibody binding site of FcεRIα, and FcεRIβ and FcεRIγ involved in the downstream signal (von Bubnoff *et al.*, 2003). This study examined that resveratrol blocked the FcεRIγ induction by DNP-HSA antigen leading to the release of Th2 cytokines. As shown in Fig. 4A, the cellular level of FcεRIγ subunit was very rapidly elevated at 2 min following the DNP-HSA stimulation of RBL-2H3 cells. Resveratrol at the doses of 1–25 μM dose-dependently diminished the elevated expression of FcεRIγ subunit in RBL-2H3 cells

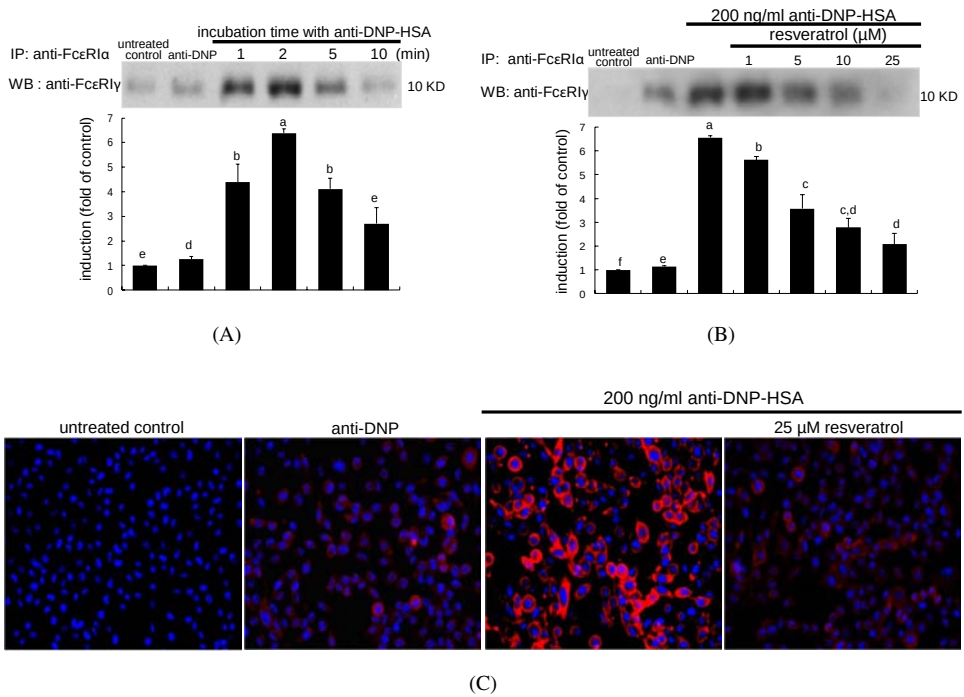


Figure 4. Western blot analyses showing time course responses of FcεRIγ expression (A) and its inhibition by resveratrol (B and C) in anti-DNP-sensitized rat basophilic leukemic RBL-2H3 cells. Cells were sensitized with 0.5 μg/ml anti-DNP and stimulated with 200 ng/ml of DNP-HSA in the absence and presence of 1–25 μM resveratrol for 10 min. Cell lysates were immunoprecipitated with FcεRIα and subject to 12% SDS-PAGE and Western blot analysis with a primary antibody against FcεRIγ (3 separate experiments). The bar graphs (mean ± SEM) represent quantitative densitometric results of upper bands. Means without a common small alphabetical letter differ, $p < 0.05$. Immunofluorescence analyses showing inhibition of FcεRIγ expression by resveratrol in IgE-sensitized rat basophilic leukemic RBL2H3 cells (C). FcεRIγ identified as red staining was visualized with a Cy3-conjugated secondary antibody and nuclear staining was done with DAPI. Magnification: 200-fold.

exposed to DNP-HSA for 2 min (Fig. 4B). Resveratrol may block the initiation of the downstream signal due to cross-linking of the FcεRIα via IgE-antigen complexes.

Consistent with western blot data, the immunocytochemical data revealed that the DNP-HSA stimulation highly caused FcεRIγ induction on the surface of cells, as manifested by the reddish staining with an immunofluorescent Cy3-conjugated anti-FcεRIγ (Fig. 4C). When 25 μM resveratrol was treated to sensitized and DNP-HSA-stimulated RBL-2H3 cells, the reddish staining was strikingly diminished (Fig. 4C).

Suppressive Effect of Resveratrol on c-Src-Paxillin Activation

This study tested whether the c-Src kinase activation was induced in sensitized RBL-2H3 cells in response to FcεRI aggregation and this induction was inhibited by resveratrol. The c-Src phosphorylation was highly and rapidly elevated within 5 min following the stimulation of DNP-HSA antigen, and the activation was substantially sustained up to 2 h (Fig. 5A). In contrast, the c-Src phosphorylation was allayed in a dose-dependent manner by adding resveratrol to anti-DNP-sensitized mast cells (Fig. 5B). These data strongly

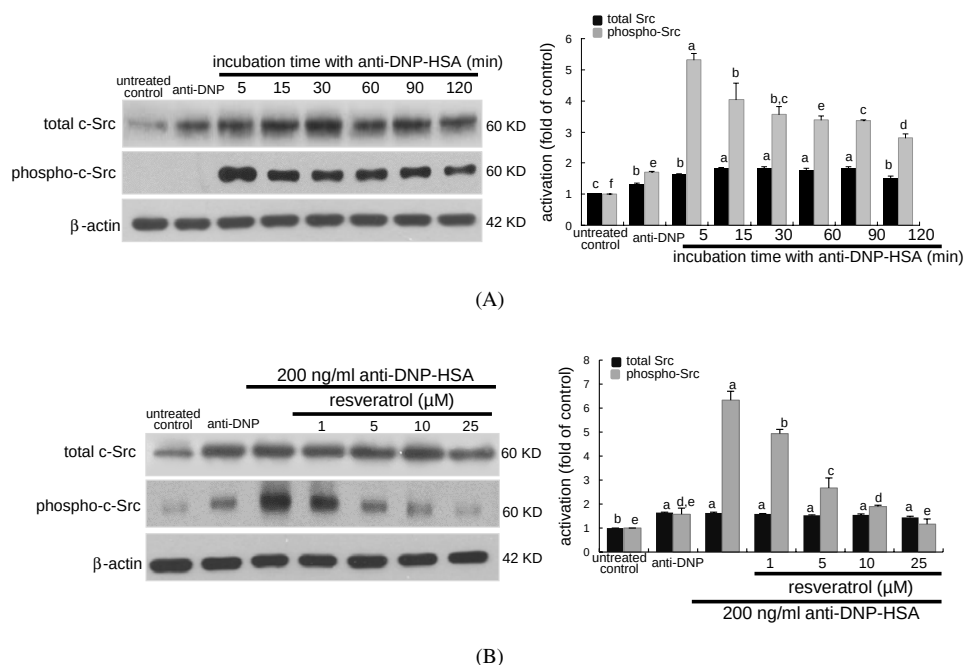


Figure 5. Time course response of Src phosphorylation (A) and inhibition of its activation by resveratrol (B) in anti-DNP-sensitized rat basophilic leukemic RBL-2H3 cells. Cells were stimulated with 200 ng/ml of DNP-HSA for 1 h with and without 1–25 μM resveratrol for 120 min in sensitized cells with 0.5 μg/ml anti-DNP. Equal amounts of cell lysate proteins were subject to 12% SDS-PAGE and Western blot analysis with a primary antibody against total Src and phospho-Src (3 separate experiments). β-Actin protein was used as an internal control. The bar graphs (mean ± SEM, $n = 3$) represent quantitative densitometric results of left bands. Means without a common small alphabetical letter differ, $p < 0.05$.

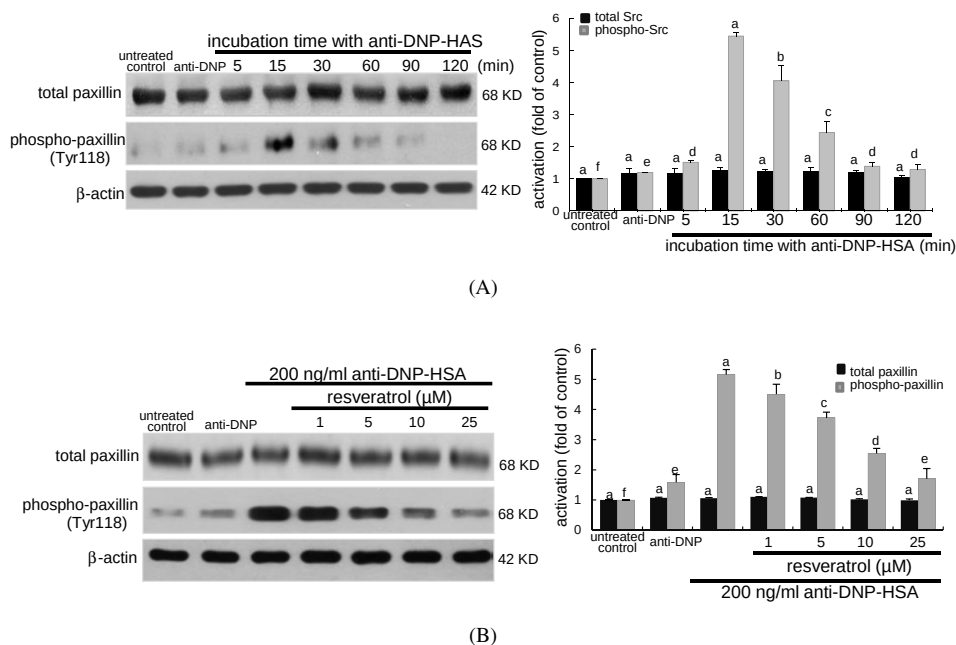


Figure 6. Temporal response of paxillin activation (A) and inhibition of its phosphorylation by resveratrol (B) in anti-DNP-sensitized rat basophilic leukemic RBL-2H3 cells. Cells were stimulated with 200 ng/ml of DNP-HSA for 1 h in the absence and presence of 1–25 μ M resveratrol for 120 min in sensitized cells with 0.5 μ g/ml anti-DNP. Equal amounts of cell lysate proteins were subject to 12% SDS-PAGE and Western blot analysis with a primary antibody against total paxillin and phospho-paxillin (3 separate experiments). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM) represent quantitative densitometric results of left bands. Means without a common small alphabetical letter differ, $p < 0.05$.

indicate resveratrol may regulate allergic responses in antigen-exposed mast cells via c-Src activation.

It has been reported that the focal adhesion protein paxillin associated with clustered IgE-Fc ϵ RI has a positive effect on Fc ϵ RI signaling (Michael and Schaller, 2001; Torres *et al.*, 2008). Paxillin can be tyrosine-phosphorylated by focal adhesion kinase and Src upon integrin engagement (Deakin and Turner, 2008). The tyrosine phosphorylation of paxillin was rapidly induced within 15 min following the antigen stimulation in RBL-2H3 cells (Fig. 6A). When resveratrol was treated with antigen-stimulated mast cells, the paxillin phosphorylation was dose-dependently attenuated (Fig. 6B). This study further examined that the MAPK signaling was responsible for DNP-HSA antigen-promoted activation of paxillin generally involved in the actin cytoskeleton rearrangement. The inhibition of p38 MAPK and ERK dampened the paxillin activation, while the JNK activation was less involved (Fig. 7A). Accordingly, the antigen-triggered paxillin signaling may entail the MAPK activation of p38 MAPK and ERK1/2 possibly responsible for the allergic responses of cytoskeleton reorganization. Furthermore, the immunohistochemical data showed that a noticeable increase in the paxillin activation (pinkish staining) was

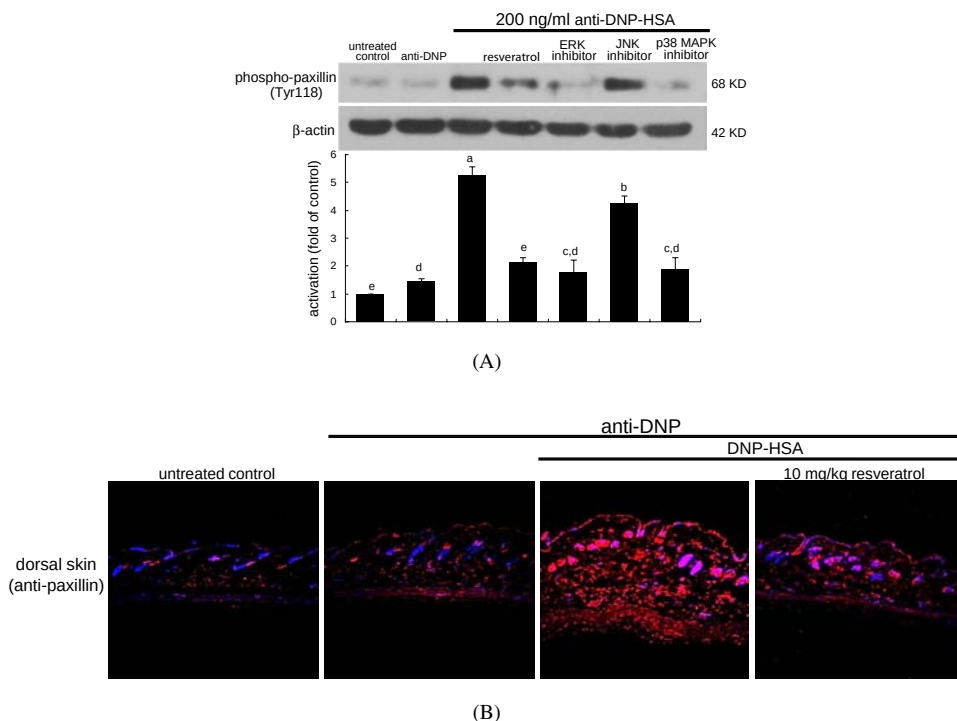


Figure 7. Modulation of paxillin activation by resveratrol and MAPK inhibition in IgE-sensitized rat basophilic leukemic RBL-2H3 cells (A) and inhibition of paxillin activation by resveratrol in sensitized mice challenged with DNP-HSA (B). Cells were stimulated with 200 ng/ml of DNP-HSA for 1 h in the absence and presence of 25 μ M resveratrol for 120 min in sensitized cells with 0.5 μ g/ml anti-DNP. In the murine allergic model, the antigen DNP-HSA was applied to the mouse dorsal skin (see Materials and Methods). Equal amounts of cell lysate proteins were subject to 12% SDS-PAGE and Western blot analysis with a primary antibody against phospho-paxillin (3 separate experiments). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM) represent quantitative densitometric results of upper bands. Values not sharing a small alphabetical letter refer to significant different at $p < 0.05$. In immunohistochemical analyses phospho-paxillin identified as red staining was visualized with a Cy3-conjugated secondary antibody and nuclear staining was done with DAPI. Each photograph is representative of 4 mice. Magnification: 200-fold.

observed in DNP-HSA-challenged mouse dorsal skin compared with mice challenged with only anti-DNP (Fig. 7B). In marked contrast, the paxillin activation in antigen-exposed mice supplemented with 10 mg/kg resveratrol declined, almost similar to that of anti-DNP-alone mice (Fig. 7B). It should be noted that the DNP-HSA challenge increased epidermal thickening in the anti-DNP-sensitized and DNP-HSA antigen-exposed mice, which was diminished by the oral treatment with resveratrol (Fig. 7B).

Attenuation of Tyk2-STAT1 Activation and Allergic Inflammation by Resveratrol

Tyk2 has been reported to be involved in the regulation of allergic inflammation in the airways (Seto *et al.*, 2003). This study investigated that the Tyk2-STAT1 signaling was

activated in DNP-HSA-challenged mice. In antigen-exposed mice, the plasma levels of TNF- α and IL-4 were highly enhanced, while resveratrol attenuated the cytokine induction (Fig. 8A). Western blot data showed that the antigen challenge activated the Tyk2-STAT1 signaling in mouse dermal tissues (Fig. 8B). In contrast, the oral administration of 10 mg/kg resveratrol diminished the tissue levels of phosphorylated Tyk2 and STAT1. This study attempted to confirm that resveratrol alleviated the allergic inflammation in an

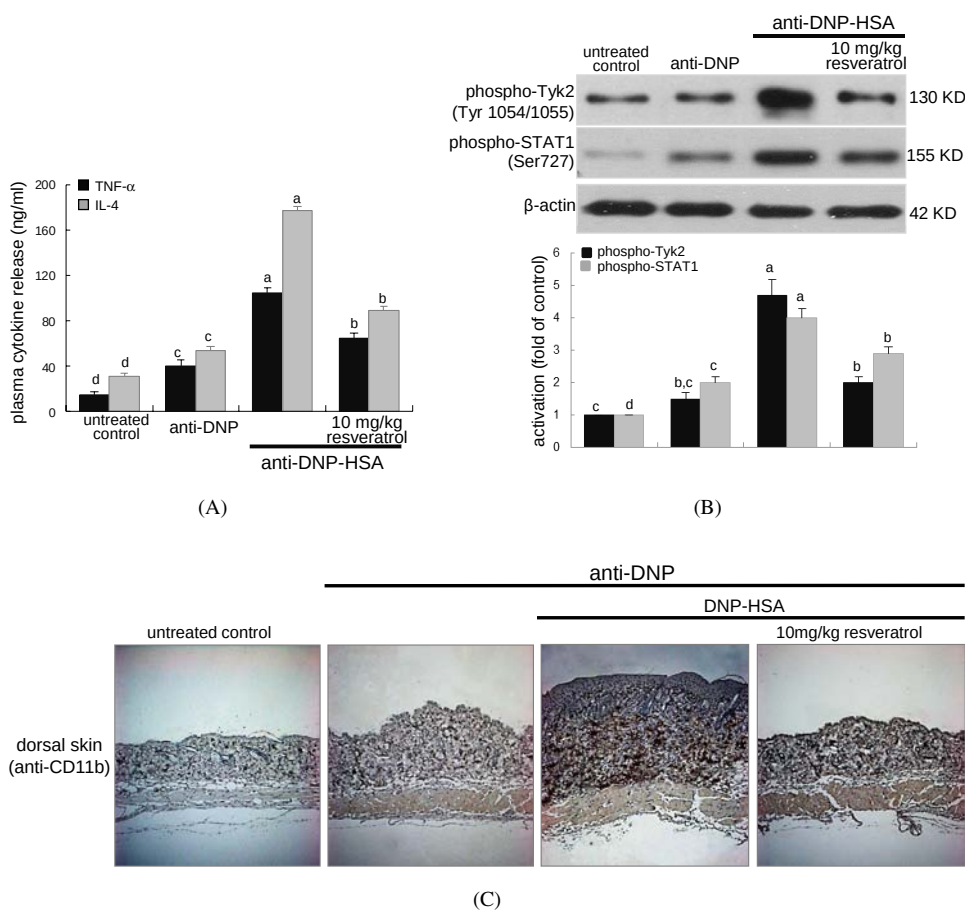


Figure 8. Reduction of plasma release of TNF- α and IL-4 (A), inhibition of Tyk2 and STAT1 activation (B) and dermal inflammation (C) by resveratrol in sensitized mice challenged with DNP-HSA. The antigen DNP-HSA was applied to the mouse dorsal skin for dermal inflammation (see Methods). The release of these cytokine was measured using ELISA analyses (A). The ELISA data represent mean $\pm$ SEM from 3 independent experiments with multiple estimations. Equal amounts of tissue proteins were subject to 10% SDS-PAGE and Western blot analysis with a primary antibody against phospho-Tyk2 and phospho-STAT1 (3 separate experiments). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM) represent quantitative densitometric results of upper bands. Values not sharing a small alphabetical letter refer to significant different at $p < 0.05$. Immunohistochemical analysis of mouse dorsal skin tissues was conducted by staining with 3,3'-diaminobenzidine (C). Each photograph is representative of 4 mice. Magnification: 200-fold.

antigen-challenged murine animal model. In mice, the CD11b (Mac-1 α ; integrin α M) chain antigen is expressed on monocytes/macrophages and microglia (Lehmann *et al.*, 2014). In the current study, the antigen stimulation enhanced the CD11b induction (dark brown spots) and such induction was diminished by the resveratrol treatment, as evidenced by DAB staining (Fig. 8C). Again, the epidermal thickening increased by the DNP-HSA challenge was restored to that of normal resveratrol-treated mice.

Discussion

This study observed 6 major findings. (i) The antigen DNP-HSA markedly enhanced the release of Th2 cytokines and TNF- α in IgE-sensitized RBL-2H3 cells, which was dose-dependently reduced by nontoxic resveratrol at 1–25 μ M. (ii) Resveratrol dampened the antigen-triggered release of these cytokines by disturbing p38 MAPK- and ERK1/2- and JNK-responsive signaling. (iii) The marked activation of these disparate MAPK were all diminished in sensitized and DNP-HSA-stimulated RBL-2H3 cells treated with resveratrol. (iv) The cellular expression of Fc ϵ RI γ subunit on the cell surface was up-regulated following the exposure of sensitized cells to DNP-HSA for 2 min, which was diminished by resveratrol. Resveratrol may block the initiation of the downstream signal of the Fc ϵ RI cross-linking. (v) The antigen-promoted phosphorylation of c-Src and paxillin was allayed by adding resveratrol to anti-DNP-sensitized mast cells, indicating that this compound may regulate allergic responses of mast cells via activation of p38 MAPK- and ERK1/2-dependent paxillin. (vi) When resveratrol was orally administrated to DNP-HSA-challenged mice displaying CD11b-linked allergic inflammation, the activation of paxillin, Tyk2 and STAT1 was inhibited in the mouse dorsal skin. Accordingly, resveratrol encumbered allergic responses of cytokine release and cytoskeleton reorganization by disturbing MAPK-paxillin-responsive and Tyk2-STAT1-responsive signaling pathways.

Allergic reactions are mediated by immunological mechanisms of excessive activation of mast cells and basophils by environmental substances known as allergens such as foods, dust, mites, and pollen (Matsuoka *et al.*, 2013; Naik and Wala, 2013). Antigens called as allergens induce the production of antigen-specific IgE that binds to high affinity-IgE receptor Fc ϵ RI on the surface of mast cells or basophils (Siraganian, 2003; von Bubnoff *et al.*, 2003; Stone *et al.*, 2010). Mast cells are involved in both immediate early phase regulated by the aggregation of Fc ϵ RI by antigens, and late-phase reaction concomitant with the infiltration of inflammatory cells (White, 1999; Barnes, 2011). Mast cell activation by Fc ϵ RI stimulation immediately releases preformed pro-inflammatory mediators through degranulation and results in later production of various cytokines and lipid mediators (White, 1999; Hansen *et al.*, 2004). These cells also express various other surface receptors that can lead to their activation, as well as inhibitory receptors, like the Fc γ RIIB, that dampen their activation (Shik and Munitz, 2010). Cytokines play important roles in the induction of inflammatory cells in the late-phase reaction. In the present study anti-DNP-sensitized basophilic mast cells highly produced Th2 cytokines of IL-3, IL-4, and IL-13 as well as TNF- α in a temporal response manner following the DNP-HSA antigen stimulation. The cytokines of IL-4 and TNF- α were initially released within 8 h,

while the release of IL-3 and IL-13 was continuously enhanced after the exposure of mast cells to antigen. However, the regulatory mechanisms for cytokine genes in mast cells are not fully defined.

The molecular and cellular mechanisms mediating the allergic inflammatory pathways involve multiple mediators, cell types, and pathways (Broide, 2001; Harvima *et al.*, 2014). In particular, IL-4 regulates differentiation of naïve Th0 cells to develop a Th2 phenotype and stimulates B cells to produce IgE. However, novel endogenous anti-inflammatory mechanisms by the potent actions of mediators have also been proposed (Serhan, 2007). A better understanding of the pathophysiologic mechanisms of allergic inflammation has led to the discovery of molecular therapeutic targets involved in the initial and late events of the inflammatory cascades. Potential targets for the development of novel therapies for allergic disease include IgE receptors, cytokines and signaling pathways involved in allergic responses. Many new pharmacological agents that target the various steps in the effector cell and mediator pathways implicated in allergic inflammation are being investigated (Barnes, 2006; Holgate and Polosa, 2008). Some of these agents are designed to exploit molecular mechanisms to inhibit effector cell activation during allergic inflammation (Gilfillan and Tkaczyk, 2006; Kraft and Kinet, 2007). Recently, it has been reported that numerous natural compounds potentially target against disorders associated with allergic inflammation (Huang *et al.*, 2008; Naik and Wala, 2013). In this study, resveratrol inhibited the antigen-triggered cytokine secretion in mast cells through disturbing the MAPK signaling of all three p38 MAPK, ERK1/2, and JNK.

Resveratrol, a polyphenol found richly in skin of red grapes and a constituent of red wine, exerts anticancer, anti-inflammatory, blood-sugar-lowering and other beneficial cardiovascular effects (Nabavi *et al.*, 2014). A recent study shows that resveratrol is detected inside cells and is taken up by vascular endothelial cells via both passive diffusion and a sodium-dependent glucose transporter 1-mediated process (Chen *et al.*, 2013). It has been shown that the anti-inflammatory effects of resveratrol appear to be associated with its immune-regulatory properties (Vial *et al.*, 2003; Xuzhu *et al.*, 2012; Han *et al.*, 2013). Accordingly, the inflammatory cells such as mast cells and basophils are ideal targets for controlling allergic reactions. Since resveratrol reduced the FcεRIγ induction on mast cells for the early phase response of IL-4 and TNF-α, it appeared to disrupt the cross-linking by DNP-HSA of IgE affixed to high-affinity FcεRI. Subsequently, this compound inhibited the release of cytokines of IL-3 and IL-13, which may contribute to the late phase responses and allergic inflammation. Nevertheless, how resveratrol disrupted the cross-linking needs to be taken into account.

Other signaling pathways of mast cell responses linked with the immunoreceptor FcεRI, presumably an exclusive determinant of the magnitude of the response to allergens, is still largely undefined. This study revealed that resveratrol inhibited the paxillin phosphorylation dependent of p38 MAPK and ERK1/2 signaling as well as the c-Src activation. Paxillin is tyrosine-phosphorylated by a nonreceptor protein tyrosine kinase such as focal adhesion kinase and c-Src (Deakin and Turner, 2008). Also, paxillin serves as a platform for the recruitment of numerous regulatory and structural proteins that together regulate the dynamic changes in cell adhesion, cytoskeletal reorganization and gene expression for cell

migration and survival (Deakin and Turner, 2008). Since the cytoskeleton rearrangement is one of the mast cell-associated pathologies (Liu *et al.*, 2007), resveratrol may allay paxillin-mediated cytoskeletal reorganization immediately after antigen stimulation. One investigation showed that the Fc ϵ RI-induced tyrosine phosphorylation of paxillin and MAPK proteins was independent of focal adhesion kinase (Vial *et al.*, 2003).

Cytokines responsible for the induction of the late-phase reaction regulate a variety of cell functions by signaling by their receptors linked to Janus kinases and STAT proteins (Ihle, 1995). Cytokine receptor signaling contributes to the diversity of cellular responses through tyrosine phosphorylation of signaling proteins recruited to the receptor complex (Kaiko *et al.*, 2008). It was shown that airway inflammation entailed modulating Tyk2-STAT1/3 signaling responsive to IL-8 in endotoxin-exposed airway epithelium and in asthmatic mice (Gong *et al.*, 2013). The present study showed that the DNP-HSA antigen increased the plasma levels of TNF- α and IL-4 in sensitized mice, which was inhibited by oral treatment of 10 mg/kg resveratrol. Concomitantly, resveratrol allayed epidermal thickening and inflammation associated with the CD11b induction in macrophages and reduced the tissue levels of Tyk2 and STAT1 in active forms. Accordingly, it can be assumed that the cytokines released by the antigen following Fc ϵ RI stimulation induced allergic inflammation in the epidermis through activating the Tyk2-STAT1 signaling downstream of their receptors. Consequently, the oral administration of resveratrol may dampen the migration and function of leukocytes in allergic inflammation by disturbing the receptor signaling of Tyk2-STAT1 of the cytokines released by antigen-exposed mast cells. Other investigation shows that resveratrol down-regulates interferon- γ -inducible inflammatory genes in macrophages by blocking the JAK/STAT-1 pathway (Chung *et al.*, 2011). In addition, resveratrol reduced the lipopolysaccharide-induced IL-8 production and pro-inflammatory response in THP-1 cells by blocking MAPK phosphorylation and NF- κ B activation (Oh *et al.*, 2009). These mechanistic actions of resveratrol would be involved in antagonizing DNP-HSA antigen-mediated allergic inflammation.

In summary, the current study investigated the preventive effects of resveratrol on allergic responses of cytokine secretion and inflammation in DNP-HSA antigen-challenged mast cells and mice. Resveratrol suppressed the release of Th2 cytokines as well as TNF- α via blockade of early signaling of MAPK-paxillin and later signaling of Tyk2-STAT1 following exposure of sensitized mast cells to DNP-HSA antigen. In addition to the inhibition of allergic inflammation, it was assumed that resveratrol may block the cytoskeleton rearrangement induced by the antigen stimulation through disturbing CD11b integrin-c-Src-paxillin signaling. Therefore, the dietary compound resveratrol is effective in ameliorating allergic and inflammatory diseases due to the exposure to antigens.

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