



Inhibitory Effect of Eriodictyol on IgE/Ag-Induced Type I Hypersensitivity

Jung-Min YOO, Ji-Hee KIM, Sae-Jin PARK, Yeo-Jin KANG, and Tack-Joong KIM[†]

Division of Biological Science and Technology, College of Science and Technology, Yonsei University, Wonju 220-710, Korea

Received December 12, 2011; Accepted March 26, 2012; Online Publication, July 7, 2012

[doi:10.1271/bbb.110952]

Mast cells are the principal effector cells involved in the allergic response, through the release of histamine. We investigated the effect of eriodictyol, derived from the painted maple and yerba santa, on mast cell degranulation and on an allergic response in an animal model. We also investigated its effect on the expression of the ceramide kinase (CERK) involved in calcium-dependent degranulation, and on ceramide activation by multiple cytokines. Eriodictyol suppressed the release of beta-hexosaminidase, a marker of degranulation, and the expression of interleukin (IL)-4 mRNA. It inhibited the expression of CERK mRNA, reduced the ceramide concentration in antigen-stimulated mast cells, and suppressed the passive cutaneous anaphylaxis (PCA) reaction in mice in a dose-dependent manner. These results suggest that eriodictyol can inhibit mast cell degranulation through inhibition of ceramide kinase, and that it might potentially serve as an anti-allergic agent.

Key words: mast cells; allergic response; eriodictyol; passive cutaneous anaphylaxis

Mast cells play important roles in immediate hypersensitivity reactions and allergic conditions that are associated with immunoglobulin E (IgE). They express the high affinity IgE receptor (FcεRI) on cell surfaces and are activated by aggregation of FcεRI when antigen binds the receptor-IgE complex.^{1,2} Activated mast cells release various preformed mediators, including histamine and proteases, from cytoplasmic granules in the process of degranulation.³⁻⁵ Degranulation occurs when antigen binding activates an FcεRI-associated protein kinase,^{6,7} and the associated signaling cascade increases the intracellular Ca²⁺ level.^{8,9} Thus control of degranulation and the allergic response might be achieved through control of a protein tyrosine kinase.

Intracellular Ca²⁺ is a key factor in the control of mast-cell degranulation. Ceramide kinase (CERK), which phosphorylates ceramide to ceramide 1-phosphate (C1P), is activated in a Ca²⁺-dependent manner, and CERK modulates Ca²⁺-dependent degranulation.^{10,11} Ceramide, a sphingolipid, is a pro-apoptotic factor activated by various cytokines, including tumor necrosis factor-α (TNF-α), IL-1β, and IL-6. C1P is involved in cell proliferation and the inflammatory response,^{12,13} in part by activating the arachidonic acid cascade to synthesize prostaglandins and leukotrienes.^{10,13} It is

thus plausible that inhibition of Ca²⁺-dependent CERK activation alleviates allergic response.

Eriodictyol is a unique constituent of the painted maple and yerba santa. Pharmacological activities attributed to eriodictyol include the control of blood vessel permeability in arthralgia and fracture, and antioxidant and antimicrobial effects,^{14,15} but its anti-allergenic activity has not been evaluated.

We investigated the anti-allergenic activity of eriodictyol in an animal model and in a mast cell line. Our study focused on the roles of sphingolipids such as ceramide, and on CERK, which activates ceramide, rather than on the protein tyrosine kinase activities involved in inflammation. We found that eriodictyol suppressed the CERK-mediated activation of ceramide, which in turn mediates Ca²⁺-dependent degranulation. Importantly, it inhibited degranulation and the release of inflammatory cytokines in mast cells and inhibited anaphylactic shock in an animal model. These findings justify further testing of eriodictyol as a potential anti-allergenic agent.

Materials and Methods

Reagents. Eriodictyol was purchased from Elcom Science (Seoul, Korea); mouse monoclonal anti-DNP IgE antibody, dinitrophenol-conjugated human serum albumin (DNP-HSA), and Evans blue from Sigma-Aldrich (St. Louis, MO); *N*-oleoyl-D-erythro-sphingosine (C₁₇ base) from Avanti Polar Lipids (Alabaster, AL); and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide from MP Biomedicals (Solon, OH).

Cell culture. The rat mast cell line RBL-2H3 was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in Eagle's minimal essential medium (WelGENE, Daegu, Korea) containing 10% (v/v) fetal bovine serum and 100 units/mL of penicillin-streptomycin (Lonza Walkersville, Walkersville, MD) at 37 °C in a humidified 5% CO₂ atmosphere. The cells were detached with trypsin-EDTA solution, washed with phosphate-buffered saline (PBS, pH 7.2), and resuspended in fresh medium for use in subsequent experiments.

Animals. Male BALB/C mice (aged 7 weeks) were purchased from Orient Bio (Gangneung, Korea) and housed in wire cages at 20–22 °C at a relative humidity of 40–50%. The animals had free access to a commercial pellet diet purchased from Samyang (Wonju, Korea) and drinking water. The Institutional Animal Care and Use Committee (IACUC) of Yonsei University approved the protocol for this study. The investigation conformed to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

[†] To whom correspondence should be addressed. Tel: +82-33-760-2242; Fax: +82-33-760-2183; E-mail: ktj@yonsei.ac.kr

Abbreviations: PCA, passive cutaneous anaphylaxis; CERK, ceramide kinase; IgE, immunoglobulin E; C1P, ceramide 1-phosphate; TNF-α, tumor necrosis factor-α

Passive cutaneous anaphylaxis (PCA) analysis. The experimental animals were divided into five groups: normal control (vehicle, $n = 5$), control (Ag stimulated group, $n = 5$), and low (10 mg/kg, $n = 5$), middle (30 mg/kg, $n = 5$) and high eriodictyol dose (50 mg/kg, $n = 5$). An anti-DNP-specific IgE antibody (0.5 μ g) was injected intradermally into one ear of each mouse. After 24 h, the mice were challenged with an intravenous injection of DNP-HSA (200 μ g; antigen, Ag) in 200 μ L of PBS 3% Evans blue. One h later eriodictyol was administered orally at a dose of 10 to 50 mg/kg. At 2 h after Ag challenge, the mice were euthanized and the treated ear was excised to measure the amount of dye that had extravasated in response to Ag. The dye was extracted from the ear in 500 μ L of formamide at 63 °C overnight and quantified by measuring the dye absorbance at 620 nm.

β -Hexosaminidase release assay. As a marker of degranulation, we measured the release of β -hexosaminidase. RBL-2H3 cells were distributed into 24-well plates (2×10^5 cells/well), and were sensitized overnight with anti-DNP-specific IgE at 200 ng/mL. The IgE-sensitized cells were washed twice with PIPES buffer (25 mM PIPES pH 7.2, 110 mM NaCl, 4 mM KCl, 0.4 mM MgCl₂, 40 mM HCl, 5.6 mM glucose, 1 mM CaCl₂, and 0.1% BSA), and then treated for 30 min at 37 °C with eriodictyol in PIPES buffer at the indicated concentrations. They were then stimulated with DNP-HSA (25 ng/mL) at 37 °C for 15 min and chilled on ice to put a stop to stimulation. To measure β -hexosaminidase release, the supernatant from the Ag-stimulated cells in PIPES buffer and β -hexosaminidase substrate (*p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) were mixed in 96-well plates and this was incubated at 37 °C for 1 h. The reaction was terminated by adding 0.1 M carbonate buffer (pH 10.5), and the absorbance at 405 nm was measured with a microplate reader.

Cell viability assay. Cell viability was assessed with a Ez-Cytox Enhanced Cell Viability Kit (Daeil Labservice, Seoul, Korea) following the manufacturer's instructions. In brief, RBL-2H3 cells (2×10^4 cells/well) were seeded into 96-well plates and incubated overnight with 200 ng/mL of anti-DNP-specific IgE. After incubation, the medium was replaced with serum-free medium, and the cells were treated with graded concentrations of eriodictyol and 25 ng/mL of DNP-HSA for 23 h. The Ez-Cytox Kit reagent was added to the medium, and the cells were incubated at 37 °C for 1 h. Cell viability was determined from the absorbance at 450 nm as measured with a microplate reader.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). RBL-2H3 cells (8×10^5 cells/well) were seeded into 6-well plates and incubated overnight in a medium containing 200 ng/mL of anti-DNP-specific IgE. They were washed twice, resuspended in PIPES buffer, and stimulated with DNP-HSA (25 ng/mL) for 1 h with and without eriodictyol. After incubation, they were washed twice with ice-cold PBS. Total RNA was isolated using a TRI reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. The concentration of total RNA was determined by spectrophotometer. Total RNA (1 μ g) was used as template for cDNA synthesis and PCR using an Accupower RT/PRC premix kit (Bioneer, Daejeon, Korea). The following primers were used: rat IL-4 sense 5'-ACCTTGCTGTCACCCTGTTC-3'; rat IL-4 antisense 5'-TTGTGAGCGTHACTCATTC-3'; rat TNF- α sense 5'-CAAGGAGGAGAAGTCCCAA-3'; TNF- α antisense 5'-CGGACTCCGTGATGTCTAAG-3'; rat CERK sense 5'-AGGAGACTTTATACGAGATCA-3'; rat CERK antisense 5'-GACTCGATAAAGCTTCAACGAA-3'; beta-actin sense 5'-ATGCC-ATCCTGCGTCTGGACCTGGC-3'; and beta-actin antisense 5'-AGC-ATTGCGGTGCACGATGGAGGG-3'. The denaturation, annealing, and extension conditions and cycle numbers were as follows: for IL-4, 94 °C for 1 min, 50 °C for 45 s, 72 °C for 45 s, at 35 cycles; for TNF- α , 94 °C for 1 min, 49 °C for 45 s, 72 °C for 45 s, at 35 cycles; and for CERK, 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, at 35 cycles. PCR products were electrophoresed in 2% agarose gel and visualized by adding ethidium bromide. The gels were examined using a transilluminator (Vilber Lourmat, France).

Measurement of ceramide. RBL-2H3 cells were washed twice with PBS and harvested. The cell pellet was solubilized with 0.2 N NaOH. Total lipid was extracted with ethanol and then incubated with C₁₇

ceramide (*N*-oleoyl-D-erythro-sphingosine) as internal standard for 1 h at 37 °C. The extract was centrifuged at 12,000 rpm for 10 min. The supernatant was dried on a centrifugal vacuum concentrator. The dried residue was dissolved in methanol, spotted on a high-performance thin-layer chromatography silica-gel plate (Merck, Darmstadt, Germany) and developed in isopropylether/methanol/29% NH₄OH (40/10/1, v/v/v). The plates were dipped in 10% H₂SO₄ and dried at 180 °C. Then the areas in the sample lanes corresponding to the band of ceramide standard were cut and ceramide was eluted with methanol. The eluates were incubated for 1 h and dried in a centrifugal vacuum concentrator. The ceramide residues were mixed with reaction buffer (pH 7.5) containing 25 mM Tris-HCl, 1% sodium cholate, and 15% fatty acid-free BSA. These mixtures were incubated with 500 μ U of sphingolipid ceramide *N*-deacylase (SCDase; Takara, Ohtsu, Japan) overnight at 37 °C and dried in a centrifugal vacuum concentrator. The resulting extracts were dissolved in methanol with OPA reagent (50 mg of OPA, 1 mL of ethanol, 200 μ L of β -mercaptoethanol, and 50 mL of 3% boric acid, pH 10.5) and incubated for 30 min for derivatization. The isocratic mobile phase for subsequent HPLC consisted of methanol/water/triethylamine (85:15:0.1, v/v/v) and the flow rate was 1 mL/min. An intelligent fluorescence detector was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Statistical analysis. Experimental results were expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) was followed by Dunnett's test for multiple comparisons. *p* values of <0.05 and <0.01 were considered statistically significant, as indicated.

Results

Effect of eriodictyol on IgE/Ag-mediated passive cutaneous anaphylaxis

The structure of eriodictyol is shown in Fig. 1. We tested the effect of eriodictyol on the allergic response in the passive cutaneous anaphylaxis (PCA) reaction. The PCA animal model is widely used to evaluate the localized mast cell-mediated allergic reaction *in vivo*.^{6,16,17} We induced the PCA reaction by injecting IgE locally into the mouse ear and then injecting the antigen systemically (intravenously). As shown in Fig. 2, eriodictyol suppressed the PCA reaction in a dose-dependent manner.

Effect of eriodictyol on IgE/Ag-mediated degranulation in mast cells

In view of the inhibitory effect of eriodictyol on the PCA reaction, we tested its effects on mast cells. The RBL-2H3 mast cell line was established from a rat basophilic leukemia.¹⁷ We evaluated the release of preformed allergic mediators using β -hexosaminidase as a biomarker of degranulation in the mast cells and then measured cell viability.¹⁸ First, the release of β -hexosaminidase was measured following stimulation of the IgE-Ag complex. Eriodictyol significantly and dose-dependently inhibited IgE/Ag-induced β -hexosaminidase release (Fig. 3A). Using the Ez-Cytox kit in the presence and the absence of Ag (25 ng/mL), we also

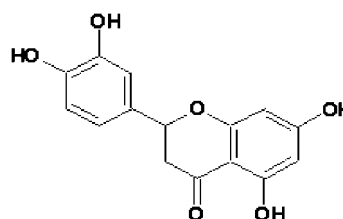


Fig. 1. Chemical Structure of Eriodictyol.

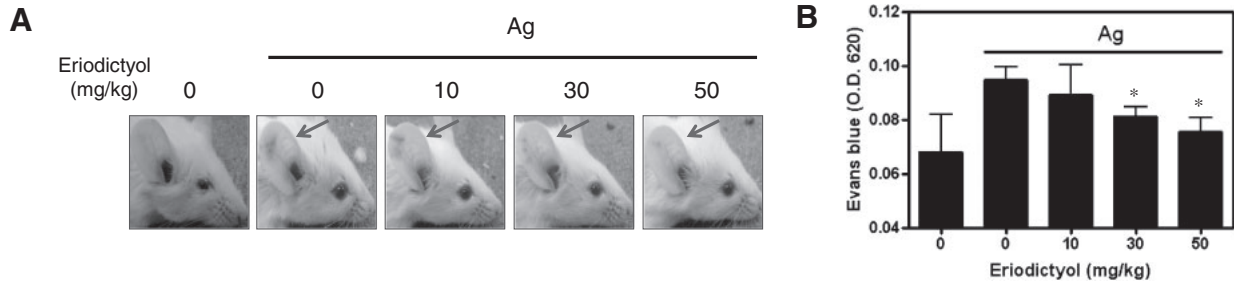


Fig. 2. Effect of Eriodictyol on IgE/Ag-Induced Passive Cutaneous Anaphylaxis (PCA).

Eleven-week-old Balb/c mice were injected intravenously with 200 μ g of antigen containing 3% Evans blue 24 h after intradermal injection of DNP-IgE (0.5 μ g) into an ear. Eriodictyol was administered orally 1 h before the antigen. A, Representative pictures of ears are shown. B, The dye was extracted overnight in 500 μ L of formamide at 63 $^{\circ}$ C, and light absorbance was measured at 620 nm. Data are expressed as mean $\pm$ SD (n = 5). * p < 0.05.

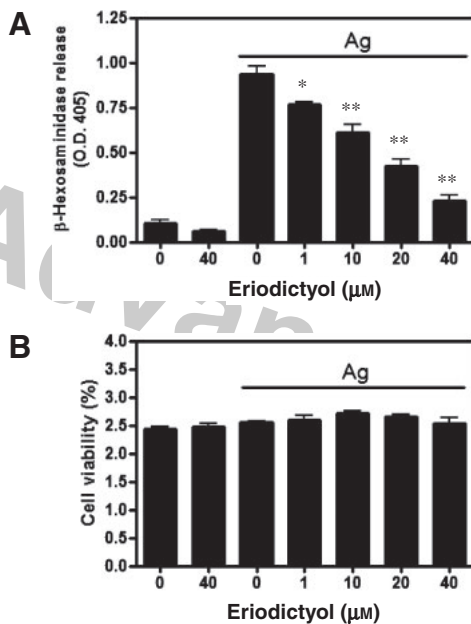


Fig. 3. Effect of Eriodictyol on IgE/Ag-Stimulated Mast-Cell Degranulation.

A, Cells were incubated overnight in 24-well plates with 200 ng/mL of DNP-specific IgE. The medium was replaced with PIPES buffer containing the indicated concentrations of eriodictyol, and the cells were then challenged with 25 ng/mL of DNP-HAS. After 15 min, β -hexosaminidase release was determined from the absorbance measured at 405 nm. B, To assess cytotoxicity, RBL-2H3 cells were incubated overnight in medium containing 200 ng/mL of DNP-specific IgE, and then were treated with eriodictyol at various concentrations for 24 h. Cell viability was determined with a Ez-Cytox kit from the absorbance at 450 nm. Data are expressed as mean $\pm$ SD (n = 3). * p < 0.05, ** p < 0.01.

found that neither induction of the IgE/Ag reaction (Ag, 25 ng/mL) nor eriodictyol at various concentrations produced cytotoxic effects in the mast cells (Fig. 3B).

Effect of eriodictyol on cytokine expression in RBL-2H3 cells

Proinflammatory cytokines, such as IL-4 and TNF- α are instrumental effectors in the allergic response.^{19,20} IL-4 induces IgE production in B cells and promotes the switch from naive T cells to Th2 cells. TNF- α is a potent inflammatory mediator secreted by mast cells in response to IgE challenge.^{17,20} Accordingly, we tested the effects of eriodictyol on the expression of IL-4 and TNF- α in IgE/Ag-stimulated RBL-2H3 cells. As shown in Fig. 4, it significantly inhibited IgE/Ag-induced expres-

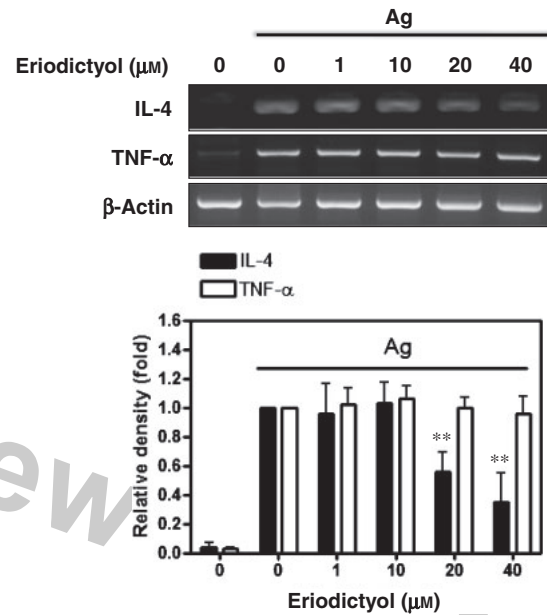


Fig. 4. Effects of Eriodictyol on the Expression of TNF- α and IL-4 in IgE/Ag-Sensitized Mast Cells.

IgE/Ag-sensitized RBL-2H3 cells were treated with various doses of eriodictyol and then challenged with 25 ng/mL of DNP-HSA for 1 h. Total RNA was isolated and analyzed by RT-PCR, which was performed to assess IL-4, TNF- α , and β -actin expression. The data from triplicate experiments were quantified by densitometry. Data are expressed as mean $\pm$ SD (n = 3). ** p < 0.01.

sion of IL-4 mRNA in a dose-dependent manner. From this, we concluded that eriodictyol inhibits IgE production in B cells by regulating down IL-4 expression, and that this suppresses IgE/Ag-mediated degranulation.

Effect of eriodictyol on CERK expression in RBL-2H3 cells

The release of proinflammatory mediators from mast cells that elicits an allergic response depends on the mobilization of Ca^{2+} .¹⁸⁻²¹ Cross-linking of Fc ϵ RI leads to Ca^{2+} release from the endoplasmic reticulum, which in turn increases the cytosolic Ca^{2+} concentration.^{6,22} A protein tyrosine kinase phosphorylation cascade can also mobilize Ca^{2+} .²³ Thus the availability of cytosolic Ca^{2+} for mast-cell degranulation is assured.

The calcium-dependent trigger for mast-cell degranulation might reside in the enzyme CERK, which phosphorylates ceramide (a central intermediary sphingolipid) to C1P.^{10,11} The formation of C1P induces the

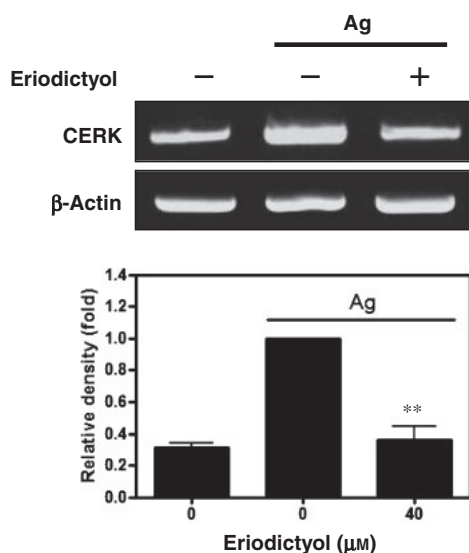


Fig. 5. Effect of Eriodictyol on CERK Expression in IgE/Ag-Sensitized Mast Cells.

IgE/Ag-sensitized RBL-2H3 cells were challenged with 25 ng/mL of antigen after treatment with 40 μM eriodictyol or no treatment. Total mast cell RNA was isolated and analyzed by RT-PCR, which was performed to assess CERK and β-actin expression. The data from triplicate experiments were quantified by densitometry. Data are expressed as mean ± SD (n = 3). ***p* < 0.01.

release of arachidonic acid and the synthesis of its inflammatory metabolites, and activates MAPK in a Ca^{2+} -dependent manner.^{10,12,13} This behavior is consistent with the role of CERK as a mediator of Ca^{2+} -dependent mast cell degranulation.¹⁰ Hence, in this study we investigated CERK and the generation of C1P in allergic response.

Mast cell stimulation with IgE/Ag for 1 h induced an increase in CERK mRNA, as determined by RT-PCR. In the eriodictyol-treated cells, however, the CERK mRNA level was significantly lower than in the cells stimulated with IgE/Ag only (Fig. 5). From this, we concluded that eriodictyol inhibited a Ca^{2+} -dependent process in mast-cell degranulation.

Effect of eriodictyol on ceramide levels in IgE/Ag-stimulated mast cells

To explore further the inhibitory mechanism of eriodictyol in degranulation, we measured ceramide levels in the mast cells. Ceramide is a sphingolipid and an effector in proinflammatory processes. In activated mast cells, ceramide activation is amplified by the synthesis of inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and by enhanced production of prostaglandins and PGE₂.¹² CERK phosphorylates ceramide to C1P, which can further stimulate arachidonic acid release and the synthesis of PGE₂ and proinflammatory cytokines.^{10,11,13} These functions link ceramide and C1P to mast-cell degranulation and the allergic response. Accordingly, we tested the effect of eriodictyol on ceramide levels in IgE/Ag-stimulated mast cells by HPLC. Following exposure to an antigen, the sensitized mast cells contained higher ceramide concentrations than non-stimulated mast cells. Eriodictyol treatment decreased the ceramide concentrations significantly in the IgE/Ag-stimulated mast cells, to levels similar to those in non-stimulated mast cells (Fig. 6). Thus the

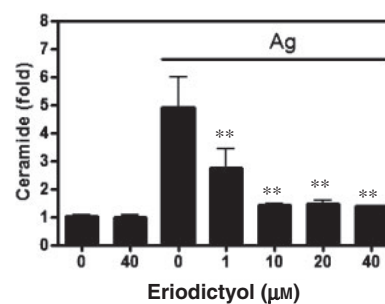


Fig. 6. Effect of Eriodictyol on Ceramide Levels in IgE/Ag-Sensitized Mast Cells.

Mast cells sensitized with IgE/Ag were challenged with 25 ng/mL of antigen. Lipids were extracted from the mast cell pellets with ethanol containing C₁₇-ceramide as internal standard. The C₁₇- and C₁₈-ceramides in the extract were separated by thin-layer chromatography and deacylated with ceramidase. The C₁₇ and C₁₈ sphingosines released were derivatized with OPA fluorescent dyes and analyzed by HPLC. Data are expressed as mean ± SD (n = 3). ***p* < 0.01.

inhibitory effect of eriodictyol on mast-cell degranulation might occur primarily through inhibition of ceramide metabolism.

Discussion

The debilitating effects of and reduction in quality of life due to allergic diseases are well known.²² Steroid medications have potent anti-allergic action but can also cause serious systemic side effects. Since a variety of natural substances induce allergic reactions, it is plausible to seek natural substances with anti-allergic properties for therapy. Drugs derived from natural products are potentially effective with fewer serious side effects than powerful synthetic drugs.

Mast cells are bone marrow-derived white blood cells with prominent roles in allergic response and anaphylaxis, as well as in wound healing and pathogen defense. Allergy and anaphylaxis begin with sensitization of mast cells through the binding of an antigen-specific IgE to FcεRI receptors on the mast-cell surface. Subsequent cross-linking of FcεRI receptors by antigen binding to IgE stimulates the release of proinflammatory cytokines, including histamine and β-hexosaminidase, from storage sites in cytoplasmic granules.^{1,17,24,25} Multiple protein tyrosine kinase cascades orchestrate these events. For example, crosslinking of FcεRI-bound IgE by an antigen can activate a Src-family tyrosine kinase receptor which subsequently phosphorylates Lyn and Syk. This cascade can recruit adapter proteins such as LAT and Gab-2 to activate downstream effectors, releasing inflammatory mediators.^{5–7,16,26}

One important target of such cascades is the intracellular Ca^{2+} concentration,²⁷ as is evident in that the Ca^{2+} chelator EGTA abolishes the Ca^{2+} mobilization and mast cell degranulation that follow FcεRI cross-linking.^{9,28} The cytoplasmic Ca^{2+} concentration is regulated by PI3K-mediated activation of phospholipase C-γ (PLC-γ). PLC-γ activates diacylglycerol (DAG) and the formation of inositol-1,4,5-triphosphate (IP3), which govern Ca^{2+} mobilization.^{3,7,9,26} The cytoplasmic Ca^{2+} concentration regulates a variety of signal-mediated processes. For example, CERK converts ceramide to C1P in a Ca^{2+} -dependent manner and activates

MAPK signaling, which can invoke ERK 1/2, JNK and p38. These signaling cascades lead to degranulation and cytokine release in IgE/Ag-stimulated mast cells, which induce and sustain the allergic response.^{10–13} It is thus plausible that modulators of intracellular Ca^{2+} in mast cells might be useful in preventing and relieving the distress of allergic disease.

In the present study, we investigated the effects of eriodictyol on the IgE/Ag-mediated PCA in mice and on the corresponding effectors in RBL-2H3 cells. The mouse ear PCA reaction is a well-established experimental system for evaluating a localized immediate allergic response *in vivo*.^{17,18,24,29} In this study, eriodictyol administered orally to the mice 2 h before antigen challenge effectively suppressed local allergic reactions (Fig. 2). Substantial inhibition was observed at 30 mg/kg of eriodictyol and complete suppression was observed with 50 mg/kg. This suggests that eriodictyol might be applied pharmacologically in treating allergic disease.

The release of chemical mediators and enzymes plays an important role in the allergic response. After stimulation with IgE/Ag, mast cells release β -hexosaminidase from storage in granules.^{4,18} Assay of β -hexosaminidase thus provides a convenient method to monitor mast-cell degranulation and activation.¹⁷ Eriodictyol significantly suppressed IgE/Ag-stimulated degranulation in a dose-dependent manner in RBL-2H3 cells (Fig. 3A). At the concentrations used in these experiments, it induced no cytotoxicity (Fig. 3B). These results offer further support for eriodictyol as a potential anti-allergenic agent.

Aggregation of IgE-bound Fc ϵ RI by Ag binding induces various inflammatory cytokines, notably IL-4 and TNF- α , which mediate the symptoms in an allergic reaction.^{19,20} IL-4 is central, because it is involved in IgE production, Th2 cell induction, and the inhibition of MAPK expression involved in Ca^{2+} influx.¹⁹ TNF- α is also an important inflammatory cytokine that mast cells produce in response to IgE challenge *via* ERK1/2 signaling.^{17,19} In this study, eriodictyol suppressed IL-4 expression in IgE/Ag-stimulated mast cells, but did not affect the expression of TNF- α , which begin upstream of IL-4 transcription factor binding (Fig. 4). This suggests that eriodictyol inhibits the allergic response not by reducing TNF- α production but by suppressing IL-4. This assumes that IL-4 inhibition reduces the IgE level in the blood, possibly through the effect of eriodictyol on intracellular Ca^{2+} levels, which in turn regulate IL-4.

To elucidate further the molecular mechanisms of eriodictyol, we investigated its effect on expression of CERK mRNA and the levels of ceramide and sphingolipid metabolites during Ca^{2+} -dependent degranulation in IgE/Ag-stimulated RBL-2H3 cells. As mentioned above, CERK converts ceramide to C1P, dependent on the cytosolic Ca^{2+} concentration,^{10,11} and increased cytoplasmic C1P stimulates MAPK, leading to degranulation and the release of inflammatory mediators.^{10,13} Following release from more complex membrane lipids such as sphingomyelin, ceramide can participate in diverse signaling pathways. For instance, it induces the release of proinflammatory cytokines and activates MAPK, which promotes the production of inflammatory mediators and adhesion molecules.¹² In the present

study, eriodictyol suppressed the expression of CERK mRNA and ceramide concentrations in IgE/Ag-stimulated mast cells (Figs. 5 and 6). Recently, we reported that CERK is a mediator of calcium-dependent degranulation in mast cells. Exogenous introduction of CERK into permeabilized RBL-2H3 cells caused degranulation.¹⁰ Thus eriodictyol appeared to inhibit mast-cell degranulation by inhibiting sphingolipid metabolism. These results are consistent with the effect of eriodictyol on intracellular Ca^{2+} levels, since it inhibited the expression of IL-4 and CERK mRNA.

Taken together, the present findings indicate that eriodictyol significantly suppressed IgE/Ag-induced PCA in mice, inhibited β -hexosaminidase release, a marker of degranulation, and downregulated CERK and IL-4, Ca^{2+} -dependent factors in degranulation. It also decreased the mast-cell concentrations of ceramide and sphingolipid metabolites. Hence, eriodictyol can suppress the IgE/Ag-induced allergic response primarily by inhibiting ceramide metabolism in mast cells.

Acknowledgments

We are grateful to Dr. Sang-Hyun Sung (Seoul National University, Seoul, Korea) for providing us with eriodictyol. This work was supported by a grant from the Next-Generation BioGreen 21 Program (no. PJ0080152012) of the Rural Development Administration, Republic of Korea.

References

- 1) Tiwari N, Wang CC, Brochetta C, Ke G, Vita F, Qi Z, Rivera J, Soranzo MR, Zabucchi G, Hong W, and Blank U, *Blood*, **111**, 3665–3674 (2008).
- 2) Sakai S, Sugawara T, Matsubara K, and Hirata T, *J. Biol. Chem.*, **284**, 28172–28179 (2009).
- 3) Bansal G, Xie Z, Rao S, Nocka K, and Druey K, *Nat. Immunol.*, **9**, 73–80 (2008).
- 4) Itoh T, Ninomiya M, Yasuda M, Koshikawa K, Deyashiki Y, Nozawa Y, Akao Y, and Koketsu M, *Bioorg. Med. Chem.*, **17**, 5374–5379 (2009).
- 5) Itoh T, Ohguchi K, Iinuma M, Nozawa Y, and Akao Y, *Bioorg. Med. Chem.*, **16**, 7592–7598 (2008).
- 6) Lee JH, Kim JW, Ko NY, Mun SH, Her E, Kim BK, Han JW, Lee HY, Beaven MA, Kim YM, and Choi WS, *J. Allergy Clin. Immunol.*, **121**, 1225–1231 (2008).
- 7) Amir-Moazami O, Alexia C, Charles N, Launay P, Monteiro RC, and Benhamou M, *J. Biol. Chem.*, **283**, 25514–25523 (2008).
- 8) Ryu SD, Lee HS, Suk HY, Park CS, and Choi OH, *Cell Calcium*, **45**, 99–108 (2009).
- 9) Baba Y, Nishida K, Fujii Y, Hirano T, Hikida M, and Kurosaki T, *Nat. Immunol.*, **9**, 81–88 (2008).
- 10) Mitsutake S, Kim TJ, Inagaki Y, Kato M, Yamashita T, and Igarashi Y, *J. Biol. Chem.*, **279**, 17570–17577 (2004).
- 11) Baumruker T, Bornancin F, and Billich A, *Immunol. Lett.*, **96**, 175–185 (2005).
- 12) Masini E, Giannini L, Nistri S, Cinci L, Mastroianni R, Xu W, Comhair SA, Li D, Cuzzocrea S, Matuschak GM, and Salvemini D, *J. Pharmacol. Exp. Ther.*, **324**, 548–557 (2008).
- 13) Chalfant CE and Spiegel S, *J. Cell Sci.*, **118**, 4605–4612 (2005).
- 14) Vandeputte OM, Kiendrebeogo M, Rasamiravaka T, Stévigny C, Duez P, Rajaonson S, Diallo B, Mol A, Baucher M, and El Jaziri M, *Microbiology*, **157**, 2120–2132 (2011).
- 15) Lee JK, *Arch. Pharm. Res.*, **34**, 671–679 (2011).
- 16) Lee JH, *Exp. Biol. Med.*, **233**, 1271–1279 (2008).
- 17) Han EH, Park JH, Kim JY, Chung YC, and Jeong HG, *Food Chem. Toxicol.*, **47**, 1069–1075 (2009).

- 18) Itoh T, Oyama M, Takimoto N, Kato C, Nozawa Y, Akao Y, and Linuma M, *Bioorg. Med. Chem.*, **17**, 3189–3197 (2009).
- 19) Matsubara M, Masaki S, Ohmori K, Karasawa A, and Hasegawa K, *Biochem. Pharmacol.*, **67**, 1315–1326 (2004).
- 20) Park HH, Lee S, Son HY, Park SB, Kim MS, Choi EJ, Singh TS, Ha JH, Lee MG, Kim JE, Hyun MC, Kwon TK, Kim YH, and Kim SH, *Arch. Pharm. Res.*, **31**, 1303–1311 (2008).
- 21) Textor B, Licht AH, Tuckermann JP, Jessberger R, Razin E, Angel P, Schorpp-Kistner M, and Hartenstein B, *J. Immunol.*, **179**, 6873–6880 (2007).
- 22) Jung ID, Lee HS, Lee HY, and Choi OH, *Cell. Signal.*, **21**, 1698–1705 (2009).
- 23) Vennekens R, Olausson J, Meissner M, Bloch W, Mathar I, Philipp SE, Schmitz F, Weissgerber P, Nilius B, Flockerzi V, and Freichel M, *Nat. Immunol.*, **8**, 312–320 (2007).
- 24) Kopeć A, Panaszek B, and Fal AM, *Arch. Immunol. Ther. Exp.*, **54**, 393–401 (2006).
- 25) Chen HJ, Shih CK, Hsu HY, and Chiang W, *J. Agric. Food Chem.*, **58**, 2596–2601 (2010).
- 26) Parravicini V, Gadina M, Kovarova M, Odom S, Gonzalez-Espinosa C, Furumoto Y, Saitoh S, Samelson LE, O'Shea JJ, and Rivera J, *Nat. Immunol.*, **3**, 741–748 (2002).
- 27) Vig M and Kinet JP, *Nat. Immunol.*, **10**, 21–27 (2009).
- 28) Zaitzu M, Narita S, Lambert KC, Grady JJ, Estes DM, Curran EM, Brooks EG, Watson CS, Goldblum RM, and Midoro-Horiuti T, *Mol. Immunol.*, **44**, 1977–1985 (2007).
- 29) Itoh T, Tsukane M, Koike M, Nakamura C, Ohguchi K, Ito M, Akao Y, Koshimizu S, Nozawa Y, Wakimoto T, Nukaya H, and Suwa Y, *J. Agric. Food Chem.*, **58**, 7149–7157 (2010).

Advance View Proofs