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RESEARCH ARTICLE

Kaempferol Isolated from *Nelumbo nucifera* Stamens Inhibits Phosphorylation of ERK 1/2, Syk, and Lyn in FcεRI-mediated Allergic Reaction

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Abstract Cross-linking of FcεRI with multivalent allergen and specific IgE on mast cells and initiates activation of signal cascades that lead to IgE-mediated allergic responses. Kaempferol is a phenolic compound contained in natural substances including the stamens of flavonoid-rich *Nelumbo nucifera* stamens. In this study, the effects of kaempferol on allergic reaction induced by an FcεRI α chain antibody were investigated. Western blot analysis revealed that the protein expression of FcεRI and the phosphorylation of Syk, Lyn, and extracellular signal-regulated kinases (ERK) 1/2 were dose-dependently inhibited by kaempferol. β-Hexosaminidase was reduced to 97.9, 89.1, 75.1, and 55.8% when treated with 5, 10, 20, and 40 μM kaempferol, respectively. From the results, the negative regulation of basophil activation with kaempferol via the suppression of FcεRI expression and degranulation may be suggested to be through inhibition of Lyn and Syk, and ERK 1/2 activation.

Keywords: *Nelumbo nucifera* stamens, kaempferol, Syk, Lyn, ERK 1/2

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Introduction

Nelumbo nucifera Gaertn is a perennial aquatic plant of the Nymphaeaceae family, which is distributed throughout Asia, the Middle East, and Egypt. All parts of *N. nucifera,* including the leaves, flowers, stamens, embryos, and rhizomes, have been utilized as traditional medicines for the treatment of diarrhea, gastritis, insomnia, and nervous prostration (1-3), and as hemostatic agents (4). This plant is also rich in flavonoids, a group of natural polyphenols known to have a number of pharmacological properties, including hepatoprotective (5), anti-HIV (6), anti-oxidative (7,8), anti-hyperlipidemic (9), anti-obesitic (10), anti-inflammatory (11,12), anti-diabetic (13), anti-allergic (14), and anti-cancer (15) effects.

The high-affinity IgE receptor, FcεRI, is expressed on the surface of human mast cells and basophils, and is involved in IgE-mediated allergic reactions (16,17). The aggregation of FcεRI by multivalent allergen-specific IgE complexes or by anti-FcεRI antibody is the major stimulus for mast cells and basophils activation, and initiation of the activation signal cascade, and triggers degranulation, resulting in the release of inflammatory mediators, including histamine, which in turn induces allergic responses, such as asthma, atopic dermatitis, and allergic rhinitis (18,19). FcεRI consists of an Fc portion-binding α chain and signal transducing β- and γ chains, which contain immunotyrosine-based activation motifs (ITAM) in their cytoplasmic domains (20,21). Cross-linking of FcεRI-bound IgE antibody with multivalent allergens activates downstream molecules, including the protein tyrosine kinases (PTK) such as Lyn and Syk, and mitogen-activated protein kinases (MAPK), including ERK 1/2, c-jun N-terminal kinase (JNK), and p38 MAPK (22-25). It is reported that neferine from *Nelumbo nucifera* inhibits the MAPK activation in cancer

cells (25,26). However, regulation of signaling in FcεRI-mediated allergic reaction by kaempferol from *Nelumbo nucifera* stamens has not been studied.

Activation of signaling pathways after the cross-linking of FcεRI-bound IgE antibody with a multivalent allergen initially depends on the interaction of FcεRI with the Src kinase, Lyn, and subsequent activation of Syk and other tyrosine kinases (22-25). Furthermore, the MAPK signaling cascades are important for the differentiation, activation, proliferation, degranulation, and migration of various immune cells, including basophils and mast cells. Thus, the down-regulation of MAPK signaling may lead to the attenuation of the FcεRI-mediated allergic reaction in basophils and mast cells.

Recently, a number of studies demonstrated the suppression of FcεRI expression and reduced allergic activity following treatment with a green tea catechin (27), (−)-epigallocatechin 3-*O*-gallate (28,29). We previously found that kaempferol from the stamens of *N. nucifera* stamens can reduce FcεRI expression and negatively regulate basophil activation (14). To continue identifying the inhibitory effects of kaempferol on FcεRI-mediated allergic reaction, we determined the regulation of PTK and MAPK pathways involved in FcεRI in human basophilic KU812F cells by the kaempferol isolated from *N. nucifera* stamens in the present study.

Materials and Methods

Materials Kaempferol, isolated from *N. nucifera* stamens (5.9 mg/kg) as described by Lim *et al*. (13), was kindly provided by Prof. Jae-Sue Choi, Pukyong National University, Busan, Korea. Kaempferol was stored at −20°C, and dissolved in DMSO prior to use.

Cell culture, treatment The human basophilic KU812F cell line was obtained from American Type Culture Collection, and maintained in RPMI-1640 (HyClone, Logan, UT, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone). The cells were treated with different concentrations of kaempferol in serum-free RPMI-1640 medium, and the cells were stimulated with an anti-FcεRI antibody (CRA-1).

Cell viability assay Cell viability was assayed using the Celltiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. KU812F cells were seeded in 96-well plates and incubated with serum-free media in the presence of compounds. After treatment for 24 h, reagents were added, and cells were incubated for 4 h. Dehydrogenase enzymes in live cells convert MTS tetrazolium compound into colored formazan products, and the absorbance is measured at 490 nm using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was calculated compared with the absorbance of the untreated cells (30,31).

Western blot analysis Expression of FcεRI protein and phosphorylation of tyrosine kinases and MAPK were assessed by Western blot analysis. The treated and stimulated cells were lysed in cell lysis buffer containing 20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, and a protease inhibitor cocktail (Roche, Penzberg, Germany). The protein samples were then separated using 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted using CRA-1, and anti-phosphorylated Lyn, Syk, ERK 1/2, p38, or JNK antibodies, followed by HRP-conjugated secondary antibodies. For detection, the chemoreactive proteins were visualized using enhanced chemiluminescence detection reagents (Perkin Elmer, Waltham, MA, USA), in accordance with the manufacturer's instructions. The membrane was exposed to x-ray film, and quantified with a Molecular Imager® Gel Doc™ XR System (Bio-Rad, Hercules, CA, USA).

β-Hexosaminidase release assay The treated and stimulated cells were centrifuged, and the β-hexosaminidase activity in the supernatant was determined spectrophotometrically (32). Briefly, the supernatant or cell lysates were aliquoted and 2 μM NP-GlcNAc (in 0.4 M citrate and 0.2 M phosphate buffer, pH 4.5) was added. The color was developed, and the enzyme reaction was terminated by adding 0.2 M glycine-NaOH, pH 10.7. Absorbance was measured on a microplate reader (VersaMax; Molecular Devices) at 405 nm. The cells were lysed with 0.1% Triton X-100, and the β-hexosaminidase activity of the extracts was measured. The percentage of β-hexosaminidase released was calculated as follows: (absorbance of the supernatant of stimulated cells)/(absorbance of the total lysates)×100.

Statistical analysis All measurements were conducted independently at least in triplicate. The data were expressed as the mean±SD. The statistical differences between the control and kaempferol groups were determined with Student's *t*-test using SPSS statistical software (version 12.0; SPSS Inc., Chicago, IL, USA). The differences were considered significant at $p<0.05$.

Results and Discussion

Kaempferol inhibits FcεRI expression Recently, a great deal of attention has been focused on the search for

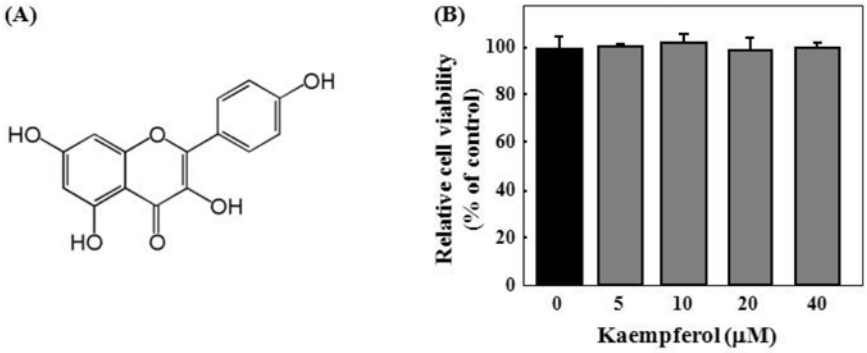

Fig. 1. Chemical structure and cytotoxic effect of kaempferol. (A) Kaempferol isolated from *N. nucifera* stamens; (B) Cytotoxicity of kaempferol treatment (0, 5, 10, 20, 40 μM) on KU812F cells after 24 h.

biologically active anti-allergic compounds, which down-regulate FcεRI expression. In the current study the molecular mechanism including PTK and MAPK activation on on FcεRI signaling was evaluated in human basophilic KU812F cells with kaempferol isolated from *N. nucifera* stamens (Fig. 1A), a well-known Oriental herb used in traditional medicine for treatment of diarrhea, gastritis, insomnia, and nervous prostration (1-4). Treatment of KU812 cells with varying doses of kaempferol (1-40 μM), did not induce cytotoxic effects over 24 h (Fig. 1B) and no reduction in viability was observed even at high concentration (data not shown). Mast cells and basophils express a high affinity IgE receptor, FcεRI, on the cell surface, which is crucial for IgE-mediated allergic reactions (16,17). We previously found that kaempferol suppressed cell surface expression and mRNA levels of FcεRI (9). In the present study, the inhibitory effects of kaempferol on FcεRI α chain expression in KU812F cells were confirmed by measuring the protein levels of the FcεRI α chain via western blot analysis (Fig. 2). The decrease in FcεRI expression by kaempferol was dose-dependent.

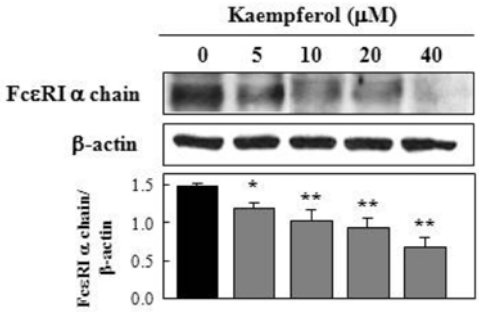

Fig. 2. Effects of kaempferol on the protein expression of FcεRI. Cells treated with various concentrations of kaempferol were harvested and the cellular lysates were isolated. Western blot analysis was conducted using CRA-1 and β-actin. The amount of protein in each band was quantified by densitometry. The upper panel shows a representative Western blot, and the lower panel shows the relative levels of FcεRI α chain corrected to β-actin. Results are presented as the mean±SD of three independent experiments. *p<0.05, **p<0.01 represent significant differences from the control.

The FcεRI molecule has α Fc portion-binding α chain and signal transducing β- and γ chains containing ITAM in their cytoplasmic domains (22-25). As previously reported (14), kaempferol treatment lowered the mRNA levels of FcεRI α and γ chains, but not the level of FcεRI β chain, in KU812F cells.

Kaempferol negatively regulates phosphorylation of PTK and MAPK The signaling pathway activated by Fc receptors in mast cells and basophils has been extensively characterized. Initial FcεRI stimulation on these cells activates a signaling cascade that includes activation of PTK (Syk and Lyn) and MAPK (ERK 1/2, p38 and JNK) (17-20). Many studies reported that flavonoids are the most crucial polyphenolic compounds widely distributed in edible plants, and of these polyphenol compounds, (−)-epigallocatechin-3-O-gallate (EGCG), chrysin, and apigenin suppress the expression of FcεRI via inhibition of ERK 1/2 phosphorylation (22-25). Kaempferol has been known to exert preventive effects against cancer and inflammation, and MAPK have been suggested to be possible targets for kaempferol (11,15). However, the effect of kaempferol on regulation of FcεRI-mediated PTK and MAPK in allergic responses has not been well characterized.

To assess the effect of kaempferol on activation of PTK and MAPK, KU812F cells were treated with various concentration of kaempferol for 24 h, and stimulated with CRA-1. As shown in Fig. 3, western blot analysis indicated that the phosphorylation of PTK, such as Lyn and Syk, was profoundly and dose-dependently suppressed by kaempferol. Interestingly, kaempferol is known to have anti-cancer and anti-inflammatory effects, and MAPK have been suggested as possible targets for kaempferol (11). To further analyze the modulation of MAPK pathways by kaempferol, we assessed the effects of kaempferol on CRA-1-induced MAPK activation. Kaempferol treatment attenuated CRA-1-induced ERK 1/2 phosphorylation in a dose-dependent

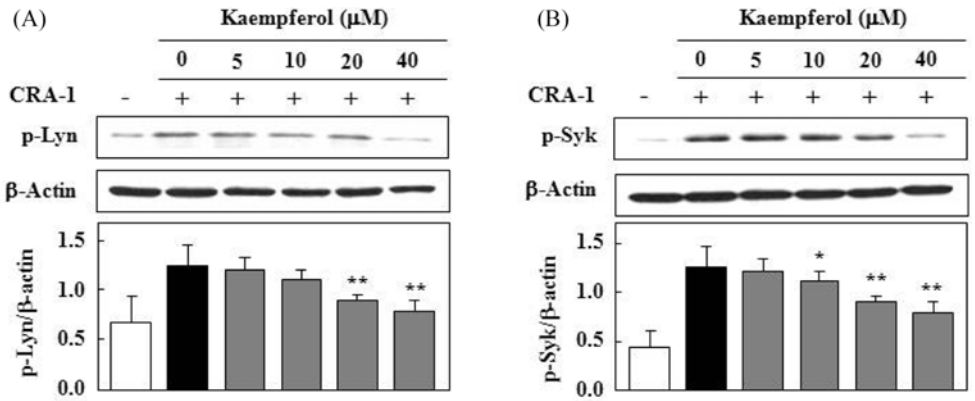

Fig. 3. Effects on FcεRI-mediated PTK phosphorylation. Cells were treated with kaempferol and stimulated with CRA-1. The cellular lysates were obtained, and the phosphorylation of Lyn and Syk was assessed via western blot analysis using anti-phosphorylated Lyn and Syk, and β-actin antibodies. The amount of protein in each band was quantified by densitometry. The upper panel shows a representative western blot, and the lower panel presents the relative levels of p-Lyn and p-Syk corrected to β-actin. Results are presented as the mean±SD of three independent experiments. *p<0.05, **p<0.01 represent significant differences from the control.

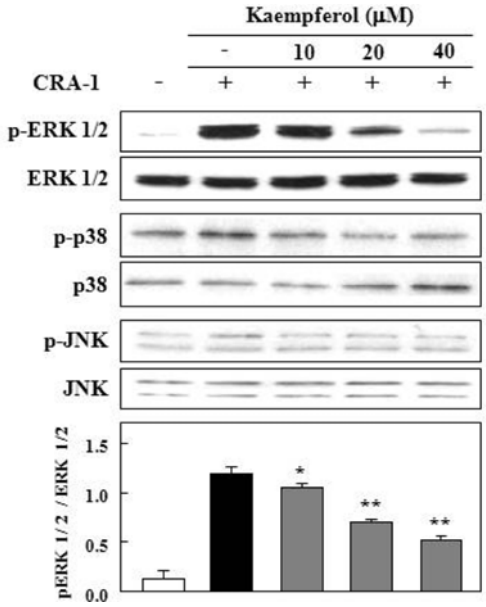

Fig. 4. Effects on FcεRI-mediated MAPK phosphorylation. Cells were treated with kaempferol, and stimulated with CRA-1. The cellular lysate was obtained, and the protein expression was assessed via western blot analysis using p-ERK, p38, JNK and β-actin antibodies. Results are presented as the mean±SD of three independent experiments. *p<0.05, **p<0.01 represent significant differences from the control.

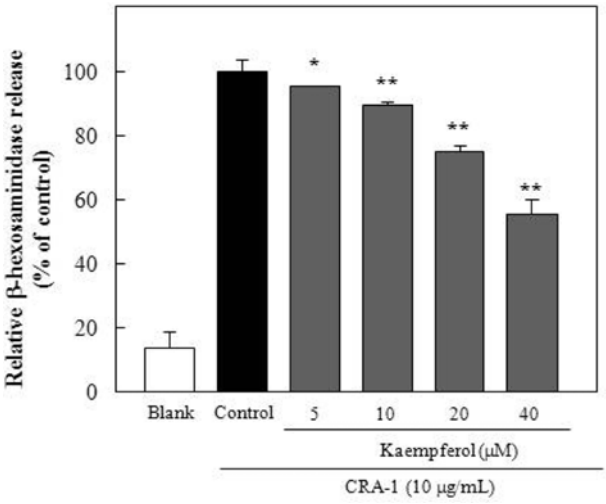

Fig. 5. Effects on FcεRI-mediated β-hexosaminidase release. Cells treated with kaempferol were stimulated for 30 min with CRA-1, and then β-hexosaminidase content was measured. Results are presented as the mean±SD of three independent experiments. *p<0.05, **p<0.01 represent significant differences from the control.

manner, but did not affect the phosphorylation of p38 MAPK and JNK (Fig. 4). These results may indicate that ERK 1/2 is a downstream regulator of PTK and that the inactivation of ERK 1/2 by kaempferol resulted in Syk and Lyn inactivation.

Considering the role of FcεRI in IgE-mediated allergic reaction, the suppression of allergen-IgE-FcεRI complex formation by kaempferol should be useful in the prevention of allergic diseases. In basophils, the aggregation of FcεRI induces the activation of proteins downstream of Syk, including phospholipase Cγ and phosphatidylinositol-3 kinases. Therefore, further studies on the regulation of other signaling pathway factors by kaempferol would be necessary.

Inhibitory effects of β-hexosaminidase release FcεRI cross-linking stimulates the elevation of intracellular calcium concentrations and degranulation in IgE-mediated allergic reaction. Among the inflammatory mediators, histamine and β-hexosaminidase are potent markers of allergic disease (32,33). We previously found that the kaempferol in from the stamens of *N. nucifera* inhibited the CRA-induced calcium influx and histamine release (14). To confirm the inhibitory effects of kaempferol on FcεRI-mediated

degranulation, we measured β-hexosaminidase release from human basophilic KU812F cells after the cross-linking of FcεRI with the CRA-1. β-Hexosaminidase was reduced from 97.9, 89.1, 75.1, and 55.8% when treated with kaempferol at 5, 10, 20, and 40 μM, respectively (Fig. 5).

In FcεRI-mediated allergic reaction, cytokine production, release of inflammatory mediators, cell survival, and calcium influx are related to the activation of MAPK (34,35).

Our findings demonstrated that kaempferol evidenced anti-allergic activity via the inhibition of FcεRI expression and the inactivation of Lyn, Syk, and ERK 1/2. Further studies on molecular mechanism of another pathway by kaempferol in FcεRI signaling are needed.

These results suggest that the inhibition of FcεRI-mediated allergic reactions is responsible for the kaempferol isolated from *N. nucifera* stamens, which likely contribute to the pharmacological action, and may be useful for the prevention and treatment of allergic diseases.

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Disclosure The authors declare no conflict of interest.

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