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Anti-allergic effect of fisetin on IgE-mediated mast cell activation *in vitro* and on passive cutaneous anaphylaxis (PCA)

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Abstract

Fisetin (3,7,3',4'-tetrahydroxyflavone), a naturally occurring bioactive flavonoid, has been shown to inhibit inflammation. However, little is known about the effect of fisetin on immunoglobulin E (IgE)-mediated allergic responses. In this study, the effect of fisetin on rat basophilic leukemia (RBL-2H3) cell-mediated allergic reactions was investigated. Fisetin inhibited β -hexosaminidase release, and decreased the level of interleukin-4 (IL-4) and tumor necrosis factor- α (TNF- α) mRNA in IgE/antigen (IgE/Ag)-stimulated RBL-2H3 cells. To elucidate the anti-allergic mechanism, we examined the levels of signaling molecules responsible for degranulation and release of inflammatory cytokines. Fisetin decreased the levels of activated spleen tyrosine kinase (Syk), Gab2 proteins, linker of activated T cells (LAT), ERK1/2 in the IgE/Ag stimulated RBL2H3 cells, NF κ B and STAT3 proteins activated in the ear tissue of mice with PCA. In addition, fisetin significantly lowered of Fc ϵ RI α -subunit mRNA expression. Consistent with the cellular data, fisetin markedly suppressed RBL-2H3 cell-dependent passive cutaneous anaphylaxis (PCA) in IgE/Ag-sensitized mice. These results suggest that fisetin may have potential as a therapeutic agent for the treatment of allergic diseases.

Keywords: IgE-mediated allergic diseases; mast cell; basophil; fisetin; Syk kinase; cytokines; passive cutaneous anaphylaxis

1. Introduction

The population of allergic disease patients has drastically increased in recent decades [1]. Among allergic diseases, type I hypersensitive reactions is defined as a severe, life-burdening, or systemic hypersensitivity reaction [2]. IgE-mediated allergic diseases include eczema, asthma, allergic rhinitis and atopic dermatitis [3]. Although the lifetime prevalence rate estimated at 0.05-2%, the rate of occurrence is rapidly increasing. The incidence of type I hypersensitivity allergic disease is rapidly increasing worldwide nowadays [4]. Type I reactions (ie, immediate hypersensitivity reactions) involve immunoglobulin E (IgE)-mediated release of histamine and other mediators from mast cells and basophils. Mast cells and basophils play the key role in evoking IgE-mediated allergic reaction [5]. Activated IgE receptor (FcεRI) signaling pathway in mast cells or basophils lead to the overproduction of inflammatory mediators that are responsible for allergic diseases [6, 7]. IL-4 promotes Th2 cellular response development and promotes B cells to produce IgE [8]. IFN γ can inhibit the production of specific IgE [9]. IL-10 suppresses monocyte/macrophage activation, reducing the production of pro-inflammatory cytokines such as TNF- α [10]. Approaches to the treatment of allergic diseases are largely based on allergen-specific immunotherapy, DNA vaccination, antihistamine drugs and steroids treatments [11]. Commonly small-molecule inhibitors to treat allergic diseases are mainly antagonists to leukotriens (LTs) or histamine receptors, but they produce undesirable side-effects, including drowsiness, stomach upset chest congestion, and dry mouth [12]. Recently, many practitioners and researchers are interested in natural products as anti-allergic drug candidates since many are known to modulate immune responses with no adverse effects [13].

Flavonoids exhibit many biological activities, including anti-inflammatory, antioxidant, antibacterial, and anti-allergic effects. Among bioactive flavonoids, fisetin (3,7,3',4'-tetrahydroxyflavone) can be isolated from plants, such as *Rhus verniciflua*, and from fruits and vegetables, such as strawberry, apple, persimmon, grape, onion, and cucumber [14, 15]. Fisetin has been shown to exert anticancer [16], antioxidative [17], and anti-inflammatory activities [18]. It substantially inhibits IgE/Ag-induced degranulation of basophils and mast cells [19]. Additionally, fisetin attenuates LPS-induced lung inflammation in mice by a mechanism involving the reduction of iNOS and TNF- α levels [18]. Previously our group reported that fisetin is a bioactive compound in *Rhus verniciflua* bark, which suppresses Type IV hypersensitivity response in mice [14]. However, anti-allergic effect of fisetin from *Rhus verniciflua* against type I hypersensitive reactions has not been studied *in vivo*. In this study, we investigated whether fisetin inhibits IgE-mediated passive cutaneous anaphylaxis (PCA), an immediate hypersensitivity reaction in the dermis and allergic reactions in mast cells.

2. Materials and Methods

2.1 Materials and Regents.

Fisetin, 2,4-Dinitrophenyl (DNP)-specific IgE, DNP-bovine serum albumin (BSA), Evans blue, cetirizine (CZ), 4-nitrophenyl N-acetyl- β -D-glucosaminide (NP-GlcNAc), and piperazine-N,N'-bis(ethanesulfonic acid) (PIPES) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, and Eagle's minimum essential

medium (EMEM) were obtained from Invitrogen (Carlsbad, CA, USA). PP2 was obtained from Calbiochem (La Jolla, CA, USA), and β -actin antibody was obtained from Santa Cruz (Dallas, TX, USA). Phospho-Syk (Tyr525/526) antibody, Syk antibody, phospho-LAT (Tyr191) antibody, phospho-ERK1/2 antibody, phospho-Gab2 (Tyr452) antibody, phospho-NF κ B p65 (Ser536) antibody, NF κ B p65 antibody, phospho-Stat3 antibody (Tyr705), phospho-Stat3 antibody (Ser727) and Stat3 antibody were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Gab2 antibody was obtained from abcam (Cambridge, UK). LAT antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

2.2 Cell culture.

Rat basophilic leukemia cells (RBL-2H3, catalog number KCLB no. 22256) were obtained from the ATCC Biological Resource Center (Manassas, VA, USA), and were cultured in EMEM supplemented with 15% FBS and 100 U/ml penicillin. The cells were grown in 75 cm² culture flasks at 37.1°C in a humidified incubator with an atmosphere of 5% CO₂.

2.3 Cell viability assay.

Cell viability was measured using the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Viability data were expressed as a percentage relative to viability in control cell cultures.

2.4 β -hexosaminidase (β -hex) release assay.

RBL-2H3 cells (5×10^4 cells/well) were cultured overnight in medium containing 200 ng/mL of DNP-specific IgE. After washing with PIPES buffer (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM CaCl_2 , 5.6 mM glucose, 40 mM NaOH, and 0.1% BSA), cells were treated with or without fisetin or PP2 at the indicated concentrations. PP2 is a Src kinase inhibitor, which inhibits degranulation in antigen stimulated RBL2H3 cells [20]. After incubation for 30 min at 37°C, cells were challenged with 200 ng/mL of DNP-BSA for 15 min at 37°C. The extent of degranulation was determined by the measurement of the released β -hexosaminidase as previously described [21].

2.5 Reverse transcriptase polymerase chain reaction (RT-PCR).

Reverse transcriptase PCR was performed as previously described [21]. Total RNA was isolated from the RBL-2H3 cells using RNA-Bee Reagent (Tel-test, Friendwood, Texas) and reverse transcribed using Revertra Ace qPCR-RT kit (Toyobo Biologics Inc., Osaka, Japan). The polymerase chain reaction was performed at 94°C for 2 min, at 94°C for 30 s, at 55°C for 30 s and at 68°C for 1min for 30 cycles. The following primers were used: rat primer; TNF- α (QT00178717), IL-4 (QT01590316), and GAPDH (QT01658692), obtained from Qiagen (Valencia, CA, USA), rat primer; IL-10 (5'-AGG CGC TGT CAT CGA TTT CT-3' (forward) and 5'-AGG AAG AAC CCC TCC CAT CA-3'(reverse)) and Interferon

gamma (IFN- γ) obtained from Bioneer Corporation (Seoul, Korea). TNF- α , IL-4, IL-10 and IFN- γ mRNA levels were normalized relative to GAPDH mRNA levels

2.6 Western blotting.

Proteins in cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gels containing 10% polyacrylamide (10% SDS-PAGE), and then transferred to nitrocellulose membranes (Millipore; Billerica, MA, USA). The membranes were incubated in 5% non-fat milk for 1 h to block non-specific binding sites, and then probed with the primary antibodies for the following proteins: phospho-LAT, phospho-Gab2, phospho-Syk, phospho-extracellular signal-related kinases (ERK1/2), and β -actin. The membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse conjugates obtained from Pierce (Rockford, IL, USA). Bands corresponding to the proteins were detected using enhanced chemiluminescence (SuperSignal[®] West Femto Enhancer Kit, Pierce, Rockford, IL, USA). Densitometry analysis was performed using odyssey LCI Image software (LI-COR Biosciences, Lincoln, Nebraska, USA) and Image J (National Institutes of Health, Bethesda, Maryland, USA)

2.7 Experimental animals.

Six-week-old male BALB/c mice were purchased from Orient Bio (Eumsung, Korea). Mice were housed in a controlled barrier facility at constant temperature (23 $\pm$ 2°C), humidity (35-60%), and 12 h light/12 h dark cycle. All experiments were authorized by, and performed

in compliance with, the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Gachon University (Seoul, Korea) (GIACUC-R2015014). In addition, all animal care was in accordance with standards set by the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC, USA).

2.8 Passive cutaneous anaphylaxis (PCA).

PCA was induced in BALB/c mice as previously described [22]. Briefly, mice were administered intradermal DNP-specific IgE (0.5 μ g) in the ear, followed 24 h later by an intravenous injection of 1mg/ml of antigen (DNP-BSA) containing 1% Evans blue. For the evaluation of anti-allergic activity, 30 or 100 mg/kg fisetin, or 10 mg/kg CZ, was administered 1 h before antigen administration. The mice were euthanized 1 h after treatment with the antigen, and each ear was excised to measure the amount of extravasated dye. The dye was extracted from the ear in 700 μ L of formamide at a temperature of 63°C. The absorbance was measured at a wavelength of 620 nm using a microplate spectrophotometer (Tecan, Mannedorf, Switzerland).

2.9 Hematoxylin and eosin (H&E) stain.

Formalin-fixed ear tissue was embedded in paraffin blocks, and then cut into 5 μ m sections. Each section was stained with hematoxylin (Polysciences, Inc., Pennsylvania, USA) and eosin (Shimakyu's Pure Chemicals, Osaka, Japan), and then observed under a light microscope for measurement of ear thickness. Digital photomicrographs were taken from

representative areas at a fixed magnification of 200 $\times$. Ear thickness was measured using Axiovision image analysis 4.7 software (Carl Zeiss Vision GmbH, Munich, Germany).

2.10 Toluidine blue stain.

Tissue sections were stained with toluidine blue (Sigma-Aldrich, St. Louis, MO, USA) for measurement of mast cell numbers. Sectioned tissues were stained with toluidine blue solution for 2-3 min, dehydrated in alcohol, and cleared in xylene. The total number of mast cells, and the number of degranulated mast cells per field of view was determined under the light microscope at both magnifications of 400 $\times$ and 1,000 $\times$.

2.12 Statistical analysis.

Results are presented as mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Dunnett's *t*-test were used to assess significant differences between treatment groups. Data were analyzed using the statistical package for the social sciences (SPSS)-12 software (one-way analysis of variance; ANOVA, Chicago, USA).

3. Results

3.1 Fisetin suppresses degranulation in IgE/Ag-stimulated RBL-2H3 cells.

To investigate the anti-allergic activity of fisetin, we evaluated its effect on the degranulation of IgE/Ag-stimulated RBL-2H3 cells. The rate of β -hexosaminidase release was measured as a marker of degranulation [23]. Fisetin inhibited degranulation in IgE/Ag-stimulated RBL-2H3 cells in a dose-dependent manner (Fig. 1). The half-maximal inhibition value (IC_{50}) for the inhibition of degranulation by fisetin was $13.61 \pm 0.17 \mu M$ (Fig. 1B). At concentrations in the range 3 to 30 μM , fisetin showed no cytotoxic effects (Fig. 1C)

3.2 Inhibition of pro-inflammatory cytokine gene expression by fisetin in IgE/Ag-stimulated RBL-2H3 cells.

Allergic responses are aggravated by pro-inflammatory cytokines such as IL-4 and TNF- α . We therefore determined whether fisetin suppressed IL-4 and TNF- α mRNA gene expression in IgE/Ag-stimulated RBL-2H3 cells, using RT-PCR. At concentrations of 10 μM and 30 μM , fisetin showed strong inhibition of IL-4 and TNF- α gene expression (Fig. 2).

3.3 Fisetin inhibited activation of MAPKs, NF κ B and STAT3 in IgE/Ag-stimulated RBL-2H3 cells.

To elucidate the anti-allergic mechanism of fisetin, we evaluated whether fisetin could affect the level of activated Syk protein and its downstream signaling molecules. As

shown in Fig. 3, the level of phosphorylated Syk protein was suppressed by fisetin in a dose-dependent manner. Phosphorylation of the downstream signaling molecules, Gab2 and LAT, also exhibited significant and dose-dependent inhibition by fisetin (Fig. 3). In addition, we examined the effects of fisetin on MAP kinases, because of their known role in the production of TNF- α and IL-4. The IgE/Ag-induced phosphorylation of ERK1/2 was found to be inhibited by fisetin in a dose-dependent manner (Fig. 3). Previously it was reported that NF κ B was activated in the Fc ϵ RI-dependent signaling pathway [24]. Our data indicate that fisetin reduced the level of phosphorylated NF κ B protein. Previous report described that the level of phosphorylated STAT3 (Tyr705 or Ser727) is increased during basophils and mast cell degranulation. Next we determined the level of phosphorylated STAT3 since ERK1/2 phosphorylates STAT3 [25]. Phosphorylated STAT3 (Tyr705 or Ser727) was also reduced by fisetin in a dose-dependent manner. Meanwhile, the levels of total Syk, Gab-2, LAT, ERK and STAT3 protein were not altered. These results indicate that fisetin inhibits IgE/Ag-induced Fc ϵ RI-mediated degranulation by decreasing the phosphorylation of Syk, Gab2, LAT, ERK, NF κ B and STAT3.

3.4 Fisetin inhibited Fc ϵ RI receptor α subunit mRNA expression in RBL-2H3 cells.

To determine whether fisetin is able to suppress the cell surface expression of Fc ϵ RI α , β , or γ subunits, we examined the effect of fisetin on the mRNA expression levels for each subunit (Fig. 4). Cells treated with 30 μ M fisetin exhibited a significantly lower level of Fc ϵ RI α -subunit mRNA expression than that in the control cells. These data suggest that

fisetin may exert its anti-allergic properties via down-regulation of FcεRI α subunit expression.

3.5 Inhibitory effect of fisetin on IgE-mediated PCA in BALB/c mice.

Mast cells play prominent roles in allergic responses and anaphylaxis. The binding of antigen-specific IgE to IgE receptors on mast cells induces the PCA reaction, an IgE-mediated type I allergic response in the dermis. In this study, to test whether fisetin suppresses IgE-mediated type I allergic response *in vivo*, fisetin was orally administered to mice with PCA, which immediate hypersensitivity reaction in the dermis is caused by IgE-dependent degranulation of mast cells [26]. To investigate whether fisetin inhibited allergic responses, we used a mast cell-dependent PCA model. IgE was injected subcutaneously into mouse ear tissue, followed by injection of DNP-BSA containing 5% Evans Blue. Oral administration with fisetin at doses of 30 mg/kg and 100 mg/kg significantly suppressed IgE/Ag-induced PCA as observed by impaired extravasation of Evans blue dye in the ear. (Fig. 5A and 5B). The dose of fisetin having the half-maximal effect (EC_{50} value) was 94 mg/kg. The effect of fisetin was similar to that of 10 mg/kg CZ, a typical antihistamine drug, which was used as a positive control.

3.6 Effects of fisetin on histopathological changes of ear tissues in mice with PCA.

For histopathological analysis, we cut the ear from fisetin-treated mice and stained it with hematoxylin/eosin dye. The ear tissues of mice with PCA exhibited a massive infiltration of inflammatory cells (Fig. 5C) in the epidermis and dermis, which was coincident with the earlobe swelling. Oral administration of fisetin reduced the number of infiltrated inflammatory cells and ear thickness (Fig. 5C). Mast cells are important effector cells, involved in allergic contact dermatitis (ACD). These cells are subsequently releasing granular mediators, such as prostaglandin, leukotrienes and a large variety of cytokine [27]. To check whether fisetin diminished the infiltration and degranulation of mast cells in the skin lesion, we stained the ear tissue with toluidine blue dye. The numbers of granulated (5.0 ± 1.4 and 5.5 ± 0.7) and degranulated (0.5 ± 0.7 and 3.0 ± 2.8) mast cells decreased after fisetin oral administration (30 and 100 mg/kg/day) compared to those (granulated (8.5 ± 2.1) and degranulated (9.5 ± 2.1) mast cells) (Fig. 5D).

3.7 Inhibitory effect of fisetin against cytokine expression and activated NF κ B and STAT3 expression in mouse ear with PCA symptoms.

To confirm the anti-allergic activity of fisetin, we checked the level of IFN- γ , IL-10 mRNA expression and the level of phosphorylated NF κ B and STAT3 protein expression. We observed that fisetin was increased the level of IFN- γ and IL10 mRNA expression in the ear tissue of PCA mice. In addition, the level of phosphorylated NF κ B and STAT3 protein expression and the level of TNF- α protein expression was decreased (Fig. 6). These data indicated that fisetin decreased IgE/Ag-mediated PCA symptoms mice.

4. Discussion

In the present study, we showed that fisetin inhibited type I hypersensitive reactions using both *in vivo* and *in vitro* models. Type I immediate hypersensitivity reactions involve immunoglobulin E (IgE)-mediated release of histamine and other inflammatory mediators from mast cells and basophils [28]. Examples include anaphylaxis and allergic rhinoconjunctivitis. Many reports have described that IgE/Ag-stimulation of mast cells and basophils initiate the activation of a signal transduction pathway, leading to degranulation. The effect of fisetin was tested in the IgE/Ag-induced passive cutaneous anaphylaxis reaction. Cetrazine (CZ), used as a positive control in this study, is a medication used in the clinic for relief of the cutaneous symptoms of anaphylaxis, e.g. urticaria, pruritus, and angioedema. We observed that fisetin-administered mice showed decreased PCA reaction. These findings suggest that fisetin could be useful for treating allergic reactions.

To elucidate the anti-allergic effect of fisetin that we observed in our animal study, we evaluated the effect of fisetin in IgE/Ag-stimulated RBL-2H3 cell cultures. Basophils and mast cells are the primary effector cells involved in IgE/Ag-mediated allergic responses, during which they secrete cytokines and other inflammatory mediators. FcεRI signaling is involved in the amplification of degranulation in mast cells or basophils [29]. Among the pro-inflammatory cytokines, TNF-α is a major cytokine stored and released by mast cells and basophils. It promotes inflammation, leukocyte infiltration, and chemotaxis of both neutrophils and T cells. TNF-α is also involved in eosinophil survival, thereby contributing to chronic inflammation [30]. IL-4 is produced by Th2 cells, as well as mast cells and basophils, and plays important roles in allergic inflammation, including mast cell and basophils activation, and isotype switching to IgE synthesis in B cells [31]. We observed inhibition of degranulation by fisetin in IgE/Ag-stimulated RBL-2H3 cells. Fisetin significantly inhibited

TNF- α and IL-4 gene expression in IgE/Ag-stimulated RBL-2H3 cells (Fig. 2). Most of previous studies with fisetin demonstrated its anti-allergic activity in cellular level only, but *in vivo* testing against Type I hypersensitivity reaction is a critical step for clinical application and current study investigated this hypothesis. The ear tissue of mice with PCA symptoms showed the number of inflammatory cells and the ear thickness. Our data support this hypothesis that fisetin showed less number of infiltrated inflammatory cells and degranulated mast cells and the ear thickness than housed without fisetin treatment (Fig. 5).

Consistent with *in vitro* data, fisetin decreased the level of TNF- α protein expression in the ear tissue with PCA reaction. Several groups reported that IL-10 producing B cells were suppressing IgE mediated anaphylaxis in mice [32, 33]. Also, IL-10 act as a potential anti-inflammatory cytokine that suppresses allergic inflammation [34]. IFN- γ reduced allergic inflammation and inhibit IgE synthesis [9], [10]. Fisetin increased the level of IL-10 and IFN-gamma mRNA expression in the ear tissue (Fig. 6). Using dose scaling as advised in FDA guidance the human equivalent dose of fisetin (oral doses of 30 mg/kg and 100 mg/kg) in our *in vivo* study translated to approximately 2.4mg/kg to 8mg/kg fisetin in a 60 kg adult and represents a reasonable starting point for future clinical investigations.

In mast cells and basophils, intracellular allergic signaling is initiated when IgE and antigen bind to the Fc ϵ RI receptor, which is a tetrameric complex of protein subunits [35]. Fc ϵ RI receptor signaling is transduced by spleen tyrosine kinase (Syk). Syk becomes phosphorylated after binding to the immunoreceptor tyrosine-based activation motifs (ITAMs) of the Fc ϵ RI β and γ subunits. Phosphorylated tyrosine residues on Syk then act as docking sites for adaptor molecules, including Gab2 and LAT, which are then phosphorylated by Syk to propagate downstream signaling [22, 36]. Signal transduction events downstream of activated Syk subsequently lead to degranulation. The tyrosine residues on the adaptor

proteins, Gab2 and LAT, also serve as docking sites for the assembly of additional signaling molecules [21]. In addition, it is reported that activated NF κ B signaling pathway in mast cells lead to the generation of allergic reaction, including PCA [24]. Inhibition of STAT3 signaling reduced the degranulation in activated mast cells [37]. We showed that fisetin inhibited the phosphorylation of Syk, Gab2, and LAT, NF κ B and STAT3 in the IgE/Ag stimulated RBL2H3 cell (Fig. 3) and the level of phosphorylated NF κ B and STAT3 proteins. Consistent with *in vitro* data, fisetin decreased the level of activated NF κ B and STAT3 protein expression. These data suggest that the anti-allergic activity of fisetin is due to the inhibition of activated Syk, Gab2, LAT, NF κ B and STAT3 proteins.

The tetrameric structure of Fc ϵ RI consists of one α , one β , and two γ protein subunits. The α chain extends into the extracellular space and binds with high affinity to the Fc portion of IgE, while the γ chains participate in signal transduction. The β chain is an amplifier of signal intensity, and stimulates the expression of unoccupied receptors [38]. In Fc ϵ RI α -deficient mice, it has been demonstrated that IgE cannot bind to the cell surface of mast cells. As a result, degranulation could not be induced by IgE [38]. Fisetin-mediated suppression of degranulation may therefore depend on the inhibition of Fc ϵ RI α subunit expression, resulting in decreased Fc ϵ RI receptor levels and diminished IgE/Ag-mediated allergic responses. The mechanism by which fisetin downregulates Fc ϵ RI α subunit mRNA expression remains unclear. In further studies, it would be worth examining the effect of fisetin on the transcriptional activity of specific transcription factors required for expression of the Fc ϵ RI subunit genes (Fig. 4).

In conclusion, fisetin suppressed degranulation, and inhibited the release of pro-inflammatory cytokines in IgE/Ag-stimulated RBL-2H3 cells. This is supported by *in vivo*

experiment that fisetin attenuated the PCA reaction, which is introduced by IgE/Ag-stimulation in the ear of BALB/c mice. In IgE/Ag-stimulated RBL-2H3 cells, fisetin inhibited the phosphorylation of downstream signaling molecules that were activated by the FcεRI receptor, including Syk, LAT, Gab2, ERK1/2, NFκB and STAT3. In addition, fisetin decreased mRNA expression of the FcεRI α subunit. Thus, fisetin inhibited the signal transduction cascade responsible for allergic responses. Our findings suggest that fisetin has potential as a therapeutic agent for preventing or treating IgE-mediated allergic diseases.

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Conflict of Interests

The authors declare no conflict of interests.

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Figure legends

Fig. 1. Effect of fisetin on the degranulation in IgE/Ag-stimulated RBL-2H3 cells.

(A) Chemical structure of fisetin. (B) The degree of degranulation in RBL-2H3 cells was determined by measuring the amount of β -hexosaminidase released. RBL-2H3 cells were treated with 0, 10, or 30 μ M, fisetin, or 10 μ M PP2, for 30 min before antigen stimulation. (C) Cell viability in the presence of 0, 3, 10, or 30 μ M fisetin was determined using an EZ-CyTox kit. Data are expressed as mean $\pm$ SEM (n = 3). One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test ($^{$$$}p < 0.001$ vs. control; $^{***}p < 0.001$ vs. IgE/Ag-stimulated control).

Fig. 2. Effect of fisetin on the level of TNF- α and IL-4 mRNA expression in IgE/Ag-stimulated RBL-2H3 cells.

RBL-2H3 cells were treated with 0, 10, or 30 μ M fisetin, or 10 μ M PP2 for 30 min, and then stimulated with antigen for 4 h. TNF- α and IL-4 mRNA expression levels were evaluated by RT-PCR. (A) Representative results from three or more independent experiments are shown. (B-C) The results of the densitometric analysis of gel images are shown. Data are expressed as mean $\pm$ SEM (n=3). One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test ($^{$$}p < 0.01$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. IgE/Ag-stimulated control)

Fig. 3. Effect of fisetin on the intracellular signaling pathways in IgE/Ag-stimulated RBL-2H3 cells.

(A) RBL-2H3 cells were treated with 0, 10 or 30 μ M fisetin, or 10 μ M PP2, for 30 min, then stimulated with antigen for 10 min. Total protein lysates were analyzed by immunoblotting analysis for phosphorylated – and total forms of Syk, Gab2, LAT, ERK, NF κ B, and STAT3 proteins. Actin was used as the internal control. (B) The results of the densitometric analysis of gel images are shown. Data are expressed as mean $\pm$ SEM (n = 3). One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test ($^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. control; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. IgE/Ag-stimulated control).

Fig. 4. Effect of fisetin on Fc ϵ RI α , β , and γ mRNA expression levels in IgE/Ag-stimulated RBL-2H3 cells.

RBL-2H3 cells were treated with 0, or 30 μ M fisetin, for 24 h. Fc ϵ RI α , β and γ mRNA expression levels were determined by RT-PCR Representative results from three or more independent experiments are shown.

Fig. 5. Effect of fisetin in a murine model of IgE/Ag-mediated passive cutaneous anaphylaxis (PCA).

Seven-week-old BALB/c mice were given intradermal injections of 0.5 μ g DNP-IgE into an ear, and 24 h later were injected intravenously with 200 μ g of antigen containing 1% Evans blue. Fisetin or CZ was administered orally 1 h before antigen injection. (A) Representative photographs of ears are shown. (B) The Evans blue dye was extracted in 700 μ L of

formamide by overnight incubation at a temperature of 63°C, and absorbance was measured at a wavelength of 620 nm. Data are expressed as mean $\pm$ SEM (n = 3). One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test ($^{$$$}p < 0.001$ vs. control; $^*p < 0.05$ vs. IgE/Ag-mediated PCA). (C) The histological changes of ear tissues were observed after H&E staining. Scale bar = 20 μ M. (D) Toluidine blue stained tissue was used to monitor the changes in the number of degranulated mast cells after oral gavage of fisetin (0, 30, 100 mg/kg). Scale bar = 20 μ M. Data are expressed as mean $\pm$ SEM (n = 3). One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test ($^{$$$}p < 0.001$ and $^{\#}p < 0.05$ vs. control; $^*p < 0.05$ vs. IgE/Ag-mediated PCA).

Fig. 6. Effect of fisetin on the level of phosphorylated NF κ B and STAT3 protein expression and cytokine mRNA expression in the ear tissue with PCA symptoms.

(A) The levels of phosphorylated- NF κ B and -STAT3 protein expression were determined by western blot in the ear tissue with PCA after oral gavage of fisetin (0, 30, 10 mg/kg). (B) The level of IL-10 and IFN- γ mRNA expression were determined by RT-PCR analysis (upper panel). The graph shows the densitometer quantification of immunoblots from three independent experiments (down panel). Data are expressed as mean $\pm$ SEM and representative of at least 3 independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test ($^{\#}p < 0.05$ vs. control; $^*p < 0.05$ vs. IgE/Ag-mediated PCA).

Fig.1. Effect of fisetin on the degranulation in IgE/Ag-stimulated RBL-2H3 cells.

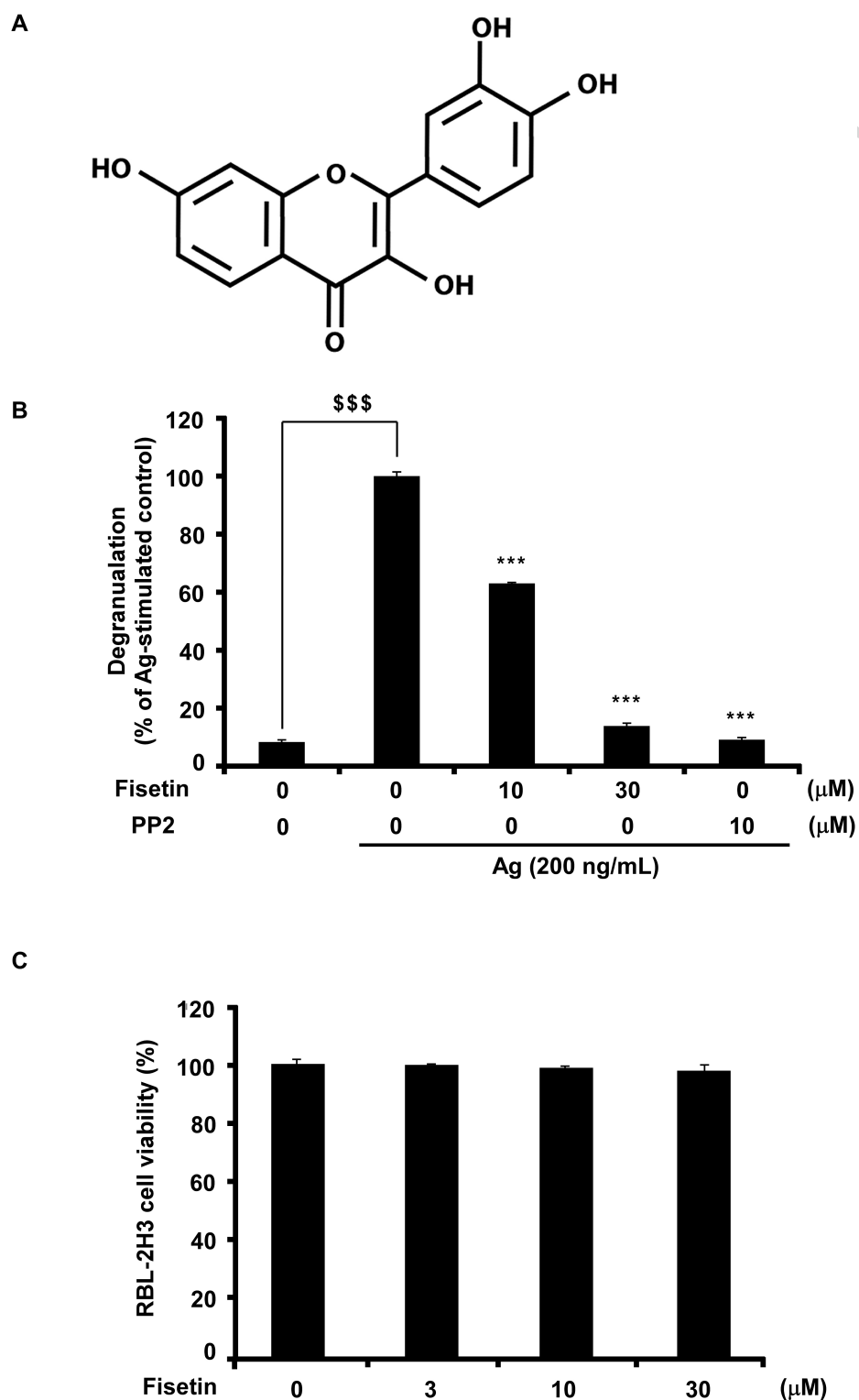


Fig. 2. Effect of fisetin on TNF- α and IL-4 mRNA expression levels in IgE/Ag-stimulated RBL-2H3 cells.

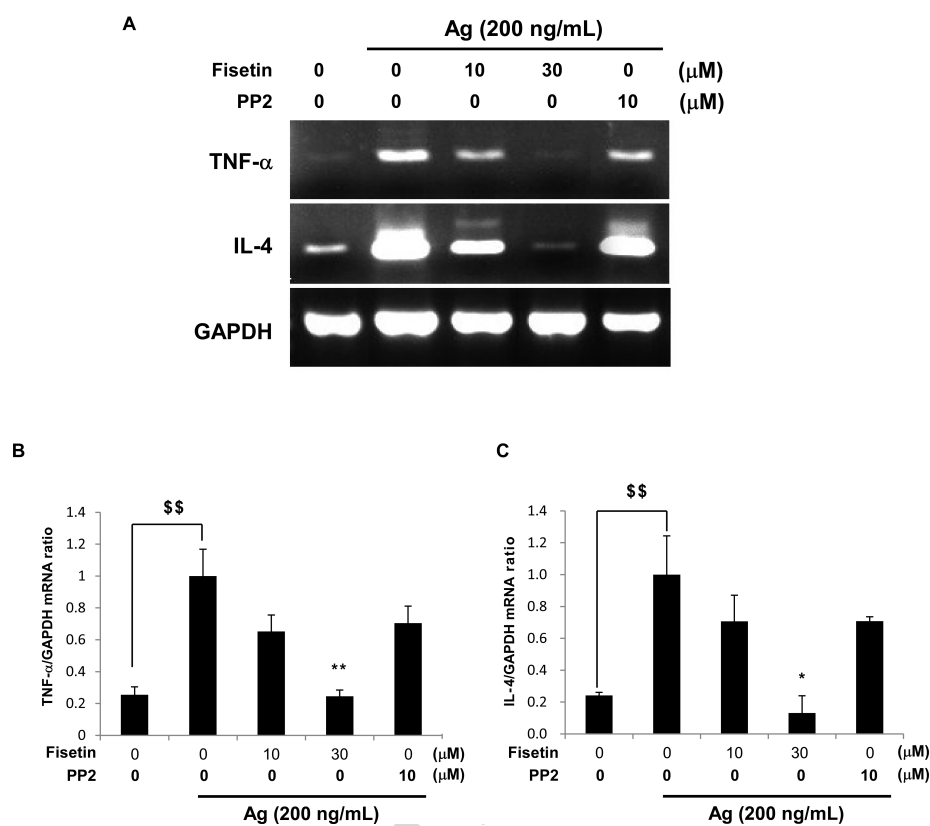
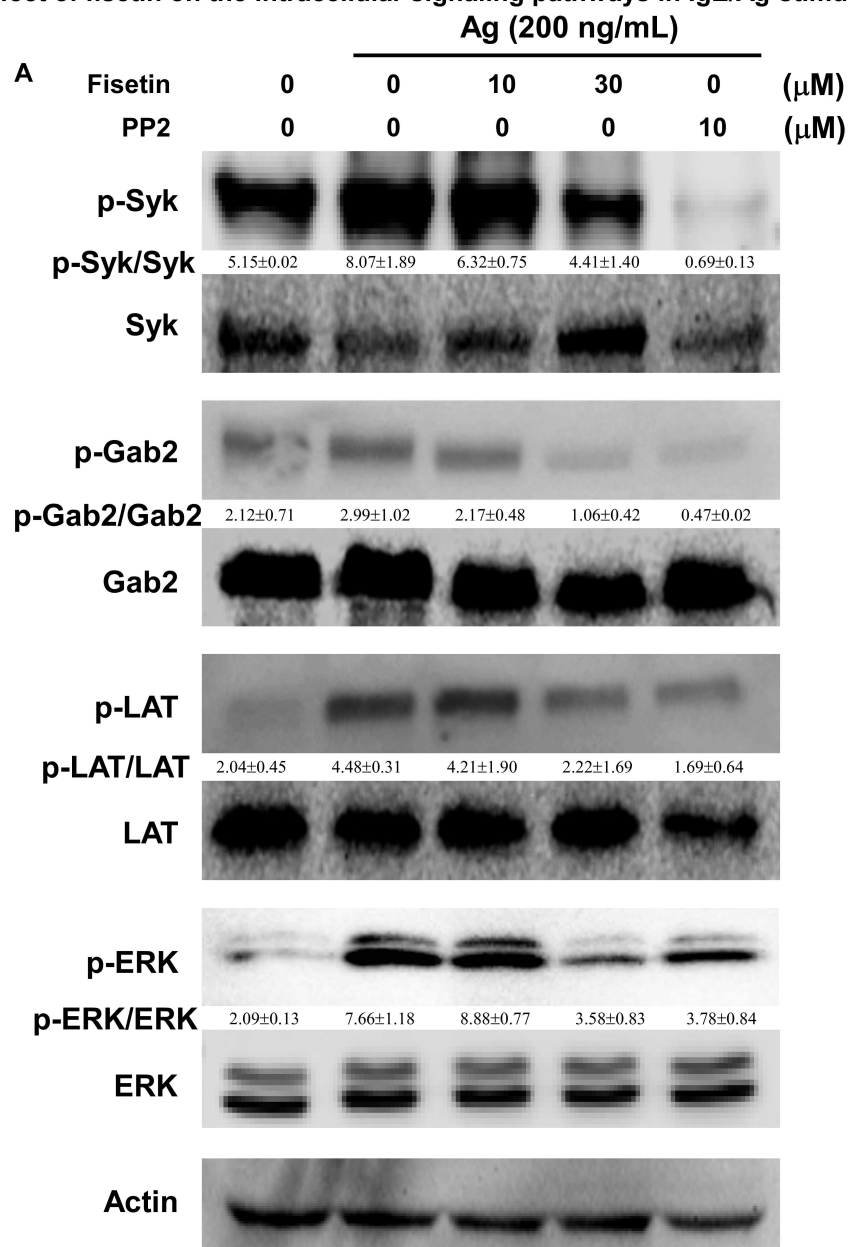


Fig. 3. Effect of fisetin on the intracellular signaling pathways in IgE/Ag-stimulated RBL-2H3 cells.



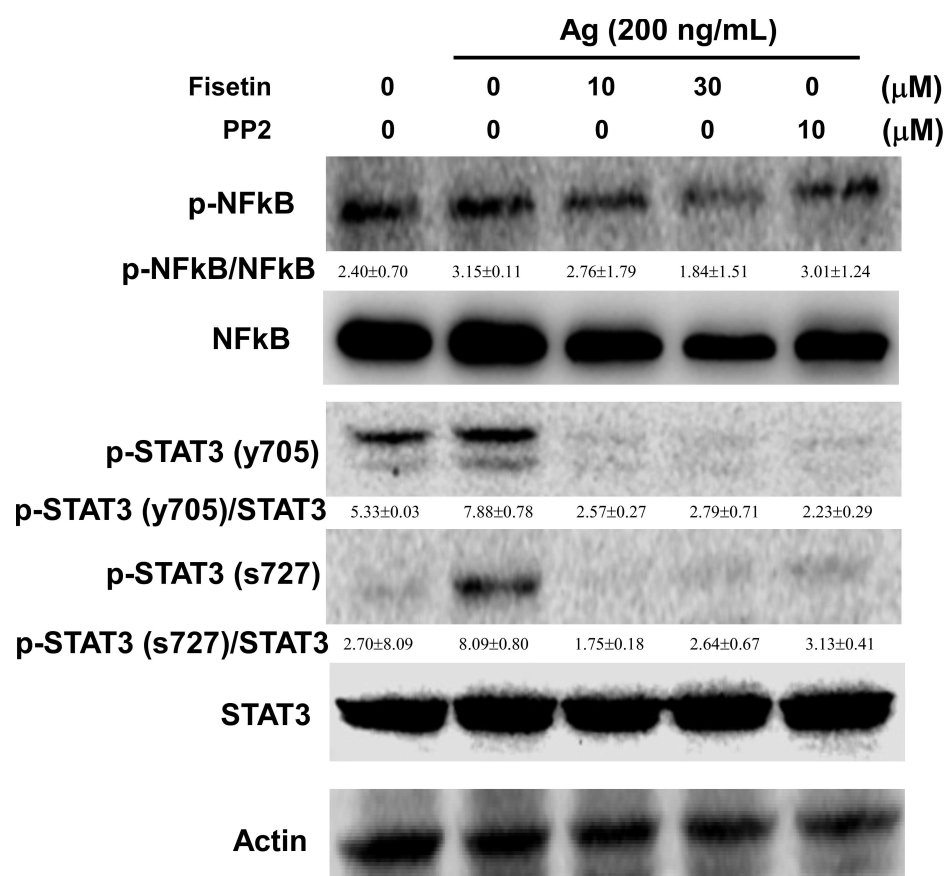


Figure 3-2

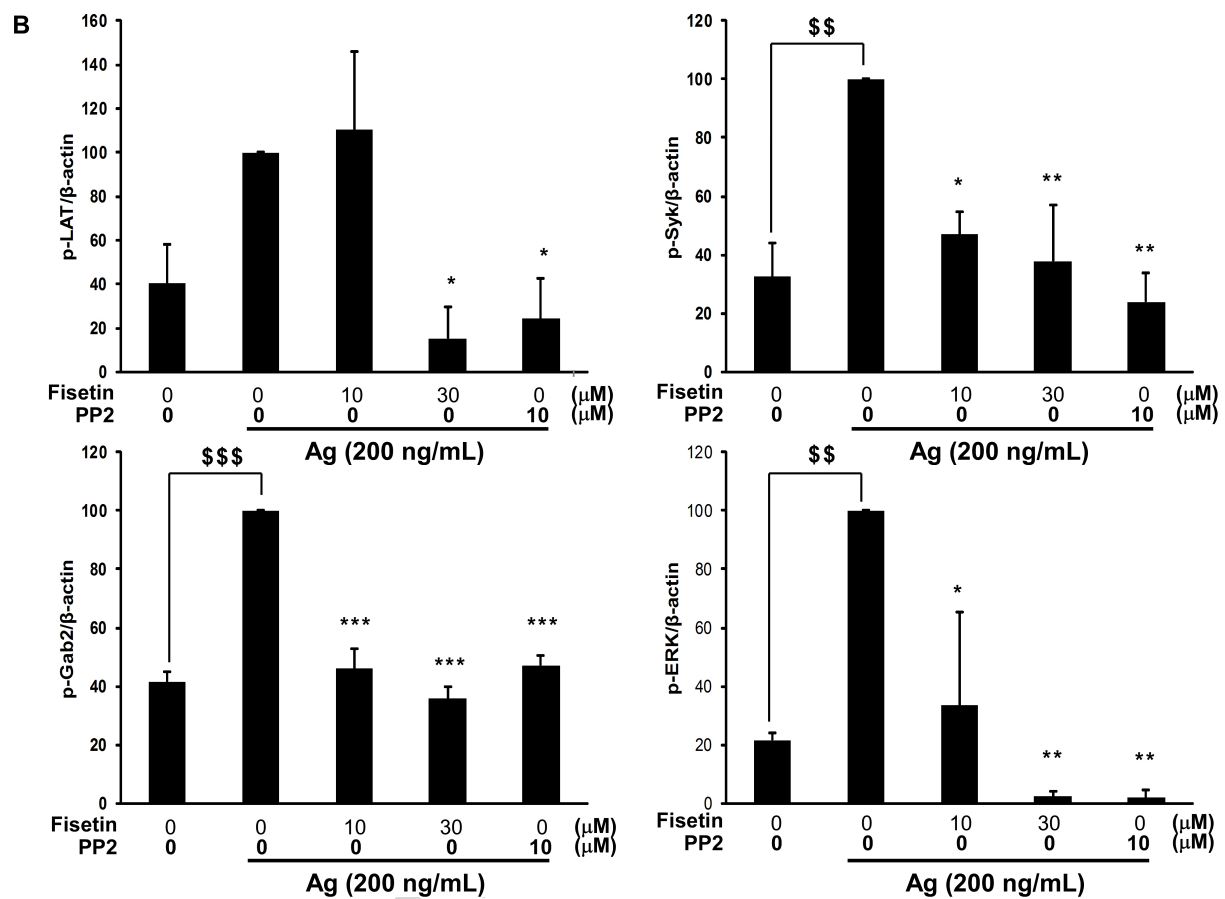


Figure 3-3

Fig. 4. Effect of fisetin on Fc ϵ RI and mRNA expression levels in IgE/Ag-stimulated RBL-2H3 cells.

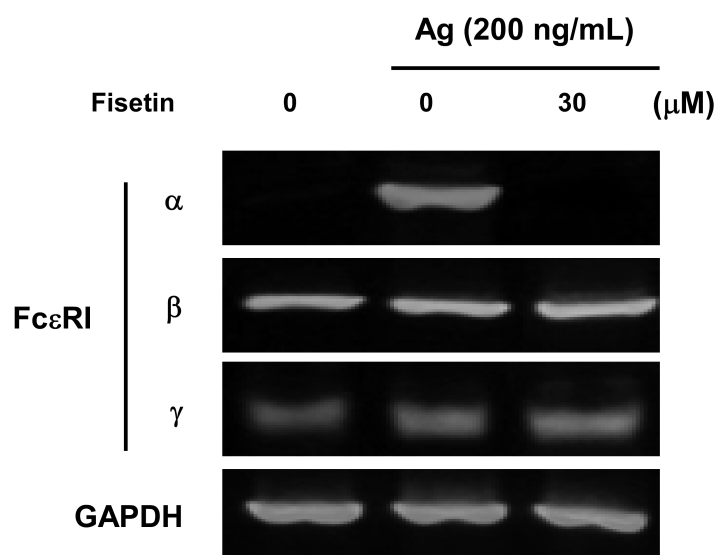
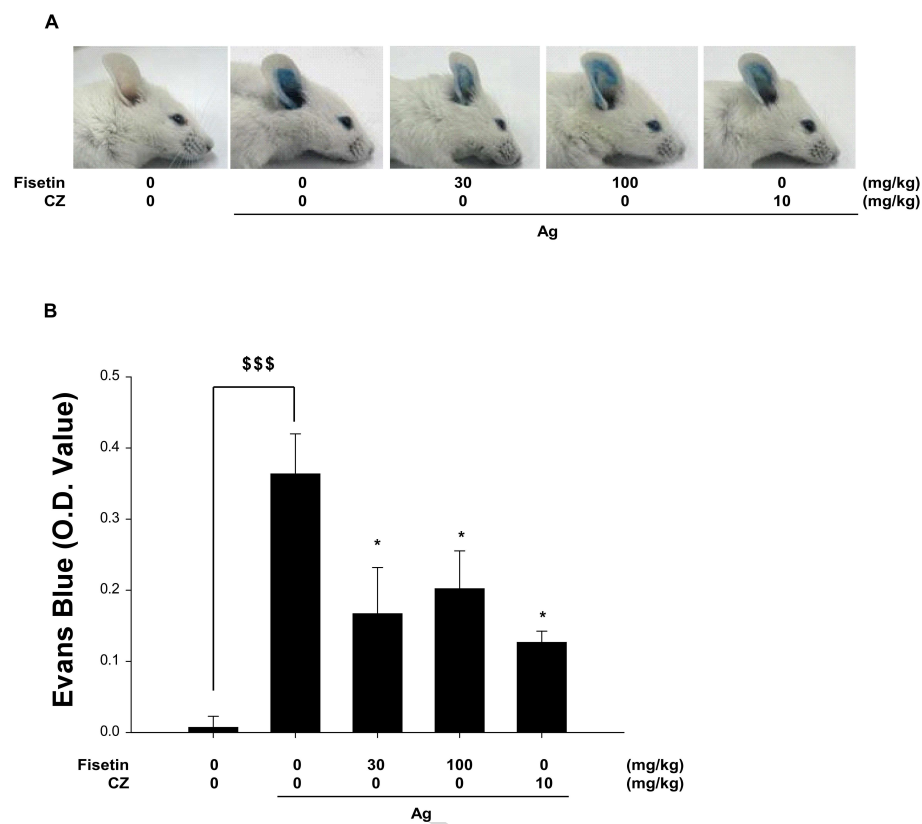


Fig. 5. Effect of fisetin in a murine model of IgE/Ag-mediated passive cutaneous anaphylaxis (PCA).



C

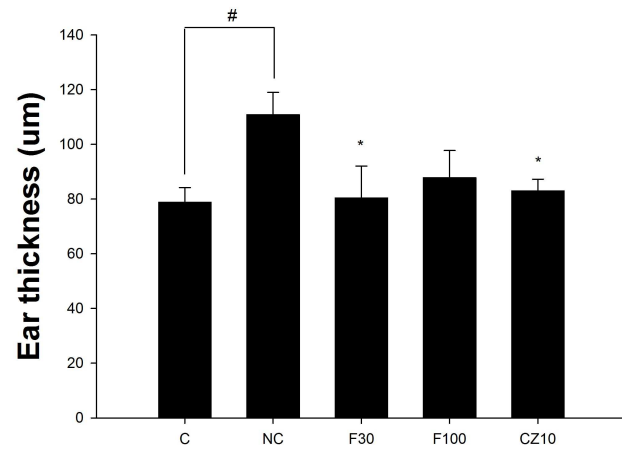
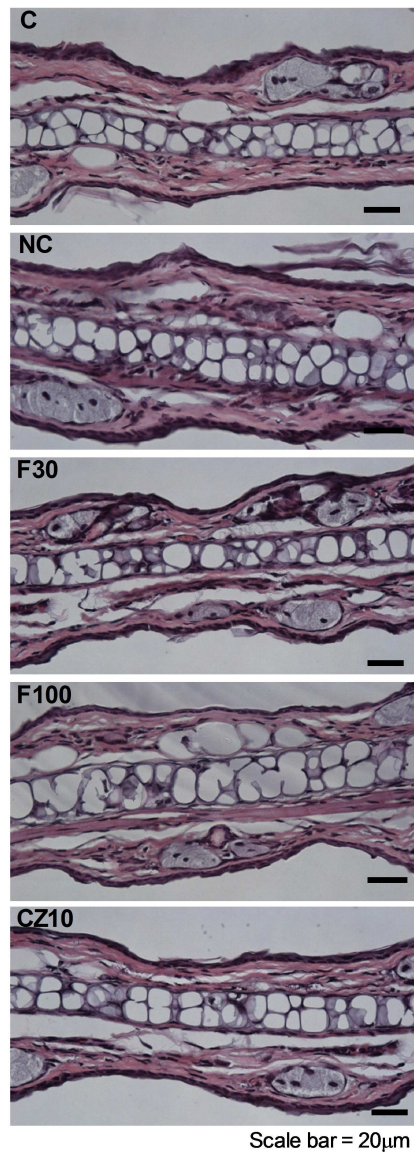


Figure 5-2

D

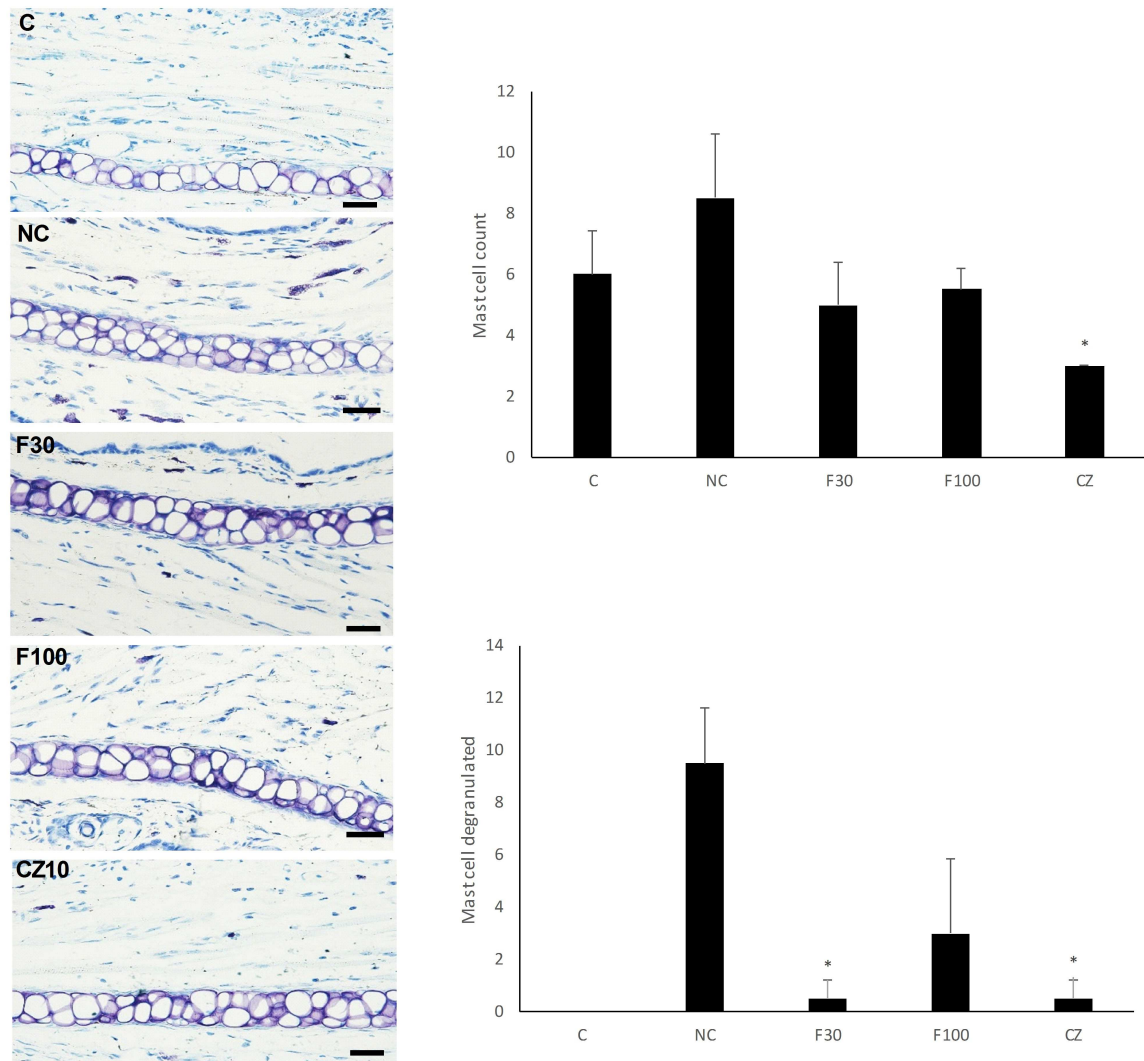
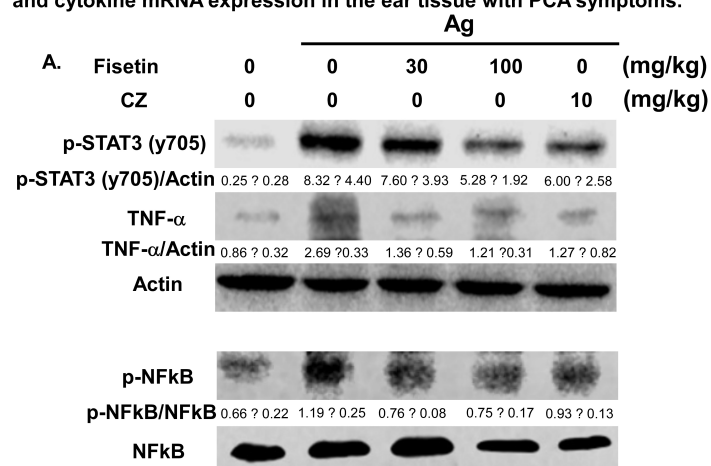


Figure 5-3

Fig. 6. Effect of fisetin on the level of phosphorylated NFkB and STAT3 protein expression and cytokine mRNA expression in the ear tissue with PCA symptoms.



B.

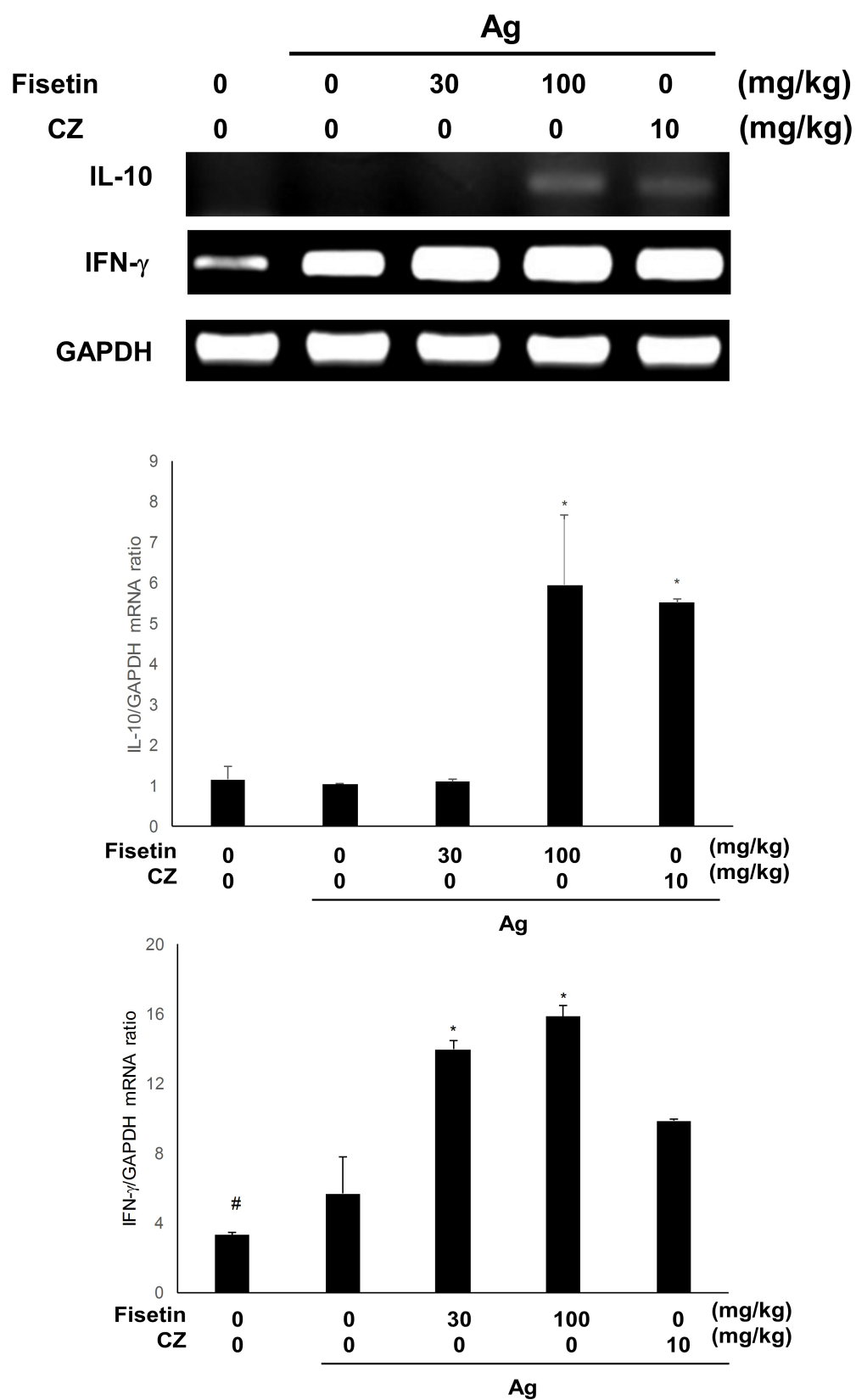
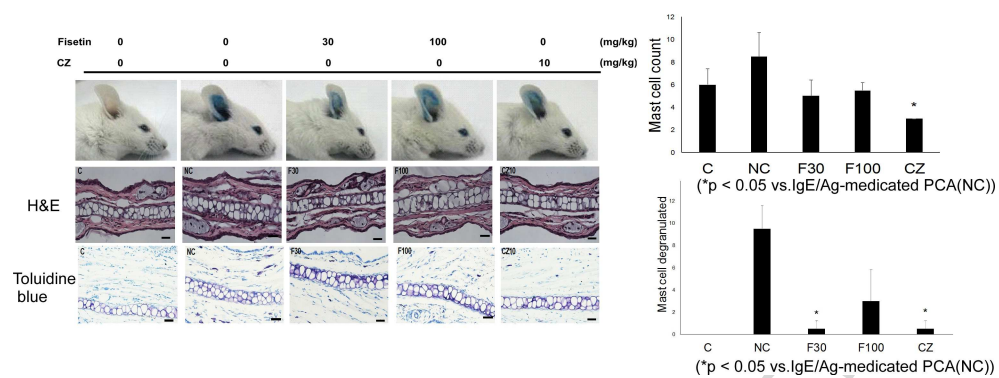


Figure 6-2



Graphical abstract