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Down-regulation of T helper 2-associated Cytokine Expression and Selective Transcription Factors by Fisetin

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Fisetin, a flavonol, has been known as an anti-allergic agent having inhibitory effects on T helper 2 cytokine gene expression in inflammatory immune cells. However, its molecular mechanisms for suppressive effects of fisetin on interleukin (IL)-4 and IL-13 in activated mast cells and basophils have been incompletely understood. In this study we found that fisetin at the concentrations having no effect on cell viability significantly inhibited the phorbol 12-myristate 13-acetate and ionomycin induced production of IL-4 and IL-13 in bone marrow-derived mast cells and RBL-2H3 basophilic cells. The levels of mRNA were dramatically decreased by fisetin, indicating the suppression might be regulated at the transcriptional levels. Transient transfection experiments using luciferase reporter plasmids expressing IL-4 or IL-13 promoter revealed that fisetin inhibited the activation of the promoters in a dose-dependent manner. Western blot analysis of the nuclear expression of various transcription factors involved in the promoter activation indicated that suppressions of c-Fos and CCAAT/enhancer-binding protein alpha were prominent together with significant down-regulations of nuclear factor of activated T cell (NF-AT) and NF-κB. Furthermore, nuclear expression of GATA-1 and GATA-2, and the mRNA expression were significantly down regulated by fisetin, whereas cyclosporine A had no significant effects on GATA transcription factors. Taken together, fisetin has suppressive effects on IL-4 and IL-13 gene expressions through the regulation of selective transcription factors.

Key words: basophils, fisetin, interleukin (IL)-4, IL-13, mast cells, transcription factors

Mast cells and basophils have been regarded as critical effecter cells in allergic disease such as asthma and atopic dermatitis. Mast cells are derived from the bone marrow as progenitors and migrate to peripheral tissues such as the skin, intestine, mucosa, and airway where they differentiate into mature mast cells [Gilfillan and Tkaczyk, 2006]. In contrast, basophils complete their maturation in the bone marrow before entering blood stream and circulate as mature granulocytes. When mast cells and basophils are activated by the cross-linking of cell-bound IgE and high-affinity immunoglobulin E receptor (FcεRI) complex with antigens or IgE-independent stimuli, they release *de novo* synthesized T helper (Th) 2 cytokines including interleukin (IL)-4 and IL-13 [Rivera and Gilfillan, 2006]. Basophils are efficient IL-4 producers [Oh *et al.*, 2007]. IL-4 mediates Th2 polarization in lymph node, leading to allergic status, and also initiates allergic inflammation by inducing IgE isotype switching in B cells and IgE-mediated degranulation. IL-13 directly induces bronchial smooth muscle contraction and eosinophil accumulation in asthmatic respiratory tracts, resulting in amplification of allergic symptoms [Wills-Karp and Chiaramonte, 2003; Monticelli *et al.*, 2004]. Acute atopic dermatitis skin lesions are associated with an increased expression of IL-13, suggesting IL-13 could play a role in the pathogenesis of atopic dermatitis [Hamid *et al.*, 1996]. Therefore, suppression of Th2 cytokine gene expression by mast cell and basophil appears to be a strategy for control of allergic diseases.

Fisetin (3,7,3',4'-tetrahydroxyflavone), a flavonol, has been known as an anti-allergic agent having inhibitory effects on Th2 cytokine productions in human basophils, and inhibits proinflammatory cytokine productions in mast cells [Higa *et al.*, 2003; Park *et al.*, 2007]. However,

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studies on its molecular mechanisms for the suppressive effects on IL-4 and IL-13 in mast cells and basophils are scarce. Expressions of IL-4 and IL-13 gene are coordinately regulated by several selective transcription factors including activator protein 1 (AP-1), nuclear factor of activated T cells (NF-AT), NF-κB, as well as GATAs and CCAAT/enhancer-binding protein alpha (C/EBPα) [Qi *et al.*, 2011] in a cell type-specific manner. The major component of AP-1 is a heterodimer of c-Jun and c-Fos, and AP-1 activation is dependent on the induction of c-Jun and c-Fos genes and the phosphorylation of c-Jun and c-Fos protein. Recently, it has been reported that cross-linking of the high affinity receptor for IgE induces activation of AP-1 through protein kinase D for the IL-13 production in RBL-2H3 cells [Yamashita *et al.*, 2010]. NF-ATc1 and NF-ATc2 regulated by Ca^{2+}-dependent phosphatase calcineurin and nuclear localization, are known to be critically involved in the expression of mast cell-derived IL-13 [Klein *et al.*, 2006]. Furthermore, NF-AT complex is associated with mast cell IL-4 transcription complex [Weiss *et al.*, 1996]. NF-κB, a heterdimeric transcription factor composed of p65 and p50, is known to be activated by FcεRI cross-linking to produce IL-13 but not IL-4 in human intestinal mast cells [Lorentz *et al.*, 2003]. The regulatory mechanisms for IL-4 and IL-13 expressions in basophils have been seldom studied. Recently, C/EBP has been reported as a critical transcription factor for IL-4 expression [Qi *et al.*, 2011] in basophil. GATA-1 and -2, which are mainly expressed in myeloid cells including mast cells and basophils, have been known to play crucial roles in the expression of IL-4 and IL-13 in activated mast cells [Henkel and Brown, 1994; Masuda *et al.*, 2004]. Here, we show that fisetin suppresses IL-4 and IL-13 gene expressions at the transcriptional levels in mast cells as well as basophils, which could be associated with the down-regulation of selective transcription factors involved in the promoter activation.

Materials and Methods

Reagents and cell culture. Fisetin, phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma (St Louis, MO). Cyclosporine A (CsA) was obtained from Calbiochem (La Jolla, CA), lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA) and luciferase substrate solution was purchased from Promega (Madison, WI). RBL-2H3, a rat basophilic leukemia cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Eagle's minimal essential medium (EMEM) medium (Lonza, Walkersville, MD) supplemented with 15% fetal bovine serum (Invitrogen) and 100 mg/mL penicillin-streptomycin (Lonza) at 37°C in a 5% CO_2-humidified air atmosphere.

Bone marrow-derived mast cell (BMMC) culture. Six-week-old female BALB/c mice were obtained from Samtako Bio Korea (Osan-si, Korea). BMMCs were derived from femoral bone marrow cells of the mice. Cells were cultured with IL-3 (10 ng/mL) for 3 weeks and then cultured with IL-3 plus stem cell factor (SCF) (10 ng/mL each) for 1 week. Complete differentiation took 4–6 weeks. c-Kit positive, a mast cell marker, made up 98% of the cells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) assay. BMMCs (2×10^6 cells/mL) and RBL-2H3 cells (7×10^5 cells/mL) were grown in the presence of fisetin at 7.5 to 120 μM at 37°C in 5% CO_2 incubator. After 24 h incubation, viable cells were stained with 50 μL of MTT (3 mg/mL) for 4 h at 37°C. The medium was removed, and the formazan crystals were dissolved in 50 μL of DMSO. Absorbance was measured at 540 nm using an ELISA microplate reader.

ELISA. BMMCs (2×10^6 cells/mL) and RBL-2H3 cells (3×10^5 cells/mL) were treated with PMA (50 ng/mL) and ionomycin (0.5 μM) for 16 h in the absence or presence of fisetin. The levels of IL-4 and IL-13 in the culture supernatant were determined using a commercially available ELISA kit (BD Science, San Diego, CA; Invitrogen) according to manufacturer's instructions.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). BMMCs (2×10^6 cells/mL) and RBL-2H3 cells (3×10^5 cells/mL) were treated with PMA (50 ng/mL) and ionomycin (0.5 μM) for 6 h in the absence or presence of fisetin. Total RNAs were isolated by the single-step method using the TRIzol reagent (Invitrogen). Each sample was reverse-transcribed to cDNA for 60 min at 42°C using a cDNA synthesis kit (iNtRON Biotechnology, Seongnam-si, Korea). The sequences of PCR primer are as follows: mouse IL-4 sense, 5'-ATG GGT CTC AAC CCC CAG C-3'; mouse IL-4 antisense, 5'-GCT CTT TAC GCT TTC CAG GAA GTC-3'; mouse IL-13 sense, 5'-GGA GCT GAG CAA CAT CAC ACA-3'; mouse IL-13 antisense, 5'-GGT CCT GTA GAT GGT GGC ATT GCA-3'; mouse β-actin sense, 5'-ACC GTG AAA AGA TGA CCC AG-3'; mouse β-actin antisense, 5'-TGT CAG CTG TGG TGG TGA AG-3'; rat IL-4 sense, 5'-ACC TTG CTG TCA CCC TGT TC-3'; rat IL-4 antisense, 5'-TTG TGA GCG TGG ACT CAT TC-3'; rat IL-13 sense, 5'-GAC CCA GAG GAT ATT GCA TG-3'; rat IL-13 antisense, 5'-CCA GCA AAG TCT GAT GTG AG-3'; rat β-actin sense, 5'-TAA CCA ACT GGG ACG ATA TG-3'; rat β-actin antisense, 5'-ATA CAG GGA CAG CAC AGC CT-3'. The PCR reactions were run for 32 cycles at

94 (30 s), 57 (30 s), and 72 (30 s). After the amplification, the RT-PCR products were separated in 1.2% (w/v) agarose gel and stained with ethidium bromide. The size of PCR products for mouse IL-4, IL-13, β-actin were 397, 141, 272 bp and rat IL-4, IL-13, β-actin were 346, 213, 200 bp, respectively.

Quantitative Real-time PCR. BMMCs (2×10^6 cells/mL) and RBL-2H3 cells (3×10^5 cells/mL) were treated with PMA (50 ng/mL) and ionomycin (0.5 μM) for 30 min in the absence or presence of fisetin. Total RNAs were isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. The RNA samples were used for cDNA synthesis using PrimeScript™ RT reagent kit (TAKARA, Shiga, Japan). Real-time PCR was conducted on 7300 real-time PCR system (Applied biosystem, Foster City, CA) using iQ SYBR green supermix (Bio-Rad, Hercules, CA) with rat GATA-1, GATA-2, and GAPDH-specific primers under the following conditions: initial denaturation for 2 min at 50°C, denaturation for 10 min at 95°C, annealing for 15 s at 95°C, and extension for 1 min at 60°C for 40 cycles. Rat GATA-1 forward, 5'-GGA AAA AGA AGC GGG GCT CA-3'; reverse, 5'-GCA GTG CCC AGT GTC AAG CC-3'; rat GATA-2 forward, 5'-CAG CCT CCA GCT TCA CCC CT-3'; reverse, 5'-TGA TGA GCG GCC GAT TCT GT-3'; rat GAPDH primer sets; Endogenous Control (VIC®/MGB Probe, Primer Limited, Carlsbad, CA) from Applied Biosystems (4352338E).

Transient transfection and luciferase assay. RBL-2H3 cells (3×10^5 cells/mL) were transfected with the pGL4.14-IL-4 and pGL4.14-IL-13 plasmid in the presence of lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendation. Briefly, the mixture of DNA (0.4 μg) and lipofectamine™ 2000 (1 μL) was added to each well of 48-well cell culture plate. Following 12 h incubation, the cells were treated with fisetin for 1 h and stimulated with 50 ng/mL of PMA and 0.5 μM of ionomycin for 16 h. Subsequently, cells were washed with PBS three times and extracted with 100 μL of lysis reagent (Promega, Madison, WI) at 4°C for 20 min, and centrifuged at 12,000 rpm at 4°C for 1 min. The supernatants were mixed with 100 μL of luciferase substrate solution, and the emitted light was measured using Victor3 1420 multilabel counter (Perkin-Elmer Precisely, Waltham, MA). Normalized luciferase activities in each study were expressed as mean ± SEM for three independent experiments.

Western blot analysis. RBL-2H3 cells (3×10^5 cells/mL) were treated with PMA (50 ng/mL) and ionomycin (0.5 μM) for 1 or 3 h in the absence or presence of fisetin. Cytoplasmic extract and nuclear extract were obtained using Nuclear extract kit (Active Motif, Carlsbad, CA) following manufacturer's recommendations. In brief, cells were washed with ice-cold phosphate buffered saline (PBS) and centrifuged at 400 × g for 5 min at 4°C, resuspended in 250 μL hypotonic buffer. Cells were allowed to swell on ice for 15 min, and lysed gently with 12.5 μL detergent reagent. After centrifugation at 14000 × g for 1 min at 4°C, the supernatant was obtained in the form of cytoplasmic extract. The nuclear extract was subsequently gathered by resuspending the nuclear pellet in 25 μL complete lysis buffer. After 30 min on ice, the suspension was centrifuged for 10 min at 14000 × g and 4°C, the supernatant was obtained in the form of nuclear extract. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biothechnology, Rockford, IL). Equal amounts (60 μg) of proteins were heated to 95°C for 5 min in sample buffer, chilled on ice, and then separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, PA) and blocked with 2% skim milk in PBS/T buffer for 1 h. They were then probed overnight with primary antibodies specific to GATA-1, GATA-2, NF-ATc1, C/EBPα (Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB p65, c-Fos (Cell signaling, Danvers, MA), and c-Jun (Gene Tex, San Antonio, TX). Blots were then incubated with streptavidin-horseradish peroxidase (HRP)-conjugated secondary antibody. HRP was detected using a Chemiluminescent HRP Substrate (Millipore).

Statistical analysis. The results are presented as mean ± SD. Each experiment was repeated at least three times. Differences in mean values between groups were analyzed by Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

Suppressive effects of fisetin on IL-4 and IL-13 expressions in mast cells and basophils. To examine the suppressive effects of fisetin on IL-4 and IL-13 expressions in mast cells, BMMCs, which were differentiated from the bone marrow of mice under the presence of IL-3 and SCF were first used for 6 weeks. Ninety-eight percent of the cells were c-Kit positive (CD117), a mast cell marker (Fig. 1A). The cells were treated with fisetin at various concentrations without affecting cell viability for 1 h and were then activated for 16 h with PMA and ionomycin (PI), which mimics cross-linking of FcεRI. The BMMCs stimulated by PI greatly produced IL-13 on the scale of several hundred nanograms per milliliter, whereas a relatively small amount of IL-4 in a nanogram scale per milliliter was produced. In the presence of fisetin, the

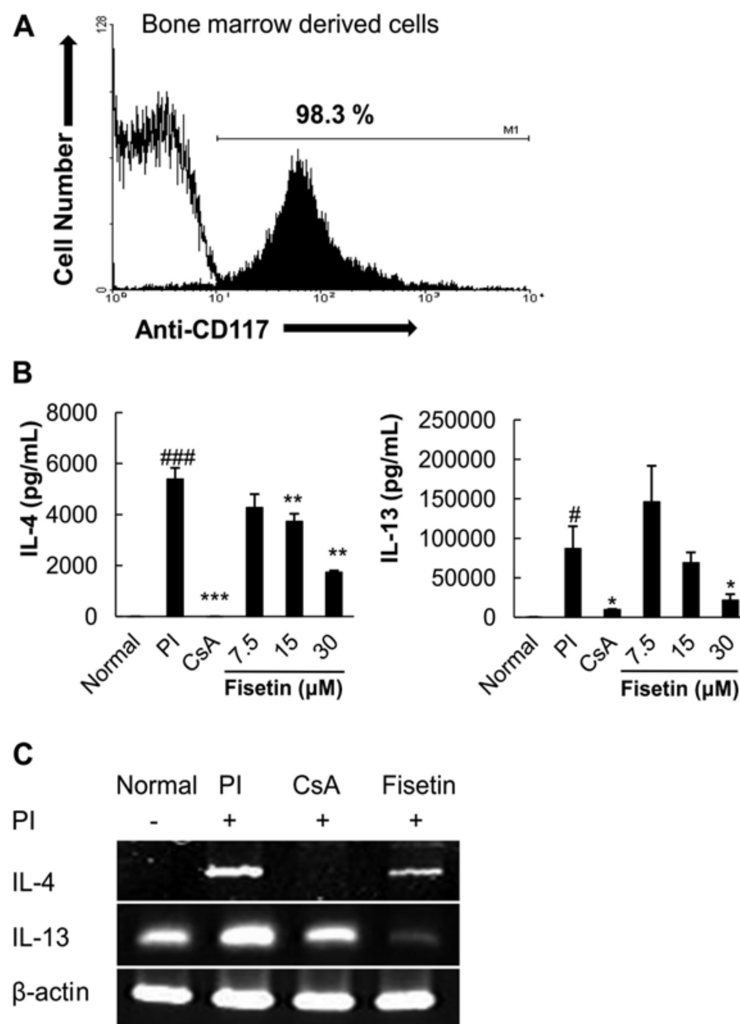

Fig. 1. Suppressive effects of fisetin on IL-4 and IL-13 cytokine production and gene expression in BMMCs. The cells were pre-treated with fisetin, 1 μM of CsA for 1 h, and then stimulated with PI for 16 or 6 h. (A) Ninety-eight percent of BMMCs was c-Kit positive, a mast cell marker. (B) The levels of IL-4 and IL-13 in BMMCs were determined by ELISA. (C) Cellular RNA from each treatment was extracted, and the mRNA expression for cytokine in BMMCs was analyzed by RT-PCR. The β-actin gene was amplified as an internal housekeeping control. Values are expressed as means ± SD from three-independent experiments. $^{\#}p$ <0.05; $^{\#\#\#}p$ <0.001 *vs*. normal group (student's *t*-test). **p* <0.05; ***p* <0.01; ****p* <0.001 *vs*. PI-treated group (student's *t*-test).

levels of IL-4 and IL-13 were dramatically decreased in a dose-dependent manner, and at the concentration of 30 μM fisetin lowered the levels down to approximately 67 and 75%, respectively, compared with those of the PI-stimulated control (Fig. 1B), followed by harboring of RBL-2H3 basophilic cells. When the cells were activated by PMA and ionomycin, large amounts of IL-4 and IL-13 were produced. The treatment with fisetin greatly decreased the cytokine production in a dose-dependent manner resulting in almost complete inhibition at 30 μM (Fig. 2A). Cyclosporine A, a well known immunosuppressive drug used as a positive control in the present study, showed a significant decrease in IL-4 and IL-13 productions. The effects of fisetin at the concentration of 30 μM were comparable to those of 1 μM of cyclosporine A (Figs. 1B and 2A). To further investigate whether fisetin suppresses IL-4 and IL-13 gene expressions at the transcriptional level, the mRNA level of each cytokine was determined by RT-PCR. In BMMCs, PI stimulation increased the levels of IL-4 and IL-13 mRNA to 7.5 and 1.3 fold, respectively. In the presence of fisetin, the mRNA expressions of IL-4 and IL-13 were significantly decreased (Fig. 1C) to 32.5 and 72.8%, respectively, during the 6 h activation period. The effects of fisetin in RBL-2H3 basophilic cells were very similar to those shown in BMMCs. The levels of mRNA were dramatically decreased by fisetin (Fig. 2B). These results suggested that fisetin suppresses IL-4 and IL-13 productions and mRNA expressions in activated mast cells and basophils.

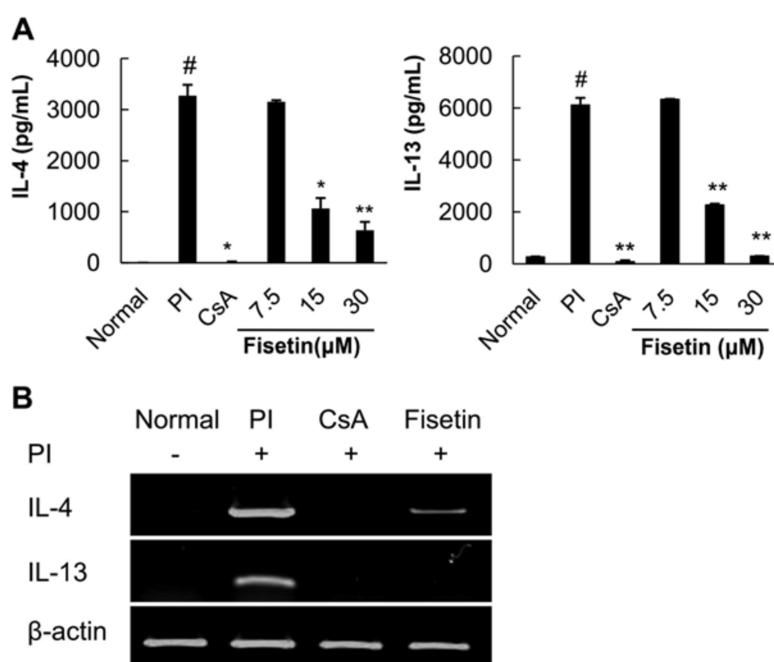

Fig. 2. Suppressive effects of fisetin on IL-4 and IL-13 cytokine production and gene expression in RBL-2H3 cells. The cells were pre-treated with fisetin, 1 μM of CsA for 1 h, and then stimulated with PI for 16 and 6 h. (A) The levels of IL-4 and IL-13 in RBL-2H3 cells were determined by ELISA. (B) Cellular RNA from each treatment was extracted, and the mRNA expression for cytokine in RBL-2H3 cells was analyzed by RT- PCR. The â-actin gene was amplified as an internal housekeeping control. Values are expressed as means ± SD from three independent experiments. $^{\#}p<0.05$ *vs*. normal group (student's *t*-test). $*p<0.05$; $**p<0.01$; *vs*. PI-treated group (student's *t*-test).

Suppressive effects of fisetin on IL-4 and IL-13 promoters. Because the expressions of IL-4 and IL-13 are regulated by the proximal promoter activities (Fig. 3A) at the transcriptional level, the effects of fisetin on the promoters were further examined. When RBL-2H3 stable cell lines constitutively expressing the promoter reporter plasmids, previously generated by our group [Choi *et al.*, 2007; 2009], were stimulated with PI, the luciferase activities of IL-4 and IL-13 promoters were greatly increased compared with the control, leading to 40-and 7-fold increase, respectively (Fig. 3B). The treatment of fisetin decreased the activities in a dose-dependent manner, indicating that fisetin could down-regulate the promoter activities of IL-4 and IL-13 in activated basophils.

Effects of fisetin on various transcription factors involved in the proximal promoters. Because fisetin presumably down-regulated IL-4 and IL-13 expressions through the promoter at the transcriptional levels, the effect of fisetin on the activities of transcription factors involved in the regulation of the promoters was examined. The nuclear expression of several selective transcription factors were analyzed by Western blot analysis. In the case of NF-κB p65, the nuclear expression was slightly increased by PI treatment, and fisetin suppressed the expression, whereas cyclosporine A seemed to rarely inhibit the expression of p65 (Fig. 4A), which was consistent with the data from a previous study of Park *et al.* [2007]. The expressions of NF-ATc1 in the nucleus were significantly increased by PI stimulation by 5-fold and were greatly decreased by fisetin as well as cyclosporine A (Fig. 4B). When the two major components of the AP-1 protein complex (c-Jun and c-Fos) were examined, the results indicated that PI stimulation dramatically increased the nuclear expression of c-Fos by approximately 9-fold, whereas that of c-Jun was slightly induced by 1.7-fold. Fisetin treatment almost completely decreased the nuclear expression of c-Fos and reduced c-Jun to the normal level. In contrast, cyclosporine A showed no notable effect on the c-Fos and c-Jun expressions (Fig. 4C and D). These data suggested that fisetin has significant suppressive effects on the nuclear expressions of transcription factors including AP-1 and NF-ATc1, and slight suppression on NF-κB, which is involved in the activation of IL-4 and IL-13 promoters. In the case of C/EBPα, there was some increase in the nuclear expression by PI, but fisetin treatment significantly decreased the expression, (Fig. 4E). Because GATA-1 and GATA-2 proteins were known to play pivotal roles in the expressions of IL-4 and IL-13 in mast cells by regulating chromatin remodeling processes as well as activation of promoters [Hural *et al.*, 2000; Ozawa *et al.*, 2001; Letting

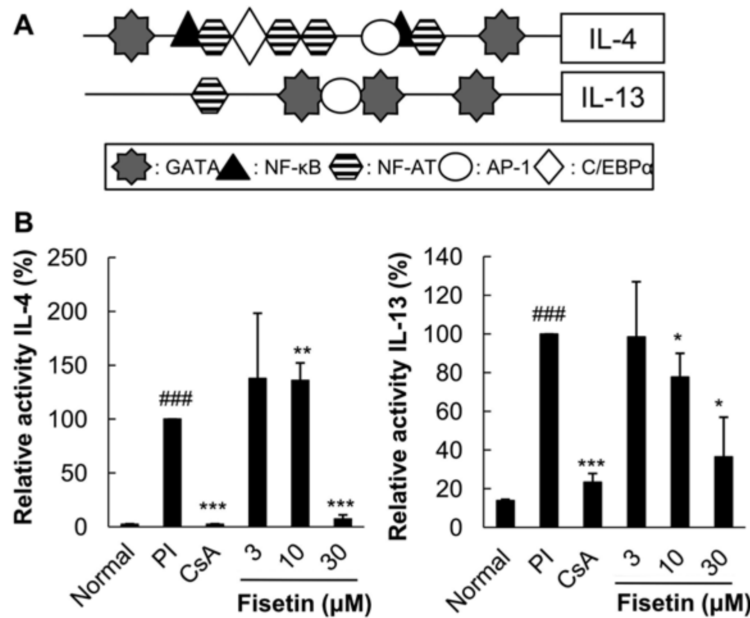

Fig. 3. Suppressive effects of fisetin on IL-4 and IL-13 promoters. RBL-2H3 cells transiently transfected with pGL4.14-IL-4 and pGL4.14-IL-13 were generated by inserting the mouse IL-4 or IL-13 promoters into the pGL4.14 plasmid. (A) Overview of the regulatory regions in the IL-4 and IL-13 promoters. (B) RBL-2H3 cells were pre-treated with various concentrations of fisetin and 1 μM of CsA for 1 h, and then stimulated with PI for 16 h, and the luciferase activities were measured by luminometer. Relative activity was calculated as relative luciferase activities compared to those of the PI-treated group. $^{###}p$ <0.001 *vs*. normal group. *p <0.05; **p <0.01; ***p <0.001 *vs*. PI-treated group.

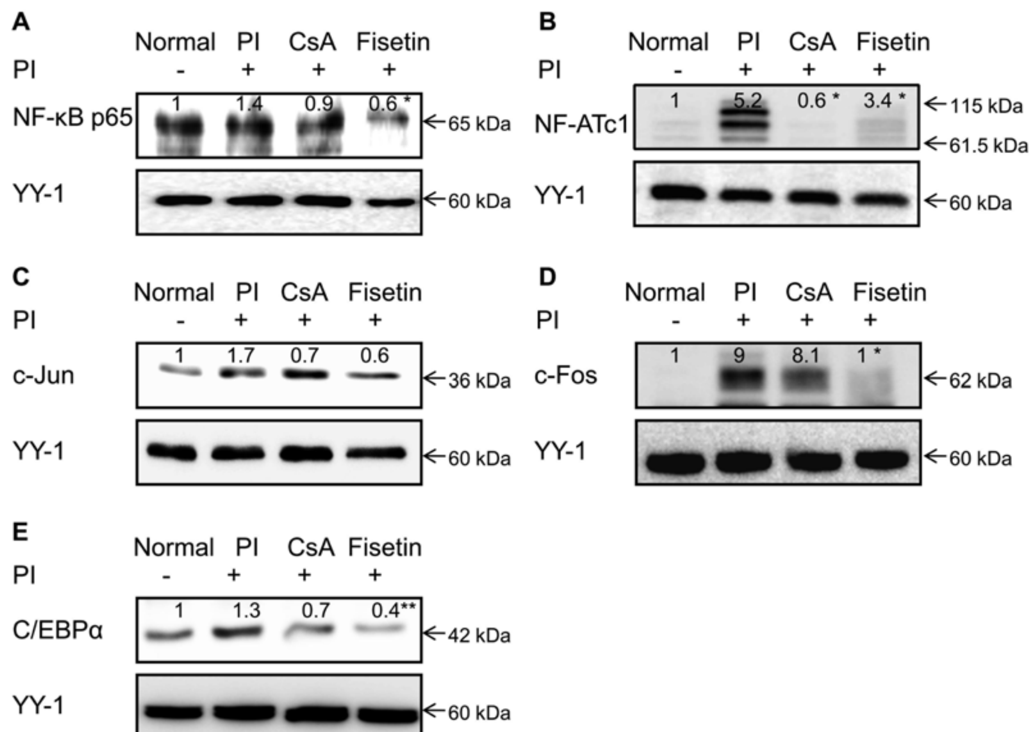

Fig. 4. Effects of fisetin on PI-induced activation of transcription factors involved in the expression of the cytokines expression. RBL-2H3 cells were pre-treated with 30 μM of fisetin and 1 μM of CsA for 1 h, and then stimulated with PI for 3 h. Nuclear extracts were isolated and separated by SDS-PAGE. The level of each transcription factor, (A) NF-κB p65 (B) NF-ATc1, (C) c-Jun, (D) c-Fos, and (E) C/EBPα was determined by Western blot analysis using specific antibodies. YY-1 was used as a loading control. The data presented are representative of three independent experiments. The numeric values indicate average density of bands from these experiments. The protein expression was quantitated by densitometric analysis with that of the control being 1 fold. Values are expressed as means ± SD from three independent experiments. *p <0.05; **p <0.01 *vs*. PI-treated group.

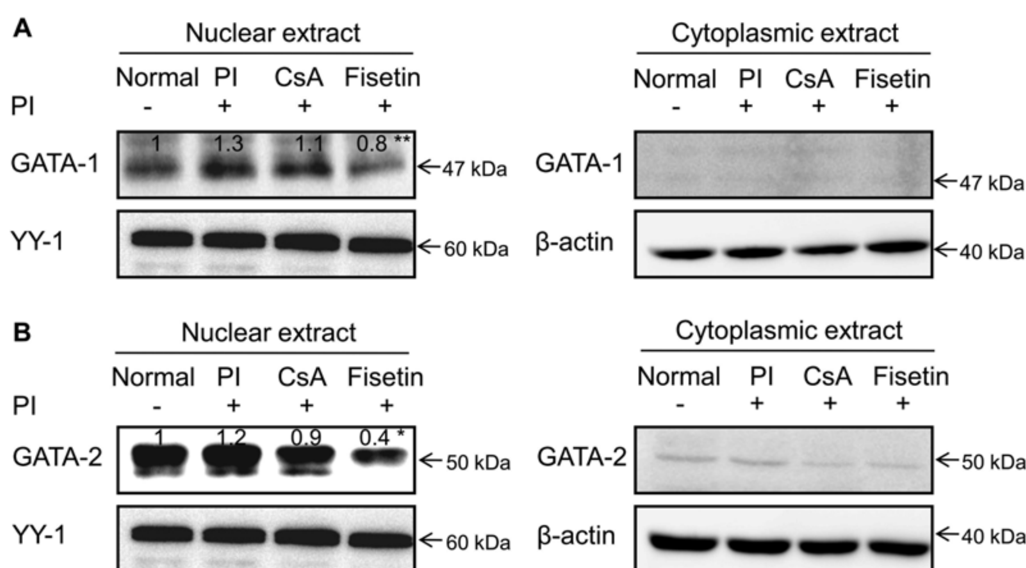

Fig. 5. Suppressive effects of fisetin on GATA transcription factors. RBL-2H3 cells were pre-treated with 30 μM of fisetin and 1 μM of CsA for 1 h, and then stimulated with PI for 1 h. Nuclear and cytoplasmic extracts were isolated and separated by SDS-PAGE. The level of (A) GATA-1 and (B) GATA-2 was determined by Western blot analysis using specific antibodies. YY-1 and β-actin were used as loading controls for the nucleus and cytoplasm, respectively. The data presented are representative of three independent experiments. The numeric values indicate average density of bands from these experiments. The protein expression was quantitated by densitometric analysis with that of the control being 1 fold. Values are expressed as means ± SD from three-independent experiments. **p* <0.05; ***p* <0.01 *vs*. PI-treated group.

et al., 2003], and there was no report on the effects of fisetin on these transcription factors in mast cells, whether fisetin controls the activation of GATA-1 and GATA-2 was examined using Western blot analysis. GATA-1 and GATA-2 constitutively exist in the nucleus of cells rather than cytoplasm (Fig. 5). The levels of GATA-1 and GATA-2 appeared to be slightly increased, but were not significantly affected by PI stimulation; however, treatment of fisetin significantly decreased the levels of GATA-1 and GATA-2 in the nucleus by approximately 37 and 61%, respectively, whereas cyclosporine A showed no significant effect on the levels of GATA proteins. Not only are the levels of GATA-1 and GATA-2 protein very low in the cytoplasm, but there were also no changes in the levels; thus, the possibility that fisetin blocked the translocation of GATA proteins into the nucleus to reduce the nuclear expression of GATA was excluded. Instead, whether fisetin down regulated the mRNA levels of GATA-1 and GATA-2 was examined (Fig. 6). Consistent with the data from Western blot analysis, PI stimulation negligibly increased GATA-1 and GATA-2 mRNA expressions, and cyclosporine A exerted no significant effects. Fisetin decreased the mRNA levels of GATA-1 and GATA-2 up to 50.3 and 38.3%, respectively (Fig 6). These data indicated that fisetin could suppress nuclear expressions of GATA-1 and GATA-2 by down-regulation of constitutive mRNA expressions.

Discussion

Intake of naturally occurring flavonoids widely distributed in phytomedicines and vegetables have been proposed to have potency for the improvement of allergic disease. Kotani *et al.* [2000] reported that oral administration of flavonoids such as astragalin and kaemperol suppresses the onset of allergic disease in the atopic dermatitis of NC/Nga mice, and that the intake of flavonoids in asthmatic models leads to a significant suppression of symptoms including bronchial hyperactivity and bronchoconstriction. Intake of human traditional vegetarian diet containing flavonoids has been reported to reduce symptoms of atopic dermatitis [Knekt *et al.*, 2002; Garcia *et al.*, 2005]. Although fisetin has suppressive effects on Th2 cytokines in human basophils [Higa *et al.*, 2003], its molecular mechanisms for the suppression of Th2 cytokine production in basophils and mast cells has been incompletely elucidated. In the present study, the suppressive effects of fisetin on IL-4 and IL-13 expressions in BMMCs was examined, and, to the best of our knowledge, for the first time we found that fisetin has prominent inhibitory effects on the nuclear expression of c-Fos protein and C/EBPα in RBL-2H3 basophilic cells. Furthermore, in GATA-1 and GATA-2, tissue-restricted transcription factors were down-regulated by fisetin. The GATA family consisting of six members (GATA-1–GATA6) is characterized by

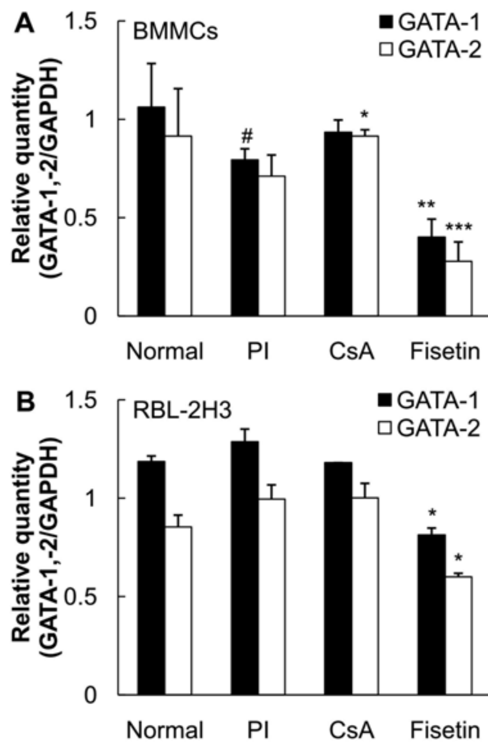

Fig. 6. Suppressive effects of fisetin on GATA mRNA expression. (A) BMMCs and (B) RBL-2H3 cells were pre-treated with 30 μM of fisetin and 1 μM of CsA for 1 h, and then stimulated with PI for 30 min. The levels of GATA-1 and GATA-2 mRNA expression were determined by quantitative real-time PCR. The GAPDH gene was amplified as an internal housekeeping control. The data shown are representative of three independent experiments. Values are expressed as means ± SD from three-independent experiments. $^{\#}p < 0.05$ *vs*. normal group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs*. PI-treated group.

recognition of the W(A/T)GATAR(A/G) motif and their DNA-binding domains including highly conserved homologous zinc fingers Cys-X2-Cys-X17-Cys-X2-Cys. These results suggest that fisetin could exert potent regulatory effect on mast cell as well as basophil, because GATA-1 and GATA-2 are expressed in mast cells and basophils, whereas not lymphocytes such as T cells, which are known to play critical roles in mast cell differentiation, survival, and activation [Pevny *et al.*, 1991; Migliaccio *et al.*, 2003]. GATA consensus sequences are present in the promoter regions of IL-4 and IL-13, and ectopic expression of GATA-1 and/or GATA-2 activates the promoter activities of these genes [Masuda *et al.,* 2007]. In addition, the regulation of mast cell IL-4 expression was reported to be dependent on NF-AT binding sites of proximal promoters as well as mast cell-specific introns containing GATA-1 and GATA-2 binding sites [Weiss *et al.,* 1996]. In the second intron of the IL-4 gene, GATAs cooperate with other transcription factors such as PU.1 and STAT5 to control histone acetylation, methylation, and chromatin remodeling, resulting in the increase of IL-4 locus accessibility [Kwan *et al.*, 2005]. In basophils, C/EBPα with NF-AT play critical roles on IL-4 gene transcription [Qi *et al.*, 2011]. Although the mechanisms that regulate IL-13 production in mast cells and basophils remain largely unknown, it has been reported that direct interaction between AP-1 and GATA proteins increases *IL-13* transcription in mast cells [Masuda *et al.*, 2004]. NF-ATs are expressed in mast cells and directly bind the IL-13 promoter as well as synergically interact with GATA proteins in the IL-13 promoters to induce IL-13 transcription [Masuda *et al.*, 2004; Monticelli *et al.*, 2004].

Results of the present study indicated that for the suppression of IL-4 and IL-13 expressions in mast cells and basophils fisetin facilitates NF-AT dependent calcineurin pathway and NF-κB inhibition as proposed in previous researches using different cell types and stimuli [Park *et al.*, 2007; Sung *et al.*, 2007]. Higa *et al.* [2003] reported that fisetin suppressed the expression of Th2 cytokines in A23187-stimulated human basophils, which could be mediated by decrease in NF-AT-DNA binding activities. Furthermore, through the suppression of NF-κB and mitogen activated protein kinases (MAPKs), fisetin decreased the proinflammatory cytokine production in human HMC-1 mast cell lines [Park *et al.*, 2007]. In the present study, we showed that AP-1, C/EBPα as well as GATA transcription factors could be also involved in the suppression of IL-4 and IL-13. Although both cyclosporine A and fisetin have strong suppressive effects on IL-4 and IL-13 expressions in basophils and mast cells, they seem to use different mechanisms. Cyclosporin A did not affect c-Fos and C/EBPα and GATA proteins (Fig. 4D, E, and 5). Further investigations on as to how fisetin is able to control diverse selective transcription factors according to cell types and which signaling molecule(s) and/or pathway(s) is critical for the regulation of the transcription factors remain to be elucidated. Furthermore, we do not exclude the possibility that fisetin directly binds with a critical molecule(s) to shut down several signaling pathways leading to activation of the diverse transcription factors. Moreover, investigation should be investigated as to how fisetin down-regulates the expressions of GATA-1 and GATA-2 mRNAs and whether fisetin is able to post-translationally modify GATA transcription factors as they use post translational modification including acetylation and phosphorylation to control their own expressions [Hernandez-Hernandez *et al.*, 2006]. Taken together, considering the prevalence of allergic disease worldwide and the strong suppressive effects of fisetin, further studies on possible use as therapeutics are definitely needed.

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