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Flavones suppress type I IL-4 receptor signaling by down-regulating the expression of common gamma chain

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ABSTRACT

Immunoglobulin E (IgE) production is induced by interleukin (IL)-4 signaling mediated by type I IL-4 receptor (IL-4R) in B cells. We found that flavones inhibited IL-4-induced ϵ germline transcription which is essential for IgE class switching, and the phosphorylation of signal transducer and activator of transcription 6, janus kinase 3, and IL-4R α , whereas IL-4 signaling mediated through type II IL-4R was unaffected by flavones. Furthermore, flavones reduced the expression of common gamma chain, a characteristic constituent subunit of type I IL-4R, suggesting that flavones suppress type I IL-4R signaling.

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1. Introduction

Immunoglobulin (Ig) E was previously identified as a key molecule mediating type I hypersensitivity reactions [1]. Interleukin (IL)-4 controls the specificity of immunoglobulin class switching and induces B cells to produce IgE [2]. For IgE class switching, expression of ϵ germline transcript (ϵ GT), which binds to the appropriate heavy chain switch region sequence, is induced by IL-4 [3]. IL-4R is divided into two types by the difference of subunits. Type I IL-4R uses IL-4R α and common gamma chain (γ c) as a component of their receptors and type II IL-4R consists of IL-4R α and IL-13R α 1 [4]. Because γ c expression is restricted to hematopoietic cells, type I IL-4R is mainly expressed in hematopoietic cells, whereas type II IL-4R is ubiquitously expressed [5]. γ c has no ability to bind IL-4 directly but is important for signaling of the type I receptor [4].

Abbreviations: IL-4, interleukin-4; IgE, immunoglobulin E; IL-4R, interleukin-4 receptor; ϵ GT, ϵ germline transcript; γ c, common gamma chain; STAT, signal transducer and activator of transcription; JAK, Janus kinase; PPAR γ , peroxisome proliferator-activated receptor

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Flavonoids are the diverse family of chemicals commonly found in many fruits and vegetables. Flavonoids are plant polyphenolic compounds which have a diphenylpropane skeleton (C6C3C6) structure which includes monomeric flavanols, flavanones, flavones, and flavonols. Some flavonoids have been found to possess various clinically relevant properties such as anti-tumor and anti-inflammatory activities [6,7]. Our previous reports demonstrated that dietary apigenin, one of flavones, could suppress serum IgE production in C57BL/6N mice [8] and antigen-specific IgE production in BALB/c mice immunized by ovalbumine [9] and in NC/Nga mice sensitized with hapten [10]. In this study, we investigated the molecular basis for the flavones functions that reduce IgE production. Here we show that flavones can suppress type I IL-4R signaling by reducing the cell surface expression of γ c.

2. Materials and methods

2.1. Reagents

Chrysin and apigenin were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO). Anti-STAT6, anti-JAK3, anti-IL-4R α , anti-PPAR γ , and anti-phosphotyrosine (PY20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human recombinant IL-4,



Fig. 1. Effect of flavones on IL-4-induced ϵ GT expression. DND39 cells were treated with indicated concentrations of flavones and IL-4 (25 U/ml) for 48 h. Total mRNA was isolated from cells and the expression of ϵ GT was analyzed by RT-PCR.

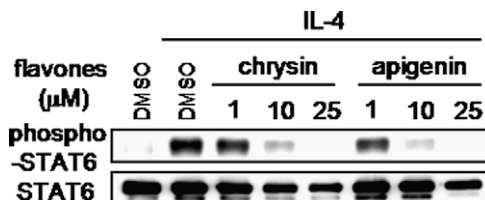


Fig. 2. Effect of flavones on IL-4-induced STAT6 phosphorylation. DND39 cells were treated with IL-4 (100 U/ml) for 30 min after treatment with flavones for 24 h. Total cell lysate was immunoprecipitated using anti-STAT6 antibodies and then immunoblotted using anti-phosphotyrosine and anti-STAT6 antibodies.

FITC-conjugated anti-human γ c antibody and anti-human γ c antibody were purchased from R&D Systems (Minneapolis, MN).

2.2. Cells and cell culture

The human mature B cell line DND39 was cultured at 37 °C under humidified 5% CO₂/95% air in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel), 100 U/ml penicillin G, 100 mg/ml streptomycin, and 10 mM HEPES buffer. As vehicle, 0.1% of DMSO was added to control cells.

2.3. Analysis of ϵ GT expression

DND39 cells were treated with IL-4 (25 U/ml) with or without flavones for 48 h. The expression level of ϵ GT was measured with the reverse transcriptase-polymerase chain reaction (RT-PCR) method as previously described [11].

2.4. Immunoprecipitation and immunoblotting

After treatment with or without flavones for 24 h, DND39 cells were stimulated with IL-4 (100 U/ml) for 30 min. Then, assessment

of the phosphorylation of STAT6, JAK3, and IL-4R α were performed by immunoprecipitation and immunoblotting as previously described [11].

2.5. Silencing of PPAR γ

Peroxisome proliferator-activated receptor (PPAR) γ -silencing DND39 cells were constructed by the method of RNA interference using psiRNA-hH1hygro G2 Kit (Invivogen, San Diego, CA). The target sequence for short hairpin RNAs for PPAR γ is as follows: 5'-GTT TGA GTT TGC TGT GAA G-3'. PPAR γ -shRNA expression vector was transfected into the cells by electroporation and the cells were selected by Hygromycin B (Roche). The PPAR γ expression level was assessed by immunoblotting analysis.

2.6. Fluorescent immunostaining of IL-4

Cell surface binding of IL-4 was assessed by fluorescent immunostaining. After the treatment of flavones, DND39 cells were incubated with biotinylated human IL-4 and were incubated with quantum dot 655 streptavidin conjugates (Invitrogen, Carlsbad, CA). Detection of IL-4 on the cell surface was performed using fluorescent microscope (BZ-HIA/HIT2/HIM2, Keyence, Osaka, Japan).

2.7. Analysis of γ c expression

The cell surface expression of γ c was assessed by flow cytometry using anti-human CD132/ γ c monoclonal antibody following the appended protocol. Mouse IgG_{2A} isotype control-CFS (R&D Systems) was used as the isotype-matched negative control. The protein expression level of γ c was assessed by immunoblotting analysis.

3. Results

3.1. Flavones inhibited IL-4-induced ϵ GT expression

Expression of ϵ GT is essential for IL-4-induced IgE class switching [3]. We examined the effect of chrysin and apigenin on IL-4-induced ϵ GT expression in DND39 cells. Both flavones dose-dependently inhibited the expression of ϵ GT (Fig. 1).

3.2. Flavones inhibited IL-4-induced STAT6 phosphorylation

IL-4 activates the signal transducer and activator of transcription (STAT) 6 transcription factor which interacts with the promoter region for ϵ GT [12]. STAT6 plays an essential role in IL-4-induced ϵ GT expression [13], and IgE class switching cannot be seen in mice with a disrupted STAT6 gene [14]. We examined the

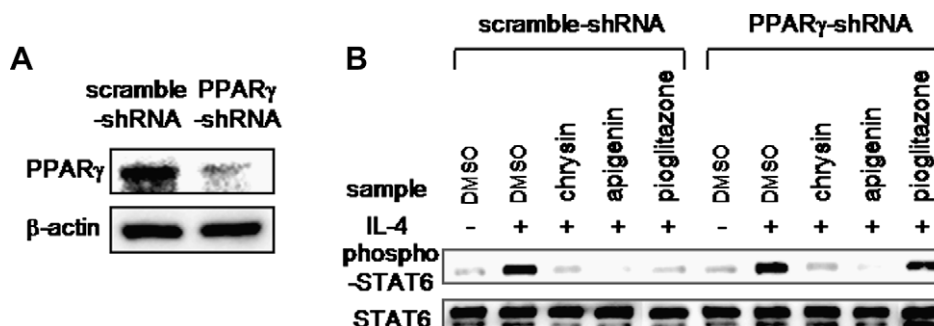


Fig. 3. Involvement of PPAR γ on the inhibitory effect of flavones on IL-4-induced STAT6 phosphorylation. (A) Scramble-shRNA or PPAR γ -shRNA expression vector were stably transfected in DND39, and the expression of PPAR γ was examined by immunoblotting. (B) PPAR γ -silencing DND39 cells were treated with IL-4 (100 U/ml) for 30 min after treatment with flavones (10 μM) or pioglitazone (5 μM) for 24 h. Total cell lysate was immunoprecipitated using anti-STAT6 antibodies and then immunoblotted using anti-phosphotyrosine and anti-STAT6 antibodies.

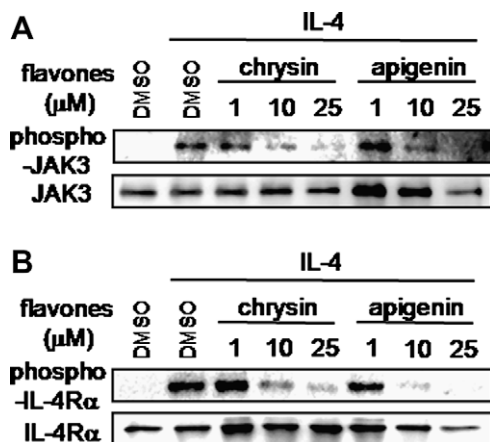


Fig. 4. Effects of flavones on IL-4-induced phosphorylation of JAK3 and IL-4Rα. DND39 cells were treated with IL-4 (100 U/ml) for 30 min after treatment with flavones for 24 h. Total cell lysate was immunoprecipitated using (A) anti-JAK3 or (B) anti-IL-4Rα antibodies and then immunoblotted.

effect of the flavones on IL-4-induced STAT6 phosphorylation. As shown in Fig. 2, chrysin and apigenin dose-dependently inhibited STAT6 phosphorylation induced by IL-4.

3.3. Flavones inhibited IL-4-induced STAT6 phosphorylation independently of PPARγ

PPARγ is a member of the nuclear hormone receptors superfamily. Recently, it has been reported that some flavonoids including chrysin and apigenin exhibit PPARγ agonist activities [15]. In addition, we reported that PPARγ ligands inhibit IL-4-induced εGT expression in DND39 cells [16]. Therefore, we investigated whether the inhibitory activity of flavones on IL-4-induced STAT6 phosphorylation is mediated through PPARγ. Western blot analysis indicated that stable RNAi for PPARγ specifically silenced PPARγ protein expression (Fig. 3A). Pioglitazone, one of PPARγ ligands, suppressed IL-4-induced STAT6 phosphorylation, and this inhibitory activity was completely abrogated in the PPARγ-silencing cells. On the other hand, the inhibitory activity of the flavones on IL-4-induced STAT6 phosphorylation was not affected in the PPARγ-ablating cells (Fig. 3B), suggesting that flavones suppress IL-4 signaling independently of PPARγ.

3.4. Flavones inhibited IL-4-induced JAK3 and IL-4Rα phosphorylation

Activation of Janus kinases (JAKs) results in phosphorylation of the cytoplasmic tyrosines in IL-4Rα, leading to the recruitment of STAT6 to the receptor, followed by STAT6 phosphorylation and activation [4]. We examined the effect of flavones on the IL-4-induced phosphorylation of JAK3 and IL-4Rα in DND39 cells. As shown in Fig. 4, the phosphorylation of JAK3 and IL-4Rα induced by IL-4 were inhibited by flavones.

3.5. IL-4 binding was unaffected by flavones

Next, we examined whether flavones inhibit IL-4 binding to IL-4R using fluorescent immunostaining. Cell surface binding of IL-4 was unaffected by flavones (Fig. 5), suggesting that the inhibitory activity of flavones on IL-4 signaling is not due to the inhibition of IL-4 binding to IL-4R.

3.6. Flavones had no effect on IL-4-induced STAT6 phosphorylation in HeLa cells

We investigated whether flavones suppress type II IL-4 signaling in type II IL-4R-expressing HeLa cells. Interestingly, IL-4-induced STAT6 phosphorylation in HeLa cells was unaffected by flavones (Fig. 6), suggesting that γc may be a critical target for the inhibitory activity of flavones on IL-4 signaling.

3.7. Flavones down-regulated common gamma chain expression

We examined the effect of flavones on the cell surface expression of γc in DND39 cells. The two flavones dose-dependently reduced the cell surface expression level of γc (Fig. 7A). We also found that flavones reduced the protein level of γc (Fig. 7B). These results suggest that the inhibitory activity of flavones on IL-4-induced εGT expression may be in part due to the downregulation of γc protein.

4. Discussion

IgE plays a key role in the pathogenesis of allergic disease [1]. IL-4 is critical for the synthesis of IgE in B cells [2]. Inhibition of IL-4 signaling may therefore be effective in inhibiting allergy disease. εGT expression induced by IL-4 is essential for IgE class switching [3]. Here we identified flavones as inhibitory molecules that inhibit IL-4-induced εGT expression.

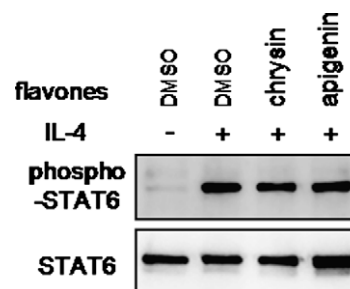


Fig. 6. Effect of flavones on IL-4-induced STAT6 phosphorylation in type II IL-4R. HeLa cells were treated with IL-4 (100 U/ml) for 30 min after treatment with 25 μM of flavones for 24 h. Total cell lysate was immunoprecipitated using anti-STAT6 antibodies and then immunoblotted using anti-phospho tyrosine and anti-STAT6 antibodies.

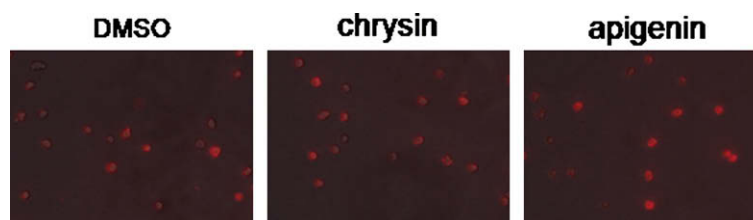


Fig. 5. Effect of flavones on IL-4 binding. DND39 cells were treated with biotinylated human IL-4 for 60 min after treatment with 25 μM of flavones for 24 h. Then cells were mixed with Qdot-streptavidin and IL-4 on cell surface was detected by using fluorescence microscope.

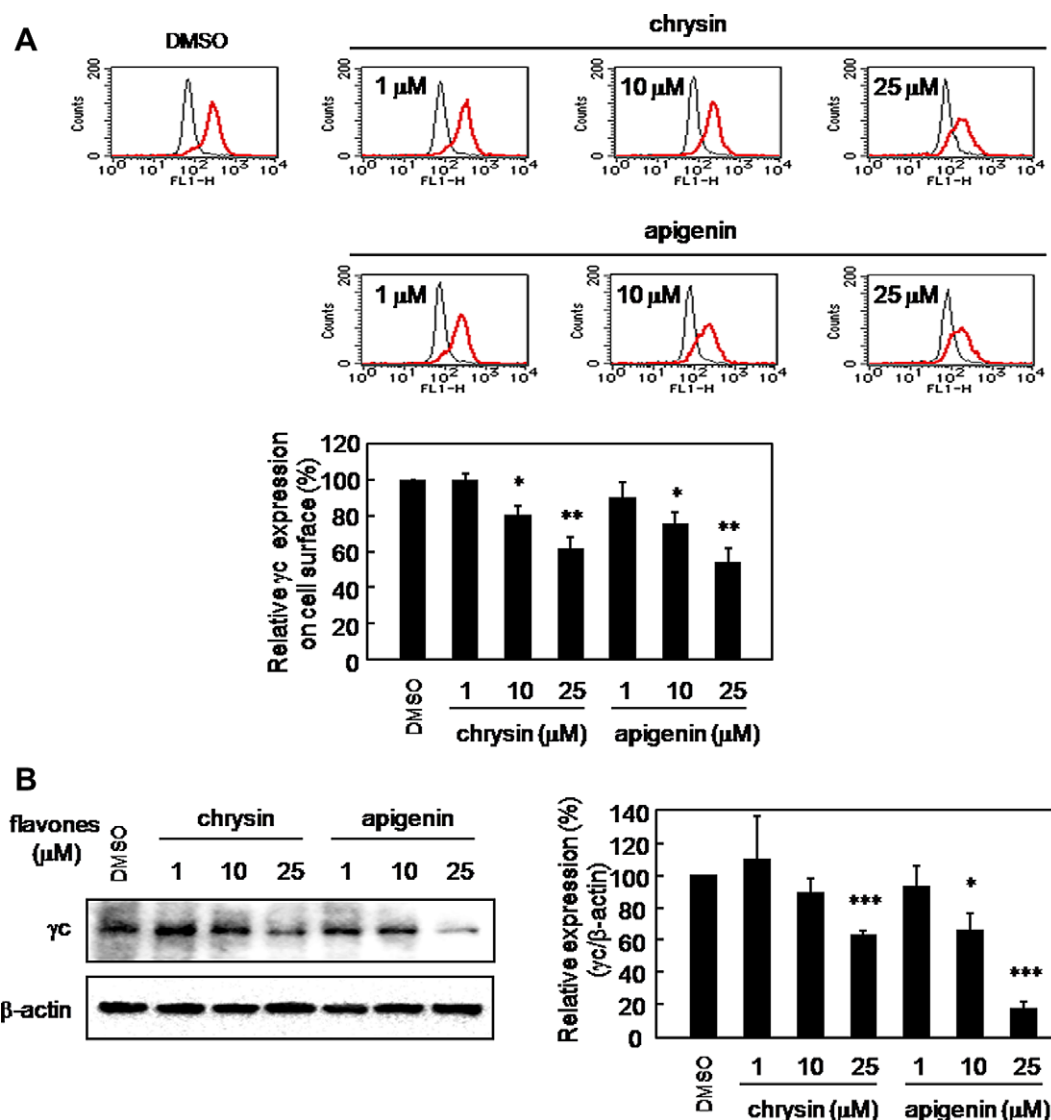


Fig. 7. Effect of flavones on the expression of γc . DND39 cells were treated with flavones for 24 h. (A) The expression of γc on the cell surface was assessed using flow cytometry analysis. Results were assessed as the Normalized Mean Fluorescence Index (NMFI). (B) The intracellular expression of γc was detected by immunoblot analysis. The relative level of γc was determined by intensity of signals normalized with β -actin. Data are means $\pm$ S.E. of three independent experiments. Statistical analysis was analyzed by Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

It has been reported that some flavonoids, including chrysin and apigenin, have PPAR γ agonist activities [15]. In addition, we previously reported that PPAR γ ligands can inhibit IL-4-induced ϵ GT expression in DND39 cells [16]. We found that pioglitazone, a synthesized PPAR γ ligand, inhibits IL-4-induced STAT6 phosphorylation through PPAR γ , whereas the inhibitory effect of flavones on STAT6 phosphorylation is independent of PPAR γ . These results suggest that PPAR γ is not a target for the preventive effect of flavones on IgE class switching.

γc is common to the receptor complexes for six different interleukin receptors (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptor) at present [17]. γc does not bind IL-4 directly but is important for signaling of the type I receptor [4]. In this study, we found that flavones significantly reduce the cell surface and protein expression of γc , suggesting that γc is a target molecule for the inhibitory effect of flavones on IL-4 signaling. However, the potency of flavones to inhibit STAT6 and JAK3 phosphorylations is stronger than their potency to reduce γc expression. This discrepancy raised a possibility

that there is other mode of action for the inhibitory effect of flavones on IL-4 signaling. We examined whether flavones have an inhibitory effect on heterodimerization of IL-4R α and γc by the method of co-immunoprecipitation. We found that flavones had no inhibitory effect on the association of γc and IL-4R α (data not shown). Enzymatic JAK3 inhibition that has been shown by a flavonoid kaempferol [18] may be involved in the anti-IL-4 signaling action of flavones. It has been reported some natural compounds such as stricninin, polyphenol in tea, and astragalin, major constituent of flavonoids in persimmon leaf extract, inhibit antigen-specific IgE production [12,19]. There are some pharmaceuticals which target IgE production or JAK3 activity for preventing allergic diseases [20,21]. To our knowledge, this is a first report of chemical compounds that induce downregulation of γc expression.

In conclusion, we demonstrate here that flavones such as chrysin and apigenin can suppress IL-4 signaling by reducing the cell surface expression of γc . Further studies are needed to elucidate the mechanisms for negative regulation of γc expression by flav-

ones. We expect that the results described here may contribute to the development of means to prevent and cure in type I allergic diseases.

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