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Isoflavones Suppress the Expression of the FcεRI High-Affinity Immunoglobulin E Receptor Independent of the Estrogen Receptor

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ABSTRACT: Isoflavones found in soybeans and soy products possess clinically relevant properties. However, the anti-allergic effect of isoflavones has been poorly studied. We examined the effects of isoflavones, genistein, daidzein, and equol, on the expression of the high-affinity immunoglobulin E (IgE) receptor, FcεRI, which plays a central role in IgE-mediated allergic response. Flow cytometric analysis showed that all of these isoflavones reduced the cell surface expression of FcεRI on mouse bone-marrow-derived mast cells and human basophilic KU812 cells. All isoflavones decreased the levels of the FcεRIα mRNA in the cells. Genistein reduced the mRNA expression of the β chain, and daidzein and equol downregulated that of the γ chain. The suppressive effects of isoflavones on FcεRI expression were unaffected by ICI 182,780, an estrogen receptor antagonist, suggesting that these effects were independent of estrogen receptors.

KEYWORDS: *FcεRI, isoflavone, mast cell, basophil, estrogen receptor*

INTRODUCTION

Mast cells are resident cells of several types of tissues and contain many granules, such as histamine, which is a chemical mediator, causing some of the symptoms of an allergic reaction. Basophils have similar characteristics to mast cells in that they store and secrete histamine. Immunoglobulin E (IgE) produced by B cells is a key molecule to trigger the release of chemical mediators from these cells. Mast cells and basophils express high-affinity IgE receptor (FcεRI) on their cell surface, and cross-linking of allergen-specific IgE bound to FcεRI leads to the release of inflammatory mediators. Therefore, FcεRI is key for the triggering of an IgE-mediated allergic reaction, such as those seen in atopic dermatitis, bronchial asthma, allergic rhinitis, and food allergy.[1,2] FcεRI is a tetrameric structure consisting of an α, a β, and two γ chains. In humans, the tetrameric structure is not obligatory because an alternative form is present, comprising an αγ2 trimer.[3] The α chain mostly extends out to the extracellular region and binds with high affinity to the Fc portion of IgE, while the γ chains transduce the signals.[3] The β chain is an amplifier of signal strength[4,5] and enhances the expression of unoccupied receptors on the cell surface.[6] Analysis of FcεRIα-deficient mice demonstrated that IgE cannot bind to the cell surface of mast cells, and consequently, degranulation through IgE-binding cannot be induced.[7] Thus, it is expected that the downregulation of FcεRI expression in mast cells and basophils can lead to the attenuation of IgE-mediated allergic symptoms. However, the evaluations of anti- or proallergic factors in foodstuffs performed thus far are almost entirely based on the inhibitory effect on chemical mediator release.[8−10]

Flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which have a diphenylpropane skeleton (C6C3C6) structure, and include monomeric flavanols, flavanones, flavones, flavonols, and isoflavones. Some flavonoids possess various clinically relevant properties, such as antitumor, antiplatelet, anti-ischemic, and anti-inflammatory activities.[11,12] Moreover, some flavonoids were previously shown to inhibit histamine release from human basophils and rat mast cells.[13,14] Isoflavones, found mainly in soy and soy foods, exist as glycoside in nature, an inactive form to which a sugar is conjugated. Glycosides are not absorbed in small intestine and require hydrolysis for bioavailability and subsequent metabolism.[15] Ingested isoflavone glycosides are hydrolyzed by intestinal glucosidases, producing the aglycones, genistein, daidzein, and glycitein. Daidzein is converted to equol by intestinal bacteria after undergoing metabolism to dihydrodidzein[16] (Figure 1). These isoflavones are well-established as phytoestrogens because they have avidity for estrogen receptors (ERs).[17−19] They inhibit the reduction in bone mass induced by estrogen deficiency, being effective for the prevention of osteoporosis,[20] and they have been associated with reduced incidences of breast and prostate cancers and cardiovascular diseases.[21] However, the anti-allergic effect of isoflavones has been poorly studied. In the present study, we examined the effect of isoflavones, genistein, daidzein, and equol, on the expression of FcεRI in mouse mast cells and human basophils.

MATERIALS AND METHODS

Reagents. Genistein and daidzein were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). (*R*,*S*)-Equol was purchased

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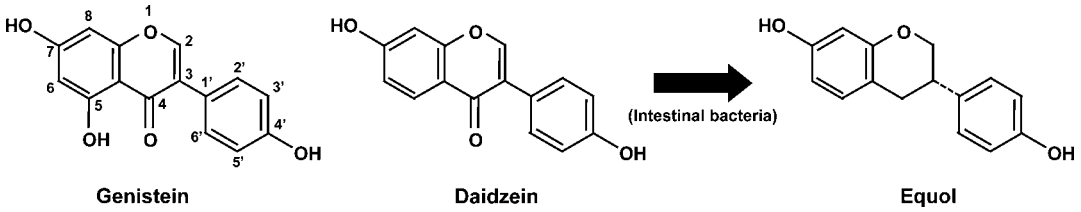

Figure 1. Chemical structure of soy isoflavones. Genistein and daidzein are two major isoflavones in soybean. Equol is converted from daidzein by intestinal bacteria.

from LC Laboratories (Woburn, MA), and (*S*)-equol and (*R*)-equol were obtained from Cayman Chemical (Ann Arbor, MI). Stock solutions of genistein, daidzein, (*R*,*S*)-equol, (*S*)-equol, and (*R*)-equol were prepared in dimethylsulfoxide (DMSO). 17β-Estradiol (E2) was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO. ICI 182,780 was purchased from Tocris Bioscience (Bristol, U.K.) and dissolved in ethanol (EtOH). Mouse anti-human FcεRIα monoclonal antibody CRA-1 was obtained from Kyokuto Seiyaku (Tokyo, Japan). Negative-control mouse IgG2b antibody was purchased from DakoCytomation Denmark (Glostrup, Denmark). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody was purchased from Protos Immunoresearch (Burlingame, CA). Mouse anti-phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), rabbit anti-ERK1/2, and mouse anti-ERα antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit antibodies were obtained from Zymed Laboratories, Inc. (San Francisco, CA) and ICN Pharmaceuticals, Inc. (Aurora, OH), respectively.

Cell Culture. KU812 cells were obtained from the Japanese Cancer Resources Bank (Tokyo, Japan) and were maintained in RPMI-1640 culture medium (Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY), 100 units/mL penicillin G (Meiji Seika, Tokyo Japan), 100 mg/mL streptomycin (Meiji Seika), and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, Wako Pure Chemical Industries, Osaka, Japan) buffer. KU812 cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Preparation of Mouse Bone-Marrow-Derived Mast Cells (BMMCs). Femurs and tibias were harvested from 13-week-old male BALB/c mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan). Both ends were severed, and they were steeped in RPMI-1640 medium. Bone marrow cells were harvested from the medium by filtration and washed in RPMI-1640 medium. The bone marrow cells were cultured in medium containing 5 ng/mL mouse interleukin-3, minimal essential medium (MEM) non-essential amino acid solution, and 10% fetal bovine serum (FBS) for 3 weeks to differentiate into mast cells. The BMMCs were used for experiments after confirmation that more than 80% of cells were treated with toluidine blue (Sigma Chemical Co.), which stains mast cells reddish purple.

Flow Cytometric Analysis of Cell Surface FcεRI Expression. The cell surface expression of FcεRI was assessed by flow cytometry. BMMCs and KU812 cells cultured with or without isoflavones were incubated with the anti-FcεRI α chain mouse monoclonal antibody for 60 min at 4 °C. The cells were then washed once in phosphate-buffered saline (PBS) and exposed to the FITC-conjugated $F(ab')_2$ goat anti-mouse IgG for 60 min at 4 °C. The cells were washed in PBS again, and then detection of the cell surface FcεRI α chain was performed using a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA). Mouse IgG2b antibody was used as the isotype-matched negative control.

Cytotoxicity Assay. Cell viability was analyzed with the trypan blue dye exclusion assay. After the exposure to 10 or 25 μM isoflavones for the indicated times (24, 48, or 72 h), cells were collected, suspended in a trypan blue solution, and counted using a hemocytometer. The cells with or without blue dye staining were recorded as dead or alive, respectively.

Quantitative Reverse-Transcription Polymerase Chain Reaction (PCR). Cells were incubated with or without 25 μM isoflavones for 24 h. Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. First-strand cDNA was synthesized from total RNA (400 ng) with PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan). Quantitative real-time PCR was performed using a thermal cycler dice real-time system (Takara Bio, Inc.). Specific primer sequences for each gene were as follows: human FcεRIα, sense 5′-GCAAAGTGTGGCAGCTGGACTA-3′ and antisense 5′-CTGTGTCCACAGCAAACAGAATCA-3′; human FcεRIβ, sense 5′-GCTTTATTTAATTGTAGGGCCTGAG-3′ and antisense 5′-CATGCCATGGAAGTGATGTG-3′; human FcεRIγ, sense 5′-CTCCAGCCCAAGATGATTCCA-3′ and antisense 5′-GCATCCAG-GATATAGCAGCAGAGCTGA-3′; human β-actin, sense 5′-TGGCAC-CCAGCACAATGAA-3′ and antisense 5′-CTAAGTCATAGTCCGCC-TAGAAGCA-3′.

Immunoblot Analysis. Cells were rinsed once with PBS and lysed in 1% Triton X-100 lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM

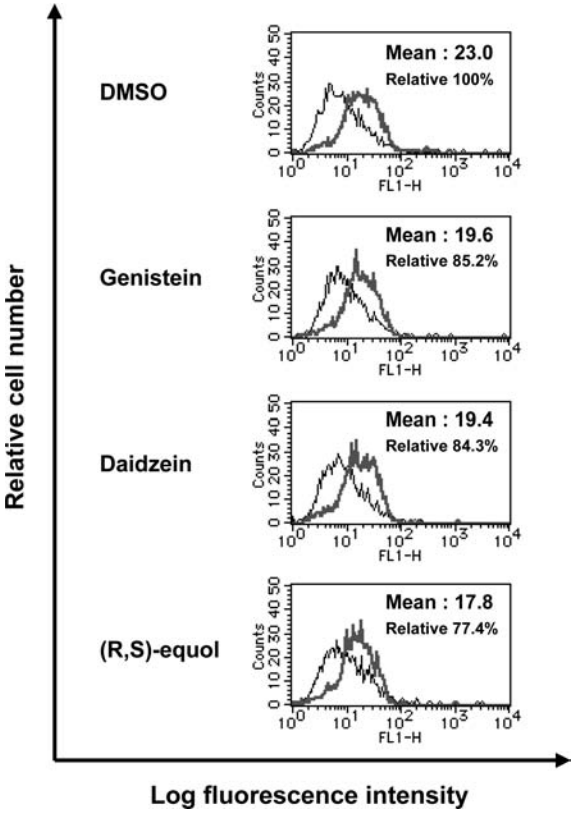

Figure 2. Effect of isoflavones on cell surface expression levels of FcεRI in mast cells. Mouse BMMCs were cultured with 20 μM genistein, daidzein, or (*R*,*S*)-equol for 24 h in 5% FBS−RPMI-1640 medium. FcεRIα expression was assessed by flow cytometric analysis. The left curves in the data indicate the fluorescence used with isotype control antibody, and the right curves are those with anti-FcεRI antibody. The mean values indicate the average of fluorescence intensity of FcεRI, and relative values represent the ratio of the mean values compared to that of DMSO-treated cells.

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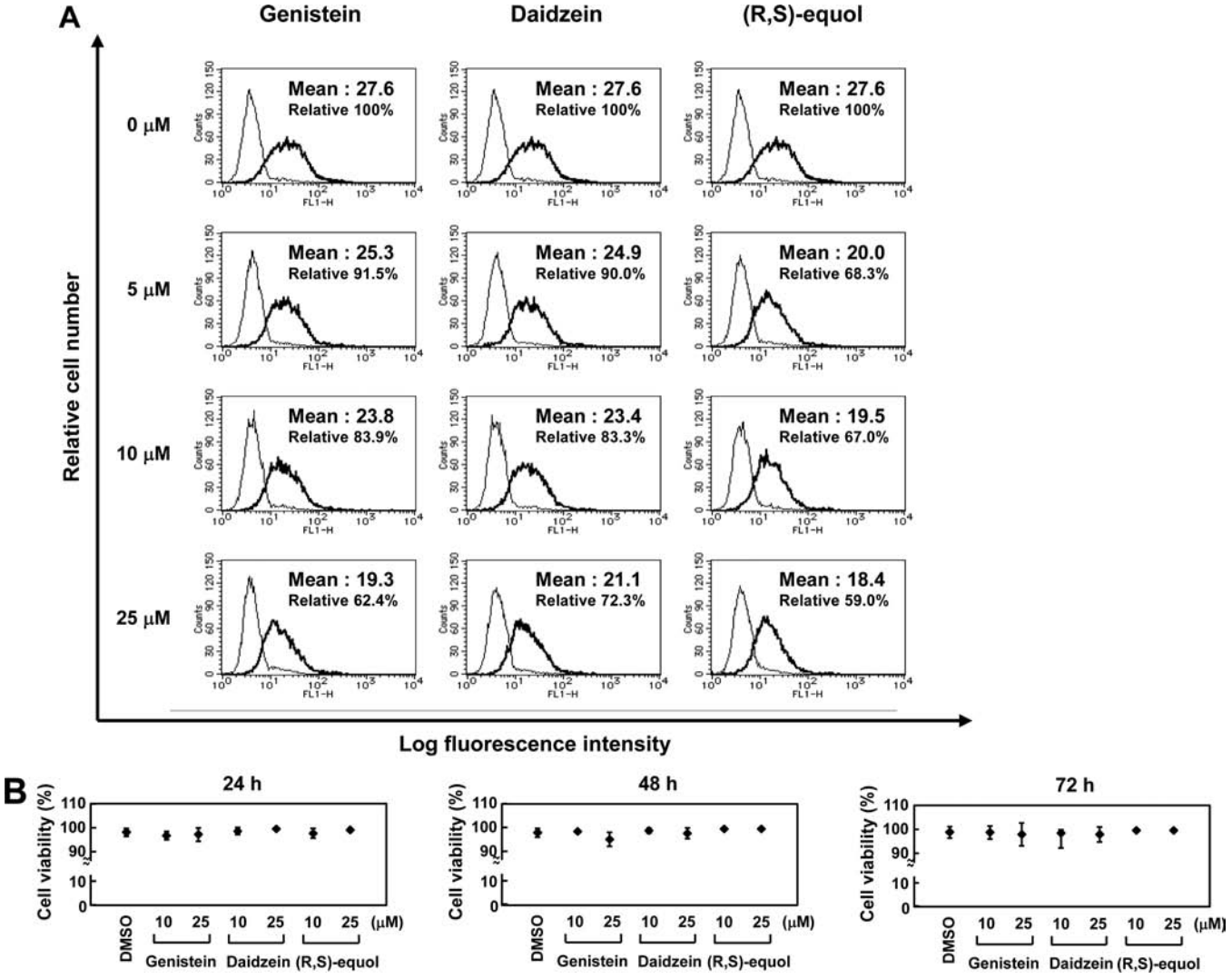

Figure 3. Effect of isoflavones on the cell surface expression levels of FcεRI in basophils. (A) Human basophilic KU812 cells were cultured with 5, 10, or 25 μM genistein, daidzein, or (*R*,*S*)-equol for 24 h in 5% FBS−RPMI-1640 medium. Then, FcεRIα expression level in the cells was assessed by flow cytometric analysis, as noted in Figure 2. (B) KU812 cells were cultured with 10 or 25 μM genistein, daidzein, or (*R*,*S*)-equol for 24, 48, or 72 h in 5% FBS−RPMI-1640 medium. Then, the cell viability was assayed by trypan blue staining. Data are presented as the mean ± SD, with $n = 3$.

NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL aprotinin]. Insoluble material was removed by centrifugation at 12000*g* for 10 min at 4 °C. Prior to analysis, total protein in the cell lysates was measured using a colorimetric bicinchoninic acid protein assay (Pierce Biotechnology, Inc., Rockford, IL) against bovine serum albumin (BSA) standards. The proteins were eluted by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer. The cell lysates were run on a 10% SDS−PAGE gel and blotted onto nitrocellulose membranes. After blocking, proteins were identified using mouse anti-phosphorylated ERK1/2, rabbit anti-ERK1/2, mouse anti-FcεRIα, or mouse anti-ERα antibodies. The bands were visualized with HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibodies, followed by visualization using the ECL system (GE Healthcare, Little Chalfont, U.K.).

Statistical Analysis. For all of the sections, the experiments were performed at least 3 times and the representative data were shown. Results are expressed as the mean ± standard deviation (SD) in Figures 4−6A. A Student's *t* test was used to determine statistical significance between the control and test groups. *p* values of <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Isoflavones Suppress Cell Surface Expression of FcεRI in Mouse BMMCs. We examined whether isoflavones reduce cell surface expression of FcεRI in mouse BMMCs by culturing them with genistein, daidzein, or (*R*,*S*)-equol for 24 h before analysis by flow cytometry. The levels of cell surface FcεRIα were lower in the cells treated with the isoflavones when compared to control cells (Figure 2), suggesting that these three isoflavones have a suppressive effect on the cell surface expression of FcεRIα in mouse BMMCs. The mean fluorescence intensity of FcεRIα expression in (*R*,*S*)-equol-treated cells was lower compared to genistein- or daidzein-treated cells, indicating that the suppressive effect of (*R*,*S*)-equol on FcεRIα expression is stronger than that of genistein or daidzein.

Isoflavones Suppress the Cell Surface Expression of FcεRI in a Human Basophilic Cell Line. Next, we addressed whether isoflavones had inhibitory effects on cell surface expression of FcεRI in the human basophil cell line KU812. All of the isoflavones tested were shown to suppress the cell surface

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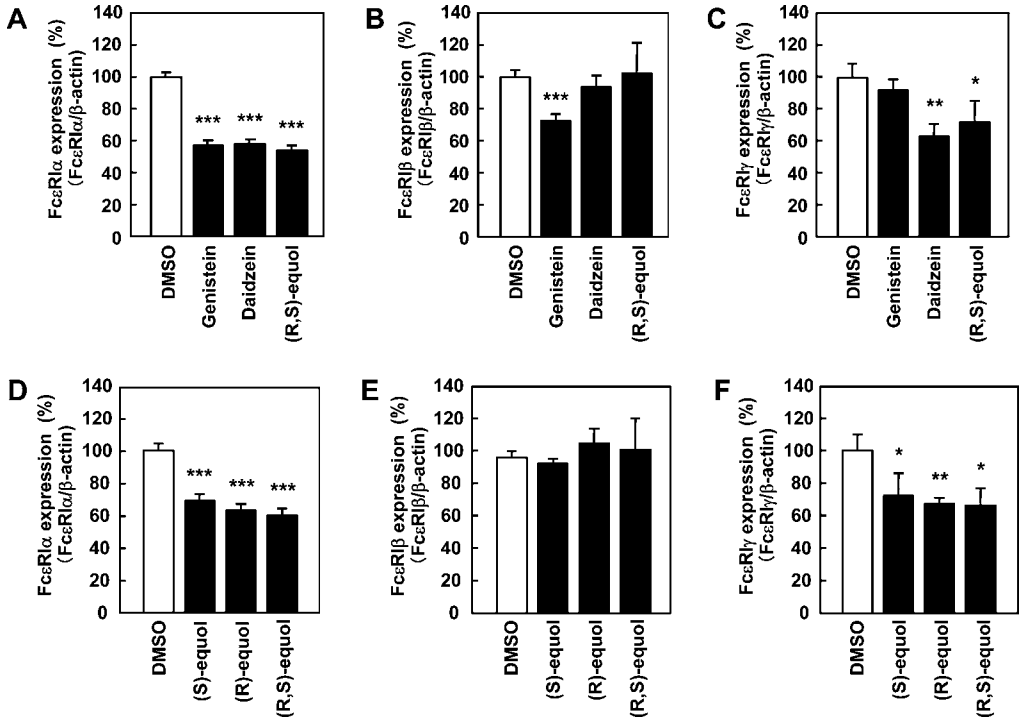

Figure 4. Effect of isoflavones on the mRNA expression levels of FcεRI*α*, FcεRI*β*, and FcεRI*γ* chains in basophils. KU812 cells were cultured in the presence of 25 μM genistein, daidzein, (*R*,*S*)-equol, (*S*)-equol, or (*R*)-equol for 24 h in 5% FBS−RPMI-1640 medium. After total mRNA isolation from the cells was performed, mRNA expression levels of (A and D) FcεRI*α*, (B and E) FcεRI*β*, and (C and F) FcεRI*γ* chains were analyzed by quantitative RT-PCR. Each expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Data are presented as the mean ± SD, with $n = 3$. (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$.

expression of FcεRI*α* at concentrations higher than 5 μM (Figure 3A). Among three isoflavones, (*R*,*S*)-equol most powerfully suppressed the FcεRI*α* expression in KU812 cells, similar to the result in mouse BMMCs. Although the concentrations of isoflavones tested were slightly higher than the physiological concentrations, they had no effect on the cell viability at concentrations lower than 25 μM, which was assayed by trypan blue stain (Figure 3B). These data suggest that isoflavones suppress the cell surface expression of FcεRI*α* in KU812 cells without cytotoxicity.

Isoflavones Suppress the mRNA Expression of FcεRI Subunits in a Human Basophilic Cell Line. To elucidate the molecular mechanism by which these isoflavones are able to suppress the cell surface expression of FcεRI*α*, we examined the effect of the isoflavones on the mRNA expression level of FcεRI*α* (Figure 4A). All of the cells treated with genistein, daidzein, and (*R*,*S*)-equol exhibited a significantly lower mRNA expression level of FcεRI*α*, as compared to the control cells, suggesting that the isoflavones reduce the amount of cell surface expression of FcεRI*α* by reducing the mRNA expression level. However, other effects would also be involved in their suppressive effect on the cell surface expression of FcεRI*α* because the intensity of their effect on the mRNA level of FcεRI*α* disagrees with that on the cell surface expression level of FcεRI*α*. Furthermore, we addressed the mRNA expression levels of FcεRI*β* and FcεRI*γ* (panels B and C of Figure 4). Only genistein reduced the mRNA expression level of FcεRI*β*. On the other hand, daidzein and equol decreased the FcεRI*γ* mRNA expression level, whereas genistein had little effect. These data suggest that the difference between the effects of these isoflavones on the mRNA expression of *β* and *γ* chains might be involved in the presence or absence of the hydroxyl

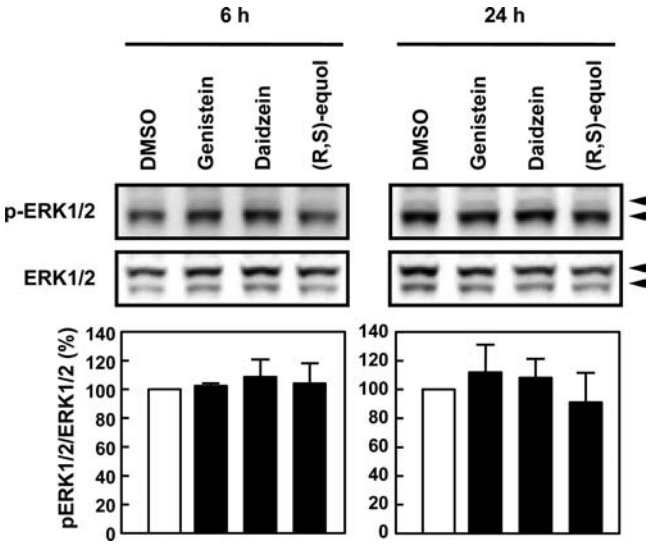

Figure 5. Effect of isoflavones on ERK1/2 phosphorylation. KU812 cells were cultured in the presence of 25 μM genistein, daidzein, or (*R*,*S*)-equol for 6 or 24 h in 5% FBS−RPMI-1640 medium. The cell lysates were prepared and subjected to 10% SDS−PAGE. Phosphorylated ERK1/2 was detected by immunoblotting using an anti-phospho-ERK1/2 antibody. Data are presented as the mean ± SD, with $n = 3$.

group at position 5 of A ring and/or the tyrosine kinase inhibitory effect characteristic of genistein.[22] It has been reported that FcεRI*β* is involved in the pathogenesis of atopic disease,[23] and oral genistein suppresses the development of atopic dermatitis in NC/Nga mice.[24] Genisetein might suppress the atopic disease by downregulation of FcεRI*β*. A mechanism for downregulation of the mRNA expression of FcεRI subunits

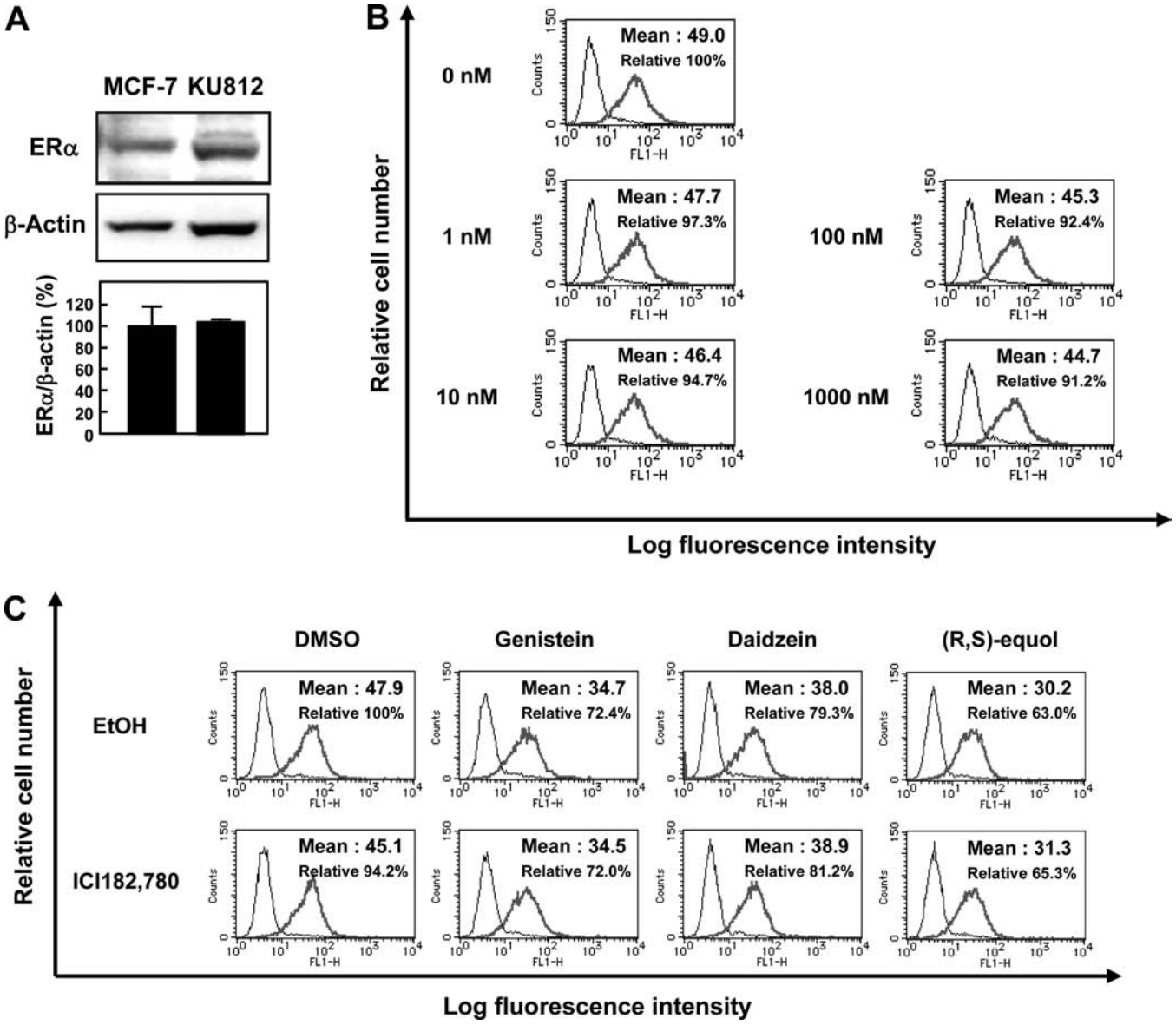

Figure 6. Involvement of ERs in the suppressive effects of isoflavones on the cell surface expression levels of FcεRI. (A) Whole cell extracts were analyzed by western blotting with antibodies against ERα and β-actin. Data are presented as the mean ± SD, with n = 3. (B) KU812 cells were cultured with E2 for 24 h in 5% FBS−RPMI-1640 medium. The FcεRI expression level was assessed by flow cytometric analysis, as noted in Figure 2. (C) KU812 cells were preincubated with ICI 182,780 (1 μM) in 5% FBS−RPMI-1640 medium for 30 min. After preincubation, cells were cultured with 25 μM genistein, daidzein, or (*R*,*S*)-equol in 5% FBS−RPMI-1640 medium for 24 h. FcεRI expression was assessed by flow cytometric analysis, as noted in Figure 2. Relative values represent the ratio of mean values compared to that of DMSO- and EtOH-treated cells.

by isoflavones remains unclear. It would be worth examining the effect of isoflavones on the transcriptional activity of specific transcription factors of FcεRI subunit genes.[25−28]

Equol is a chiral molecule that can exist in two enantiomeric forms, (*S*)-equol and (*R*)-equol, and the enantiomer produced by metabolic reduction from isoflavones is known to be (*S*)-equol.[29] It is reported that they have different affinities for ERs,[30] and (*R*)-equol inhibits chemically induced tumorigenesis of breast cancer *in vivo*, whereas (*S*)-equol has no effect.[31] We assessed the effect of (*S*)-equol and (*R*)-equol on the mRNA expression levels of FcεRIα, FcεRIβ and FcεRIγ chains. Both enantiomers equally reduced the mRNA level of α and γ chains (panels D and F of Figure 4), and neither of them had any effect on the β chain mRNA expression level (Figure 4E). These data suggest that (*S*)- and (*R*)-equol act on FcεRI subunits in the same manner.

Isoflavones Had No Effect on ERK1/2 Phosphorylation. We previously found that green tea polyphenol EGCG and flavones, including chrysin and apigenin, suppressed the level of FcεRIα expression, and those suppressive effects were associated with a reduction in the phosphorylation of ERK1/2.[32−34] Thus, we assessed the effect of the isoflavones on the phosphorylation levels of ERK1/2 in KU812 cells (Figure 5). There was little change in ERK1/2 phosphorylation after isoflavone treatment, suggesting that the downregulation of FcεRIα expression by isoflavones does not involve modulation of ERK1/2 phosphorylation different from EGCG and flavones.

Suppressive Effect of Isoflavones on the Cell Surface Expression of FcεRI Is Independent of Estrogen Receptors. Isoflavones are often referred to as phytoestrogens because they have an affinity for ERs[17−19] and are reported to function through ERs.[35−37] ERs can be detected in a broad

spectrum of tissues, with ERα mainly expressed in uterus, prostate, ovary, testes, epididymis, bone, breast, brain, liver, and white adipose tissue.[38] We examined ERα expression levels in KU812 cells and found that levels were comparable to those of the MCF-7 human breast cancer positive-control cells (Figure 6A). Next, we examined whether the suppressive effects of isoflavones on the expression of FcεRIα were dependent upon ERs. E2, an agonist for ERs, had little effect on the cell surface FcεRIα expression level (Figure 6B). The suppressive effects of genistein, daidzein, and (*R*,*S*)-equol were unaffected by ICI 182,780, an antagonist for ERs (Figure 6C). These results suggest that the suppressive effects of isoflavones on FcεRIα expression are independent of ERs. Although a majority of the mechanisms elucidated for the physiological actions of isoflavones are believed to involve ERs, the existence of ER-independent effects has recently been suggested. Genistein has been reported to decrease thymus weight in ovariectomized adult mice through both ER and non-ER mechanisms.[39] Genistein also induces apoptosis in zebrafish embryos in an ER-independent manner.[40] The rapid vascular relaxation effect of equol in human aortic and umbilical vein endothelial cells has been reported to be independent of ERs.[41] Equol inhibits nuclear factor-κB activation and the subsequent gene expression of tumor necrosis factor-α in mouse macrophages independent of ER.[42] We demonstrated a suppressive effect on FcεRIα expression as a novel ER-independent activity of isoflavones in the present study. Further studies are needed to understand the detailed mechanisms and the molecular targets of ER-independent effects of isoflavones.

In summary, we demonstrated that isoflavones, such as genistein, daidzein, and equol, suppress the expression of FcεRIα partially as a result of reducing the mRNA expression of FcεRIα in an ER-independent manner. Besides, genistein reduces the mRNA level of FcεRIβ, and daidzein and equol downregulate that of FcεRIγ. It would be worth considering the effect of soy-based foods that contain isoflavones on the expression of FcεRI. Our results should help applications of isoflavones to therapeutic potential, preventing various allergic diseases.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

FcεRI, high-affinity IgE receptor; ER, estrogen receptor; BMMC, bone-marrow-derived mast cell; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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