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Dietary apigenin attenuates the development of atopic dermatitis-like skin lesions in NC/Nga mice[☆]

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Abstract

One of the flavones, apigenin has various physiological functions including anti-inflammatory activities. Atopic dermatitis (AD) is a chronically relapsing inflammatory disorder that is characterized by pruritic and eczematous skin lesions. To evaluate the anti-allergic effect of apigenin in vivo, we examined the effect of dietary apigenin on picrylchloride (PiCl)-induced AD-like pathology in NC/Nga mice. NC/Nga mice were fed experimental diets containing apigenin from Day 18 after sensitized with PiCl for 4 weeks. Dietary apigenin significantly alleviated the development of skin lesions, accompanied by lower serum immunoglobulin (Ig) G1 and IgE levels in NC/Nga mice. Interferon (IFN)-γ mRNA expression level in spleen cells from NC/Nga mice was reduced by apigenin feeding. Moreover, interleukin 4-induced signal transducers and activators of transcription 6 phosphorylation in primary spleen cells from BALB/c mice was inhibited by treatment with apigenin. These results suggest that apigenin attenuates exacerbation of AD-like symptoms in part through the reduction of serum IgE level and IFN-γ expression in NC/Nga mice.
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Keywords: Apigenin; NC/Nga mice; Atopic dermatitis; Picrylchloride; IgE; IFN-γ; STAT6

1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory disorder that is characterized by pruritic and eczematous skin lesions often associated with elevated serum immunoglobulin (Ig) E level. AD has been recently increasing in industrialized countries and onset is usually during early infancy and childhood, but can also occur in adulthood [1,2]. Although topical steroids, emollients and oral antihistamines are used as the first-line therapy for AD, many patients are still worried about long term use of these agents [3]. The therapy and prevention of AD through dietary intervention is currently receiving considerable attention. Recently, it has been reported that intake of diet containing lactic acid bacteria and natural polyphenols such as astragalin and genistein suppresses AD-like skin lesions [4–6].

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. One of the flavones, apigenin has been found to possess various clinically relevant properties such as anti-tumor, anti-platelet and anti-inflammatory activities [7–10]. Our previous report demonstrated that dietary apigenin could reduce serum IgE level in BALB/c mice immunized by ovalbumin (OVA) [11], suggesting that the intake of apigenin may alleviate allergic symptoms.

Signal transducers and activators of transcription 6 (STAT6) is a critical transcription factor that regulates interleukin (IL)-4-mediated immune responses. STAT6 is phosphorylated and activated through an IL-4 receptor-mediated signal. IL-4-induced STAT6 activation results in transcriptional events including IgE class switching [12,13].

NC/Nga mice are accepted as a suitable model for human atopic dermatitis [14]. Repeated applications of

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picrylchloride (PiCl) to NC/Nga mice cause a simple and reproducible hapten-induced AD-like skin disease even under specific pathogen-free conditions [4,15,16]. In this study, we evaluated the effect of apigenin on PiCl-induced AD-like symptoms in NC/Nga mice. Further, to study molecular mechanisms for anti-allergic effect of apigenin, we investigated the effect of apigenin on IL-4-induced STAT6 activation in primary spleen cells.

2. Materials and methods

2.1. Chemicals

Apigenin was purchased from Sigma-Aldrich (St. Louis, MO, USA). MF diet was obtained from Oriental Yeast (Tokyo, Japan). 2,4,6-Trinitrochlorobenzene [picrylchloride (PiCl)] was purchased from Tokyo Chemical Industry (Tokyo, Japan), and olive oil was purchased from J-Oil Mills (Tokyo, Japan). Anti-mouse CD3ε antibody was obtained from R&D Systems (Minneapolis, MN, USA), and anti-mouse CD28 antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Protein A sepharose 6MB was purchased from GE Healthcare UK (Buckinghamshire, UK). Anti-STAT6 antibody and horseradish peroxidase-conjugated p-Tyr (PY20) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Experimental animals and diets

Male 5-week-old NC/Nga mice bred under specific pathogen-free (SPF) conditions were purchased from Charles River Laboratories Japan (Yokohama, Japan). The mice were kept at the Biotron Institute of Kyushu University in a 12-h light/12-h dark cycle (light on 8 a.m.–8 p.m.) in an air-conditioned room (20°C and 60% humidity under SPF conditions). This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese government. After preliminary breeding for 1 week, all mice were provided with control diet (MF diet) at 5 g/day for 18 days. Then, the mice divided in two groups were provided with either of the following diets: control diet or apigenin diet (MF diet containing 0.05% apigenin) at 5 g/day for 4 weeks. Body weight was measured before the experimental feed started and every fourth day during the experimental period. Blood samples were collected from the abdominal aorta under light anesthesia with diethylether at the end of the feeding period. Serum was obtained by centrifugation at 1000×*g* for 15 min at 4°C and stored at −80°C until use.

Male 8-week-old BALB/c mice were purchased from Charles River Laboratories Japan.

2.3. Induction and observation of AD

Dermatitis induced by PiCl was developed in NC/Nga mice according to standard instructions as previously described [15,16]. Briefly, the back and ears were sensitized with 5% PiCl dissolved in an ethanol and acetone mixture (4:1). Four days after sensitization, the back and ears were challenged with 1% PiCl dissolved in olive oil. This challenge was repeated five times at 1-week intervals.

The observation items were the following four symptoms: flare and haemorrhage, oedema, xerosis and incrustation, excoriation and erosion. The dermatitis scores were expressed as the sum of the individual score grades from 0 to 3 (no sign, 0; mild, 1; moderate, 2; severe, 3) for each item [5,16–18].

2.4. Serum levels of Ig's and cytokine

Serum levels of total IgG1 and IgE were measured by using mouse IgG1 and IgE enzyme-linked immunosorbent assay (ELISA) Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's protocol.

As described in our previous paper [19], 40 cytokine proteins in mice sera were assessed using a commercially available RayBio Mouse Inflammation Antibody Array 1 (Ray Biotech, Norcross, GA, USA). The density of each band was quantified using a computer program obtained from the US National Institutes of Health.

2.5. Preparation of spleen cells

Cell suspensions were prepared from spleens. Red cells were lysed with the ammonium-chloride potassium carbonate buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA·2Na, pH 7.4). Nucleated cells were washed 3 times with RPMI 1640 medium.

2.6. Real-time reverse transcriptase-polymerase chain reaction

The levels of IL-4 and interferon (IFN) -γ mRNA were measured with the real-time reverse transcriptase-polymerase chain reaction method. Spleen cells from NC/Nga mice (2×10^6 cells/mL) were cultured in RPMI 1640 medium with anti-CD3ε antibody (1 μg/mL) and anti-CD28 antibody (1 μg/mL) at 37°C for 24 h. Total RNA was extracted from the cells using TRIZOL (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (5 μg) with an oligo$(dT)_{20}$ primer and moloney mouse leukemia virus-reverse transcriptase (GE Healthcare UK). The real-time quantitative PCR was performed on a Thermal Cycler Dice Real Time System TP800 machine (Takara Bio, Shiga, Japan) using SYBR Premix Ex Taq according to the manufacturer's protocol. Specific primer sequences for each gene were as follows: mouse IL-4, sense 5′-TCTCGAATGTACCAGGAGCCATATC-3′, and antisense 5′-AGCACCTTGGAAGCCCTACAGA-3′; mouse IFN-γ, sense 5′-CGGCACAGTCATTGAAAGCCTA-3′, and antisense 5′-GTTGCTGATGGCCTGATTGTC-3′; mouse β-actin, sense 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and antisense 5′-ATGGAGCCACCGATCCACA-3′.

2.7. Immunoprecipitation and immunoblot analysis for STAT6 phosphorylation

Primary spleen cells (2×10^7 cells/mL) from BALB/c mice were cultured with apigenin for 3 h under serum-free conditions. Cells were rinsed once with phosphate-buffered saline (PBS) and lysed in 1% Triton X-100 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 1 mM pervanadate). Insoluble material was removed by centrifugation at 12,000×*g* for 20 min at 4°C. Prior to analysis, total protein in the cell lysates was measured using a colorimetric BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) against bovine serum albumin standards. Whole cell lysates were immunoprecipitated with anti-STAT6 antibody pre-bounded to protein A sepharose 6MB for 4 h at 4°C. The beads were washed three times with lysis buffer and PBS respectively, and then the bound proteins were eluted with Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Immunoprecipitates were run on 8% SDS-PAGE gel and blotted onto nitrocellulose membranes. After blocking, proteins were identified using HRP-conjugated PY20 antibody and the bands were visualized using the ECL system (GE Healthcare UK).

2.8. Statistical analysis

Data were analyzed using the Wilcoxon signed-rank test. Mean values were significantly different from those of the control group at $P<.05$.

3. Results

3.1. Dietary apigenin suppresses PiCl-induced AD-like skin lesions in NC/Nga mice

NC/Nga mice have been shown to develop AD-like skin lesions by repeated application of PiCl under SPF conditions. In the present study, dermatitis was noted from 1 week and gradually increased from 2 weeks after the start of induction treatment. To examine whether apigenin diet could attenuate the development of dermatitis symptoms, apigenin diet was provided for 4 weeks after control diet for 18 days (Fig. 1A). The clinical score of the apigenin group was low as compared with that of the control group from Day 42 (Fig. 1B). As shown in Fig. 1C, AD-like skin lesions of the ears in mice fed apigenin were remarkably suppressed. In addition, there was no significant difference in body weight between two groups during the experimental period (Fig. 1D). These results suggest that dietary apigenin is beneficial for attenuation the development of dermatitis symptoms without changes of body weight.

3.2. IgG1, IgE and cytokine levels in NC/Nga mice sera

It has been reported that the dermatitis induced by PiCl in an SPF environment is caused by a Th2 reaction in NC/Nga mice [16]. Th2 cells produce cytokine such as IL-4 and IL-13, which are implicated mainly in B cell, help for IgG1 and IgE production. To investigate whether alteration of IgG1 and IgE levels in mice sera are involved in the inhibitory effect of apigenin on the exacerbation of dermatitis, we assessed their levels at day 47 after sensitization. As shown in Fig. 2, both serum IgG1 and IgE levels were significantly low in the apigenin group as compared with that in the control group. These results suggest that dietary apigenin inhibits the development of dermatitis through the suppression of IgG1 and IgE hyperproduction.

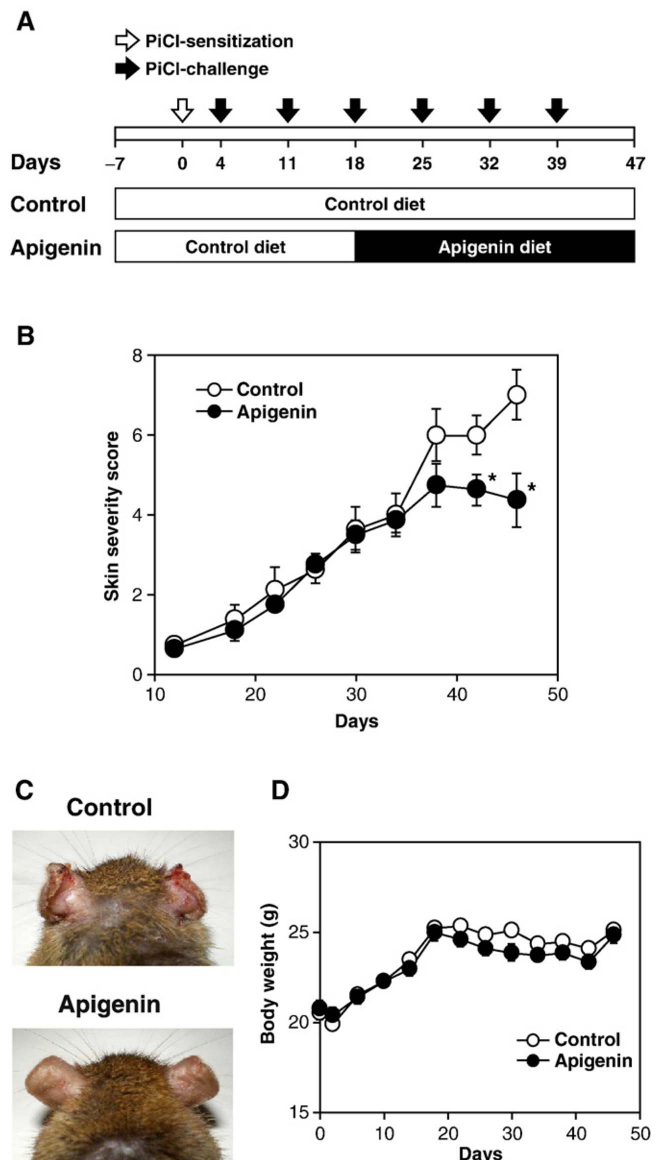

Fig. 1. Effect of dietary apigenin on clinical scores of PiCl-induced skin symptoms. Schematic representation of the experiment (A). Data are means ±S.E. for eight mice in both groups. Statistical analysis was performed using the Wilcoxon signed-rank test. Mean values are significantly different from control group: *$P<.05$ (B). Representative photographs at day 46 (C). Body weights in both groups of NC/Nga mice were monitored every fourth day during the experimental period. Data are means±S.E. for eight mice in both groups (D).

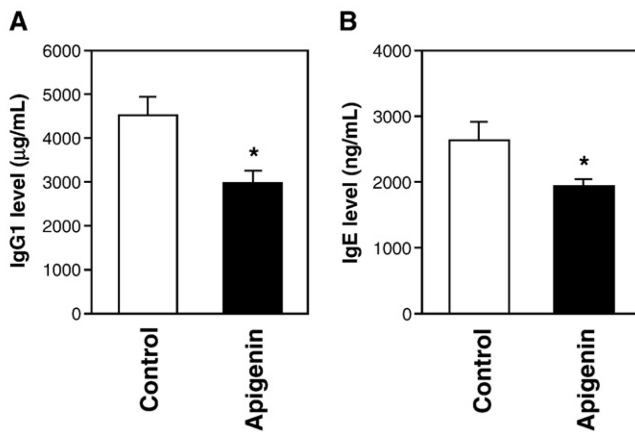

Fig. 2. Total serum IgG1 and IgE levels in NC/Nga mice. Serum concentration of IgG1 (A) and IgE (B) at the end of the experiment was measured by ELISA. Data are means±S.E. for six mice in both groups. Statistical analysis was performed using the Wilcoxon signed-rank test. Mean values are significantly different from control group: $^*P<.05$.

To clarify the mechanisms involved in the suppression of the IgE production in mice fed apigenin diet, we evaluated cytokine levels in sera using inflammation cytokine array.

Table 1
Effects of dietary apigenin on cytokine levels in NC/Nga mice sera

Cytokines	Apigenin	Cytokines	Apigenin
BLC	1.06	IL-17	N.D.
CD30L	1.14	I-TAC	N.D.
Eotaxin	1.11	KC	1.18
Eotaxin-2	1.16	Leptin	1.07
Fas ligand	N.D.	LIX	0.89
Fractalkine	1.18	Lymphotactin	1.01
GCSF	1.09	MCP-1	1.03
GM-CSF	1.09	MCSF	1.12
IFN-γ	1.08	MIG	N.D.
IL-1α	1.15	MIP-1α	1.08
IL-1β	N.D.	MIP-1γ	1.35
IL-2	1.12	RANTES	1.43
IL-3	1.03	SDF-1	1.46
IL-4	1.06	TCA-3	1.62
IL-6	N.D.	TECK	1.45
IL-9	1.10	TIMP-1	1.27
IL-10	N.D.	TIMP-2	1.22
IL-12 p40 p70	0.97	TNF-α	N.D.
IL-12 p70	1.23	sTNFRI	1.31
IL-13	1.38	sTNFRII	1.04

The relative levels of cytokines were determined by intensity. The densities of signals were normalized with background and positive control.
BLC, B lymphocyte chemoattractant; CD30L, cluster of differentiation 30 ligand; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; I-TAC, IFN-inducible T-cell alpha chemoattractant; KC, CXC ligand 1; LIX, lipopolysaccharide-induced CXC chemokine; MCP, monocyte chemoattractant protein; MCSF, macrophage colony-stimulating factor; MIG, monokine induced by interferon-γ; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T cell expressed and secreted; SDF, stromal cell-derived factor; TCA, thymus-derived chemotactic agent; TECK, thymus-expressed chemokine; TIMP, tissue inhibitor of metalloprotease; TNF, tumor necrosis factor; sTNFR, soluble tumor necrosis factor receptor; N.D., not detected.

However, no cytokine was remarkably up-regulated or down-regulated in the apigenin groups as compared with the control group (Table 1).

3.3. Dietary apigenin suppresses IFN-γ mRNA expression in spleen cells from NC/Nga mice

It has been reported that not only the Th2 cytokine such as IL-4 but also the Th1 cytokine such as IFN-γ plays important roles in the inflammation and hypertrophy of the skin in AD [20]. Both the T cell receptor (TCR/CD3) and the CD28 molecule costimulation signal in T cells augment the proliferation and secretion of multiple T cell cytokines such as IL-2, IFN-γ and IL-4 [21,22]. Therefore, we examined effects of dietary apigenin on IL-4 and IFN-γ mRNA expression induced by anti-CD3 and anti-CD28 antibodies costimulation in spleen cells at day 47 after sensitization. As shown in Fig. 3, the mRNA expression of IFN-γ in the apigenin group was significantly reduced as compared with that in the control group. On the other hand, there was no significant difference in IL-4 mRNA expression between the two groups. These results suggest that apigenin suppressed AD-like skin lesions at least in part through down-regulation of IFN-γ.

3.4. Apigenin suppresses IL-4-induced STAT6 tyrosine phosphorylation

To examine the mechanism for the suppression of IgG1 and IgE production by apigenin, we investigated the effect of apigenin on IL-4-induced STAT6 tyrosine phosphorylation in primary spleen cells from BALB/c mice. Cells were precultured for 3 h with apigenin followed by IL-4 stimulation for 30 min. Apigenin suppressed IL-4-induced phosphorylation of STAT6 (Fig. 4). This result suggests that

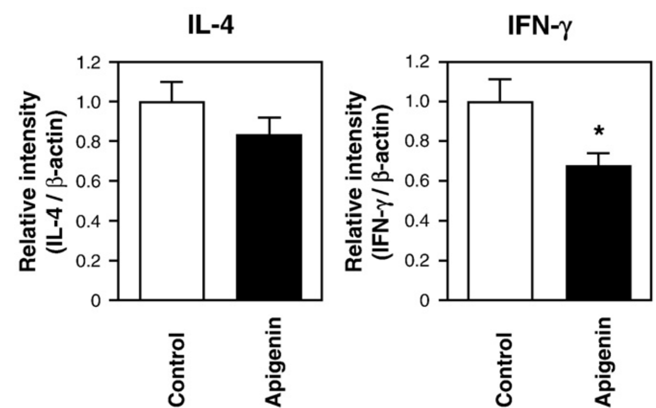

Fig. 3. Effect of dietary apigenin on IL-4 and IFN-γ mRNA expression in spleen cells from NC/Nga mice. After spleen cells from NC/Nga mice fed apigenin diet were cultured with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies for 24 h, total mRNA isolation from the cells was performed, and then IL-4, IFN-γ and β-actin mRNA were analyzed by real-time PCR. Data are means±S.E. for six mice in both groups. Statistical analysis was performed using the Wilcoxon signed-rank test. Mean values are significantly different from control group: $^*P<.05$.

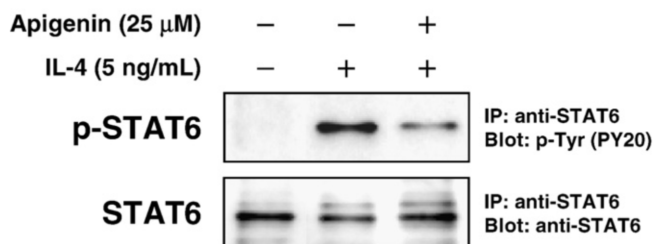

Fig. 4. Apigenin suppresses IL-4-induced STAT6 tyrosine phosphorylation in primary spleen cells from BALB/c mice. After spleen cells from BALB/c mice were cultured with apigenin (25 μM) for 3 h followed by treatment with IL-4 (5 ng/mL) for 30 min, STAT6 was immunoprecipitated, separated on an 8% SDS-PAGE and immunoblotted with the anti-phosphotyrosine antibody.

the reduction of IgG1 and IgE levels by apigenin may result from the suppression of STAT6 tyrosine phosphorylation.

4. Discussion

In this report, we demonstrated that dietary apigenin alleviates AD symptoms induced by PiCl in NC/Nga mice. Further, the improvement in clinical aspects was accompanied with the reduction of serum IgG1 and IgE levels and the down-regulation of IFN-γ expression induced by anti-CD3 and anti-CD28 antibodies costimulation in splenocytes. These results suggest that apigenin has therapeutic potential for AD.

A high-serum IgE concentration is one of the critical characteristics of atopic diseases, such as AD, asthma and allergic rhinitis. Clinical observations have suggested a correlation between serum IgE levels and the extent and/or the severity of a disease, especially with cutaneous lesions [23,24]. Therefore, IgE production is an important therapeutic target for AD. Recently, we have reported that two flavones, chrysin and apigenin, suppress IgE biosynthesis through the suppression of IL-4 and IL-13 expression in OVA-sensitized mice [11]. In this study, we show that dietary apigenin improves AD-like skin lesions and suppresses increase of serum IgE levels induced by PiCl. These findings indicate that apigenin exerts antiallergic effects by reducing IgE production.

There was no significant difference in serum levels of any cytokines between the control and the apigenin groups in this study. It has been suggested that T cells, mast cells, monocytes/macrophages and eosinophils infiltrated into AD-like skin lesions play important roles in the development of AD by releasing various cytokines/chemokines [23]. It is possible that dietary apigenin decreases cytokine levels not in serum but in skin lesions. The lymphocytes infiltrating into the skin lesions of AD have been shown to produce not only Th2 cytokines but also Th1 cytokines such as IFN-γ in later stages of the disease [23]. Furthermore, spontaneous IFN-γ production has been reported to associate with the development of skin lesions in NC/Nga mice [25]. Here, we show that dietary apigenin reduced IFN-γ mRNA expression induced by anti-CD3 and anti-CD28 antibodies in splenocytes from NC/Nga mice, suggesting that apigenin may suppress delayed-type hypersensitivity reaction.

It is known that the transcription factor STAT6 plays an essential role in IL-4 signaling. Furthermore, IgG1 and IgE are commonly induced by CD40 ligand and IL-4 through the STAT6 activation [12,26]. Moreover, it has been reported that some flavonoids including apigenin could inhibit IL-4-induced STAT6 phosphorylation in B cell line [27]. In our study, we show that dietary apigenin reduces both IgG1 and IgE levels without affecting IL-4 level in serum and its mRNA expression level in spleen cells. On the other hand, apigenin suppressed IL-4-induced STAT6 phosphorylation in primary spleen cells from BALB/c mice. These findings suggest that apigenin does not suppress Th2 differentiation and IL-4 production from T cells but inhibit IgG1 and IgE production from B cells through the suppression of IL-4-induced STAT6 activation.

Apigenin accumulates in a bound form as apigenin 7-*O*-glucoside and various acylated derivatives. After ingestion, the apigenin glucoside is hydrolyzed by both intestinal mucosal and bacterial β-glucosidases releasing the aglycone. In a recent study, absorption and excretion of apigenin after the ingestion of apigenin-rich food, i.e., parsley, was tested; the result was, apigenin is enriched in the human circulation. However, maximum plasma concentrations were comparably low (0.34 μmol/L), and on average, only 0.22% of the ingested apigenin dose was found in the 24-h urine samples [28]. The main part of the ingested apigenin may be either unabsorbed or remain in various tissues after absorption. In case of quercetin (3,3′,4′,5,7-pentahydroxyflavone), it has been reported that its major metabolite in human plasma are incorporated into the target cells (such as macrophages), converted to aglycone (and further to the methylated form) and exert the antiatherosclerotic activities [29]. In the future, it needs to be investigated how dietary apigenin is metabolized and whether it localizes to tissues.

Many studies have demonstrated that apigenin has various physiological functions [7–11]. Recently, we have reported that the 67-kDa laminin receptor (67LR) is a molecular target for the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) and EGCG triggers signaling pathway for cancer prevention through 67LR [30,31]. It will be also important to identify the molecular targets of apigenin to elucidate the mechanisms of its physiological activities.

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