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Buckwheat extracts (*Fagopyrum tataricum*) and rutin attenuate Th2 cytokines production and cellular allergic effects *in vitro* and *in vivo*

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ABSTRACT

The key feature of food allergy is a T helper-2 cell (Th2)-predominant allergen-specific immune response. Rutin can increase PPAR- γ expression to improve diabetes; however, this effect whether attenuating Th2 signal is still unknown. The 75% ethanol extracts of buckwheat (EEB) and rutin (major active compound) significantly suppress interleukin-4 (IL-4) secretion from splenocytes in ovalbumin (OVA)-induced sensitive Balb/c mice; therefore, the inhibitory effects of EEB and rutin on Th2 signaling pathway in PMA/ionomycin-induced EL4 T cells were subsequently investigated. At the transcriptional level, rutin reduced the expression of Th2 markers such as GATA3 and p-STAT6 in PMA/ionomycin-induced EL4 T cells. Moreover, we used the PPAR- γ antagonist GW9662 to confirm that rutin skewed the immunological function toward Th2 activity through regulation of PPAR γ expression. Taken together, EEB and rutin may have the potential to regulate immune responses, thereby deeming them useful in reducing food hypersensitivities and improving anti-cellular allergic responses.

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1. Introduction

The worldwide occurrence of allergic disorders, in particular, food hypersensitivities, is increasing with time. Recent epidemiologic studies suggest a rising incidence of allergies in the Western nations, affecting up to 8% and 2% of the child and adult populations, respectively, in industrialized countries (Hugh & Sampson, 2004). Although there is enormous diversity in the human diet, a limited number of foods account for the majority of food allergies (Hugh & Sampson, 2004). The clinical manifestations of food allergies vary from mild symptoms such as oral allergy syndrome or mild urticaria to severe anaphylactic reactions with fatal consequences. Although food allergies are a major cause of severe hypersensitivity reactions, the primary management still consists of

strictly avoiding relevant allergens. Recently, attempts to use specific immunotherapy for treatment of food allergies have been proposed as a promising alternative to allergen avoidance (Reese, Ballmer-Weber, Wangorsch, Randow, & Vieths, 2007).

Activation of peroxisome proliferator-activated receptor- γ (PPAR- γ) has been reported to inhibit IL-4 production in phorbol 12-myristate 13-acetate (PMA)/ionomycin-induced T cells and increases the levels of regulatory CD4 T cells (Lee et al., 2003). When naive CD4 T cells encounter an antigen on antigen-presenting cells (APCs), they develop into at least 2 distinct T helper (Th) cells: (1) Th1 cells secreting interleukin (IL)-2, interferon (IFN)- γ , tumor necrosis factor (TNF)- α ; and (2) Th2 cells secreting IL-4, IL-5, and IL-13. Allergy patients exhibit high serum levels of immunoglobulin (Ig) E, as well

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as production of IL-4, IL-5, and IL-13 by allergen-specific T helper (Th2) cells (Neurath, Finotto, & Glimcher, 2002). In T cells, IL-4 gene expression is regulated at the transcriptional level by both ubiquitous and cell type-specific factors, including nuclear factor of activated T cells (NF-AT), c-Maf, GATA-3, Stat6, JunB, and other transcription factors (Brown & Hural, 1997; Szabo, Glimcher, & Ho, 1997). Dysregulated production of IL-4 by Th2 cells has been linked with autoimmune and allergic diseases (Choi & Reiser, 1998).

Fagopyrum tataricum (buckwheat) is an herbaceous plant belonging to the Polygonaceae family. It has been introduced as a part of human diet in many countries, because the seeds of this herb are healthy and nutritionally important. Rutin has been found to be the major ingredient of buckwheat (Holasova et al., 2002), and has recently been shown to possess anti-allergic activity by inhibiting peptide presentation by the APCs (Gong & Chen, 2003) and suppressing IgE-mediated mast cell activation, thereby reducing histamine production and secretion (Chen et al., 2000a). The findings of these studies, however, did not reveal the effects of rutin on T cells. Therefore, we evaluated the effects of buckwheat extracts and rutin on ovalbumin (OVA)-induced sensitive Balb/c mice, as well as their effects on the regulation of Th2 cytokine production in phorbol myristate acetate (PMA)/ionomycin- and IL-4-treated EL4 T cells in this study.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, Hank's balanced salt solution (HBSS), penicillin, streptomycin, and glutamine were purchased from Invitrogen (Carlsbad, CA, USA). Peroxisome proliferator-activated receptor- γ (PPAR γ) antagonist (GW9662) was purchased from Cayman chemical (Ann Arbor, MI, USA). Fetal bovine serum (FBS), phytohemagglutinin, brefeldin A, ionomycin, ovalbumin (OVA), phorbol myristate acetate (PMA), and rutin were purchased from Sigma (St. Louis, MO, USA). Antibodies were purchased as follow: anti-GATA3 antibody (Abcam, Cambridge, MA, USA), anti-p-STAT6 antibody Abcam, Cambridge, MA, USA), anti-NFAT1 antibody (Cell Signaling Technology, Beverly, MA, USA), anti-PPAR γ antibody (Cayman Chemical, Ann Arbor, MI), and anti-GAPDH antibody (Cell Signaling Technology, Beverly, MA, USA). Anti-interleukin-4 (IL-4) antibody and anti-IFN- γ were purchased (Abcam, Cambridge, MA, USA), and IL-4 ELISA kits was purchased from eBioscience (San Diego, CA, USA).

2.2. Preparation of sample

The seeds of buckwheat (*F. tataricum*) were provided by Taiwan Golden Buckwheat Limited company, Chiayi, Taiwan), and then were freeze-dried and ground. Approximately 2.5 kg of the buckwheat powder were extracted by 25 L of 75% ethanol at 4 °C for 2 days. After the extraction, the ethanolic extracts were vacuum concentrated and freeze-dried. The extract powder was stored at –20 °C until used. The 75% ethanol extracts of buckwheat was abbreviated to be EEB in this

study. The quercetin and rutin of EEB were assayed by HPLC, and results suggested that 228.8 mg/g of rutin and 58.6 mg/g of quercetin were present in EEB (Lee, Hsu, Shen, Cheng, & Wu, 2012).

2.3. Animals

Female Balb/c mice (4-week-old) (BioLASCO, Co., Ltd., Yilan, Taiwan) were used in this study. Animals were provided with food (MF-18, BioLASCO, Taiwan) and water *ad libitum*. Animals (42 mice) were subjected to 12 h light/dark cycle with a maintained relative humidity of 60% and a temperature of 25 °C. The experiments were carried out in a qualified animal breeding room in the animal center of our institute. Mice (42 mice) were divided into groups (7 groups) receiving the following treatments: (1) sham sensitization by phosphate-buffered saline (PBS) (intraperitoneal injection; ip) ($n = 6$) and (2) 36 mice were sensitization by OVA (10 mg/kg bw) emulsified with alum adjuvant (ip) (Sigma, St. Louis, MO, USA) at week-0 and week-2 (once time per week) (Perez-Berezo et al., 2009). The animals by OVA sensitization were randomly divided into 6 groups, including (a) OVA injection ($n = 6$), (b) OVA + GW9662 (2 mg/kg bw) ($n = 6$), (c) OVA + GW9662 + rutin (11.5 mg/kg bw) ($n = 6$), (d) OVA + rutin (11.5 mg/kg bw) ($n = 6$), (e) OVA + GW9662 + EEB (50 mg/kg bw) ($n = 6$), and (f) OVA + EEB groups ($n = 6$). The dosage of rutin was equivalent to EEB administration group, and samples were administered from 0-week to 4-week. At 4 weeks after immunization, mice sacrificed to carry out following measurements. EEB and rutin were orally administered to animals and GW9662 was administered by intraperitoneal injection. All animal procedures were followed as per the experimental protocols approved by the institutional animal ethics committee (Chiayi University, Chiayi, Taiwan, ROC).

2.4. Assay for IL-4 from PBMCs

Peripheral blood mononuclear cells (PBMCs) were separated from mice peripheral blood by density gradient centrifugation (300g for 15 min) with Ficoll-Hypaque (1.077 g/mL). PBMCs were washed with PBS three times, and was incubated in RPMI 1640 medium supplemented 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). PBMCs (1×10^6 /well in 6 cm dish) were stimulated with phytohemagglutinin (PHA; 5 μ g/mL) for 24 h, respectively; and brefeldin A (2 μ g/mL) was added into medium prior 4–6 h for assay. Cells were collected and washed by PBS three times, and IL-4 (Th2) and IFN- γ (Th1) were in turn assayed by Western blot (Park et al., 2006).

2.5. EL4 T cells culture

EL4 murine T-lymphoma cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). EL4 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS (without antibiotics). Confluent cells were subcultured at a ratio of 1:3 in 10 cm-dish, and media were changed twice a week. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. EL4 T cells were induced

Th2 cytokines production by PMA (10 µg/mL) and ionomycin (400 nM) treatment with or without rutin (5–30 µM) for 48 h.

2.6. Western blot

Cells were lysed in ice-cold lysis buffer containing 20 mM of Tris-HCl (pH 7.4), 1% of Triton X-100, 0.1% of SDS, 2 mM of EDTA, 10 mM of NaF, 1 mM of phenylmethylsulfonyl fluoride (PMSF), 500 µM of sodium vanadate, and 10 µg/mL of aprotinin overnight. And then the cell lysates were sonicated with ice cooling (four times each 15 s), and then centrifuged (12,000g, 10 min) to recover the supernatant. The supernatant was taken as the cell extract. The protein concentration in the cell extract was determined using a Bio-Rad protein assay kit (Bio-Rad Lab. Inc., Hercules, CA, USA). The nuclear and cytosolic fractions of EL4 cells were extracted by the kit (Biovision, Palo Alto, CA, USA). The samples (25–60 µg of protein) were subjected to 10% dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The protein spots were electrotransferred to a polyvinylidene difluoride (PVDF) membrane by blotting buffer (9.68 g of Tris-base, 45.04 g of glycine, 400 mL of methanol, and 3.6 L of deionized water). The membrane was incubated with block buffer (PBS containing 0.05% of Tween-20 and 5% (w/v) nonfat dry milk) for 1 h, washed with PBS containing 0.05% Tween-20 (PBST) three times, and then probed with primary-antibody overnight at 4 °C. In addition, the intensity of the blots probed with 1:1000 diluted solution of mouse monoclonal antibody to bind GAPDH (Cell Signaling Technology, Beverly, MA, USA) was used as the control to ensure that a constant amount of protein was loaded into each lane of the gel. The membrane was washed three times each for 5 min in PBST, shaken in a solution of HRP-linked anti-rabbit IgG secondary antibody, washed three more times each for 5 min in PBST. The expressions of proteins were detected by enhanced chemiluminescent (ECL) reagent (Millipore, Billerica, MA, USA) and image reader (LAS-3000, Fuji, Tokyo, Japan).

2.7. PPAR γ knockdown of EL4 cells by specific siRNA

For PPAR γ knockdown assay, PPAR γ interference of EL4 cells was performed with Mirus-siRNA transit-TKO transfection reagent (Mirus Bio. LLC., Madison, WI, USA) followed by the protocols provided by the kit. The sequence of specific small interfering RNA (siRNA) for PPAR γ , and the control group (transfected with scramble siRNA) were purchased from Santa Cruz Biotechnology Inc. (Delaware Ave, CA, USA). Whole cell lysates were subjected to Western blotting with anti-PPAR γ antibody to confirm the effect of siRNA on alteration of PPAR γ transcriptional expression.

2.8. Assay for DNA binding activity of PPAR γ and NF-AT

Nuclear extracts were prepared from EL4 T cells by means of a commercially available transcription factor ELISA kit (Trans-Binding PPAR γ assay and NFAT assay) (Panomics, Redwood City, CA, USA) and performed according to the instructions of the manufacturer. Briefly, oligonucleotides encoding for the PPAR γ consensus binding site were bound to microplates. Subsequently, the transcriptional activity of activated PPAR γ

and nuclear factor of activated-T cells (NFAT) (in the prepared nuclear extracts) was determined by PPAR γ and NFAT binding to the immobilized DNA and confirmed with PPAR γ and NFAT antibody using ELISA technology.

2.9. Statistical analysis

Experimental results were averaged triplicate analysis. The data were recorded as mean $\pm$ standard deviation (SD) and analysis by statistical analysis system (SAS Inc., Cary, NC, USA). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of EEB and rutin on IL-4 and IFN- γ production in OVA-induced mice

As shown in Fig. 1, OVA significantly increased IL-4 production in the PBMCs stimulated by PHA for 24 h. OVA treatment did not significantly affect the level of IFN- γ in PBMCs. Administration of EEB as well as rutin inhibited IL-4 production from PBMCs, while it did not affect the levels of IFN- γ . In addition, the PPAR γ antagonist GW9662 prevented the inhibition of IL-4 by EEB and rutin. These findings suggest the importance of PPAR γ in attenuating IL-4 production.

3.2. Effect of rutin on Th2 signals and transcription factors in EL4 cells

Previous studies have demonstrated that PMA/ionomycin can induce Th2 cytokine production in the EL4 T cell line (Aherne & O'Brien, 2008; Chung, Kang, & Kim, 2003). We also found that PMA/ionomycin significantly increased the expression of NF-AT and GATA3. Rutin (5–30 µM) markedly suppressed NFAT and GATA3 expression, and attenuated PMA/ionomycin induced STAT6 phosphorylation, which is required for responsiveness to IL-4 and IL-13 (Fig. 2A). In addition, rutin significantly decreased the level of IL-4 production in PMA/

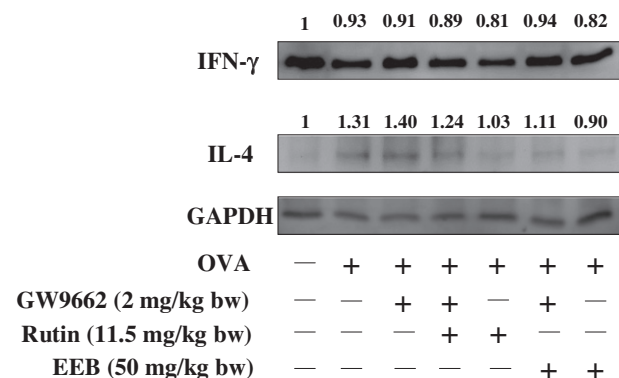


Fig. 1 – The inhibitory effects of EEB and rutin on Th1 and Th2 cytokines production in PBMCs of OVA-induced mice for 4 weeks administration. PBMCs were obtained from 6 mice, and these cells were mixed and lysed to assay cytokines by Western blot.

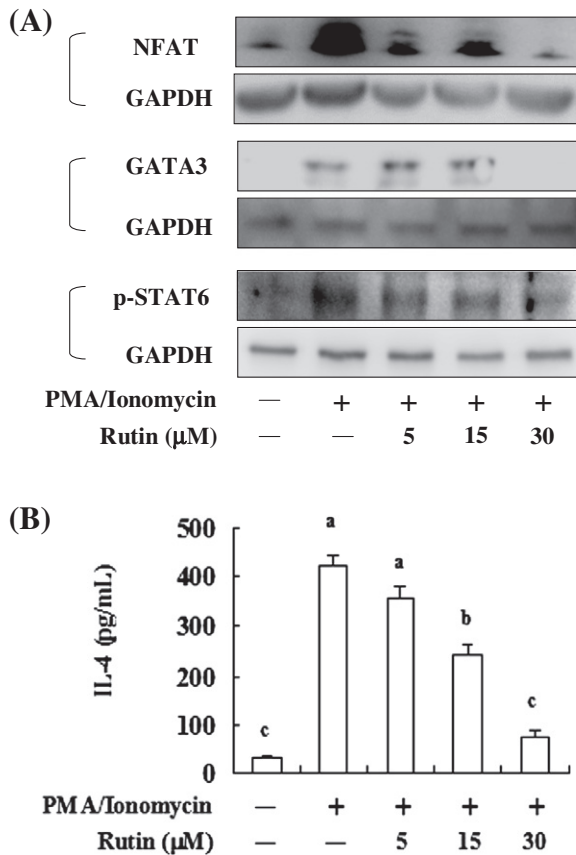


Fig. 2 – The inhibitory effects of EEB and rutin on NF-AT, GATA3, and STAT6 phosphorylation (A); and the suppression of IL-4 production (B) in PMA/ionomycin induced-EL4 T cells for 48 h. Significant difference was indicated with different symbol ($p < 0.05$).

ionomycin-treated EL4 T cells in a dose-dependent manner (Fig. 2B).

3.3. Effects of GW9662 on rutin inhibited Th2 signal and transcription factors in EL4 cells

Inhibition of PPAR γ with GW9662 reduced the production of regulatory T cells and IL-4 in the spleen and blood of OVA-induced mice; therefore, we further investigated whether GW9662 also affects Th2 signals and transcription factors in PMA/ionomycin-induced EL4 T cells. Our results indicate that the expression of NF-AT associated with cytokine production, and the expression of GATA3 and p-STAT6 associated with Th2 signaling were both promoted by GW9662 treatment in PMA/ionomycin-induced EL4 T cells (Fig. 3A). We found that the inhibition of NF-AT, GATA3, and p-STAT6 were attenuated by GW9662 in the group treated with rutin. Similarly, suppression of IL-4 production by rutin was abolished by GW9662 treatment (Fig. 3B).

3.4. Rutin promotes PPAR γ activation

We found that PMA/ionomycin treatment increased cytosolic PPAR γ and decreased nuclear PPAR γ in EL4 T cells, suggesting

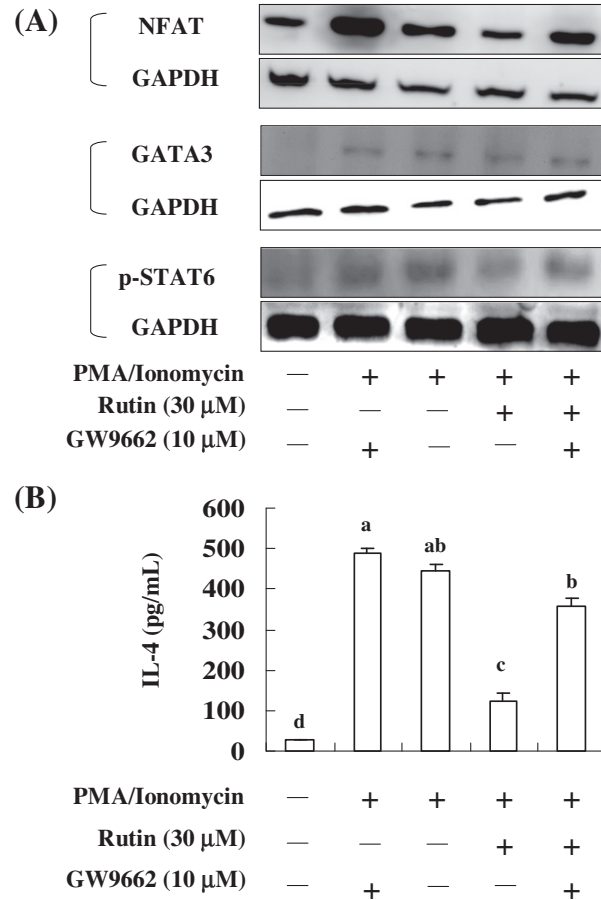


Fig. 3 – The suppressions of NF-AT, GATA3, and p-STAT6 (A), and IL-4 production (B) by EEB and rutin treatment were abolished by PPAR γ antagonist (GW9662) treatment in PMA/ionomycin-induced EL4 T cells for 48 h. Significant difference was indicated with different symbol ($p < 0.05$).

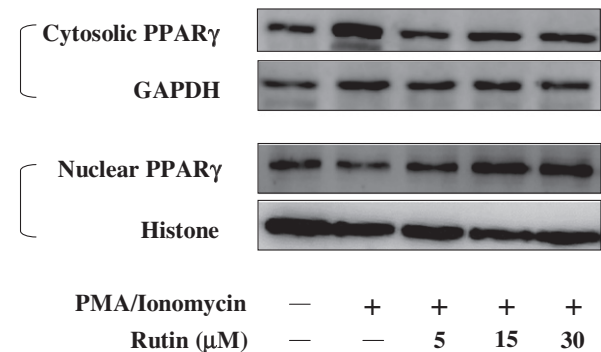


Fig. 4 – The activation of PPAR γ in PMA/ionomycin-induced EL4 T cells treating with EEB and rutin for 48 h.

that the PMA/ionomycin treatment inhibited PPAR γ translocation, thereby reducing its activity (Fig. 4). These results indicated that rutin markedly promotes PPAR γ activation by decreasing cytosolic PPAR γ and increasing nuclear PPAR γ after 48 h of treatment. We confirmed this effect of rutin in EL4 T cells by knocking down PPAR γ with specific siRNA. We found

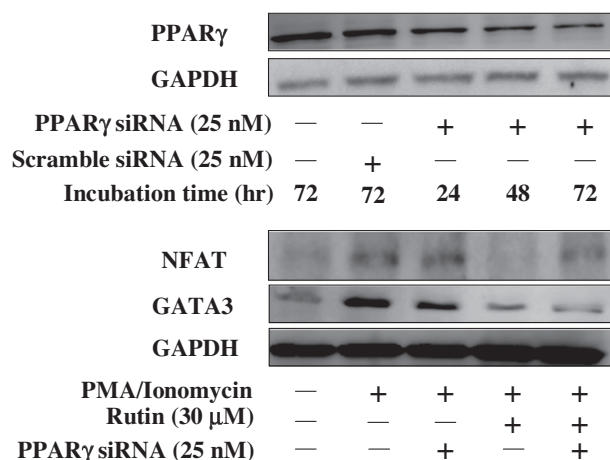


Fig. 5 – The inhibition of PPAR γ by specific siRNA treating EL4 T cells (A). The effects of knocking down PPAR γ on NFAT and GATA3 in PMA/ionomycin-induced EL4 T cells treating with EEB and rutin for 48 h (B).

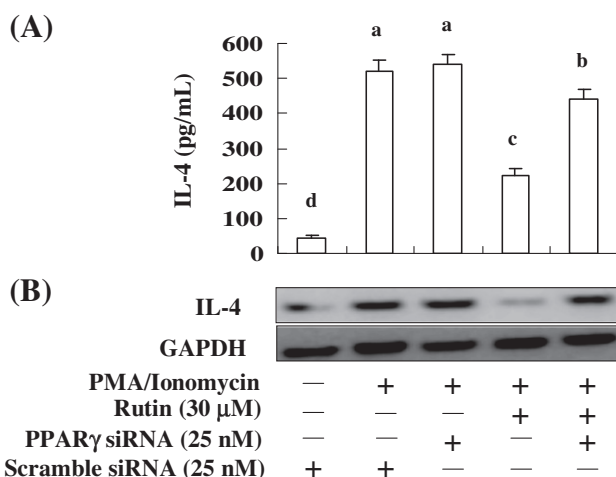


Fig. 6 – The effects of EEB and rutin on IL-4 production by ELISA assay (A) and by Western blot assay (B) in PPAR γ knockdown-PMA/ionomycin-induced EL4 T cells for 48 h. Significant difference was indicated with different symbol ($p < 0.05$).

that rutin reduced the expression of NFAT and GATA3 induced by PMA/ionomycin in EL4 T cells, and that this effect was also attenuated by PPAR γ siRNA (Fig. 5). A similar result was displayed in IL-4 production (Fig. 6), suggesting that rutin inhibited IL-4 and Th2 signaling in a PPAR γ -dependent manner.

3.5. Effects of rutin on the DNA-binding activity of NFAT and PPAR γ

We measured the inhibition of the DNA binding of NFAT in response to rutin treatment of PMA/ionomycin-induced EL4 T cells (Fig. 7A), and this inhibitory activity of rutin on the NFAT-DNA binding activity was reduced by GW9662. In addition, we also found that rutin significantly elevated the

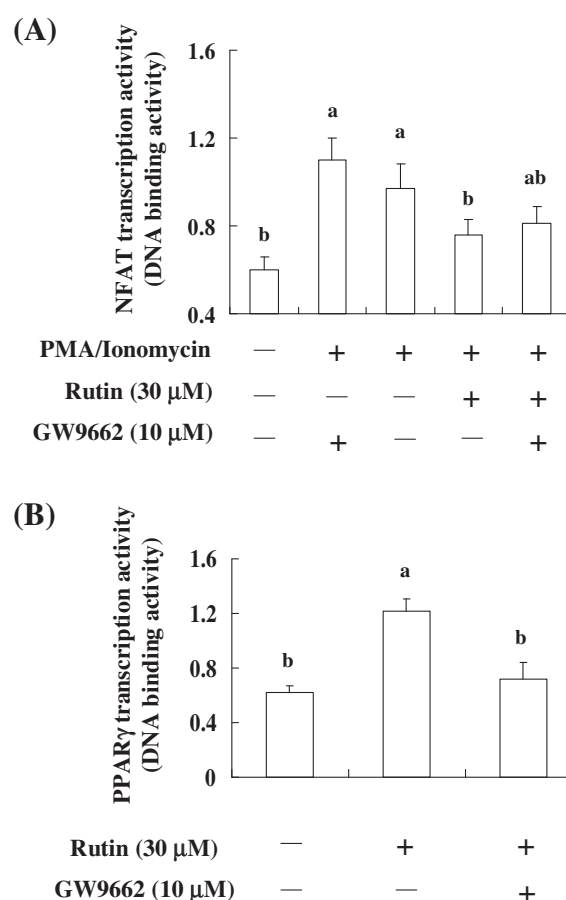


Fig. 7 – The inhibition of NFAT binding to DNA by EEB and rutin treatment in PMA/ionomycin-induced EL4 T cells (A). The promotion of PPAR γ binding to DNA by EEB and rutin treatment in EL4 T cells (B) for 48 h.

PPAR γ -DNA binding activity, and GW9662 treatment markedly attenuated this binding activity (Fig. 7B).

4. Discussion

In allergic individuals, allergen-specific T cells have longer lifespan. Foods such as milk, eggs, peanuts, soy, and wheat account for approximately 90% of the hypersensitivity reactions in children (Bohle, 2004). Several studies have addressed the characterization of the cellular response to allergens from hen egg, cow milk, and peanuts. Allergen-reactive Th2 cells play a central role in the pathophysiology of food allergy (Bohle, 2004; Brown & Hural, 1997; Hugh & Sampson, 2004).

IL-4 is synthesized by Th2 cells, and plays an important role in allergic reactions. It is essential for IgE production, induces the differentiation of naive CD4 T cells into Th2 cells, and inhibits the functioning of Th1 cells. The cytokine IFN- γ is produced primarily by the natural killer and Th1 cells. It induces the differentiation of naive CD4 T cells into Th1 cells, and inhibits the proliferation of Th2 cells (Szabo, Sullivan, Peng, & Glimcher, 2003).

A recent study has shown that rutin can inhibit peptide presentation by APCs (Gong & Chen, 2003) as well as suppress

histamine secretion from mast cells (Chen et al., 2000a). In addition, the anti-allergic and immunopharmacological activities of buckwheat have been investigated by Yanagihara and Koda (1979a, 1979b). However, there are no references reports the regulation of the effects of rutin and buckwheat extracts on Th1/Th2 cytokines yet. In this study, we aimed to determine whether the effects of buckwheat extracts and rutin are downregulated in the OVA-induced mice and the PMA/ionomycin-induced EL4 T cells. Our findings indicate that the administration of EEB and rutin both reduced the levels of IL-4 (from the splenocytes and PBMCs), while it did not affect IFN- γ production (Th1 cytokine), suggesting that EEB and rutin modulate immune responses through inhibition of Th2 cytokine secretion.

The expression of T cell-specific transcription factors and components of mitogen-activated protein kinase (MAPK) pathways, which are known to play critical roles in T cell activation (Boulton, Gregory, & Cobb, 1991; Su et al., 1994) and affect the gene expression of Th2 cytokines. GATA3 belongs to the GATA family of transcription factors, which is an important regulator of T cell development, and plays a role in endothelial cell biology. GATA-3 promotes the secretion of IL-4, IL-5, and IL-13 from Th2 cells (Joulin et al., 1991). NF-AT is a general name applied to a family of transcription factors that play an important part in immune response. One or more members of the NF-AT family are expressed in most cells of the immune system, and this transcription factor promotes cytokine expression and T cell activation (Macian, 2005). Several transcription factors critical for IL-4 transcription, including Stat6 (Kaplan, Schindler, Smiley, & Grusby, 1996), NF-AT (Ranger et al., 1998), c-Maf (Ho, Hodge, Rooney, & Glimsher, 1996), GATA3 (Zhang, Cohn, Ray, Bottomly, & Ray, 1997), and JunB (Faith et al., 2003) have been identified. To date, NFAT has been shown to directly bind to and/or activate the IL-4 promoter (Chen et al., 2000a).

The transcription factor NF-AT is known to play an essential role in the inducible transcription of the IL-4 gene during T cell activation, since human and murine IL-4 gene promoters contain at least 4 NF-AT sites that control their induction in T cells. In this study, we found that rutin significantly inhibited NF-AT and GATA3 expression in PMA/ionomycin-induced EL4 T cells, thereby exerting anti-allergic activity; however, this activity was abolished by treatment with the PPAR γ antagonist GW9662, suggesting that the anti-allergic effect of rutin was dependent on PPAR γ activation in the PMA/ionomycin-induced EL4 T cells. PPAR γ ligand has also been shown to promote its activity, thereby inhibiting NF-AT transcriptional activity and suppressing IL-4 production (Chung et al., 2003).

Several flavonoids such as rutin and quercetin also elevate the expression of PPAR γ mRNA, which attenuates inflammation and insulin resistance (Chuang et al., 2010). Several lines of evidence indicate that MAPK kinases inhibit PPAR γ function via direct phosphorylation at serine residues, thus affecting DNA-binding activity and increasing PPAR γ degradation via the ubiquitin-proteasome-dependent pathway (Diaz-Delfin, Morales, & Caelles, 2007). On the other hand, PPAR γ can inhibit inflammatory gene expression via several mechanisms, including the formation of nuclear-cytoplasmic shuttling complexes with the p65 subunit of NF κ B (Chawla et al., 2001), as well as the formation of direct complexes with

the members of the AP-1 and NF κ B families (Lee et al., 2003). In this study, we found that rutin significantly prevented an increase in the DNA-binding activity of NF-AT, and effectively elevated the DNA-binding activity of PPAR γ . These findings indicated that rutin could promote PPAR γ activation, thereby inhibiting the transcriptional activity of NF-AT.

Several allergens from buckwheat have been identified for three decades (Yanagihara & Koda, 1979a and 1979b). However, allergen protein did not to be extracted in 75% ethanol extracts of buckwheat (EEB) thereby antioxidants of EEB playing important role for Th2 signal attenuation.

In addition, antioxidants have been reported to elevate the levels of regulatory T cell (CD4/CD103/Foxp3), thereby exerting anti-allergy capacity (Sato et al., 2010). Moreover, PPAR γ activation also promotes the conversion of CD4 T cells into Foxp3 regulatory T cells (Chung et al., 2003; Lei, Hasegawa, Matsumoto, & Yasukawa, 2010; Wang, Zhu, Zhu, & Ma, 2010). Elevation of PPAR γ by rutin has been reported (Chuang et al., 2010), and rutin suppresses APCs activation for peptide presentation to exert anti-allergy (Gong & Chen, 2003); in addition, this compound also reduces mast cell activation by IgE induction thereby attenuating histamine production and secretion (Chen, Gong, Liu, & Mohammed, 2000b). These results indicated that rutin has potentials for down-regulating allergy by multi-manners; however, the affects of rutin for T cell and Th cytokine production remains unknown yet. We found that rutin and EEB extracts could significantly inhibit Th2 cytokines in EL4 T cells in this study. But the anti-allergic activity of rutin in acute-hyper-response in human is unknown.

In summary, our findings support the hypothesis that rutin regulates immune responses through the suppression of IL-4, inhibition of NF-AT and GATA3, and reduction of Th2 signaling (p-STAT6) in PMA/ionomycin-induced EL4 T cells. Furthermore, the present study shows that OVA-induced IL-4 production is inhibited by rutin and EEB. Thus, buckwheat extracts and rutin may function as anti-allergic agents. Further studies with established immune cell lines are needed to elucidate the molecular and cellular mechanisms of these activities.

5. Conflict of interest

The authors have declared no conflict of interest.

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