

Note

Nobiletin Enhances Induction of Antigen-Specific Immune Responses in BALB/c Mice Immunized with Ovalbumin

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Summary We examined the effect of nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) on immune response in ovalbumin (OVA)-immunized mice. Treatment with nobiletin increased OVA-specific IL-4 and IL-10 production. In addition, mice that received nobiletin showed higher levels of OVA-specific IgE, IgG and IgG1 production than did control mice. The antibody response to the thymus-independent antigen 2,4,6-trinitrophenyl-Ficoll was not different in the control and nobiletin groups, suggesting that nobiletin does not directly stimulate antibody production. An in vitro study showed that treatment with nobiletin enhanced the ability of antigen presentation of bone marrow-derived dendritic cells. The in vivo and in vitro results indicate that nobiletin regulates immune function.

Key Words polymethoxylated flavonoid, immunization, antigen presentation, citrus fruits, dendritic cells

Nobiletin, known as 5,6,7,8,3',4'-hexamethoxyflavone, is a dietary polymethoxylated flavonoid found in citrus fruits. Nobiletin is one of the most extensively studied flavonoids and has been shown to possess a multifunctional pharmaceutical agent. Nobiletin has various biological effects including anti-obesity (1), anti-tumor (2) and neuroprotective effects (3, 4). The immune function of nobiletin has also been examined in some studies. An in vitro study showed that nobiletin decreased LPS-induced production of prostaglandin E2 and proinflammatory cytokines including IL-1 α , IL-1 β , TNF- α and IL-6 in mouse J774 macrophage cells (5). It was also shown that nobiletin suppresses LPS- and IgE-mediated activation of human intestinal mast cells (6). An in vivo study showed that nobiletin attenuates endotoxic shock status through inhibiting TNF- α and IL-6 (7). In other studies, nobiletin was shown to ameliorate experimental colitis by reducing inflammation in a 2,4,6-trinitrobenzene sulfonic acid-induced colitis and IL-10-deficient mouse model (8, 9).

Vaccination is a useful strategy for preventing infectious diseases. When individuals are immunized with an exogenous antigen (Ag), the administered Ag is taken up by Ag-presenting cells such as dendritic cells and B

cells and presented on MHC class II molecules to helper T cells, resulting in Ag-specific humoral and cellular immune responses (10, 11). In this study, we conducted an experiment to determine how nobiletin affects Ag-specific humoral and cellular immune responses in ovalbumin (OVA)-immunized mice. Furthermore, to understand the underlying mechanism, the effect of nobiletin on Ag-presenting function in vitro was examined.

Materials and Methods

Mice and diets. Eight-week-old female BALB/c mice (Japan SLC, Inc., Shizuoka, Japan) and C57BL/6 mice (Japan SLC) were maintained under specific pathogen-free conditions with a 12-h light:dark cycle at $25 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ relative humidity. The mice were given free access to water and food throughout the experiment. The mice were maintained on a control diet (No. D10012G; Research Diets Inc., NJ, USA). All studies were performed in accordance with the ethical guidelines for animal experimentation by the Graduate School of Biomedical Sciences, Tokushima University, Japan and were approved by the institution review board of the animal ethics committee (T28-82).

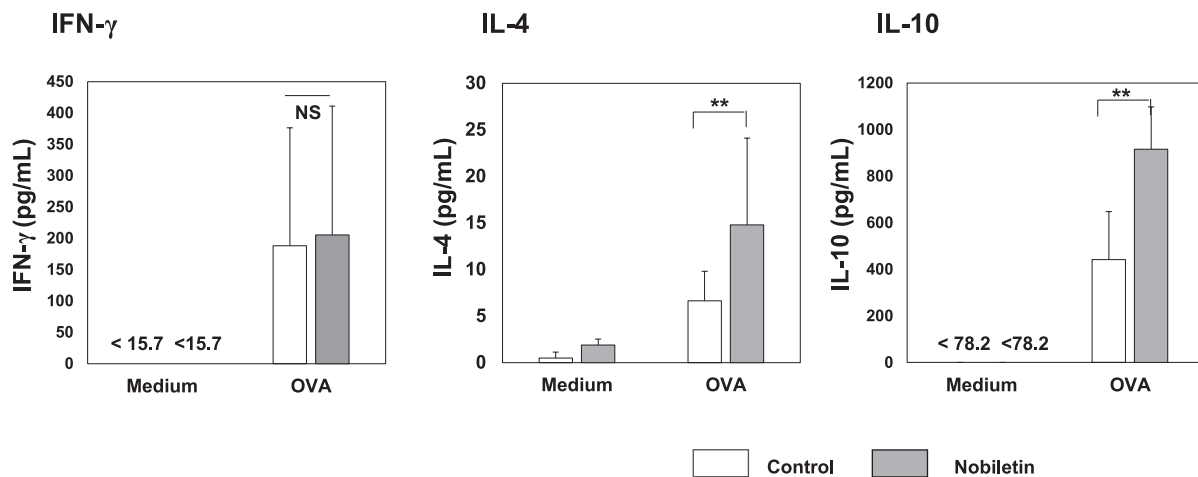
Nobiletin treatment. Nobiletin was provided by Ushio-Chemix Co. (Shizuoka, Japan). The purity of nobiletin used in this study was $>98\%$. For oral administration, nobiletin was dissolved in dimethyl sulfoxide (DMSO). Nobiletin solution was prepared daily in 20 mmol/L of Na₂CO₃. BALB/c mice were administered 200 μL of nobiletin solution containing 20 mg nobiletin/kg body weight by gavage for 35 d. Control mice were treated with a corresponding volume of DMSO solution with

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Abbreviations: Ab, antibody; Ag, antigen; BM, bone marrow; DC, dendritic cell; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; m, monoclonal; MLR, mixed lymphocyte reaction; OVA, ovalbumin; TdR, thymidine deoxyribose; TNP, 2,4,6-trinitrophenyl.

(A)



(B)

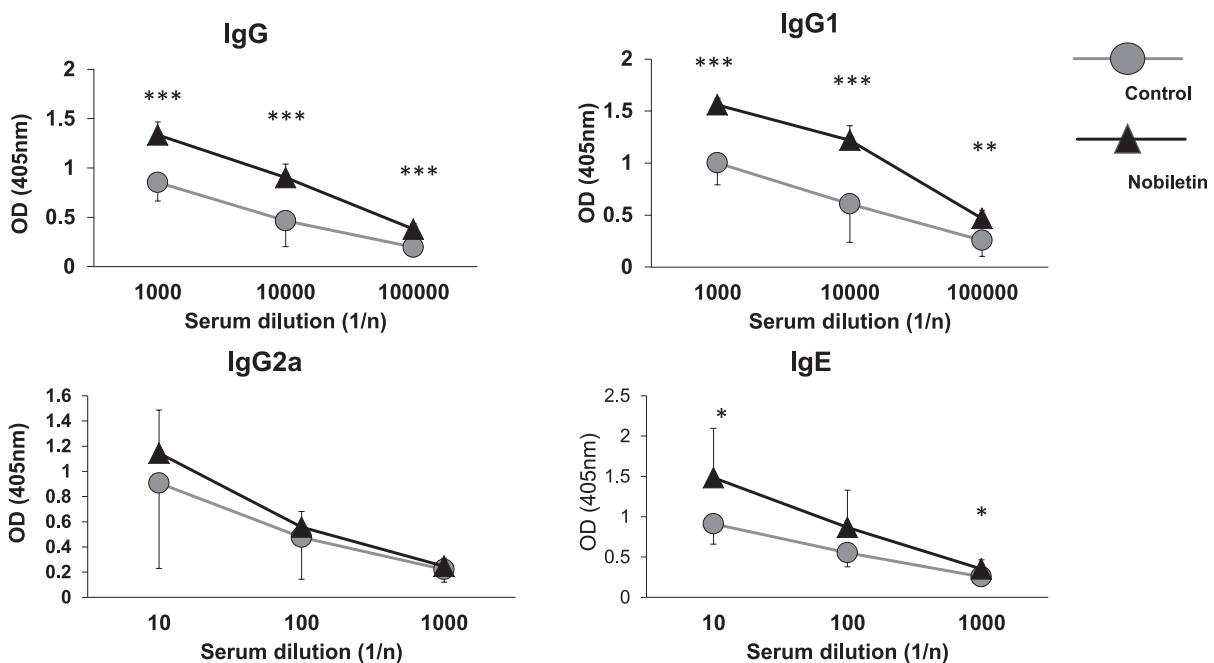


Fig. 1. Nobiletin enhances antigen-specific cytokine production and Ab production in OVA-immunized mice. BALB/c mice were administered nobiletin by gavage and were immunized with OVA twice as described in "Materials and Methods." (A) Splenocytes from OVA-immunized mice were stimulated with OVA for 72 h. Concentrations of IFN- γ , IL-4 and IL-10 were determined by ELISA methods. Each value is the mean $\pm$ SD of 7 mice/group. Statistical differences between the control and nobiletin groups were analyzed. ** p < 0.01. (B) Levels of IgG1, IgG, IgE and IgG2a in diluted serum were measured by ELISA methods. Statistical difference between control and nobiletin groups was analyzed at the same serum dilution level. Each value is the mean $\pm$ SD of 7 mice/group. * p < 0.05, ** p < 0.01, *** p < 0.001.

200 μ L of 20 mmol/L Na_2CO_3 . During the administration period, immunization was done twice and the spleen and serum were obtained from each mouse at 35 d.

Immunization. BALB/c mice were immunized intraperitoneally with 500 μ L of OVA solution containing 10 μ g of OVA (Sigma Chemical Co., MO, USA) absorbed in 2 mg of aluminium hydroxide gel adjuvant (Sigma-Aldrich Co., MO, USA) on days 7 and 21 after the start of nobiletin administration. In another experiment, BALB/c mice were immunized with 25 μ g of 2,4,6-tri-

nitrophenyl (TNP)-Ficoll (Sigma Chemical) in the same manner as that of OVA immunization.

Cytokine production. Splenocytes (2.5×10^6 cells/well) were stimulated with 400 μ g/mL OVA or plate-bound anti-CD3 monoclonal (m) Ab and anti-mouse CD28 (coated overnight at 1 μ g/mL) in a 48-well flat-bottom plate at 37°C under 5% CO_2 for 72 h. After the culture, culture supernatants were collected and stored at -30°C until used. Interferon (IFN)- γ , interleukin (IL)-2, IL-4 and IL-10 in the supernatants were quantified using mouse IFN- γ (eBioscience, CA, USA), IL-4 (Bio-

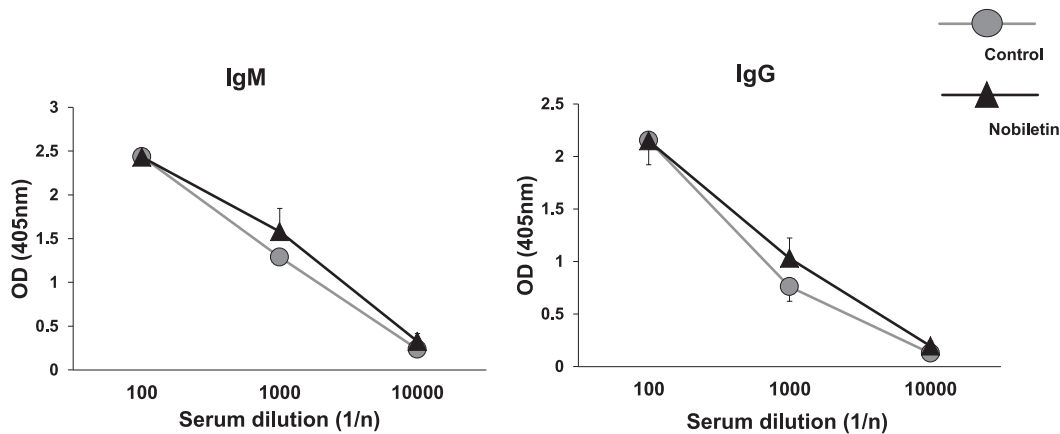


Fig. 2. Nobiletin does not affect Ab production in response to thymus-independent Ag. BALB/c mice were administered nobiletin by gavage and were immunized with TNP-Ficoll twice as described in the "Materials and Methods." Levels of IgM and IgG in diluted serum were measured by ELISA methods. Each value is the mean $\pm$ SD of 5 or 6 mice/group.

Legend, San Diego, CA) and IL-10 (eBioscience) enzyme-linked immunosorbent assay (ELISA) kits according to the instructions of the manufacturers.

Measurement of Ag-specific antibody (Ab). OVA-specific IgG, IgG1, IgG2a and IgE were measured by ELISA as described previously (12).

In vitro dendritic cell experiment. Bone marrow (BM) cells were collected from the femurs of BALB/c mice. The BM cells (1×10^6 cells/mL) were cultured with GM-CSF (10 ng/mL) for 7 d at 37°C under 5% CO₂. CD11c⁺ cells were purified using CD11c⁺ Micro Beads (Miltenyi Biotec Inc., Auburn, CA, USA) and then the cells (2×10^6 cells/mL) were cultured in a 24-well plate with lipopolysaccharide (LPS) (100 ng/mL) and nobiletin (0, 12.5, 25 or 50 μ M) for 24 h. For determination of Ag presentation ability, BM cell-derived CD11c⁺ dendritic cells (BMDC) (3.3×10^4 cells) were cultured with C57BL/6 mouse-derived CD4⁺ cells (10^5 cells) in a 96-well round-bottom plate for 72 h. For the last 8 h of the culture, 37 kBq of [³H] thymidine deoxyribose (TdR) was added to the wells and the amount of [³H]TdR incorporated was measured by a scintillation counter (Aloka Co., Japan).

Statistics. Data were analyzed using Student's *t*-test for the OVA-immunized mouse experiment and using ANOVA followed by post-hoc comparisons tests (Tukey) for the in vitro Ag-presentation experiment. Data are expressed as means $\pm$ SD. Differences were considered significant at $p < 0.05$.

Results and Discussion

BALB/c mice were treated intraperitoneally with OVA and were administered nobiletin by gavage. We examined the ex vivo proliferative responses of splenocytes by co-culture with OVA or anti-CD3 mAb. The proliferative responses to OVA and anti-CD3 mAb were not significantly different in the control and nobiletin groups (data not shown). We also examined the levels of IFN- γ , IL-4 and IL-10 production. When splenocytes were stimulated with OVA, the levels of IL-4 and IL-10 production were increased in the nobiletin group compared to those in the control group, whereas the levels

of IFN- γ production were not significantly different (Fig. 1A). In addition to cellular immune response, humoral immune response was investigated. The levels of OVA-specific IgG1, IgG and IgE were significantly increased in the nobiletin group compared with those in the control group, whereas the levels of OVA-specific IgG2a in the two groups were not different (Fig. 1B). Flow cytometric analysis showed that the percentages of CD4⁺ cells, CD8⁺ cells and CD44^{low} CD62L^{high} cells in CD4⁺ cells (naïve phenotype) and the percentage of CD44^{high} CD62L^{low} cells in CD4⁺ cells (memory phenotype) were not different in the control and nobiletin groups (data not shown).

To examine the possibility that nobiletin directly stimulates B cells, resulting in increased Ab production, we further analyzed the effect of nobiletin on B cell response by using a thymus-independent Ag, TNP-Ficoll. Mice were treated with TNP-Ficoll and were administered nobiletin by gavage. The levels of TNP-specific IgM and IgG were not significantly different between the control and nobiletin groups (Fig. 2). These results suggest that the regulatory effect of nobiletin on Ag-specific immune responses is not a result of a direct effect on B cells but might be mediated by helper T cells.

The effect of nobiletin on the function of dendritic cells was examined by the mixed lymphocyte reaction (MLR) assay because Ag presentation was a crucial step in evoking induction of immune responses. In these assays, we observed the proliferation response of C57BL/6 mouse CD4⁺ cells (H-2^b) to allogenic BALB/c-derived BM dendritic cells (H-2^d). Treatment with nobiletin increased the MLR response in a dose-dependent manner in LPS-stimulated BMDCs. A significant difference was observed between LPS-stimulated BMDC treated with 0 μ M nobiletin and those treated with 50 μ M nobiletin (Fig. 3).

Naïve helper T cells differentiate into type 1 (Th1), Th2, Th17 and regulatory T cells depending on the circumstance of Ag presentation and then regulate immune functions in the body (13, 14). In this study, we found that nobiletin enhanced Ag-specific IL-4 and

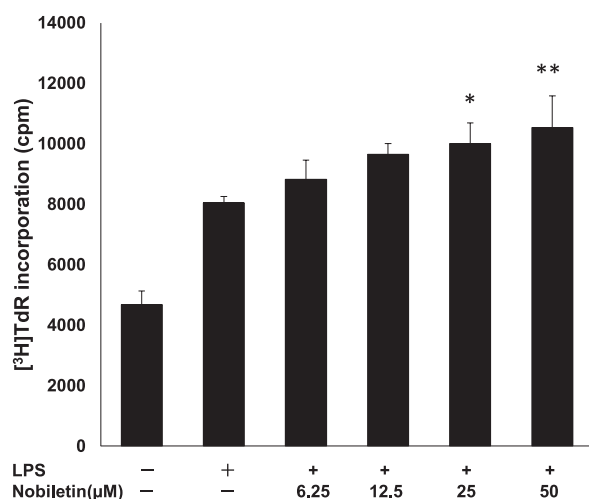


Fig. 3. Nobiletin enhances the ability of Ag presentation in GM-CSF-induced dendritic cells in vitro. BM cells were cultured with GM-CSF for 7 d and the cells were cultured with LPS and/or nobiletin for 24 h. Purified BALB/c-derived CD11c⁺ DCs were cultured with C57BL/6 mouse CD4⁺ cells at a ratio of 1/3 or 1/10 : 1 for 72 h. Ag-presenting ability was assessed by incorporation of [³H]TdR. Statistical differences between LPS only and LPS plus nobiletin treatment were shown. **p*<0.05, ***p*<0.01; LPS vs LPS+nobiletin. Statistical differences were observed between the groups treated with LPS and not treated with LPS but are not shown in this figure.

IL-10 production and also antigen-specific IgG1 and IgE Ab responses (Fig. 1). These observations indicate the possibility that nobiletin preferentially induces a Th2 immune response. The observed preferential Th2 induction might be related to the mouse strain and adjuvant because the BALB/c mouse strain and alum adjuvant have been shown to induce a dominant Th2 response (15, 16). T cell differentiation to Th1 or Th2 cells has been shown to crucially depend on the activation of redox-sensitive signaling cascades, in which oxidative conditions support Th1 development, while antioxidative stress leads to a shift to Th2 responses (17, 18). In the RAW 264.7 macrophage-like cell line, nobiletin has been shown to inhibit LPS-induced intracellular production of reactive oxygen species (19). It seems likely that redox regulation of nobiletin is one of the mechanisms for immune regulation. Let us discuss the subject from the viewpoints of activities of polyphenols and Th1/Th2 differentiation. Other than nobiletin, the polyphenols quercetin and genistein have been shown to be potent antioxidants (20, 21). How do these polyphenols regulate Ag-specific immune responses? In a murine model of asthma, quercetin reduced the level of IL-4 and increased the level of IFN- γ in OVA-sensitized and -challenged mice (22). Genistein treatment decreased the production of both OVA-specific IFN- γ and IL-4 in OVA-immunized mice (23). The results of these studies suggest that the antioxidants regulate IFN- γ and IL-4 productions. However, each antioxidants does not affect of Th1/Th2 regulation uniformly. A recent study

has shown that nobiletin regulates the differentiation of CD4⁺ cells in a phorbol-13 acetate-induced skin lesion mouse model (24). From our study, it is not known whether nobiletin directly regulates CD4⁺ cell function. However, the results of our in vitro study (Fig. 3) indicate the possibility that nobiletin regulates dendritic cell function, resulting in augmentation of both humoral and cellular immune responses in vivo.

Citrus polymethoxyflavones are flavones that bear two or more methoxy groups on their basic benzo- γ -pyron skeleton with a carbonyl group at the C-4 position (25). The number and positions of methoxy groups might influence the biological function of polymethoxyflavones. Nobiletin possesses a unique structure in which there are 6 methoxy groups. Recently, we have shown that the polymethoxyflavones sudachitin that have 3 methoxy groups, 5,7,4'-trihydroxy-6,8,3'-trimethoxyflavone, also regulate both humoral and cellular immune responses in mice (26). 5,7,4'-Trihydroxy-6,8,3'-trimethoxyflavone induces dominant Th2 responses as indicated by IL-4, IgG1 and IgE levels. This result is similar to the observation in the experiment using nobiletin. Concerning the methoxy group, the 6,3'-methoxy position is a common structure in nobiletin and sudachitin. To verify that the 6,3'-methoxy position contributes to the induction of a Th2 response, other methoxyflavones containing the 6,3'-methoxy position should be examined.

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Author contributions

A.N., M.M., E.S., Y.N., and T.S. designed the research, M.M., A.N., K.U., A.M., and T.S. performed the experiment, M.M., A.N., and M.N. analyzed data, and M.M., A.N., and T.S. wrote the manuscript. All authors have read and approved the final manuscript.

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