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Nobiletin, a polymethoxy flavonoid, exerts anti-allergic effect by suppressing activation of phosphoinositide 3-kinase

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ABSTRACT

Nobiletin is a polymethoxy flavonoid present in fruit tissues of citrus species and has been reported to suppress degranulation of RBL-2H3 cells. However, the detailed mechanism is still unknown. In this study the mechanism of suppressive effect of nobiletin on degranulation of RBL-2H3 cells was examined. Nobiletin suppressed degranulation of both RBL-2H3 cells and bone marrow-derived mast cells induced by antigen stimulation. Nobiletin also suppressed degranulation stimulated by the calcium ionophore A23187 and thapsigargin, a specific inhibitor of the Ca^{2+} -dependent ATPase. Elevation of intracellular free Ca^{2+} concentration was also suppressed by nobiletin when RBL-2H3 cells were stimulated by either antigen or A23187. Immunoblot analysis revealed that the inhibitory effect results from down-regulated phosphorylation of both phosphatidylinositol 3-kinase (PI3K) and phospholipase $\text{C}\gamma 1$ in the signaling pathways related to degranulation stimulated by antigen. Furthermore, oral administration of nobiletin suppressed vascular permeability in passive cutaneous anaphylaxis model mice. The tendency in all experiments obtained by treating with nobiletin was highly similar to that obtained by wortmannin, a specific inhibitor of PI3K. These findings suggest that nobiletin suppresses Ca^{2+} influx and consequently exerts the anti-allergy activity in a similar way to wortmannin.

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1. Introduction

Prevalence of allergic diseases such as atopic dermatitis, asthma, hives, and hay fever is a serious health problem. These

diseases are classified into the type I allergy caused by release of the granule-stored substances such as histamine, proteases, lipid mediators, and cytokines from mast cells or basophils. One of the potent stimuli for activation of mast cells or

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Abbreviations: BMMC, bone marrow-derived mast cell; BSA, bovine serum albumin; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; DNP, dinitrophenol; FBS, fetal bovine serum; FcεRI, high-affinity IgE receptor; HRP, horseradish peroxidase; HSA, human serum albumin; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% Tween 20; PCA, passive cutaneous anaphylaxis; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; SD, standard deviation 1756-4646/\$ - see front matter © 2013 Published by Elsevier Ltd.

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basophils is initiated by the binding of antigen to IgE bound to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. Activation of mast cells or basophils induces exocytosis and fusion of cytoplasmic granules with the plasma membrane, followed by release of various granule-stored mediators. Prevention of allergic diseases using food ingredients has been intensively studied, and evidences of preventive effect of dietary polyphenols on allergic diseases have been published (Singh, Holvoet, & Mercenier, 2011).

Nobiletin (Fig. 1), a citrus flavonoid containing six methoxy groups, is a major component in citrus fruits (Nogata et al., 2006). Nobiletin has been reported to display anti-inflammatory (Wu, Zhou, Tao, & Li, 2006), anti-tumour (Miyata, Sato, Yano, & Ito, 2004), and anti-diabetes (Lee et al., 2010) activities. Polymethoxyflavones isolated from *Citrus reticulata* with similar structures to nobiletin suppressed degranulation via mainly down-regulation of the tyrosine kinase Syk, phospholipase C (PLC) γ , the protein kinase C pathway, and Ca^{2+} influx (Itoh, Ohguchi, Iinuma, Nozawa, & Akao, 2008). Nobiletin has also been reported to suppress degranulation of rat basophilic leukemia RBL-2H3 cells via down-regulation of phosphorylation of Akt (Kobayashi & Tanabe, 2006), although its detailed mechanism of action is still unknown.

In this study, we investigated the detailed mechanism of nobiletin on degranulation of RBL-2H3 cells that are mucosal mast cells functioning similar to primary mast cells and normal basophils and have been used as a pertinent model for studying the anti-allergic activity (Choi, Kim, & Hwang, 2012; Metcalfe, Peavy, & Gilfillan, 2009; Yamaki et al., 2007). In addition, the effect of nobiletin on the IgE-mediated passive cutaneous anaphylaxis (PCA) in mice was evaluated. Herein, we describe suppressive effects of nobiletin on degranulation of RBL-2H3 cells and on PCA in mice in a similar way to the specific phosphoinositide 3-kinase (PI3K) inhibitor wortmannin.

2. Materials and methods

2.1. Reagents

Nobiletin (3',4',5,6,7,8-hexamethoxyflavone; Fig. 1), 4-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside, wortmannin, thapsigargin, and Tween 20 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nobiletin was dissolved in dimethyl sulfoxide (DMSO) to make a 20 mM stock solution and used for the experiments described below. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, penicillin, streptomycin, bovine serum albumin (BSA), mouse anti-dinitrophenol (DNP) monoclonal IgE, DNP-human

serum albumin (HSA), a calcium ionophore A23187, Evans blue, and Triton X-100 were products of Sigma (St. Louis, MO, USA). Goat anti-actin antibody (sc-1616) and horseradish peroxidase (HRP)-labeled anti-goat IgG antibody (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PI3K p85 antibody (#4257), anti-phosphorylated PI3K p85/p55 antibody (#4228), anti-Akt (#4691), anti-phosphorylated Akt (#4060), a B cell signaling antibody sampler kit (#9768) including antibodies against Syk, phosphorylated Syk, Lyn, and phosphorylated Lyn, phosphorylated PLC γ antibody sampler kit (#3860) including antibodies against PLC γ 1, phosphorylated PLC γ 1, PLC γ 2, and phosphorylated PLC γ 2, and HRP-labeled anti-rabbit IgG antibody (#7074) were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cells and cell culture

2.2.1. RBL-2H3 cells

RBL-2H3 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 5% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS, USA) at 37 °C under humidified 5% CO₂–95% air.

2.2.2. Bone marrow-derived mast cells (BMMCs)

Five-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and kept in a specific pathogen-free facility. After acclimatization to their housing environment, BMMCs were generated from femurs of 6- to 8-week-old mice according to the method of Lee et al. (2010) with some modifications. Bone marrow cells were cultured in RPMI-1640 medium supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 10% FBS, and 10 ng/mL of recombinant murine IL-3 (PeproTech, Rocky Hill, NJ, USA) in a 24- or 96-well culture plate (BD Falcon, Franklin Lakes, NJ, USA) for 4 weeks at 37 °C under humidified 5% CO₂–95% air. The culture medium was replaced once a week. The resulting BMMCs were used for the degranulation assay as described below. All animal experiments described herein were carried out in accordance with the protocol approved by the Laboratory Animal Care Committee of Ehime University (approval number: 08-U-2-1, validity date: 31 March 2014). Mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

2.3. Degranulation assay

2.3.1. Antigen-induced degranulation assay using RBL-2H3 cells

The assay was performed as previously described (Ishida, Nishi, Watanabe, & Sugahara, 2013). Briefly, RBL-2H3 cells were seeded at 5.0×10^4 cells/well in a 96-well culture plate and cultured at 37 °C overnight. The cells were subsequently sensitized for 2 h with anti-DNP IgE (50 ng/mL) diluted in the medium. After washing with the modified Tyrode's buffer (20 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% BSA, pH 7.4), the cells were treated for 10 min with various concentrations of nobiletin or DMSO (vehicle control) diluted in the modified Tyrode's buffer.

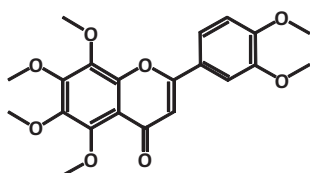


Fig. 1 – Chemical structure of nobiletin.

The cells were then stimulated for 30 min by adding 10 μ L of DNP-HSA diluted in the modified Tyrode's buffer at the final concentration of 50 ng/mL. After each supernatant was collected, the cells were sonicated in 130 μ L of the modified Tyrode's buffer containing 0.1% Triton X-100 for 5 s on ice. Both supernatant and cell lysate were transferred to a new 96-well microplate at 50 μ L/well and incubated at 37 °C for 5 min. Then, 100 μ L of 3.3 mM 4-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside dissolved in 0.1 M citrate buffer (pH 4.5) were added to each well and incubated at 37 °C for 25 min. The enzyme reaction was terminated by adding 100 μ L of 2 M glycine buffer (pH 10.4), and the absorbance was read at 405 nm using a microplate reader (SH-8000Lab, Corona Electric, Ibaraki, Japan). A β -hexosaminidase release rate was calculated as $100 \times [(A_{\text{supernatant}} - A_{\text{blank of supernatant}}) / \{(A_{\text{supernatant}} - A_{\text{blank of supernatant}}) + (A_{\text{cell lysate}} - A_{\text{blank of cell lysate}})\}]$, in which "A" is the absorbance of each well.

2.3.2. Antigen-induced degranulation assay using BMMCs
BMMCs cultured in a 24-well culture plate were sensitized for 5 h with anti-DNP IgE (100 ng/mL) diluted in the culture medium. After washing with the modified Tyrode's buffer, the cells were treated with various concentrations of nobiletin or DMSO diluted in the modified Tyrode's buffer for 30 min. The cells were then stimulated for 60 min with DNP-HSA (500 ng/mL) diluted in the modified Tyrode's buffer. After incubation, the supernatant was each collected, and the cells were sonicated in 500 μ L of the modified Tyrode's buffer containing 0.1% Triton X-100 for 10 s on ice. The following procedure was performed as described in the method of antigen-induced degranulation assay using RBL-2H3 cells.

2.3.3. A23187 or thapsigargin-induced degranulation assay
RBL-2H3 cells were seeded in a 96-well culture plate and treated with nobiletin or DMSO as described above, except for sensitization with anti-DNP IgE. To assess the effect of PI3K inhibition on the degranulation event, RBL-2H3 cells were treated for 30 min with wortmannin diluted in the modified Tyrode's buffer instead of nobiletin. Then, the cells were subsequently stimulated by adding 10 μ L of A23187 or thapsigargin diluted in the modified Tyrode's buffer at the final concentration of 3 μ M or 1 μ M, respectively, to each well, and the cells were incubated at 37 °C for 30 min. Then, the β -hexosaminidase release rate was measured as described above.

2.4. Measurement of cell viability

Cytotoxicity of nobiletin on RBL-2H3 cells and BMMCs were examined using a WST-8 assay kit (Kishida Chemical, Osaka, Japan) according to manufacturer's instructions. RBL-2H3 cells were seeded at 5.0×10^4 cells/well in a 96-well culture plate and cultured at 37 °C overnight. BMMCs were cultured in a 96-well culture plate. After sensitization with anti-DNP IgE, the cells were treated with various concentrations of nobiletin or DMSO and subsequently stimulated with DNP-HSA as described above. After washing with phosphate-buffered saline (PBS, pH 7.4) once, the culture medium containing 10% of the WST-8 solution was added to each well of the cell culture plate and incubated for 30 min at 37 °C. Then,

the absorbance was read at 450 nm using a microplate reader (SH-8000Lab, Corona Electric).

2.5. Antigen–antibody binding assay

One hundred microlitres of DNP-HSA diluted in 50 mM carbonate buffer (50 mM NaHCO₃, 50 mM Na₂CO₃, pH 9.6) at 0.25 μ g/mL were added to each well of a 96-well microplate and kept at 4 °C overnight. The plate was washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 300 μ L of PBS containing 5% skim milk at 4 °C overnight. After washing 3 times with PBS-T, 50 μ L of various concentrations of nobiletin or DMSO alone diluted in PBS were added and incubated for 20 min at 37 °C. Then, 0.5 μ g/mL of DNP-IgE diluted in PBS was added to each well at 50 μ L/well and incubated for 1 h at 37 °C. After washing with PBS-T 3 times, each well was treated with 100 μ L of the biotinylated rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) diluted at 0.5 μ g/mL in 5% skim milk-PBS for 1 h at 37 °C. After washing 3 times with PBS-T, each well was treated with the HRP-streptavidin conjugate (BioSource International, Camarillo, CA, USA) diluted in 3000 times in PBS containing 1% BSA for 1 h at 37 °C. Then, 0.3 mg/mL of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt dissolved in 0.03% H₂O₂–0.05 M citrate buffer (pH 4.0) was added at 100 μ L/well, and the absorbance was measured at 415 nm using the microplate reader after addition of 100 μ L of 1.5% oxalic acid to terminate the coloring reaction.

2.6. Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]_i)

The [Ca²⁺]_i was measured using a Calcium kit-Fluo 3 (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer's instructions. RBL-2H3 cells were seeded to a white 96-well culture plate (Nunc, Roskilde, Denmark) and treated with anti-DNP IgE as described above. After washing with PBS twice, the IgE-sensitized cells were incubated with 100 μ L of Fluo-3 AM for 1 h. After washing with PBS, the cells were treated with 120 μ L of nobiletin (50 μ M) or DMSO alone at 37 °C for 10 min. Then, the cells were stimulated by addition of 10 μ L of DNP-HSA or A23187 diluted in the modified Tyrode's buffer at the final concentration of 0.625 μ g/mL or 3 μ M, respectively, and the fluorescent intensity was immediately monitored with an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a microplate reader (SH-8000Lab, Corona Electric).

2.7. Immunoblot analysis

RBL-2H3 cells were seeded at 2.5×10^6 cells to 100 mm culture dishes (BD Falcon) and treated with anti-DNP IgE as described above. The cells were washed with the modified Tyrode's buffer and treated with 50 μ M of nobiletin, 5 μ M of wortmannin, or DMSO alone diluted in the modified Tyrode's buffer for 10 min. The cells were subsequently stimulated with DNP-HSA at the final concentration of 0.625 μ g/mL for 2 min. Then, total cell lysates were immediately prepared and immunoblotted with various antibodies as previously described (Ishida et al., 2013).

2.8. IgE-mediated PCA in mice

The assay was performed by the method of Knoop, Louahed, Van Snick, and Renaud (2005) with some modifications. Five-week-old female BALB/c mice (Japan SLC) were kept in a specific pathogen-free facility. After acclimatization to their housing environment for 1 week, the mice were injected with 100 ng of anti-DNP IgE diluted in PBS and with PBS alone into left and right ears, respectively, using a 50 μ L gas-tight syringe (Hamilton, Reno, NV, USA). After 24 h, 200 μ L of DNP-HSA diluted in 200 μ L of PBS containing 0.5% Evans blue were injected into the tail vein using a 0.5 mL gas-tight Hamilton syringe. To assess the effect of nobiletin and wortmannin, the mice were orally administered with 50 mg/kg body weight of nobiletin or 10 mg/kg body weight of wortmannin diluted in 40 μ L of mineral oil or with 40 μ L of mineral oil alone 1 h before injection of DNP-HSA. The amount for oral administration was determined according to the method by Kim, R  dinger, and Gilfillan (2008). The mice were euthanized 30 min after treatment with DNP-HSA, and the treated ears were excised. Then, the extravasated dye was extracted from each ear with 500 μ L of formamide at 70 $^{\circ}$ C overnight, and the

absorbance of the dye was read at 620 nm using a Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.9. Statistical analysis

Data obtained were expressed as the mean $\pm$ standard deviation (SD). Dunnett's test was used to assess the statistical significance of the difference against control. Values with $^*p < 0.05$ or $^{**}p < 0.01$ were considered to be statistically significant.

3. Results

3.1. Effect of nobiletin on antigen-mediated degranulation of RBL-2H3 cells and BMMCs

At first, the effect of nobiletin on degranulation of RBL-2H3 cells was examined. Anti-DNP IgE-sensitized RBL-2H3 cells were treated with nobiletin, and degranulation was induced by DNP-HSA. β -Hexosaminidase release was used as a marker of degranulation. As shown in Fig. 2A, degranulation was

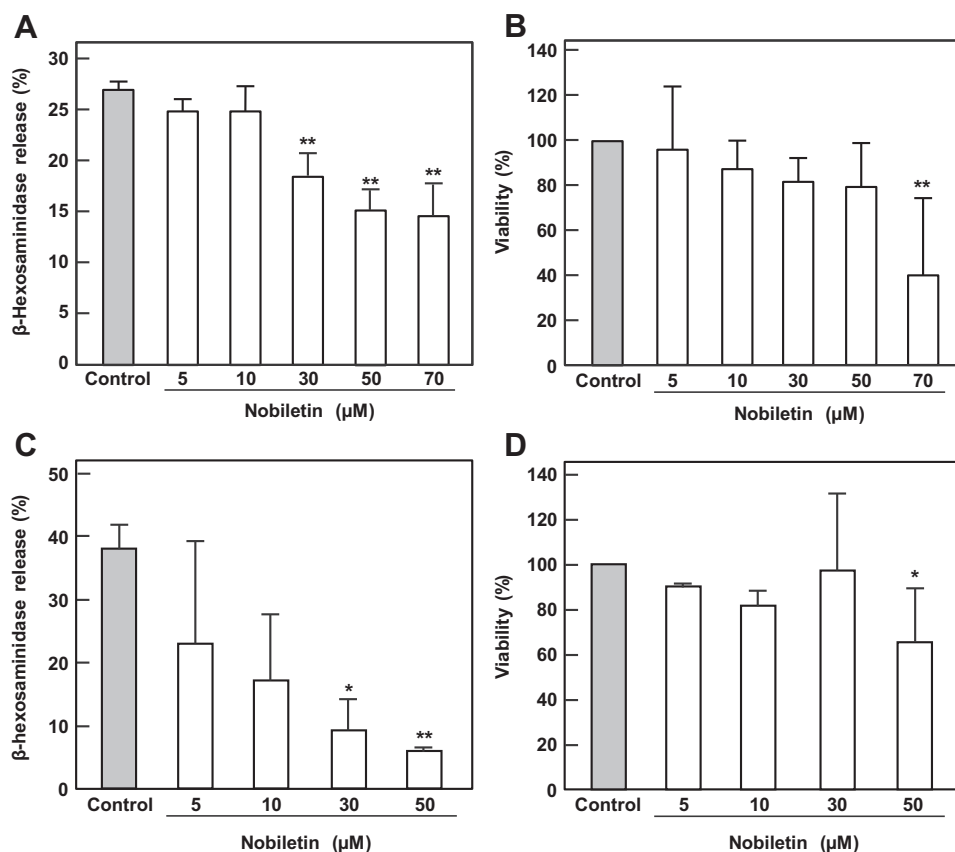


Fig. 2 – Effects of nobiletin on degranulation and cell viability of RBL-2H3 cells and BMMCs. RBL-2H3 cells (A) and BMMCs (C) were sensitized with anti-DNP IgE. The cells were subsequently treated with various concentrations of nobiletin or DMSO alone (vehicle control), and degranulation was induced with DNP-HSA. Released β -hexosaminidases were used as a marker of degranulation. Cell viability was assessed using the WST-8 solution after various concentrations of nobiletin or DMSO alone (vehicle control) were treated to anti-DNP IgE-sensitized RBL-2H3 cells (B) and BMMCs (D) followed by stimulation with DNP-HSA. Cell viability of the control cells was expressed as 100%. Data were represented as the mean $\pm$ SD ($n = 3$). Dunnett's test was used to assess the statistical significance of the difference against control. $^*p < 0.05$ and $^{**}p < 0.01$ against control.

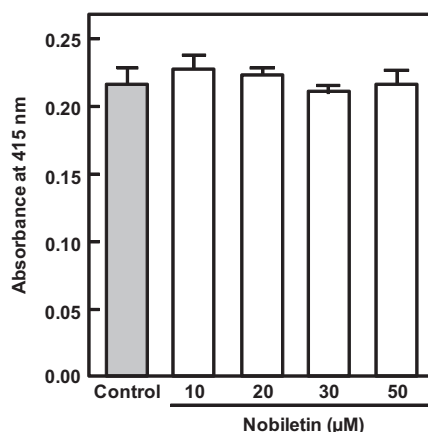


Fig. 3 – Effects of nobiletin on antigen–antibody binding. Various concentrations of nobiletin or DMSO alone (vehicle control) were added to a 96-well microplate coated with DNP-HSA. After addition of anti-DNP IgE, the biotinylated anti-mouse IgE and streptavidin-HRP were added. Then, the coloring substrate was added, and the absorbance was measured at 415 nm. Data were represented as the mean $\pm$ SD ($n = 3$).

suppressed by nobiletin in a dose-dependent manner. Significant differences were observed against the control in the β -hexosaminidase release rate at 30 μ M or higher concentrations of nobiletin. Cytotoxicity of nobiletin on RBL-2H3 cells was assessed by the WST-8 assay. Although nobiletin showed cytotoxicity on RBL-2H3 cells at 70 μ M, no cytotoxicity was observed in lower concentrations than 70 μ M (Fig. 2B). These results suggested that nobiletin suppresses degranulation of RBL-2H3 cells stimulated by antigen with no cytotoxic effect.

For further experiments, 50 μ M nobiletin was used because of the high inhibitory effect on degranulation without cytotoxicity.

The effect of nobiletin on degranulation was also examined using BMMCs, a primary mast cell model. As shown in Fig. 2C, nobiletin significantly suppressed degranulation of BMMCs. Although there was no statistically significant difference at 5 or 10 μ M against the control, the suppressive effect of nobiletin seemed to be stronger in BMMCs than in RBL-2H3 cells. Cytotoxicity of nobiletin was also examined using BMMCs. The result showed that nobiletin has a cytotoxic effect on BMMCs at 50 μ M (Fig. 2D), indicating that BMMCs were more sensitive to nobiletin than RBL-2H3 cells. These results suggested that nobiletin suppresses the IgE-mediated degranulation response of mast cells.

3.2. Effect of nobiletin on binding of antigen to antibody

Next, whether nobiletin inhibits the binding between antigen and antibody was examined by an enzyme-linked immunosorbent assay, which is the first response on degranulation of mast cells. Nobiletin and anti-DNP IgE were added to wells of a microplate coated with DNP-HSA, and the coloring reaction was performed. If nobiletin inhibits the antigen–antibody binding, the resulting absorbance would decrease in the presence of nobiletin. As shown in Fig. 3, the absorbance was not affected regardless of the nobiletin concentration, suggesting that nobiletin does not inhibit the antigen–antibody reaction.

3.3. Effect of nobiletin on Fc ϵ RI-independent degranulation of RBL-2H3 cells

Elevation of $[Ca^{2+}]_i$ is the most common cause of degranulation. To examine whether nobiletin is involved in the

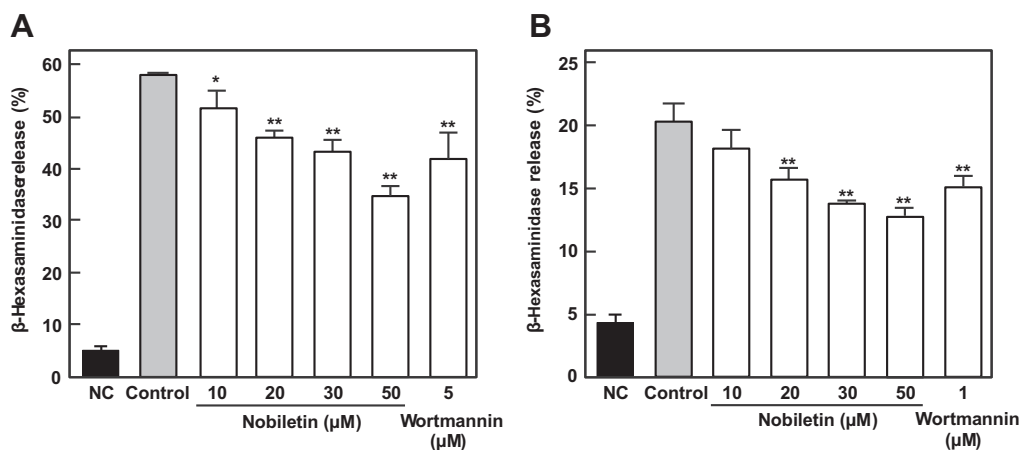


Fig. 4 – Effects of nobiletin on degranulation induced by A23187 or thapsigargin. (A) RBL-2H3 cells were treated with various concentrations of nobiletin, 5 μ M of wortmannin, or DMSO alone (vehicle control) and subsequently stimulated with 3 μ M of A23187 for 30 min to induce degranulation. The cells in negative control (NC) was treated with DMSO and not stimulated with A23187. Released β -hexosaminidases were used as a marker of degranulation. (B) RBL-2H3 cells were treated with various concentrations of nobiletin, 1 μ M of wortmannin, or DMSO alone (control) and subsequently stimulated with 1 μ M of thapsigargin for 30 min to induce degranulation. The cells in NC was treated with DMSO and not stimulated with thapsigargin. Released β -hexosaminidases were used as a marker of degranulation. Data were represented as the mean $\pm$ SD ($n = 3$). Dunnett's test was used to assess the statistical significance of the difference against control. * $p < 0.05$ or ** $p < 0.01$ against control.

FcεRI-independent degranulation of mast cells, A23187 and thapsigargin were used. A23187, a calcium ionophore, carries Ca^{2+} into the cytosol across the plasma membrane and induces degranulation. Because wortmannin, an inhibitor of PI3K, has been reported to suppress degranulation induced by A23187 (Marquardt, Alongi, & Walker, 1996), the compound was used as a positive control. The result showed that nobiletin significantly suppressed degranulation in a dose-dependent manner (Fig. 4A). Wortmannin also suppressed degranulation as reported previously. Thapsigargin is a specific inhibitor of the Ca^{2+} -dependent ATPase, which pumps Ca^{2+} from the cytosol into endoplasmic reticulum. As well as wortmannin, nobiletin significantly inhibited degranulation induced by thapsigargin (Fig. 4B). These results indicated that nobiletin suppresses Ca^{2+} influx to the cytosol.

3.4. Effect of nobiletin on $[\text{Ca}^{2+}]_i$

Stimulation of FcεRI on mast cells by antigen leads to the Ca^{2+} entry with the subsequent degranulation. To investigate the effect of nobiletin on the Ca^{2+} response, $[\text{Ca}^{2+}]_i$ was monitored using Fluo-3 AM. As shown in Fig. 5A, $[\text{Ca}^{2+}]_i$ in the control cells rapidly increased after stimulation with DNP-HSA. On the other hand, elevation of $[\text{Ca}^{2+}]_i$ in the cells treated with nobiletin was suppressed compared with the control. The effect of nobiletin on the FcεRI-independent Ca^{2+} response was further examined using A23187. Elevation of $[\text{Ca}^{2+}]_i$ was suppressed by nobiletin; however, the suppressive effect was not as strong on the A23187-induced degranulation as on the antigen-induced degranulation (Fig. 5B). These results indicated that nobiletin inhibits elevation of $[\text{Ca}^{2+}]_i$ not only by the FcεRI-dependent pathway but also by the FcεRI-independent pathway.

3.5. Effect of nobiletin on FcεRI-mediated signaling pathway

Nobiletin has been reported to suppress phosphorylation of Akt (Kobayashi & Tanabe, 2006). Then, the effect of nobiletin on phosphorylation of PI3K locating upstream of Akt was examined by immunoblotting. As shown in Fig. 6A and B, nobiletin as well as wortmannin inhibited phosphorylation of both PI3K and Akt. In addition, the effect of nobiletin on phosphorylation of proteins on the signaling pathway involved in degranulation was investigated. As shown in Fig. 6C, phosphorylation of PLCγ1 locating downstream of PI3K was suppressed by nobiletin, although phosphorylation of Lyn, Syk, or PLCγ2 was not suppressed. Interestingly, the tendency to inhibit the protein phosphorylation by nobiletin was highly similar to that by wortmannin.

3.6. Effect of nobiletin in PCA model mouse

The anti-allergic activity of nobiletin *in vivo* was evaluated using female BALB/c mice. The IgE-mediated PCA was successfully induced by the sequential injections of IgE and DNP-HSA. The PCA reaction was significantly suppressed by oral administration of nobiletin or wortmannin at a dose of 50 mg/kg or 10 mg/kg, respectively (Fig. 7A, B). The result indi-

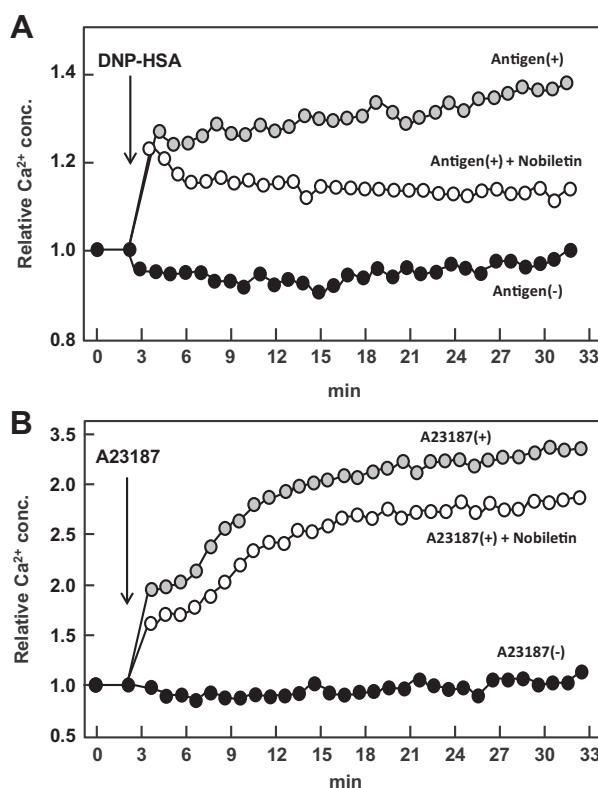


Fig. 5 – Effects of nobiletin on $[\text{Ca}^{2+}]_i$ of RBL-2H3 cells. (A) Anti-DNP IgE-sensitized cells were pre-incubated with Fluo-3 AM for 1 h and subsequently incubated with 50 μM of nobiletin or DMSO alone (vehicle control) for 10 min. The treated cells were then stimulated with antigen (DNP-HSA) and the fluorescence intensity was monitored. Closed gray circle; DMSO-treated cells stimulated with DNP-HSA, open circle; nobiletin-treated cells stimulated with DNP-HSA, closed black circle; DMSO-treated cells not stimulated with DNP-HSA. **(B)** Anti-DNP IgE-sensitized cells were pre-incubated with Fluo-3 AM for 1 h and subsequently incubated with 50 μM of nobiletin or DMSO alone (control) for 10 min. Then, RBL-2H3 cells were stimulated with 3 μM of A23187 and the fluorescence intensity was monitored. Closed gray circle; DMSO-treated cells stimulated with A23187, open circle; nobiletin-treated cells stimulated with A23187, closed black circle; DMSO-treated cells not stimulated with A23187. Data were represented as the mean ($n = 3$).

cated that nobiletin exerts the anti-allergy activity *in vivo* by oral administration.

4. Discussion

Nobiletin has been reported to suppress release of both histamine and β -hexosaminidase from RBL-2H3 cells at 100 μM or higher concentration of nobiletin (Kobayashi & Tanabe, 2006). We herein confirmed that 50 μM of nobiletin significantly suppresses degranulation without affecting the viability of RBL-2H3 cells (Fig. 2A, B). Nobiletin also suppressed degranulation

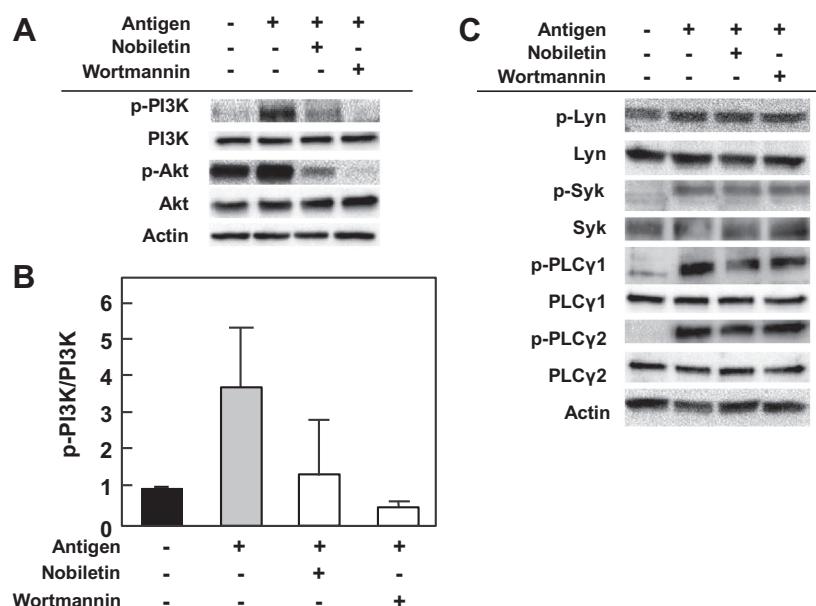


Fig. 6 – Effects of nobiletin on signaling pathways involved in degranulation of RBL-2H3 cells stimulated by antigen. (A) Nobiletin (50 μ M), wortmannin (5 μ M), or DMSO alone was added to anti-DNP IgE-sensitized cells, and degranulation was induced with DNP-HSA. Then, each cell lysate was subjected to immunoblot analysis. The p-PI3K and p-Akt represent phosphorylated PI3K and phosphorylated Akt, respectively. A representative blot from two independent experiments is shown. **(B)** The ratio of p-PI3K/PI3K in each cell lysate relative to that in the intact cells (antigen; –, nobiletin; –). Data were represented as the mean $\pm$ SD ($n = 2$). **(C)** Nobiletin was added to anti-DNP IgE-sensitized cells at 50 μ M, and degranulation was induced with DNP-HSA. Then, each cell lysate was subjected to immunoblot analysis. The p-Lyn, p-Syk, and p-PLC represent phosphorylated Lyn, phosphorylated Syk, and phosphorylated PLC, respectively. A representative blot from two independent experiments is shown.

of BMMCs (Fig. 2C), suggesting that nobiletin affects not only a specified cell line but also unspecified mast cells.

Degranulation was caused by the antigen–antibody reaction inducing the Fc ϵ RI-mediated signaling transduction cascade and thereby raises $[Ca^{2+}]_i$. To clarify the mechanism of action, the suppressive effect of nobiletin on degranulation was examined using RBL-2H3 cells in detail. Nobiletin was confirmed not to affect the antigen–antibody reaction that is the first step for degranulation (Fig. 3). PI3K plays an important role in the intracellular trafficking actin polymerization and in the growth factor signaling (Cohen, Liu, Drucker, Roberts, & Schaffhausen, 1990; Jullien et al., 1993; Kundra et al., 1994). PI3K is also responsible for common signaling pathways after stimulation by antigen (Turner & Kinet, 1999). The enzyme activity is necessary for releasing the granular contents from mast cells. A PI3K inhibitor wortmannin has been reported to suppress the calcium ionophore A23187-induced degranulation (Marquardt et al., 1996; Yano et al., 1993). In addition, it has been reported that degranulation induced by thapsigargin is also dependent on PI3K (Huber, Hughes, & Krystal, 2000). PI3K inhibitors decrease the Ca^{2+} entry to the cytosol when cells were stimulated by thapsigargin, an inhibitor of the store-operated channels (Lam et al., 2008). Interestingly, nobiletin was found to suppress degranulation induced by either A23187 or thapsigargin like a PI3K inhibitor such as wortmannin (Fig. 4A, B). Elevation of $[Ca^{2+}]_i$ was suppressed by treating with nobiletin after stimulation by anti-

gen (Fig. 5A, B). Because activation of PI3K leads to phosphorylation of PLC γ 1/2 (Barker, Lujan, & Wilson, 1999; Kim et al., 2008; Koyasu, 2003), inhibition of PI3K would result in the decreased phosphorylation of PLC γ 1/2. From these results, nobiletin was found to suppress the Ca^{2+} entry into cells and indicated to down-regulate phosphorylation of both of PI3K and PLC γ 1/2 in the signaling pathways because of the similar behavior of nobiletin to wortmannin.

The signaling pathways mediated through Fc ϵ RI are initiated from aggregation of α -chains of Fc ϵ RI by binding of IgE to antigen (Turner & Kinet, 1999). Then, phosphorylation of the Src family non-receptor tyrosine kinase Lyn is induced and the activated Lyn phosphorylates β - and γ -chains of ITAM locating in the cytoplasmic domain of individual receptor subunits (Pribluda, Pribluda, & Metzger, 1994; Cambier, 1995). The activated γ -ITAM recruits and in turn phosphorylates the Syk kinase to initiate the signaling cascade (Kopeć, Panaszek & Fal, 2006; Kraft & Kinet, 2007; Metcalfe et al., 2009; Rivera & Gilfillan, 2006; Turner & Kinet, 1999). As shown in Fig. 6C, nobiletin did not suppress phosphorylation of Lyn or Syk. In addition, wortmannin did not inhibit phosphorylation of Lyn or Syk in the antigen-stimulated RBL-2H3 cells as previously reported by Barker et al. (1995). Thus, it was indicated that nobiletin does not affect the initial signaling event mediated by Fc ϵ RI as is the case with wortmannin.

PI3K is activated by the adapter protein Gab2 by phosphorylation (Gu et al., 2001; Kraft & Kinet, 2007). The activated PI3K

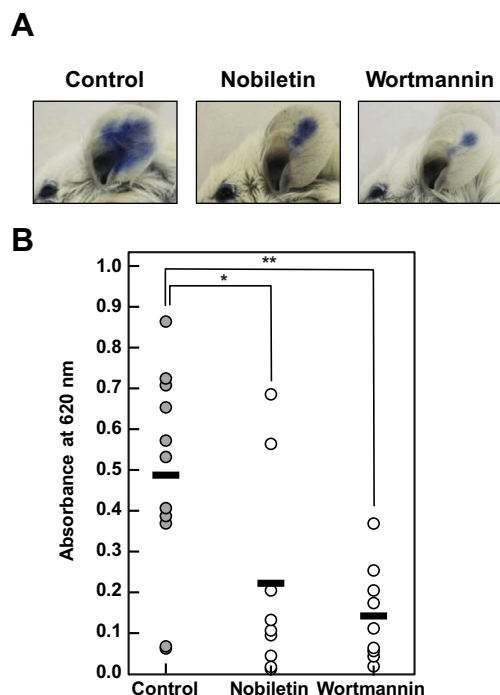


Fig. 7 – Effects of nobiletin on IgE-mediated PCA in mice. Six-week-old female BALB/c mice were intravenously injected with 200 μ g of DNP-HSA with 0.5% Evans blue 24 h after intradermal administration of 100 ng of anti-DNP IgE and sterilized PBS into left and right ears of mice, respectively. Nobiletin (50 mg/kg body weight), wortmannin (10 mg/kg body weight), or mineral oil alone (control) was orally administrated 1 h before administration of DNP-HSA. (A) Photographs were representative left ears from mice in each administration group 30 min after administration of DNP-HSA. (B) Evans blue was extracted from each ear and the absorbance of the dye was measured at 620 nm. The absorbance of the dye extracted from left ear was subtracted with that from right ear. Control group; $n = 11$, nobiletin-administrated group; $n = 8$, wortmannin-administrated group; $n = 9$. Dunnett's test was used to assess the statistical significance of the difference against control. * $p < 0.05$ or ** $p < 0.01$ against control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

converts phosphatidylinositol 4,5-diphosphate to phosphatidylinositol 3,4,5-trisphosphate and leads to phosphorylation of PLC γ 1/2 (Barker et al., 1999; Kim et al., 2008; Koyasu, 2003). Activation of PI3K also results in phosphorylation of the serine kinase Akt (Fruman and Cantley, 2002). Nobiletin has been reported to suppress phosphorylation of Akt locating downstream of PI3K when degranulation was stimulated by antigen in RBL-2H3 cells (Kobayashi & Tanabe, 2006). In this study, we found, by immunoblot analysis, that nobiletin suppresses phosphorylation not only of Akt but also of PI3K (Fig. 6A, B). Wortmannin has also been reported to suppress phosphorylation of PLC γ 1 but not PLC γ 2 (Barker, Caldwell, Pfeiffer, & Wilson, 1998). Phosphorylated PLC γ 1 is translo-

cated to the membrane fraction after cross-linking of Fc ϵ RI (Atkinson, Kaliner, & Hohman, 1992; Park, Min, & Rhee, 1991). As shown in Fig. 6C, nobiletin suppressed phosphorylation of PLC γ 1 but not PLC γ 2. Thus, nobiletin also exhibited a similar tendency to wortmannin on phosphorylation of both PI3K and PLC γ 1/2 after stimulation by antigen.

Overall results demonstrated that nobiletin suppresses degranulation of RBL-2H3 cells in a similar manner to that of the PI3K inhibitor wortmannin, suggesting that nobiletin also inhibits PI3K like wortmannin. Thus, an enzyme inhibition assay was performed using a commercially available purified PI3K. The result described that nobiletin does not inhibit the enzyme activity of PI3K even at 50 μ M, although wortmannin completely inhibited at 5 μ M (data not shown). Our data suggested that nobiletin suppresses degranulation of RBL-2H3 cells in a similar way to wortmannin, but is not a PI3K inhibitor.

In a PCA mice model, nobiletin exerted the anti-allergic activity in vivo by oral administration. Release of chemical mediators such as histamine, serotonin, and leukotrienes from mast cells by degranulation results in type I allergic reaction. In this study, nobiletin and wortmannin were found to suppress the IgE-dependent PCA reaction by oral administration (Fig. 7). It has been reported that nobiletin is metabolized to demethylated derivatives in vivo and that the anti-inflammatory effect of the nobiletin metabolites was greater than the parent compound in RAW 264.7 macrophages (Li et al., 2007). Because the concentration of nobiletin in vivo was assumed to be much less than 50 μ M, the anti-allergic activity of nobiletin found in the PCA model mice was considered to result from the more bioactive metabolites converted from the parent compound nobiletin.

In conclusion, nobiletin suppressed the antigen-induced degranulation in RBL-2H3 and BMMC cells. The inhibitory effect of nobiletin was shown to result from the suppressed Ca^{2+} mobilization through the down-regulated phosphorylation of both PI3K and PLC γ 1. In addition, oral administration of nobiletin suppressed the PCA reaction in mice. Although further investigations would be required, overall findings indicated that nobiletin has a potential as a natural remedy to treat allergic responses by suppressing degranulation of mast cells through down-regulation of phosphorylation of PI3K. The behaviour of nobiletin was very much similar to wortmannin in each experiment. Taken together, our findings suggest that nobiletin has an anti-allergic effect and would be valuable as a functional food factor.

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