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Nobiletin and tangeretin ameliorate scratching behavior in mice by inhibiting the action of histamine and the activation of NF-κB, AP-1 and p38

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ABSTRACT

Nobiletin and tangeretin are polymethoxy flavonoids that are abundantly present in the pericarp of *Citrus unshiu* (family Rutaceae) and the fruit of *Citrus depressa* (family Rutaceae). They exhibit various biological activities, including anti-inflammatory and anti-asthmatic effects. To evaluate the anti-allergic effects of nobiletin and tangeretin, we measured their inhibitory effects in histamine- or compound 48/80-induced scratching behavioral mice. Nobiletin and tangeretin potently inhibited scratching behavior, as well as histamine-induced vascular permeability. Furthermore, they inhibited the expression of the allergic cytokines, IL-4 and TNF-α as well as the activation of their transcription factors NF-κB, AP-1 and p38 in histamine-stimulated skin tissues. They also inhibited the expression of IL-4 and TNF-α and the activation of NF-κB and c-jun in PMA-stimulated RBL-2H3 cells. Furthermore, nobiletin and tangeretin inhibited protein kinase C (PKC) activity and the IgE-induced degranulation of RBL-2H3 cells. These agents showed potent anti-histamine effect through the Magnus test when guinea pig ileum was used. Based on these results, nobiletin and tangeretin may ameliorate scratching behavioral reactions by inhibiting the action of histamine as well as the activation of the transcription factors NF-κB and AP-1 via PKC.

1. Introduction

Pruritus, which is an unpleasant cutaneous sensation that provokes the desire or reflex to scratch, can be local or widespread and is associated with many diseases, such as atopic dermatitis, urticaria, cholestasis, and uremia. Many endogenous amines, proteases, growth factors, neuropeptides, opioids, eicosanoids, and cytokines act as a pruritogen [1–3]. For example, histamine, substance P or compound 48/80 significantly induces scratching in mice [1,4]. Therefore, histamine, substance P, or compound 48/80 is used as a pruritogen for pruritic animal models. Pruritus can cause skin lesions and contribute to severe psychological disturbances [5]. Thus, inhibiting pruritic responses is beneficial for improving the quality of life. However, no specific remedy is available for this common symptom.

Nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) and tangeretin (5,6,7,8,4′-pentamethoxy flavone) are polymethoxy flavonoids (PMFs) that are abundantly present in the pericarp of *Citrus unshiu* and the fruit of *Citrus depressa* (Taiwan tangerine or Shiikuwasa, family Rutaceae) [6,7]. They exhibit several biological activities, including anticancer [8], anti-inflammatory [9,10], neuroprotective [11], hypolipidemic [12,13] and anti-obesity effects [14]. PMFs also suppress scavenger receptor expression in monocytes [15]. Furthermore, nobiletin attenuates ovalbumin-induced eosinophilic airway inflammation in asthmatic rats [16] and Type II collagen-induced arthritis [17]. To our knowledge, the ability of these PMFs to inhibit scratching behavior has not been studied. Therefore, we evaluated the inhibitory effects of nobiletin and tangeretin in histamine- or compound K-induced scratching behavior in mice.

2. Materials and methods

2.1. Materials

Histamine, compound 48/80, phorbol 12'-myristate 13'-acetate (PMA), Evans blue and azelastine hydrochloride were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) kits for cytokines were purchased from R&D Systems (Minneapolis, MN, U.S.A.). ELISA kit for protein kinase C

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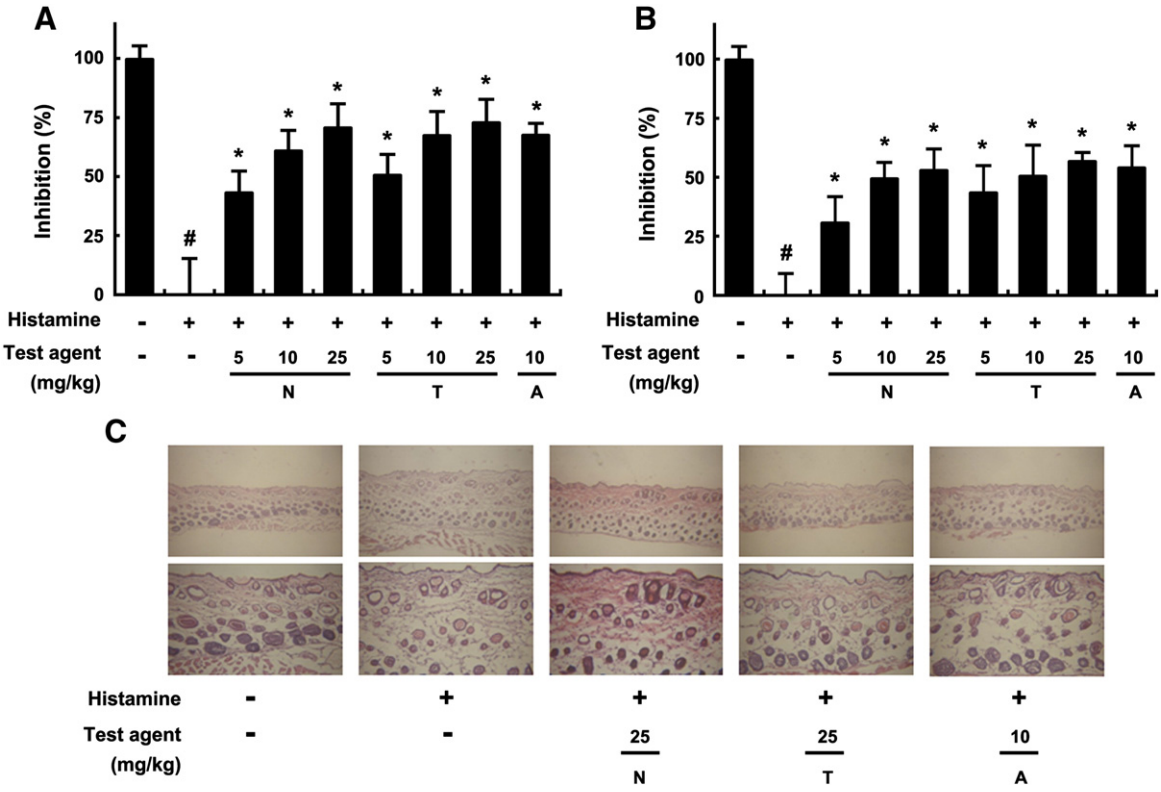

Fig. 1. Inhibitory effects of nobiletin and tangeretin on histamine- or compound 48/80-induced scratching behavior in mice. (A) Effect on histamine-induced scratching behavior. (B) Effect on compound 48/80-induced scratching behavior. The scratching behavior frequency of a normal control treated with saline alone, and histamine- or compound 48/80-induced control group for 1 h was 2 ± 1, 82 ± 5 and 321 ± 27, respectively. (C) Histological exams. The skin of mice that were treated with histamine in the absence or presence of test agents was stained using hematoxylin–eosin and viewed under a light microscope. The test agents (nobiletin at 5, 10, or 25 mg/kg; tangeretin at 5, 10, or 25 mg/kg; and azelastine at 10 mg/kg, dissolved in 2% cremophor) were orally administered 1 h before treatment with the scratching agent. Normal control mice were treated with the vehicle alone instead of test agents or histamine. The values indicate the mean ± SD ($n = 6$). #Significantly different from the normal control group ($P < 0.05$). *Significantly different from the group treated with histamine alone ($P < 0.05$).

(PKC) activity was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, U.S.A.)

2.2. Isolation of nobiletin and tangeretin

Nobiletin and tangeretin were isolated from the fruit of *Citrus depressa* (family Rutaceae) according to the previously reported methods [6,7]. The isolated nobiletin and tangeretin were identified by comparing these spectral data and physical properties of standard nobiletin and tangeretin (Wako Pure Chemical Industries, Japan), respectively.

Nobiletin - colorless needles; mp 137–138 °C; EI-MS, *m/z* 402 (M^+).
Tangeretin - light yellow needles; mp 153–154 °C; EI-MS, *m/z* 372 (M^+).

2.3. Animals

Male ICR mice (5 weeks-old, 20–25 g) and male Hartley guinea pigs (270–330 g) were supplied from Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20 – 22 °C and 50 ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center) and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

2.4. Assay of scratching behavioral frequency

The behavioral experiments were all performed as previously described [18]. Before the experiments, mice were acclimated in the acrylic cages (22 × 22 × 24 cm) for about 10 min and then divided into groups of 6 mice. The rostral part of the skin on the back of each mouse was clipped, and histamine (300 μg/50 μL) or compound 48/80 (50 μg/50 μL) for each mouse was intradermally injected with the use of a 29 gauge needle. The scratching agents were dissolved in saline. Normal control mice received a saline injection in the place of the scratching agent. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage and, for the observation of scratching, their behaviors recorded using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea) under unmanned conditions. Scratching of the injected site by the hind paws was counted and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches for 1 s, and a series of these behaviors were counted as one incident of scratching for 60 min. The test agent, nobiletin, tangeretin or azelastine (5, 10 or 25 mg/kg, dissolved in 2% cremophor), was orally administered 1 h before treatment with the scratching agent in mice.

2.5. Assay of vascular permeability

The increase in vascular permeability caused by histamine was assessed as reported previously [18]. After the intradermal injection of histamine (300 μg/50 μL) into the rostral part of the back of each mouse, the injected site (1 × 1 cm) was outlined with an indelible red marker and 0.2 mL of 1% Evans blue solution in saline was injected

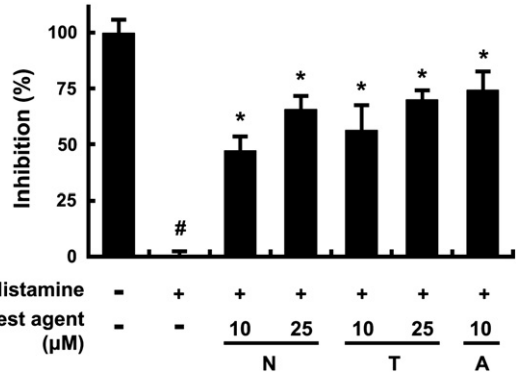

Fig. 2. Inhibitory effects of nobiletin and tangeretin on histamine-induced vascular permeability in mice. The vascular permeability was increased by histamine in mice. The mice were treated with or without the oral administration of test agents 1 h before the intradermal injection of histamine (300 μg/50 μL) into the skin on the backs of mice. The amount of Evan blue extravasated from the dorsal skin (1 cm × 1 cm) of the control group stimulated with histamine and normal group treated with vehicle alone was 17 ± 4 μg and 4 ± 2 μg, respectively. The test agents (nobiletin at 10 or 25 mg/kg; tangeretin at 10 or 25 mg/kg; and azelastine at 10 mg/kg, dissolved in 2% cremophor) were orally administered 1 h before treatment with the scratching agent. Normal control mice were treated with vehicle alone instead of test agents or histamine. The values indicate the mean ± S.D. ($n = 6$). #Significantly different from the normal control group ($P < 0.05$). *Significantly different from the group treated with histamine alone ($P < 0.05$).

intravenously. Nobiletin, tangeretin or azelastine, (10 or 25 mg/kg, dissolved in 2% cremophor) was orally administered 1 h before treatment with histamine. Mice were sacrificed 60 min later by anesthesia with ether and the scratching agent-injected site (1 × 1 cm) immediately excised. The skin specimen was dissolved in 1 mL of 1 M KOH solution by overnight incubation, and 4 mL of a mixture of 0.2 M phosphoric acid solution-acetone (5:13) was added. After vigorous shaking, the precipitates were filtered off and the amount of dye was measured colorimetrically at 620 nm.

2.6. Histopathologic examination

The skin specimen injected with histamine was post-fixed in 50 mM phosphate buffer (pH 7.4) containing 4% paraformaldehyde overnight and then immersed in 30% sucrose solution (in 50 mM phosphate buffered saline). Frozen specimen was sectioned in a cryostat at 30 μm and stained with hematoxylin–eosin, and then assessed under light microscopy.

2.7. ELISA and immunoblotting

After the intradermal injection of histamine (300 μg/50 μL) into the rostral part of the back of each mouse, the injection site (1 × 1 cm) was outlined with indelible red marker. Nobiletin, tangeretin or azelastine (dissolved in 2% cremophor) was orally administered 1 h before treatment with histamine. Mice were sacrificed 1 h after histamine injection, and then the scratching agent injection site (1 × 1 cm) was excised.

For the ELISA of IL-4, TNF-α and PKC, the skin tissues were homogenized in 1 mL of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (15,000 ×g, 4 °C) for 10 min, and the supernatant was transferred to 96-well ELISA plates. IL-4 and TNF-α were measured according to the manufacturer's protocol.

For immunoblotting of c-Jun, p-c-Jun, p-p65, p65, and β-actin, the skin tissues or the cultured cells were carefully homogenized to obtain viable single cells, which were resuspended in 1 mL of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor

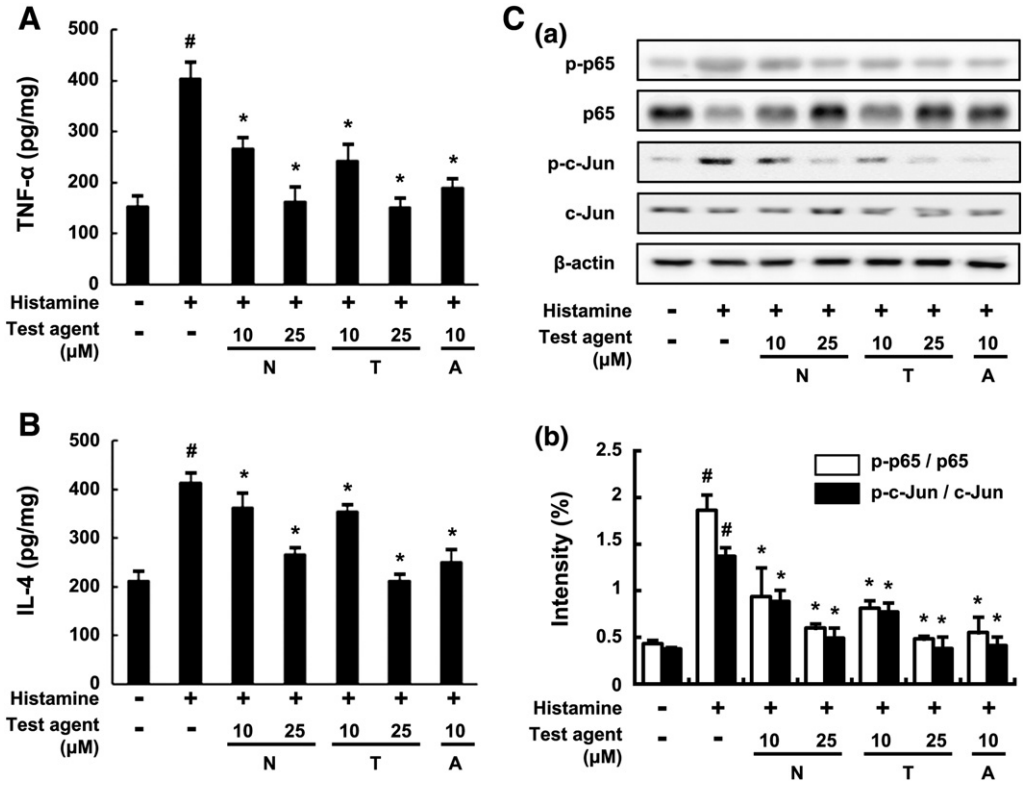

Fig. 3. Inhibitory effects of nobiletin and tangeretin on the protein expression of TNF-α (A) and IL-4 (B) and their transcription factors NF-κB and c-Jun (C) in histamine-induced mouse skin tissues. TNF-α and IL-4 levels were assayed by ELISA and NF-κB (p-p65 and p65), AP-1 (p-c-Jun and c-Jun) and β-actin were assayed by immunoblotting. Intensity of the immunoblotted bands is represented as the ratio of p-p65/p65 and p-c-jun/c-jun. Nobiletin, tangeretin and azelastine were orally administered to mice: The test agents (nobiletin at 10 or 25 mg/kg; tangeretin at 10 or 25 mg/kg; and azelastine at 10 mg/kg, dissolved in 2% cremophor) were orally administered 1 h before treatment with the scratching agent. Normal control mice were treated with the vehicle alone instead of test agents or histamine. The values indicate the mean ± S.D. ($n = 6$). #Significantly different from the normal control group ($P < 0.05$). *Significantly different from the group treated with histamine alone ($P < 0.05$).

cocktail. The protein from collected cells was subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to nitrocellulose membrane. Immunodetection was performed using an enhanced chemiluminescence detection kit.

2.8. Culture of RBL-2H3 cells

RBL-2H3 cell, a basophilic leukemia cell line, was cultured in DMEM. For the assay of TNF-α, IL-4, p65, p-p65, c-jun (AP-1), p-c-jun, p38, p-p38, and β-actin, the cells (2×10^5 cells) were stimulated with 20 nM PMA. The cells (1.8 mL) were exposed to 0.2 mL of nobiletin, tangeretin or azelastine (10 and 25 μM, dissolved in 0.5% dimethyl sulfoxide) for 0, 0.5 and 1, followed by treatment with 0.2 mL DNP-HSA (1 μg/mL) for 40 min at 37 °C. For the assay of TNF-α and IL-4, the supernatant (50 μL) was transferred to 96-well ELISA plates.

For the assay of p65, p-p65, c-jun, p-c-jun, p-c-jun, p38, p-p38, and β-actin, the collected cells were lysed with RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail and then analyzed by immunoblotting.

The degranulation of RBL-2H3 cells was evaluated by measuring the release of β-hexosaminidase from RBL-2H3 cells stimulated by IgE–antigen complex according to the method of Choo et al. [18].

2.9. Anti-histamine action assay

Guinea pigs were sacrificed by exsanguination and the ilea were prepared in cold Tyrode's solution. The prepared ileal strip was then suspended in a 10 mL Magnus tube (32 °C, 95% O_2 + 5% CO_2) containing Tyrode's solution. Each test agent was added to the preparation 30 s before treatment with histamine (1×10^{-6} M). The percentage contraction is shown as a percentage of the maximal response to histamine.

2.10. Statistic analysis

All the data were expressed as the mean ± standard deviation, and statistical significance was analyzed by one-way ANOVA followed by Student–Newman–Keuls test ($P < 0.05$).

3. Results

To evaluate the anti-allergic effects of nobiletin and tangeretin, which exhibit anti-inflammatory and anti-asthmatic effects, we measured their inhibitory effects in histamine- or compound 48/80-induced scratching behavior in mice. Nobiletin and tangeretin potently inhibited the histamine-induced scratching behavior (Fig. 1A). At a dose of 25 mg/kg, they inhibited the scratching behavior by 71% and 73%, respectively. Nobiletin and tangeretin also inhibited compound 48/80-induced scratching behavior (Fig. 1B). They (dose, 25 mg/kg) inhibited the compound 48/80-induced scratching behavior by 53% and 57%, respectively. We also investigated their effects in histological exams (Fig. 1C). Although histamine caused severe inflammation manifested as thick and erythematous skin, these inflammatory symptoms were inhibited. The inhibitory effect of tangeretin was superior to that of nobiletin. The anti-scratching behavioral effect of tangeretin (dose, 25 mg/kg) was comparable to that of azelastine (dose, 10 mg/kg), which is a commercially available anti-histamine drug.

To understand the anti-scratching behavioral effects of nobiletin and tangeretin, their inhibitory effects against histamine-induced vascular permeability were measured (Fig. 2). Nobiletin and tangeretin (25 mg/kg) inhibited histamine-induced vascular permeability by 45% and 51%, respectively. Their inhibitory effects were proportional to their anti-scratching behavioral effects. They also inhibited the expression of IL-4 and TNF-α in mouse skin that was stimulated by histamine (Fig. 3). Although histamine increased protein expression of IL-4 and TNF-α, nobiletin and tangeretin (25 mg/kg) inhibited the IL-4 expression by 84% and 96%, respectively, and the TNF-α expression by 94% and 96%, respectively. Furthermore, nobiletin and tangeretin inhibited the histamine-induced activation of transcription factors NF-κB, which regulates the TNF-α expression [19], and AP-1, which regulates the IL-4 expression [20]. Its inhibitory effects were comparable to that of azelastine.

Next, we applied nobiletin or tangeretin to PMA-stimulated RBL-2H3 cells and measured the expression of TNF-α (Fig. 4) and IL-4 by ELISA (Fig. 4B). PMA significantly increased the expression of IL-4 and TNF-α as well as the activation of NF-κB, which is induced via PKC [21]. Nobiletin and tangeretin (25 μM) significantly inhibited PMA-induced IL-4 expression by 55% and 61%, respectively, and TNF-α expression by 46% and 52%, respectively. They also inhibited the histamine-induced activation of the transcription factors NF-κB, c-Jun, and p38 (Fig. 5). Furthermore, they inhibited purified PKC activity (Fig. 5D). They (10 μM) inhibited PKC by 51% and 55%, respectively. No cytotoxic effects of nobiletin and tangeretin (25 μM for 48 h) were observed under the conditions that were used in the present experiments (Fig. 5E). Nobiletin and tangeretin inhibited the contraction of guinea pig ileum that was used by histamine as well as IgE-induced degranulation of RBL-2H3 cells, although their inhibitory potencies were weaker than that of azelastine (Fig. 6). The anti-histamine action of tangeretin against the ileum of the guinea pig was more potent than that of nobiletin.

4. Discussion

Pruritus, which is associated with various allergic diseases, has been treated using anti-histamines such as azelastine, steroids such as betamethasone, and immunosuppressants such as FK-506 and cyclosporine A [22,23]. Azelastine is an H1-receptor antagonist. It also inhibits the release of mediators from mast cells and basophils. Betamethasone is a potent corticosteroid that is used to treat allergic diseases such as

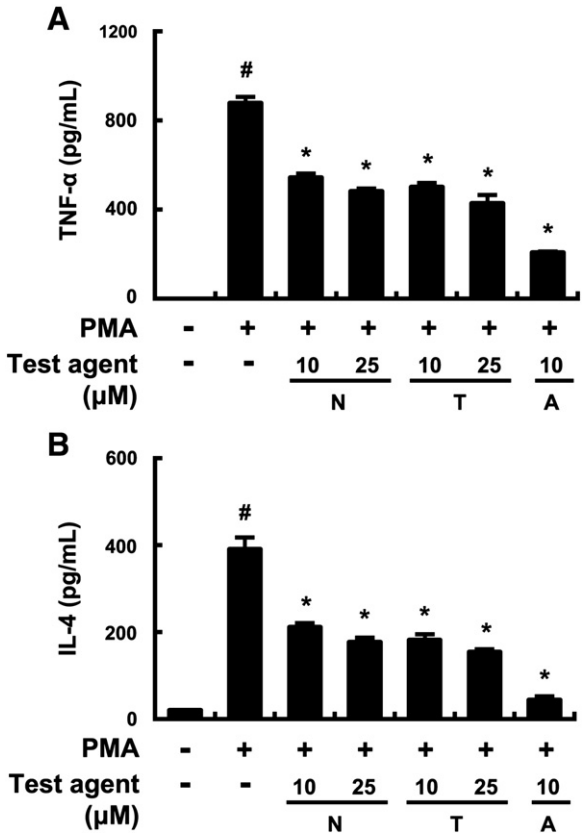

Fig. 4. Inhibitory effects of nobiletin and tangeretin on the protein expression of TNF-α and IL-4 in PMA-induced RBL-2H3 cells. TNF-α (A) and IL-4 (B) were determined by ELISA. The values indicate mean ± SD. ($n = 3$). #Significantly different from the normal control group ($P < 0.05$). *Significantly different from group treated with PMA alone ($P < 0.05$).

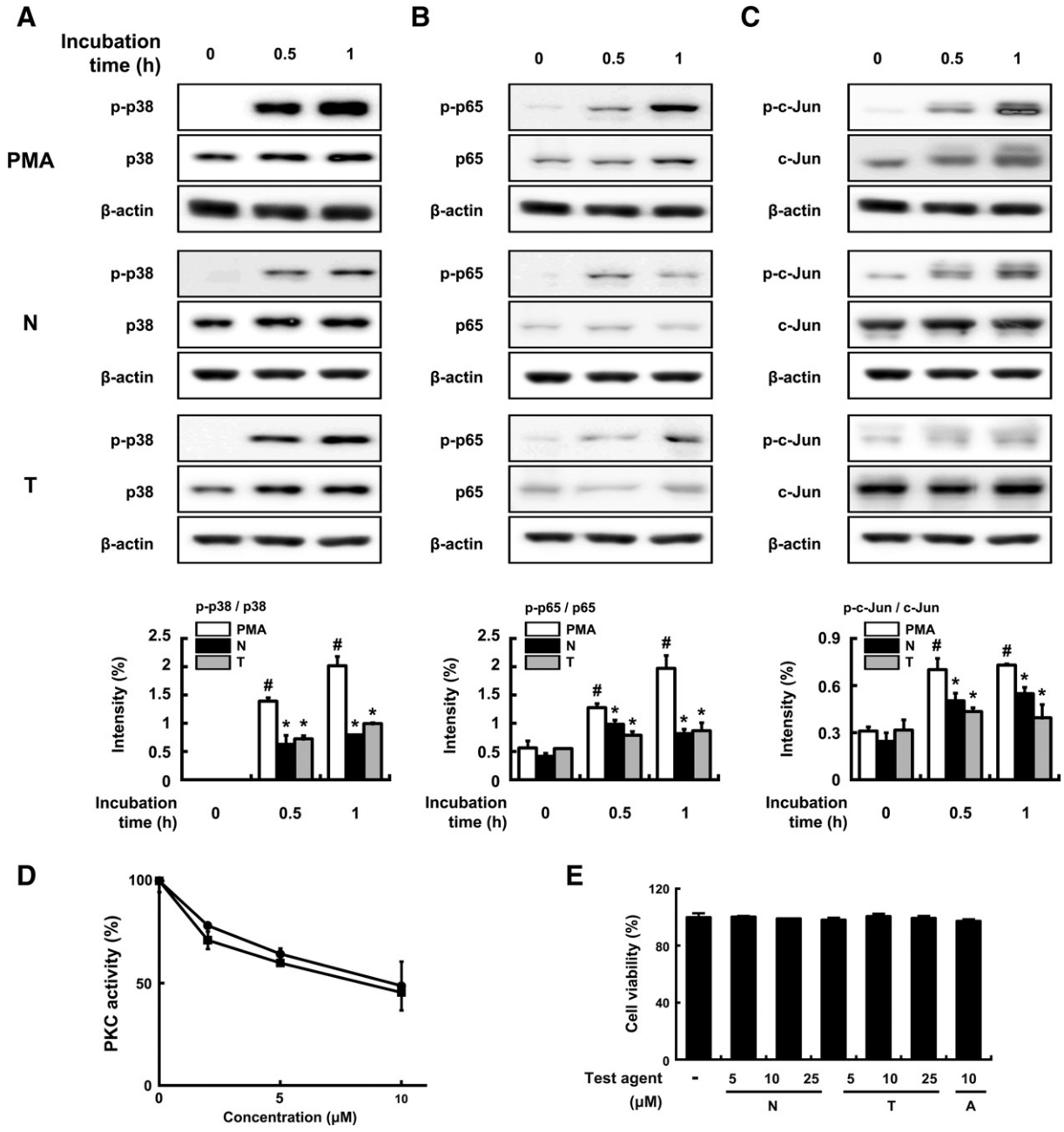

Fig. 5. Inhibitory effects of nobiletin and tangeretin on NF-κB, c-Jun and p-38 activation and protein kinase C (PKC) activity in PMA-induced RBL-2H3 cells. NF-κB (p-p65 and p65) (A), AP-1 (p-c-Jun and c-Jun) (B), p38, p-p38 and β-actin (C) were periodically assayed by immunoblotting. Intensity of the immunoblotted bands is represented as the ratio of p-p65/p65 and p-c-jun/c-jun and p-p38/p38. PKC activity (D) was assayed by ELISA. For the assay of cell viability, RBL-2H3 cells were treated with test agents for 48 h and the cytotoxicity (E) was determined by crystal violet method. The values indicate mean ± S.D. ($n = 3$). #Significantly different from the normal control group ($P < 0.05$). *Significantly different from group treated with PMA alone ($P < 0.05$).

psoriasis and other skin disorders. Unfortunately, these agents have side effects, such as severe nephrotoxicity and neurotoxicity [24]. Therefore, new agents for clinical uses were developed from herbal medicines [25].

Among PMFs, nobiletin inhibits the PI3K/AKT pathway in IgE-stimulated basophils, NF-κB and MAPK pathways in LPS-induced microglia [26] and RAW264.7 cells [27], and COX-2 and iNOS expression in TPA-stimulated murine skin inflammation [28]. Tangeretin inhibits COX-2 expression in human lung carcinoma cells [10] and ultraviolet B-induced mouse epidermal cells by regulating AKT and/or MAPK signaling pathways [29]. Nevertheless, their inhibitory effects against allergic diseases, such as itching, have not been studied. We evaluated the anti-scratching behavioral effects of nobiletin and tangeretin on the histamine- or compound 48/80-induced scratching behavior in mice. Nobiletin and tangeretin potently inhibited histamine-induced scratching behavior. The anti-scratching behavioral effects of nobiletin and tangeretin were proportional to their inhibitory effects against histamine-induced vascular permeability. These agents also inhibited scratching behavior that was induced by compound 48/80, which causes mast cell-independent scratching behavior [30]. Although the anti-scratching behavioral effects of nobiletin and tangeretin were not comparable to that of azelastine, which is a positive agent, these agents also showed anti-histamine action in the ileum of guinea pig. These results suggest that their anti-scratching behavioral effects might be due to decreased vascular permeability caused by anti-histamine and other unknown actions.

In this study to understand the anti-scratching behavioral mechanisms of nobiletin and tangeretin, we found that they inhibited the expression of TNF-α and IL-4 as well as the activation of the transcription

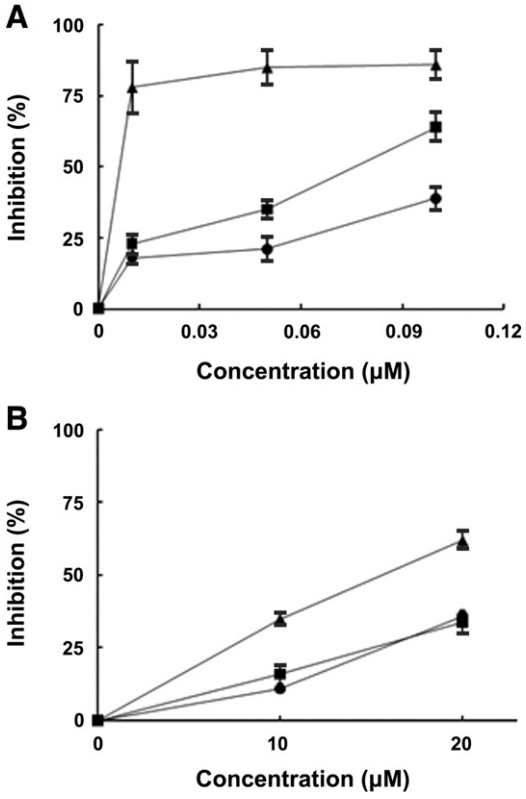

Fig. 6. Inhibitory effects of nobiletin and tangeretin on histamine action (ileum contraction) in the ileum of guinea pig and on IgE–antigen complex-induced degranulation in RBL-2H3 cells. (A) Anti-contraction effect in Magnus test using guinea pig ileum. The ileal strip was set in a 10 mL Magnus tube (32 °C, 95% O_2 + 5% CO_2) containing Tyrode's solution. Each test agent [10, 50 and 100 μM of nobiletin (circle), tangeretin (square) or azelastine (triangle) dissolved in 2% Triton X-l00] was added to the preparation 30 s before treatment with histamine (1×10^{-6} M). (B) Anti-degranulation effect in IgE-induced RBL-2H3 cells. RBL-2H3 cells (5×10^5 cells/well), which were grown in DMEM supplemented with 10% fetal bovine serum and L-glutamine, were dispensed into 24 well plates, and sensitized using 0.5 μg/mL of mouse monoclonal IgE, washed, exposed to 40 μL of various concentrations of each agent for 20 min, and treated with 20 μL of antigen (DNP-HSA, 1 μg/mL) for 10 min at 37 °C. The degranulation of RBL-2H3 cells was determined by the released β-hexosaminidase activity. The β-hexosaminidase activity (%) released by the IgE–antigen complex was 72 ± 5%. The values indicate mean ± S.D. ($n = 3$).

factors NF-κB and c-jun (AP-1) in skin stimulated with histamine. NF-κB is an important signal in immune responses of allergic diseases [19]. Histamine activates NF-κB in skin tissues. Furthermore, nobiletin and tangeretin inhibited the activation of p38, which is a representative MAPK [8]. PMA also activates NF-κB via PKC [21]. In the present study, nobiletin and tangeretin inhibited histamine-induced activation of NF-κB, AP-1 and p38 in mice and PMA-stimulated RBL-2H3 cells. Furthermore, nobiletin and tangeretin inhibited purified PKC activity, although we could not measure the PKC kinase-inhibitory effects of nobiletin and tangeretin in PMA-stimulated RBL-2H3 cells. Notably, AP-1 regulates the expression of IL-4, which is an IgE-switching cytokine [20]. These results suggest that nobiletin and tangeretin may inhibit TNF-α and IL-4 expression by regulating the activation of their transcription factors, NF-κB, AP-1 and p38 via PKC.

On the basis of these findings, nobiletin and tangeretin may reduce scratching in skin by inhibiting vascular permeability and allergic cytokine expression via NF-κB, AP-1, and p38 signal pathways.

Conflict of interest

The authors have no conflict of interest.

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