Inhibitory Effect of Citrus Nobiletin on Phorbol Ester-induced Skin Inflammation, Oxidative Stress, and Tumor Promotion in Mice

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ABSTRACT

The intake of citrus fruits has been suggested as a way to prevent the development of some types of human cancer. Nitric oxide (NO) is closely associated with the processes of epithelial carcinogenesis. We attempted a search for NO generation inhibitors in Citrus unshiu. The active component was traced by an activity-guiding separation. NO and superoxide (O$_2^-$) generation was induced by a combination of lipopolysaccharide and IFN-γ in mouse macrophage RAW 264.7 cells, and by 12-O-tetradecanoylphorbol-13-acetate (TPA) in differentiated human promyelocyte HL-60, respectively. Expression of inducible NO synthase and cyclooxygenase 2 proteins were detected by Western blotting. The in vivo anti-inflammatory and antitumor promoting activities were evaluated by topical TPA application to ICR mouse skin with measurement of edema formation, epidermal thickness, leukocyte infiltration, hydrogen peroxide production, and the rate of proliferating cell nuclear antigen-stained cells. As a result, nobiletin, a polymethoxyflavonoid, was identified as an inhibitor of both NO and O$_2^-$ generation. Nobiletin significantly inhibited two distinct stages of skin inflammation induced by double TPA application (first stage priming (leukocyte infiltration) and second stage activation (oxidative insult by leukocytes)) by decreasing the inflammatory parameters. It also suppressed the expression of cyclooxygenase-2 and inducible NO synthase proteins and prostaglandin E$_2$ release. Nobiletin inhibited dimethylbenz[a]anthracene (0.19 nmol)/TPA (1.6 nmol)-induced skin tumor formation at doses of 160 and 320 nmol by reducing the number of tumors per mouse by 61.2% (P < 0.001) and 75.7% (P < 0.001), respectively. The present study suggests that nobiletin is a functionally novel and possible chemopreventive agent in inflammation-associated tumorigenesis.

INTRODUCTION

The intake of citrus fruits has been found to be beneficial for cancer prevention by epidemiological survey (1). Citrus fruit contains several classes of chemopreventive agents, e.g., limonoids and their glycosides (2, 3), d-limonene (4, 5), several flavonoids (6) such as hesperidin (7, 8), and glycerolglycolipids (9). Our group has demonstrated that aurapentine, which is a coumarine-related compound widely occurring in citrus juices (e.g., grapefruit), exhibited a marked inhibition of TPA$_2$-induced O$_2^-$ and hydroperoxide production and inhibited skin tumor promotion in mice by topical application (10). Oral administration of aurapentine also inhibited 4-nitroquinoline-1-oxide-induced oral carcinogenesis in rats (11) and azoxymethane-induced aberrant crypt (12) and adenocarcinoma formation (13) in the rat colon.

Nitric oxide (NO), a gaseous free radical, is released by a family of enzymes, constitutive NO synthase and an inducible one (iNOS; Ref. 14) with the formation of stoichiometric amounts of l-citrulline from l-arginine (15). iNOS-mediated excessive and prolonged NO generation has attracted attention because of its relevance to epithelial carcinogenesis (16–18). NO has been reported to cause mutagenesis (19) and deamination of DNA bases (20) and to form carcinogenic N-nitrosoamines (21). With respect to the role of NO in the postinitiation phase, it should be noted that it plays important roles in such inflammatory responses as edema formation and hyperplasia, as well as in papilloma development in mouse skin (22, 23). NO is also involved in the production of VEGF (24), the overexpression of which induces angiogenesis, vascular hyperpermeability, and accelerated tumor development (25). Topical application of TPA to mouse led to edema and papilloma formation by enhancing COX-2 protein expression. Specific COX-2 inhibitors (26, 27) could counteract these biological events. Collectively, suppression of enzyme induction and the activities of iNOS/COX-2 is a new paradigm for the prevention of carcinogenesis in several organs, including the stomach and colon. In fact, some dietary compounds, including chemopreventive agents (28–31) and the extracts from edible plants (32), have recently been reported to suppress iNOS-mediated NO generation.

The present study was undertaken to search for NO generation inhibitors in Citrus unshiu (satsuma mandarin), the most popular citrus fruit in Japan, by activity-guiding fractionation and purification, using a combined LPS- and IFN-γ-induced NO generation test in mouse macrophage RAW 264.7 cells. As a result, we identified a polymethoxyflavonoid, nobiletin, to be a dual inhibitor of both NO and O$_2^-$ generation. Nobiletin significantly inhibited two distinct stages of skin inflammation induced by double TPA application (first stage priming (leukocyte infiltration) and second stage activation (oxidative insult by leukocytes)) by decreasing the inflammatory parameters. It also suppressed the expression of cyclooxygenase-2 and inducible NO synthase proteins and prostaglandin E$_2$ release. Nobiletin inhibited dimethylbenz[a]anthracene (0.19 nmol)/TPA (1.6 nmol)-induced skin tumor formation at doses of 160 and 320 nmol by reducing the number of tumors per mouse by 61.2% (P < 0.001) and 75.7% (P < 0.001), respectively. The present study suggests that nobiletin is a functionally novel and possible chemopreventive agent in inflammation-associated tumorigenesis.

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3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; COX, cyclooxygenase; DMBA, 7,12-dimethylbenz[a]anthracene; HPLC, high-performance liquid chromatography; IL, interleukin; iNOS, inducible NO synthase; IR, inhibitory rate; LPS, lipopolysaccharide; O$_2^-$, superoxide; PCNA, proliferating cell nuclear antigen; PG, prostaglandin; ROS, reactive oxygen species; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
inhibitory effect of nobiletin on mouse skin tumor promotion using a two-stage carcinogenesis model was investigated.

MATERIALS AND METHODS

Chemicals, Cells, and Animals. TPA was obtained from Research Biochemicals International (Natick, MA). DMEM and fetal bovine serum were purchased from Gibco RBL (Grand Island, NY). LPS (Escherichia coli sere-type 0127, B8) was purchased from Difco Labs (Detroit, MI) and IFN-γ from Genzyme (Cambridge, MA). Cytochrome c was obtained from Sigma (St. Louis, MO. Luteolin was obtained from Extrasyntese (Genay, France). All other chemicals were purchased from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). HL-60 cells were purchased from American Type Culture Collection (Manassas, VA). RAW 264.7 cells were kindly donated by Ohtsuka Pharmaceutical Co. Ltd. (Ohtsu, Japan). Female ICR mice, ages 6–7 weeks, were obtained from Japan SLC (Shizuoka, Japan).

Isolation and Identification of Nobiletin. Whole fruit of C. unshiu (10-kg fresh weight), harvested in Wakayama Prefecture, Japan, were processed by an FMC in-line citrus juice extractor (FMC Corporation, Chicago, IL) to produce cold-pressed oil (150 ml). Most of the monoterpene, δ-limonene being the major constituent (~90%), were removed from the oil by evaporation under reduced pressure at 80°C. Then, the terpene-less oil was separated into useful fractions by monitoring the inhibition of NO generation. The active fraction was separated by silica gel column chromatography (α-hexane/ethanol, stepwise method), then by preparative TLC (α-hexane/ethyl acetate, 1:1), and finally by HPLC (column: μBondapak C18 Waters, Milord, MA; 19 × 150 mm; elute: 70% methanol in water; flow rate: 7.0 ml/min; detection: U254 nm) to produce nobiletin (84 mg; retention time at 16.9 min; purity >98%). The chemical identity was confirmed by comparison of its spectral data with those reported previously (35).

NO Generation Test. Murine macrophage cell line RAW 264.7 cells grown confluent in 2 ml of DMEM on a 60-mm dish were treated with LPS (100 ng/ml), tetradehydrobipterin (10 mg/ml), IFN-γ (100 units/ml), L-arginine (2 mM), and a test compound (0, 25, 50, or 100 μM). After 18 h, the levels of NO2− and NO3− were measured by Griess assay. Cytotoxicity was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each experiment was done independently in duplicate twice, and the data are expressed as mean ± standard deviation (SD) values.

Western Blotting. Confluent RAW 264.7 cells were stimulated and incubated in the same manner as described above. After the cells were washed, a boiling lysis solution was added to the cells, which were then scraped from the dish, sonicated, and boiled for 10 min. Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad Laboratories). BSA was used as the standard. Ten-μg proteins were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, MA). After blocking, the membranes were incubated with the primary antibody (antimouse INOS, 1:1000 dilution; Affinity Biosystems, Inc., Golden, CO), and then with a secondary antibody (peroxidase-conjugated swine antirabbit IgG, 1:1000 dilution; Dako, Glostrup, Denmark). The blots were developed using an ECL detection kit (Amersham Life Science, Buckinghamshire, United Kingdom). The antibodies were stripped and the blots were successively reprobed with each primary antibody. The first incubation was with: (a) goat antirat COX-2 antibody [cross-reacts with mouse counterparts, 1:1000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)]; or (b) rabbit polyclonal anti-β-actin antibody (1:1000 dilution; Biochemical Technologies, Stoughton, MA). Each membrane was then incubated with a corresponding secondary antibody: (a) peroxidase-conjugated rabbit antigoat IgG (1:1000 dilution; Dako); and (b) peroxidase-conjugated swine antirabbit IgG (1:1000 dilution; Dako). The levels of iNOS or COX-2 bands were detected using those of β-actin as an internal standard. Each experiment was done independently in duplicate twice, and the data are expressed as mean ± SD values.

Double TPA Treatment of Mouse Skin. The double TPA treatment experiment was performed as reported previously (36). Briefly, the back of each mouse was shaved using surgical clippers 2 days before each experiment; each experimental group consisted of five mice. Nobiletin (810 nmol in 100 μl of acetone) was applied topically to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 μl of acetone). In the double-treatment protocol, the same doses of TPA and the test compounds or acetone were applied twice at an interval of 24 h. We divided the mice into five groups as follows: group 1 (acetone × 2/acetone × 2); group 2 (acetone-TPA/acetone-TPA); group 3 (nobiletin-TPA/nobiletin-TPA); group 4 [nobiletin (priming)-TPA/acetone-TPA]; and group 5 [acetone-TPA/nobiletin (activation)-TPA].

Measurement of H2O2 and Edema Formation in Mouse Skin. Measurement of the levels of H2O2 and edema formation was done as reported previously (36). In brief, mice treated by the double-treatment protocol were sacrificed 1 h after the second TPA treatment. Skin punches (epidermis and dermis) were obtained with an 8-mm-diameter cork borer and were weighed with an analytical balance. The skin punches were minced in 3 ml of 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide and then homogenized for 30 s twice. The homogenate was centrifuged at 10,000 × g for 20 min at 4°C. The H2O2 content was determined by the phenol red–horseradish peroxidase method.

Histological Examination. Histological examinations were done as reported previously (36). Briefly, excised skin was fixed in 10% buffered formalin and then embedded in paraffin. For each skin section (3 μm) stained with H&E, the thickness of the epidermis from the basal layer to the stratum corneum was measured at five equidistant interfollicular sites using a Leica Q500IW-EX image analysis system (Leica, Co. Ltd., Tokyo, Japan) with a Leica DMRE HC microscope (Leica, Co. Ltd.). The number of infiltrating leukocytes was counted at five different areas.

PCNA Immunohistochemistry. A PCNA immunohistochemistry was done as reported previously (36). Briefly, skin sections were treated with 1.2% H2O2 in absolute methanol for 30 min and were stained by the indirect abuin-biotin-horseradish peroxidase method (ABC standard; Vector laboratories, Burlingame, CA). Color development with diaminobenzidine (Vector laboratories) was monitored by the appearance of normal PCNA brown staining in the normal epidermis. The primary antibody to PCNA (PC10; Boehringer Mannheim, Mannheim, Germany) was applied at a 1:300 concentration overnight at 4°C. The PCNA index was counted at six different areas of each section using the image analysis system and was expressed as the number of positive squamous cells divided by the total number of squamous cells × 100.

O2− Generation in Differentiated HL-60 Cells. An inhibitory test of TPA-induced O2− generation was performed as reported previously (36). Briefly, differentiated HL-60 cells were treated with 100 nm TPA, appropriate concentrations of the test compounds, and 1 mg/ml cytochrome c. After incubation at 37°C for 30 min, the level of extracellular O2− was measured by cytochrome c reduction. Each experiment was done in duplicate independently twice, and the data are expressed as mean ± SD values.

Cellular Uptake. Differentiated HL-60 cells were incubated and treated in the same manner as described above. For extracellular measurement, after the cell suspension was centrifuged, supernatant was collected, and the cells were washed with media three times to combine the media used for washing with the supernatant. The combined solution (4 ml) was partitioned between distilled water (1 ml) and ethyl acetate (5 ml). For intracellular measurement, cells were suspended in ice-cold water and ethyl acetate (5 ml each) and were sonicated for 10 s twice on ice. The ethyl acetate fractions from the intracellular and extracellular samples were separately concentrated in vacuo and subjected to HPLC analysis [column: YMC-Pack ODS H-80 J (4.6 × 150 mm); Yamamura Chemical Co. Ltd., Kyoto, Japan]; detection: UV254 nm at 45°C with a flow rate of 1.0 ml/min]. Nobiletin and luteolin were detected using 40% acetonitrile in water as an elute at 8.1 min and 25% acetonitrile in water at 3.9 min, respectively. Their incorporation efficiencies were determined by the intracellular:extracellular ratio. Each experiment was done in duplicate independently twice, and the data are expressed as mean ± SD values.

PGEx Determination. RAW 264.7 cells, grown confluent in 2 ml of DMEM on a 60-mm dish, were treated with LPS (100 ng/ml), tetrahydropripterin (10 mg/ml), IFN-γ (100 units/ml), L-arginine (2 mM), and a test compound (0, 25, 50, or 100 μM) dissolved in DMSO (0.5%, v/v). After 18 h, the concentrations of PGE2 in the media were measured using a commercial experimental kit (Cayman, Ann Arbor, MI), and protein concentrations were determined using a DC Protein Assay kit (Bio-Rad Laboratories). BSA was used as the standard. Each experiment was done in duplicate independently twice, and the data are expressed as mean ± SD values.

Two-Stage Carcinogenesis Experiment in Mouse Skin. The antimutagenic activity of nobiletin was examined by a standard initiation-promo-
tion protocol with DMBA and TPA, as reported previously (10). One group was composed of 15–17 female ICR mice. These mice were given commercial rodent pellets and fresh tap water ad libitum, both of which were freshly exchanged twice a week. The back of each mouse was shaved with a surgical clipper 2 days before initiation. Mice at 6 weeks old were initiated with DMBA (0.19 μmol in 100 μl of acetone). One week after initiation, the mice were promoted with TPA (1.6 nmol in 100 μl of acetone) twice a week for 20 weeks. In the other four groups, the mice were treated with nobiletin (40, 80, 160, or 320 nmol in 100 μl of acetone) 40 min before each TPA treatment. The antitumor-promoting activity was evaluated by both the ratio of tumor-bearing mice and the number of tumors that measured more than 1 mm in diameter, per mouse. The data were statistically analyzed using Welch’s t test for the average number of tumors per mouse and by the χ² test for the rates of tumor-bearing mice.

Statistical Analysis. The statistical significance of differences between groups in each assay was assessed by Welch’s t test, Student’s t test, or χ² test.

**RESULTS**

**Isolation and Identification of Nobiletin.** Whole fruit of satsuma mandarins was processed by an FMC in-line citrus juice extractor, which is widely used in fruit juice factories, to produce cold-pressed oil. d-Limonene was removed from the oil to be further separated into novel active compounds. The terpene-less oil thus obtained was then separated into useful fractions by monitoring the inhibitory activities toward NO generation. The active fraction was chromatographed on silica gel and finally purified by HPLC to give nobiletin (Fig. 1), the chemical identity of which was elucidated by comparison of the spectral data with those reported previously (35).

**Inhibition of NO Generation.** Stimulation of RAW 264.7 cells with LPS/IFN-γ for 18 h resulted in NO generation and then nitrite (NO₂⁻) accumulation in the media (40.3 ± 7.3 μM). Nobiletin, at a concentration range of 25–100 μM, concentration-dependently suppressed NO₂⁻ production by 18.6–58.1% (Fig. 2). The inhibitory effect of a red wine polyphenol, resveratrol (37–39), was also examined for comparison of activity, and its inhibitory potency was found to be similar to that of nobiletin. iNOS protein expression was then detected using Western blotting. As also shown in Fig. 2, although being hardly detectable in the cellular lysate without stimulation, iNOS protein was remarkably up-regulated 25-fold 18 h after stimulation. Whereas nobiletin showed a dose-dependent suppression at a concentration range of 25–100 μM (IRs, 32.7–82.0%), resveratrol showed marked inhibition only at a concentration of 100 μM, and activity drastically declined at the concentrations of 50 and 25 μM. Notable cytotoxicity was not observed in any of the experiments (data not shown).

**Anti-inflammatory and Antioxidative Activities in Mouse Skin.** We then examined whether nobiletin inhibits the priming and/or activation stages using the double-TPA-application model. As shown in Table 1, double TPA applications, at a dose of 8.1 nmol each with a 24-h interval, led to notable edema formation (42.3 ± 2.6 mg/punch in group 2) as compared with the control (24.7 ± 4.5 mg/punch in group 1; P < 0.001 versus group 2). Double pretreatment with nobiletin (group 3) 30 min prior to each TPA application suppressed edema formation by 54.5% (P < 0.05). Pretreatment in the priming phase (group 4; IR, 72.1%; P < 0.01) was found to be more effective for edema suppression than in the activation phase (group 5: IR, 29.5%; P < 0.001) and was comparable or higher in double pretreatment. In accordance with the TPA-induced enhancement of edema formation, an increase in epidermal thickness was observed with double TPA applications (Table 1 and Fig. 3, A and B; 12.2 ± 1.6 μm in group 1 versus 29.1 ± 3.3 μm in group 2; P < 0.001). Pretreatment with nobiletin in the priming stage (Table 1 and Fig. 3D; IR, 42.0%; P < 0.001 in group 4) was also more suppressive than that in the activation stage (Table 1 and Fig. 3E; IR, 59.2%; P < 0.001 in group 5). Double nobiletin pretreatment showed the highest inhibition (Table 1 and Fig. 3C; IR, 81.7%; P < 0.001 in group 3).

As shown in Table 1 and Fig. 3, A and B, a greater number of leukocytes were found to have infiltrated the dermis by double TPA application as compared with the control (26.2 ± 8.7/mm² in group 1 versus 396.3 ± 9.9/mm² in group 2; P < 0.001; 15-fold). Double pretreatment with nobiletin-inhibited leukocyte infiltration by 76.9% (P < 0.001; Table 1 and Fig. 3C) and a single pretreatment in either the priming or activation phase also suppressed infiltration by 58.0 and 47.2%, respectively (Table 1 and Fig. 3, D and E).

Notably, the double TPA application dramatically increased the level of H₂O₂ in the mouse epidermis and dermis 7.5-fold (Table 1: 0.73 ± 0.15 nmol/punch in group 1 versus 5.47 ± 0.28 nmol/punch in group 2; P < 0.001). A higher inhibition by nobiletin was again observed when applied in the priming stage (IR, 57.2% in group 4; P < 0.001) than in the activation phase (IR 44.5% in group 4; P < 0.001). Double pretreatment with nobiletin showed the highest...
CITRUS NOBILETIN INHIBITS OXIDATIVE STRESS

Table 1  Inhibitory effects of nobiletin (NOB) on TPA-induced biological and histological parameters in mouse skin

Five ICR mice were used in one experimental group. Nobiletin [810 nmol in 100 μl of acetone (Ac)] was topically applied to the shaved area of dorsal skin 30 min before application of a TPA solution (8.1 nmol/100 μl of acetone). After 24 h, the same doses of nobiletin or acetone were applied 30 min prior to a second TPA application. Methods for measurement of biological activities are shown in “Materials and Methods.” Statistical analysis was done by Student’s t-test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema (mg/punch)</th>
<th>Epidermal thickness (μm)</th>
<th>Infiltrated leukocytes (No./mm²)</th>
<th>H₂O₂ (nmol/punch)</th>
<th>PCNA-labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Ac/Ac→Ac/Ac)</td>
<td>24.7 ± 4.5</td>
<td>12.2 ± 1.6</td>
<td>26.2 ± 8.7</td>
<td>0.73 ± 0.15</td>
<td>15.7 ± 2.4</td>
</tr>
<tr>
<td>2 (Ac/TPA→Ac/TPA)</td>
<td>42.3 ± 2.6</td>
<td>29.1 ± 3.3</td>
<td>396.3 ± 9.9</td>
<td>5.47 ± 0.28</td>
<td>52.0 ± 4.1</td>
</tr>
<tr>
<td>3 (NOB/TPA→NOB/TPA)</td>
<td>32.7 ± 6.4</td>
<td>15.3 ± 1.0</td>
<td>111.7 ± 6.2</td>
<td>2.22 ± 0.39</td>
<td>24.0 ± 1.4</td>
</tr>
<tr>
<td>4 (NOB/TPA→Ac/TPA)</td>
<td>29.6 ± 4.9</td>
<td>19.1 ± 2.8</td>
<td>181.7 ± 73.1</td>
<td>2.78 ± 0.49</td>
<td>25.3 ± 2.9</td>
</tr>
<tr>
<td>5 (Ac/TPA→NOB/TPA)</td>
<td>37.1 ± 2.1</td>
<td>22.0 ± 2.2</td>
<td>221.7 ± 39.2</td>
<td>3.38 ± 0.35</td>
<td>31.3 ± 10.4</td>
</tr>
</tbody>
</table>

*P < 0.001 (versus group 1).
*P < 0.05 (versus group 2).
*P < 0.001 (versus group 2).
*P < 0.01 (versus group 1).
*P < 0.02 (versus group 2).

Inhibition of H₂O₂ production among the three inhibitory groups (IR, 69.1% in group 3; P < 0.001).

The PCNA-labeling index, a marker for cell proliferation, in the epidermis of the group 2 mice increased by 3.3-fold over that of group 1 (Table 1 and Fig. 4, A and B; 15.7 ± 2.4% in group 1 versus 52.0 ± 4.1% in group 2; P < 0.001). Pretreatment with either a single or double nobiletin application significantly reduced the PCNA-labeling indices (IRs, 57.0–73.6%; Table 1 and Fig. 4, C–E).

Inhibition of O₂⁻⁻ Generation from Differentiated HL-60 Cells.

We then examined the inhibitory effects of nobiletin in differentiated HL-60 cells, which are known to generate O₂⁻⁻ with TPA stimulation. The inhibitory effect of a polyhydroxyflavonoid, luteolin, was compared with that of nobiletin, because resveratrol was unable to suppress O₂⁻⁻ generation up to a concentration of 100 μM (data not shown). As shown in Fig. 5A, TPA stimulation of differentiated HL-60 cells for 30 min led to marked extracellular O₂⁻⁻ generation (27.9 ± 3.4 μm in the media). Nobiletin demonstrated a much higher level of inhibition than did luteolin toward O₂⁻⁻ generation accompanied by a complete suppression at concentrations of 20 and 100 μM.

The IC₅₀ value of nobiletin (IC₅₀, 1.2 μM) was approximately eight times lower than that of luteolin (IC₅₀, 9.8 μM). No cytotoxicity was detected in any experiments (data not shown). As shown in Fig. 5B, the intracellular and extracellular ratios of nobiletin and luteolin, at a 100 μM concentration 45 min after incubation, were found to be 0.108 ± 0.032 and 0.048 ± 0.010 (intracellular:extracellular), respectively. The intracellular luteolin was not detected at a concentration of 20 μM.

Suppression of PGE₂ Production and COX-2 Protein Expression.

Stimulation of RAW cells with LPS and IFN-γ for 18 h led to PGE₂ production in the media (15.1 ng/ml/mg protein; Fig. 6). Nobiletin suppressed PGE₂ production at the concentrations of 50 and 100 μM (IRs, 35.8 and 41.2%, respectively), whereas resveratrol exhibited higher suppressive rates at the 25–100 μM concentrations (IRs, 48.6–100%). Stimulation of RAW 264.7 cells also led to a dramatic up-regulation of COX-2 protein expression by 90.9-fold (Fig. 6), which was hardly detectable in the control. Whereas nobiletin showed a dose-dependent suppression at a concentration range of 25–100 μM (IRs, 50.7–90.4%), resveratrol showed marked inhibition only at a...
concentration of 100 μM, and its activity declined at a concentration range of 50 and 25 μM. Notable cytotoxicity was not observed in any of the experiments (data not shown).

Antitumor-promoting Activity in Mouse Skin. We then examined the inhibitory effects of a topical application of nobiletin, at a dose range of 40–320 nmol, on tumor formation in DMBA (0.19 μmol)-initiated and TPA (1.6 nmol)-promoted mouse skin. As shown in Table 2, the tumor incidence in the control group was 47.1% 20 weeks after promotion. In the four nobiletin-pretreated groups, the incidence was reduced by 33.5–43.3% (statistically not significant). The average number of tumors per mouse in the control was 2.06 ± 1.00, and pretreatment with nobiletin at 160 and 320 nmol dose-dependently reduced them by 61.2% (P < 0.01) and 75.7% (P < 0.01), respectively.

DISCUSSION

Polymethoxyflavonoids including nobiletin occur exclusively in citrus fruit, and the Dancy tangerine has the highest total, containing approximately 5 times the amount found in the peel of sweet orange varieties (e.g., Citrus sinensis; Ref. 35). Nobiletin and tangeretin, lacking a single methoxyl group that is present in nobiletin, occur at the concentrations of 46 and 7 ppm, respectively, in Valencia orange juice (40). Nobiletin has previously been reported to induce differentiation of mouse myeloid leukemia cells (41), to exhibit antiproliferative activity toward a human squamous cell carcinoma cell line (42), to exert antimutagenic activity (43), and to suppress the induction of matrix metalloproteinase-9 (44). In addition, tangeretin was reported to inhibit tumor invasion and metastasis (45) and to induce apoptosis in HL-60 cells (46). The present study shows that nobiletin suppresses both LPS and IFN-γ-induced NO2 production in a dose-dependent manner (Fig. 2). Whereas resveratrol was reported to suppress iNOS expression through down-regulation of nuclear factor-κB activity (37), the action mechanism of nobiletin for iNOS suppression is unclear. In addition, issues of whether nobiletin directly inhibits iNOS activity and affects both neural and endothelial NOS functions remain to be addressed.

Fig. 4. Suppression by nobiletin of the TPA-induced increase in PCNA-positive cells in mouse epidermis. Three mice were used in each experimental group. Nobiletin (810 nmol/100 μl of acetone) was topically applied to the shaved area of dorsal skin 30 min before application of a TPA solution (8.1 nmol/100 μl of acetone). After 24 h, the same dosages of nobiletin or acetone were applied 30 min prior to a second TPA application. Methods for PCNA staining are described in “Materials and Methods.” Ac, acetone; NOB, nobiletin.

Fig. 5. Inhibitory effects of nobiletin and luteolin on TPA-induced O2\(^2\) generation in differentiated HL-60 cells and their cellular uptake. In A, differentiated HL-60 cells (1 × 10^6 cells/ml) were stimulated by 100 nM TPA to induce O2\(^2\), the extracellular concentration of which was measured by the cytochrome c method. Test compounds were added at concentrations of 0.8, 4, 20, and 100 μM. □, nobiletin; ▼, luteolin. *1, P < 0.001; *2, P < 0.01 versus the group treated only with TPA in Student’s t test. In B, nobiletin (□, 100 or 20 μM each) was added to 1 ml of differentiated HL-60 cell suspension (1 × 10^6 cells/ml), and the mixture thus obtained was incubated at 37°C for 45 min. Intracellular and extracellular contents of the test compounds were determined using HPLC. *, P < 0.001 versus the group treated with luteolin in Student’s t test. Each experiment was done independently in duplicate twice, and the data are shown as mean ± SD values.
cells release TNF-α, IL-1β, and IL-6 (50), and, recently, we found that nobiletin inhibited the release of both LPS-induced and IFN-γ-induced TNF-α and IL-1β from RAW 264.7 cells (data not shown). Additional studies on the influence of nobiletin on cytokine regulations may elucidate the action mechanism(s) by which nobiletin suppresses leukocyte infiltration.

Because nobiletin did not significantly scavenge O$_2^·$ generated from the xanthine/xanthine oxidase system nor inhibit xanthine oxidase activity up to a concentration of 500 μM (data not shown), it may inhibit the assembly or activity of a multicomponent NADPH oxidase system in differentiated HL-60 cells (Fig. 5A). This inhibitory mode, i.e., not radical scavenging but generation inhibition, is also found in NO generation. A chemical characteristic of nobiletin, which bears none of the hydroxyl groups that are indispensable for O$_2^·$ scavenging activity, explains such an inhibitory mode. Possessing no hydroxyl groups may occasionally give some advantages to nobiletin in terms of antioxidation because, although being reported to exhibit antioxidative activities in rat liver microsomes, some polyhydroxyflavonoids change to become pro-oxidants to generate H$_2$O$_2$ (51).

Nobiletin possesses a much higher ability to inhibit O$_2^·$ generation than does luteolin (Fig. 5A). A predominance of polymethoxyflavonoids over polyhydroxyflavonoids was also observed in antitumor activities using a cell culture system (42). Differences in their activities may be derived from the relatively greater membrane uptake efficiencies of polymethoxyflavonoids than those of polyhydroxyflavonoids because methoxylation of the phenolic hydroxyl groups, in general, increases the molecular hydrophobicity that regulate incorporation rates. In fact, intracellular content of nobiletin was approximately two times higher than that of luteolin in differentiated HL-60 cells when incubated at a concentration of 100 μM (Fig. 5B).

Double-TPA-application-induced H$_2$O$_2$ production in vivo was markedly inhibited by the pretreatment(s) of nobiletin (Table 1). Reduced levels of H$_2$O$_2$ may be attributable to the inhibition of O$_2^·$ generation because H$_2$O$_2$ is mostly derived from O$_2^·$ as a function of O$_2^·$ dismutase or nonenzymatically. Nobiletin, a dual inhibitor of both O$_2^·$ and NO radical generation, can be recognized as a potent, naturally occurring anti-inflammatory agent, because peroxyxynitrite, a coupling product of O$_2^·$ and NO, enhances COX-2 activity (52), involving inflammatory processes and thereby leading to carcinogenesis. By contrast, resveratrol was a selective inhibitor of NO generation (Fig. 2).

Certain ROS, such as H$_2$O$_2$, and proinflammatory cytokines including TNF-α are reported to cause cell death, which is occasionally attributable to apoptosis (53). Conversely, cell injury and cell death are manifest in TPA-applied mouse skin. Compensatory growth is subsequently necessary and is induced by growth factors such as transforming growth factor α and β and keratinocyte growth factor, all of which are inducible by TPA (54–56). Double TPA applications promoted cell growth, as revealed by increased PCNA-labeling indices, and resulted in epidermal hyperplasia and edema formation (Table 1, and Fig. 4, A and B). Pretreatment with nobiletin decreased cell growth rates (Table 1, and Fig. 4, C–E), possibly because of the escaping of epidermal cells from oxidative cellular death, which diminished the growth factor production in surviving cells.

### Table 2 Antitumor-promoting activity of nobiletin in DMBA-initiated ICR mouse skin

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence, %</th>
<th>No. of tumours/mouse (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA + TPA</td>
<td>47.1</td>
<td>2.06 ± 1.00</td>
</tr>
<tr>
<td>+ nobiletin (40 nmol)</td>
<td>31.1 (33.5)</td>
<td>1.63 ± 0.87 (20.9)</td>
</tr>
<tr>
<td>+ nobiletin (80 nmol)</td>
<td>31.1 (33.5)</td>
<td>1.94 ± 0.98 (5.8)</td>
</tr>
<tr>
<td>+ nobiletin (160 nmol)</td>
<td>26.7 (43.3)</td>
<td>0.80 ± 0.48 (61.2)</td>
</tr>
<tr>
<td>+ nobiletin (320 nmol)</td>
<td>31.1 (33.5)</td>
<td>0.50 ± 0.22 (75.7)</td>
</tr>
</tbody>
</table>

*P < 0.001 in Welch’s t test.
As predicted by the suppressive efficacies of biochemical markers relating to oxidative stress and inflammation (Figs. 2–6), topical application of nobiletin at doses of 160 and 320 nmol inhibited the multiplicity of skin tumors in a dose-dependent manner (Table 2). The chemopreventive efficacy of nobiletin in the mouse skin model may be superior to that of resveratrol, which inhibited tumor formation at the dose range of 1–25 μmol although experimental conditions were different (39). In any case, this is the first report demonstrating the chemopreventive ability of nobiletin in an animal model.

In conclusion, our approach, i.e., searching for iNOS induction inhibitors by activity-guiding separation, may be a promising strategy for discovering effective chemopreventive agents. Citrus flavonoid nobiletin was found to be a functionally novel antitumor promoter by working in both the priming and activation stages in mouse skin. It is also notable that it exclusively occurs in citrus fruits, is chemically stable, and is suitable for mass preparation for a variety of rodent studies. Nobiletin is currently being investigated by our collaborative research group for its chemopreventive efficacies in other inflammation-associated carcinogenesis models.

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REFERENCES


Inhibitory Effect of Citrus Nobiletin on Phorbol Ester-induced Skin Inflammation, Oxidative Stress, and Tumor Promotion in Mice

Akira Murakami, Yoshimasa Nakamura, Koji Torikai, et al.

Cancer Res 2000;60:5059-5066.

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