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Characteristics of nobiletin-mediated alteration of gene expression in cultured cell lines

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ABSTRACT

Nobiletin, a polymethoxylated flavonoid that is highly contained in the peels of citrus fruits, exerts a wide variety of beneficial effects, including anti-proliferative effects in cancer cells, repressive effects in hyperlipidemia and hyperglycemia, and ameliorative effects in dementia at *in vitro* and *in vivo* levels. In the present study, to further understand the mechanisms of these actions of nobiletin, the nobiletin-mediated alterations of gene expression in three organ-derived cell lines – 3Y1 rat fibroblasts, HuH-7 human hepatocarcinoma cells, and SK-N-SH human neuroblastoma cells – were first examined with DNA microarrays. In all three cell lines, treatments with nobiletin (100 μM) for 24 h resulted in more than 200% increases in the expression levels of five genes, including the endoplasmic reticulum stress-responsive genes *Ddit3*, *Trib3*, and *Asns*, and in less than 50% decreases in the expression levels of seven genes, including the cell cycle-regulating genes *Ccna2*, *Ccne2*, and *E2f8* and the oxidative stress-promoting gene *Txnip*. It was also confirmed that in each nobiletin-treated cell line, the levels of the DDIT3 (DNA-damage-inducible transcript 3, also known as CHOP and GADD153) and ASNS (asparagine synthetase) proteins were increased, while the level of the TXNIP (thioredoxin-interacting protein, also known as VDUP1 and TBP-2) protein was decreased. All these findings suggest that nobiletin exerts a wide variety of biological effects, at least partly, through induction of endoplasmic reticulum stress and suppressions of oxidative stress and cell proliferation.

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1. Introduction

Nobiletin (Fig. 1), a citrus polymethoxyflavonoid with six methoxy groups, is highly contained in the peels of citrus fruits. There is accumulating evidence that nobiletin exerts a wide variety of beneficial activities, including anti-dementia [1–8], anti-tumor [9–23], anti-metabolic syndrome, including anti-obesity, anti-hyperlipidemia, and anti-diabetes [18,21,24–30], and anti-inflammatory [31–35] activities at *in vivo* and *in vitro* levels. Nobiletin is thus expected to be clinically applicable for the treatment of these respective diseases. It has therefore become increasingly important to clarify the mechanisms of nobiletin-mediated biological effects, including adverse (toxic) effects.

The aim of the present study was to look closely into the nobiletin-mediated alterations of gene expression to further understand the mechanisms underlying nobiletin's effect on cellular events. We performed DNA microarray analyses by using total RNAs prepared from three different organ-derived cell lines: 3Y1 rat fibroblasts, HuH-7 human hepatocarcinoma cells, and SK-N-SH human neuroblastoma cells. Cells of each line were treated with 100 μM nobiletin, the concentration used in many previous studies, for 24 h. The results showed that five up-regulated and seven down-regulated genes were commonly identified in all three treated cell lines.

2. Materials and methods

2.1. Nobiletin

Nobiletin was extracted and isolated from *Citrus reticulata* peels as described previously [1,36]. This compound was dissolved at a concentration of 100 mM in dimethyl sulfoxide (DMSO) and then stored at −20 °C.

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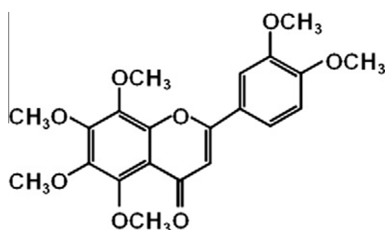

Fig. 1. Chemical structure of nobiletin.

2.2. Cell culture and treatments

The rat 3Y1-B clone 1–6 (3Y1) fibroblast and human HuH-7 hepatocellular carcinoma cell lines were obtained from the Japanese Cancer Research Resources Bank. The human SK-N-SH neuroblastoma cell line was obtained from RIKEN Cell Bank. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, and 60 μg/ml kanamycin at 37 °C in a saturated humidity atmosphere of 95% air and 5% CO_2. Appropriate numbers of cells were incubated with 0.1% (v/v) DMSO as a vehicle (control) or 100 μM nobiletin for the appropriate time at 37 °C in the culture medium.

2.3. DNA microarray analysis

Total RNA was extracted from cells treated with vehicle and 100 μM nobiletin for 24 h using an RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. One total RNA sample per nobiletin-treated cell line and two samples per vehicle (DMSO)-treated cell line were supplied for DNA microarray analysis. Total RNA (500 ng) was labeled with Cyanine-3 using a Quick-Amp Labeling Kit (Agilent Technologies, Palo Alto, CA, USA). Fluorescently labeled targets were hybridized to a SurePrint G3 Rat GE 8 × 60 K DNA microarray for 3Y1 cells and a SurePrint G3 Human GE 8 × 60 K DNA microarray for HuH-7 and SK-N-SH cells (Agilent Technologies). Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for the image analysis and data extraction processes. Subio Platform (Subio, Tokyo, Japan) computer software was used to exclude the genes showing inappropriate expression values and to select genes whose expression levels in all nobiletin-treated cell lines were more than double or fewer than half the average expression levels in the corresponding vehicle controls. In other words, the genes whose raw signals were lower than 40 in both the nobiletin- and vehicle-treated cells of each cell line were excluded. When one of the two vehicle-treated samples was selected as a control, the genes whose expression levels in another vehicle-treated sample were more than double or fewer than half the expression levels in the control were also excluded.

2.4. Western blotting

Cells were lysed with lysis buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing phenylmethylsulfonyl fluoride (PMSF), aprotinin, sodium orthovanadate and protease inhibitor cocktail, and the protein amounts in the obtained lysates were determined using BCA™ Protein Assay Reagent (Thermo Fisher Scientific Inc.). Fifteen micrograms per lane of total protein was subjected to SDS–PAGE (12.5% e-PAGEL; ATTO, Tokyo, Japan) and subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare). The membranes were blocked with TBST buffer (150 mM NaCl, 0.1% Tween20, and 10 mM Tris–HCl, pH 7.6) containing 5% skim milk or BSA for 1 h at room temperature, followed by incubation overnight at 4 °C with monoclonal anti-DDIT3 (2000:1; Cell Signaling), monoclonal anti-ASNS (1000:1; Santa Cruz),monoclonal anti-TXNIP (1000:1; MBL), or polyclonal anti-β-ACTIN (5000:1; Cell Signaling) antibodies. Detection utilized anti-mouse IgG horseradish peroxidase (HRP)-linked antibody(Cell Signaling) for DDIT3 (5000:1), ASNS (10,000:1), and TXNIP (5000:1), and anti-rabbit IgG HRP-linked antibody (Cell Signaling) for β-ACTIN (10,000:1), and the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.).

2.5. Alamar blue proliferation test

For evaluation of the effect of nobiletin on the proliferation, an Alamar blue assay kit (Invitrogen) was used. Cells were seeded at 5000 cells per well (0.5 ml) in 24-well microplates. Four wells were used for each time point. After 21 h, control cells (0 day) were applied to Alamar blue assay, while after 24 h the cells in the other wells were treated with DMSO (vehicle) or 100 μM nobiletin. After 21, 45, 69, and 93 h of the treated cells were applied to Alamar blue assay. The assays were basically carried out according to the manufacturer's instructions. Briefly, 50 μl of Alamar blue stock solution was added to the wells. The resazurin reduction in the cultures was determined after 3 h incubation with Alamar blue at 5% CO_2 and 37 °C by measuring the absorbance at an excitation wavelength of 550 nm and emission wavelength of 590 nm using a Wallac 1420 ARVOsx multilabel counter (Perkin Elmer). The fluorescence obtained for control cells (0 h) of each cell line was set to a value of 100, indicating 100% viability, and that for variously treated cells of the corresponding cell line was calculated accordingly.

3. Results

3Y1 rat fibroblast, HuH-7 human hepatocarcinoma, and SK-N-SH human neuroblastoma cells were treated with 100 μM nobiletin or with DMSO (vehicle control) for 24 h and the DNA microarray analyses were performed by use of total RNAs prepared from the treated cells. The genes whose expression levels in all nobiletin-treated cell lines were more than double or fewer than half the expression levels of the corresponding vehicle controls were selected and are listed in Table 1.

Among the listed genes, the expression levels of proteins encoded by the *Ddit3*, *Asns*, and *Txnip* genes were estimated by Western blotting. As shown in Fig. 2, the results demonstrated that the amounts of the DDIT3 and ASNS proteins were higher in all nobiletin-treated cell lines than in the corresponding vehicle controls, while the TXNIP protein level was lower in all the treated cell lines than in the controls, which was consistent with the results from DNA microarray analysis.

The effect of 100 μM nobiletin-treatment on the cell growth of the three cell lines was estimated by Alamar blue assay. The results showed that the growth of all cell lines was inhibited for 1–4 days by the treatment (Fig. 3).

4. Discussion

In the present study, DNA microarray analysis revealed that five genes were up-regulated (*Ddit3*, *Trib3*, *Asns*, *Slc6a9*, *Tmem116*) and seven genes were down-regulated (*Ccna2*, *Ccne2*, *Txnip*, *Depdc1*, *E2F8*, *Hist1h1b*, *Kif11*) in all three cell lines, 3Y1, HuH-7, and SK-N-SH cells, treated with 100 μM nobiletin for 24 h. Clarification of the significant roles played by these gene alterations may help

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