Hesperetin Induced G1-Phase Cell Cycle Arrest in Human Breast Cancer MCF-7 Cells: Involvement of CDK4 and p21

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Abstract: This study was to investigate the effects of hesperetin on cell proliferation and cell cycle arrest and explored the mechanism for these effects in breast carcinoma MCF-7 cells. Hesperetin significantly inhibited cell proliferation in a dose-dependent manner after treatment for 48 and 72 h and resulted in significant cell cycle arrest in the G1 phase. In the G1-phase related proteins, hesperetin down-regulates the cyclin-dependent kinases (CDKs) and cyclins and upregulates p21Cip1 and p27Kip1 in cells treated with hesperetin for 48 h and 72 h. After 72 h treatment, these phenomena were more pronounced. Hesperetin treatment at high concentration for 72 h resulted in a decrease in CDK2 and CDK4 together with cyclin D. In addition, hesperetin increases the binding of CDK4 with p21Cip1 but not p27Kip1 or p57Kip2. Taken together, our data suggest for the first time that the regulation of CDK4 and p21Cip1 may participate in the anticancer activity pathway of hesperetin in MCF-7 cells.

Introduction

Flavonoids are the most abundant natural antioxidants in food. Several epidemiological studies have supported the hypothesis that the antioxidant action of flavonoids may reduce the risk of developing cancer and cardiovascular disease (1–4). Reports based on the in vitro action of flavonoids on cancer cells have found various anticancer effects such as the inhibition of cell proliferation and kinase activity and the induction of apoptosis (4,5–7).

Flavonoids have been shown to inhibit the proliferation of cultured human cancer cell lines (8–9). The observed antiproliferative property of flavonoids suggests that these compounds may inhibit the cell cycle or induce apoptosis (10). However, many of the mechanisms underlying the potential anticancer activity of flavonoids are not fully understood.

Hesperetin (3',5,7-trihydroxy-4-methoxyflavanone), a member of the flavanone subclass of flavonoids, is found in fruit sources including various citrus species (11). Hesperetin occurs as hesperidin (its glycoside form) in nature. Dietary hesperidin is deglycosylated to hesperetin by intestinal bacteria prior to absorption (12), and hesperidin may be considered a pro-drug, which is metabolized to hesperetin (13). In several animal studies of the absorption, bioavailability, and pharmacokinetics of hesperidin, hesperidin has not been observed in plasma, bile, or urine (14–16). Several reports have described the beneficial biological effects of hesperidin. However, these studies may have inadvertently investigated the biological effects of hesperidin because hesperidin is not found in the body. To understand the biological activities of hesperidin, it is necessary to study the molecule’s biologically active form. Moreover, relatively little has been reported about hesperetin in comparison with hesperidin, and only a few in vitro studies have assessed it. Hesperetin is reported to be a powerful radical scavenger and to promote cellular antioxidant defense-related enzyme activity (17–18). Hesperetin has also shown potential anticarcinogenic activity as an antiangiogenic agent in mES cells (19). Thus, hesperetin may be useful in nutritional therapy and chemotherapy. We investigated the possible anticarcinogenic effects of hesperetin through the inhibition of cell proliferation and cell cycle arrest in human breast carcinoma MCF-7 cells, which are regarded as an in vitro breast cancer model.

Materials and Methods

The Cells Culture and Hesperetin Treatment

MCF-7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were routinely maintained in RPMI 1640 (Gibco BRL, MD), supplemented with 10% fetal bovine serum and antibiotics (50 U/ml of penicillin and 50 μg/ml streptomycin; Gibco) at 37°C in a humidified atmosphere containing 5% CO2. Cells were treated with hesperetin ranging from 1 to 100 μM for 24 h, 48 h, and 72 h. Hesperetin was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO; final concentration 0.1% in medium). All experiments were done in triplicate or quadruple.
Cell Proliferation and Cell Death Assay

Cell proliferation was determined using the methyl thiazolyl tetrazolium (MTT) assay. At 24 h, 48 h, and 72 h point, the cells exposed to hesperetin were added to MTT. DMSO was added 4 h later to each well to dissolve the resulting formazan crystals, and then absorbance was recorded at 490 nm in a microplate reader SpectraMax Plus; Molecular Devices, CA).

Cell Cycle Distribution

Cells were then harvested, washed with cold phosphate buffered solution (PBS), and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at –20°C for later analysis. The fixed cells were centrifuged at 1,000 rpm and washed with cold PBS twice. Ribonuclease A (20 µg/ml final concentration) and propidium iodide staining solution (50 µg/ml final concentration) was added to the cells and incubated for 30 min at 37°C in the dark. The cells were analyzed a FACS Calibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest version 3.3 software. ModFit LT version 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Immunoblotting and Immunoprecipitation Assay

Cells were lysed in radio-immunoprecipitation assay buffer (1% NP-40, 150 mM NaCl, 0.05% 4-chloro-2,5-dimethoxyamphetamine, 1% sodium dodecyl sulfate (SDS), 50mM Tris, pH 7.5) containing protease inhibitor for 1 h at 4°C. The supernatant was separated by centrifugation, and protein concentration was determined by Bradford protein assay kit II (Bio-rad Laboratories, CA). Proteins (25 µg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45 µm). The membranes were blocked with a 1% bovine serum albumin solution for 3 h, washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4°C. Antibodies against cyclin-dependent kinases (CDKs) CDK2, CDK4, CDK6, cyclin D, cyclin E, p16INK4a, p18INK4c, p21Cip1, p27Kip1, p57Kip2, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used to probe the separate membranes. The next day, the immunoreaction was continued with the secondary goat antirabbit horseradish-peroxidase–conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-rad Laboratories).

For CDK4-CDK inhibitors binding assay, cells were treated with vehicle or 100 µM of hesperetin for 48 h and 72 h. CDK4 from cell lysates (250 µg protein) was immunoprecipitated using anti-CDK4 antibody (4 µg) and precleared protein A/G-plus agarose beads (Santa Cruz) for overnight at 4°C. The precipitates were washed with lysis buffer, dena-

Figure 1. Effect of hesperetin on cell proliferation of MCF-7 cells. Cells were exposed to either vehicle (0.1% dimethyl sulfoxide in medium) or hesperetin (1–100 µM) and incubated for 24 h, 48 h, and 72 h. All data are reported as the percentage change in comparison with the vehicle-only group, which were arbitrarily assigned 100% viability. *, P < 0.001, significantly different from the vehicle-only group (hesperetin concentration = “0”).

Statistical Analyses

All data were expressed as percent compared with vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test (Sigma Stat, Jandel, San Rafael, CA). For all comparisons, differences were considered statistically significant at P < 0.05.

Results

Inhibition of Cell Proliferation by Hesperetin

The effects of hesperetin on proliferation of human breast cancer MCF-7 cells were measured using an MTT assay. The treatment of MCF-7 cells with hesperetin for 24 h did not affect cell proliferation (Fig. 1). Hesperetin significantly decreased cell proliferation after 48 h and 72 h of treatment in a dose- and time-dependent manner (P < 0.05). Cell proliferation was decreased up to 21.07% with 100 µM hesperetin for 72 h compared to the control.

Hesperetin-Induced Cell Cycle Arrest

Hesperetin-induced cell cycle arrest in MCF-7 cells was investigated (Fig. 2A). Dose-dependent, hesperetin-induced cell cycle arrest in the G1 phase was observed when MCF-7 cells were treated with hesperetin for 48 h or 72 h but not for 24 h. A significant increase in the number of G1-phase
cells was seen with hesperetin treatment at concentrations of 50 and 100 µM (61.6% and 67.2% of the cell population compared with control value 49.2%, respectively, \( P < 0.05 \)). After exposure to 10, 50, and 100 µM hesperetin for 72 h, we observed that the proportion of MCF-7 cells in the G1 phase increased from 50.9% to 62.1%, 50.9% to 65.3%, and 50.9% to 77.0%, respectively, and the proportion of S-phase cells decreased from 39.8% to 26.8%, 39.8% to 25.8%, and 39.8% to 15.4%, respectively.

The effect of hesperetin on the expression of G1-involved cell cycle regulatory proteins are shown in Fig. 3A. Hesperetin treatment resulted in a marked reduction in the expression of CDK4 in a dose-dependent manner after 48 and 72 h of treatment. Similarly, the expression of cyclin D was inhibited after 48 h of 50 µM hesperetin. A reduction in the expression of CDK2 and cyclin E was observed at the maximum concentration of 100 µM hesperetin for 72 h. The inhibitors of CDK 4 (INK4), p16INK4a, and p18INK4c did not differ compared with vehicle-treated MCF-7 cells. The expression levels of the p21Cip1 and p27Kip1 were increased in response to hesperetin treatment compared with vehicle-treated MCF-7 cells. When cells were treated with hesperetin for 24 h, expression of cell cycle regulatory proteins were not affected (data not shown).

Also, the formation of CDK4–p21Cip1 in response to hesperetin treatment was increased in MCF-7 cells, although CDK4–p27Kip1 and CDK4–p57Kip2 complex levels did not change (Fig. 3B).

### Hesperetin-Induced Apoptosis

To investigate whether apoptosis was induced by hesperetin in MCF-7 cells, we measured the expression of Bcl-2 and Bcl-2 associated x protein (Bax) as markers of apoptosis (Fig. 4). Hesperetin decreased the expression of Bcl-2 and increased that of Bax in a dose-dependent manner at 48 h. More pronounced changes were observed in cells treated with hesperetin for 72 h.

### Discussion

It is well known that flavonoids act as general cell growth inhibitors (20). This biological capacity of flavonoids suggests their potential use in cancer chemotherapy. Hesperetin inhibits vessel structure formation (an antiangiogenic effect)
Hesperetin-induced cytotoxicity was observed, even at high concentrations (100 µM). Thus, we investigated the anticancer effects of hesperetin on breast carcinoma MCF-7 cells in which hesperetin inhibits cell proliferation by causing cell cycle arrest.

To assess whether the hesperetin-induced inhibition of cell growth was mediated via alterations in cell cycle progression, cells were exposed to either vehicle (0.1% DMSO) or various concentrations of hesperetin (1, 5, 10, 50, 100 µM). Hesperetin significantly inhibited cell proliferation in a dose- and time-dependent manner. Hesperetin can inhibit the proliferation of both estrogen receptor-negative MDA-MB-435 and estrogen receptor-positive MCF-7 human breast cancer cells in vitro (21–22). Our results supported these observations; the exposure of MCF-7 cells to hesperetin resulted in a significant inhibition of cell proliferation.

We evaluated the mechanism by which hesperetin inhibited cell proliferation using a cell cycle analysis. The eukaryotic cell cycle is controlled by catalytic complexes of CDK/cyclin that coordinate internal and external signals at several key checkpoints (23). G1 arrest leads cells to undergo repair or to follow the apoptosis pathway (24). Activated CDK/cyclin complexes can be changed to an inactive state by the binding of CDK inhibitory subunits (CKIs). The CKIs can be divided into 2 classes. One class contains p21Cip1, p27Kip1, p57Kip2, and related proteins, with a preference for CDK2 and CDK4/cyclin complexes; the other class contains the INK4 family, including p16INK4a, p15INK4b, p18INK4c, and p19INK4d and closely related CKIs specific for CDK4 and CDK6/cyclin complexes (25–26).

Hesperetin induced a decrease in cyclinD and CDK4, together with an increase in p21Cip1 and p27Kip1 associated with CDK4. CDK4 appears to play a key role in the G1 cell cycle arrest observed in response to hesperetin.

We assessed the effect of hesperetin on the interaction between CDK4 and its inhibitors using immunoprecipitation. An increase in the binding of CDK4—p21Cip1, but not of CDK4—p27Kip1 or CDK4—p57Kip2, was observed in MCF-7 cells exposed to hesperetin. Additionally, hesperetin induced G1-phase cell cycle arrest through the regulation of CDK4 and p21Cip1, indicating that the inhibition of cell proliferation and the contribution to the apoptotic process is a possible mechanism by which hesperetin acts on cancer cells.

Moreover, the G1-phase cell cycle arrest induced by hesperetin resulted in apoptosis in MCF-7 cells. Hesperetin induced a dose- and time-dependent decrease in Bcl-2 expression and increase in Bax expression in MCF-7 cells. Apoptosis is a multistep process important in controlling cell number and proliferation as part of normal development; the Bax genes and members of the Bcl-2 family such as bcl-2 are involved in the control of apoptotic pathways. Decreased expression of Bcl-2 and increased expression of Bax are correlated with the response to anticancer compounds for chemotherapy in cancer cell lines. In addition, the loss of Bcl-2 expression can promote the induction of apoptosis (27–29). An anticancer drug that initiates apoptosis may be useful as a new category of chemotherapeutic agent.

In conclusion, our results indicate that hesperetin treatment leads to the inhibition of cell proliferation, the induction of cell cycle arrest at the G1 phase, and apoptosis. Additionally, our data suggest for the first time that the regulation of CDK4 and p21Cip1 may participate in the anticancer activity of hesperetin in MCF-7 cells. Therefore, we suggest that hesperetin is worthy of further study to assess its potential as an anticancer drug; it remains to be determined whether hesperetin has anticarcinogenic activity in vivo.

Acknowledgments and Notes

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, KRF–2006–311–F00127). Address correspondence to EJ Choi, Ph.D., Plant Resource Research Institute, Duksung Women’s University, 419 Ssangmun-dong, Dobong-ku, 132–714, Seoul, South Korea. Phone: +82-2-901-8663. FAX: 82-2-901-8661. E-mail: ejchoi@duksung.ac.kr.

Submitted 14 January 2007; accepted in final form 27 March 2007.

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