Distinct Effects of Naringenin and Hesperetin on Nitric Oxide Production from Endothelial Cells

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Diets rich in citrus and citrus-based products have been negatively correlated with the risk of cardiovascular disease, but so far no studies have been conducted to determine whether naringenin and hesperetin, two major flavanones in citrus plants, influence endothelium nitric oxide (NO) production. The aim of this study is to clarify estrogenic activities of naringenin and hesperetin and to examine whether they affect endothelial NO production via estrogen receptor (ER) activation. Both naringenin and hesperetin were observed to promote growth of MCF-7 cells under greatly reduced estrogen conditions and to suppress estrogen-induced response. Naringenin activated both ERα and ERβ, whereas hesperetin exhibited stronger potential to activate ERα rather than ERβ. Hesperetin, but not naringenin, increased NO releases from human umbilical vein endothelial cells in a dose-dependent manner. Hesperetin-induce responses were suppressed by ICI 182 780 and actinomycin D. Real-time reverse transcription polymerase chain reaction (RT-PCR) and western-blotting analysis revealed that hesperetin up-regulated endothelium nitric oxide synthase (eNOS) expression. These results suggested that hesperetin exerts an antiatherogenic effect, in part, via ER-mediated eNOS expression and subsequent increase of endothelial NO production. Distinct effects of naringenin and hesperetin on NO production also imply that ERα might play the major role in estrogen-induced eNOS expression. However, the inefficacy of naringenin on NO production remains to be elaborately studied. Our findings add more proof to the molecular explanations for the health benefits of citrus used to prevent cardiovascular disease, especially for postmenopausal women.

KEYWORDS: Citrus flavonoid, naringenin, hesperetin, nitric oxide, endothelium NO synthase, estrogen receptor

INTRODUCTION

Dietary supplementation with fruits, especially citrus fruits, has been associated with a reduced risk of cardiovascular disease (1, 2). Although numerous studies have evaluated the benefits of citrus and citrus-based products, their mechanisms of action remain unclear. Citrus fruit products are rich in diverse phytochemicals, such as flavonoids (3). Of particular interest are naringenin and hesperetin and their glycosides, which exhibit hypolipidemic, antioxidant, anti-inflammatory, and antiatherogenic properties (4–6). Naringenin and hesperetin are flavanones and share the same backbone, except for minor differences on the B-ring (Figure 1). The two flavanones in citrus usually occur as glycosides, which are hydrolyzed to active aglycones by intestinal bacteria (7).

Naringenin is known as an estrogen-like compound. Estrogenic activity of naringenin has been demonstrated using recombinant estrogen receptor gene assays (8, 9). It was also found to have uterotrophic effects in female mice at human relevant doses (10). In some investigations, naringenin exhibits antiestrogenic activity. It significantly decreases 17β-oestradiol (E2)-induced uterine weight increase in the immature rodent and proliferation of breast cancer cells, MCF-7 (11). Combinational administration of estrogen and naringenin results in up-regulation of nitric oxide synthase (12). On the other hand, estrogenic activity of hesperetin remains uncertain.

Estrogen has long been considered to play an important role in cardiovascular protection, especially for postmenopausal women (13). Recent evidence suggests that the vascular actions of estrogen are partly mediated by up-regulation of nitric oxide (NO) production from vascular endothelia cells (14). Endothelium-derived NO supports the pivotal role in the regulation of blood pressure, platelet aggregation, leukocyte adhesion, and migration and proliferation of smooth muscle cells (15). In atherosclerotic lesions, regulatory function of endothelium-dependent relaxation is impaired, which has been linked to a decreased production and/or biological activity of endothelium-derived NO (16). Some dietary phenolic compounds with estrogenic activity, such as daidzein and resveratrol have been demonstrated to exert antiatherogenic effects by rapidly activating endothelium NO synthase (eNOS) or up-regulating eNOS expression (17). Although numerous studies have evaluated the benefits of citrus...
against coronary heart disease, there is no direct evidence linking naringenin and hesperetin with the influencing of the eNOS system as yet.

Because citrus or citrus-based products are consumed in daily life, beneficial effects of citrus flavonoids may be partly attributed to the longer-term action on endothelium. The aim of this study is to clarified estrogenic activities of naringenin and hesperetin (especially for the latter) and to examine whether they affect endothelial NO production and eNOS expression via ER activation. Our finding will significantly contribute to the growing knowledge about citrus for the prevention of cardiovascular disease.

MATERIALS AND METHODS

Reagents. Naringin, E₂, actinomycin D (act-D), endothelial cell growth supplement (EGCS), recombinant anti-human β-Actin antibody, and horseradish peroxidase-conjugated IgG were purchased from Sigma Chemical (St. Louis, Missouri, USA). Hesperidin was purchased from the Control of Pharmaceutical and Biological Products (Beijing, China). Naringenin (Nar) and hesperetin (Hes) were purified from the acid hydrolysate of naringin or hesperidin, their purity were above 97% (based on HPLC analysis). ICI 182 780 was purchased from Tocris Cookson Inc. (Ballwin, Missouri, USA). A23187 was purchased from Calbiochem (San Diego, CA, USA.). 2,3-diaminonaphthalene (DAN) was from Cayman (Ann Arbor, Michigan, USA.). Rabbit anti-human eNOS antibody was from Affinity Bioreagents (Golden, Colorado, USA.). Collagenase I and all cell culture reagents were purchased from Gibco (Carlsbad, California, USA.).

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord with collagenase I according to Marin’s description (18). The cells were cultured on gelatin-coated flasks in medium 199, containing 10% FBS and 10% charcoal-treated FBS supplemented with 10% charcoal-treated FBS (to minimize the effect of estrogen) at 37°C and 100 units/mL of penicillin and 0.1 mg/mL of streptomycin, at 37°C in a humidified incubator with 5% CO₂. The supernatant was decanted and 100 µL of DMSO was added to each well. The absorbance (550nm) of DAN reagent for 10 min. After the incubation period, 20 µL of 2.8 mol/L NaOH solution was added. The fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 430 nm with a fluorescence microplate reader. Standard curves with NaNO₂ were performed daily. HUVECs were also preincubated with various regents: E₂ (10 nM), ICI 182 780 (100 nM), A23187 (5 µM), and act-D (1 µg/mL), respectively. Each assay was performed in quadruplicate.

Fluorescence Analysis of HUVECs NO Release. HUVECs were seeded into 96-well tissue culture plate at 2 × 10⁵ cell/well and cultured for 24 h. NO production was measured by determination of nitrite in culture medium using a fluorometric assay (19). A 5 µL portion of culture medium was diluted to 100 µL with pure water and then incubated with 10 µL of DAN reagent for 10 min. After the incubation period, 20 µL of 2.8 mol/L NaOH solution was added. The fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 430 nm with a fluorescence microplate reader. Standard curves with NaNO₂ were performed daily. HUVECs were also preincubated with various regents: E₂ (10 nM), ICI 182 780 (100 nM), A23187 (5 µM), and act-D (1 µg/mL), respectively. Each assay was performed in quadruplicate.

RNA Extraction and Real-Time RT-PCR. Total cellular RNA was extracted using an RNeasy mini kit according to manufacturer’s instructions. RNA was quantified by measuring absorbance at 260 nm. Reverse transcription of mRNAs from human liver. Amplified cDNAs were cloned into pcDNA3.1 (Invitrogen, Carlsbad, California) and sequenced to confirm the right reading frames. Luciferase reporter plasmid was constructed by insertion of an annealed oligonucleotide containing three copies of the ER response element to the upstream of the luciferase gene in pGL3-promoter vector (Promega, Madison, Wisconsin, USA.). pCMVβGal was purchased from Clontech (Palo Alto, California, USA.). U-2OS Cells were seeded in 96-well tissue-culture plates the day before transfection to give a confluency of 50–80% at transfection. A total of 50 ng of DNA containing 10 ng of pCMVβGal, 10 ng of the indicated nuclear receptor expression vectors, and 30 ng of corresponding reporters were cotransfected per well using the Fugene transfection reagent (Roche, Indianapolis, Indiana, USA) following the manufacturer’s instructions. After 24 h, tested samples in fresh media supplemented with 10% charcoal-treated FBS were added. The vehicle (DMSO) was added to control media to produce the same final solvent concentration (typically 0.2%) in all wells. After incubation with the tested samples for 24 h, cells were collected with Cell Culture Lysis buffer (Promega, Madison, Wisconsin, USA.). Luciferase activity and β-galactosidase activity were measured using the corresponding assay kits according to the manufacturer’s instructions. The relative luciferase activity was normalized by β-galactosidase activity. Each assay was performed in quadruplicate.

Luciferase Reporter Gene Assay. Human ERα and ERβ expression vectors were obtained by PCR amplification using cDNA synthesized by reverse transcription of mRNA from human liver. Amplified cDNAs were cloned into pcDNA3.1 (Invitrogen, Carlsbad, California) and sequenced to confirm the right reading frames. Luciferase reporter plasmid was constructed by insertion of an annealed oligonucleotide containing three copies of the ER response element to the upstream of the luciferase gene in pGL3-promoter vector (Promega, Madison, Wisconsin, USA.). pCMVβGal was purchased from Clontech (Palo Alto, California, USA.). U-2OS Cells were seeded in 96-well tissue-culture plates the day before transfection to give a confluency of 50–80% at transfection. A total of 50 ng of DNA containing 10 ng of pCMVβGal, 10 ng of the indicated nuclear receptor expression vectors, and 30 ng of corresponding reporters were cotransfected per well using the Fugene transfection reagent (Roche, Indianapolis, Indiana, USA) following the manufacturer’s instructions. After 24 h, tested samples in fresh media supplemented with 10% charcoal-treated FBS were added. The vehicle (DMSO) was added to control media to produce the same final solvent concentration (typically 0.2%) in all wells. After incubation with the tested samples for 24 h, cells were collected with Cell Culture Lysis buffer (Promega, Madison, Wisconsin, USA.). Luciferase activity and β-galactosidase activity were measured using the corresponding assay kits according to the manufacturer’s instructions. The relative luciferase activity was normalized by β-galactosidase activity. Each assay was performed in quadruplicate.
RESULTS

Estrogenic Activity of Naringenin and Hesperetin. The estrogenic activity of naringenin and hesperetin was examined in ER positive MCF-7 breast cancer cells under greatly reduced estrogen conditions (10% eFBS). In a primary experiment, a significant response was not observed at the concentration below 12.5 µM for both compounds (data not shown). Naringenin and hesperetin promoted proliferation of MCF-7 cells in a dose-dependent manner in the dose range from 12.5 to 100 µM, as shown in Figure 2A. It also illustrated that 10 nM E2 induced a 150% increase in cell proliferation, and a similar increase was induced by naringenin or hesperetin at high concentrations (50–100 µM). Complete antagonist of ER, ICI 182 780 (100 nM) significantly inhibited their activities (Figure 2B), indicating that naringenin and hesperetin promoted MCF-7 growth via ER activation. Meanwhile, naringenin or hesperetin inhibited E2-induced cell proliferation was also observed, indicating the two flavanones also acted as antagonists of ER.

Ligand binding to the ER initiates transcriptional activation through the specific estrogen response element (ERE) in certain target genes. To determine whether the two flavanones are able to induced ER-dependent gene expression, U-2OS cells were cotransfected with an ERα or ERβ expression vector together with a plasmid containing the luciferase reporter gene controlled by ERE. Naringenin, hesperetin, or E2 (10 nM) as a positive control, were tested for their induction of luciferase activity. As shown in Figure 2, panels C and D, E2 at 10 nM increased luciferase activity about 5 fold for ERα and 12-fold for ERβ, respectively. Naringenin was observed to activate both ERα and ERβ through a range of concentrations (6.25–50 µM), whereas hesperetin exhibited weak activity to induced ERβ controlled luciferase expression even at 50 µM, implying that hesperetin selectively activated ERα. Generally, naringenin showed slightly more efficacy in ER-dependent response (including MCF-7 proliferation assay and gene reporter assay) than hesperetin.

Effect of Naringenin and Hesperetin on NO Release from Endothelial Cells. To evaluate the potency to stimulate NO release from HUVECs, naringenin or hesperetin at a range of concentrations (12.5–100 µM), or A23187 (5 µM) as a positive control, was incubated with the cells for 24 h. As a result, hesperetin, but not naringenin, was found to increase

Western-blotting Analysis. Total cellular protein was obtained by lysing cells with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM Navanadate, 5 µg/mL leupeptin, 0.2% Triton X-100, and 0.3% NP-40). Protein concentration of the lysates was determined using a 2-D Quant kit (Amersham Biosciences). SDS–PAGE resolved protein bands were transferred to nitrocellulose membranes (BioRad). After blocking with 5% skimmed milk, the membrane was incubated with primary antibodies (1:1000 dilution) for 3 h, followed by horseradish peroxidase-conjugated IgG milk, the membrane was incubated with primary antibodies (1:1000 dilution) for 3 h, followed by horseradish peroxidase-conjugated IgG (1:10000 dilution). Target proteins were visualized with an EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel). Semi-quantifications were performed with densitometric analysis by Quantity One software (BioRad). The eNOS protein bands were normalized using the respective β-Actin protein bands. Samples harvested from two independent cultures were analyzed.

Statistical Analysis. All experiments were performed by two or three times. The results are expressed as mean ± SD and they were evaluated statistically using the one-way analysis of variance (ANOVA) followed by the Dunnett t-test. The level of significance was taken as p ≤ 0.05.

Figure 2. Estrogenic activity of naringenin and hesperetin. (A) MCF-7 cells were treated with increasing doses (12.5–100 µM) of naringenin and hesperetin and E2 (10 nM) for 72 h. (B) Effects of E2 (10 nM) and ICI 182 780 (100 nM) on naringenin- or hesperetin-induced MCF-7 cell proliferation. Cells were pretreated with the indicated compounds prior to naringenin or hesperetin for 15 min and then incubated for 72 h. Asterisk indicates P ≤ 0.05 vs the group treated with naringenin alone, # indicates P < 0.05 vs the group treated with hesperetin alone (n = 4). (C and D) Activation of the estrogen receptors by naringenin and hesperetin. U-2OS cells were transfected with the expression vectors either for ERα (C) or ERβ (D), together with the reporter plasmids containing the corresponding response element. Cells were incubated with different compounds at the indicated concentrations for 24 h, and luciferase activity was determined (n = 4).
NO release from HUVECs in a dose-dependent manner (Figure 3A). NO production in HUVECs was doubled by hesperetin when the concentration of the compound was 100 µM. Because the two flavonones appeared to have an inhibitory effect on HUVECs growth/viability beyond 150 µM (data not shown); the concentration used here was not higher than 100 µM. ICI 182 780 (100 nM) and transcription inhibitor act-D (1 µg/mL) on hesperetin-induced NO by HUVECs. Cells were pretreated with indicated compounds prior to naringenin or hesperetin for 15 min and then incubated for 24 h. An asterisk indicates P ≤ 0.05 vs the control group, and # P ≤ 0.05 vs the group treated with hesperetin alone (n = 4).

Effect of Naringenin and Hesperetin on eNOS Expression. To study the effect of the two flavonones on the eNOS mRNA abundance, eNOS mRNA expression was measured in HUVECs treated with naringenin or hesperetin for 24 h using real-time PCR. The expression level of GAPDH mRNA was used as an internal control. Because naringenin or hesperetin at 50 µM induced comparable responses to E2 (10 nM), this dose was selected for the following analysis. As shown in Figure 4A, the relative eNOS mRNA expression was significantly up-regulated in cells treated with E2 (10 nM) or hesperetin, but not naringenin, as compared to the eNOS mRNA level seen in the control. Subsequently, eNOS protein level was semiquantified by western-blotting analysis. The level of β-actin. Data is denoted as mean ± SD of n = 2. An asterisk indicates P < 0.05 vs the control group.

DISCUSSION
In the present study, we assessed estrogenic activity of naringenin and hesperetin using MCF-7 proliferation assay and ER-controlled reporter gene assays. Unlike previous reports (20), both naringenin and hesperetin were observed to promote growth of MCF-7 cells under greatly reduced estrogen conditions and to suppress E2-induced response, indicating that naringenin and hesperetin can be considered as partial estrogen receptor (ER) agonists. Interestingly, although the close analogs presented similar potential to increase MCF-7 growth and activate ERα-induced gene expression, their action on ERβ was significantly different. Hesperetin exhibited stronger potential to activate ERα rather than ERβ.

We next evaluated their effects on NO production and eNOS expression in cultured endothelial cells. As a result, hesperetin,
but not naringenin, promoted NO releases from HUVECs in a dose-dependent manner, and hesperetin-induced responses were suppressed by ER antagonist ICI 182 780 and transcription inhibitor act-D. It promoted NO release from HUVECs via up-regulation of eNOS expression, also evidenced by real-time RT-PCR and western-blotting analysis. These results implied activation of ERβ is not a requirement for ER-mediated up-regulation of eNOS. ERα has been shown to mediate acute response of estrogen by coupling to eNOS in caveolae (21), but its role in genomic effects of estrogen is not completely understood. As compared with WT mice, eNOS protein in ERα knockout mice is reduced, indicating that estrogen modulation of eNOS protein content is mediated in part through ERα (22). Recent study has shown that ERα activates the ERE and eNOS promoter-dependent luciferase activity in COS-7 cells and bovine pulmonary artery endothelial cells (23). On the basis of these findings, we suggested that ERα might play the major role in estrogen-induced eNOS expression.

Recently, much attention has been focused on some food-derived phenolic compounds with estrogenic activities that may be beneficial for the prevention of cardiovascular disease by influencing the eNOS system (17). Soy protein-rich diet and genistein has been demonstrated to enhance eNOS promoter activity and NO output from endothelial cells after long-term administration in vitro (24, 25). Long-term incubation with red wine polyphenols or resveratrol leads to an increase of eNOS protein expression and eNOS-derived NO in endothelial cells (26). In this work, hesperetin was shown to increase NO release from endothelial cells and to up-regulate eNOS expression. Because hesperetin with its glycosides exist in a relative high abundance in citrus, it is highly possible that those actions of the hesperetin contribute to the beneficial effect of citrus products on the cardiovascular system. Accordingly, it is reasonable to deduce that estrogenic compounds are potent for elevating endothelium-derived NO at the low endogenous estrogen level. However, our study provided an exception for the inference. Although naringenin was shown to have estrogenic activities in MCF-7 proliferation assays and reporter-gene activities of chemicals and complex mixtures using in vitro recombinant receptor-reporter gene assays. Can. J. Physiol. Pharmacol. 1996, 74 (2), 216–222.


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