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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

Diets rich in citrus and citrus-based products have been negatively correlated with the risk of cardiovascular disease (*1, 2*). The health benefits are largely attributed to their high content of flavonoids. Until now, more than 60 citrus flavonoids have been isolated and structurally determined (*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 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-estradiol (E_2)-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 down-regulation of hepatic expression of estrogen-regulated mRNA stabilizing factor (*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 plays 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

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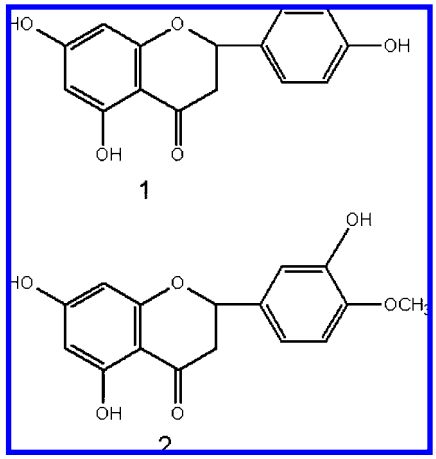

Figure 1. Chemical structures of naringenin (**1**) and hesperetin (**2**).

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, E_2, actinomycin D (act-D), endothelial cell growth supplement (ECGS), recombinant antihuman 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 (Bejing, 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 antihuman eNOS antibody was from Affinity Bioreagents (Golden, Colorado, USA.). Collagenase I and all cell culture reagents were purchased from Gibco (Carlsbad, California, USA.). SuperScript II RNase H^- Reverse Transcriptase, Oligo $(dT)_{12-18}$ and RNase inhibitor were purchased from Invitrogen (Carlsbad, California, USA.). RNeasy mini kit and QuantiTect SYBR Green PCR Kit were purchased from Qiagen (Valencia, 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 with ECGS (20 μg/mL), 100 μg/mL of streptomycin, and 100 units/mL of penicillin. Umbilical vein endothelial cells were confirmed by their cobblestone morphology and positive immunoreactivity to VIII factor. MCF-7, a human breast cancer cell line, was cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum supplemented with streptomycin and penicillin, at 37 °C in a humidified incubator with 5% CO_2. U-2OS, an osteosarcoma cell line, was cultured in McCoy's 5A medium containing 10% fetal bovine serum supplemented with streptomycin and penicillin.

MCF-7 Proliferation Assay. MCF-7 cells were seeded into 96-well tissue-culture plates at 5×10^3 cells/well and incubated for 24 h. Cell culture medium was replaced by phenol-free medium 199 supplemented with 10% charcoal-treated FBS for 24 h and then treated with naringenin or hesperetin at a dose range of 12.5~100 μM or 10 nM E_2, respectively. Untreated cells with equal concentration of vehicle were used as controls. Cells were cultured for 72 h. To measure cell growth, the methyl-tetrazolium (MTT) assay was used. The medium was removed and replaced with MTT at 5 mg/mL in DMEM. The plates were incubated for 4 h at 37 °C. The supernatant was decanted and 100 μL of DMSO was added to each well. The absorbance (550nm) of the formazan product was measured directly in the 96-well plates with a microplate reader. 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 were sequenced to confirm the right reading frames. Luciferase reporter plasmid ERE-luc 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 Fugen6 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.

Fluorescence Analysis of HUVECs NO Release. HUVECs were seeded into 96-well tissue culture plate at 2×10^4 cell/well and cultured for confluent. Then the mediums were replaced by phenol-free medium 199 supplemented with 5% charcoal-treated FBS (to minimize the effect of estrogen) at the presence or absence of test samples 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 the 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 $NaNO_2$ were performed daily. HUVECs were also preincubated with various regents: E_2 (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 2 μg total RNA was performed in 20 μL reaction volumes containing 200 units of SuperScript II transcriptase, 40 units of RNase inhibitor, 500 ng of Oligo $(dT)_{12-18}$, and 0.5 mM dNTP for 60 min at 42 °C. A 0.6 μL portion of total cDNA was used as a template to determine the relative amount of mRNA by real-time PCR (Mastercycle Realplex^4, Eppendorf) using a specific primer, eNOS, 340 bp (forward primer: 5′-CAAGTTCCCTCGTGTGAAGAACTG-3′; reverse primer: 5′-TAAAGGTCTTCTTCCTGGTGATGCC-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; reverse primer: 5′-TCCTTGGAGGCCATGTGGGCCAT-3′) as a reference housekeeping gene. Following a 15 min Tag activation step at 95 °C, reactions were subjected to 40 cycles of 30 s denaturation at 95 °C, 30 s of annealing at 49 °C, and 30 s of extension at 72 °C. The threshold cycle (Ct) was determined, and the relative gene expression was expressed as fold change $= 2^{-\Delta\Delta Ct}$. Samples harvested from three independent cultures were analyzed.

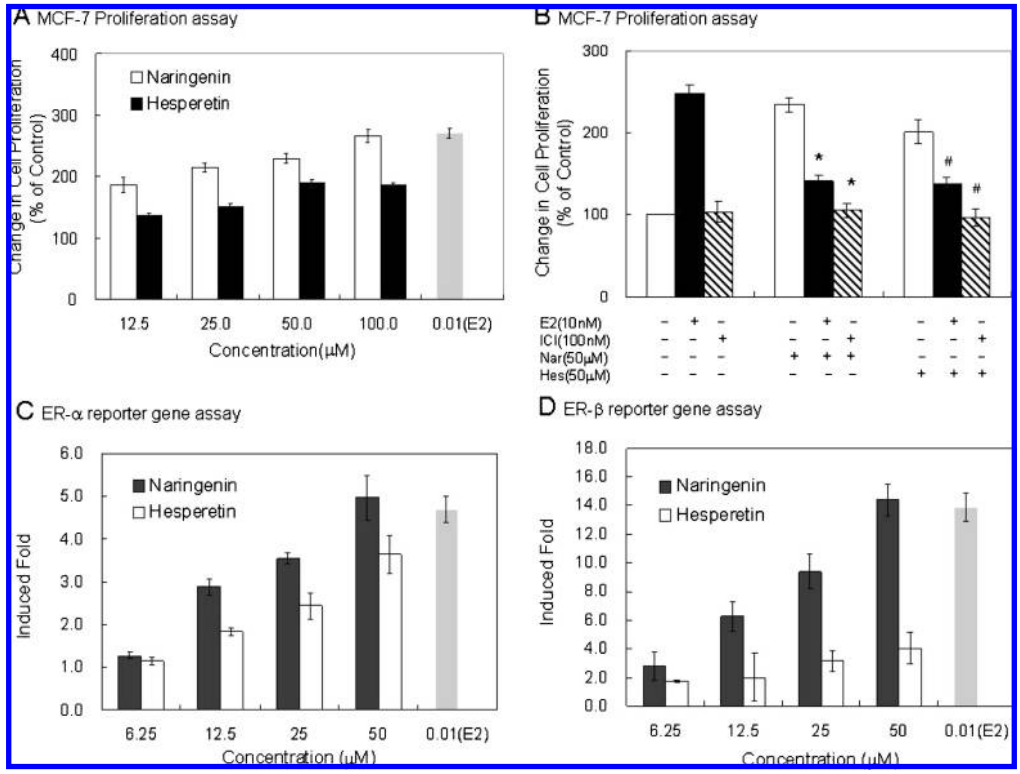

Figure 2. Estrogenic activity of naringenin and hesperetin. (**A**) MCF-7 cells were treated with increasing doses (12.5∼100 μM) of naringenin and hesperetin and E_2 (10 nM) for 72 h. (**B**) Effects of E_2 (10 nM) and ICI 182 780 (100 nM) on naringenin- or hesperetin-induced MCF-7 cell proliferation. Cells were pretreated with the indicated compounds prior to naringenin or hesperetin for 15 min and then incubated for 72 h. Asterisk indicates $P \leq 0.05$ 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 ($n = 4$).

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 (1:10000 dilution). Target proteins were visualized with an EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel). Semiquantifications 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 $p \leq 0.05$.

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% cFBS). 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 E_2 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 E_2-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 E_2 (10 nM) as a positive control, were tested for their induction of luciferase activity. As shown in **Figure 2**, panels **C** and **D**, E_2 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

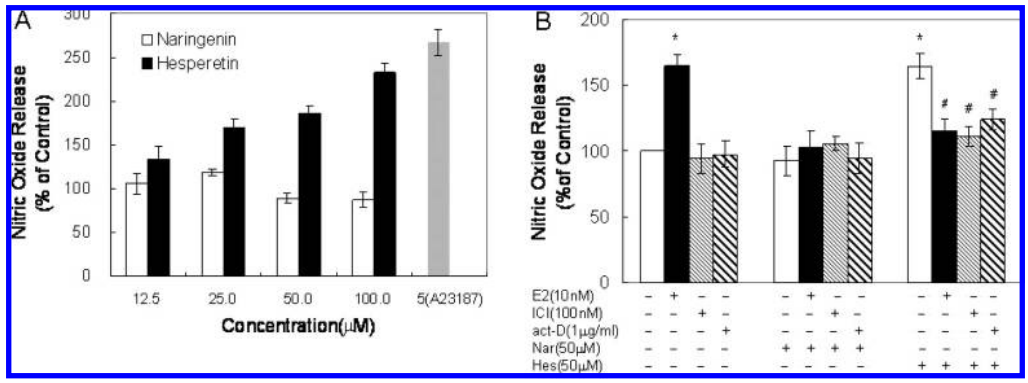

Figure 3. Effects of naringenin and hesperetin on NO release from HUVECs. NO release by endothelial cells was measured using the DAN fluorescence assay. (**A**) Cells were treated with naringenin or hesperetin in the range of concentration (12.5∼100 μM) for 24 h. A23187 (5 μM) was used as a positive control ($n = 4$). (**B**) Effects of E_2 (10 nM), ICI 182 780 (100 nM), and 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 $P \leq 0.05$ vs the control group, and # $P \leq 0.05$ vs the group treated with hesperetin alone ($n = 4$).

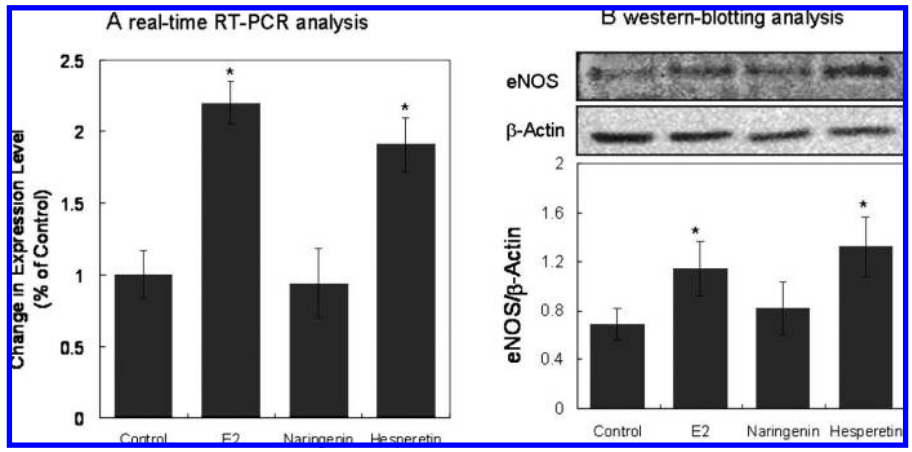

Figure 4. Effects of naringenin and hesperetin on eNOS transcription and protein expression. (**A**) Real-time PCR mRNA levels of eNOS and GAPDH in HUVECs treated with E_2 (10 nM), naringenin(50 μM), or hesperetin(50μM) for 24 h. The transcription level of GAPDH mRNA was used as an internal control. Results were expressed as fold change $= 2^{-\Delta\Delta Ct}$. Data is denoted as mean ± SD of $n = 3$. An asterisk indicates $P < 0.05$ vs the control group. (**B**) Western-blotting analyzes eNOS and β-actin protein in HUVECs treated with indicated compounds. The results were normalized by the level of β-actin. Data is denoted as mean ± SD of $n = 2$. An asterisk indicates $P < 0.05$ vs the control group.

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 flavanones 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) inhibited hesperetin-induced NO production (**Figure 3B**), indicating that the action of hesperetin on endothelial cells was involved in estrogen receptor mediated gene expression. However, combinational treatment of estrogen and naringenin or hesperetin resulted in significant suppression of NO release induced by E_2.

Effect of Naringenin and Hesperetin on eNOS Expression. To study the effect of the two flavanones 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 to correct for intersample variations in total RNA abundance. Because naringenin or hesperetin at 50 μM induced comparable responses to E_2(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 E_2 (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 was used as an internal control. The results illustrated in **Figure 4B** show that only E_2 and hesperetin significantly increase the eNOS protein, which were consistent with the changes in mRNA. These finding indicated that the distinct effects of naringenin and hesperetin on endothelial NO production were attributed to their abilities to up-regulate eNOS expression.

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 E_2-indcued 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 assays, it was unable to promote NO release from HUVECs under the presented experiment condition. We propose that the actions of naringenin on endothelial cell are involved not only in estrogenic receptors but also in some unknown signaling pathway, which may compromise its NO elevation effect. However, the exact underlying molecular mechanism remains to be revealed.

In summary, our study found that the citrus flavonoids, naringenin and hesperetin, exhibited distinct effects on NO production from endothelial cells, although they possessed similar chemical structures and estrogenic activities. The findings in the present study add more proof to the molecular explanations for the health benefits of citrus used to prevent cardiovascular disease, especially for postmenopausal women. However, the inefficacy of naringenin to effect NO production remains to be elaborately studied, and further investigative work is underway.

ABBREVIATIONS USED

Act-D, actinomycin D; cFBS, charcoal-treated fetal bovine serum; DAN, 2,3-diaminonaphthalene; E_2, 17β-estradiol; ECGS, endothelial cell growth supplement; eNOS, endothelium nitric oxide synthase; ER, estrogen receptor; Hes, hesperetin; HUVECs, human umbilical vein endothelial cells; Nar, naringenin; NO, nitric oxide

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