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Hesperetin, a Bioflavonoid, Inhibits Rat Aortic Vascular Smooth Muscle Cells Proliferation by Arresting Cell Cycle

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Abstract Diet can be one of the most important factors that influence risks for cardiovascular diseases. Hesperetin, a flavonoid present in grapefruits and oranges, is one candidate that may benefit the cardiovascular system. In this study, we have investigated the effect of hesperetin on the platelet-derived growth factor (PDGF)-BB-induced proliferation of primary cultured rat aortic vascular smooth muscle cells (VSMCs). Hesperetin significantly inhibited 50 ng/ml PDGF-BB-induced rat aortic VSMCs proliferation and [^{3}H]-thymidine incorporation into DNA at concentrations of 5, 25, 50, and 100 μM. In accordance with these findings, hesperetin revealed blocking of the PDGF-BB-inducible progression through G_0/G_1 to S phase of the cell cycle in synchronized cells. Western blot showed that hesperetin inhibited not only phosphorylation of retinoblastoma protein (pRb) and expressions of cyclin A, cyclin D, cyclin E, cyclin-dependent kinase 2 (CDK2) as well as proliferating cell nuclear antigen (PCNA) protein, but also downregulation of cyclin-dependent kinase inhibitor (CKI) p27^{kip1}, while did not affect CKI p21^{cip1}, p16^{INK4}, p53, and CDK4 expressions as well as early signaling transductions such as PDGF beta-receptor, extracellular signal-regulated kinase (ERK) 1/2, Akt, p38, and JNK phosphorylation. These results suggest that hesperetin inhibits PDGF-BB-induced rat aortic VSMCs proliferation via G_0/G_1 arrest in association with modulation of the expression or activation of cell-cycle regulatory proteins, which may contribute to the beneficial effect of grapefruits and oranges on cardiovascular system. J. Cell. Biochem. 104: 1–14, 2008. © 2007 Wiley-Liss, Inc.

Key words: hesperetin; bioflavonoid; p27^{kip1}; vascular smooth muscle cell; cell cycle

In cardiovascular diseases and cancer, abnormal or unregulated cell proliferation has long been considered as an important etiological factor [Ross, 1990; Schwartz, 1997], which can be triggered by various extracellular mitogenic stimuli, such as platelet-derived growth factor (PDGF)-BB and basic fibroblast growth factor (bFGF) etc., that activate a variety of signaling events involved in the regulation of cell growth and division [Blenis, 1993; Heldin et al., 1998; Ahn et al., 1999]. The cell cycle, as a final common pathway of proliferative signaling cascade, is shared by various mitogenic stimuli. The modulated expression of the cell cycle regulatory genes is one of the important mechanisms of cell growth inhibition [Dzau et al., 2002]. Generally the cell cycle is regulated by the coordinated action of cyclin-dependent kinases (CDKs) in association with their specific regulatory cyclin proteins. Thus, functional activation of CDK–cyclin kinase activities is required for cell cycle progression [Dzau et al., 2002; Braun-Dullaeus et al., 2004]. The kinase activity of these CDK–cyclin complexes is further regulated negatively by a number of

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cyclin dependent kinase inhibitors (CKIs) [Sherr and Roberts, 1999], which are grouped into two classes. The members of the INK4 family ($p16^{INK4a}$ and $p15^{INK4b}$) inhibit only CDK4 and CDK6 [Ortega et al., 2002], and the members of cip family ($p21^{cip1}$ and $p27^{kip}$) inhibit all CDKs [Coqueret, 2003a,b].

Flavonoids are a large group of low molecular weight polyphenolic compounds, and have been shown to be one of the most important classes in free states and as glycosides. Large amounts of bioflavonoids are ingested because of their abundance and wide distribution in foods and beverages. A dietary antioxidant that has received attention with regard to antioxidant effects is the polyphenolic compounds hesperidin (hesperetin 7-rhammnoglucoside) and its aglycone hesperetin (3′, 5, 7-trihydroxy-4′-methoxy flavanone), both flavonoids present extensively in the plant kingdom especially in many citrus fruits such as grapefruits and oranges, and are commonly used in traditional medicines. Hesperetin, a prominent component of citrus fruits [Garg et al., 2001], is also present in several plants (e.g., *Buddleja madagascariensis*, *Rosmarinus officinalis*, etc.), and displays a number of biological effects such as antiinflammatory, anticarcinogenic, antihypertensive, and antithrombotic activities [Galati et al., 1996; Garg et al., 2001]. Hesperetin has been reported to inhibit low-density lipoprotein oxidation in vitro [Shin et al., 1999]. It has also been reported that hesperetin inhibits HMG-CoA reductase and lowers the plasma cholesterol level in rats [Bok et al., 1999]. The role of hesperetin and structurally related naringenin citrus flavanone in the prevention and treatment of atherogenic disease and cancer has recently received considerable attention, with particular interest in the use of these flavanones as anticancer and anti-atherogenic compounds [Joshipura et al., 1999; Wilcox et al., 1999]. Previously we have reported that hesperetin displayed a potent antiplatelet effect, and this was mediated by inhibition of PLCγ2 phosphorylation and cyclooxygenase-1 activity [Jin et al., 2006]. However, the antiproliferative effect of hesperetin on cell proliferation has not yet been extensively studied.

In the present study, we have investigated the antiproliferative activity of hesperetin on various growth factors-stimulated primary cultured rat aortic vascular smooth muscle cells (VSMCs) and fibroblasts by measurements of cell proliferation and [^{3}H]-thymidine incorporation into DNA. In particular, the influences of hesperetin on the PDGF-BB-inducible cell cycle progression as well as PDGF-BB-mediated early signal transductions such as PDGF-Rβ and ERK1/2, JNK, Akt and p38, and cell cycle-regulatory proteins such as cyclins, CDKs and CKIs, as part of the early G_0/G_1–S interphase transition induced in quiescent rat aortic VSMCs by PDGF-BB stimulation were also investigated.

MATERIALS AND METHODS

Materials and Reagents

Hesperetin and bovine serum albumin (BSA) were from Sigma Chemical Co. (MO). Cell culture materials were purchased from Gibco-BRL (MD). Phospho-ERK1/2, ERK1/2, phospho-Akt, Akt, phospho-JNK, JNK, phospho-p38, p38, phospho-Rb, CKI $p21^{cip1}$, $p27^{kip1}$, and p53 antibodies were purchased from New England Biolabs (MA). PDGF-Rβ and phospho-PDGF-Rβ antibodies were obtained from Upstate Biotechnology (NY). α-Actin, cyclin D1, cyclin E, cyclin A, CDK2, CDK4, and CKI $p16^{INK4}$ antibodies were purchased from Santa Cruz (MA). All other chemicals were of analytical grade.

Cell Culture

Rat aortic VSMCs were isolated by enzymatic dispersion according to the modified method of Chamley et al. [1977]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES, and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator. The purity of VSMCs culture was confirmed by immunocytochemical localization of α-smooth-muscle actin. The passage of rat aortic VSMCs used in this experiment was 4–8.

Measurement of Cell Proliferation

The proliferation of rat aortic VSMCs was measured by direct counting and non-radioactive colorimetric WST-1 assay (Premix WST-1, Takara, Japan). In brief, for direct cell counting, rat aortic VSMCs were seeded in 12-well culture plates at 1×10^5 cells/ml and cultured in DMEM with 10% FBS at 37°C for 24 h. Under these conditions, a cell confluence of ~70% was reached. The medium was then replaced by serum-free medium and incubated

for 24 h, then changed with serum-free medium containing various concentrations of hesperetin and incubated for 24 h. The cells were then stimulated with 50 ng/ml PDGF-BB. After 24 h the cells were trypsinized by trypsin-EDTA and counted by using hemocytometer under microscopy. Rat aortic VSMCs proliferation and viability were also determined by WST-1 assay; all of the experimental procedure was performed as recommended by the manufacturer's instructions, and the results were expressed as percentage of control. We also examined which time-point of the PGDF-BB-induced cellular proliferation become resistant to hesperetin's action. After a cell confluence of ~70% was reached, the medium was replaced by serum-free medium and incubated for 48 h. Rat aortic VSMCs were stimulated with 50 ng/ml PDGF-BB for another 24 h, and hesperetin was added at various time-points (0, 3, 6, 9, 12, and 15 h) after PDGF-BB stimulation.

Measurement of DNA Synthesis

DNA synthesis was determined by [^{3}H]-thymidine incorporation assay as previously described [Kim et al., 2005a]. In brief, rat aortic VSMCs were seeded in 24-well culture plates under the same conditions. The medium was then replaced by serum-free medium and incubated for 24 h, then changed with serum-free medium containing various concentrations of hesperetin and incubated for 24 h. The cells were then stimulated with 50 ng/ml PDGF-BB, and 2 μCi/ml of [^{3}H]-thymidine was added to the medium. The reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). Acid-insoluble [^{3}H]-thymidine was extracted into 250 μl of 0.5 M NaOH/well, and this solution was mixed with 3 ml scintillation cocktail (Ultimagold, Packard Bioscience, CT), and quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

Cell Cycle Progression Analysis

Cell cycle was determined as previously described [Kim et al., 2005a]. In brief, rat aortic VSMCs were seeded in 60 mm^2 cell culture dish at 1×10^5 cells/ml and cultured in DMEM with 10% FBS at 37°C for 24 h. Under these conditions, a cell confluence of ~70% was reached. The medium was then replaced by serum-free medium and incubated for 24 h, then changed with serum-free medium containing various concentrations of hesperetin and incubated for 24 h. Rat aortic VSMCs were then stimulated with 50 ng/ml PDGF-BB for another 24 h. To evaluate the change of cell cycle progression in various time points in the presence or absence of hesperetin (100 μM), rat aortic VSMCs were stimulated with PDGF-BB for scheduled time (0, 6, 9, 12, 15, 20, 24, 27, and 30 h). We also examined which time-point in the G_0/G_1–S–G_2/M transitions of the cell cycle become resistant to hesperetin's action. After a cell confluence of ~70% was reached, the medium was replaced by serum-free medium and incubated for 48 h. Rat aortic VSMCs were stimulated with 50 ng/ml PDGF-BB for another 24 h, and hesperetin were added at various time-points (0, 3, 6, 9, 12, and 15 h) after PDGF-BB stimulation. The cells were trypsinized and were centrifuged at 1,500*g* for 7 min. The centrifuged pellets were suspended in 1 ml of 1× PBS and washed twice. The centrifuged pellets were suspended by 70% ethanol and fixed overnight at 4°C. The fixed cells were briefly vortexed and centrifuged at 15,000*g* for 5 min. The ethanol was discarded and the pellets were stained with 0.4 ml propidium iodide (PI) solution (50 μg/ml PI in sample buffer containing 100 μg/ml of RNase A). Before analysis by flow cytometry, each sample was incubated at room temperature for 1 h. The PI-DNA complex in each cell nucleus was measured with FACSCalibur (Becton & Dickinson Co.). The rate of the cell cycle within G_0/G_1, S and G_2/M phase was determined by analysis with the computer program Modfit LT (Verity Software House, Inc.).

Western Blot Analysis and Immunoprecipitation

The immunoblotting of rat aortic VSMCs was performed as previously described [Kim et al., 2005a]. Briefly, rat aortic VSMCs were seeded in 12-well culture plates at 1×10^5 cells/ml and cultured in DMEM with 10% FBS at 37°C for 24 h. Under these conditions, a cell confluence of ~70% was reached. The medium was then replaced by serum-free medium and incubated for 24 h, then changed with serum-free medium containing various concentrations of hesperetin and incubated for 24 h. Then rat aortic VSMCs were stimulated with 50 ng/ml PDGF-BB for 1 min for PDGF-Rβ, 5 min for ERK 1/2, 15 min for Akt and p38, and JNK phosphorylation

assays. For the assay of cyclin A, cyclin D1, cyclin E, CDK2, CDK4, proliferating cell nuclear antigen (PCNA), p53, $p21^{cip1}$, $p16^{INK4}$, and $p27^{kip1}$ expressions and pRb phosphorylation, rat aortic VSMCs were stimulated with 50 ng/ml PDGF-BB for different lengths of time. Rat aortic VSMCs were then lysed with SDS lysis buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT) and protease inhibitor Cocktail Tablet (Roche, Mannheim, Germany). For immunoprecipitation, cell extracts were prepared from rat aortic VSMCs treated with hesperetin (100 μM) followed by PDGF-BB-stimulation for 24 h in ice-cold lysis buffer (complete Lysis-M, Roche, Germany) with protease inhibitor Cocktail Tablet. Lysates were centrifuged in cold temperature for 10 min in a microcentrifuge at maximum speed. Protein determination of the supernatant was performed using a BCA Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer's manual using BSA as a standard. Cell extracts containing 500 μg of the total protein were incubated at 4°C for 2 h under constant rotation containing 500 ng of anti-CDK2 antibodies and 20 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunoprecipitates were washed twice with 500 μl of PBS and lysed in 40 μl 2× SDS-lysis buffer. The lysates were centrifuged at 13,000*g* for 10 min, and the supernatants were collected. Proteins were separated in 7.5–15% SDS polyacrylamide gel (SDS–PAGE) using a Mini-Protein III System (Bio-Rad, CA). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 250 mA with a transfer buffer containing 25 mM Tris–HCl, 192 mM glysine and 20% methanol (pH 8.3). PVDF membrane was blocked with 5% BSA in TBS-T at room temperature for 4 h. The membrane was washed using TBS-T and incubated with primary phospho-ERK1/2 (1:1,000); phospho-Akt (1:1,000); phospho-PLCγ1 (1:1,000); phospho-PDGF-Rβ (1:1,000); CDK2 (1:1,000); CDK4 (1:1,000); cyclin D1 (1:1,000); cyclin E (1:1,000); cyclin A (1:1,000); $p21^{cip1}$ (1:1,000); phospho-pRb (1:1,000); $p27^{kip1}$ (1:1,000); PCNA (1:1,000) and p53 (1:1,000) antibodies at 4°C for overnight and horseradish peroxidase-conjugated IgG secondary antibody (New England Biolabs) at 1:5,000 dilution in BSA/TBS-T buffer at 4°C for 3 h, respectively. After washing, the membrane was detected by chemiluminescent reaction (ECL plus kit, Amersham Pharmacia Biotech, Buckinghamshire, UK) followed by exposure of the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech). The detected proteins were normalized by α-actin or respective total proteins, respectively. The intensities of bands were quantified using a Scion-Image for Window Program (Scion Corporation, MA).

Statistical Analysis

The experimental results were expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was used for multiple comparison (Sigma Stat®, Jandel Co., San Rafael, CA). If there was a significant variation between treated-groups, Dunnett's test was applied. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of Hesperetin on PDGF-BB-Induced Proliferation of Rat Aortic VSMCs

Hesperetin inhibited PDGF-BB-induced proliferation of rat aortic VSMCs in a concentration-dependent manner (Fig. 1A). The number of cells was significantly increased after 50 ng/ml PDGF-BB-treatment ($32.2 \pm 3.6 \times 10^4$ cells/well) compared to the non-stimulated group ($18.1 \pm 2.6 \times 10^4$ cells/well). And the increased cells were significantly reduced to 29.2 ± 1.9, 24.1 ± 2.1, 21.8 ± 2.0, and $18.4 \pm 1.7 \times 10^4$ cells/well at concentrations of 5, 25, 50, and 100 μM, respectively. Similarly the results of WST-1 assay showed the same inhibitory pattern on rat aortic VSMCs proliferation (Fig. 1B). In addition, treatment of rat aortic VSMCs with various concentrations of hesperetin (5, 25, 50, and 100 μM) for 24 h did not show any cytotoxicity to rat aortic VSMCs in serum free medium (Fig. 1C) or apoptotic effect in rat aortic VSMCs after PI and annexin V double staining (data not shown), indicating that the antiproliferative effect of hesperetin on rat aortic VSMCs was not resulted from cytotoxicity or apoptosis.

Effect of Hesperetin on PDGF-BB-Induced DNA Synthesis of Rat Aortic VSMCs

The effect of hesperetin on DNA synthesis was assayed by using [^{3}H]-thymidine incorporation. As shown in Figure 2, 50 ng/ml PDGF-BB potently increased [^{3}H]-thymidine

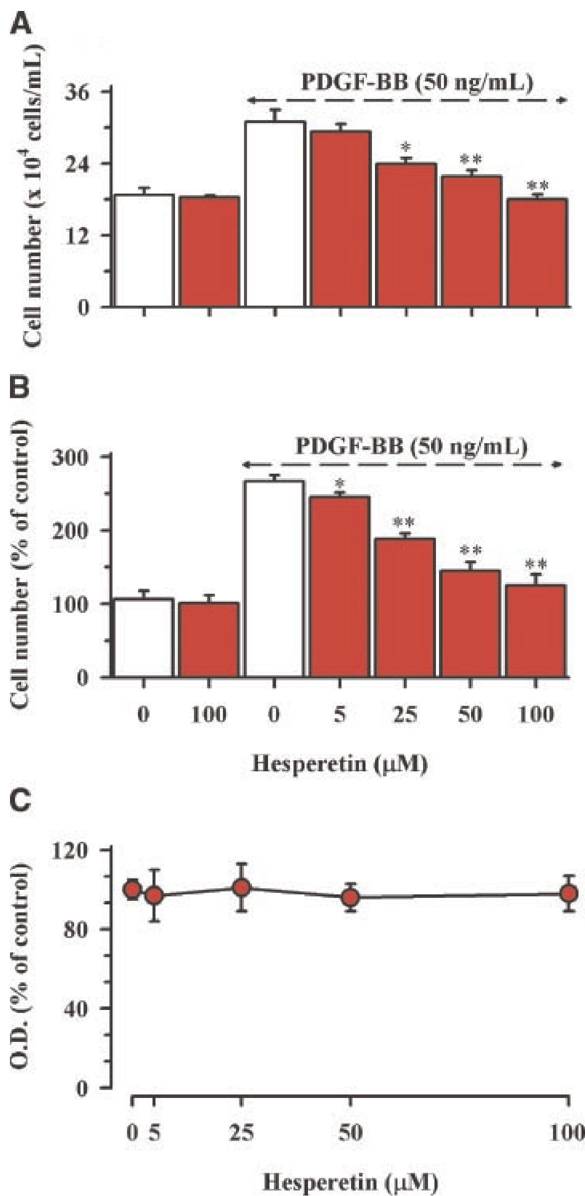

Fig. 1. Effect of hesperetin on PDGF-BB-induced rat aortic VSMCs proliferation and cell viability. **A**: Rat aortic VSMCs were pre-cultured in serum-free medium in the presence or absence of hesperetin (5–100 μM) for 24 h, and then stimulated by 50 ng/ml PDGF-BB for a further 24 h. Then the cells were trypsinized and counted using a hemocytometer. Data are expressed as mean ± SEM (n = 4, $^*P < 0.05$, $^{**}P < 0.01$ vs. stimulus control). **B**: WST-1 reagent was added at 20 h and further incubated for 4 h. Then the absorbance was determined using an ELISA at a wavelength of 450 nm. Cell numbers are expressed as percentage of control (n = 8, $^*P < 0.05$, $^{**}P < 0.01$ vs. stimulus control). **C**: Rat aortic VSMCs viability was determined in the presence of various concentrations of hesperetin in serum starvation by WST-1 assay. Data are expressed as mean ± SEM (n = 8). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

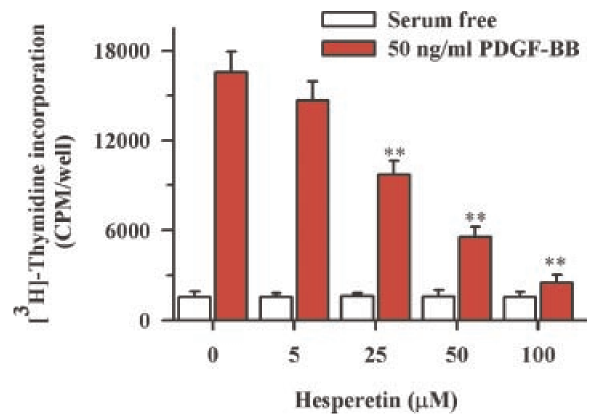

Fig. 2. Effect of hesperetin on [^{3}H]-thymidine uptake into rat aortic VSMCs stimulated by PDGF-BB. After confluence, the cells were cultured in serum-starved medium in the presence or absence of hesperetin (5–100 μM) for 24 h, and then exposed to 50 ng/ml PDGF-BB for 20 h before 2 μCi/ml [^{3}H]-thymidine was added to the medium. Four hours later, labeling reaction was terminated and quantified using a liquid scintillation counter. Data are expressed as mean ± SEM (n = 4, $^{**}P < 0.01$ vs. stimulus control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

incorporation from 1,563 to 16,540 cpm/well. Hesperetin significantly inhibited the PDGF-BB-induced [^{3}H]-thymidine incorporated into DNA in a concentration-dependent manner. The inhibition percentages were 11.2 ± 8.4%, 41.2 ± 6.2%, 66.5 ± 4.2%, and 85.1 ± 2.3% at the concentrations of 5, 25, 50, and 100 μM, respectively.

Effect of Hesperetin on PDGF-BB-Induced PDGF-Rβ Phosphorylation in Rat Aortic VSMCs

After pre-incubation of rat aortic VSMCs with various concentrations of hesperetin for 24 h, rat aortic VSMCs were stimulated with PDGF-BB (50 ng/ml) for 1 min, which caused a marked phosphorylation of PDGF-Rβ. However, treatment of the rat aortic VSMCs with hesperetin had no significant effect on PDGF-Rβ phosphorylation (Fig. 3).

Effects of Hesperetin on PDGF-BB-Induced ERK1/2, Akt, JNK, and p38 Phosphorylations in Rat Aortic VSMCs

To demonstrate whether hesperetin affects the downstream intracellular signal transduction pathways of PDGF-BB, parallel determination of the phosphorylations of ERK1/2, Akt, JNK, and p38 were performed. As shown in Figure 4, hesperetin had no effect on PDGF-BB-stimulated ERK1/2, Akt, JNK, and p38

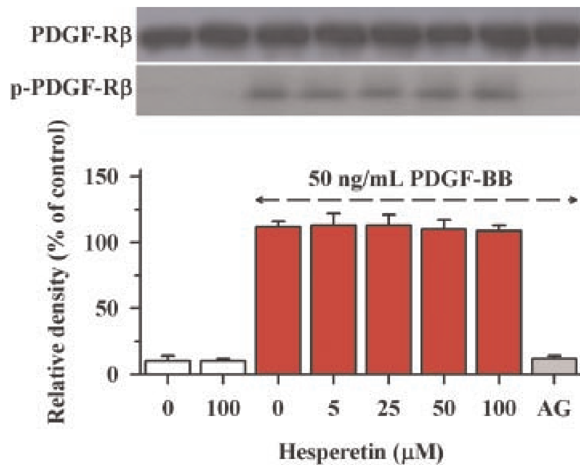

Fig. 3. Effect of hesperetin on PDGF-BB-induced PDGF-Rβ phosphorylation in rat aortic VSMCs. Confluent cells were precultured in the presence or absence of hesperetin (5–100 μM) or AG1295 (20 μM) in serum-free medium for 24 h, and then stimulated by 50 ng/ml PDGF-BB at 37°C for 1 min. The cells were lysed, and proteins were analyzed using 7.5% SDS–PAGE and immunoblotting. Total PDGF-Rβ was used for normalization. After densitometric quantification, data are expressed as mean ± SEM (n = 3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

phosphorylations, indicating that the antiproliferative effect of hesperetin was not involved in early signaling transductions.

Effect of Hesperetin on PDGF-BB-Induced Cell Cycle Progression in Rat Aortic VSMCs

Effect of hesperetin on cell cycle progression was also analyzed (Fig. 5A). The serum-deprivation of rat aortic VSMCs in primary culture for 24 h resulted in an approximately $94.5 \pm 0.6\%$ synchronization of the cell cycle in the G_0/G_1 phase. The percentage of cells in S phase was increased from 1.26 ± 0.5 to $14.7 \pm 1.4\%$ for 24 h after PDGF-BB (50 ng/ml) addition. In contrast, hesperetin-treated cells showed a significant blocking of cell cycle progression. Hesperetin at concentrations of 5, 25, 50, and 100 μM decreased the percentages of cells in S phase to $11.7 \pm 2.5\%$, $8.8 \pm 1.5\%$ ($P < 0.05$, n = 3, duplicate) $4.8 \pm 0.5\%$ ($P < 0.01$, n = 3, duplicate), and $1.2 \pm 1.1\%$ ($P < 0.01$, n = 3, duplicate), respectively. This finding indicates that hesperetin may act at the early events of the cell cycle to be effective against DNA synthesis.

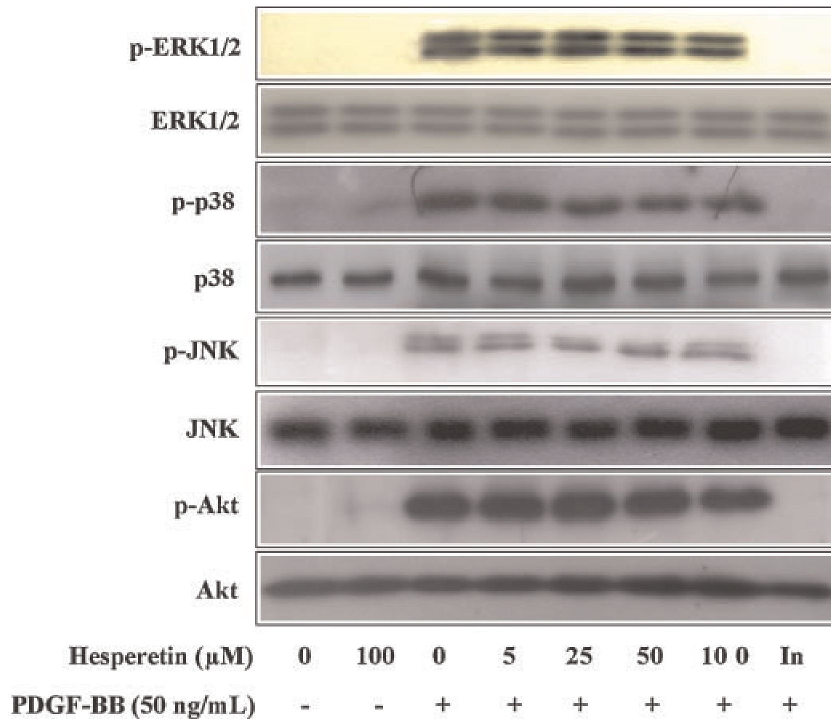

Fig. 4. Effect of hesperetin on ERK1/2, Akt, p38, and JNK phosphorylations in rat aortic VSMCs. Confluent cells were precultured in the presence or absence of hesperetin (5–100 μM), U0126 (an ERK1/2 inhibitor, 10 μM), LY294002 (an Akt inhibitor, 50 μM), SP600125 (a JNK inhibitor, 20 μM), or SB203580 (a p38 inhibitor, 20 μM) in serum-free medium for 24 h. The cells were stimulated by 50 ng/ml PDGF-BB for 5 min for ERK1/2, 15 min for Akt and p38, and 30 min for JNK assay at 37°C, respectively. The cells were lysed, and proteins were analyzed using SDS–PAGE and immunoblotting. Respective total protein were used for normalization. (n = 3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The time-course assay of PDGF-BB-induced cell cycle progression can be observed in Figure 5B, the cell cycle progressions in various time-points (0, 6, 9, 12, 15, 20, 24, 27, and 30 h) after PDGF-BB-stimulation were examined; after PDGF-BB-treatment, the cells were keeping in G_0/G_1 phase at the time-point of 12 h, transiting into S phase and starting DNA synthesis since the time-point of 15 h, reaching a peak of DNA synthesis about 20 h, and finishing a cycle at the time-point of 30 h in control group. In the presence of hesperetin (100 μM), the cell cycle distribution did not change following PDGF-BB-stimulation, indicating the cell cycle progression was completely blocked. As Figure 5C,D shows, treatments of hesperetin at 0, 3, and 6 h after PDGF-BB stimulation were able to completely block cell cycle progression from G_0/G_1 to S phase and cell proliferation; however, when treatments of hesperetin at 9 h or later after PDGF-BB stimulation, the cell cycle progressions and cell proliferation were revealed to be resistant to hesperetin's action. Therefore, when taken these results together, it is clear that hesperetin must be targeting on some signaling transductions evoked in the G_0/G_1–S interphase rather than those involved in S or G_2/M phase.

Effects of Hesperetin on PDGF-BB-Induced Cyclins, CDKs, and PCNA Expressions as well as Rb Protein Phosphorylation in Rat Aortic VSMCs

Cell cycle progression is tightly regulated through a complex network of positive and negative cell cycle regulatory molecules, such as CDKs and cyclins. To characterize the mechanism of hesperetin-induced cell cycle arrest, the effects of hesperetin on cell cycle events such as cyclin D, cyclin E, CDK2, and CDK4 expressions were determined. Cyclin D was barely detectable in quiescent rat aortic VSMCs and was induced 3 h after addition of PDGF-BB (50 ng/ml), while cyclin E was constitutively expressed in serum-starved quiescent rat aortic VSMCs and was upregulated since the addition of PDGF-BB for 12 h. Treatment of hesperetin inhibited the expressions of cyclin D and cyclin E in a time- and concentration-dependent manner. As shown in Figure 6, CDK2 was constitutively expressed in serum-starved quiescent rat aortic VSMCs and was induced increasingly by addition of PDGF-BB (50 ng/ml). Treatment of hesperetin only inhibited the expression of CDK2 in a concentration-dependent manner, while has no effect on CDK4 expression (data not shown).

In late G_1 phase, cells reach the so-called restriction point. Beyond this point, cells are

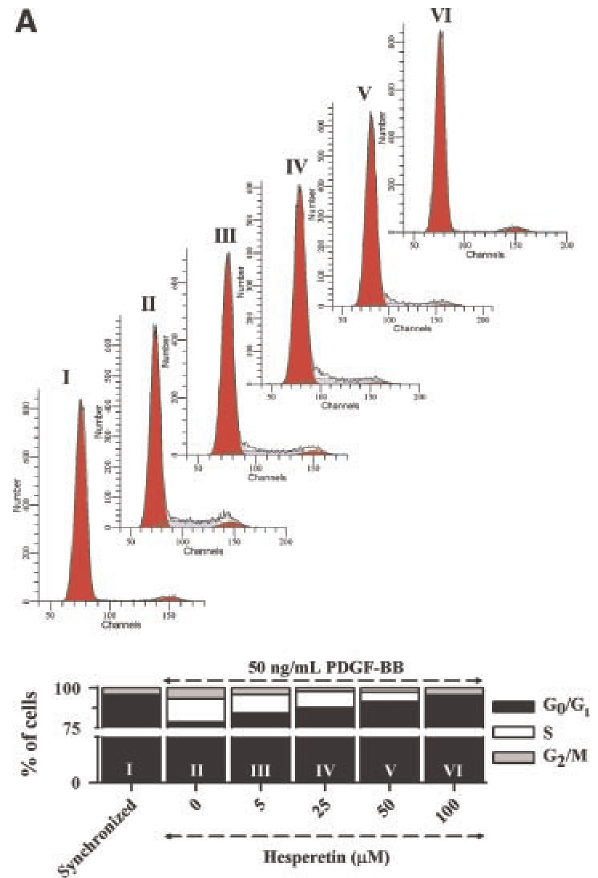

Fig. 5. Effect of hesperetin on PDGF-BB-induced cell cycle progression in rat aortic VSMCs. Rat aortic VSMCs were seeded in six-well culture plates and cultured in DMEM with 10% FBS at 37°C for 24 h. **A**: The medium was then replaced by serum-free medium and incubated for 24 h, then changed with serum-free medium containing various concentrations of hesperetin and incubated for 24 h. The cells were then stimulated with 50 ng/ml PDGF-BB for 24 h. **B**: After being treated with hesperetin for 24 h, rat aortic VSMCs were stimulated with PDGF-BB for various times (0, 6, 9, 12, 15, 24, 27, and 30 h). **C**: After cultured in serum-free and incubated for 48 h, rat aortic VSMCs were stimulated with 50 ng/ml PDGF-BB for 24 h and hesperetin (100 μM) were added in various time-points (0, 3, 6, 9, 12, and 15 h) after PDGF-BB-stimulation. The cells were treated as described in Materials and Methods Section and the individual nuclear DNA content was reflected by fluorescence intensity of incorporated propidium iodide. **D**: After cultured in serum-free and incubated for 48 h, rat aortic VSMCs were stimulated with 50 ng/ml PDGF-BB for 24 h and hesperetin (100 μM) were added in various time-points (0, 3, 6, 9, 12, and 15 h) after PDGF-BB-stimulation. The cells were collected and counted under a hemocytometer. Each item is derived from a representative experiment, where data from at least 11,000 events were obtained. Results are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

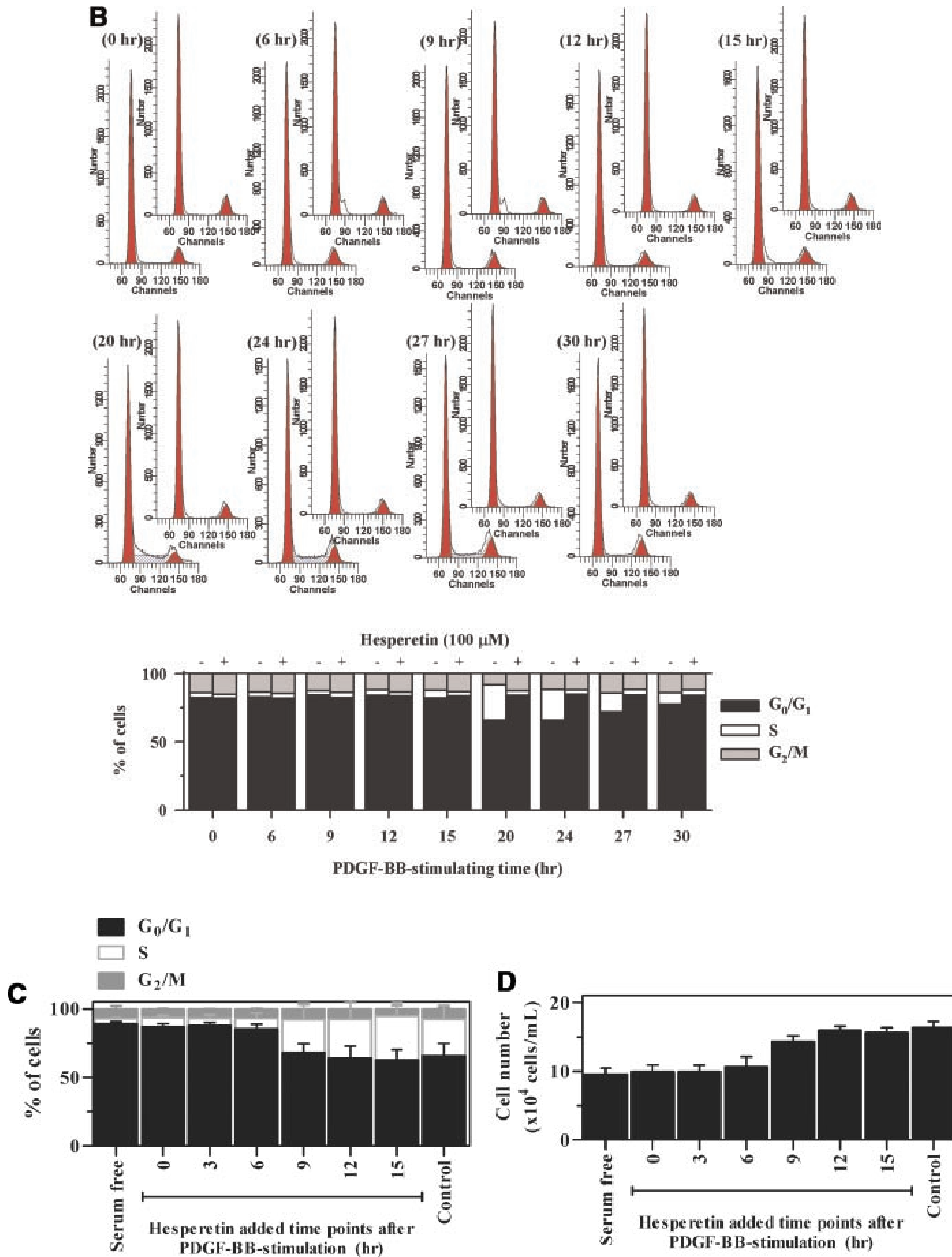

Fig. 5. (*Continued*)

committed to DNA replication, and further cell cycle progression proceeds independent of growth factor stimulation. Rb protein is a key component of the molecular network controlling the restriction point. Hypophosphorylated pRb binds the E2F family of transcription factors, and thus inhibits transcription of E2F-responsive genes necessary for cell cycle progression. Therefore, we investigated the impact of hesperetin on pRb hyperphosphorylation. As shown

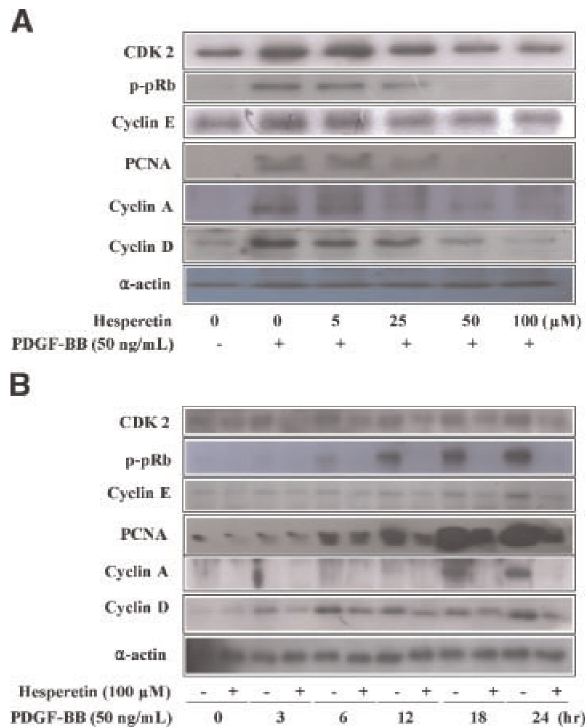

Fig. 6. Effects of hesperetin on expressions or activations of cell-cycle regulatory proteins in rat aortic VSMCs treated with PDGF-BB. **A**: Quiescent rat aortic VSMCs were stimulated with PDGF-BB in the absence or presence of various concentrations of hesperetin for 24 h. Then the cells were lysed, and proteins were analyzed using SDS–PAGE and immunoblotting. **B**: Quiescent rat aortic VSMCs were stimulated with PDGF-BB in the absence or presence of 100 μM hesperetin for indicated times over a 3–24 h period. The cells were lysed, and proteins were analyzed using SDS–PAGE and immunoblotting. Total α-actin was used for normalization. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in Figure 6, PDGF-BB stimulated a continuous increase of pRb hyperphosphorylation over 18 h, first detectable after 6 h, and pretreatment of cells with hesperetin (100 μM) completely blocked Rb protein hyperphosphorylation. At time point of 24 h, hesperetin also caused a concentration-dependent inhibition of Rb protein hyperphosphorylation.

Induction of cyclin A synthesis in response to PDGF-BB occurs as cells enter S phase and the synthesis of cyclin A is mainly controlled at the transcriptional level, involving E2F and other transcription factors. As shown in Figure 6, in the absence of hesperetin, there was a robust induction of cyclin A at 18 h after PDGF-BB (50 ng/ml) addition. However, no induction of cyclin A was detected in the presence of hesperetin at 18 and 24 h after addition of PDGF-BB. In addition, at 24 h after PDGF-BB addition, cyclin A expression was inhibited by hesperetin in a concentration-dependent manner. These results further confirm that the inhibition of Rb protein hyperphosphorylation by hesperetin leads to an inhibition of cyclin A induction. Furthermore, expression of PCNA, which is synthesized as a phospho-pRb-mediated gene product in the early G_0/G_1 and S phase of the cell cycle, was also inhibited by hesperetin in the same pattern as the inhibition of Rb protein phosphorylation.

Effect of Hesperetin on CKIs Expressions in PDGF-BB-Stimulated Rat Aortic VSMCs

The effects of hesperetin on the expressions of CKIs such as $p16^{INK4}$, $p21^{cip1}$, $p27^{kip1}$, and p53 were further determined. The $p27^{kip1}$ protein level was rapidly downregulated by PDGF-BB and fell to undetectable level at 24 h. In contrast, $p27^{kip1}$ level was unaffected in the presence of hesperetin and, even at 24 h after PDGF-BB addition and comparable with that in the quiescent cells (Fig. 7). The total $p27^{kip1}$ protein showed a decrease at 24 h after PDGF-BB-stimulation in the absence of hesperetin but not in its presence. To evaluate CDK2 activation, we have performed immunoprecipitation assay, it can be observed that CDK2-bound $p27^{kip1}$ protein levels dropped markedly at 24 h after PDGF-BB stimulation in the absence of hesperetin. However, for the hesperetin-treated samples, CDK2-bound $p27^{kip1}$ protein levels were comparable with those in serum-free starved rat aortic VSMCs. Consistent with this finding, Rb protein phosphorylation was high in PDGF-BB-stimulated rat aortic VSMCs but remained low in rat aortic VSMCs treated with hesperetin and PDGF-BB. These results indicate that the inhibition of Rb protein phosphorylation observed may be due to a block in CDK2 activities by high level of $p27^{kip1}$ in the presence of hesperetin. The $p21^{cip1}$ level was induced at 24 h after PDGF-BB addition. Pretreatment of hesperetin did not show any changes in this pattern, indicating that $p21^{cip1}$ is not regulated by the presence of hesperetin. Accordingly p53, an upstream regulator of $p21^{cip1}$, was also not affected by the presence of hesperetin (data not shown). Furthermore, $p16^{INK4}$, which is a relative weak CDK inhibitor in the regulation of VSMCs growth, was constitutively expressed in VSMCs. Treatment of PDGF-BB did not change $p16^{INK4}$ level, even after

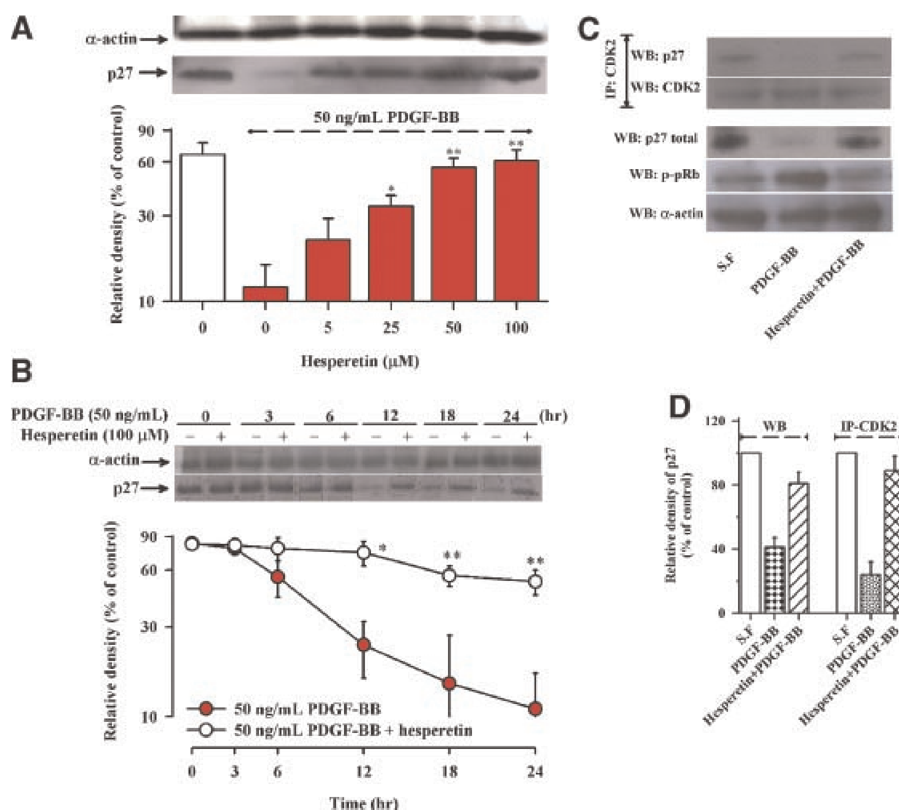

Fig. 7. Effects of hesperetin on expression of CKI $p27^{kip1}$ in rat aortic VSMCs treated with PDGF-BB. **A**: Quiescent rat aortic VSMCs were stimulated with PDGF-BB in the absence or presence of various concentrations of hesperetin for 24 h. **B**: Quiescent rat aortic VSMCs were stimulated with PDGF-BB in the absence or presence of 100 μM hesperetin for indicated times over 3–24 h period. The cells were lysed, and proteins were analyzed using SDS–PAGE and immunoblotting. **C**,**D**: To determine the association between CDK2 and $p27^{kip1}$, 500 μg of protein extracts were first immunoprecipitated with anti-CDK2 antibody and then the immunoprecipitates were examined by Western blot analysis using anti-$p27^{kip1}$ antibodies. $p27^{kip1}$ total and phospho-pRb were determined directly by Western blot. Total α-actin was used for normalization. After densitometric quantification, data are expressed as mean ± SEM (n = 4, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. stimulus control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

24 h stimulation, and hesperetin had no effect on the $p16^{INK4}$ expression (data not shown).

DISCUSSION

Many epidemiological studies have found an inverse relationship between fruit and vegetable intake and the risk of several chronic proliferative diseases, including cardiovascular diseases, and cancer [Ross and Kasum, 2002; Arts and Hollman, 2005; Scalbert et al., 2005; Stangl et al., 2007]. A number of in vitro and in vivo studies on the flavonoids, the major biological constituents present in fruit and vegetable, have revealed that their beneficial effect on such proliferative diseases may be due to the modulation of abnormal cell proliferation [Ahn et al., 1999; Kim et al., 2002; Rosenkranz et al., 2002; Surh, 2003; Kim et al., 2005a]. In the present study, therefore, we have investigated the antiproliferative effect of hesperetin, a bioflavonoid, on rat aortic VSMCs. In parallel, the cell type- and mitogen-specific inhibition of hesperetin was also characterized. We demonstrated that hesperetin could inhibit mitogenic stimulation of VSMC and other cells in culture and its effect varied with cell type and with nature of mitogen; in particular, the inhibition of PDGF-BB-induced rat aortic VSMCs proliferation might be due to a G_0/G_1 arrest in association with the upregulation of $p27^{kip1}$ expression and downregulation of cyclin A, cyclin D, cyclin E, CDK2, and PCNA protein expressions as well as retinoblastoma protein phosphorylation.

In the proliferation assay, we clearly demonstrated that hesperetin potently inhibited proliferation of rat aortic VSMCs (Fig. 1A,B) and DNA synthesis (Fig. 2) induced by PDGF-BB. The inhibitory effect of hesperetin on incorporation of [^{3}H]-thymidine into the cells gradually

declined as the delay between the rat aortic VSMCs treated with or without hesperetin in the presence of PDGF-BB. In addition, the same inhibitory effect of hesperetin can be also observed in bFGF-induced rat aortic VSMCs as well as PDGF-BB- and EGF-stimulated fibroblast in the same concentration ranges (supplement data). However, the antiproliferative effect of hesperetin was not due to cellular cytotoxicity or apoptosis, which was demonstrated by WST-1 assay (Fig. 1C) and flow cytometry assay (data not shown). Our results were also in agreement with Choi et al. reports that hesperetin inhibited vessel structure formation in mouse embryonic stem cells without any cytotoxicity in the same concentration ranges as we used (1–100 μM) [Choi et al., 2006]. It has been reported that hesperetin can induce significant apoptosis in the promyelocytic cells HL-60, but has no effect in the mature monocytic cells THP-1 and human PMN cells [Chen et al., 2003]. On the contrary, Pollard et al. [2006] have also reported that hesperetin could protect peroxynitrite-induced cytotoxicity in firoblasts. Therefore, it seems hesperetin can inhibit mitogenic stimulation of VSMC and other cells in culture, and its effects vary with cell type and with nature of stimuli.

Previous studies have suggested that some flavonoid compounds such as EGCG, catechins, luteolin, and kaempferol inhibited PDGF-BB-mediated VSMCs proliferation by prevention of PDGF-Rβ phosphorylation [Ahn et al., 1999; Rosenkranz et al., 2002; Sachinidis et al., 2002; Weber et al., 2004; Kim et al., 2005a,b]. Even at the highest concentration tested (100 μM, Fig. 3), hesperetin did not affect PDGF-Rβ phosphorylation in PDGF-BB-stimulated rat aortic VSMCs. This indicates that hesperetin may affect downstream signal transduction instead of PDGF-Rβ phosphorylation.

Mitogen-activated protein (MAP) kinases, including ERK1/2, JNK, and p38, are important players in the early intracellular mitogenic signal transductions for cell growth and have been implicated in proliferation and migration of various cell types induced by PDGF-BB [Graf et al., 1997]. Hesperetin failed to inhibit PDGF-BB-stimulated ERK1/2, JNK, and p38 phosphorylations (Fig. 4) and also had no effect on Akt phosphorylation, which is another downstream signal involved in PDGF-BB-mediated proliferation [Inui et al., 1994; Stabile et al., 2003]. These results indicate that the PDGF-BB-mediated early signals activations such as ERK1/2, JNK, p38, and Akt may be not involved in the inhibition of hesperetin on rat aortic VSMCs proliferation.

To further investigate the pattern of the antiproliferative effect of hesperetin, flow cytometry analysis was performed. As revealed by flow cytometry assay, the antiproliferative effect of hesperetin was associated with an accumulation of cells in G_0/G_1 phase of the cell cycle (Fig. 5A). Whereas PDGF-BB-stimulated cells transited into S phase at 15 h, hesperetin (100 μM) completely blocked PDGF-BB-induced cell cycle progression (Fig. 5B). Moreover, hesperetin added at 0, 3, or 6 h after PDGF-BB stimulation also completely blocked cell cycle progression from G_0/G_1 to S phase and cell proliferation (Fig. 5C,D), but hesperetin treatment at 9 h after PDGF-BB addition had no effect. Taken together, these results indicate that hesperetin must be targeting a signaling transductions event(s) evoked in the G_0/G_1–S interphase rather than those involved in S or G_2/M phase.

Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle regulatory proteins (including CDKs, cyclins, CKIs, p53, and pRb) provides an important mechanism for inhibition of cell growth [Sherr, 1996; Sherr and Roberts, 1999; Sherr, 2000; Braun-Dullaeus et al., 2004]. The fact that hesperetin permitted VSMCs to arrest in G_0/G_1 phase suggests that modulation of several cell cycle regulatory proteins may occur after hesperetin treatment. CDK2 and CDK4 are key mediators during the G_0/G_1 to S phase progression of the cell cycle by forming complexes with cyclin A, E, and D1 [Sherr and Roberts, 1999; Jirawatnotai et al., 2004; Martin et al., 2005]. These complexes phosphorylated a large number of proteins, resulting in hyperphosphorylation of pRb, which then releases transcription factors that promote DNA synthesis [Sherr, 1996; Dzau et al., 2002]. As shown in Figure 6, hesperetin not only inhibited CDK2, cyclin A, cyclin D, and cyclin E expressions but also suppressed Rb protein phosphorylation in a concentration-dependent manner, whereas it had no effect on CDK4 expression (data not shown). Although a number of CDKs are known to phosphorylate pRb, the suppression of CDK2 alone may be sufficient to prevent Rb protein hyperphosphorylation [Akiyama

et al., 1992; Connell-Crowley et al., 1998]. In addition, several reports suggest that inhibition of CDK4 may not be necessary to arrest cell cycle progression [Sasaguri et al., 1996; Brooks et al., 1997]. Therefore, the inhibition of CDK2, cyclin A, cyclin D, and cyclin E expressions as well as Rb protein phosphorylation may be sufficient to achieve cell cycle arrest. According with above finding, expression of PCNA, which is synthesized as a phospho-pRb-mediated gene product in the early G_0/G_1 and S phase of the cell cycle [Tomita et al., 2005], was also inhibited by hesperetin in a concentration-dependent manner.

The CDK inhibitors such as $p16^{INK4}$, $p21^{cip1}$, and $p27^{kip1}$ are important regulators of cyclin-CDK complexes. They can tightly bind and inhibit the kinase activities of several cyclin-CDK complexes such as cyclin D-CDK4/6, cyclin E-CDK2, and cyclin A-CDK2, and arrest cell growth at the G_0/G_1 and G_1/S boundary [Sherr and Roberts, 1999; Dzau et al., 2002; Ortega et al., 2002; Coqueret, 2003a; Braun-Dullaeus et al., 2004; Martin et al., 2005]. Interestingly, the expressions of p53, $p21^{cip1}$, and $p16^{INK4}$ were relatively unaffected by hesperetin (data not shown). In contrast, hesperetin prevented the downregulation of $p27^{kip1}$ after PDGF-BB stimulation (Fig. 7A). Interestingly, there was an inverse correlation between cell cycle progression and $p27^{kip1}$ downregulation (Figs. 5B and 7B), which indicated an essential role of $p27^{kip1}$ in the antiproliferative effect of hesperetin. Total $p27^{kip1}$ protein was decreased at 24 h after PDGF-BB-stimulation in the absence of hesperetin but not in its presence (Fig. 7C). Immunoprecipitation assays showed that CDK2-bound $p27^{kip1}$ protein levels dropped markedly at 24 h after PDGF-BB stimulation in the absence of hesperetin, but were comparable with those in serum-free starved rat aortic VSMCs for hesperetin-treated samples. Consistent with this finding, Rb protein phosphorylation was high in PDGF-BB-stimulated rat aortic VSMCs but remained low in rat aortic VSMCs treated with hesperetin and PDGF-BB (Fig. 7C,D). These results clearly indicate that the elevated levels of $p27^{kip1}$ protein contribute to the inhibition of CDK2 activity in the presence of hesperetin. It has been reported that $p27^{kip1}$ abrogated the activity of both CDK2 and CDK4 as well as VSMCs proliferation in vitro and in vivo after balloon-injury in pig [Tanner et al., 2000]. It has also been reported that some drugs with upregulation of $p27^{kip1}$ expression exhibited potent antiproliferative activity on VSMCs proliferation [Chen et al., 1997; Marra et al., 2000; Tanner et al., 2000]. Thus, our finding that hesperetin selectively upregulates $p27^{kip1}$ expression is consistent with its greater inhibitory effects on multiple cell cycle proteins expressions and activations. Further evaluation of in vivo efficiency of hesperetin on VSMCs proliferation in rat carotid artery injury models is currently in preparation.

It has been reported that volunteers given a commercial orange juice reached maximum plasma concentration of 1.28–2.72 μM hesperetin [Manach et al., 2003; Nielsen et al., 2006]. In addition, a recent study revealed that orally administrated-hesperetin at a dose of 135 mg was rapidly absorbed and its concentration in plasma reached a peak of 4.2 μM in human subjects [Kanaze et al., 2007]. Concerning the strong inhibitory effect of hesperetin on VSMCs proliferation, this effect is related to the high concentrations of hesperetin treatment (>10 μM). This raises the question of whether the observed antiproliferative effect of hesperetin at higher concentrations is exact and of value in vivo. Because hesperetin can bind to membrane proteins and partition within cells, however, the effective cellular concentration achieved remains to be determined. In fact, we and others have observed that oral administered hesperetin in rat significantly inhibited platelet aggregation ex vivo [McGregor et al., 1999; Jin et al., 2007]. In addition, it has also been reported that micronized purified flavonoids fraction (MPFF, which was commercialized as Daflon® 500 mg, or Arvenum® 500, Capiven, Detralex, Variton, Ardium, and Venitol), consisting of 90% diosmin and 10% hesperidin, significantly inhibited tumor size in vivo [Tanaka et al., 1997a,b].

In summary, our study demonstrates that hesperetin could inhibit mitogenic stimulation of VSMC and other cells in culture and its effect varied with cell type and with nature of mitogen; in particular, hesperetin inhibits PDGF-BB-induced rat aortic VSMCs proliferation via G_0/G_1 arrest in association with the downregulation of the expressions of retinoblastoma protein and cyclin A, cyclin D, cyclin E, CDK2 as well as PCNA proteins, and upregulation of $p27^{kip1}$. This beneficial property of hesperetin may be of importance in its

beneficial effect on cardiovascular diseases and cancer, and may contribute to the beneficial effects of grapefruits and oranges in vivo.

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