

Effects of hesperetin on platelet-derived growth factor-BB-induced pulmonary artery smooth muscle cell proliferation

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Abstract. Hesperetin is a natural flavonoid, which has been reported to exert various biological activities and positive health effects on mammalian cells. The present study aimed to investigate the effects of hesperetin on the proliferation of primary cultured rat pulmonary artery smooth muscle cells (PASMCs), and to elucidate the possible underlying molecular mechanisms. The results of the present study indicated that hesperetin was able to inhibit the proliferation and DNA synthesis of platelet-derived growth factor-BB (PDGF-BB)-induced PASMCs in a dose- and time-dependent manner, without exerting cell cytotoxicity. In addition, hesperetin blocked the progression of the cell cycle from G₀/G₁ to S phase, which was correlated with the decreased mRNA expression levels of cyclin D1, cyclin E, cyclin-dependent kinase (CDK)2 and CDK4, and the increased mRNA expression levels of p27. Furthermore, the anti-proliferative effects of hesperetin were associated with suppression of the AKT/glycogen synthase kinase (GSK)3 β and p38 signaling pathway, but were not associated with the extracellular signal-regulated kinases 1/2 and c-Jun N-terminal kinases signaling pathways. These results suggested that hesperetin may inhibit PDGF α -BB-induced PASMC proliferation via the AKT/GSK3 β signaling pathway, and that it may possess therapeutic potential for the treatment of pulmonary vascular remodeling diseases.

Introduction

Pulmonary arterial hypertension (PAH) is a complex, multifactorial disorder in which pulmonary arterial obstruction increases pulmonary vascular resistance, resulting in an overload of the right ventricle and progressive right-sided heart dysfunction (1,2). Excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) is a hallmark feature of pulmonary vascular remodeling, which is a critical factor in the pathogenesis and development of PAH (3,4). Various stimuli are able to induce the proliferation of PASMCs, resulting in arterial wall remodeling and PAH (5). As a potential mitogen and chemoattractant, platelet-derived growth factor (PDGF) has previously been demonstrated to have a crucial role in the pathogenesis of PAH. In addition, the upregulated expression levels of PDGF and PDGF receptor have been correlated with PAH in various experimental animal models (6,7) and in humans (8,9). There are numerous members of the PDGF protein family: A, B, C and D. PDGF-BB is a potent inducer of vascular smooth muscle cell (VSMC) proliferation, and has been proposed to have important roles in the development of atherosclerosis, lung fibrosis and PAH (10-12).

Hesperetin is a member of the flavanone subclass of flavonoids, which exists naturally in its glycoside form, hesperedin. Hesperetin has previously been reported to exert various biological activities, including anti-cancer, anti-oxidative, anti-inflammatory and neuroprotective effects (13-19). A previous study demonstrated that hesperetin was able to alleviate the inhibitory effects of high-level glucose on the osteoblastic differentiation of periodontal ligament stem cells by regulating the levels of reactive oxygen species, and the phosphoinositide 3-kinase/AKT and β -catenin signaling pathways (20). In addition, hesperetin may induce G₁ phase cell cycle arrest in MCF-7 human breast cancer cells by regulating cyclin-dependent kinase (CDK)4 and p21 expression (15), and may inhibit the proliferation of breast cancer cells by downregulating the expression of glucose transporter 1, and the phosphorylation of the insulin receptor- β subunit and AKT (13). However, the effects of hesperetin on the proliferation of PASMCs remain largely unknown.

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The present study aimed to investigate whether hesperetin could inhibit the proliferation of PDGF-BB-induced PSMCs, and to examine the underlying molecular mechanisms.

Materials and methods

Materials. Hesperetin (98% purity, as determined by high-performance liquid chromatography analysis) was purchased from Shanghai Winherb Medical S&T Development Co., Ltd. (Shanghai, China). Recombinant human PDGF-BB was obtained from Peprotech, Inc. (Rocky Hill, NJ, USA). Cell Counting kit (CCK)-8 was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Cell proliferation enzyme-linked immunosorbent assay (ELISA) and bromodeoxyuridine (BrdU) proliferation assay kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany). TRIzol[®] was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibodies targeting total (T) and phosphorylated (P) forms of extracellular signal-regulated kinases (ERK)1/2 (cat. nos. 4695 and 4370, respectively), T-p38 (cat. no. 92120), P-p38 (cat. no. 45110), T-c-Jun N-terminal kinases (T-JNK) (cat. no. 9258), P-JNK (cat. no. 4668), P-AKT (cat. no. 4691), T-AKT (cat. no. 4060), T-glycogen synthase kinase (T-GSK)3 β (cat. no. 9315), P-GSK3 β (cat. no. 9322) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. 2118) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Adult male Sprague Dawley rats (weight, 150–200 g) were obtained from Wuhan University Center for Animal Experiment (Wuhan, China). Animals were kept under a 12-h light/dark cycle and maintained at a constant temperature of 22°C with free access to food and water. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Renmin Hospital of Wuhan University (Wuhan, China).

PASMC isolation and cell culture. Sprague-Dawley rats (weight, 150–200 g) were intraperitoneally administered 1% sodium pentobarbital (50 mg/kg; Sinopharm Chemical Reagent Co., Shanghai, China). Subsequently, the pleura of each rat were rapidly sectioned, and the heart and lungs were removed and transferred to a petri dish filled with phosphate-buffered saline, in order to wash off the residual blood. To separate the pulmonary artery, the outer fibrous arterial connective tissue was stripped under a microscope (CKX41; Olympus, Tokyo, Japan) using tweezers, and was rinsed several times in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific). The artery was subsequently cut into 1 mm sections and was digested in a centrifuge tube pre-filled with 0.2% collagenase I. Every 20 to 30 min, the mixture was observed and gently agitated. The total duration of the digestion was 2–4 h. When the arterial fragments had been digested, the samples were centrifuged at 195 x g for 5 min, and the supernatant was discarded. The pellet was rinsed with DMEM/F12 supplemented with 20% fetal bovine serum (FBS) (both from Gibco) and was cultured at 37°C in an incubator containing 5% CO₂. After 4–5 days, the cells were passaged and grown in DMEM/F12 supplemented with 10% FBS. The purity of the PASMC cultures was assessed by immunocytochemical localization of α -smooth muscle

actin according to a previously described procedure (21). The cells from passages 4 and 10 were used in the present study. The PSMCs were grown to 70–80% confluence in 96-well microplates and were then subjected to serum starvation for 24 h prior to experimentation.

Drug administration. After 24 h serum starvation, the cells were pretreated with various concentrations of hesperetin for 1 h. They were subsequently treated with PDGF-BB (20 ng/ml) for 12, 24 and 48 h in the presence of hesperetin.

Measurement of cell proliferation and DNA synthesis. Cell proliferation was determined using the CCK-8 assay, according to the manufacturer's protocol. The cells were treated with PDGF-BB (20 ng/ml) for 12, 24 and 48 h in the presence of hesperetin, and incubated with CCK-8 for the final 3 h. Cell proliferation was determined by measuring the absorbance at 450 nm. DNA synthesis was determined using a BrdU assay. Briefly, BrdU labeling mixture was added to each well and the cells were incubated for 2 h. Finally, DNA synthesis was determined by measuring the incorporation of BrdU, using a cell proliferation ELISA kit as previously described (21) and a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Evaluation of cell viability. The toxicity of hesperetin on PSMCs was determined using the trypan blue exclusion method (Sinopharm Chemical Reagent Co.). After 12-, 24- and 48-h incubations with hesperetin (12.5–100 μ M), the PSMCs were removed from the culture and the cells that excluded 0.4% trypan blue were counted in an automated cell counter (Invitrogen; Thermo Fisher Scientific, Inc.) (21).

Cell cycle analysis. The cell cycle distribution was determined using a Cell Cycle and Apoptosis Analysis kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) were performed. Once the PSMCs had reached 70–80% confluence in six-well plates, they were subjected to serum starvation for 24 h. The cells were subsequently pre-incubated with hesperetin (100 μ M) for 1 h and subsequently treated with PDGF-BB (20 ng/ml) for 24 h prior to analysis. The cells were then trypsinized (Hyclone Laboratories, Inc., Logan, UT, USA) and fixed with 70% ethanol overnight. The fixed cells were collected by centrifugation at 438.75 x g for 5 min, washed once in phosphate-buffered saline, incubated with 0.5 ml PI staining buffer and analyzed using FACS. Cell cycle distributions were analyzed using Multicycle AV software, version 2.50 (Phoenix Flow Systems, San Diego, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (qPCR). After 24 h serum starvation and preincubation with 100 μ M hesperetin for 1 h, the cells were treated with PDGF-BB for 24 h in the absence or presence of hesperetin. Total RNA was extracted from the PSMCs using TRIzol[®] (Invitrogen) and its yield and purity was spectrophotometrically estimated via the A260/A280 and A230/260 ratios using a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The RNA (2 μ g) was reverse-transcribed into cDNA

using oligo (dT18) primers from the Transcriptor First Strand cDNA Synthesis kit (cat. no. 04896866001; Roche Diagnostics GmbH) using oligo(dT) primers. qPCR was conducted using the LightCycler 480 SYBR Green 1 Master mix (Roche Diagnostics GmbH) and the LightCycler 480 qPCR system (Roche Diagnostics GmbH). The PCR cycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Target gene mRNA expression was normalized to the GAPDH internal control and was expressed relative to the control group using the $\Delta\Delta CT$ method (22). The primer sequences (Sangon Biotech, Shanghai, China) used were as follows: Cyclin D1 forward, 5'-GAGACCATCCCCCTGACGGC-3' and reverse, 5'-TCTTCC TCTCCTCGGCGGC-3'; Cyclin E forward, 5'-GTCCTGGCT GAATGTATACATGC-3', and reverse, 5'-CCCTATTTTGT CAGACAACATGGC-3'; CDK2 forward, 5'-GCTTTCTGC CATTCTCATCG-3' and reverse, 5'-GTCCCAGAGTCCGA AAGAT-3'; CDK4 forward, 5'-ATGTTGTCCGGCTGATGG-3' and reverse, 5'-CACCAGGGTTACCTTGATCTCC-3'; p27 forward, 5'-TGCAACCGACGATTCTTCTACTCAA-3' and reverse, 5'-CAAGCAGTGATGTATCTGATAACAAGGA-3'; GAPDH forward, 5'-ATTCCATGGCACCGTCAAGG-3' and reverse, 5'-AATTCGTTGTCATACCAGGA-3'.

Western blot analysis. Confluent serum-starved PSMCs were treated with hesperetin (100 μM) for 1 h following exposure to 20 ng/ml PDGF-BB for 5, 10 or 15 min. The cells were lysed in radioimmunoprecipitation assay buffer supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and were centrifuged at 3,362 x g for 30 min at 4°C. The protein concentration was assessed using a bicinchoninic acid assay (Thermo Fisher Scientific). The protein extracts (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto Immobilon-FL transfer membranes (Millipore, Billerica, MA, USA). The blotted membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 for 2 h and were subsequently incubated with primary antibodies against T-ERK1/2, P-ERK1/2, T-p38, P-p38, T-JNK, P-JNK, T-GSK3 β , P-GSK3 β , T-AKT, P-AKT and GAPDH overnight at 4°C. After three washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with the secondary antibody, IRdye 800 anti-rabbit (cat. no. 926-32211; LI-COR Biosciences, Lincoln, NE, USA) at a dilution of 1:2,500 for 1 h. The bands were quantified using the Odyssey infrared imaging system (LI-COR Biosciences). The protein expression levels were normalized to the expression levels of the GAPDH internal control.

Statistical analysis. The results are expressed as the mean \pm standard deviation. Differences among the groups were assessed using one-way analysis of variance or unpaired two-tailed t-tests. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of hesperetin on the proliferation of PDGF-BB-induced PSMCs. As compared with the control cells, PDGF-BB

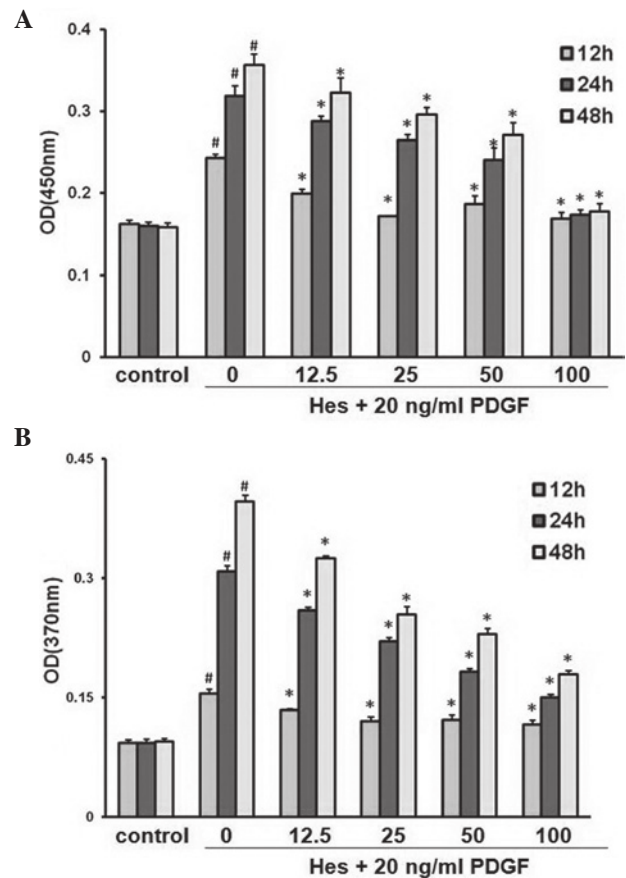


Figure 1. Effects of Hes on (PDGF)-BB-induced PSMC proliferation and DNA synthesis. PSMCs were pre-cultured in serum-free medium for 24 h, and then incubated in the absence or presence of Hes (12.5-100 μM) alongside 20 ng/ml PDGF-BB for a further 12, 24 or 48 h. Cell proliferation was examined using (A) the Cell Counting kit-8 test, and (B) bromodeoxyuridine incorporation was determined using an enzyme-linked immunosorbent-based assay. Data are expressed as the mean OD \pm standard deviation. # $P < 0.05$, vs. the control group; * $P < 0.05$ vs. cells exposed to PDGF-BB alone; n=5. Hes, hesperetin; PDGF, platelet-derived growth factor; PSMC, pulmonary artery smooth muscle cell; OD, optical density.

significantly increased the optical density (OD) of the cells in a time-dependent manner, which represented the time-dependent proliferation of PSMCs. In addition, pretreatment with hesperetin significantly suppressed the PDGF-BB-induced proliferation in a concentration-dependent manner, and the greatest suppression of proliferation was observed when the cells were treated with 100 μM hesperetin (Fig. 1A). Similarly, treatment with PDGF-BB increased DNA synthesis in PSMCs in a time-dependent manner, whereas treatment with hesperetin significantly suppressed the increase in DNA synthesis in a dose- and time-dependent manner (Fig. 1B).

Toxicity of hesperetin on PSMCs. Treatment with hesperetin at concentrations between 12.5 and 100 μM did not induce significant levels of cell necrosis in PSMCs after 12, 24 or 48 h, as compared with the untreated cells (Fig. 2). Regardless of whether the cells were treated with hesperetin or not, cell viability remained at $\sim 95\%$. This result suggests that hesperetin is not cytotoxic at the tested concentrations.

Effects of hesperetin on cell cycle progression. As shown in Fig. 3, the percentage of cells in S phase increased from

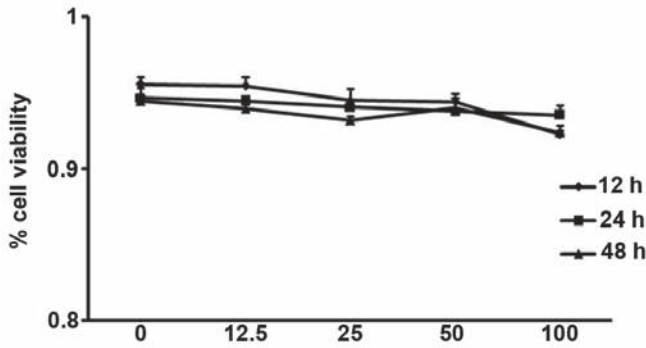


Figure 2. Effects of hesperetin on the viability of pulmonary artery smooth muscle cells. Cell viability was evaluated by counting the number of cells in a trypan blue exclusion test, in the absence or presence of various concentrations of hesperetin for 12, 24 or 48 h. (P>0.05 vs. the control group; n=4).

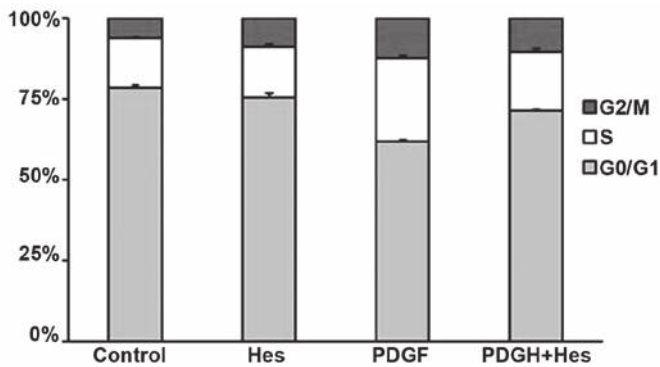


Figure 3. Effects of Hes on PDGF-BB-induced cell cycle progression. PSMCs were treated with Hes (100 μ M) in the absence or presence of 20 ng/ml PDGF-BB for 24 h. Quantification of the number of PSMCs in the G₀/G₁, S and G₂/M phases was determined using flow cytometric evaluation (n=3). Hes, hesperetin; PDGF, platelet-derived growth factor; PSMC, pulmonary artery smooth muscle cell.

15.3 to 25.7% following PDGF-BB stimulation. In addition, the percentage of cells in G₀/G₁ phase decreased from 78.7 to 62.0%. These effects were markedly reversed by pretreatment with 100 μ M hesperetin. The percentage of cells in S phase decreased to 17.7%, whereas the percentage of cells in G₀/G₁ phase increased to 69.7%. These results indicate that hesperetin affects G₀/G₁ to S phase transition; however, no effect was detected on the transition between S and G₂/M phase.

Effects of hesperetin on associated gene expression. Treatment with PDGF-BB significantly increased the mRNA expression levels of cyclin D1, cyclin E, CDK2 and CDK4. Conversely, pretreatment with hesperetin significantly suppressed the PDGF-BB-induced upregulation of the studied genes (Fig. 4). Cyclin-CDK complexes, which are formed during cell cycle progression, are regulated by CDK inhibitors (CKIs), including p27, which lead to cell cycle arrest at the G₁ and G₁/S boundary (23). As shown in Fig. 4, hesperetin upregulated the expression levels of p27.

Involvement of mitogen-activated protein kinases (MAPK) and AKT/GSK3 β signaling in the hesperetin-induced inhibition of PSMCs proliferation. Significant activation of ERK1/2, p38, JNK and AKT/GSK3 β was observed 5, 10 and 15 min after

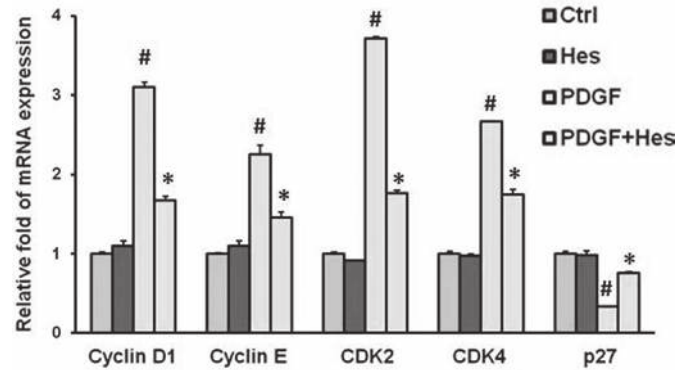


Figure 4. Effects of Hes on the expression levels of various cell cycle regulatory genes in PSMCs. PSMCs were pretreated with Hes (100 μ M) for 1 h prior to 24 h culture with 20 ng/ml PDGF-BB. The mRNA expression levels of cell cycle regulatory genes were analyzed. Experiments were performed in triplicate and repeated a minimum of three times. #P<0.05, vs. the control group; *P<0.05 vs. PDGF-BB-treated cells; n=3. CDK, cyclin-dependent kinase; Hes, hesperetin; PDGF, platelet-derived growth factor; PSMC, pulmonary artery smooth muscle cell.

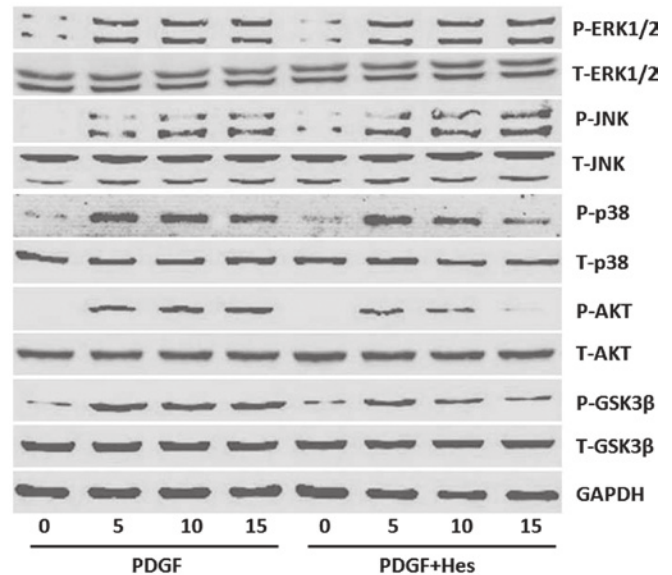


Figure 5. Effects of Hes on the activation of signaling pathways in PDGF-BB-stimulated PSMCs. PSMCs were pretreated with Hes (100 μ M) for 1 h prior to treatment with 20 ng/ml PDGF-BB. The protein expression levels of P-ERK1/2, T-ERK1/2, P-JNK, T-JNK, P-p38, T-p38, P-AKT, T-AKT, P-GSK3 β and T-GSK3 β were determined after 5, 10 and 15 min by western blot analysis. One representative image out of three independently performed experiments is shown. T, total; P, phosphorylated; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; GSK, glycogen synthase kinase; PDGF, platelet-derived growth factor; PSMCs, pulmonary artery smooth muscle cells; Hes, hesperetin.

PDGF treatment; however, the total levels of these molecules were not affected, as determined by western blotting (Fig. 5). In addition, hesperetin significantly reduced the phosphorylation of AKT/GSK3 β and p38, but did not exhibit any inhibitory effects on the phosphorylation of ERK1/2 and JNK (Fig. 5).

Discussion

PAH, which is characterized by a sustained increase in pulmonary artery pressure and pulmonary vascular remodeling,

is a progressive disease that is associated with a poor prognosis (24,25). PAH is associated with the excessive proliferation of PASMCs; therefore, inhibition of PASMC proliferation may be considered an effective therapeutic strategy. The present study demonstrated that hesperetin inhibited PDGF-induced PASMC proliferation and DNA synthesis in a concentration- and time-dependent manner, without exerting cell cytotoxic effects.

Cell proliferation is tightly regulated by the cell cycle, and hesperetin has been reported to induce cell cycle arrest in cancer cells (15). In the present study, flow cytometric analysis demonstrated that treatment with 100 μ M hesperetin for 24 h led to a significant increase in the number of cells in G₀/G₁ phase and a decrease in the number of cells in S phase, without any significant effect on the number of cells in G₂/M phase. According to these results, hesperetin suppressed the PDGF-BB-induced progression of PASMCs from G₀/G₁ to S phase. Progression through the cell cycle is regulated by the coordinated activities of cyclin-CDK complexes. Regulation of these cyclin-CDK complexes may be provided by their binding to CKIs (26). CDK2 and CDK4 form complexes with cyclin E and cyclin D1, which are essential for the cell cycle progression from G₀/G₁ phase to S phase (27,28). In addition, as a CKI, p27 phosphorylation has been reported to inhibit CDK2-containing complexes and cyclin D-CDK4 complexes (29,30).

In the present study, the expression levels of cell cycle regulatory genes in PASMCs were detected in response to PDGF-BB. Hesperetin decreased the mRNA expression levels of cyclin D1, cyclin E, CDK2 and CDK4, and increased the mRNA expression levels of p27. These results suggested that the anti-proliferative activity of hesperetin has multifaceted effects on numerous target molecules involved in growth inhibition.

MAPK families (including ERKs, p38 and JNK) and the AKT pathway have an important role in the regulation of cell proliferation. It has previously been reported that PDGF-BB significantly stimulates the phosphorylation of ERK1/2, JNK, p38 and AKT/GSK3 β in PASMCs (31). AKT is involved in the regulation of various downstream signaling pathways, including those associated with metabolism, cell proliferation, survival, growth and angiogenesis; GSK3 β is one of these critical downstream molecules. The results of the present study demonstrated that hesperetin was able to inhibit the phosphorylation of AKT/GSK3 β after PDGF-BB induction. These results are consistent with previous studies, which have reported that hesperetin may inhibit the AKT pathway (13,17). However, other studies have reported conflicting results, in which hesperetin has been shown to stimulate the AKT pathway (20,32). In rat aortic vascular smooth muscle cells, hesperetin exerted anti-proliferative activities; however, it had no effect on PDGF-BB-stimulated ERK1/2, AKT, JNK and p38 phosphorylation (33). Whether these differences are associated with the various types of cells used, different biological effects or different concentrations remains to be elucidated.

Cyclin D1 is regulated by GSK3 β (34,35). Activation of GSK3 β has been reported to regulate cyclin D1 export from the nucleus to the cytoplasm for proteolysis, thus downregulating the expression of cyclin D1 (36). Furthermore, inhibition of the phosphorylation of the AKT/GSK3 β signaling pathway

has been reported to decrease the expression levels of cyclin D1 in cultured VSMCs (37). Inhibition of GSK3 β has also been shown to decrease the expression levels of the CKI, p27 (38). These results suggested that hesperetin may have an anti-proliferative effect on PASMCs via the AKT/GSK3 β signaling pathway.

The present study also examined the effects of hesperetin on the activation of MAPKs, including ERK1/2, p38 and JNK, which are important factors implicated in the proliferation of PASMCs. p38 has an important role in the proliferation of PASMCs, whereas hesperetin may inhibit the activation of p38 MAPK. Therefore, it was hypothesized that the anti-proliferative effects of hesperetin may be associated with the inhibition of p38, thus suggesting that hesperetin may inhibit the PDGF-BB-induced proliferation of PASMCs via regulating p38 MAPK signal transduction.

In conclusion, the present study demonstrated that hesperetin was able to inhibit the PDGF-BB-induced proliferation of PASMCs. Notably, this process appears to be associated with an inhibition of cyclin D1 expression and an increased expression of p27, via suppression of the AKT/GSK3 β and p38 signaling pathway. These results suggest that hesperetin may be a potential therapeutic strategy for the treatment of pulmonary vascular remodeling diseases.

Acknowledgements

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