Caffeic Acid Phenethyl Ester Inhibits Proliferation and Migration, and Induces Apoptosis in Platelet-Derived Growth Factor-BB-Stimulated Human Coronary Smooth Muscle Cells

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Key Words

Caffeic acid phenethyl ester • Human coronary smooth muscle cells • Apoptosis • Antiproliferative effect • Antimigratory effect

Abstract

Background/Aims: Restenosis after a percutaneous coronary intervention (PCI) during treatment for coronary artery disease is closely related to smooth muscle cell (SMC) proliferation and migration. In this study, we investigated the effects of caffeic acid phenethyl ester (CAPE) and its underlying mechanism on human coronary SMCs (HCSMCs) after platelet-derived growth factor-BB (PDGF-BB) stimulation in vitro. **Methods and Results:** The results showed that CAPE inhibited proliferation and migration, and induced apoptosis. Concomitantly, CAPE inhibited activation of AKT1, MEK1 and ERK1/2 signaling molecules at 10–60 min after CAPE treatment. As revealed by flow cytometry, DNA fragmentation and TUNEL assay, the cells accumulated at the sub-G₁ phase, and cell apoptosis was observed after 30 and 90 μ M CAPE treatment for 72 h. CAPE triggered the release of cytochrome c from mitochondria to cytosol, upregulated the proapoptotic gene *Bax* and downregulated the antiapoptotic gene *Bcl-2*. Upregulation of caspase-9 and caspase-3 indi-

cated that CAPE precipitated the mitochondrion-dependent apoptotic signaling pathway. **Conclusions:** These results provide a molecular explanation for the antiproliferation, antimigration and proapoptotic effects of CAPE on HCSMCs after PDGF-BB stimulation.

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Introduction

Percutaneous coronary intervention (PCI) is the major therapeutic strategy for coronary artery disease. However, restenosis after PCI still challenges both the vascular biologist and interventional cardiologist. Restenosis is associated with vascular smooth muscle cell (SMC) proliferation and migration as well as high resistance to apoptosis in the intima, reflecting failure of the apoptotic pathway in the cells [1, 2]. SMCs re-enter the cell cycle and become able to migrate due to release of cytokines and growth factors from recruited platelets and impaired endothelial cells in

the vessel walls following injury [3]. Among the various growth factors that can trigger SMC proliferation and migration, platelet-derived growth factors (PDGF)-BB possess the most potent mitogenic and inflammatory effects [4]. PDGF-BB can activate several signaling cascades, including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/AKT [5]. It has been reported that in animals, increased levels of PDGF-BB result in neointimal thickening [6].

In the past, various pharmacologic tools, such as rapamycin/sirolimus-coated stents, have demonstrated potential in overcoming restenosis [7]. However, the problems have not been completely resolved. Recently, natural compounds rich in polyphenolic constituents have been a new field of research for preventing restenosis due to evidence of a decreasing risk of cardiovascular disease in epidemiological studies [8, 9]. Caffeic acid phenethyl ester (CAPE), the active component of propolis, has antioxidative, anti-inflammatory antiproliferative and antineoplastic biological properties [10, 11]. We have demonstrated that using a local infusion system, CAPE was able to reverse iliac artery intimal thickening in rabbits after balloon angioplasty [12]. It has been reported that CAPE may lead to a reduction of neointima formation by inhibiting nuclear transcription factor kappa-B (NF- κ B) activation after balloon angioplasty in a rat model [13]. The underlying mechanism, however, is still not clear. In this study, we investigated the effects of CAPE on HCSMCs after PDGF-BB stimulation *in vitro* in order to mimic a post-PCI PDGF-BB-releasing condition. Meanwhile, we have examined the expression of signaling molecules and apoptotic target genes in cells to explore the underlying mechanisms of the effects of CAPE on PDGF-BB-stimulated HCSMCs.

Methods

Cell Culture

HCSMCs (PromoCell GmbH, Heidelberg, Germany) were cultured at 37°C in T-25 flasks (Corning Glassworks, Corning, N.Y., USA) in Medium 199 (Gibco, New York, N.Y., USA) supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 U/ml, Sigma, St. Louis, Mo., USA) in a 5% CO₂/95% air atmosphere. The culture medium was replaced every 2 days. Once the cells reached 70–80% confluence, they were trypsinized and seeded on 6-well plastic dishes for the following experiments. Passage 3–9 HCSMCs were used in the experiment.

Cell Growth Assay

HCSMCs were seeded on 6-well plastic dishes at a concentration of 1×10^5 per well. Prior to the experiment, 60–70% confluence cells were washed twice with phosphate-buffered saline (PBS), stimulated with PDGF-BB (10 μ M) (Sigma) [14] and incu-

bated with serum medium supplemented with various concentrations (0, 3, 10, 30, 90 μ M) of CAPE (Sigma) for 0, 6, 12, 24, 48 and 72 h, respectively. Cells were then stained with trypan blue and cell numbers were counted under an inverted microscope. Data are presented as the means $\pm$ SDs of 3 replicates from 6 separate experiments.

Cell Migration Assay

HCSMCs were plated at an initial density of 1×10^5 cells/ml to form a monolayer. During the last 4 h, cytosine β -D-arabino-furanoside (Sigma) was added at a final concentration of 4 μ M to prevent SMC proliferation. Cells were washed twice with PBS and incubated with 10 μ M PDGF-BB and serum medium containing various concentrations (0, 3, 10, 30 and 90 μ M) of CAPE for 0, 6, 12, 24, 48 and 72 h, respectively. Then, cells were wounded by scraping with a pipette tip to make an approximately 400- μ m gap in the cell monolayer. The images of cell migration were observed at different time intervals and photographed at 5 marked locations on each dish using a phase-contrast microscope. The number of migrated cells was counted and averaged. All experiments were carried out in triplicate and repeated at least 6 times.

Western Blot Analysis

HCSMCs were treated with 10 μ M PDGF-BB at various concentrations of CAPE (0, 3, 10, 30, 90 μ M) for various time intervals. After incubation, the cells were washed 3 times with PBS and lysed with RIPA lysis buffer (Pierce, Rockford, Ill., USA) with a Protease Inhibitor Cocktail Kit (Thermo, Rockford, Ill., USA) at 4°C for 30 min. The lysates were then centrifuged at 10,000 g at 4°C to obtain solubilized cellular proteins. The supernatant protein concentration was measured with a bicinchoninic acid protein assay (Pierce). Proteins (50 μ g) were separated by 10 or 12% SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane. Blots were probed with phosphorylated rabbit anti-human AKT1 (1:1,000, v/v), phosphorylated rabbit anti-human MEK (1:1,000), phosphorylated rabbit anti-human-extracellular signal-regulated kinase (ERK) (1:1,000), phosphorylated rabbit anti-human-I κ B (1:1,000), mouse anti-human caspase-8 (1:1,000, v/v), rabbit anti-human caspase-9, rabbit anti-human caspase-3 (Cell Signaling Technology, Boston, Mass., USA), followed by HRP-conjugated goat anti-rabbit IgG (1:5,000, v/v) (Zymed, South San Francisco, Calif., USA) or HRP-conjugated goat anti-mouse IgG (1:5,000, v/v) (Zymed). After washing with PBS containing 0.5% Tween-20, peroxidase activity was assessed using enhanced chemiluminescence (Perkin-Elmer Life Science, Boston, Mass., USA). For an internal control, the same membrane was reprobed with a monoclonal antibody directed against β -actin (1:10,000, v/v) (Sigma). The intensities of the reaction bands were analyzed with the Image Gauge System (Fuji, Tokyo, Japan).

Flow Cytometry Analysis

Cell density was adjusted to 1×10^5 cells/ml, then treated with 10 μ M PDGF-BB and various concentrations (0, 10, 30, 90 μ M) of CAPE for 48 or 72 h. Both detached and attached cells were collected, washed with PBS and fixed with ice-cold 70% ethanol at 4°C overnight. Cells were then washed with PBS, incubated with propidium iodide (40 μ g/ml) (Molecular Probes, Eugene, Oreg., USA) and DNase-free RNase A (100 μ g/ml) for 1 h at 37°C in the dark. The DNA content of the nuclei was analyzed with a flow cytometer (Partec GmbH, Münster, Germany).

DNA Fragmentation Analysis

HSCMCs were treated with 10 μ M PDGF-BB and various concentrations of CAPE (0, 3, 10, 30, 90 μ M) for 48 or 72 h. Both detached and attached cells were collected and washed with PBS. The analysis was performed using the Tissue & Cell Genomic kit (GeneMark, Taiwan) according to the manufacturer's instructions. Fragmented DNA was extracted and electrophoresed on a 1.8% agarose gel containing 0.1 μ g/ml ethidium bromide.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Staining

Cell apoptosis was identified by a TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay according to the manufacturer's instructions (Roche, USA). Briefly, the cells grown on a coverslip in a 6-well plate were incubated with 10 μ M PDGF-BB and various concentrations (0, 10, 30, 90 μ M) of CAPE for 48 or 72 h. The cells were then chilled on ice, washed 3 times with cold 0.3% Triton-X-100-PBS and fixed in 2% paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed 3 times with cold 0.3% Triton X-100-PBS and coverslip cultures were incubated with a TUNEL reaction mixture in a humidified atmosphere for 60 min at 37°C in the dark. After incubation, the cells were washed 3 times with cold 0.3% Triton X-100-PBS and then coverslip cultures were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and examined with an LSM 510 confocal microscope (Zeiss, Germany). Quantifications of positive cells in TUNEL staining were performed by taking images in 6 regions of cover slides at a magnification of 400 $\times$. The percentages of TUNEL-positive cells per total numbers of cells were counted and analyzed.

Analysis of Mitochondrial and Cytosolic Cytochrome c in HSCMCs

Mitochondrial and cytosolic protein fractions of cells were extracted as previously described [12] with some modifications. Briefly, PDGF-BB-stimulated cells after treatment with or without CAPE for 48 or 72 h were collected and homogenized in ice-cold 1 $\times$ Cytosol Extraction Buffer Mix (BioVision, Mountain View, Calif., USA) with a Protease Inhibitor Cocktail Kit (Thermo) for 10 min and then centrifuged at 700 g for 10 min at 4°C to remove the pellets. The supernatants were further centrifuged at 10,000 g for 30 min at 4°C to separate the cytosolic (in the supernatant) and the mitochondrial (in the pellets) fractions. Finally, the pellets were resuspended with Mitochondrial Extraction Buffer Mix (BioVision) containing Protease Inhibitor Cocktail Kit (Thermo), vortexed for 10 s to obtain the mitochondrial fraction and stored at -80°C until use. The purity of mitochondrial and cytosolic fractions was verified by immunoblotting for a mitochondrial marker, prohibitin. Fifty micrograms of proteins from each extract was analyzed by Western blotting for cytochrome c. The ratio of cytosolic to total (cytosolic and mitochondrial) abundance of cytochrome c was determined by densitometry [15].

Statistical Analysis

Data were expressed as means $\pm$ SDs. The two groups were compared via a two-tailed t test using SAS software. The cell growth and cell migration were analyzed via ANOVA followed by post-hoc analysis. $p < 0.05$ was considered statistically significant.

Fig. 1. Inhibition of cell growth of PDGF-BB-stimulated HSCMCs by CAPE. The cells were treated at the indicated concentrations of CAPE and at 10 μ M PDGF-BB for the indicated times. Cell viability was determined by a trypan blue dye exclusion assay. Data are presented as means $\pm$ SDs of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$, compared to the control group.

Results

Inhibition Effect of CAPE on PDGF-BB-Stimulated Proliferation of HSCMCs

To test the effect of CAPE on cell growth in PDGF-BB-stimulated HSCMCs, the growth rate was measured using a trypan blue exclusion assay. The results showed that the cell number of the group without CAPE treatment increased from 12 to 72 h after stimulation with 10 μ M PDGF-BB; however, treatment with CAPE reduced or inhibited PDGF-BB-stimulated HSCMC growth (fig. 1).

Inhibition Effect of CAPE on PDGF-BB-Stimulated Migration of HSCMCs

To assess the effects of CAPE on PDGF-BB-stimulated HSCMC migration, the images of the cell culture after wound-healing experiments were photographed and analyzed at different time intervals. The results showed that the number of migrated HSCMCs was significantly increased at 12 h and beyond without CAPE treatment. The nonviable cells apparently increased and floated in the

Fig. 2. Inhibition of cell migration of PDGF-BB-stimulated HCSMCs by CAPE. Migration was assessed following mechanical wound healing. **a** Cell migration was evaluated at 0, 6, 12, 24, 48 and 72 h after treatment with various doses of CAPE. Cell migration was strongly inhibited from 12 to 72 h after CAPE

treatment (10–90 μM). **b** Quantitative data showed that cell migration was significantly repressed by CAPE treatment at doses above 10 μM . Data are presented as means $\pm$ SDs of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$, compared to the control group.

culture medium from 12 to 72 h after treatment with 10, 30 and 90 μM CAPE (fig. 2a). Quantitative data indicated that the PDGF-BB-stimulated HCSMC migration was strongly inhibited in the cells treated with 10, 30 and 90 μM CAPE (fig. 2b).

Inhibition Effect of CAPE on the Expression of Activated AKT1, MEK1, ERK1, but Not on Phosphorylated I κ B

To study the signaling molecules involved in CAPE-mediated changes in the proliferation and migration of HCSMCs, we analyzed the cellular expression of phosphorylated AKT1, MEK1, ERK1/2 and I κ B (fig. 3a). The decrease of phosphorylated AKT1, MEK1 and ERK1/2 after treatment with higher doses of CAPE for 10, 30 and 60 min indicated that the inhibitory effects of CAPE on the growth and migration of PDGF-BB-stimulated HCSMCs were associated with a downregulation of AKT and MEK signaling pathways. The expression of phosphorylated I κ B did not change after treatment with CAPE

in PDGF-BB-stimulated HCSMCs. The average quantitative results on the level of a specific protein are presented in figure 3b.

Enhancement of the Apoptotic Effect of CAPE on PDGF-BB-Stimulated HCSMCs

To determine the effect of CAPE on cell apoptosis in PDGF-BB-stimulated HCSMCs, we investigated the apoptotic effects of CAPE by performing a flow-cytometric assay, DNA fragmentation analysis and a TUNEL assay. In flow cytometry, the apoptotic cell population was significantly increased and accumulated at the sub- G_1 phase after treatment with 30 or 90 μM CAPE for 72 h (fig. 4a). DNA fragmentation was also observed in the cells at 72 h after treatment with 30 or 90 μM CAPE (fig. 4b). Cell apoptosis was further confirmed by the TUNEL assay. Few to no TUNEL-positive cells were detected in the cells at zero or low-dose CAPE treatment (10 μM) (fig. 4c, d). The percentages of TUNEL-positive cells were 42 ± 3 and $64 \pm 2\%$, respectively, after treatment with 30 and

Fig. 3. CAPE inhibited the activation of AKT1, MEK1 and ERK1/2 but displayed no effect on IκB activation in PDGF-BB-stimulated HCSMCs. **a** Phosphorylated (p) AKT1, ERK1/2, MEK1/2 and IκB expression was detected by Western blotting using specific antibodies. Phosphorylated AKT1, MEK1 and ERK1/2 cellular expres-

sion decreased at 10–90 min after 30 or 90 μM CAPE treatment, whereas phosphorylated IκB expression was not changed. **b** Quantitative results on the level of specific proteins. * $p < 0.05$, ** $p < 0.01$, compared to the control group.

90 μM CAPE for 72 h (fig. 4e, f). These results indicate that CAPE induced apoptosis of the PDGF-BB-stimulated HCSMCs at high concentrations (30 and 90 μM).

Abundance of Mitochondrial and Cytosolic Cytochrome c in HCSMCs

The release of cytosolic cytochrome c has been reported as the initiation of apoptosis [16], which triggers the activation of effector caspases [17]. To speculate whether CAPE modulated the apoptotic factors in PDGF-BB-induced HCSMCs, the distribution of cytochrome c in mitochondrial and cytosolic compartments of HCSMCs was assessed. Immunoblot analysis showed that cytosol-

ic cytochrome c increased from about 25 to 90% after CAPE treatment (fig. 5a, b). These results indicate that CAPE can induce PDGF-BB-triggered cytochrome c release in HCSMCs.

CAPE Induced Proapoptotic and Suppressed Antiapoptotic Effects on Bcl-2 Family Proteins and Caspase Activation

It has been reported that the integrity of the outer mitochondrial membrane is mediated by Bcl-2 family proteins in SMCs [18–20]. Since CAPE enhanced cytochrome c release from mitochondria to cytosol, the balance among Bcl-2 family proteins was investigated. Immu-

Fig. 4. Effect of CAPE on cell cycle and DNA fragmentation in PDGF-BB-stimulated HCSMCs. **a** DNA distribution histogram of PDGF-BB-stimulated HCSMCs by flow-cytometric analysis. The sub- G_1 peak (apoptotic cells) was observed at 72 h after 30 or 90 μM CAPE treatment. **b** DNA fragmentation analysis demonstrated that CAPE sensitized apoptotic cells at 72 h after 30 or 90 μM CAPE treatment. TUNEL assay and DAPI staining (blue fluorescence) to assay the effect of CAPE on cell apoptosis in PDGF-BB-stimulated HCSMCs. **c, d** No TUNEL-positive cells were detected in cells at 72 h after treatment with 0 and 10 μM CAPE. **e, f** TUNEL-positive cells (green fluorescence) were detected at 72 h after treatment with 30 and 90 μM CAPE. Arrows indicate TUNEL-positive cells. Scale bar: 15 μm .

Fig. 5. Effects of CAPE on mitochondrial cytochrome c release in PDGF-BB-stimulated HCSMCs. **a** Immunoblotting data indicate that CAPE increased the abundance of cytochrome c in the cytosolic fraction and decreased its amount in the mitochondrial fraction at 48 h after treatment. The purity of the mitochondrial and

cytosolic protein extracts was confirmed by the mitochondrial marker prohibitin, which was present in the mitochondrial but not in the cytosolic fractions. **b** Quantitative analysis indicates that the cytosolic fraction of cytochrome c increased from about 25 to 90% after CAPE treatment.

Fig. 6. Immunoblotting analysis of anti-apoptotic effects on Bcl-2 family proteins and caspase activation. **a, b** The expression of the antiapoptotic molecule Bcl-2 was downregulated and the proapoptotic molecule Bax was upregulated in cells treated with 30 and 90 μM CAPE compared with the non-CAPE-treated cells at 48 h. **a, c** Moreover, the cellular amount of cleaved caspase-9 and -3 but not caspase-8 was significantly increased after 10, 30 and 90 μM CAPE treatment at 48 h. * $p < 0.05$, ** $p < 0.01$, compared to the control group.

noblotting analyses indicated that expression of the anti-apoptotic molecule Bcl-2 decreased and the proapoptotic molecule Bax increased in CAPE-treated cells compared with those in the non-CAPE-treated group at 72 h (fig. 6a, b). Several key factors in the execution of mitochondrial apoptosis, caspase-9 and -3, were activated in the intrinsic pathway (fig. 6a, c). The expression of caspase-8, however, was not activated (fig. 6a, c).

Discussion

Proliferation, migration and high resistance to apoptosis of coronary SMCs play important roles in the development of atherosclerotic diseases and restenosis after angioplasty. A natural compound, rich in polyphenolic constituents, administered via drug-eluting stents, may prevent arterial restenosis. A number of in vitro and in vivo studies on polyphenolic compounds have revealed

that their beneficial effect on vascular diseases may be due to the modulation of abnormal cell proliferation, migration and apoptosis [21]. The underlying cellular mechanisms, however, have not been elucidated. In this study, we examined the molecular mechanisms of CAPE on the growth, migration and apoptosis of HCSMCs after PDGF-BB stimulation in an in vitro model mimicking the action of HCSMCs triggered in the PDGF-BB-releasing condition after PCI.

Various growth factors released from impaired endothelial cells and recruited platelets and macrophages during vascular injury can trigger SMC proliferation and migration. Some reports demonstrated that proliferation and apoptosis of SMCs are closely related to the activation of NF- κ B [13, 22]. However, in our study, no such effects of phosphorylated I κ B on SMC proliferation, migration and apoptosis were observed in CAPE-treated cells (fig. 3a). We suggested that CAPE inhibits proliferation and migration, and induces apoptosis via NF- κ B-independent pathways [23, 24]. PDGF-BB exerts the most potent mitogenic and inflammatory effects. It has been reported that PDGF-BB can induce both proliferation and directed migration in vitro, activate several signaling cascades, promote cell survival, and inhibit the intrinsic apoptotic pathway [25]. PDGF-BB-stimulated HCSMCs have been suggested to be associated with upregulation of the PI3K/AKT and ERK signaling pathways [26]. Dephosphorylation of ERK/MAPK signaling molecules induces apoptotic activity. Downregulation of SMC apoptosis is a key contributor to intimal lesions [27]. In this study, we demonstrated that CAPE effectively inhibited proliferation and migration, and induced apoptosis in PDGF-BB-stimulated HCSMCs. Concomitantly, phosphorylated AKT1, MEK1 and ERK1/2 decreased in a dose-dependent manner after CAPE treatment within 10, 30 and 60 min (fig. 3).

Arterial SMCs normally stay in the G₀ phase of the cell cycle and keep in a quiescent nonproliferative state. They re-enter the cell cycle after balloon angioplasty; marked induction of cyclins A and E, CDK2 and proliferating cell nuclear antigen protein expression was observed with an increase in the level of CDK2 kinase activity [28]. We have demonstrated that the expressions of cyclin A, cyclin E, CDK2, cyclin D1, CDK4, cyclin B1 and CDK1 decreased in the PDGF-BB-stimulated HCSMCs after CAPE treatment (data not shown). To further investigate the pattern of the antiproliferative effect of CAPE on PDGF-BB-stimulated SMCs, flow-cytometric analysis was performed in this study. The results showed that CAPE treatment at the concentrations of 30 and 90 μ M for 72 h resulted in the induction of cell accumulation at

the sub-G₁ phase. In the meantime, cell apoptosis was also observed by DNA fragmentation and a TUNEL assay (fig. 4, 5). Our previous study had indicated that without stimulation of PDGF-BB, cell cycle arrest occurred in the S phase and no apoptosis was found in the cells after CAPE treatment [12]. It has been reported that vascular SMCs from injured and uninjured arteries show different growth properties in cultures [28]. The present study demonstrated that CAPE is more effective at inducing apoptosis in vascular SMCs after growth factor stimulation than in nonstimulated SMCs. We assumed that CAPE can inhibit cell proliferation and migration and induce apoptosis more efficiently by inhibiting signal transduction kinases, thereby activating the caspase pathways.

PDGF-BB can activate the cell-signaling molecules and inhibit the interaction between Bcl-2 and Bim, Bcl-2 and Bax, thereby inhibiting cell apoptosis [29]. In this study, we demonstrated that treatment of HCSMCs with CAPE after exogenous PDGF-BB stimulation induced apoptosis through the mitochondrion-dependent apoptotic pathway. Its action is accompanied by activation of caspase-3 and caspase-9, downregulation of Bcl-2 and upregulation of Bax in the cells. Caspase-8, however, was not activated by CAPE, indicating the death receptor Fas or its ligand FasL was not involved in CAPE-induced SMC apoptosis. It has been reported that apoptosis can be induced by CAPE, 6 and 20 μ g/ml, in human leukemic HL-60 cells and mouse epidermal cell line C141 cells, respectively [30, 31]. Apparently, CAPE needs a higher dose (30 μ g/ml) to induce apoptosis in primary HCSMCs than in cancer cell lines. Further investigation of the in vivo efficiency of CAPE on vascular SMCs in animal injury models should be performed.

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References

- 1 Liu MW, Roubin GS, King SB: Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. *Circulation* 1989;79:1374–1387.
- 2 Pollman MJ, Hall JL, Gibbons GH: Determinants of vascular smooth muscle cell apoptosis after balloon angioplasty injury. Influence of redox state and cell phenotype. *Circ Res* 1999;84:113–121.

- 3 Sriram V, Patterson C: Cell cycle in vasculoproliferative diseases: potential interventions and routes of delivery. *Circulation* 2001;103:2414–2419.
- 4 Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG, Ross R: Platelet-derived growth factor: distinct signal transduction pathways associated with migration versus proliferation. *Ann NY Acad Sci* 1995;12: 416–430.
- 5 Cospedal R, Abedi H, Zachary I: Platelet-derived growth factor-BB (PDGF-BB) regulation of migration and focal adhesion kinase phosphorylation in rabbit aortic vascular smooth muscle cells: roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinases. *Cardiovasc Res* 1999;41: 708–721.
- 6 Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW: Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest* 1992; 89:507–511.
- 7 Jeremias A, Kirtane A: Balancing efficacy and safety of drug-eluting stents in patients undergoing percutaneous coronary intervention. *Ann Intern Med* 2008;148:234–238.
- 8 Cherubini A, Vigna GB, Zuliani G, Ruggiero C, Senin U, Fellin R: Role of antioxidants in atherosclerosis: epidemiological and clinical update. *Curr Pharm Des* 2005;11:2017–2032.
- 9 Kyaw M, Yoshizumi M, Tsuchiya K, Izawa Y, Kanematsu Y, Tamaki T: Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases. *Acta Pharmacol Sin* 2004;25:977–985.
- 10 Jaganathan SK, Mandal M: Antiproliferative effects of honey and of its polyphenols: a review. *J Biomed Biotechnol* 2009;2009:1–13.
- 11 Orban Z, Mitsiades N, Burke TR Jr, Tsokos M, Chrousos GP: Caffeic acid phenethyl ester induces leukocyte apoptosis, modulates nuclear factor- κ B and suppresses acute inflammation. *Neuroimmunomodulation* 2000;7:99–105.
- 12 Ho HC, Hsu SL, Ting CT, Kuo CY, Yang VC: Caffeic acid phenethyl ester inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using a local delivery system. *Cell Mol Biol* 2009;55:1161–1167.
- 13 Maffia P, Ianaro A, Pisano B, Borrelli F, Capasso F, Pinto A, Ialenti A: Beneficial effects of caffeic acid phenethyl ester in a rat model of vascular injury. *Br J Pharmacol* 2002;136: 353–360.
- 14 Chen CN, Li YS, Yeh YT, Lee PL, Usami S, Chien S, Chiu JJ: Synergistic roles of platelet-derived growth factor-BB and interleukin-1 β in phenotypic modulation of human aortic smooth muscle cells. *Proc Natl Acad Sci USA* 2006;103:2665–2670.
- 15 Liu TJ, Yeh YC, Ting CT, Lee WL, Wang LC, Lee HW, Wang KY, Lai HC: *Ginkgo biloba* extract 761 reduces doxorubicin-induced apoptotic damage in rat hearts and neonatal cardiomyocytes. *Cardiovasc Res* 2008;80: 227–235.
- 16 Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:147–157.
- 17 Scorrano L: Opening the doors to cytochrome c: changes in mitochondrial shape and apoptosis. *Int J Biochem Cell Biol* 2009; 41:1875–1883.
- 18 Adams JM, Cory S: Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr Opin Immunol* 2007;19:488–496.
- 19 Green DR, Kroemer G: The pathophysiology of mitochondrial cell death. *Science* 2004; 305:626–629.
- 20 Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P: Toxic proteins released from mitochondria in cell death. *Oncogene* 2004;23:2861–2874.
- 21 Giovannini C, Scazzocchio B, Vari R, Santangelo C, D'Archivio M, Masella R: Apoptosis in cancer and atherosclerosis: polyphenol activities. *Ann Ist Super Sanita* 2007;43:406–416.
- 22 Hishikawa K, Nakaki T, Fujita T: Oral flavonoid supplementation attenuates atherosclerosis development in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2005;25:442–446.
- 23 Tatetsu H, Okuno Y, Nakamura M, Matsuno F, Sonoki T, Taniguchi I, Uneda S, Umezawa K, Mitsuya H, Hata H: *Mol Cancer Ther* 2005;4:1114–1120.
- 24 Wang C, Fu M, D'Amico M, Albanese C, Zhou JN, Brownlee M, Lisanti MP, Chatterjee VKK, Lazar MA, Pestell RG: *Mol Cell Biol* 2001;21:3057–3070.
- 25 Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME: Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 1999;286:1358–1362.
- 26 Li JM, Brooks G: Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system; potential targets for therapy. *Eur Heart J* 1999;20:406–420.
- 27 Andrés V: Control of vascular cell proliferation and migration by cyclin-dependent kinase signalling: new perspectives and therapeutic potential. *Cardiovasc Res* 2004;63: 11–21.
- 28 Walker LN, Bowen-Pope DF, Ross R, Reidy MA: Production of platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. *Proc Natl Acad Sci* 1986;83:7311–7315.
- 29 Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ: Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proc Natl Acad Sci* 2004;101:15313–15317.
- 30 Chen YJ, Shiao MS, Hsu ML, Tsai TH, Wang SY: Effect of caffeic acid phenethyl ester, an antioxidant from propolis, on inducing apoptosis in human leukemic HL-60 cells. *J Agric Food Chem* 2001;49:5615–5619.
- 31 Nomura M, Kaji A, Ma W, Miyamoto K, Dong Z: Suppression of cell transformation and induction of apoptosis by caffeic acid phenethyl ester. *Mol Carcinog* 2001;31:83–89.