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Apigenin Attenuates Neointima Formation via Suppression of Vascular Smooth Muscle Cell Phenotypic Transformation

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ABSTRACT

Abnormal proliferation, migration, and phenotypic modulation of vascular smooth muscle cells (VSMCs) are critical factors in neointima formation during restenosis. The purpose of this study is to determine the efficacy and possible cell signaling mechanisms of apigenin in VSMC activation induced by platelet-derived growth factor (PDGF)-BB and injury-induced neointima formation. Our data revealed a dose-dependent apigenin inhibition of PDGF-BB-induced proliferation of VSMCs by arresting cells in G0/G1-phase of the cell cycle as determined using 5-bromo-2′-deoxyuridine incorporation and flow cytometry. This was associated with the inhibition of cyclin-dependent kinase (CDK) 4,6 expression and an increase in p27Kip1 levels in PDGF-stimulated VSMCs. Moreover, apigenin was also found to regulate PDGF-induced migration and expression of smooth-muscle-specific contractile markers. Mechanistically, the PDGF-BB-induced phosphorylation of PDGF-receptor β (PDGF-Rβ), Akt/glycogen synthase kinase(GSK)3β, extracellular signal-regulated kinase1/2 (ERK1/2), and signal transducers and activators of transcription 3 (STAT3) is negatively modulated by apigenin. For the in vivo studies using a mouse carotid arterial injury model, the administration of apigenin resulted in a significant inhibition of the neointima/media ratio and proliferating cell nuclear antigen (PCNA)-positive cells. These results demonstrate that apigenin can suppress PDGF-induced VSMC activation and neointima hyperplasia after vascular injury; these beneficial effects are probably the result of the blockade of PDGF-Rβ phosphorylation and its downstream signal transduction, including the Akt/GSK-3β, ERK1/2, and STAT3 pathways. The results suggest that apigenin may be a potential therapeutic candidate for the prevention of restenosis. J. Cell. Biochem. 113: 1198–1207, 2012.

KEY WORDS: APIGENIN; NEOINTIMA; VASCULAR SMOOTH MUSCLE CELL; PLATELET-DERIVED GROWTH FACTOR

Restenosis is the major factor attenuating the beneficial effects of angioplasty and stenting [Karsch et al., 1991; Bauters and Isner, 1997]. The abnormal proliferation, migration and phenotypic modulation of vascular smooth muscle cells (VSMCs) are critical factors in neointima formation and vascular lumen loss during restenosis [Bauters et al., 1996; Weintraub, 2007]. All these events can be induced by cytokines and growth factors such as platelet-derived growth factor (PDGF). PDGF initiates a multitude of biological effects through the activation of intracellular signal transduction pathways that contribute to VSMC proliferation, migration, and phenotypic modulation [Owens et al., 2004]. The importance of PDGF in the development of neointima has been established in arterial injury models. Therefore, the inhibition of PDGF-stimulated VSMC proliferation, migration, and phenotypic modulation may represent an important point of therapeutic intervention in restenosis after angioplasty.

Flavonoids, a family of polyphenolic compounds with a similar structure, are synthesized by plants and have many biological and pharmacological activities such as antioxidative, anti-inflammatory, and antitumor effects. Epidemiological studies have shown that an increased consumption of flavonoids is associated with a lower risk of cancer and cardiovascular-disease-related death [Heederik et al., 1990; Knekt et al., 1997; Graf et al., 2005]. Apigenin belongs to the flavone subclass of flavonoids, which usually occur in common

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fruits, vegetables, and tea. In addition to its potent anti-inflammatory, antioxidative, and anticancer properties [Singh et al., 2004; Shanmugam et al., 2008; Shukla and Gupta, 2010], apigenin has been reported to display antimutagenic, neuroprotective, and vasorelaxant effects [Zhang et al., 2000; Siddique and Afzal, 2009; Braidy et al., 2010]. However, the role of apigenin in regulating VSMC activation is not yet clearly understood. Two research groups demonstrated that apigenin inhibited VSMC proliferation and migration [Kim et al., 2002; Lamy et al., 2008], however, in another group, apigenin was found to stimulate VSMC proliferation [Trochon et al., 2000]. In this study, we investigated the effect of apigenin on PDGF-BB-induced VSMC proliferation, migration, and phenotypic modulation in vitro and in vivo injury-induced neointima formation. The intracellular signaling pathways responsible for the role of apigenin in PDGF-BB-induced VSMC activation were also investigated. We explored the potential therapeutic effect of apigenin as a novel treatment for vascular remodeling after injury.

MATERIALS AND METHODS

MATERIALS

Apigenin was purchased from Sigma-Aldrich (St. Louis, MO), and recombinant human PDGF-BB was obtained from Prospec (Rehovot, Israel). The antibodies used to recognize the total levels and phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2), Akt, glycogen synthase kinase(GSK)3β, and signal transducers and activators of transcription 3 (STAT3) were ordered from Cell Signaling technology (Danvers, MA). Anti-smooth muscle alpha-actin (SM α-actin), SM22 α, total levels and phosphorylation of PDGF-receptor β (PDGF-Rβ) were purchased from Abcam (Cambridge, MA). Anti-desmin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cyclin-dependent kinase 4(CDK4), CDK6, p27Kip1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and proliferating cell nuclear antigen (PCNA) were purchased from Cell Signaling technology. Complete protease inhibitor, PhosSTOP, Cell Proliferation Reagent WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), and Cell Proliferation ELISA, BrdU (colorimetric) were obtained from Roche Diagnostics (Mannheim, Germany). All other reagents were from Sigma-Aldrich except where specified. For the in vitro study, apigenin was dissolved in dimethyl sulfoxide (DMSO) medium. DMSO alone (without apigenin) served as a control. The control did not show any effect on cell viability, cell proliferation, or related molecular mechanisms (data not shown).

CELL CULTURE

Primary VSMCs were isolated enzymatically from thoracic aortas of male Sprague–Dawley rats (100–150 g) and were grown in DMEM/F12 medium containing 10% fetal bovine serum. The purity of the VSMCs was assessed by cell morphology observations and positive staining of SM α-actin. Cells used in this study were between passages 5–12. VSMCs were grown to 60–80% confluency and were serum starved for 24 h. Quiescent cells were pre-treated with various concentrations of apigenin for 1 h prior to stimulation with PDGF-BB (20 ng/ml).

MEASUREMENT OF CELL PROLIFERATION AND DNA SYNTHESIS

Cell proliferation was determined using a non-radioactive colorimetric WST-1 assay according to the manufacturer's instructions. VSMCs (5×10^3/well) were grown to 60% confluency in a 96-well microplate, and growth was arrested by serum deprivation for 24 h. After preincubation with various concentrations of apigenin for 1 h, cells were treated with PDGF-BB (20 ng/ml) in the presence/absence of apigenin for 48 h and loaded with WST-1 for the last 2 h. The color intensity was read at 450 nm. DNA synthesis in the VSMCs was assessed by measuring the incorporation of BrdU. Cells were seeded and treated as in the WST-1 assay. BrdU was added for the last 2 h of the treatment. BrdU incorporation was measured using a cell proliferation ELISA kit.

CELL CYCLE PROGRESSION ASSAYS

Cell cycle progression was measured using PI staining with fluorescence-activated cell sorting (FACS). Briefly, cells at 70% confluency were synchronized by serum deprivation for 24 h, preincubated with apigenin (12.5 μM) for 1 h, and subsequently treated with PDGF-BB (20 ng/ml) for 24 h. Cells were then trypsinized and fixed with 70% ethanol overnight. Fixed cells were collected by centrifugation, washed once in PBS, incubated with 1 ml of PI staining buffer (20 μg/ml PI and 50 μg/ml RNase A), and then analyzed by FACS. Cell cycle distributions were analyzed using the Multicycle AV software (Phoenix Flow Systems, San Diego, CA).

MIGRATION ASSAY

A migration assay was performed using the Transwell system (a 6.5-mm polycarbonate membrane with 8-μm pores; Corning, NY). Cells (50 μl; 5×10^4 total) were seeded onto the upper chamber and allowed to attach for 30 min. The monolayers were then treated for 1 h by adding 50 μl of twofold concentrated apigenin solution into the upper chamber and 600 μl of the undiluted apigenin solution (1×) into the lower chamber. PDGF-BB was added to the bottom chamber as the chemoattractant. The cells were allowed to migrate through the membrane to the lower surface for 6 h. Cells on the upper surface of the membrane that had not migrated were scraped off using Q-tips, and cells that had migrated to the lower surface were fixed and stained using 0.1% crystal violet/20% methanol and counted. The numbers of migrated cells were calculated as the number of migrated cells per high-power field (HPF 200×).

WESTERN BLOTTING

VSMCs were cultured in a 6-cm-diameter dish, grown to 70–80% confluency, and then starved in serum-free medium for 24 h. The cells were then treated with apigenin (12.5 μM) for 2 h before exposure to 20 ng/ml PDGF-BB for the indicated duration. Cells were lysed in RIPA (RadioImmunoPrecipitation Assay) buffer with protease and phosphatase cocktails. Protein extract (20 μg) was used for SDS-PAGE analysis. Proteins were then transferred to an Immobilon-FL transfer membrane (Millipore) and probed with various antibodies. After incubation with a secondary IRDye® 800CW-conjugated antibody, the signals were visualized using an Odyssey Imaging System. Specific protein expression levels were

normalized to GAPDH for total protein analyses or to total proteins for phosphorylated protein measurements.

ENDOVASCULAR CAROTID ARTERY GUIDEWIRE INJURY

All animal experimentation protocols were performed under the institutional guidelines for animal welfare and in accordance with Animal Care and Use Committee of Renmin Hospital of Wuhan University. Male c57 BL/6 mice at 8 weeks of age were fed either a normal rodent chow diet or a normal chow diet containing 0.09% apigenin (w/w). This diet corresponded to 150 mg/kg apigenin if a 30-g mouse consumed 5 g of chow. The mice were placed on these diets for 14 days before they underwent endothelial denudation injuries by the insertion of a guidewire (0.38 mm diameter; No. C-SF-15-15, Cook, Bloomington, IN) as described previously [Kuhel et al., 2002]. Briefly, with the use of aseptic techniques, the left carotid artery was exposed and the distal bifurcation of the carotid artery was encircled proximally and ligated distally with 8–0 silk suture. A guidewire was introduced into the arterial lumen through a transverse arteriotomy made between the sutures, and the guidewire was then advanced toward the aortic arch and withdrawn five times. After the removal of the guidewire, the proximal 8–0 suture was ligated, and the incision was closed. Sham surgery without injury was performed on the right side. After surgery, the mice were maintained on these diets for 28 days before they were euthanized. The right and left carotid arteries were isolated, fixed in buffered formalin phosphate, and processed for morphometric and immunohistochemical analysis.

EVALUATION OF NEOINTIMA FORMATION

Paraffin-embedded sections for morphometric analyses were cut (5-μm thickness) from equally spaced intervals in the middle of the injured and control common carotid artery segments and stained with hematoxylin and eosin to demarcate the cell types. Fifteen sections from each carotid artery were reviewed and scored under blind conditions. The intimal (I) and medial (M) areas were measured using the Image-Pro Plus 6.0 program, and I/M ratios were calculated.

PROLIFERATING CELL NUCLEAR ANTIGEN IMMUNOHISTOCHEMISTRY

Immunostaining for PCNA was performed as previously described [Wang et al., 2007]. An anti-PCNA monoclonal antibody complemented by a biotinylated anti-mouse secondary antibody was applied to perfusion-fixed, paraffin-embedded tissues. The slides were treated with an avidin-biotin block, exposed to DAB (3,3′-diaminobenzidine) with hematoxylin and analyzed using light microscopy. The data were presented as the number of PCNA-positive-stained cells within the neointima.

STATISTICAL ANALYSES

The results were expressed as the means ± SEM. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by a Dunnett multiple comparison test. The mean values were considered significantly different when $P < 0.05$.

RESULTS

APIGENIN INHIBITS VSMC PROLIFERATION STIMULATED BY PDGF

The abnormal proliferation of VSMCs is known to contribute to the formation of vascular lesions, and apigenin has been shown to be an antitumor compound that leads to growth suppression in cancer cells. However, it is not clear whether apigenin has a growth suppression effect on VSMCs. Cell viability assay (WST-1) which measures metabolic activity as an indirect marker of cell proliferation, was performed to evaluate the effect of apigenin on PDGF-BB-induced cell proliferation. After 48 h incubation periods, VSMC growth significantly increased after treatment with 20 ng/ml of PDGF relative to the non-treated control (Fig. 1A). Apigenin inhibited the PDGF-BB-induced increase in VSMC numbers in a dose-dependent manner. Apigenin at concentrations of 12.5 and 25 μM completely blocked the cell proliferation induced by PDGF-BB. Cells treated with apigenin (2.5–12.5 μM) alone for 48 h in the absence of PDGF-BB showed no significant difference in the viability of VSMCs relative to the untreated cells, whereas apigenin at 25 μM resulted in a slight decrease in cell viability (Fig. 1A). The results suggested that at concentrations of 2.5–12.5 μM, apigenin showed no cytotoxity to the cells. Therefore, we used an apigenin concentration of 12.5 μM in the subsequent study. The inhibitory effect of apigenin on DNA synthesis was investigated further. Figure 1B illustrated the results from the BrdU incorporation assay that demonstrated the same inhibitory pattern on VSMC proliferation. Apigenin significantly inhibited DNA synthesis

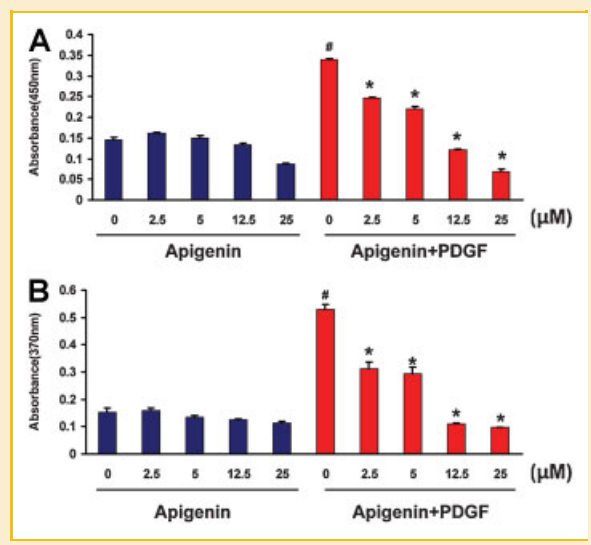

Fig. 1. Apigenin prevents the proliferation and DNA synthesis of VSMCs induced by PDGF-BB. VSMCs were treated with the indicated concentrations of apigenin (2.5–25 μM) for 48 h in the absence or presence of PDGF-BB (20 ng/ml). A: Cell viability was examined using the WST-1 test. Data are expressed as the mean OD450 ± SEM (#$P < 0.01$ vs. the control group; *$P < 0.01$ vs. PDGF alone; n = 6). B: BrdU incorporation was determined using an ELISA-based assay. DNA synthesis is expressed as the mean OD370 ± SEM (#$P < 0.01$ vs. the control group; *$P < 0.01$ vs. PDGF alone; n = 6). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

in a dose-dependent manner, and DNA replication was almost completely blocked in the VSMCs that were treated with apigenin at concentrations of 12.5 and 25 μM compared to the controls (Fig. 1B).

APIGENIN PREVENTS CELL CYCLE ENTRY/PROGRESSION IN THE G0/G1 PHASE

The cell cycle is a final common pathway of proliferative signaling cascades. To elucidate the mechanisms responsible for its antiproliferative effect, the influence of apigenin on cell cycle progression was analyzed. As shown using flow cytometry, the PDGF-BB treatment alone significantly increased the percentage of cells in the S phase while decreasing the G0/G1 populations. In contrast, apigenin at a concentration of 12.5 μM significantly increased the fraction of G0/G1-phase cells and decreased the number of S-phase cells in the VSMCs, indicating that apigenin prevented cell cycle entry/progression into the G0/G1 phase (Fig. 2A,B). This result suggests that apigenin acts during the early processes of cell cycle progression.

APIGENIN INHIBITS CDK EXPRESSION AND P27KIP1 DOWNREGULATION INDUCED BY PDGF-BB

Cell cycle progression is coordinated by CDKs, which form holoenzymes with their regulatory subunits, the cyclins [Malumbres and Barbacid, 2001]. To characterize the mechanism responsible for apigenin-induced cell cycle arrest, the effects of apigenin on cell cycle events, such as CDK expression, were analyzed using western blotting. As shown in Figure 2C, the expression of CDK4 and CDK6 was induced by PDGF-BB (20 ng/ml), whereas the treatment of apigenin (12.5 μM) significantly decreased the expression of these molecules (Fig. 2C).

Cyclin-CDK complexes are precisely regulated by cell cycle inhibitors that block their catalytic activity. One such inhibitor is p27Kip1, which inactivates the cyclin-CDK complexes in the G1 phase leading to cell cycle arrest. Subsequently, we assessed the effect of apigenin on the induction of p27Kip1. P27Kip1 was constitutively expressed in serum-starved quiescent VSMCs and was downregulated by PDGF-BB. In contrast, pretreatment with apigenin restored p27Kip1 expression to levels that were comparable to that in the quiescent cells (Fig. 2C).

APIGENIN PREVENTS PDGF-STIMULATED MIGRATION OF VSMCS

The migration of smooth muscle cells from the media to the intimal region also contributes to the formation of vascular lesions. To determine whether apigenin plays a role in the regulation of VSMC migration, we performed a transwell assay. As indicated in Figure 3A, treatment with PDGF-BB (20 ng/ml) resulted in a greater than threefold increase of VSMCs moving across the membrane; however, pretreatment with apigenin (12.5 μM) markedly inhibited

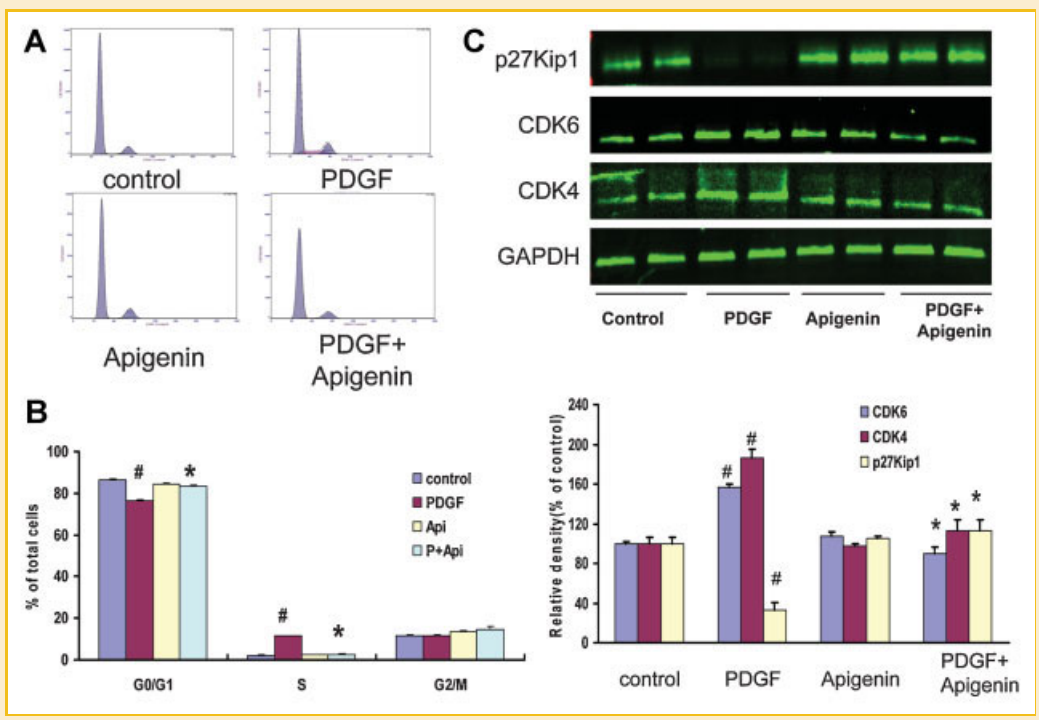

Fig. 2. Apigenin prevents cell cycle progression in VSMCs. VSMCs were grown with apigenin (12.5 μM) in the absence or presence of PDGF-BB (20 ng/ml) for 24 h. Cell cycle distribution was evaluated using flow cytometric analyses. A: Representative cell cycle profiles are shown. B: Quantification of VSMCs in the G0/G1, S, and G2/M phases as determined by flow cytometric evaluation is shown (#$P < 0.01$ vs. the control group; *$P < 0.01$ vs. PDGF alone; n = 3). C: The expression of cell cycle regulatory proteins was determined using western blotting. The detection of GAPDH served as a loading control. The upper panel showed representative western blots; and the lower panel, quantification of three independent experiments. Results are expressed as percentages of the control (#$P < 0.05$ vs. the control group; *$P < 0.05$ vs. PDGF alone). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

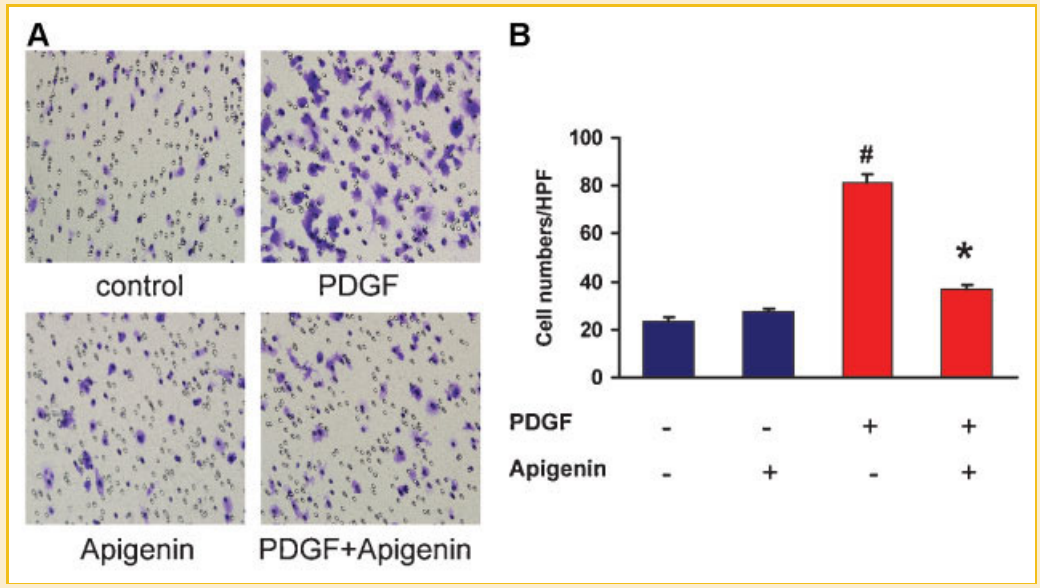

Fig. 3. Apigenin inhibits PDGF-BB-induced cell migration. A: VSMCs were cultured in a cell migration filter insert and stimulated with PDGF-BB for 6 h with or without apigenin treatment (12.5 μM). B: Cellular migration was determined by counting the cells that migrated through the pores. The results are expressed as the means ± SEM (#$P < 0.01$ vs. the control group; *$P < 0.01$ vs. PDGF alone). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

PDGF-BB-induced migration. The number of migrated cells was significantly decreased by apigenin (Fig. 3B). These results suggest that apigenin is a potent inhibitor of VSMC migration.

EFFECT OF APIGENIN ON PHENOTYPIC MODULATION OF VSMCS

Pathological conditions, such as atherosclerosis, restenosis, and hypertension, are associated with phenotypic modulation of VSMCs, which is characterized by a loss of contractility and abnormal proliferation, migration, and matrix secretion [Lagna et al., 2007]. Phenotypically modulated VSMCs exhibit a decreased expression of a variety of contractile genes including SM α-actin, SM22α, and desmin. To further evaluate the phenotypic modulation of VSMCs by apigenin, western blot analysis was used to detect differentiated phenotype markers. Following pretreatment with apigenin (12.5 μM) for 2 h, quiescent VSMCs were stimulated with PDGF (20 ng/ml) for 48 h in the presence/absence of apigenin. As indicated in Figure 4, PDGF caused a reduction in the levels of SM α-actin, SM22α, and desmin proteins. Moreover, pretreatment with apigenin partially blocked the repressive effect of PDGF, suggesting that apigenin had an effect on the maintenance of the quiescent (differentiated) state of VSMCs.

MOLECULAR MECHANISMS INVOLVED IN APIGENIN INHIBITION OF PROLIFERATION AND MIGRATION OF VSMCS

To investigate the molecular mechanisms of the phenotypic modulation of apigenin in VSMCs, PDGF-Rβ phosphorylation was evaluated to determine if it was a direct target for apigenin. The cells were treated with PDGF, and the phosphorylation of PDGF-Rβ(Tyr857) was detected. Our data showed that PDGF induced a rapid and sustained phosphorylation of PDGF-Rβ without affecting their total levels (Fig. 5A,B). We then examined the effects of apigenin on the kinetics of PDGF-induced PDGF-Rβ activation. The cells were pre-treated with 12.5 μM apigenin for 2 h and then treated with PDGF for the indicated duration. As shown in Figure 5, pre-treatment with apigennin significantly inhibited the PDGF-Rβ phosphorylation(Tyr857) that was induced by PDGF-BB. The

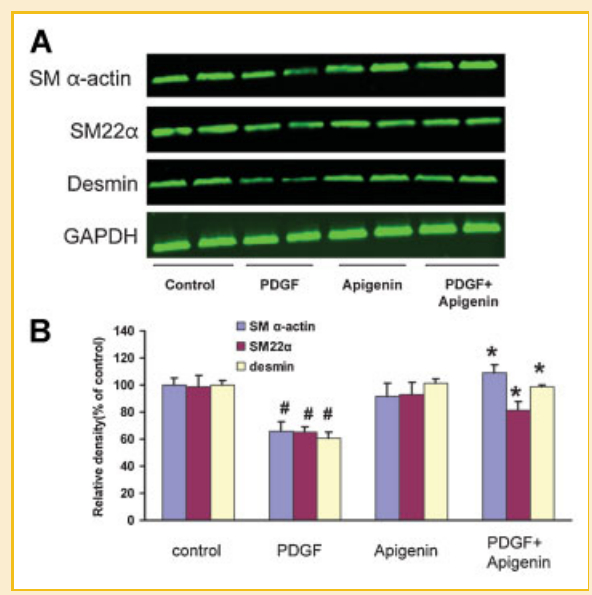

Fig. 4. Effect of apigenin on the regulation of smooth muscle gene expression. A: VSMC preincubated for 2 h with apigenin and then stimulated with PDGF (20 ng/ml) for 48 h. Levels of SM α-actin, SM22α, and desmin proteins were determined using western blot analyses and were quantified by densitometry. B: Bar graphs showing the quantification of the western blots; results are expressed as percentages of the control (#$P < 0.05$ vs. the control group; *$P < 0.05$ vs. PDGF alone). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

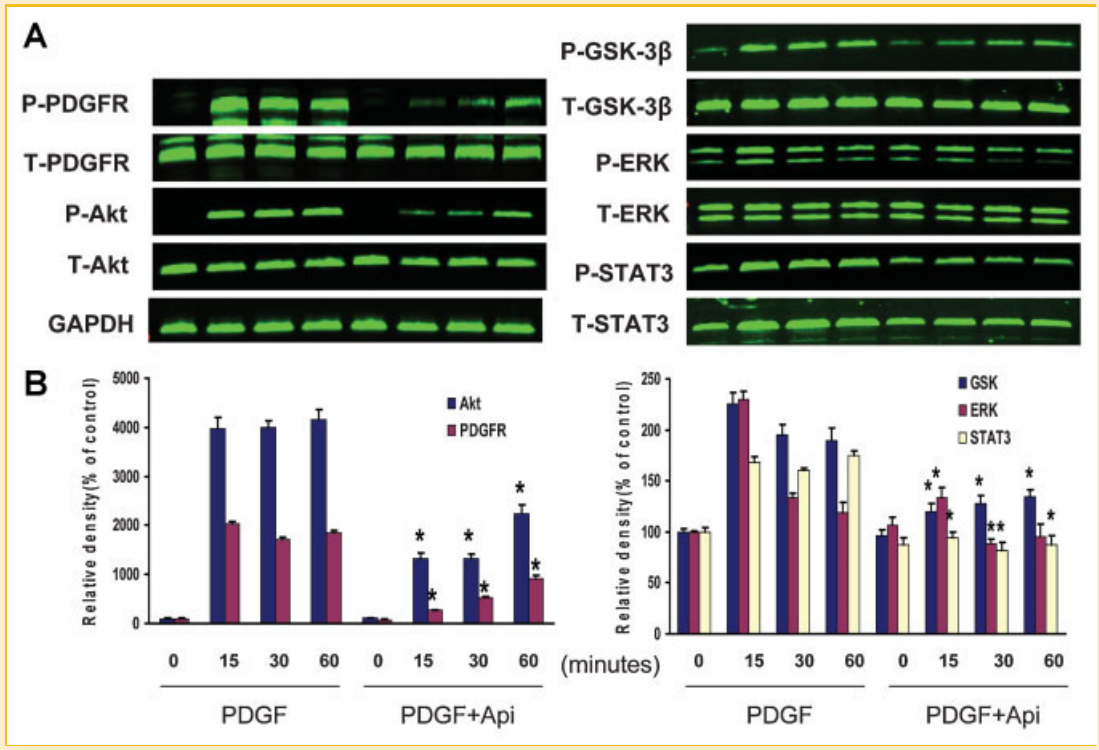

Fig. 5. Inhibitory effects of apigenin on PDGF-Rβ, Akt, GSK-3β, ERK1/2, and STAT3 activation in PDGF-BB-stimulated VSMCs. Serum-starved VSMCs were stimulated with PDGF-BB for the indicated duration in the absence or presence of apigenin (12.5 μM). Levels of phospho-PDGF-Rβ, PDGF-Rβ, phospho-Akt, Akt, phospho-GSK-3β, GSK-3β, phospho-ERK1/2, ERK1/2, phospho-STAT3, and STAT3 proteins were determined using western blot analyses. A: One representative image out of three independently performed experiments is shown. B: Bar graphs showing the quantification of the western blots; results are expressed as percentages of the control ($^*P < 0.05$ vs. PDGF alone). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

observed inhibitory effects of apigenin on PDGF-induced PDGF-Rβ phosphorylation were not due to the reduction in total protein levels.

We then evaluated the effect of apigenin on the PDGF-induced activation of the Akt/GSK3β signal transduction pathway, which is an important downstream signal pathway of PDGF-Rβ and has been demonstrated to have a critical role in VSMC proliferation and migration [Bianchi et al., 2005; Galaria et al., 2005]. Our data demonstrated that stimulation with PDGF resulted in a robust phosphorylation of Akt and GSK3β (Fig. 5A,B). Treatment with apigenin significantly impaired PDGF-induced Akt and GSK3β phosphorylation. Neither treatment with PDGF nor apigenin altered the total protein levels.

We then examined the effect of apigenin on the PDGF-induced activation of ERK1/2 pathways. We treated the VSMCs with PDGF for the indicated durations. Significant phosphorylation of ERK1/2 was first observed at 15 min after PDGF treatment and was sustained for up to 60 min relative to the control as assessed by western blot analysis. We further demonstrated that apigenin significantly inhibited PDGF-induced ERK1/2 phosphorylation (Fig. 5A,B).

STAT3 is also a downstream signal molecule of PDGF and thought to play a role in the regulation of cell growth and differentiation. Indeed, stimulation of VSMCs with PDGF-BB produced a strong activation of STAT3, as demonstrated by the phosphorylation of STAT3. In contrast, preincubation with apigenin (12.5 μM) significantly attenuated the phosphorylation of STAT-3 (Fig. 5A,B).

EFFECT OF APIGENIN ON NEOINTIMA FORMATION AND CELL PROLIFERATION IN VIVO

To evaluate the effect of apigenin on neointima formation, mouse carotid arteries were harvested 28 days after injury and subjected to morphometric analysis. Representative sections from control and apigenin-treated injured carotid arteries are shown in Figure 6A. The formation of the neointima was observed in all guidewire-injured carotid arteries, but marked differences were observed between apigenin-treated and non-treated mice. Morphometric analyses revealed a significant reduction in the neointima area in apigenin-treated carotid arteries compared to injured controls ($8{,}305.9 \pm 1{,}395.1\ \mu m^2$ vs. $16{,}906.6 \pm 2{,}529\ \mu m^2$, respectively; $n = 6$; Fig. 6B). As a result, the I/M ratio in the apigenin-treated group was approximately 50% of the control group (0.357 ± 0.053 vs. 0.716 ± 0.062, respectively; $n = 6$; Fig. 6C). To assess the growth of the VSMCs, arterial sections were stained with an anti-PCNA antibody. The apigenin treatment significantly diminished the injury-induced increase in PCNA-positive staining in neointima of the carotid arteries (165.5 ± 7.8 cells per HPF in the control, $n = 9$; 70.5 ± 10.29 cells per HPF in the apigenin group, $n = 9$) (Fig. 6D).

DISCUSSION

In the present study, we observe that apigenin has potent inhibitory effects on PDGF-BB-stimulated VSMC proliferation, migration, and

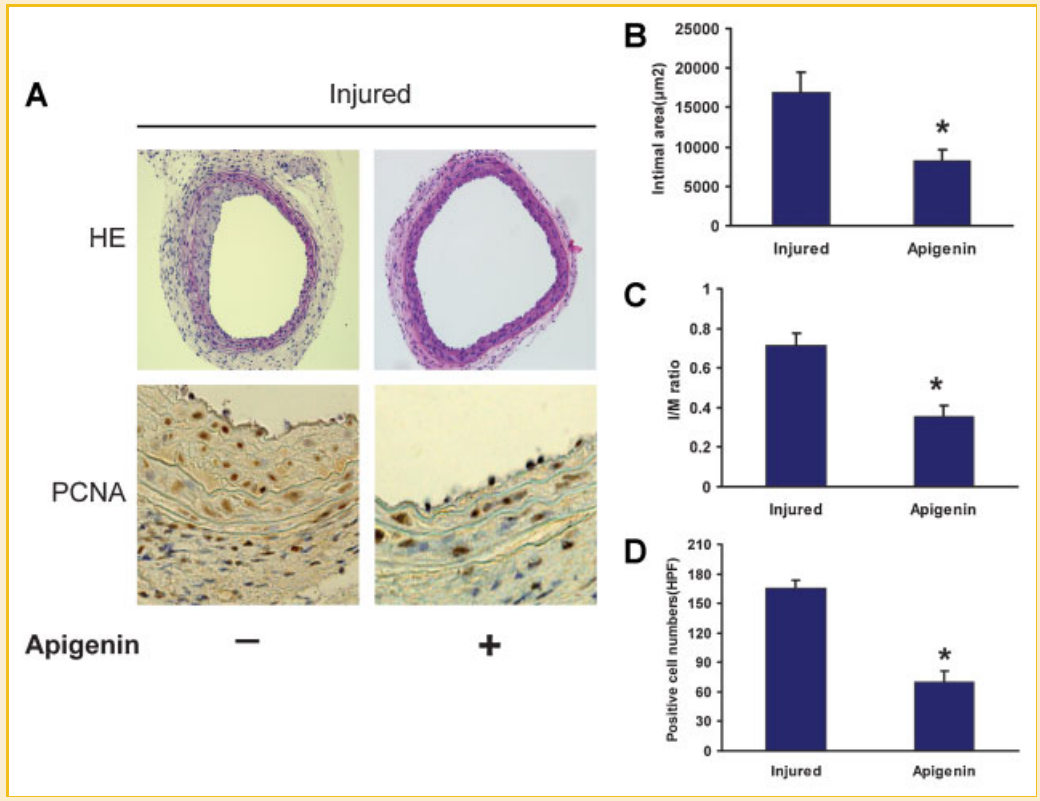

Fig. 6. The preventive effect of apigenin on neointima formation induced by guidewire injury. A: A representative section of the injured carotid artery of an animal from either the control group or the apigenin-treated group ($n = 6$; $^{*}P < 0.01$ vs. the injured control) is shown. B–D: Quantification of I/M ratios, intimal area, and PCNA-positive cells of carotid arteries of mice from either the control group or the apigenin-treated group ($n = 9$; $^{*}P < 0.01$ vs. the injured control). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

phenotypic modulation. We also demonstrate for the first time the inhibition of neointima hyperplasia induced by vascular injury. We provide evidence that apigenin restores p27Kip1 expression and downregulates CDK. These benefits of apigenin on VSMCs are in part attributable to the inhibition of PDGF-Rβ phosphorylation and its downstream signal transduction, including the Akt/GSK-3β, ERK1/2, and STAT3 pathways. Our results suggest that apigenin may prove to be a potential therapeutic agent for the prevention and treatment of restenosis.

Although apigenin has been shown to suppress cell growth in several types of cancer cells, little is known about the effect of apigenin in VSMC proliferation. In this study, we found that apigenin inhibited PDGF-BB-induced proliferation of VSMCs and DNA synthesis in a dose-dependent manner. Consistent with our results, Kim et al. [2002] demonstrated that 50 ng/ml of apigenin significantly inhibited PDGF-BB-induced proliferation of primary cultured rat VSMCs in a concentration-dependent manner as determined by cell counting. However, the effect of apigenin on cell cycle progression was not studied in their work. We performed flow cytometry analyses to further investigate the pattern of the antiproliferative effect of apigenin. As revealed by flow cytometry assay, the antiproliferative effect of apigenin was associated with cell cycle arrest in the G0/G1 phase. However, apigenin has been shown to arrest the cell cycle in different phases for different cell lines. Apigenin has been shown to induce G2/M arrest in rat neuronal cells, mouse keratinocytes, and human colon carcinoma cell lines [Sato et al., 1994; Wang et al., 2000; McVean et al., 2002], whereas apigenin treatment caused G0/G1 arrest in TF1 cells and prostate cancer cells [Shukla and Gupta, 2007; Ruela-de-Sousa et al., 2010]. The observed differences may reflect cell specificity.

In mammalian cells, the process of cell cycle passage is tightly regulated by CDKs, which are positively regulated by their corresponding regulatory cyclin subunits and negatively regulated by CDK inhibitors (CKI) [Malumbres and Barbacid, 2001; Sriram and Patterson, 2001]. Among the key regulators of G1/S-phase entry, CDK4 and CDK6, which are thought to be involved in the early G1 phase, form active complexes with D-type cyclins and promote the transition from the G1 to the S phase. p27Kip1, a well-known CKI, can bind to and inhibit a wide spectrum of cyclin/CDK complexes, including cyclin D-CDK4/6 and cyclin E-CDK2, and prevent the G1/S transition. To further characterize the mechanism responsible for apigenin-induced G1 arrest, we examined the effect of apigenin on these regulatory proteins. Our results show that apigenin significantly decreased the expression of CDK4 and CDK6 and increased p27Kip1 expression in response to PDGF-BB. Consistent with our results, several studies have also revealed that apigenin induced cell cycle arrest via induction of p27Kip1 [Gupta et al., 2002; Way et al., 2005; Shukla and Gupta, 2009]. However, these results stand in contrast to those obtained by another group using human artery

smooth muscle cells, in which apigenin was reported to stimulate VSMC proliferation and inhibit p27Kip1 expression. Whether these differences are related to a differential response of the cells of rat and human origins to these molecules or to different apigenin concentration used in the studies and experimental conditions remain to be determined.

In addition to VSMC proliferation, VSMC migration also contributes to the development of restenosis. Using a transwell migration assay, we found that pretreatment with apigenin significantly reduced PDGF-BB-induced migration, suggesting that apigenin is a potent inhibitor of VSMC migration. Lamy et al. [2008] also found a similar inhibitory effect of apigenin on migration using pulmonary aortic VSMCs. They suggested that blockade of PDGFR-β signal axis was responsible for the inhibitory effect of apigenin on smooth muscle cell migration. Over the last decade, several observations have revealed a close link between cell cycle progression and cell migration. Apart from the well-established function of proliferation suppression, p27Kip1 has been suggested to be a negative regulator of cell migration. P27Kip1 overexpression was found to reduce the migration of human vein endothelial cells (HUVECs) and rabbit aorta VSMCs in culture, and that this effect was associated with the inhibition of CDK activity [Goukassian et al., 2001; Castro et al., 2003; Diez-Juan and Andres, 2003]. Moreover, p27Kip1-null VSMCs have been shown to be more resistant than wild-type cells to the antimigratory properties of rapamycin [Sun et al., 2001]. All these results suggest that p27Kip1 inhibits VSMC migration. In this study, apigenin restored expression of p27Kip1 repressed by PDGF-BB, combined with its inhibitory effect on VSMC migration, we postulated that the effect of apigenin on p27Kip1 expression may also contribute to the inhibition of VSMC migration. Further investigations are needed to clarify the exact role and mechanism of p27Kip1 in the process of inhibition of migration by apigenin. Taken together, our study suggests that p27Kip1, CDK4, and CDK6 are the target molecules of apigenin for the modulation of vascular cell proliferation and migration in a coordinated manner. Therefore, protection against neointimal thickening by apigenin might result from the combination of growth suppression and migration blockade.

VSMC phenotypic modulation is another important event in the pathogenesis of restenosis, which is characterized by a decreased expression of contractile markers [Owens et al., 2004; Lagna et al., 2007]. PDGF-BB has been shown to be a key mediator of VSMC phenotypic switching [Dandre and Owens, 2004]. Consistent with previous studies, we observed that PDGF-BB induced proliferation and migration of VSMCs and a decrease in SM α-actin, SM22α, and desmin expression. More importantly, our in vitro study demonstrated that apigenin partially blocked the repressive effect of PDGF-BB on differentiated phenotype marker expression. These results indicate that apigenin maintains the differentiated phenotype of the VSMCs. A prior report by Ricupero et al. [2001] demonstrated that in human lung myofibroblasts, apigenin reduced basal, and transforming grow factor-β (TGF-β)-stimulated levels of SM-α actin mRNA, although a decreased phosphorylation of Akt and ERK1/2 was also observed after apigenin treatment. Different cell type studied may account for discrepant results between the two studies. Myofibrablast constitutively express low levels of SM-α actin, which could be induced by TGF-β stimulation. On the contrary, VSMCs express high levels of SM-α actin, which could be decreased by PDGF stimulation. These differences may contribute to the different response to apigenin treatment.

The mechanisms by which apigenin inhibits PDGF-BB-induced VSMC proliferation, migration, and phenotypic modulation remain largely unclear. PDGF binds to its cognate receptor, the PDGF-Rβ, leading to its phosphorylation on multiple tyrosine residues. This results in the recruitment and activation of specific signaling molecules that may mediate the migration and proliferation of VSMCs in response to injury[Heldin et al., 1998]. In the present study, we found that apigenin inhibited the phosphorylation of PDGF-Rβ on the residue of Tyr857, which suggest that apigenin directly interfere with the kinase activity of PDGFR [Lamy et al., 2008]. The activated PDGF-Rβ stimulates a plethora of events, including activation of ERK1/2, PI3K/Akt, and STAT. Apigenin has been shown to induce apoptosis through the inhibition of the function of Akt in tumor cells [Way et al., 2004]. Therefore, we evaluated the effect of apigenin on the PDGF-induced activation of Akt and its downstream substrate GSK3β in VSMCs. Our data showed that PDGF-induced Akt/GSK3β activation was significantly impaired by apigenin, suggesting that the interruption of the Akt signaling pathway contributes to the inhibitory role of apigenin in PDGF-BB-induced biological effects in VSMCs. The activation of GSK-3β has been found to regulate the stability and expression of cyclin D1 [Diehl et al., 1998; Karpurapu et al., 2010]. In addition, GSK-3β inhibition has also been shown to decrease the expression of the CDK inhibitor p27Kip1 [Tseng et al., 2006], indicating its involvement in the regulation of p27Kip1 expression. Moreover, Bianchi et al. [2005] found that GSK-3β phosphorylated serine residue of FAK to negatively modulate FAK catalytic activity, which is important in cell migration. We then investigated the effect of apigenin on the ERK1/2 signaling pathway, which is also critical to VSMC proliferation and migration. Western blot analyses showed that apigenin significantly inhibited PDGF-induced ERK1/2 phosphorylation. These findings are consistent with a study showing that apigenin results in a significant inhibition of FBS-induced phosphorylation of ERK1/2 [Kim et al., 2002]. The STAT3 signaling pathway is another key pathway thought to have a role in PDGF-BB-induced biological effects in VSMCs. STAT3 can directly regulate the transcription of cyclin D1 [Leslie et al., 2006]. Moreover, it has been shown that STAT3 activation is involved in VSMC inflammation and neointima formation [Shibata et al., 2003; Kovacic et al., 2010]. We found that PDGF-induced STAT3 activation was also significantly attenuated by apigenin. These results indicate that the inhibitory effects of apigenin in PDGF-BB-stimulated VSMCs are probably mediated by the interruption of PDGF-Rβ phosphorylation and its downstream signal transduction.

Finally, to determine whether our in vitro findings have any physiological relevance, we evaluated the effect of apigenin on neointima formation in the guidewire-injured carotid artery in vivo. Our data showed that the I/M ratios and neointima area were significantly reduced in apigenin-treated carotid arteries compared to injured controls. Moreover, apigenin significantly diminished the injury-induced increase in PCNA-positive staining in the neointima of carotid arteries. These results substantiate our in vitro findings

and strongly support the notion that apigenin confers protection against injury-induced pathological vascular remodeling.

In summary, apigenin is capable of inhibiting PDGF-BB-stimulated VSMC activation and attenuating the neointima response to experimental arterial injury, probably through the blockade of PDGF-Rβ phosphorylation and its downstream signal transduction. This study suggests that apigenin is a potential therapeutic candidate that warrants further investigation regarding its potential use in the prevention of restenosis.

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