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RESEARCH PAPER

Protective effect of nectandrin B, a potent AMPK activator on neointima formation: inhibition of Pin1 expression through AMPK activation

Sung Hwan Ki[1], Jung-Woon Lee[1], Sung Chul Lim[2], Tran Thi Hien[1,3], Ji Hye Im[3], Won Keun Oh[1], Moo Yeol Lee[4], Young Hyun Ji[5], Yoon Gyoon Kim[5] and Keon Wook Kang[3]

[1]*BK21 Project Team, College of Pharmacy, Chosun University, Gwangju, South Korea,* [2]*Department of Pathology, College of Medicine, Chosun University, Gwangju, South Korea,* [3]*College of Pharmacy* and *Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, South Korea,* [4]*College of Pharmacy, Dongguk University, Goyang, Gyeonggi-do, South Korea, and* [5]*College of Pharmacy, Dangook University, Cheonan, South Korea*

Correspondence
Keon Wook Kang PhD, College of Pharmacy, Seoul National University, San 56-1 Sillim-dong, Gwanak-gu, Seoul 151-742, South Korea. E-mail: kwkang@snu.ac.kr

First two authors (Ki SH and Lee JW) equally contributed to this work.

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BACKGROUND AND PURPOSE

Neointima is considered a critical event in the development of vascular occlusive disease. Nectandrin B from nutmeg functions as a potent AMP-activated protein kinase (AMPK) activators. The present study addressed whether nectandrin B inhibits intimal hyperplasia in guide wire-injured arteries and examined its molecular mechanism.

EXPERIMENTAL APPROACH

Neointima was induced by guide wire injury in mouse femoral arteries. Cell proliferation and mechanism studies were performed in rat vascular smooth muscle cells (VSMC) culture model.

KEY RESULTS

Nectandrin B increased AMPK activity in VSMC. Nectandrin B inhibited the cell proliferation induced by PDGF and DNA synthesis. Moreover, treatment of nectandrin B suppressed neointima formation in femoral artery after guide wire injury. We have recently shown that Pin1 plays a critical role in VSMC proliferation and neointima formation. Nectandrin B potently blocked PDGF-induced Pin1 and cyclin D1 expression and nectandrin B's anti-proliferation effect was diminished in Pin1 overexpressed VSMC. PDGF-induced phosphorylation of ERK and Akt was marginally affected by nectandrin B. However, nectandrin B increased the levels of p53 and its downstream target p21 and, also reversibly decreased the expression of E2F1 and phosphorylated Rb in PDGF-treated VSMC. AMPK inhibition by dominant mutant form of adenovirus rescued nectandrin B-mediated down-regulation of Pin1 and E2F1.

CONCLUSIONS AND IMPLICATIONS

Nectandrin B inhibited VSMC proliferation and neointima formation via inhibition of E2F1-dependent Pin1 gene transcription, which is mediated through the activation of an AMPK/p53-triggered pathway.

Abbreviations

ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, adenosine 5′-monophosphate-activated protein kinase; BrdU, 5-bromo-2′-deoxy-uridine; CDK, cyclin-dependent kinase; DN-AMPK, dominant negative mutant of AMPK; E2F1, E2 transcription factor1; MEF, mouse embryonic fibroblast; MSCV, murine

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stem cell viral; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI3K, phosphatidylinositol 3-kinase; Pin1-VSMC, Pin1-overexpressing VSMC; Rb, retinoblastoma; VSMC, vascular smooth muscle cells; WT-AMPK, wild-type AMPK

Introduction

Restenosis after balloon angioplasty surgery is a critical clinical problem (Choy *et al.*, 2001). Neointimal hyperplasia, defined as abnormal growth of the intimal layer of blood vessels, is considered a critical event in the development of restenosis and atherosclerosis (Fischman *et al.*, 1994). After vascular injury, vascular smooth muscle cells (VSMCs) acquire a new phenotype, migrate more to the site of injury, and then show proliferation and adhesion at the intimal layer. Although the exact mechanism of these complicated events is not fully understood, inhibition of VSMC proliferation is considered a potential therapeutic interruption, one that decreases the incidence of vascular occlusive diseases. In clinics, paclitaxel, an anti-cancer agent that acts through inhibition of cell cycle progression, has frequently been used as a coating material for stents (Herdeg *et al.*, 1998).

AMP-activated protein kinase (AMPK), a physiological sensor of cellular energy status, plays a crucial role in regulating lipid homeostasis and whole body glucose and has been suggested as a potential target for treating obesity and diabetes (Misra, 2008). AMPK activation also has a protective effect on the heart as well as on vascular endothelial and smooth muscle cells (Xu and Si, 2010). A defect in AMPK signalling is involved in the genesis of chronic cardiovascular diseases including atherosclerosis and hypertension (Xu and Si, 2010). Several studies have demonstrated that AMPK may be a therapeutic target for preventing neointimal hyperplasia and restenosis. A chemical AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), suppresses both angiotensin II-stimulated DNA synthesis in VSMC and neointimal formation in a balloon injury rat model (Nagata *et al.*, 2004). Treatment of human aortic smooth muscle cells and aortic strips with AICAR results in the blocking of cell proliferation through inhibition of cell cycle progression (Igata *et al.*, 2005). It has been also reported that AMPKα2 deletion exacerbates neointima formation by up-regulating Skp2 in VSMCs (Song *et al.*, 2011).

Pin1, a peptidyl prolyl isomerase, is an enzyme that specifically recognizes phosphorylated Ser/Thr immediately preceding a Pro residue and accelerates the isomerization of the peptide bond (Lu *et al.*, 1996). The main physiological functions of Pin1 have been studied by investigators in the field of cancer biology. In fact, Pin1 is overexpressed in most types of tumour tissues (Bao *et al.*, 2004) and Her-2/Neu-induced tumour formation was diminished in Pin1-null mice (Wulf *et al.*, 2004). We and other groups found that Pin1 is involved in the development of neurodegenerative diseases and rheumatoid arthritis (Balastik *et al.*, 2007; Jeong *et al.*, 2009). Our recent study demonstrated that Pin1 was up-regulated in the injured vascular wall, and that Pin1 overexpression is necessary for VSMC proliferation and neointimal formation (Kim *et al.*, 2010).

Phytochemicals are becoming of increasing interest as potential therapeutics against diverse cardiovascular diseases. *Myristica fragrans* (nutmeg), an aromatic evergreen tree cultivated in India, South Africa and other tropical countries, has been used as food and in traditional oriental medicine (Grover *et al.*, 2002). Extracts of its seeds possess anti-hyperlipidaemic and anti-atherosclerotic activities *in vivo* (Sharma *et al.*, 1995). We have isolated seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans from total extracts of *M. fragrans* and 5 μM nectandrin B showed a strong AMPK stimulation in differentiated C2C12 cells (Nguyen *et al.*, 2010). In the current study, we determined whether nectandrin B inhibits VSMC proliferation and neointimal formation in a wire-injured femoral artery mouse model. We also tried to clarify its molecular mechanism, focusing on signalling pathways for its AMPK activation and Pin1 down-regulation.

Methods

Materials

Antibodies against Pin1, E2 transcription factor1 (E2F1), p53, retinoblastoma (Rb) and p21 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phosphorylated form-specific or total form recognizing antibodies against Akt, ERK, AMPK and acetyl-CoA carboxylase (ACC), and antibodies for phosphorylated Rb, Cyclin D1, and HRP-conjugated anti-rabbit, anti-mouse IgGs were purchased from Cell Signaling Technology (Beverly, MA, USA). Alkaline phosphatase-conjugated donkey anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). PDGF-BB was purchased from PeproTech (Rocky Hill, NJ, USA). Anti-actin antibody and most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO, USA). Dominant-negative mutant of AMPK (DN-AMPK) and wild-type AMPK (WT-AMPK) adenoviruses were kindly donated by Dr. JH Ha (College of Medicine, Kyunghee University, Seoul, Korea). Labelling and detection kits for 5-bromo-2′-deoxy-uridine (BrdU) were purchased from Roche (Mannheim, Germany).

Nectandrin B isolation

The dried semens of *M. fragrans* were purchased at a medicinal market in Gwangju, South Korea. The sample was identified by Professor YH Moon at Chosun University, and a specimen (No. 0010) was deposited at the Department of Pharmacy, Chosun University. The ethanol extract of *M. fragrans* was subjected to an HP-20 column (10 × 60 cm), eluted with a gradient of ethanol in H_2O (60, 80, 90 and 100%, each 3 L), and finally washed with acetone (2 L) to give five fractions. Bioassay of the five fractions for AMPK activity revealed that the 80% ethanol-eluted fraction was most active as an AMPK activator. This fraction was further chromatographed

over silica gel (6 × 60 cm; 63–200 μm particle size) using a gradient of *n*-hexane/acetone (from 6:1 to 0:1), to yield five fractions (F.1–F.5) according to their profiles. Nectandrin B was purified from a part of fraction 2 by chromatography on a reverse phase ODS-A column (5.0 × 60 cm, 150 μm particle size) eluted with methanol/H_2O (1.5:1 to 2:1, each 3 L). The purity of nectandrin B was confirmed by HPLC to be >95% (Figure 1A).

Cell culture

VSMC were isolated from rat thoracic aorta as described previously (Lee *et al.*, 2009). Briefly, the aortas were removed, cut open longitudinally, cleaned of connective tissue, fat and endothelium, and digested with collagenase and elastase to remove the adventitia and to dissociate the VSMC. Individual cells were plated in a culture dish, and grown in DMEM containing 10% FBS, 100 units mL^{-1} penicillin and 100 g mL^{-1} streptomycin. Cells were passaged by trypsinization, and passages between 5 and 12 were used for experiments. Pin1-overexpressing VSMC (Pin1-VSMC) was previously established by Murine Stem Cell Viral (MSCV)-retrovirus system (Clontech, Mountain View, CA, USA) (Kim *et al.*, 2010). Pin1 cDNA was subcloned into a pMSCVhyg retroviral vector and phoenix cells (a packaging cell line) were transfected with a pMSCVhyg plasmid (control) or a pMSCVhyg-Pin1 (Pin1 overexpressed) plasmid. Supernatants containing amphotrophic replication-incompetent retroviruses were collected and then stored at –80°C until required. Thirty per cent confluent VSMCs were infected 16 times with retrovirus particles. Intensities of infection were confirmed by Western blot analysis using a specific antibody. Mouse embryonic fibroblast (MEF) cells were obtained from Dr. Kwak MK (Catholic University, Korea) and maintained in DMEM medium containing 10% fetal calf serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C in a humidified atmosphere.

Immunoblot analysis

After washing with sterile PBS, the VSMCs were lysed in lysis buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 μg·mL^{-1} leupeptin. The cell lysates were centrifuged at 10 000 *g* for 10 min to remove cell debris, and the proteins were then fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper and immunoblotted using specific antibodies. HRP- or alkaline phosphatase-conjugated anti-IgG antibodies were used as the secondary antibodies. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium or an enhanced chemiluminescence system. For chemiluminescence detection, an LAS3000-mini system (Fujifilm, Tokyo, Japan) was used.

MTT assay for cell proliferation assessment

Viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg mL^{-1}) for 4 h. Media were then removed and the formazan crystal-stained cells were dissolved in 200 μL dimethylsulfoxide. Absorbance was assayed at 540 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

BrdU assay for DNA synthesis

VSMC were incubated with BrdU labelling solution (10 μM) for 2 h. Cells were fixed with fixation solution for 30 min at room temperature and incubated with 100 μL anti-BrdU peroxidase-labelled antibody for 90 min. After three washes, substrate solution for colorimetric quantification was added at a final concentration of 100 μL mL^{-1} and left at room temperature for 5–30 min until colour development was sufficient for photometric detection. The absorbance was assayed at 405 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

Mouse femoral artery injury model and immunohistochemistry

The protocols of the animal studies were approved by the Animal Care Committee of Chosun University (Gwangju, South Korea). Transluminal mechanical injury of bilateral femoral arteries was induced by introducing a large wire as previously reported (Sata *et al.*, 2001), In brief, the left femoral arteries of imprinting control region (ICR) male mice were exposed by blunt dissection, and was looped proximally and distally with 6-0 silk suture for temporal control of blood flow during the procedure. A straight spring wire, 0.38 mm in diameter (Cook, Bloomington, IN, USA), was carefully inserted into the femoral artery towards the iliac artery via a small branch between the rectus femoris and the vastus medialis muscles. The wire was left in place for 1 min to denude and dilate the artery. Then the wire was removed, and the silk suture at the proximal portion of the muscular branch artery was secured. Blood flow in the femoral artery was restored by releasing the sutures placed in the proximal and distal femoral portions. The skin incision was closed with a 6-0 silk suture. At 21 days after the injury, mice were killed by CO_2 inhalation, and were pressure-perfused at 100 mm Hg with 0.9% sodium chloride solution, followed by pressure-fixation with a 4% paraformaldehyde solution. The femoral artery was then carefully excised and embedded in paraffin. For the immunohistochemistry of Pin1 and E2F1, a universal immunoenzyme polymer method was used for immunostaining. Sections were cut from formalin-fixed, paraffin-embedded tissue blocks, mounted on polylysine-coated slides, dewaxed in xylene, and rehydrated through a graded ethanol series. After deparaffinization, antigen retrieval treatment was performed at 121°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) and was then treated with 3% hydrogen peroxide in methanol solution for 20 min to quench endogenous peroxidase activity. To block intrinsic avidin–biotin binding, the tissue slides were treated with avidin–biotin blocking kit reagents (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 15 min. Anti-Pin1 and anti-E2F1 antibodies were used as the primary antibodies. The final products were visualized using the 3-3′diaminobenzidine tetrahydrochloride detection system (DakoCytomation, Glostrup, Denmark). All experiments were performed in duplicate.

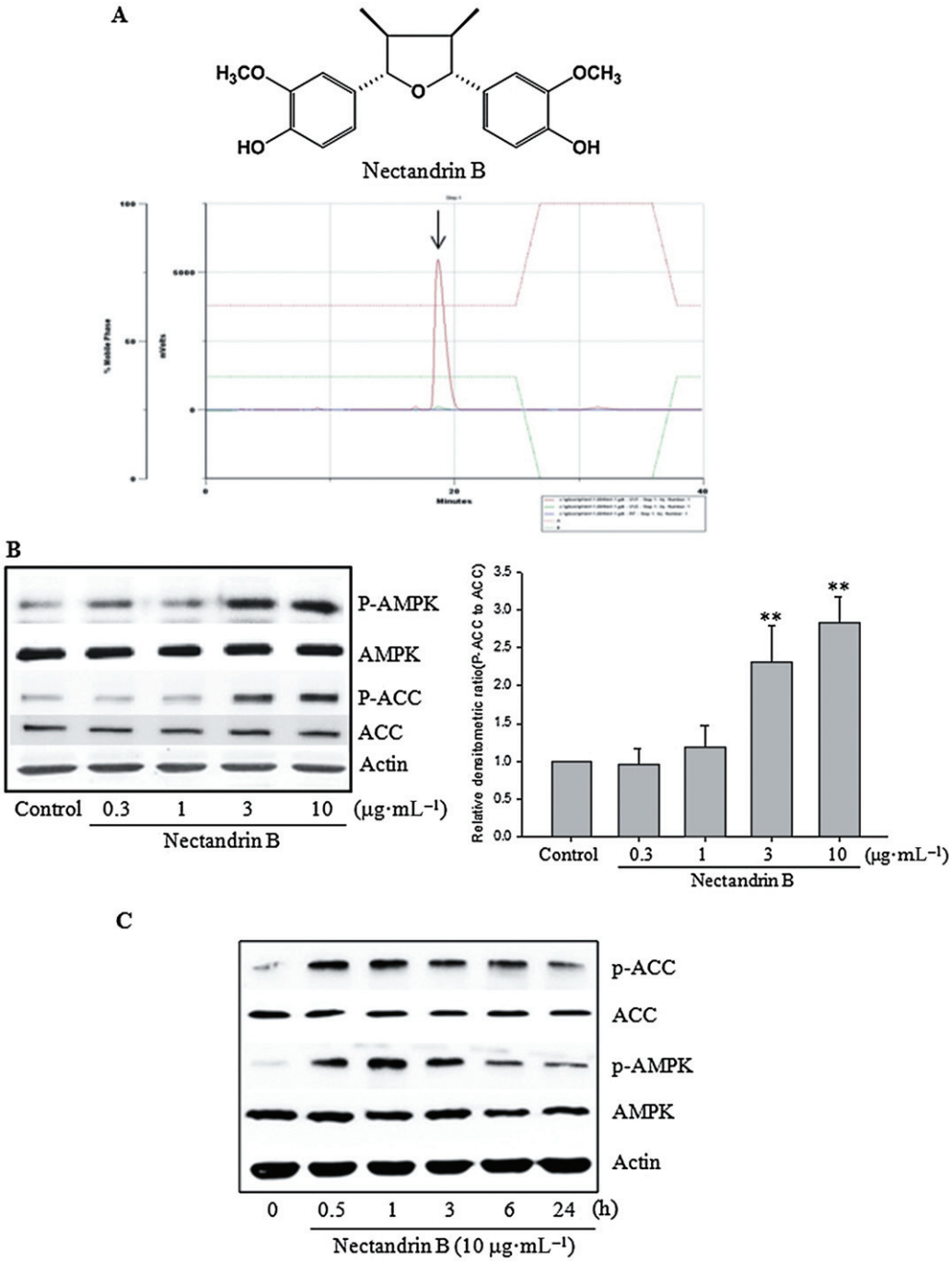

Figure 1

AMPK activation by nectandrin B. (A) Structure of nectandrin B and purity assessment by HPLC. (B) Concentration-dependent AMPK activation by nectandrin B. VSMC were serum starved for 6 h and incubated with nectandrin B (0.3–10 μg mL^{-1}) for 1 h. Left; representative immunoblots show total and phosphorylated AMPK and ACC. Right; relative changes in the ACC phosphorylation were assessed by scanning densitometry. Data represent the means ± SD of three separate experiments (significant as compared with control, $^{**}P < 0.01$; control level = 1). Equal loading of proteins were verified by actin immunoblot. (C) Time-dependent AMPK activation by nectandrin B. VSMC were serum starved for 12 h and incubated with 10 μg mL^{-1} nectandrin B for the indicated time points (30 min, 1, 3, 6 and 24 h).

Apparatus and LC-MS/MS conditions for pharmacokinetics of nectandrin B

The automated blood sampling unit consisted of a freely moving mouse containment device (RaturnTM, BASi, West Lafayette, IN, USA) and an automated blood sampler Culex™ (BASi). The blood samples were collected in a fraction collector Honey Comb™ (BASi). The LC-MS/MS system used was a UltiMate® 3000LC unit (Thermo Fisher Scientific Inc., IL,

USA) connected to a API3200 quadruple MS/MS unit (AB Sciex Inc., MA, USA). Blood samples were analysed by HPLC using Cadenza CD-C18 (Imtakt, Kyoto, Japan), 50 × 2.1 mm, 3 μm particle size main column. An isocratic mobile phase consisting of solvent A (purified water containing 0.1% formic acid) and solvent B (methanol containing 0.1% formic acid) mixed in the ratio 15/85 (v/v, A/B) was used at a flow rate of 0.3 mL min^{-1}. The column oven was maintained at 40°C and the run time was 2 min. Multiple reaction monitoring transitions and collision energy (eV) for nectandrin B and internal standard (drotaverine) were m z^{-1} 345.3 → 137.3 (33 eV) and 398.1 → 326.1 (31 eV), respectively. Scan and dwell times were 1.2 and 0.4 s, respectively. Electrospray ionization was performed at ionspary voltage of 5500 V at 180°C.

Sample preparation for pharmacokinetics of nectandrin B

ICR male mice (24–27 g) were acclimated for 1 week before the study. Upon arrival, animals were randomized and housed at three per cage under strictly controlled environmental conditions (20–25°C and 48–52% relative humidity). A 12-h light/dark cycle was used at an intensity of 150–300 Lux. After anaesthetizing mice using Zoletil 50 (10 mg kg^{-1}, i.m.), carotid artery was cannulated using CX-2052S (BASi). 0.1 mL of heparinized normal saline (20 units mL^{-1}) was injected to prevent clotting. After surgery, mice were recovered for 4–5 h until pharmacokinetics study in metabolic cage. Nectandrin B was dissolved in 40% polyethyleneglycol 400, 5% ethanol, 5% Tween 80 and 50% sterile water and was intraperitoneally injected at the dose of 20 mg kg^{-1}. The blood sampling times were 0 (before administration), 5, 15, 30, 45, 60, 90, 120 and 180 min after injection with virtually no blood loss, and sample volumes were compensating for with equal volumes of heparinized saline. At each blood sampling time, the automatic blood sample system was programmed to collect 20 μL of blood together into microvials containing 50 μL of heparinized saline (20 units mL^{-1}). The sample was centrifuged for 3 min at 12 000 rpm and 30 μL of plasma was collected and kept in –70°C until LC/MS/MS assay. After adding 5 μL of internal standard solution (100 ng mL^{-1}), 10 μL of 1 M hydrochloride and 500 μL of ethylacetate to 30 μL of mouse plasma sample, the mixture was vortexed for 30 s and centrifuged for 10 min at 12 000 rpm. Four hundred fifty microlitres of organic phase was transferred to a clean microtube. The organic phase was evaporated in nitrogen stream. After adding 100 μL of methanol to the residue, the mixture was vortexed for 30 s and centrifuged for 10 min at 12 000 rpm. Five microlitres of aliquot was injected into HPLC.

Reporter gene assay

Pin1 promoter reporter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Briefly, VSMC or MEF cells were replated in 12-well plates overnight and transiently transfected with Pin1-Luc reporter plasmids/phRL-SV plasmid (hRenilla luciferase expression for normalization) (Promega) using FuGENE® 6 (for VSMC, Roche Applied Science, Indianapolis, IN, USA) or Hilymax® reagent (for MEF cells, Dojindo Molecular Tech., MD, USA). Cells were then exposed to PDGF for 18 h, and the firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (Berthold Tech., Bad Wildbad, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to hRenilla luciferase.

Gel shift analysis

Preparation of nuclear extracts was performed as described previously (Kim *et al.*, 2008). Double-stranded oligonucleotides corresponding to the functional E2F-binding site in the promoter region of the Pin1 gene (Ryo *et al.*, 2002) was used for gel shift analysis after end-labelling the probe with [γ-^{32}P]ATP using T_4 polynucleotide kinase. The sequences of the E2F region in the Pin1 promoter was 5-CGGGAGT TTTTTGAAGCTCGCTAAAGG-3. Reaction mixtures contained 4 μL of 5 × binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg mL^{-1} poly dI-dC, 50 mM Tris-Cl (pH 7.5), 5 μg of nuclear extracts and sterile water to a total volume of 20 μL. The reaction mixtures were preincubated for 10 min. DNA-binding reactions were performed at room temperature for 20 min after adding 1 μL of probe (10^6 cpm). Binding specificity was determined with competition experiments using a 10-fold excess of an unlabelled oligonucleotide to reaction mixtures before the DNA-binding reaction. Samples were loaded onto 4% polyacrylamide gels at 100 V, and gels were dried and autoradiographs were obtained using a FLA-7000 phosphoimaging system (Fujifilm, Tokyo, Japan).

Adenovirus infection

VSMC cultured in 6-well plates were treated with DN-AMPK or WT-AMPK adenovirus for 18 h and then incubated in DMEM without serum for an additional 18 h. VSMC were then exposed to nectandrin B (1, 3, 5 and 10 μg mL^{-1}) for 30 min and treated with PDGF (30 ng mL^{-1}) for an additional 24 h.

Data analysis

One-way ANOVA procedures were used to assess significant differences between treatment groups. When treatment was found to have a significant effect, the Newman–Keuls test was used to compare multiple group means. Statistical significance was accepted at either $P < 0.05$ or $P < 0.01$.

Results

AMPK activation by nectandrin B in VSMC

In order to assess AMPK activation effects of nectandrin B, nectandrin B was added to VSMC in a concentration-dependent manner. Immunoblot analyses revealed that both the protein levels of phosphorylated AMPK and phosphorylated ACC were increased by 3 or 10 μg mL^{-1} nectandrin B (Figure 1B). We additionally determined time-course effect of nectandrin B on AMPK activity in VSMC. As shown in Figure 1C, nectandrin B activates AMPK up to 24 h. However, the maximal activity was found at 1 or 3 h after 10 μg mL^{-1} nectandrin B treatment. These data demonstrate that nectandrin B activates AMPK in VSMC.

Effects of nectandrin B on neointima formation and PDGF-induced VSMC proliferation

VSMC proliferation is a key event in the pathogenesis of vascular diseases, including atherosclerosis, post-balloon angioplasty restenosis and vein graft occlusion (Braun-Dullaeus *et al.*, 1998). PDGF, a crucial mediator of VSMC proliferation, plays an important role in intimal hyperplasia (Uchida *et al.*, 1996). MTT assays showed that PDGF (30 ng mL^{-1})-stimulated VSMC proliferation was significantly inhibited by nectandrin B (1–10 μg mL^{-1}) (Figure 2A). To confirm this effect, we did BrdU assays. Incubation of serum-deprived VSMC with PDGF (30 ng mL^{-1}) for 24 h increased DNA synthesis more than 1.8-fold versus untreated controls and the enhanced DNA synthesis was significantly suppressed by nectandrin B in a concentration-dependent manner (Figure 2B).

We then assessed whether nectandrin B inhibits neointima formation induced by guide wire-induced arterial injury. Intraperitoneal injection of nectandrin B [20 mg kg^{-1}, twice a week (Monday and Thursday) for 3 weeks] significantly inhibited intimal hyperplasia compared with the vehicle-treated group (Figure 2C). These results demonstrate that nectandrin B contains inhibitory activity against PDGF-mediated VSMC proliferation and neointima formation. We additionally performed pharmacokinetics study after intraperitoneal injection of nectandrin B. As shown in Figure 2D and Table 1, AUC_t (μg min mL^{-1}) of nectandrin B after intraperitoneal injection of 20 mg kg^{-1} nectandrin B is 39.8 ± 9.5. Hence, enough amounts of nectandrin B may reach the injured vessel in our experimental condition.

Effects of nectandrin B on PDGF-stimulated Pin1 and cyclin D1 expression

Very recently, we found that Pin1 inhibition blocked PDGF-induced VSMC proliferation and cyclin D1 expression in cultured VSMC (Kim *et al.*, 2010). Lv *et al.* also showed that lentivirus-mediated Pin1 knock-down resulted in cell cycle arrest and enhancement of apoptosis in VSMC (Lv *et al.*, 2010). Cyclin D1 is a key cell cycle regulator of the G1/S checkpoint and is important for VSMC proliferation (Goukassian *et al.*, 2001). Moreover, cyclin D1 expression is positively regulated by Pin1 through transcriptional or posttranslational mechanisms, and this seems to be required for Pin1-mediated cell proliferation (Ryo *et al.*, 2002). When we determined Pin1 and cyclin D1 expression in PDGF-treated VSMC, we found that PDGF treatment sharply increased protein levels of Pin1 and cyclin D1 in VSMC, whereas nectandrin B suppressed expression of both proteins in a concentration-dependent manner (Figure 3A).

We then tested whether Pin1 overexpression restores nectandrin B's inhibitory effect on VSMC proliferation. As previously reported (Kim *et al.*, 2010), the basal VSMCs proliferation rate under serum-free condition was increased in Pin1-overexpressing VSMC following Pin1-retroviral infection (Pin1-VSMC) (Figure 3B). In comparison with control VSMC (Con-VSMC), inhibition of VSMC proliferation by nectandrin B was diminished in Pin1-VSMC (Figure 3B). From these results, we conclude that nectandrin B inhibition of VSMC proliferation is associated with down-regulation of Pin1 expression.

Decreased Pin1 gene transcription by nectandrin B and effects on PDGF-stimulated E2F1 expression

In order to determine whether transcriptional activity of the Pin1 gene is affected by nectandrin B, a reporter gene assay was done using a Pin1-Luc reporter plasmid containing the luciferase structural gene and the human Pin1 promoter. In VSMC, Pin1 promoter activity was increased 2.7-fold by PDGF treatment and this effect was significantly decreased by 1–10 μg mL^{-1} nectandrin B (Figure 4A). Because primary cultured VSMC show low efficiency for liposome-based transfection, we further confirmed the result using MEF cells. Nectandrin B also significantly reduced Pin1 promoter activity in MEF cells (Figure 4B), which implied that nectandrin B's Pin1 inhibition is mediated through the blocking of Pin1 gene transcription. E2F transcription factors play an important role in the regulation of cell cycle progression and the function of tumour-suppressor proteins (Neuman *et al.*, 1996). Rb phosphorylation disrupts the formation of the Rb/E2F1 complex, leading to cell cycle progression (Polager and Ginsberg, 2008). Because E2F1 is crucial for the transcriptional activation of the Pin1 gene (Ryo *et al.*, 2002), we hypothesized that Rb phosphorylation and E2F1 might be affected by nectandrin B in PDGF-treated VSMC. We found that the protein expression of both E2F1 and phosphorylated Rb was diminished in the nectandrin B-treated group (Figure 4C). Moreover, both the E2F binding activity to Pin1 promoter and E2F1 mRNA levels were significantly reduced by nectandrin B treatment in VSMC (Supporting Information Figure S1A and B).

Because Pin1 expression is enhanced by growth factors and serum (Ryo *et al.*, 2002; You *et al.*, 2002), nectandrin B-mediated Pin1 down-regulation may result from the blocking of cell proliferation signalling, such as PI3K or ERK. In our previous study, it was also revealed that PI3K actively participates in Pin1 expression in tamoxifen-resistant MCF-7 cancer cells (Lee *et al.*, 2011). Thus, we examined the effects of nectandrin B on PDGF-induced activation of several kinases including ERK and PI3K in order to elucidate the signalling pathway for Pin1 down-regulation by nectandrin B. To identify the activities of both kinases, we used antibodies specific

Table 1

Pharmacokinetic parameters of nectandrin B (20 mg kg^{-1}) after intraperitoneal injection to mice

Pharmacokinetic parameter	Mean ± SD
C_{max} (μg mL^{-1})	1.18 ± 0.11
T_{max} (min)	21 ± 8
AUC_t (μg min mL^{-1})	39.76 ± 9.53
AUC_{inf} (μg min mL^{-1})	52.21 ± 30.39
Terminal $t_{1/2}$ (min)	29.8 ± 8.6

The plasma concentration–time profile after intraperitoneal injection of nectandrin B (20 mg kg^{-1}) to five mice is shown in Figure 1D.

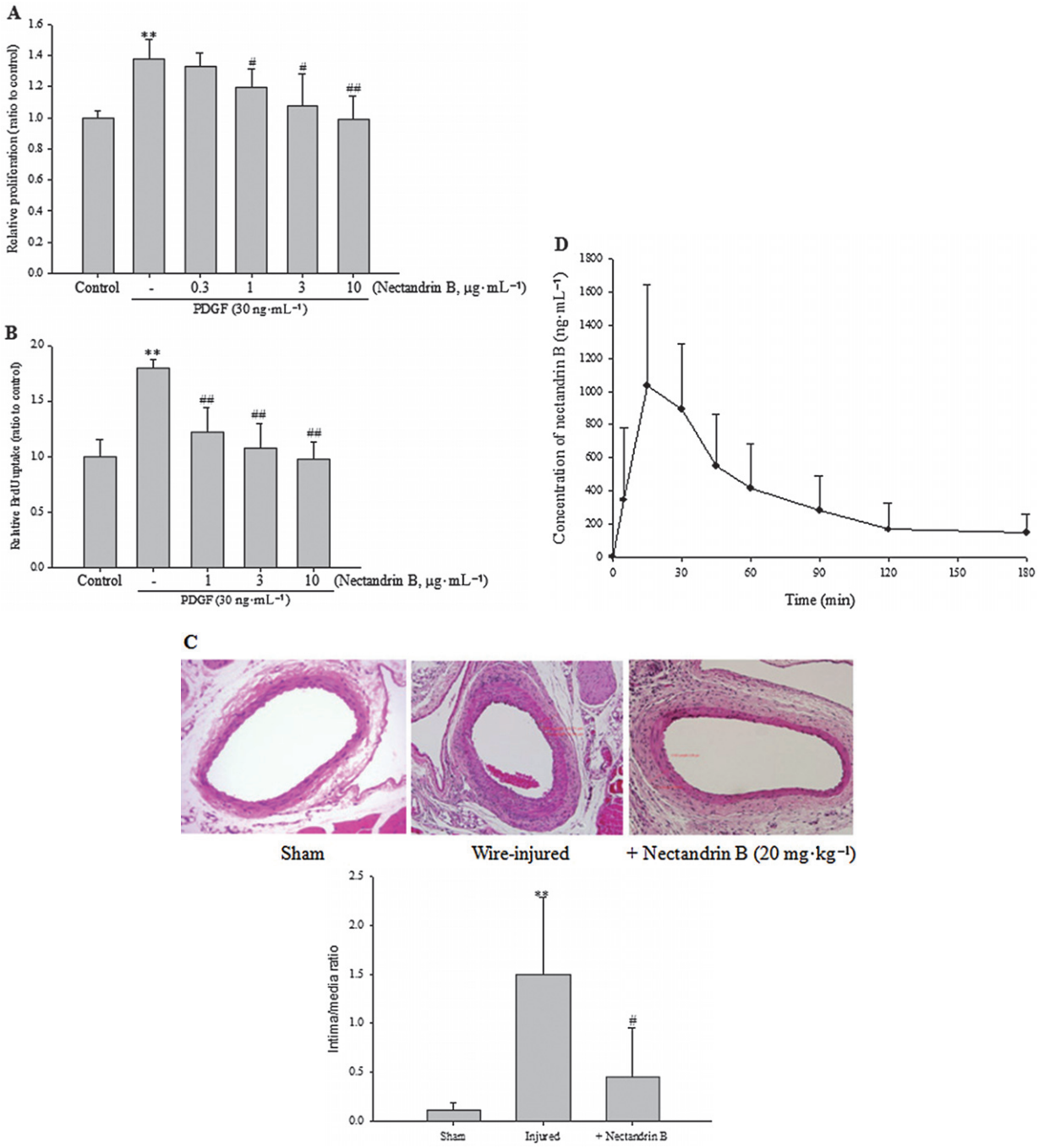

Figure 2

Suppression of neointimal formation and VSMC proliferation by nectandrin B. (A) Inhibitory effect of nectandrin B on PDGF-induced VSMC proliferation. VSMC were treated with or without PDGF (30 ng mL^{-1}) in the presence or absence of nectandrin B (0.3–10 μg mL^{-1}) for 24 h. Nectandrin B was treated 1 h before PDGF treatment. Cell proliferation was detected by using MTT assay. Data represent the mean ± SD of 14 different samples (significant as compared with control group, $^{**}P < 0.01$; significant as compared with PDGF-alone treated group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$). (B) Inhibitory effect of nectandrin B on PDGF-induced DNA synthesis. VSMC were treated same as described in panel A and BrdU assays were performed. Data represent the mean ± SD of six different samples (significant as compared with control group, $^{**}P < 0.01$; significant as compared with PDGF-alone treated group, $^{\#\#}P < 0.01$). (C) Representative pictures (magnification ×200) of haematoxylin and eosin-stained femoral arteries obtained from guide wire-injured mice (3 weeks). Nectandrin B (20 mg kg^{-1}, twice a week, intraperitoneal injection) treated group exhibits significant reduction of intimal thickness compared with vehicle-treated group. Data represent the mean ± SD of four to six different animals (significant as compared with sham-operated group, $^{**}P < 0.01$; significant as compared with wire-injured group, $^{\#}P < 0.05$). (D) The plasma concentration–time profile after intraperitoneal injection of nectandrin B (20 mg kg^{-1}) to five mice. The plasma concentration of nectandrin B rapidly increased and reached C_{max} within 30 min. The mean C_{max} was 1.2 ± 0.1 μg mL^{-1}. After reaching C_{max}, the plasma concentration of nectandrin B declined mono-exponentially with the mean terminal half-life of 29.8 min. Data represented the mean ± SD of five different mice.

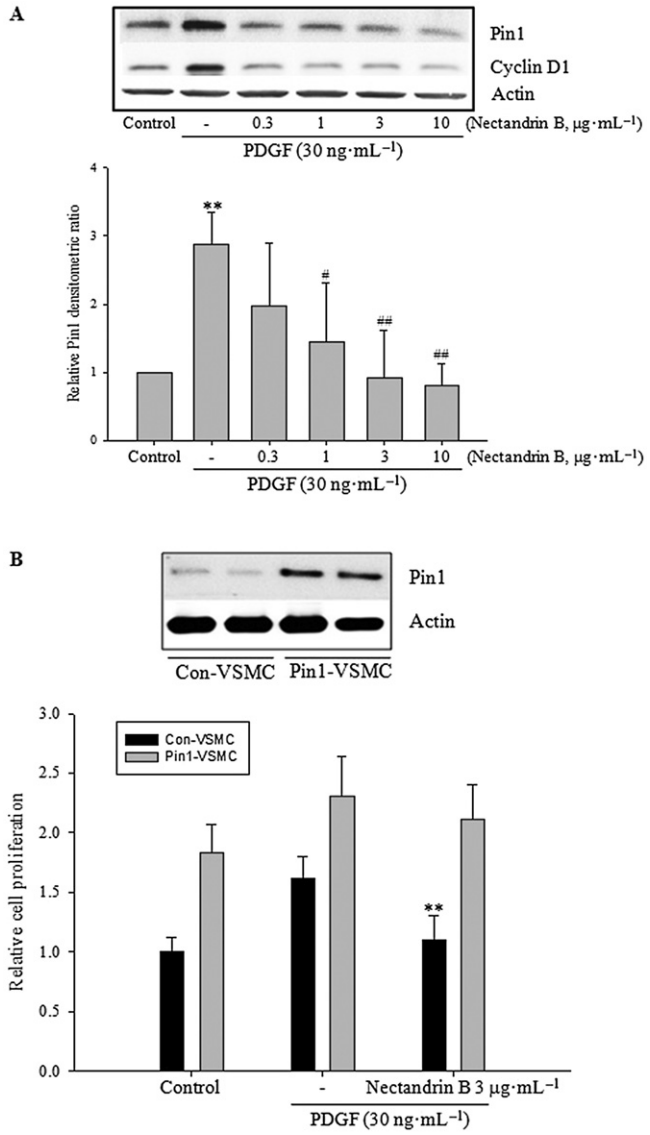

Figure 3

Role of Pin1 inhibition in the inhibitory effect of nectandrin B on VSMC proliferation. (A) Inhibition of PDGF-induced Pin1 expression by nectandrin B. Serum-starved VSMC were pretreated with nectandrin B (0.3–10 μg mL^{-1}) for 1 h and then incubated with PDGF (30 ng mL^{-1}) for additional 24 h. Upper; the protein expression of Pin1 and cyclin D1 were determined by immunoblot analyses. Lower; relative changes in the Pin1 protein expression were assessed by scanning densitometry. Data represent the means ± SD of three separate experiments (significant as compared with control, $^{**}P < 0.01$; control level = 1; significant as compared with PDGF-alone treated group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$). (B) Diminished anti-proliferation effect of nectandrin B in Pin1-overexpressing VSMC (Pin1-VSMC). Upper, Pin1 expression in Con-VSMC and Pin1-VSMC. Lower; cell proliferation. The equal number of Con-VSMC and Pin1-VSMC were seeded and incubated for 12 h and both the cell types were further incubated in the presence or absence of PDGF (30 ng mL^{-1}) and 3 μg mL^{-1} nectandrin B for additional 24 h and the relative cell numbers were obtained by MTT assays. Data represent the means ± SD of eight separate samples (significant as compared with PDGF-exposed Con-VSMC, $^{**}P < 0.01$).

for the phosphorylated (active) forms of ERK and Akt (a downstream target of PI3K). Akt and ERK were phosphorylated within 5 min after exposure of VSMC to PDGF. Although nectandrin B slightly reduced the active forms of ERK and Akt at 15 and 30 min, neither of the phosphorylated kinases were distinctly changed by 10 μg mL^{-1} nectandrin B (Supporting Information Figure S2). These results suggest that nectandrin B suppresses PDGF-mediated Pin1 gene transcription through E2F1 inactivation, but not through ERK or PI3K inhibition.

Requirement of AMPK-mediated p53/p21 activation for E2F1-dependent Pin1 down-regulation

The p53 tumour-suppressor gene, a key regulator of cell cycle progression, can be up-regulated by AMPK activation in VSMC (Igata *et al.*, 2005) and activation of p53 and its downstream target, p21$^{Waf1/Cip1}$ negatively regulate the activity of E2F1 by preventing cyclin-dependent kinase (CDK)-mediated Rb phosphorylation (Lee *et al.*, 1998). VSMC proliferation was also suppressed by induction of p53 and p21 and inhibition of E2F1/phosphorylated Rb (Chan *et al.*, 2010). Hence, we focused on whether nectandrin B's inhibitory activities on VSMC cell proliferation and Pin1 expression were regulated by the p53/p21 pathway. The results showed that the protein levels of p53 and p21 were increased by nectandrin B (Figure 5A). Moreover, p53 and p21 overexpression induced by a p53-overexpressing adenovirus itself decreased PDGF-stimulated induction of Pin1, cyclin D1 and E2F1 (Figure 5B). To confirm this result, we further determined Pin1-Luc promoter activity in PDGF-treated MEF cells. p53 Overexpression significantly suppressed PDGF-induced Pin1-Luc reporter activity in MEF cells (Figure 5C). These suggest that nectandrin B reduces Pin1 transcription in a p53/p21 pathway-dependent manner. To finally determine whether AMPK activation by nectandrin B dominates p53/p21/E2F1-dependent Pin1 expression, we used adenoviruses overexpressing DN-AMPK and WT-AMPK. We found that inhibitory effects of nectandrin B on the expression of Pin1, pRb and E2F1 were almost completely reversed by DN-AMPK overexpression (Figure 6A and B). In addition, p53-inducing effect of nectandrin B was inhibited by DN-AMPK overexpression (Figure 6A and B).

When we performed immunostainings using the injured mouse femoral arteries, the enhanced Pin1 expression in intimal hyperplasia region was reduced in the nectandrin B-treated mice (Figure 7A). Moreover, the E2F1-positive stainings found in the injured arteries were diminished by nectandrin B treatment (Figure 7B). However, in our immunostaining condition, p53- or phosphorylated ACC-positive cells were not detected in all experimental groups (data not shown). The data demonstrate that nectandrin B-mediated Pin1 gene down-regulation is associated with AMPK and subsequent p53 activation, and with an E2F1 inactivation pathway (Figure 8).

Discussion

Because abnormal VSMC proliferation and consequent intimal hyperplasia obviously participate in the development

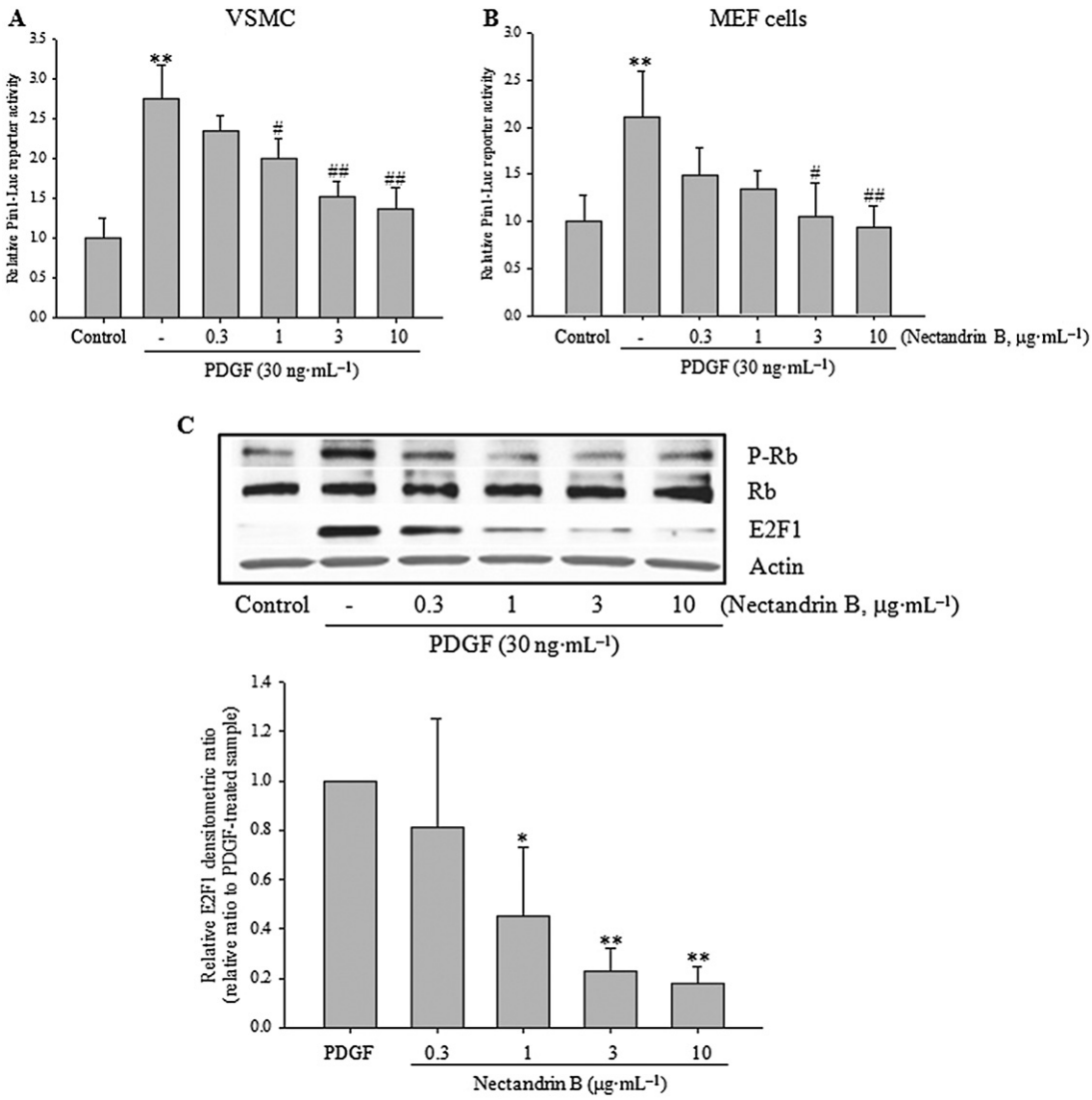

Figure 4

Effect of nectandrin B on E2F1-mediated Pin1 gene transcription. (A, B) Pin1-Luc reporter gene assay. VSMC (A) and MEF cells (B) were transfected with Pin1-Luc and phRL-SV (hRenilla luciferase) plasmids and incubated for 6 h in serum-free condition. The plasmid-transfected cells were pre-incubated with nectandrin B (0.3–10 μg mL^{-1}) for 1 h and further exposed to PDGF (30 ng mL^{-1}) for additional 24 h. Dual luciferase reporter assays were performed on the lysed cells. Activation of the reporter genes was calculated as a relative change to hRenilla luciferase activity. Data represented the mean ± SD of four different samples (significant as compared with control group, $^{**}P < 0.01$; significant as compared with PDGF-alone treated group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$). (C) Effects of nectandrin B on the expression of E2F1 and phosphorylated Rb. Serum-starved VSMC were pretreated with nectandrin B (0.3–10 μg mL^{-1}) for 1 h and then incubated with PDGF (30 ng mL^{-1}) for additional 24 h. Upper; representative immunoblots show E2F1, total and phosphorylated Rb protein expression levels. Lower; relative changes in the E2F1 protein expression were assessed by scanning densitometry. Data represent the means ± SD of three separate experiments (significant as compared with PDGF-alone treated group, $^{*}P < 0.05$; $^{**}P < 0.01$).

of atherosclerosis as well as restenosis (Schwartz, 1997; Ross, 1999), reducing VSMC proliferation is a potential approach to treat diverse, chronic cardiovascular diseases. In our previous study, we showed that Pin1 is involved in VSMC proliferation and subsequent neointima formation, and suggested that Pin1 functions as a crucial therapeutic target for several cardiovascular diseases (Kim *et al.*, 2010). In addition, lentivirus-mediated Pin1 knock-down effectively resulted in cell cycle arrest and enhancement of apoptosis in VSMC (Lv *et al.*, 2010). Hence, we hypothesized that safe compounds reducing Pin1 expression may be effective in inhibiting intimal hyperplasia and thus be useful for the treatment of chronic vascular diseases. Up-regulation of PDGF levels has commonly been found in injured arteries and the importance of PDGF in the formation of neointima has been elucidated (Miyauchi *et al.*, 1998; Heldin and Westermark, 1999). Here, we found that nectandrin B potently suppressed Pin1 induction by PDGF in cultured VSMC.

Many studies have reported that AMPK activation has beneficial physiological activities such as (i) anti-obesity and

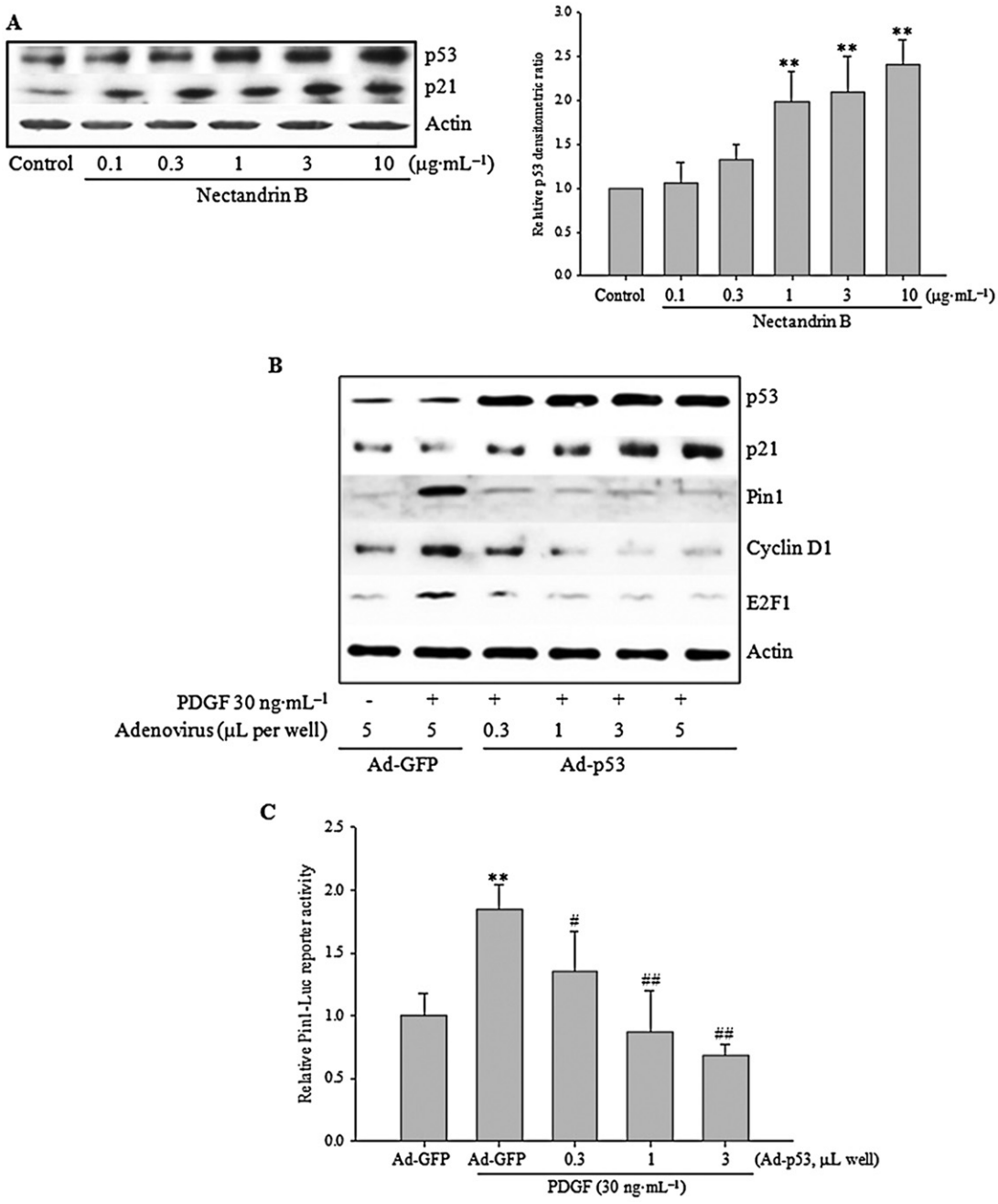

Figure 5

Involvement of p53/p21 activation in the inhibitory effect of nectandrin B on Pin1 expression. (A) Increase in the expression levels of p53 and p21 by nectandrin B. VSMC were exposed to nectandrin B (0.1–10 μg mL^{-1}) for 24 h and the total cell lysates were subjected to immunoblottings for p53 and p21. Left; representative immunoblots show the protein levels of p53 and p21. Right; relative changes in the p53 protein levels were assessed by scanning densitometry. Data represent the means ± SD of three separate experiments (significant as compared with control, $^{**}P < 0.01$; control level = 1). (B) Effect of p53 overexpression on Pin1 and E2F1 expression levels. VSMC were pretreated with adenovirus-overexpressing GFP (Ad-GFP) or -overexpressing p53 (Ad-p53) for 12 h and further incubated with 30 ng mL^{-1} PDGF for 24 h. Representative immunoblots show p53, p21, Pin1, cyclin D1 and E2F1 protein expression levels. (C) Effect of p53 overexpression on Pin1 gene transcription. MEF cells were transfected with Pin1-Luc and phRL-SV (hRenilla luciferase) plasmids and incubated for 6 h in serum-free condition. The plasmid-transfected MEF cells were pre-incubated with Ad-GFP or Ad-p53 for 6 h and further exposed to PDGF (30 ng mL^{-1}) for additional 18 h. Dual luciferase reporter assays were performed as described in the legend of Figure 4(A). Data represented the mean ± SD of four different samples (significant as compared with Ad–GFP-alone treated group, $^{**}P < 0.01$; significant as compared with Ad–GFP- and PDGF-treated group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$).

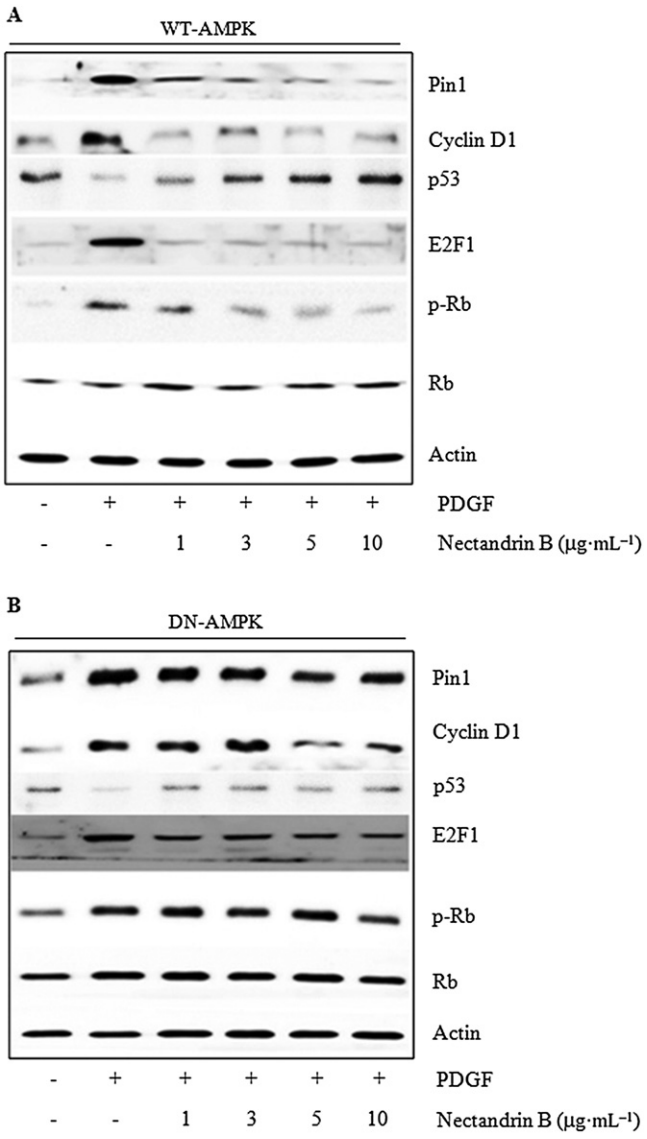

Figure 6

Role of AMPK activation in nectandrin B's inhibitory effect on E2F1-mediated Pin1 expression. Effects of adenovirus-overexpressing WT-AMPK (A) or overexpressing DN-AMPK (B) on the expression of Pin1, cyclin D1, p53, E2F1 and phosphorylated Rb. VSMC were treated with WT-AMPK or DN-AMPK and incubated for 12 h. The adenovirus-infected VSMC were preincubated with nectandrin B (1–10 ng mL^{-1}) for 1 h and then further incubated with 30 ng mL^{-1} PDGF for additional 24 h. Representative immunoblots show Pin1, cyclinD1, p53, E2F1 and total and phosphorylated Rb protein expression levels.

anti-diabetes effects through the control of lipid homeostasis and whole body glucose (Misra, 2008) and (ii) anti-vascular proliferation effects through the inhibition of cell cycle progression (Igata *et al.*, 2005). Our collaborative group recently demonstrated that nectandrin B isolated from *M. fragrans* (nutmeg) acts as a potent AMPK activator in differentiated C2C12 muscle cells and that its extracts are effective in reducing weight gains in mice fed high-fat diets (Nguyen *et al.*, 2010). In this study, nectandrin B-activated AMPK in VSMC and efficiently inhibited VSMC proliferation. Moreover, nectandrin B potently inhibited Pin1 expression via transcriptional inhibition in PDGF-exposed VSMC. Cyclin D1 regulates the G1/S checkpoint and has a crucial role in the development of vascular proliferation diseases (Quasnichka *et al.*, 2006). Pin1 can regulate cyclin D1 through at least two different mechanisms. It has been shown that Pin1 up-regulates cyclin D1 gene transcription by activating c-Jun/AP-1 and β-catenin/coactivation of T-cell factor 4 transcription factors (Ryo *et al.*, 2001; Wulf *et al.*, 2005). Moreover, Pin1 directly binds to cyclin D1 and then isomerizes the pThr286-Pro motif of cyclinD1 and makes cyclinD1 stable by preventing its nuclear export and ubiquitin-mediated degradation (Liou *et al.*, 2002). Hence, the inhibitory effect of nectandrin B on cyclin D1 expression seems to be related to its Pin1 down-regulation effect.

We raised a further question regarding how nectandrin B regulates Pin1 expression. Several reports have shown that Pin1 expression is up-regulated during cancer development and under growth stimulating conditions such as exposure to serum or growth factors (Ryo *et al.*, 2002; You *et al.*, 2002). Although PI3K and ERK are the major signal transduction molecules that have crucial roles in cell proliferation and in expression of a variety of genes (Kyriakis and Avruch, 2001); in the present study, we showed that nectandrin B did not alter the activities of ERK and PI3K (Supporting Information Figure S2). The Pin1 promoter contains three putative E2F-binding sites, and the binding of E2F family proteins to these sites activates Pin1 gene transcription (Ryo *et al.*, 2002; 2003). Thus, it is possible that deregulation of the Rb/E2F pathway after exposure to PDGF contributes to the up-regulation of Pin1 gene transcription. E2F1 is a transcription factor that plays an important role in the regulation of the cell cycle and of tumour-suppressor proteins (Neuman *et al.*, 1996). Here, we found that nectandrin B diminished both E2F1 expression and Rb phosphorylation compared with the PDGF-treated group. From these results, we can conclude that down-regulation of the Rb/E2F pathway is responsible for Pin1 inhibition by nectandrin B.

p53, a well-characterized tumour-suppressor protein, is vital in multicellular organisms where it regulates the cell cycle and thus causes genetic stability, apoptosis and inhibition of angiogenesis (Matlashewski *et al.*, 1984; Isobe *et al.*, 1986; McBride *et al.*, 1986). p53 activation stimulates the expression of several genes such as WAF1/CIP1 which encodes for p21. p21 as a CDK inhibitor, suppresses the activities of cyclin D/CDK4 and cyclin E/CDK2 complexes and functions as a critical cell cycle regulator at the S-phase (Gartel and Radhakrishnan, 2005). Several studies have shown that induction of p53 and p21 inhibits VSMC proliferation, and that a cooperative interaction between p53 and Rb is important for the regulation of VSMC proliferation (Bennet *et al.*, 1998; Xu and Si, 2010). Here, we found that p53 and p21 were induced by nectandrin B treatment in VSMC, and Pin1 and E2F1 expression were diminished by p53 overexpression. Hence, nectandrin B-mediated p53/p21 activation may be involved in the blocking of Pin1-dependent VSMC proliferation. In PDGF-treated VSMC, an AMPK activator suppresses VSMC proliferation through the induction of p53/p21 and subsequent down-regulation of cyclin D1 (Liang *et al.*, 2008). Therefore, we have hypoth-

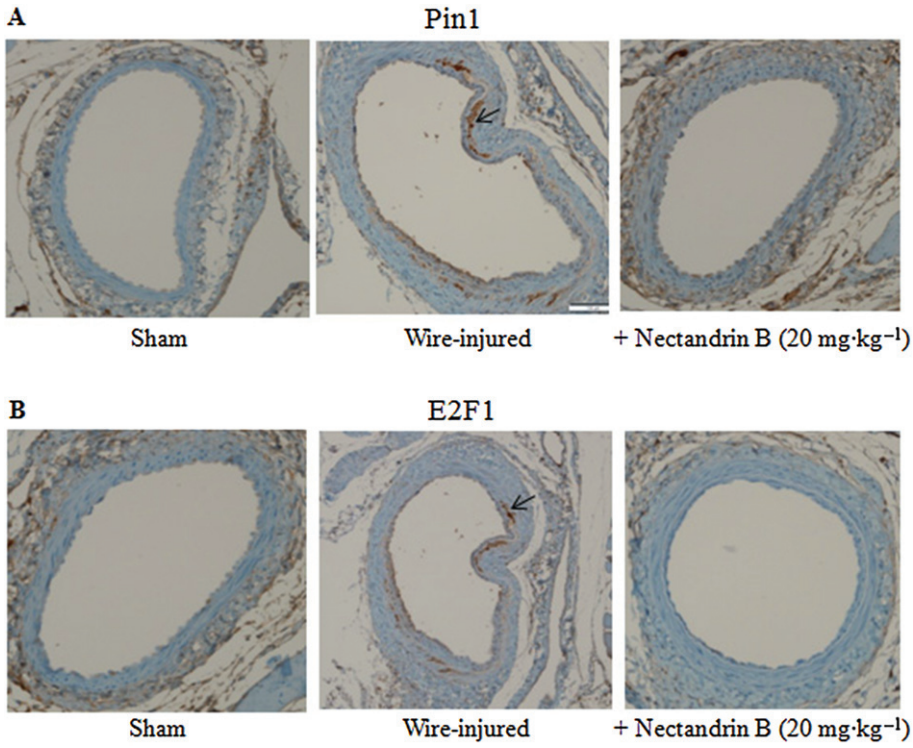

Figure 7

Immunohistochemical analyses of Pin1 and E2F1 in femoral arteries. Representative pictures (magnification ×200) of femoral arteries obtained from guide wire-injured mice (3 weeks). (A) Pin1 immunohistochemistry. The enhanced Pin1 staining in intimal hyperplasia region was decreased in the nectandrin B (20 mg kg^{-1}, twice a week, intraperitoneal injection)-treated mice. (B) E2F1 immunohistochemistry. Immunostaining pattern of E2F1 was similar to that of Pin1.

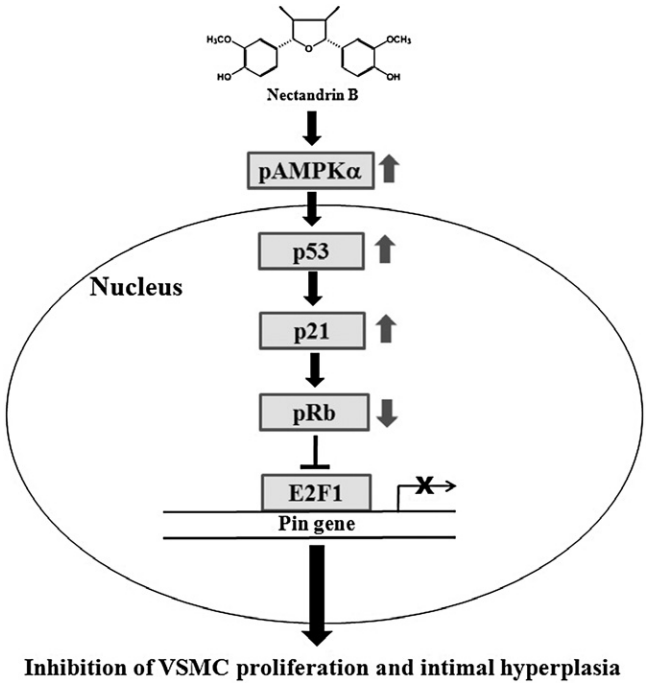

Figure 8

Proposed mechanism for nectandrin B's inhibitory effect on neointima formation.

esized that AMPK activation by nectandrin B first increases p53/p21 expression and then inactivates E2F1 through a decrease in phosphorylated Rb. In this study, AMPK inhibition by DN-AMPK adenovirus infection completely reversed the expression changes induced by Pin1, cyclinD1, E2F1 and phosphorylated Rb in response to nectandrin B.

Taken together, our data show that an AMPK activator, nectandrin B, suppresses VSMC proliferation, and this anti-proliferative effect is correlated with AMPK-mediated Pin1 down-regulation. Nectandrin B-stimulated AMPK activation induces p53, p21 and then attenuated Rb phosphorylation and E2F1 expression, and eventually reduces Pin1 expression via transcriptional inactivation. In a wire-injured femoral artery mouse model, we found that nectandrin B significantly inhibits neointimal formation. Nectandrin B could become a therapeutic agent for the treatment or prevention of vascular-proliferative diseases including restenosis and atherosclerosis. Because we could not assess neointima formation in Pin1-null mice, direct evidence for the role of Pin1 in intimal hyperplasia is still not elucidated. We are trying to establish Pin1-null mice.

Acknowledgements

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Conflict of interest

We confirm that all authors fulfilled all conditions required for authorship. We also confirm that there is no potential conflict of interest or financial dependence regarding this publication.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of nectandrin B on E2F binding to Pin1 promoter and E2F1 mRNA expression. (A) Gel shift analysis of the E2F binding to Pin1 promoter. Nuclear extracts were prepared from VSMC cultured with or without 30 ng mL^{-1} PDGF for 24 h. All lanes contained 5 μg of nuclear extracts and 5 ng of the labeled oligonucleotide (E2F binding site in Pin1 promoter). (B) Real-time PCR analysis of E2F1 mRNA expression. Glyceraldehyde 3-phosphate dehydrogenase mRNA was determined as control. Data represented the mean ± SD of 5 different samples (significant as compared with control, $^{**}P < 0.01$; significant as compared with PDGF-alone treated group, $^{\#\#}P < 0.01$).

Figure S2 Effects of nectandrin B on PDGF-stimulated PI3K and ERK activation. 18 h-serum starved VSMC were pre-incubated with or without nectandrin B (10 μg mL^{-1}) for 30 min and then exposed to 30 ng mL^{-1} PDGF for 5, 15 or 30 min. Representative immunoblots show total and phosphorylated ERK and Akt protein expression levels.