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이 정 운

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Neointima 형성은 풍선 혈관 조형술시 발생하는 가장 큰 문제 중 하나이다. 비정상적인 혈관 평활근 세포(VSMC)의 증식은 neointima 형성을 돕는다. Pin1은 특이적 Phospho-Serine/Threonine-Proline isomerase 로서 암의 치료를 위한 신규 표적으로서 주목받고 있다. 이전 연구에서 Pin1 이 혈관 평활근 세포의 비정상적인 증식에 중요한 역할을 한다는 것을 밝힌 바 있다. 본 연구에서는 *Myristica fragrans* (nutmeg, Myristicaceae)에서 분리한 강력한 AMPK 활성물질인 nectandrin B 라는 물질을 이용하였다. 본 연구는 우선 nectandrin B 가 인산화된 AMPK와 ACC를 증가시키는 것을 확인하였고 PDGF 로 인해 유도된 혈관 평활근 세포의 증식을 농도 의존적으로 억제하는 것을 보였다. PDGF는 Pin1을 증가시키고 세포주기 조절 단백질인 cyclinD1 또한 증가시키는데 이들은 모두 nectandrin B 에 의해서 강력하게 억제되는 것을 밝혔다. 본 연구는 Pin1 promoter reporter의 전사활성 또한 PDGF 를 처치한 혈관 평활근 세포에서 nectandrin B에 의해 감소하는 것을 밝혔으며 nectandrin B 가

ERK/MAPK, p38/MAPK, Akt/PI3K 같은 상위 신호전달 인산화 효소와 세포 주기를 조절하는 단백질인 p53, E2F1 그리고 인산화된 Rb에 어떠한 영향을 미치는지 연구하였다. PDGF는 혈관 평활근 세포에서 p53 을 제외한 모든 종류의 상위 신호전달 인산화 효소와 E2F1 과 인산화된 Rb를 증가시켰고, 본 연구에서는 nectandrin B가 ERK/MAPK, p38/MAPK, Akt/PI3K 같은 상위 신호전달 인산화 효소에는 영향을 끼치지 않으나, PDGF 처치 혈관 평활근 세포에서 p53을 증가시키고 E2F1 과 인산화된 Rb를 감소시킨다는 것을 밝혀냈다. 또한, nectandrin B(20mg/kg 주 2회 복강투여) 를 mouse에게 3주간 투여한 결과 guide wire injury 로 유도된 neointima 형성이 억제됨을 확인하였다.

결론적으로 강력한 AMPK 활성화 물질인 nectandrin B 는 p53의 증가와 E2F1, 과인산화된 Rb의 감소를 통하여 Pin1의 발현과 cyclinD1의 발현을 억제함을 밝혀내었다. 이러한 결과들을 종합 시, nectandrin B 는 혈관 재 협착이나 동맥경화 같은 혈관 증식 질환에 매우 효과적인 약물이 될 수 있음을 시사한다.

ABSTRACT

Inhibititory effect of nectandrin B, a potent AMPK activatior on neointimal formation.

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Neointima formation is a major problem at the balloon angioplasty surgery. Abnormal proliferation of vascular smooth muscle cell (VSMC) serve neointima formation. Pin1, a specific phosphor-Serine/Threonine-Proline isomerase, has been studied about a important target for the treatment of cancer. In our recent study, we have shown that Pin1 also plays a critical role in abnormal VSMC proliferation. Nectandrin B, isolated from *Myristica fragrans* (nutmeg, Myristicaceae), functions as a potent AMP-activated protein kinase (AMPK) activator. In this study, we first showed that nectandrin B increased phosphorylated AMPK as well as phosphorylated Acetyl CoA Carboxylase(ACC) in VSMC. Nectandrin B inhibited VSMC proliferation induced by PDGF in a concentration-dependent manner. PDGF also increased the expression of both Pin1 and cyclin D1, a cell cycle regulated protein and these were dramatically blocked by nectandrin B. Also, we verified that Pin1 promoter activity was decreased by nectandrin B in PDGF-exposed VSMC. In order to identify the signaling pathways for Pin1 and cyclinD1

down-regulation by nectandrin B, we investigated the effects of nectandrin B on the activation of ERK/MAPK, p38/MAPK, Akt/PI3K and another cell cycle regulated proteins including p53, E2F1 and phosphorylated Rb. PDGF increased the active phosphorylated forms all these kinases, E2F1 and phosphorylated Rb except p53. We found that nectandrin B increased p53 expression and reversely decreased the expression of E2F1 and phosphorylated Rb in PDGF-treated VSMC. Moreover, treatment of nectandrin B for 3 weeks (20mg/kg, two times a week, intraperitoneal injection) suppressed neointimal formation in femoral artery after guide wire-injury.

In conclusion, nectandrin B, a potent AMP-activated protein kinase (AMPK) activator, blocked PDGF-stimulated Pin1 induction through p53 activation and subsequent E2F1 inactivation in VSMC. From these results, nectandrin B may be useful as a potential curative agent for vascular proliferative disease such as restenosis and atherosclerosis.

Keywords : Nectandrin B, Pin1, AMPK, Neointima

1. Introduction

Restenosis after balloon angioplasty surgery is a critical clinical problem (Choy et al., 2001). A well known event in the narrowing of vascular lumen after

angioplasty is migration and proliferation of vascular smooth muscle cells (VSMCs). After vascular injury, VSMCs acquire a new phenotype, migrate to the site of injury and then proliferation, adhesion at the intima layer and elaborate the extracellular matrix. The mechanism of this complex series of incidents is not fully understood. Nevertheless it is generally accepted that VSMCs phenotype modulation, migration, proliferation, and extracellular matrix rearrangement are essential for neointimal formation. Among these processes, inhibition of VSMC proliferation is considered as a potential therapeutic interruption for weakening the incidence of vascular occulusive disease.

Platelet-derived growth factor (PDGF) is a potent growth factor produced by platelets, VSMCs and endothelial cells in the injured vascular wall (Majesky et el., 1990; Miyauchi at al., 1998). PDGF plays a critical role in the neointima formation eventually in restenosis.(Uchida, K et al., 1996; Chandrasekar, B et al., 2000)

Upregulation level of PDGF has commonly been found in injury arteries, which responsible for neointimal cellular proliferation (Uchida, K et al., 1996). In the arterial injury models, importance of PDGF in the formation of neointima has been elucidated (Heldin et al., 1999). Therefore it has been believed that blocking of platelet activation is a worthless therapeutic interruption for weakening the occurrence of most vascular occulusive disease.

Pin1, a peptidyl prolyl isomerase, is an enzyme that accelerate the isomerisation of the peptide bond at the proline residue (Lu et al., 2004). Pin1 has specific interaction with proteins that are phosphorylated at Ser/Thr-Pro motifs and then

regulate the cell cycle(Lu et al., 1996; Yaffe et al., 1997). CyclinD1 regulates the G1/S check point in the cell cycle and have crucial role in the development disease about the vascular proliferation (Quasnichka et al., 2006). CyclinD1 is downstream of Pin1 and Pin1 regulates CyclinD1 through at least two mechanisms (Wulf et al., 2001; Wulf et al,. 2005). Increased Pin1 expression up-regulates cyclinD1 gene transcription through activating c-jun/AP-1 and β -catenin/TCF transcription factors (Ryo et al., 2001; Wulf et al., 2001). Moreover, Pin1 directly binds to cyclin D1 and then isomerizes the pThr 286-Pro motif of cyclinD1 and makes cyclinD1 stable by preventing its nuclear export and ubiquitin-mediated degradation (Liou et al., 2002). In the most studies, pathological role of Pin1 has been focused on cancer, since Pin1 overexpression is frequently found in many types of tumor tissues. However, our recent study has demonstrated that Pin1 is upregulated in the injured vascular wall and that Pin1 overexpression is necessary for VSMC proliferation and neointima formation (Kim et al., 2009).

Many studies have shown that upregulation of AMP-activated protein kinase (AMPK) have protective effect in vascular smooth muscle cell and cardiovascular smooth muscle cell. (Xu, Q et al., 2010) And AMP-activated protein kinase (AMPK) activators inhibit the obesity and diabetes through the control of lipid homeostasis and whole body glucose. (Mirsa, p., 2008) Nectandrin B can be isolated from *Myristica fragrans* Houtt. (Myristicaceae) that is an aromatic evergreen tree cultivated in the, India, South Africa and other tropical countries. (Oh et al., 2010). Dr. WK Oh group recently revealed that the compound potently

activates AMPK in C2C12 cells.

In this study, we tested whether a potent AMP-activated protein kinase (AMPK) activator, nectandrin B inhibits VSMC proliferation and neointiima formation and tried to clarify its molecular mechanism, focusing on the signaling pathways for its Pin1 down-regulation effect.

2. Materials and Methods

2-1. Materials

Antibodies against Pin1, E2F1, p53 and p21 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated form-specific or total form recognizing antibodies against PI3K/Akt, ERK/MAPK, p38/MAPK, and antibodies for phosphorylated Rb, Cyclin D1 and horseradish peroxidase-conjugated anti-rabbit, anti-mouse IgGs were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase-conjugated donkey anti-mouse IgG was provided by Jackson Immunoresearch Laboratories (West Grove, PA). Anti-actin antibody and most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). PDGF was purchased from PeproTech (Rocky hill, NJ). Nectandrin B was kindly provided by Dr. WK Oh (Chosun University, Gwang-ju, Korea). Dominant-negative AMPK (DN-AMPK), wild-type AMPK (WT-AMPK) adeno viruses was provided by Dr. J.H. Ha (College of

Medicine, Kyung hee university, Seoul, Korea) 5-Bromo-2'-deoxy-uridine labeling and detection kit was purchased from Roche (Mannheim, Germany).

2-2. Plasmid

Pin1 plasmids were provided from Dr. H.S. Choi (College of Pharmacy, Chosun University, Gwangju, Korea) and pGL-AP1 plasmid were purchased from Stratagene (Santa Clara, CA).

2-3. Cell culture

VSMCs were isolated from rat thoracic aorta as described previously (Slodzinski et al., 1995-21). 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 Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 g/ml streptomycin. Cells were passaged by trypsinization, and passages between 5 to 12 were used for experiments.

2-4 .Immunoblot analysis

After washing with sterile PBS, the VSMCs were lysed in EBC 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 □g/ml leupeptin. The cell lysates were centrifuge at 10,000*g* for 10 min to remove the debris, and the proteins were then fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with the specific antibodies. Horseradish peroxidise- 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 (BCIP)/4-nitroblue tetrazolium (NBT) or an ECL chemiluminescence system. For chemiluminescence detection, the LAS3000-mini system (Fujifilm, Tokyo, Japan) was used.

2-5 BrdU assay for cell proliferation

Viable adherent cells were incubated with BrdU (5-bromo-2'-deoxy-uridine) labelling solution (10μM) for 2h. Cells were fixed with fixation solution for 30 min at room temperature and incubated with 100 μL anti-BrdU peroxidase-labeled antibody for 90 min. After three washings, the substrate solution for the colorimetric quantification was added at a final concentration of 100 μL/mL and left at room temperature for 5–30 min until color development was sufficient for photometric detection. The absorbance was assayed at 405nm.

2-6. MTT assay for cell viability

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

2-7. Reporter gene assay

The Pin1 minimal reporter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, mouse embryonic fibroblast (MEF) cells (3×10^5 cells/well) were replated in 12-well plates overnight and transiently transfected with the Pin1 and AP-1 minimal reporter plasmids/phRL-SV plasmid (*hRenilla* luciferase expression for normalization) (Promega, Madison, WI) using Hilymax® reagent (Dojindo Molecular Tech., MD). The 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 versus hRenilla luciferase.

2-8. Adeno virus infection.

VSMCs cultured in 6 well plates were treated with DN-AMPK or WT-AMPK adeno virus for 18 h and then incubated in DMEM without serum for

additional 18 h. VSMCs were then exposed to nectandrin B (1, 3, 5 and 10μg/ml) for 30min and treated with PDGF(30ng/ml) for 24 h.

2-9. Mouse femoral artery injury model

Transluminal mechanical injury of bilateral femoral arteries was induced by introducing a large wire as previously reported (Sata et al. 2000), In brief, the left femoral artery was 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), was carefully inserted into the femoral artery toward the iliac artery via a small branch between the rectus femoris and vastus medialis muscles. The wire was left in place for 1 minute 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 femoral artery was restored by releasing the sutures placed in the proximal and distal femoral portions. Skin incision was closed with a 6-0 silk suture. At 21 days after injury, the mice were sacrificed 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.

2-9. Data analysis

One way analysis of variance (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 $p<0.01$.

3. Results

3-1. Nectandrin B activates AMP-activated protein kinase (AMPK) in VSMC.

In order to confirm AMPK activation effect of nectandrin B, nectandrin B was treated in VSMC in a concentration-dependent manner. Immunoblot analyses revealed that both the protein levels of phosphorylated AMP-activated protein kinase (AMPK) and phosphorylated Acetyl CoA Carboxylase (ACC) were increased by nectandrin B (Fig. 1).

3-2. Effects of nectandrin B on PDGF-induced VSMC proliferation.

VSMC proliferation in neointima formation is a key event in pathogenesis of vascular disease, including atherosclerosis, post-balloon angioplasty restenosis and vein graft occlusion (Braun-Dullaeus et al., 1998). 5-Bromo-2'-Deoxy-uridine (BrdU) assays were performed to investigate the effects of nectandrin B. 24 h PDGF (30ng/ml) exposure to serum-deprived VSMCs increased DNA synthesis above 2 fold versus untreated control group. The PDGF-stimulated DNA synthesis was suppressed by nectandrin B (1 μg/ml) in a concentration-dependent manner (Fig. 2A). To confirm the effect of Nectandrin B on VSMC proliferation, we also performed MTT assay. PDGF (30ng/ml)-stimulated proliferation of VSMCs was significantly decreased by nectandrin B (1-10 μg/ml). These results demonstrate that nectandrin B contains inhibitory activity against PDGF-mediated VSMC proliferation.

3-3. Effects of nectandrin B on PDGF-stimulated Pin1, CyclinD1 expression.

Pin1 plays an important role in the neointima formation (Kim et al., 2009). And cell cycle regulated protein, Cyclin D1 is also essentially required for the proliferation of VSMC (David Goukassin et al., 2001). We examined the effects of nectandrin B on the Pin1 and CyclinD1 expression. PDGF treatment in VSMCs increased the expression levels of Pin1 and cyclinD1, while nectandrin B suppressed both the protein expression in a concentration-dependent manner (Fig. 3a). In addition we examined the transcriptional activation of Pin1 and AP-1 promoter by using luciferase assays. Because primary cultured VSMCs have extremely low efficiency to liposome-based transfection, we used MEF cells. The results showed that Pin1 promoter activity was 2 fold increased by PDGF treatment and this was significantly decreased by 1 or 3 μg/ml nectandrin B. From these results, we suppose that nectandrin B's inhibitory effect on VSMC proliferation is associated with Pin1 down-regulation.

3-4. Nectandrin B attenuates PDGF-stimulated E2F1 and retinoblastoma protein (pRb).

Because Pin1 expression could be controlled by growth factors and serum (A. Ryo et al., 2002 H; You et al., 2002) cell proliferation signalings such as PI3-kinase/Akt or MAP kinase may be involved in Pin1 induction by PDGF. Thus, to elucidate the signaling pathway for Pin1 down-regulation by nectandrin B, we first examined the effects of nectandrin B on PDGF induced activation of several kinases including ERK/MAPKs, p38 MAPK and PI3-kinase. To identify the activities of these

kinases, we used antibodies specific for the phosphorylated (active) forms of ERK/MAPKs, p38 MAPK and PI3-kinase. All of these kinases were phosphorylated within 5 minutes after PDGF treatment. However any of these kinases were changed after treatment of nectandrin B (10μg/ml)(Fig.4a).

E2F transcription factors play an important role in the regulation of cell cycle progression and the function of tumor–suppressor protein. E2F1 can bind to phosphorylated retinoblastoma protein (pRb) in a cell cycle dependent manner and also mediates both p53 dependent/independent apoptosis and cell proliferation (Neuman, E et al., 1996). Because E2F activation is crucial for the transcriptional activation of *Pin1* gene (Ryo et al., 2002), we hypothesized that E2F1 and Rb phosphorylation might be affected by nectandrin B in PDGF-treated VSMCs. Both the expression of E2F1 and phophorylated retinoblastoma was decreased by nectandrin B in comparison to PDGF alone-treated group (Fig. 4b). These results suggest that nectandrin B suppresses the PDGF-mediated Pin1 activation through E2F inactivation, but not through MAP kinases and PI3-kinase.

3-5. AMPK activator, nectandrin B induces P53 and p21.

Many studies reported that VSMC proliferation was suppressed by induction of p53 and p21 and inhibition of E2F1 and phosphorylated Rb (Chan et al., 2010). We then focused on whether nectandrin B's inhibitory activity on VSMC cell proliferation was regulated by cooperative interaction of p53 and p21. (Bennet, MR et al., 1998) Both p53 and p21 are easily activated in the serum-deprived condition, so we treated nectandrin B in the presence of serum condition.

The results showed that the protein levels of p53 and p21 were enhanced by nectandrin B (Fig. 5a). To identify the whether nectandrin B decrease the Pin1 level through AMP-activated protein kinase (AMPK), we used adenoviruses overexpressing DN-AMPK and WT-AMPK. We found that nectandrin B did not reduce the expression of Pin1, CyclinD1, E2F1 and Rb in DN-AMPK infected VSMCs (Fig. 5b). The data demonstrate that nectandrin B-mediated Pin1down-regulation is related to the AMPK and subsequent p53 activation, and E2F1 inactivation pathway.

3-6. Nectandrin B inhibits neointimal formation.

Finally, we assessed that whether nectandrin B could decrease neointimal formation in vivo. During 3 weeks, intraperitoneal injection of nectandrin B (20 mg/kg, twice a week) started after guide wire injury of mouse femoral artery. Nectandrin B treatment significantly reduced neointimal formation compared with vehicle-treated group (Fig. 6).

4. Discussion

VSMC proliferation is correlated with diverse cardiovascular disease including restenosis and atherosclerosis after balloon angioplasty surgery (Ross et al., 1999; Schwartz et al., 1997). Neointimal formation due to VSMC proliferation so reducing VSMC proliferation may be a potentially target for treatment of cardiovascular disease. In our previous study, we revealed that Pin1 plays important role in VSMC proliferation and neointimal formation and suggested that Pin1 functions as a crucial therapeutic target for several cardiovascular disease (Kim et al., 2009). Hence, we hypothesized that safe compounds that reduce Pin1 expression may function as potential therapeutics to treat chronic vascular diseases such as restenosis and atherosclerosis. To increase Pin1 expression in VSMC, we used PDGF that is kind of the growth factor and it plays critical role in VSMC proliferation, angiogenesis, atherosclerosis and neointimal formation (Ashino T et al., 2010, Paul D et al., 1971).

Many studies have reported that AMPK activation has diverse bioactivities such as anti obesity (Mirsa P, 2008), anti diabetes through the control of lipid homeostasis and whole body glucose (Mirsa P, 2008) and anti proliferation effects on VSMCs through the cell cycle progression inhibition (Igata M et al., 2005). Hence, we used a potent AMPK activator, nectandrin B isolated from *Myristica fragrans* (nutmeg)(Oh et al., 2010) and examined the effects of nectandrin B on Pin1 induction and cell proliferation in PDGF-treated VSMCs.

Here we confirmed that nectandrin B activated AMPK in VSMCs and dramatically inhibited VSMCs proliferation within the concentration ranges of AMPK activation. Moreover, nectandrin B potently inhibited Pin1 expression via transcriptional inhibition in PDGF-exposed VSMCs. And another factor that is relate with the VSMC proliferation, cyclin D1 expression was also inhibited by nectandrin B. Cyclin D1 is a downstream target protein of Pin1; Pin1 regulates function of cyclin D1 at the transcriptional activation or posttranslational stabilization (You et al., 2002, Ryo et al., 2002).

We raised further question, how nectandrin B regulates Pin1 expression. Several reports have shown that Pin1 expression is up-regulated during cancer development and growth stimulating condition such as exposure of serum or growth factor(s)(Ryo et al., 2002; You et al., 2002). Although PI3-kinase and MAP kinase are the major signal transduction molecules that have crucial role for cell proliferation and a variety of gene expression (Kyriakis et al., 2001), in the present study, we showed nectandrin B did not change the activities of MAP kinases and PI3-kinase. Pin1 promoter contains three putative E2F-binding sites and binding of E2F family proteins to these sites activates *Pin1* gene transcription (Ryo et al., 2002; A. Ryo et al.,2003). Thus, it could be possible that deregulation of Rb/E2F pathway after exposure to PDGF contributes to the up-regulation of Pin1. Hence, we focused on E2F1 expression and Rb phosphorylation in nectandrin B-treated VSMCs. The dysfunctional Rb appears in many types of cancer. E2F1 is a transcription factor that plays important role in the regulate cell cycle and tumor–

suppressor proteins (Neuman E et al., 1996). In this study, we found that nectandrin B diminished the both E2F1 expression and Rb phosphorylation compared to PDGF-treated group. From these results, we can conclude that down-regulation of Rb/E2F pathway is responsible for the Pin1 inhibition by nectandrin B.

P53 is known as tumor suppressor protein. (Matlashewski G et al., 1984, Isobe M et al., 1986, McBride OW et al., 1986) p53 is vital in multicellular organism where it regulates the cell cycle and, thus, functions as genetic stability, apoptosis, and inhibition of angiogensis eventually it can suppress the tumor. When p53 activates, it can activates expression of several genes such as WAF1/CIP1 encoding for p21. P21 is a cyclin-dependent kinase inhibitor and it inhibits the activity of cyclinD/CDK4 and cyclinE/CDK2 complexes. It is regulated by tumor suppressor protein, p53 and has function as regulator of cell cycle progression at S phase. (Gartel AG et al., 2005) Many studies have shown that induction of p53 and p21 could inhibit the VSMC proliferation and also cooperative interaction of p53 and Rb regulated VSMC proliferation. (Xu Q et al., 2010, Bennet MR et al., 1998) In PDGF-treated VSMCs, AMPK activator suppresses VSMC proliferation through the induction of p53 and p21 and subsequently down-regulates cell cycle regulators such as cyclinD1 (Liang KW et al., 2008). Hence we can conclude that AMPK activation by nectandrin B may first increase p53 and p21 expression and inactivates E2F through the decrease in phosphorylated Rb. In our studies, we found that AMPK inhibition by DN-AMPK adenovirus infection completely

reversed the expression changes of Pin1, cyclinD1, E2F1 and phosphorylated Rb in response to nectandrin B.

Taken together, our data show that AMPK activator, nectandrin B treatment suppresses the VSMCs proliferation, and this anti-proliferative effect is correlated with AMPK-mediated Pin1 down-regulation. Nectandrin B-stimulated AMPK activation induced p53, p21 and then attenuated Rb phosphorylation and E2F1 expression, and eventually reduced Pin1 expression via transcriptional inactivation. In guide wire-injured femoral artery mouse model, we elucidated that nectandrin B significantly suppressed the neointimal formation. Nectandrin B could be applicable as a therapeutic agent for the treatment or prevention of vascular-proliferative diseases including restenosis and atherosclerosis.

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6. Figure Legends

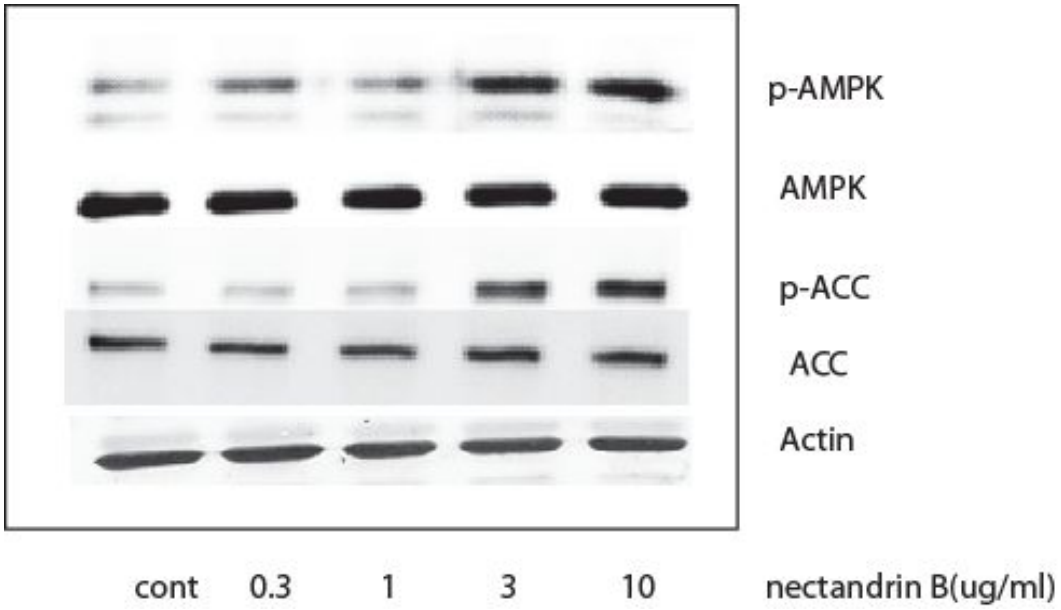
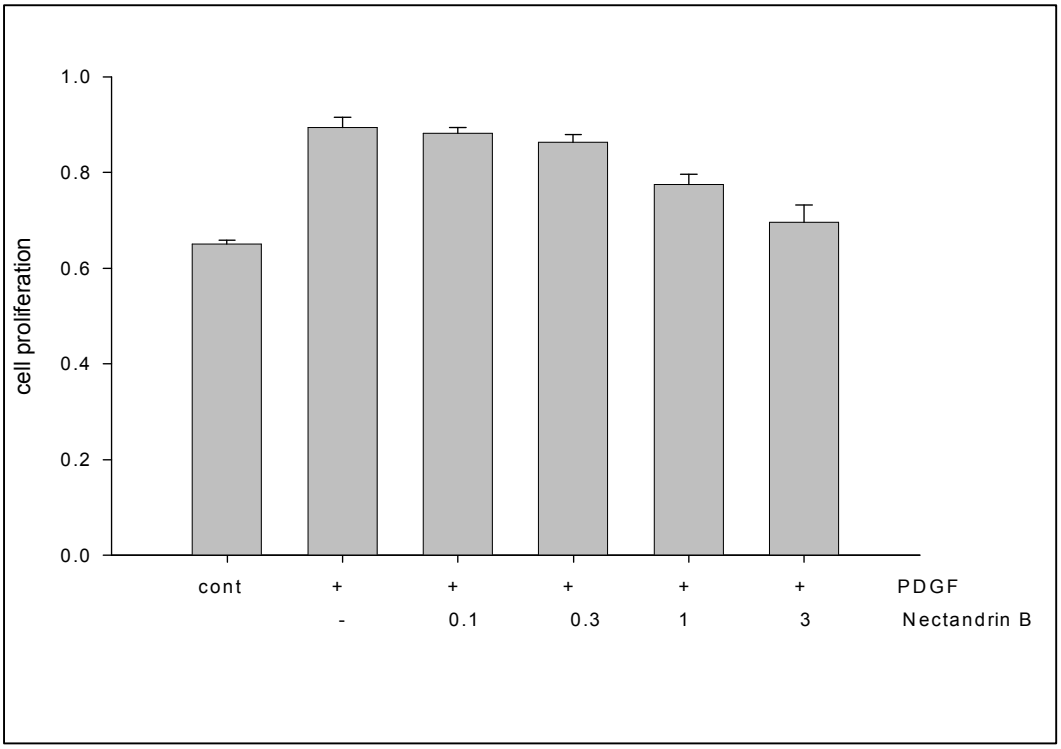


Figure 1. Nectandrin B increase phosphorylated AMP-activated protein kinase (AMPK) and phosphorylated Acetyl CoA Carboxylase (ACC) in VSMCs. To identify the whether nectandrin B is potent AMP-activated protein kinase (AMPK) activator or not, after serum starved 6h treated nectandrin B(0.3 to 10μg/ml) for 1h incubation. Representative immunoblots show total and phosphorylated AMPK and ACC were determined by using Immunoblot analysis as described under Materials and Methods. Equal loading of proteins were verified by actin immunoblot.

A)


cell proliferation
1.0
0.8
0.6
0.4
0.2
0.0
cont
+
+
+
+
+
PDGF
-
0.1
0.3
1
3
Nectandrin B

B)

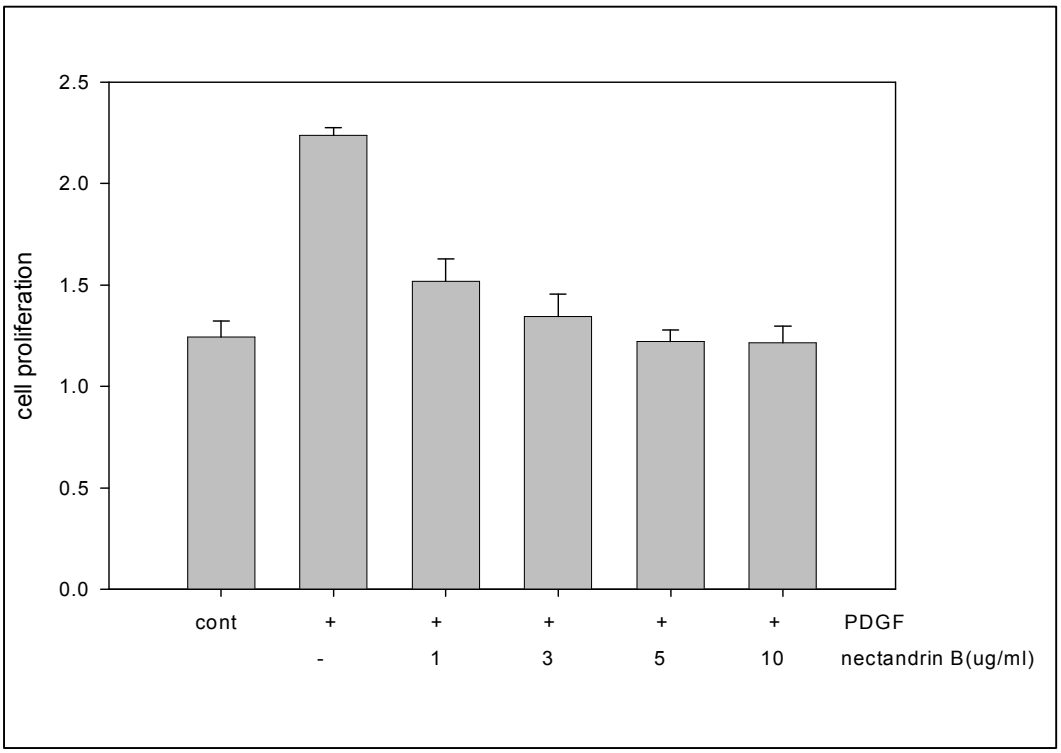
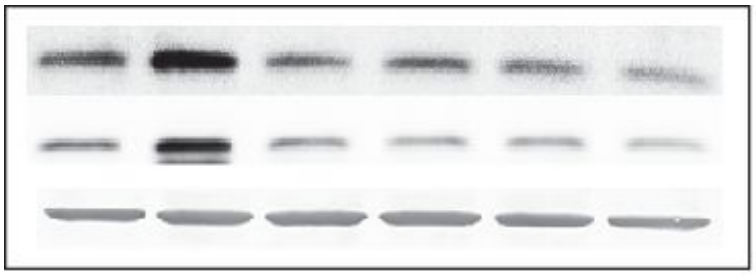

cell proliferation
2.5
2.0
1.5
1.0
0.5
0.0
cont
+
+
+
+
+
PDGF
-
1
3
5
10
nectandrin B(ug/ml)

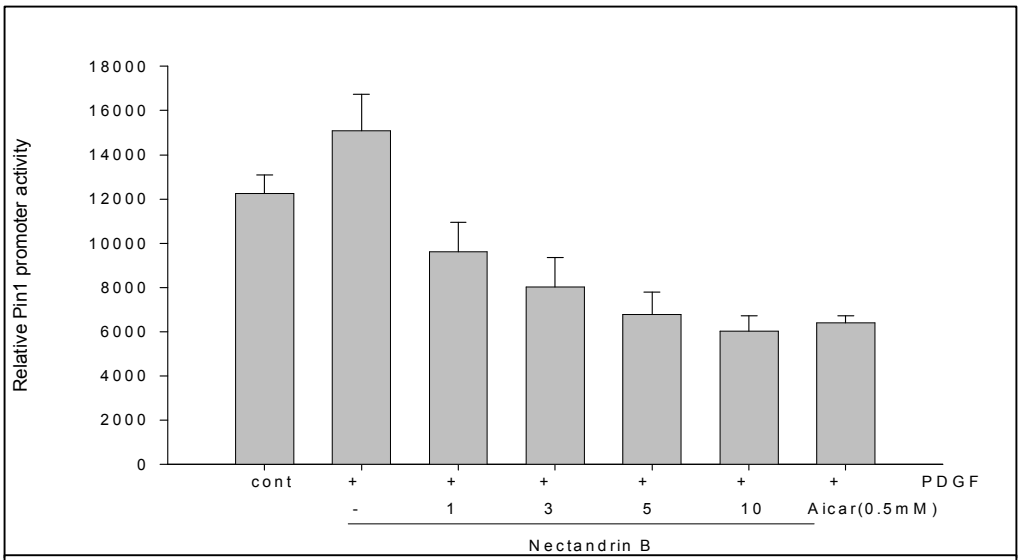
Figure 2. Nectandrin B suppresses PDGF-induced VSMCs proliferation. (A) Inhibitory effect of nectandrin B on PDGF-induced VSMCs proliferation. VSMCs were pre-treated with nectandrin B (0.3 to 10μg/ml) and incubate 30min and then VSMCs further incubated with or without PDGF (30ng/ml) for additional 24h. VSMCs proliferation was determined by using MTT assay as described under Materials and Methods. Data represented the mean± SD with 4 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$) (B) Inhibitory effect of nectandrin B on PDGF-induced VSMC proliferation. VSMCs were pre-treated with nectandrin B (0.3 to 10μg/ml) for 30 min and then VSMCs further incubated with or without PDGF (30ng/ml) for additional 24 h. VSMC proliferation was determined by using 5-Bromo-2'-Deoxy-uridine (BrdU) assay as described under Materials and Methods. Data represented the mean± SD with 4 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$)

A)



cont	+	+	+	+	+	PDGF
	-	0.3	1	3	10	nectandrin B(ug/ml)

B)



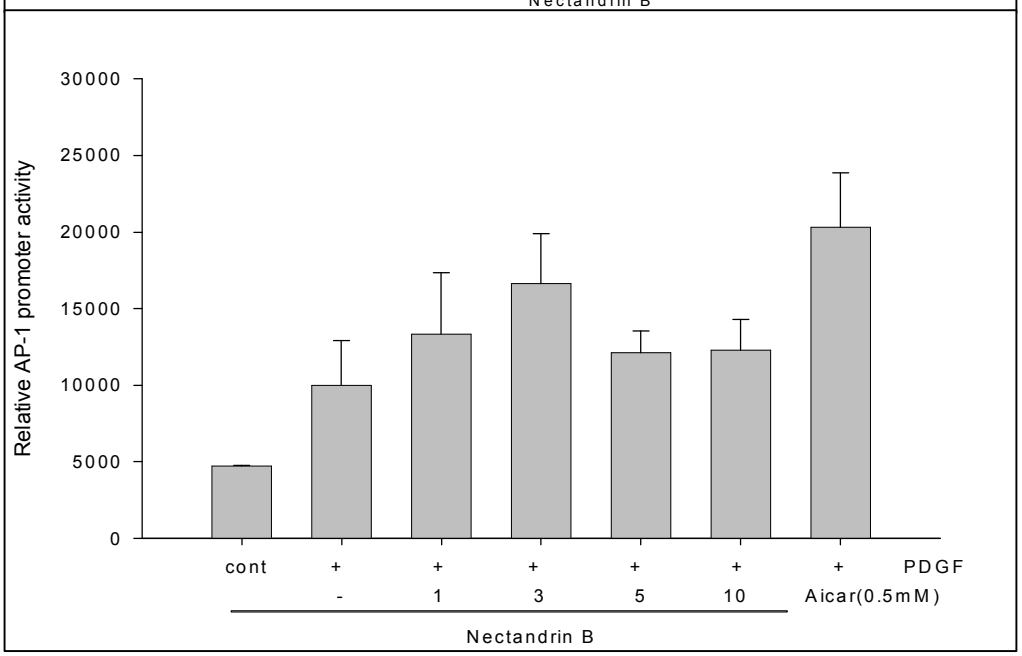
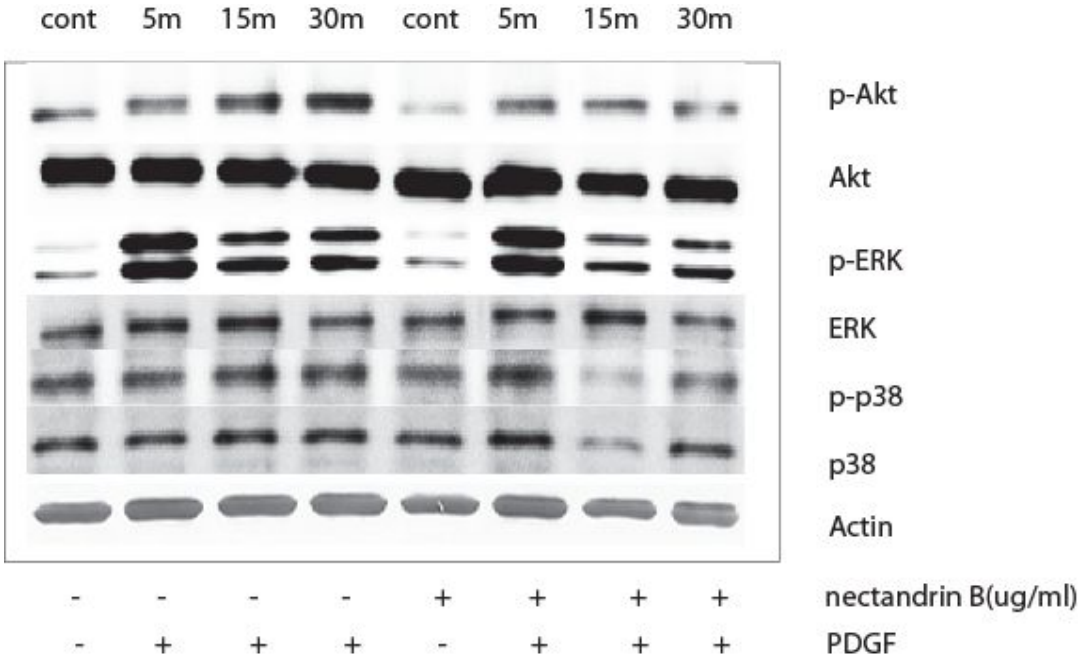


Figure 3. Nectandrin B inhibits cyclinD1 and Pin1 in PDGF-stimulated VSMCs. (A) After serum starved for 18h VSMCs were pre-treated with nectandrin B(0.3 to 10μg/ml) and incubate 30min. And then VSMCs further incubated with or without PDGF(30ng/ml) for additional 24h. Pin1 and cyclinD1 expression were determined by using Immunoblot analysis as described under Materials and Methods. Equal loading of proteins were verified by actin immunoblot. (B) MEF cells were transfected with Pin1-Luc-plasmids and AP-1-Luc-plasmids respectively for 8h, and then in the serum deprived condition Pin1 or AP-1 transfected MEF cells were pre-treated with nectandrin B(1 to 10μg/ml) and incubate 30min. And VSMCs further incubated with or without PDGF(30ng/ml) for additional 24h. Activation of Pin1 and AP-1 reporter genes were measured as described under Materials and Methods. Data represented the mean± SD with 4 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$)

A)


cont
5m
15m
30m
cont
5m
15m
30m
p-Akt
Akt
p-ERK
ERK
p-p38
p38
Actin
- - - - + + + + nectandrin B(ug/ml)
- + + + - + + + PDGF

B)

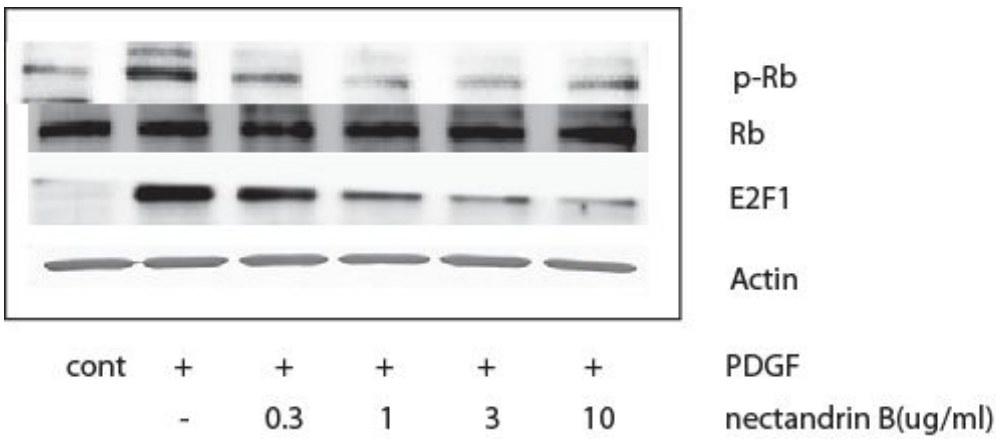
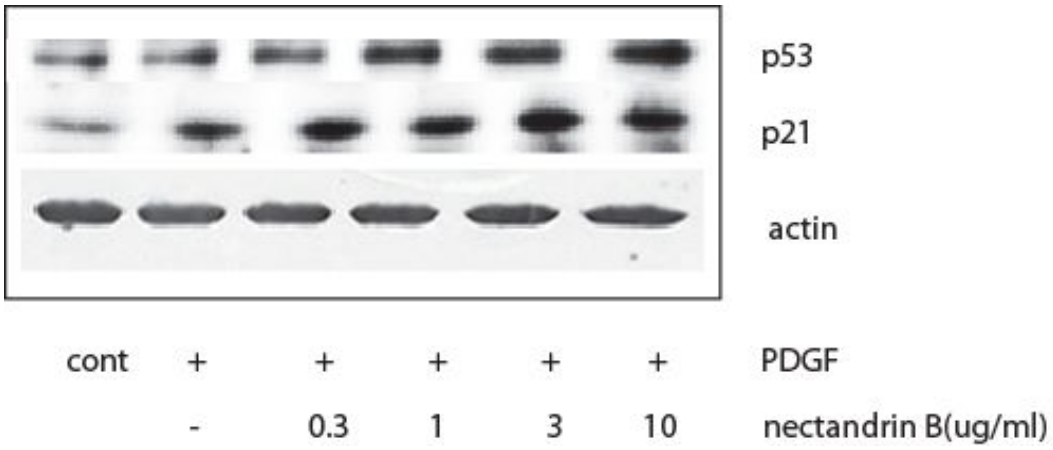

p-Rb
Rb
E2F1
Actin
cont + + + + + PDGF
- 0.3 1 3 10 nectandrin B(ug/ml)

Figure 4. Effects of nectandrin B on PDGF-stimulated E2F1, phosphorylated Rb. (A) After serum starved for 18h and VSMCs were treated with PDGF(30ng/ml) for each time.(5, 15 30min) Before 30min on PDGF treatment, some groups were pre-treated with nectandrin B.(10μg/ml) Representative immunoblots show total and phosphorylated ERK, p38, Akt protein expression levels. Equal loading of proteins were verified by actin immunoblot. (B) Effects of nectandrin B on PDGF-induced E2F1 and phosphorylated Rb. After serum starved for 18h and VSMCs were pre-treated with nectandrin B and incubate 30min. And then VSMCs further incubated with or without PDGF(30ng/ml) for additional 24h. Representative immunoblots show E2F1, total and phosphorylated Rb protein expression levels. Equal loading of proteins were verified by actin immunoblot.

A)


p53
p21
actin
cont + + + + + PDGF
- 0.3 1 3 10 nectandrin B(ug/ml)

B)

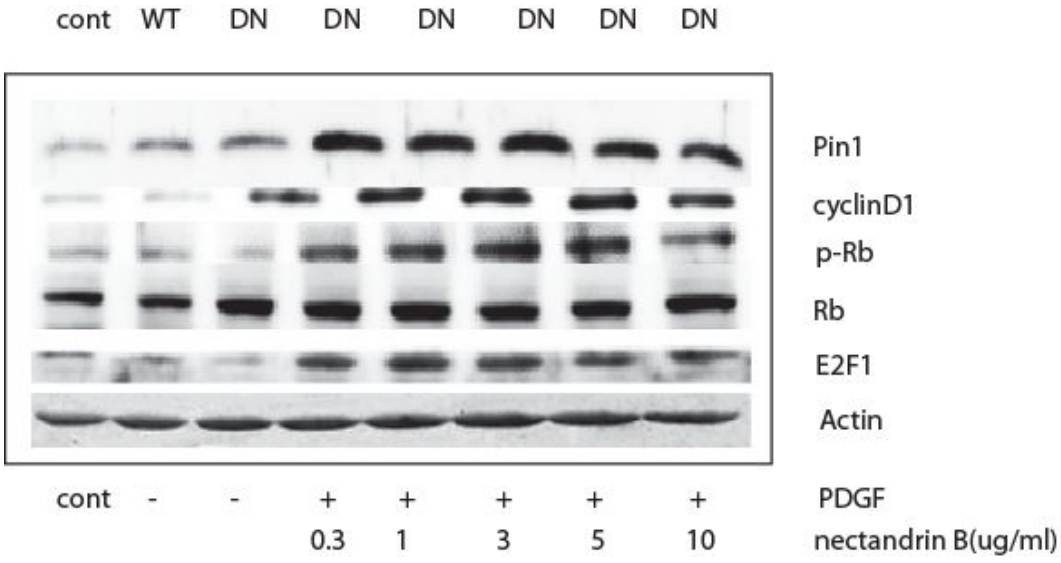
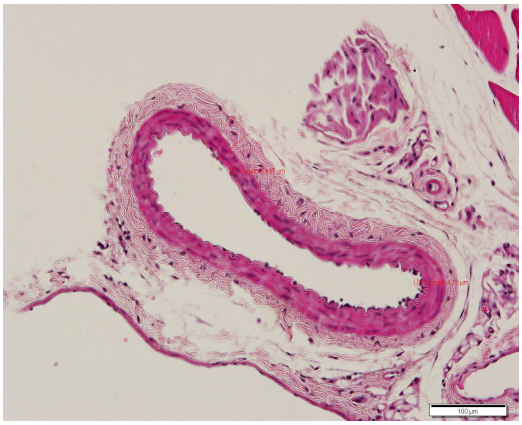
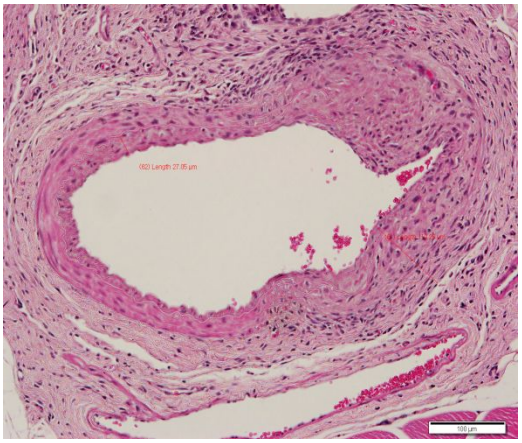

cont WT DN DN DN DN DN DN
Pin1
cyclinD1
p-Rb
Rb
E2F1
Actin
cont - - + + + + + PDGF
0.3 1 3 5 10 nectandrin B(ug/ml)

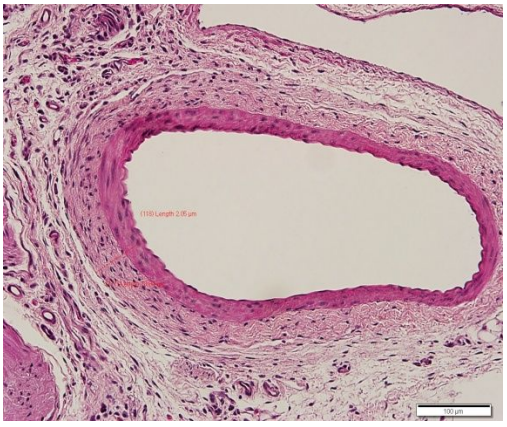
Figure 5. Induction of p53 and p21 expression by nectandrin B. (A) In the presence of serum VSMCs were treated with nectandrin B(1 to 10μg/ml) and incubate 24h. Representative immunoblots show p53 and p21 protein expression levels. Equal loading of proteins were verified by actin immunoblot. (B) To identify the whether nectandrin B inhibit Pin1 through AMP-activated protein kinase (AMPK) or not, we treated dominant-negative AMPK and wild-type AMPK adeno viruses and incubate 18h. After 18h, VSMCs were starved for 18 h and then pre-treated with nectandrin B (1 to 10μg/ml) in the treated with dominant-negative adeno viruses groups and incubate 30min. VSMCs were further incubated with or without PDGF(30ng/ml) for additional 24 h. Representative immunoblots show Pin1, cyclinD1, E2F1 and total and phosphorylated Rb protein expression levels. Equal loading of proteins were verified by actin immunoblot.



Sham



Vehicle



Nectandrin B(20mg/kg)

Figure 6. Inhibition of neointimal formation by nectandrin B. Representative pictures (magnification X 200) of H&E (Hematoxylin and eosin) – stained femoral arteries obtained from guide wire injured mice (3 weeks). Nectandrin B (20 mg/kg, twice a week) peritoneal treated group exhibits significant reduction of neointimal formation compared with vehicle-treated group.

저작물 이용 허락서

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논문제목	한글 : AMP kinase 활성화제인 nectandril에 의한 혈관내벽증식 억제효과 영어 : Inhibitory effect of nectandril, a potent AMPK activator on neointimal formation				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

20010 년 10 월 15 일

저작자: 이 정 운 (서명 또는 인)

조선대학교 총장 귀하