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Inhibition of Neointimal formation by CPP343

조선대학교 대학원

약학과

김 정 우

CPP343 에 의한
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이 논문을 약물학 석사학위신청 논문으로 제출함

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혈관내벽 증식에 의한 재 협착 현상은 혈관 풍선 확장술 후의 성공적 결과에 걸림돌이 된다. 혈관 평활근 세포 (VSMC)의 비정상적인 세포증식과 세포 이동은 혈관내벽 증식에 기여한다. Pin1 은 특이적 Phospho-Serine/Threonine-Proline isomerase 로서, 암의 치료를 위한 신규 표적으로서 주목을 받고 있다. 최근에 본 연구진은 Pin1 이 비정상적인 혈관 평활근 세포의 증식에 매우 중요한 작용을 하는 것으로

밝힌 바 있다. 머루 추출물의 stilbenoids 그리고 oligostibenoids 들이 암의 치료, 항 염증작용, 심혈관 질환 예방효과, 항 산화작용 그리고 항 세포증식효과 등 다양한 생물학적 작용을 갖는 것으로 알려져 많은 주목을 받고 있다. 이러한 이유로 우리는 몇몇 stilbenoids 와 oligostibenoids 의 혈관 평활근 세포에서 Pin1 억제효과에 대한 연구를 실행하였으며, oligostibenoids 중 하나인 CPP343 이라는 물질이 탁월한 Pin1 발현 억제효과를 가지고 있음을 밝혀냈다. CPP343 이 강력한 Pin1 의 억제물질이라는 사실 하에, 본 연구에서는 CPP343 이 Pin1 과 연관된 혈관 평활근 세포의 증식 억제효과와 혈관 내벽증식의 억제에 미치는 영향을 밝혀내고자 한다.

Platelet derived growth fator (PDGF) 에 의한 자극은 혈관 평활근 세포의 증식을 촉진한다. PDGF 로 자극된 혈관 평활근 세포의 증식이 CPP343 의 처리시에 의해 농도 의존적으로 감소하였다. 또한 PDGF 는 cyclin D1, cyclin D3 나 인산화된 Rb 같은 세포주기조절 관련 단백질의 발현을 증가시켰으며, 이러한 발현의 증가는 CPP343 에 의해 강력하게 억제 되었다. CPP343 의 혈관 평활근 세포 증식과 연관된 Pin1 발현 억제효과의 기전을 규명하기 위하여 우리는 ERK/MAPK, p38/MAPK, SAPK/JNK 그리고 Akt/PI3K 같은 상위 신호전달 인산화 효소에 CPP343 이 미치는 영향에 대해 연구하였다. PDGF 는 이러한 상위

신호전달 인산화 효소들을 모두 활성화 시켰으며, CPP343 에 의해 p38/MAPK, SAPK/JNK 및 Akt/PI3K 의 활성화 정도가 감소 되었다. 또한, PDGF 에 의해서 증가된 Pin1 의 발현이 p38/MAPK 억제제에 의하여 강력하게 차단되었다. 이러한 현상은 세포주기조절 관련 단백질의 발현에서도 동일하게 관찰 되었다. 또한 높은 농도의 CPP343 의 처리는 Caspase-3 나 PARP 와 연관된 VSMC 의 세포자연사를 증가시켰다. 마지막으로 CPP343 (3 mg/kg, 주 2 회 구강투여)을 3 주간 투여하여 wire injury 로 유도된 neointima 형성을 억제함을 보였다.

결론적으로, CPP343 은 p38/MAPK 의 활성의 억제를 통하여 Pin1 의 발현과 세포주기의 진행을 억제하며, 고농도 범위에서는 세포자연사 효과를 일으킨다. 이러한 결과들을 종합 시, CPP343 이 동맥경화나 혈관 재 협착 같은 혈관질환의 치료나 예방효과의 효과적인 약물이 될 수 있음을 시사한다.

ABSTRACT

Inhibition of neointimal formation by CPP343

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In-stent restenosis due to neointima formation is a major limitation following balloon angioplasty. Abnormal proliferation of vascular smooth muscle cell (VSMC) contributes to neointima formation. Pin1 as a specific phospho-Serine/Threonine-Proline isomerase, has been focused on a promising target for the treatment of cancer. Our recent study has also shown that Pin1 plays a key role in abnormal VSMC proliferation. More attention has been focused on stilbenoids and oligostibenoids from *vitis amurensis* extract, because they has been found to have multifaceted bioactivities such as cancer-chemopreventive, anti-inflammatory, cardio-protection, anti-oxidant

and anti-proliferative properties. In this study, we first examined whether several stilbenoids and oligostibenoids inhibits Pin1 expression in PDGF-exposed VSMCs. CPP343, one of oligostilbenoids from v*itis amurensis*, showed potent inhibitory activity on Pin1 expression. CPP343 inhibits PDGF induced VSMC proliferation in a concentration–dependent manner. PDGF also increased cell cycle regulated proteins, including cyclin D1, cyclin D3 and phosphorylated Rb, and these were completely blocked by CPP343. In order to clarify the signaling pathways for Pin1 down-regulation by CPP343, we investigated the effects of CPP343 on the activation of ERK/MAPK, p38/MAPK, SAPK/JNK and Akt/PI3K. PDGF increased the active phosphorylated forms of all these kinases, and the activities of these kinases were attenuated by CPP343 treatment in VSMCs. Moreover, PDGF-induced increases in Pin1 expression and Pin1 promoter activity were siginificantly reversed by inhibition of p38/MAPK, SAPK/JNK or Akt/PI3K, and the most potent inhibition was found by p38/MAPK inhibitor. Higher concentration ranges of CPP343 increased activity of caspase-3 and PARP, which were related with VSMCs apoptosis. At last treatment of CPP343 for 3 weeks (3mg/kg, two times a week, orally injection) suppressed neointimal formation induced by guide

wire injury.

In conclusion, CPP343 blocked Pin1 expression level and cell cycle progression mainly through the inhibition of p38/MAPK, and CPP343 induced apoptosis effect. From these results, CPP343 may have therapeutic potential for vascular proliferative diseases such as atherosclerosis and restenosis.

Keywords: CPP343, Pin1, VSMCs, Neointima formation

1. Introduction

Restanosis after angioplasty procedures is a significant clinical problem (Choy et al., 2001). A dominant cellular event in the re-narrowing of vascular lumen after angioplasty is due to vascular smooth muscle cells (VSMCs) proliferation and migration. After vascular injury, the VSMCs start to proliferate and then migrate into the developing neointima thus becoming the major cellular substrate of the restenotic tissue. The rough-surfaced endoplasmic reticulum in SMCs can express a large number of growth-regulatory molecules and extracellular matrix components, which are involved in the regulation of cell proliferation and migration. Although mechanisms responsible for proliferation and migration of VSMCs are not fully understood, several factors produced in response to vascular injury have been implicated in this process (Jackson et al., 1992).

Platelet-derived growth factor (PDGF) is a potent growth factor produced by platlets, VSMCs, and endothelial cells in the injured vascular wall (Majesky et al., 1990; Miyauchi at al., 1998). PDGF initiates various biological effects through the activation of intracellular signal transduction pathways that contribute to VSMC proliferation

(Heldin et al., 1999). The importance of PDGF in the development of neointima has been evaluated in arterial injury models (Heldin et al., 1999). Accordingly, inhibition of PDGF-stimulated VSMC proliferation represents an important point of therapeutic intervention to attenuate cellular manifestation of many vascular diseases.

Pin1, a peptidyl-prolyl isomerase, is an evolutionarily conserved enzyme that promotes the *cis-trans* isomerisation of the peptide bond at the amino side of the proline residue (Lu et al.,2004). Pin1 plays an important role in cell cycle regulation through its specific interaction with proteins that are phosphorylated at Ser/Thr-Pro motifs (Lu et al.,1996; Yaffe et al.,1997). Pin1 is essential for cell cycle progression in yeast and in mammalian cells (Lu et al.,1996). Cyclin D1 is cycle regulator of the G1/S check-point in the cell cycle and plays an important role in the development of many vascular proliferative diseases (Quasnichka et al., 2006). A series of in vitro and in vivo studies demonstrate a close correlation between Pin1 and cyclin D1 (Wulf et al., 2001). Pin1 regulates cyclin D1 gene expression by at least two different mechanisms (Wulf et al., 2001; Wulf et al,. 2005). Importantly, upregulation of Pin1 has been shown to elevate cyclin D1 gene expression by activating the c-jun/AP-1 and β-catenin/TCF

transcription factors (Ryo et al., 2001; Wulf et al., 2001). Furthermore, Pin1 directly binds cyclinD1 and isomerize the pThr 286-Pro motif of cyclin D1 thereby preventing its nuclear export and ubiquitin-mediated degradation, resulting in cyclin D1 stabilization (Liou et al., 2002). Our recent study has shown that Pin1 is induced in the injured vascular wall by PDGF release, and that Pin1 overexpression is required for the VSMC proliferation and neointima formation (Kim et al., 2009).

Recent reports have shown that stem of *vitis amurensis* contain several stilbenoids and oligostilbenoids (Kulesh et al., 2006). And these compounds have diverse bioactivities such as antioxidant (He et al., 2008), cancer-chemoprevention (Jang et al., 1997), anti-inflammation (Huang et al., 2000), anti-HIV (Dai et al., 1998), anti-fungal (Bokel et al., 1988), anti-proliferation (Berardi et al., 2009) activities. We isolated seven different stilbenoids and oligostilbenoids from stem of *vitis amurensis* and we found that CPP343 potently suppressed PDGF-stimulated Pin1 expression in VSMCs. Based on the above considerations, the purpose of the present investigation was to determine the effects of CPP343 on PDGF-stimulated proliferation, as well as the intracellular mechanisms for the inhibition of Pin1 expression by CPP343.

2. Materials and Methods

2-1. Materials

The Pin1 antibody was supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated form-specific or total form recognizing antibodies against PI3K/Akt, ERK/MAPK, p38/MAPK, SAPK/JNK and antibodies for phosphorylated Rb, Cyclin D1, Cyclin D3, cleaved caspase-3, full length PARP1 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). CPP343 was kindly provided by Dr. WK Oh (Chosun University, Gwang-ju, Korea). PD98059, LY294002, SB203580 and SP600125 were purchased from Calbiochem (San diego, CA). 5-Bromo-2'-deoxy-uridine labeling and detection kit was purchased from roche (Mannheim, Germany).

2-2. Plasmid

The mitogen-activated protein (MAP) kinase kinase 1 (MKK1) dominant-negative mutant was a gift from Dr. N. G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder, CO). The dominant negative mutant of p38 kinase (DN-p38K), dominant negative mutant of p85 kinase (DN-p85K) and c-Jun N-terminal kinase 1 (JNK1) dominant-negative mutant (DN-JNK1) were provided by Dr. N. Dhanasekaran (Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia, PA) and Dr. H.S. Choi (College of Pharmacy, Chosun University, Gwangju, Korea), respectively.

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 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 μg/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 μl 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, Pin1 +/+ MEF cells (3×10^5 cells/well) were replated in 12-well plates overnight and transiently transfected with the DN-p38K or DN-MKK1 or DN-JNK1 or DN-p85K plasmids and Pin1 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. 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. Effects of CPP343 on PDGF-stimulated Pin1 expression.

In order to screen drug candidates inhibiting VSMCs proliferation, we tested the inhibitory effects of several stilbenoids and oligostilbenoids isolated from *vitis amurensis* on Pin1 protein expression in PDGF exposed VSMCs. Immunoblot analyses revealed that 10μg/ml of CPP343, one of oligostilbenoids most potently attenuated the Pin1 expression in PDGF-stimulated VSMCs (Fig.1a). When we performed dose-response studies, CPP343 concentration-dependently inhibited PDGF mediated Pin1 induction and 10μg/ml of CPP343 completely inhibited Pin1 induction (Fig.1b). The possible involvement of CPP343 in the transcriptional activation of Pin1 gene was approached by using luciferase reporter gene containing Pin1 promoter. Because of low transfection efficiency in VSMCs, we used MEF cells for reporter gene assay. The Pin1 promoter activity was 1.5 fold increased by PDGF treatment and this was suppressed by CPP343 treatment (Fig.1c). Considering the essential role of Pin1 in cell cycle regulation and vascular proliferation, we hypothesized that CPP343 suppresses the proliferation of VSMCs.

3-2. Effects of CPP343 on PDGF-induced VSMC proliferation and the expression of cell cycle regulated proteins.

The proliferation of VSMC is a central event in pathogenesis of vascular lesions, including post-angioplasty restenosis, transplant arteriosclerosis and vein graft occlusion (Braun-Dullaeus et al.,1998). To investigate the effects of CPP343 on VSMC proliferation, we performed 5-Bromo-2'-Deoxy-uridine (BrdU) assay. Incubation of serum-deprived VSMCs with PDGF (30 ng/ml) for 24h increased DNA synthesis by 2 fold versus untreated control group. Treatment with CPP343 resulted in concentration dependent inhibition of VSMCs proliferation. There was no significant difference in proliferation of VSMCs containing 0.1μg/ml and 0.3μg/ml CPP343 compared with the control of PDGF. However, VSMC proliferation was significantly inhibited in the presence of 1-10 μg/ml CPP343 compared with PDGF alone-treated group. Furthermore, there was a good correlation between the decreased level of Pin1 protein expression and proliferation rate (fig.2a).

Progression through the cell cycle is dependent on the activities of specific cell-cycle regulator proteins. We examined the effects of CPP343 on the expression of several cell-cycle regulator proteins,

including cyclin D1, cyclin D3 and phosphorylated Rb in VSMCs. PDGF increased cyclin D1, cyclin D3 protein level, whereas CPP343 blocked reversed both the protein expression in a concentration dependent manner. In a similar manner, CPP343 also inhibited the protein level of phosphorylated Rb (Fig.2b). Taken together, the above results demonstrate that the CPP343 inhibited VSMC proliferation through the down-regulation of cell-cycle regulator proteins.

3-3. CPP343 attenuates PDGF-stimulated activation of p38/MAPK, SAPK/JNK and PI3K/Akt.

PDGF mediated Pin1 expression may be linked with cellular growth signaling such as MAPKs or PI3-kinase. In order to investigate which intracellular signaling pathway(s) is responsible for the reduction of Pin1 expression in response to CPP343, we first measured the effects of CPP343 on PDGF induced activation of PI3K and MAPKs. To determine activities of these kinases, antibodies specific for the total and phosphorylated forms of PI3K/Akt, p38/MAPK, ERK/MAPK and SAPK/JNK were used in immunoblotting analyses. PI3K and three kind of MAPKs were phosphorylated within 5-min after PDGF treatment. Reduction in phosphorylation levels of each kinase was

observed after treatment of CPP343 (10μg/ml). with exeption of ERK/MAPK. Although, ERK/MAPK is recognized as a key regulator of cell proliferation (Seger et al., 1994; Lai et al., 1996), which was not significantly changed by CPP343 (Fig.3).

3-4. Effects of MAPKs and PI3K inhibitors on PDGF induced Pin1 expression and cell-cycle associated proteins.

The possible involvement of PI3K and MAPKs in the transcriptional activation of Pin1 gene was approached by using luciferase reporter gene containing Pin1 promoter and dominant negative mutants of each kinase. The specific dominant-nagative mutant plasmids used were: DN-MKK1, DN-p85, DN-p38, DN-JNK1, respectively specific dominant-nagative mutant form of MAPKK/MEK, Akt/PI3K, P38/MAPK, SAPK/JNK. Because of low transfection efficiency in VSMCs, we also used MEF cells for reporter gene assay. The Pin1 promoter activity was 2.5 fold increased by PDGF treatment and this was most potently suppressed by DN-p38 transfection and less effectively by DN-MKK1, DN-p85, and DN-JNK1 (Fig.4a). The changes in Pin1 protein expression levels were also studied by specific kinase inhibitors. The inhibitors used were: PD98059, LY294002,

SB203589, SP600125, respectively specific inhibitor for MAPKK/MEK, Akt/PI3K, P38/MAPK, SAPK/JNK. Similar with Pin1 promoter study, all of inhibitors had inhibitory effects on Pin1 protein expression, but p38/MAPK inhibition showed the most profound inhibitory effect on Pin1 expression (Fig.4b).

Next, we analysed the inhibitors effects on PDGF-induced cyclin D proteins expression and phosphorylation of Rb. The induction of cyclin D proteins expression and Rb phosphorylation by PDGF were effectively inhibited by SB203580 but not by PD098059, and less effectively by LY294002 and SP600125, which again correlated well with the inhibitory effects on Pin1 expression by the kinase inhibitors (Fig.4b). These results suggest that PDGF-mediated p38/MAPK activation mainly leads to increased *Pin1* gene transcription, and subsequently causes increased expression of cell-cycle regulators including cyclin D1and cyclin D3.

3-5. High concentration of CPP343 exposure resulted in apoptosis of VSMCs.

CPP343 characterized by diminished PI3K/Akt (Fig.3). PI3K/Akt has been shown to regulate cell apoptosis (Brazil et al., 2002).

And this apoptotic cell death is induced by the activation of caspase-3 PARP. So we examined these proteins on CPP343 stimulated VSMCs by immunoblotting analysis. VSMCs were exposed to CPP343 (1 to 30μg/ml). Compare with control, CPP343 treatments at low concentrations (0.3μg/ml, 1μg/ml) did not produce significant increases in cleaved caspase-3 expression. However, when the exposed concentrations were high (3μg/ml to 30μg/ml), there were progressive increases in the upregulation levels of cleaved caspase-3 (Fig 5a). In contrast to these effects, CPP343 treatments at high concentrations (3μg/ml to 30μg/ml) caused decreases in the full length of PARP (Fig.5a).

Next, we performed MTT cell viability assay, to determine the VSMCs viability after CPP343 treatment. At higher concentration ranges of CPP343 (10 and 30μg/ml), decreased cell viability was observed (Fig.5b). These results indicate that high concentration of CPP343 increased activity of caspase-3 and PARP, further supporting the role of CPP343 in increasing apoptosis and it was correlated with VSMCs anti-neointimal proliferative effects.

3-6. Inhibition of neointimal formation by CPP343.

Finally, we evaluated whether CPP343 could reduce neointimal formation *in vivo*. Orally injection of CPP343 (3 mg/kg, twice a week) started after guide wire injury of mouse femoral artery and continued for 3 weeks. CPP343 treatment (3 mg/kg) significantly inhibited neointimal formation, as compared with vehicle-treated group (Fig.6).

4. Discussion

VSMC proliferation is one of the most important events in the pathogenesis of atherosclerosis and restanosis after balloon angioplasty (Ross et al., 1999; Schwartz et al., 1997). PDGF is kind of the most potent mitogens and chemoattractants for VSMCs and plays the central role in pathogenesis of various vascular disorders (Ross et al., 1993). Furthermore, this PDGF increase Pin1 expression in VSMC, which plays a critical role in VSMC proliferation and neointima formation. And importantly, it is therapeutic target for several vascular disease (Kim et al., 2009). It follows that a substance that inhibits the proliferation through the inhibition of Pin1 expression may have a potential beneficial effect on the development of restanosis or atherosclerotic disease. Many kine of stilbenoids and oligostilbenoids from *vitis amurensis* extract have diverse bioactivities such as antioxidant (He et al., 2008), cancer chemoprevention (Jang et al ., 1997), anti-inflammation (Huang et al., 2000), anti-HIV (Dai et al ., 1998), anti fungal (Bokel et al., 1988), anti-proliferation (Berardi et al., 2009) activities. Since the Pin1 function as a key signal to stimulate abnormal VSMC proliferation, in this present study, we examined the effect of these stilbenoids and oligostilbenoids on the Pin1 inhibition

effect of VSMCs. And suggesting its potential role related to neointimal formation.

We report here that CPP343, one kind of oligostilbenoids from *vitis amurensis* extract, has potent and concentration-dependent inhibitory effects on PDGF-stimulated VSMCs Pin1 protein expression and transcription. We have also shown using BrdU assay that CPP343 significantly inhibits VSMCs proliferation. Progression through the cell cycle is dependent on the activity of specific cell-cycle regulators. A regulatory role for cyclin D1 has been proposed with regard to Rb phosphorylation (Hannon et al., 1993; Dowdy at al., 1993). And cyclin D1 is a critical player in cell cycle exit (Zwijsen et al., 1996). Interactions between cyclin D1 and a variety of cyclin-dependent kinases have been reported, and the expression of D-type cyclins is regulated by growth factors (Matsushime et al., 1991). Pin1 expression can positively regulate the function of cyclin D1 at the transcriptional level and by posttranslational stabilization (You et al., 2002, Ryo et al., 2002). Given the close correlation between the induction of Pin1 and cyclin D1 as well as the Rb phosphorylation. We also revealed that CPP343 atteenuated PDGF induced cell-cycle regulators such as cyclin D1, cyclin D3 and phosphorelated Rb. Based on the inhibition by

CPP343, it seems reasonable to conclude that these inhibitory effects are at least part attributable to inhibition of VSMC neointima formation.

Intracellular signal transduction system plays a key role in several mitogen-induced cellular response, including cell proliferation, apoptosis, and a variety of gene expression. Mitogene-activated protein (MAP) kinases including extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase(JNK), p38/MAPK are the major signal transduction molecules responsible for the above-mentioned cellular response (Kyriakis et al., 2001). Specially, p38/MAPK plays an important role in growth factor-stimulated VSMC proliferation, also in neointimal formation (Protor et al,. 2008). PI3K/Akt has been shown to regulate a number of cellular processes including cell cycle progression angiogenesis, cell motility, and apoptosis (Brazil et al., 2002). The possible role of PI3K and MAPKs has been investigated since previous data have shown that in VSMCs of vascular and visceral origin these pathways are activated by PDGF (Hayashi et al., 1998, 1999). After CPP343 treatment, only p38/MAPK, SAPK/JNK and Akt/PI3K activated level is attenuated. From these result, we can conclude that p38/MAPK, SAPK/JNK and Akt/PI3K plays an important role in CPP343-obstructed VSMC proliferation and pin1 reduction effect. In

particular, the possible involvement of PI3K and MAPKs in the upregulation of Pin1 and cell-cycle regulators to PDGF is approached by selectively inhibiting the single kinases in conditions of stimulation with this growth factor. Upregulated Pin1 promoter activity was completely abolished by dominant negative mutant p38/MAPK plasmid co-transfection. Also, upregulated Pin1 protein expressions were completely blocked by p38/MAPK inhibitor treatment. Same phenomenons were observed in cell-cycle regulator proteins study, which again correlated well with the inhibitory effects on Pin1 expression by the kinase inhibitors. It is suggested that PDGF effection on Pin1 transcription and translation were primarily via p38/MAPK pathway, similar to the observation that PDGF effect on cyclin D and phosphorylated Rb proteins are primarily through p38 MAPK pathway.

Not only VSMC proliferation, but also VSMCs apoptosis can affect to neointima formation (Lemay et al., 2009). It has been show that PI3K/Akt regulated cell apoptosis (Brazil et al., 2002). And this apoptotic cell death is induced by the activation of caspase-3 PARP. Also, we shown that CPP343 attenuated PDGF induced phosphorylation levels of PI3K/Akt. Cleaved caspase-3 is the activated form of caspase-3, whereas PARP is one of the substrates of activated

caspase-3. In contrast with low concentration, high concentration of CPP343 increased activation of caspase-3 and PARP. VSMCs viablity was also decreased on a high concentration. The activation of caspase-3 and PARP caused by CPP343 treatment may result from diminution in the phosphorylation of cell survival kinases, such as PI3K/Akt. And these result indicate that CPP343 induce VSMCs apoptosis through the activation of caspase-3 and PARP. Our results showing that CPP343 treatment significantly suppresses neointimal formation in wire-injured femoral artery.

Taken together, this present study show that VSMC proliferation is blocked by CPP343 treatment, and this anti-proliferative effect is correlated with cell-cycle regulators attenuation through the Pin1 inhibition which function may result from CPP343 mediated p38/MAPK inactivation. And PARP and Caspase-3 mediated apoptosis effects on a high concentration of CPP343 treatment were also related to VSMC anti-proliferation effects. Our observation could be meaningful that CPP343, represents a potential therapeutic agent in treating or preventing vascular-proliferative disease including atherosclerosis and restenosis.

5. References

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6. Figure Legends

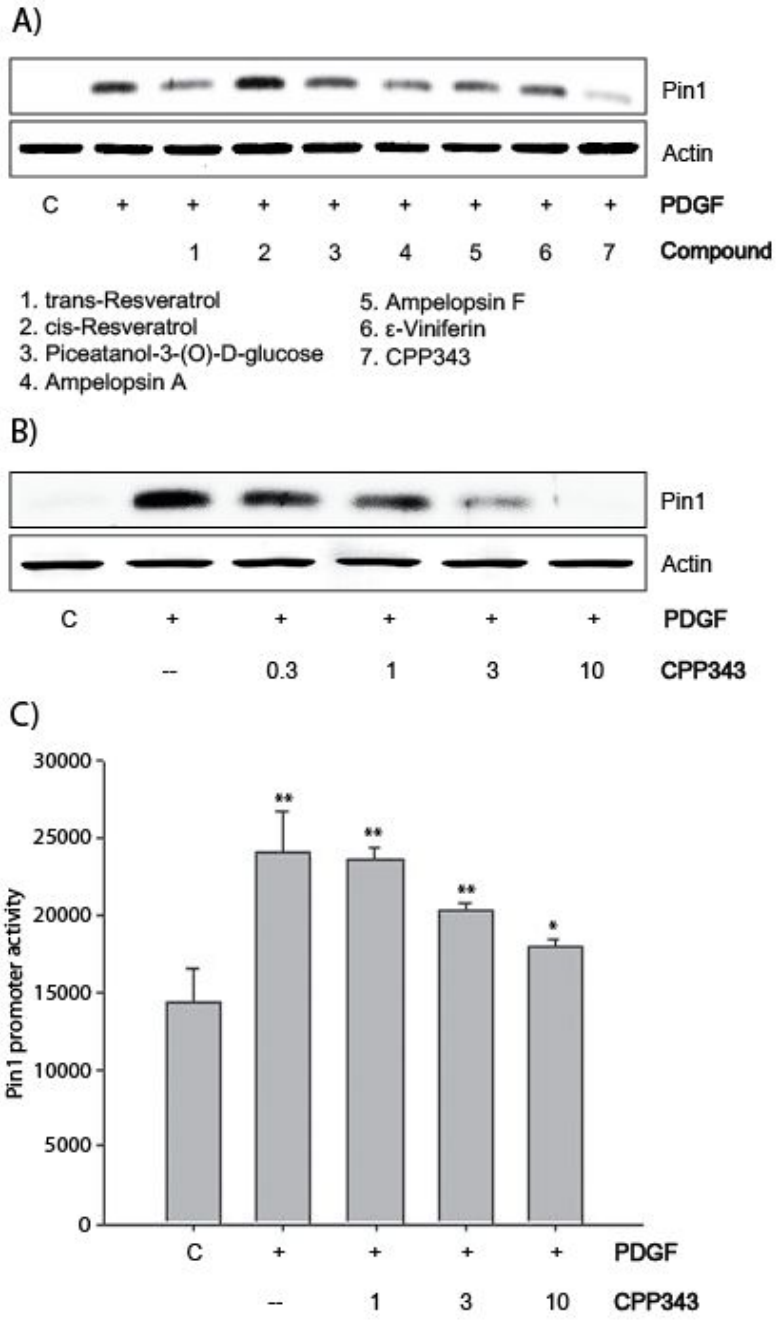
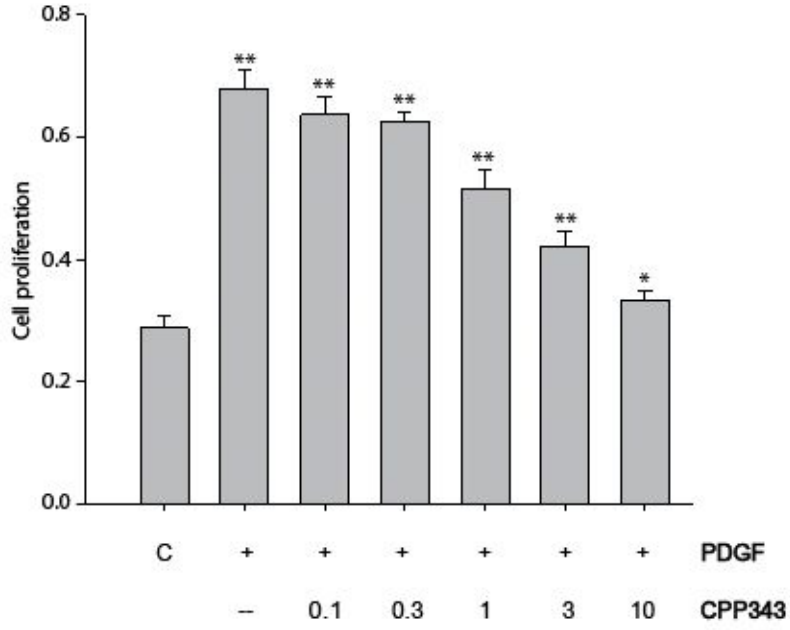


Figure 1. CPP343 attenuates PDGF-stimulated VSMCs pin1 expression. (A) Comparison of Pin1 inhibition effects between several stilbenoids and oligostilbenoids from *vitis amurensis*. After serum starved for 24h and VSMCs were pre-treated with each compound. (1 to 7, 10μg/ml) for 30m, then pre-treated VSMCs further incubated with or without PDGF (30ng/ml) for additional 24h. (B) Pin1 inhibition effects of CPP343. After serum starved for 24h and VSMCs were pre-treated with CPP343 (0.3 to 10 μg/ml) for 30m, then pre-treated VSMCs were further incubated with or without PDGF (30ng/ml) for additional 24h. The Pin1 expression was determined by using Immunoblot analysis as described under Materials and methods. Equal loading of proteins were verified by actin immunoblot. (C) CPP343 attenuated PDGF induced Pin1 promoter activity. MEF cells were co-transfected with Pin1-Luc-plasmid for 6h, then Pin1 transfected MEF cells were pre-incubated with CPP343 (0.3 to 10 μg/ml) for 30m, then pre-treated VSMCs were further incubated with or without PDGF (30ng/ml) for additional 18h. Activation of the reporter genes were performed as described under Materials and methods. Data represent the mean ± SD with 3 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$)

A)


0.8
0.6
0.4
0.2
0.0
Cell proliferation
**
**
**
**
**
*
C + + + + + + PDGF
-- 0.1 0.3 1 3 10 CPP343

B)

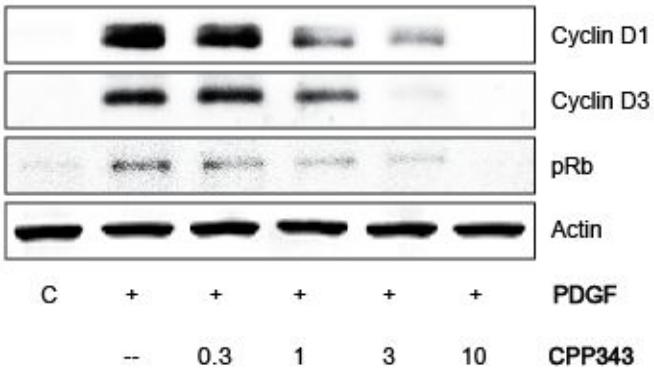

Cyclin D1
Cyclin D3
pRb
Actin
C + + + + + PDGF
-- 0.3 1 3 10 CPP343

Figure 2. CPP343 inhibits PDGF-induced VSMCs proliferation and cell cycle associated proteins. (A) Inhibitory effect of CPP343 on PDGF-induced VSMCs proliferation. VSMCs were pre-treated with CPP343. (0.1 to 10 μg/ml) and incubated 8h, then VSMCs further incubated with or without PDGF (30ng/ml) for additional 24h. VSMCs DNA synthesis 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,*$p < 0.05$, **$p < 0.01$) (B) Effects of CPP343 on PDGF-induced cell cycle regulated proteins expression. After serum starved for 24h and VSMCs were pre-treated with CPP343. (0.3 to 10 μg/ml) and incubated 30m, then pre-treated VSMCs further incubated with or without PDGF (30ng/ml) for additional 24h. Representative immunoblots show cyclin D1, cyclin D3 and phosphorylated Rb protein expression levels. Equal loading of proteins were verified by actin immunoblot.

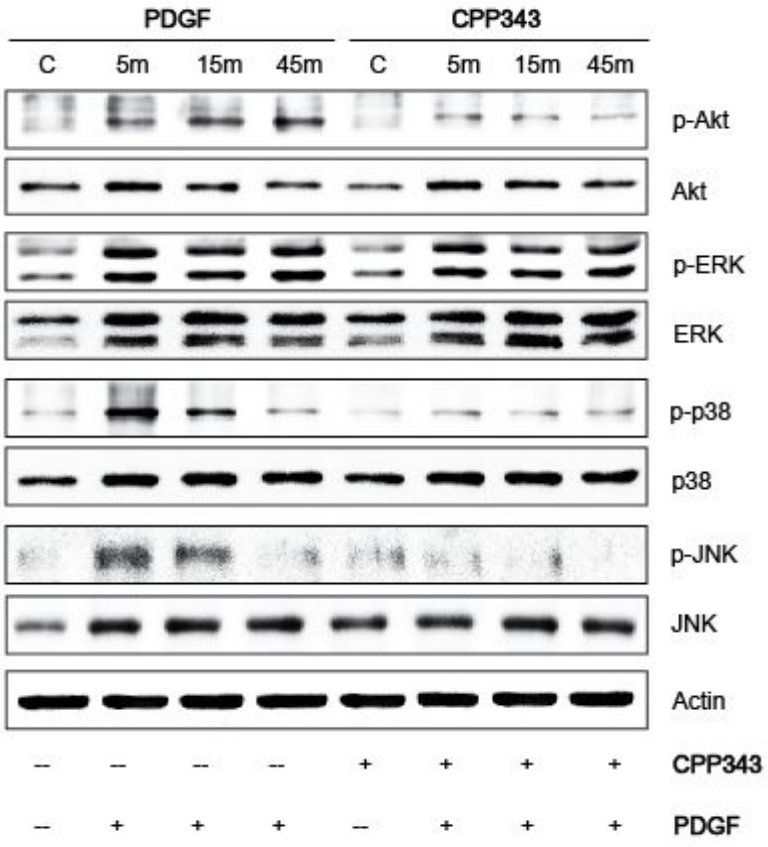

PDGF
CPP343
C
5m
15m
45m
C
5m
15m
45m
p-Akt
Akt
p-ERK
ERK
p-p38
p38
p-JNK
JNK
Actin
– – – – + + + + CPP343
– + + + – + + + PDGF

Figure 3. Effects of CPP343 on PDGF-activated PI3K and MAPKs. After serum starved for 24h and VSMCs were treated with PDGF (30ng/ml) for each times. (5m, 15m 45m) Specially, before 30m on each PDGF treatment, CPP343 (10μg/ml) were pre-treated. Representative immunoblots show total and phosphorylated Akt, ERK, p38, JNK protein expression levels. Equal loading of proteins were verified by actin immunoblot.

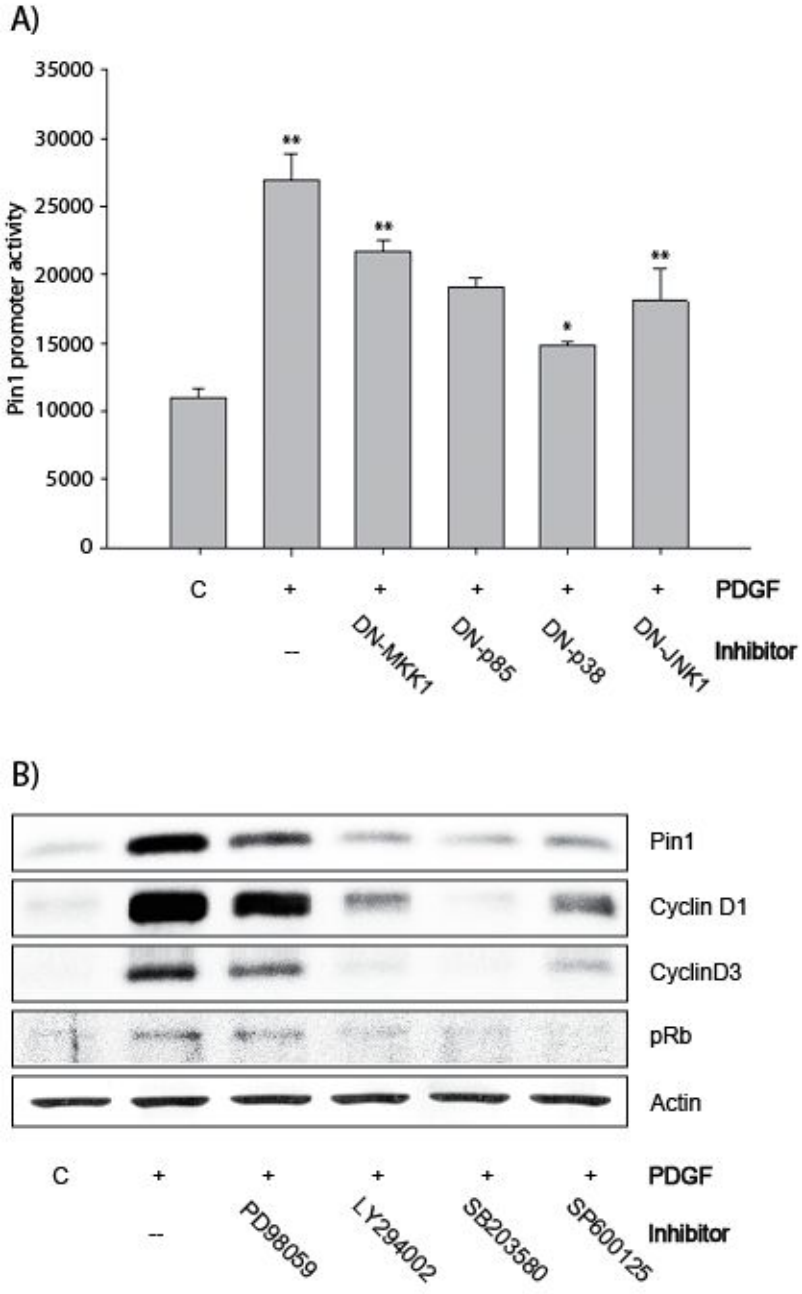

A)
35000
30000
25000
20000
15000
10000
5000
0
Pin1 promoter activity
**
**
**
*
C
+
+
+
+
+
PDGF
–
DN-MKK1
DN-p85
DN-p38
DN-JNK1
Inhibitor
B)
Pin1
Cyclin D1
CyclinD3
pRb
Actin
C
+
+
+
+
+
PDGF
–
PD98059
LY294002
SB203580
SP600125
Inhibitor

Figure 4. Effects of PI3K and MAPKs inhibitors on PDGF-induced Pin1 expression and cell cycle associated proteins. (A) PI3K and MAPKs dominant negative mutant plasmids inhibited PDGF-induced Pin1 promoter activity. MEF cells were co-transfected with Pin1-Luc-plasmid and each dominant negative plasmids (DN-MKK1, DN-p85, DN-p38, DN-JNK1) for 6h, then Pin1 transfected MEF cells were incubated with PDGF (30ng/ml) for additional 18h. Activation of the reporter genes were performed as described under Materials and methods. Data represent the mean ± SD with 3 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$) (B) PI3K and MAPKs inhibitiors inhibited PDGF-induced Pin1 and cell cycle associated proteins expression level. After serum starved for 24h and VSMCs were treated with each inhibitors. (30μM of PD98059, 10μM of LY294002, 20μM of SB203580, 10μM of SP600125) for 30m, then VSMCs were further incubated with or without PDGF (30ng/ml) for additional 24h. The Pin1, cyclin D1, cyclin D3 and phosphorylated Rb expression was determined by using Immunoblot analysis as described under Materials and methods. Equal loading of proteins were verified by actin immunoblot.

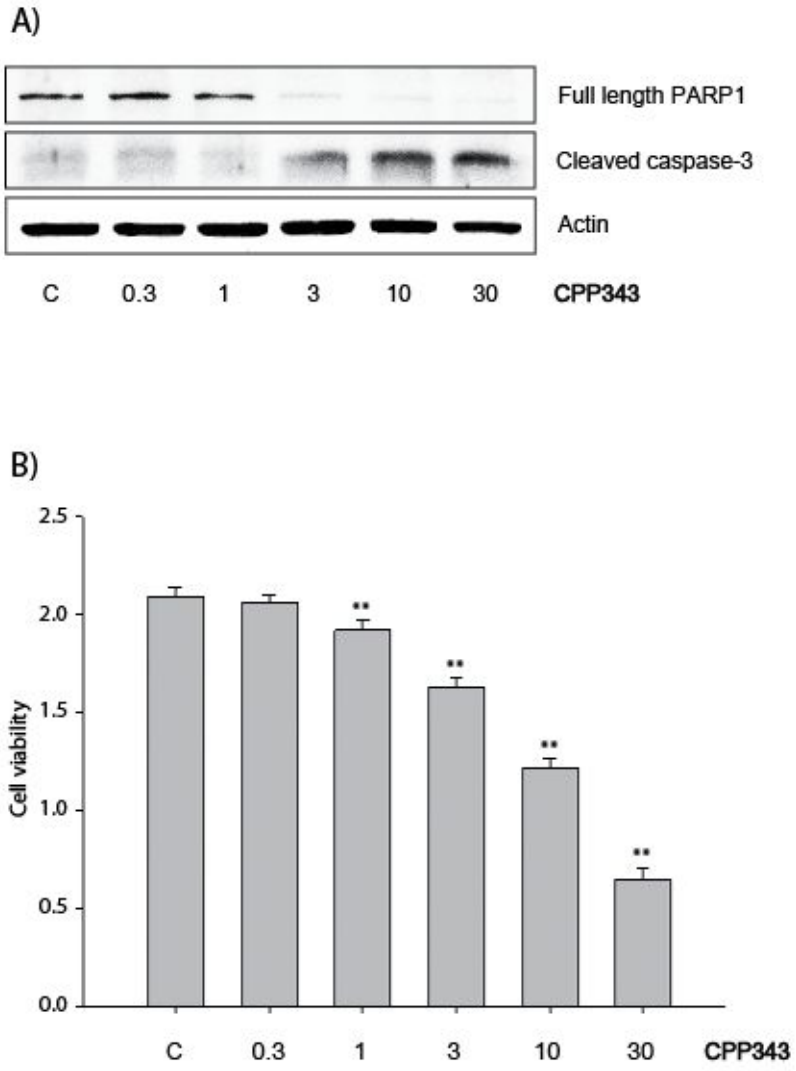
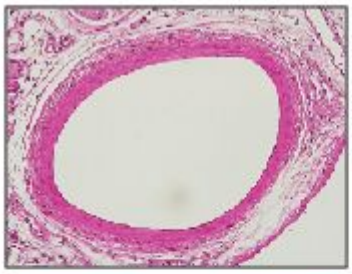
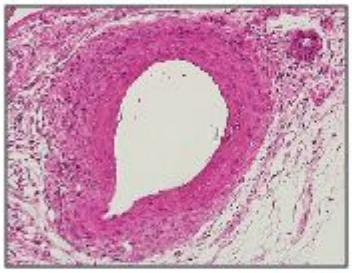

A)
Full length PARP1
Cleaved caspase-3
Actin
C
0.3
1
3
10
30
CPP343
B)
Cell viability
2.5
2.0
1.5
1.0
0.5
0.0
**
C
0.3
1
3
10
30
CPP343

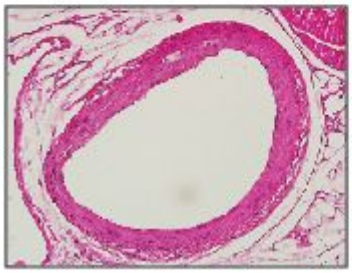
Figure 5. High concentration of CPP343 induces VSMC apoptosis. (A) Effects of CPP343 on VSMCs apoptosis. After serum starved for 24h and VSMCs were incubated with or without CPP343 (0.3 to 30 μg/ml) for 24h. Representative immunoblots show PARP1 and cleaved caspase-3 protein expression levels. Equal loading of proteins were verified by actin immunoblot. (B) The equal number of VSMC were seeded and incubated for 48 h and VSMCs were further incubated with or without CPP343 (0.3 to 30 μg/ml) for additional 24 h and the relative cell numbers were obtained by MTT assays as described under Materials and methods. Data represent the mean ± SD with 4 different samples. (significant as compared to control groups,$^{**}p < 0.01$)



Sham



Vehicle



CPP343 3mg/kg

Figure 6. Inhibition of neointimal formation by CPP343. Representative pictures (magnification X 200) of H&E (Hematoxylin and eosin) – stained femoral arteries obtained from guide wire injured mice (3 weeks). CPP343 (3 mg/kg, twice a week) orally treated group exhibits significant reduction of neointimal formation compared with vehicle-treated group.

저작물 이용 허락서

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논문제목	한글 : CPP343 에 의한 혈관내벽 증식의 억제효과 영어 : Inhibition of neointimal formation by CPP343				

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 의사 표시가 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( )

2009 년 10 월 20 일

저작자: 김 정 우 (서명 또는 인)

조선대학교 총장 귀하