Original Article

Inhibitory Effects of Cilostazol on Proliferation of Vascular Smooth Muscle Cells (VSMCs) Through Suppression of the ERK1/2 Pathway

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Aim: The abnormal proliferation of vascular smooth muscle cells (VSMCs) in arterial walls is an important pathogenic factor of vascular disorders such as atherosclerosis and restenosis after angioplasty. During atherogenesis or in response to vessel injury, VSMC proliferation is induced by a number of peptide growth factors released from platelets and VSMCs. Cilostazol is a phosphodiesterase (PDE) 3 inhibitor that increases intracellular cAMP levels and decreases intracellular Ca^{2+} levels, inhibiting platelet aggregation and inducing vasodilatation. Cilostazol is also known to have an inhibitory effect on the proliferation of VSMCs, but the anti-proliferative mechanism of cilostazol in VSMCs has not yet been established. In the present study, we investigated whether the anti-proliferative mechanism of cilostazol is associated with the suppression of extracellular signal-regulated kinases (ERK) and phosphatidylinositol 3 kinase (PI3K) signaling pathways.
Methods: To confirm the anti-proliferative effects of cilostazol on VSMCs, VSMCs were induced to proliferate by serum-induced mitogenesis and then were treated with cilostazol for 24 h. And, to investigate whether the anti-proliferative mechanism of cilostazol in VSMCs involves the suppression of the ERK and PI3K pathways, expression of the phosphorylated forms of ERK1/2, Raf, Akt, and glycogen synthase kinase (GSK)-3 were evaluated by western blot.
Results: Cilostazol inhibited VSMC proliferation in a dose-dependent manner. Phosphorylated ERK1/2 and Raf were significantly reduced in a dose-dependent manner, whereas phosphorylated Akt and GSK-3 were not changed.
Conclusion: These results suggest that suppression of the ERK pathway but not the PI3K pathway is an important mechanism in the anti-proliferative effect of cilostazol on VSMCs.

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Key words; Cilostazol, Atherosclerosis, cAMP, Anti-proliferation, ERK

Introduction

Atherosclerosis is one of the most important pathogenic mechanisms in ischemic stroke. The central events of atherosclerosis include endothelial dysfunction, inflammation, vascular smooth muscle cell (VSMC) proliferation, and matrix alteration[1]. VSMCs primarily regulate vascular tone and contraction and are an important component of the medial layer of the arteries, which is also made up of endothelial cells and a small number of myointimal cells from the intimal layer[2]. Moreover, it is well established that the proliferation of VSMCs plays a major role in the restenotic process, which is strongly related to the recurrence of ischemic stroke after arterial stenting and balloon angioplasty[3]; therefore, inhibition of VSMC proliferation may help to prevent ischemic stroke.

Cilostazol ({6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro 2(1H)quinolinone}, CLZ, **Fig. 1**) increases intracellular cyclic 3', 5'-adenosine monophosphate (cAMP) concentrations by selectively blocking phosphodiesterase type Ⅲ. Elevation of intracellular cAMP levels and decreased intracellular Ca^{2+} levels by cilostazol can induce vasodilatation and anti-plate-
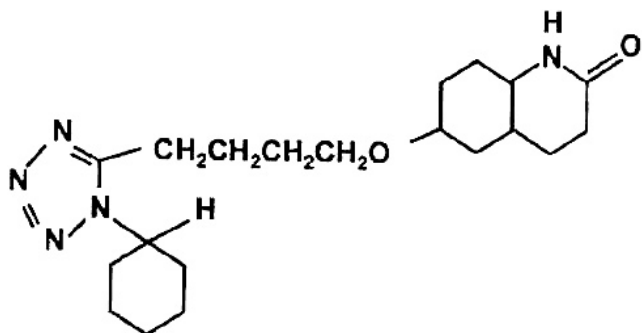
Fig. 1. Chemical structure of cilostazol.

let action[4]. Cilostazol is known to regulate NO production, transcription factor E2F, and pituitary adenylate cyclase-activating polypeptide (PAC-1)[5-7], and it has been reported that cilostazol reduces neointimal formation in balloon-injured rat carotid arteries and inhibits restenosis after percutaneous transluminal coronary angioplasty[3]. Anti-proliferative effects of cilostazol on VSMCs have also been reported[5]; however, the precise mechanisms of the anti-proliferative effects of cilostazol on VSMCs have not yet been clearly established, particularly as they relate to the ERK1/2 pathway.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 mitogen-activated protein kinase (MAPK) are subfamilies of MAPKs in mammalian cells. The activation of ERK1/2 is involved in the cell cycle, transcription, and cellular proliferation[8]. Considering the role of ERK1/2 in cells, the activation of ERK1/2 may be essential for the proliferation of VSMCs associated with atherosclerosis. This hypothesis is partially supported by findings that an increase in the active form of Raf, an immediate upstream activator of ERK1/2, was necessary to induce VSMC proliferation[9, 10]. Confirmation that cilostazol can inhibit the ERK/Raf pathway would help to explain the mechanism important to the anti-proliferative effects of cilostazol; however, reports about the effects of cilostazol on the ERK/Raf pathway are lacking.

The phosphatidylinositol 3 kinase (PI3K) pathway may play an important role in the migration of VSMCs[11]. Considering that the PI3K pathway is critical for the proliferation and migration of cells, this pathway may play an important role in VSMC proliferation. Although activated Akt has been reported to induce the differentiation of VSMCs[12, 13], it has not yet been clearly demonstrated whether the PI3K pathway is involved in the proliferation of VSMCs and

whether cilostazol affects the PI3K pathway in VSMCs.

In the present study, we examined the anti-proliferative effects of cilostazol on fetal bovine serum (FBS)-induced abnormal proliferation of VSMCs. We also investigated the mechanisms of these effects, with special focus on the effects of cilostazol on the ERK and PI3K pathways.

Materials and Methods

Materials
Cilostazol ({6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro 2(1H)quinolinone}, CLZ), generously donated by Otsuka Pharmaceutical Co. Ltd., Osaka, Japan), was dissolved in dimethyl sulfoxide to make a 10 mM stock solution. H89 (-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; cat. B1427), a PKA inhibitor, was obtained from Sigma Aldrich Chemicals.

Cell Culture
Human aortic (HA) VSMCs (passage 3) (Cascade Corp.) were cultured in DMEM (Gibco BRL, low glucose) and supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. HAVSMC purity was determined by staining with smooth muscle-specific α-actin monoclonal antibodies (Sigma). VSMCs from the seventh passage were used in all experiments.

MTT Assay
3-(4, 5-dimethylthiazolyl-2)-2, t-diphenyl tetrazolium bromide (MTT) is taken up by the cell, and formazan is then formed by the action of mitochondrial succinate dehydrogenase. The accumulation of formazan directly reflects mitochondrial activity, which functions as an indirect measurement of cell viability. Cells were plated at a density of 5×10^3 cells/well in a 96-well plate, cultured for 48 h in culture medium supplemented with 10% FBS, and treated with the indicated concentration of cilostazol prior to the addition of 50 µL of 2 mg/mL MTT (Sigma) to the 200 µL of medium present in each well. An aliquot (220 µL) of the resulting solution was removed from each well, and 150 µL dimethyl sulfoxide (DMSO) was added. After the precipitate in each well was resuspended on a microplate mixer for 10 min, optical density (OD) at 570 nm was measured using an ELISA plate reader. All results were normalized to OD values measured from an identically conditioned well without cell culture[14]. To differentiate between the death and inhibition of proliferation of VSMCs by cilostazol, VSMCs that had not been treated with

10% FBS, meaning that proliferation was not induced, were treated only with several concentrations of cilostazol. Viability was subsequently evaluated using an MTT assay.

BrdU Incorporation Assay

HAVSMCs were plated at a density of 5×10^3 cells/well in a 96-well plate and cultured for 48 h in culture medium. Various concentrations of cilostazol were then added to the culture medium, and DNA synthesis was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation (cell proliferation colorimetric ELISA System; Roche). BrdU labeling solution (BrdU concentration: 10 μM) was added to the cells and incubated for 24 h. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of FixDenat (reagent for simultaneous fixation of cells and denaturation of DNA used in anti-BrdU-based ELISA applications; Roche). After 30-min incubation at room temperature, FixDenat was removed. Subsequently, peroxidase-labeled anti-BrdU monoclonal antibody was added, and the plate was incubated at room temperature for 90 min. BrdU-antibody complexes were detected by colorimetric reaction with the substrate, and optical density was read at 370/492 nm using a microplate ELISA reader.

Western Blot

Phosphorylated and non-phosphorylated forms of the enzymes Akt, GSK-3β, ERK, and c-Raf were analyzed by Western blotting. HAVSMCs (4×10^5) were cultured in media containing 10% FBS for 48 h and then were treated with various concentrations of cilostazol for 24 h with culture media. The cells were then washed twice in cold PBS and incubated for 30 min on ice in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 50 μL/mL aprotinin, 1% igepal 630, 100 mM NaF, 0.5% sodium deoxycholate, 0.5 mM EDTA, 0.1 mM EGTA]. Cell lysates were centrifuged at 12,000 ×g for 2 min at 4℃, and the supernatants were used to evaluate phosphorylated and non-phosphorylated forms of the enzymes Akt, GSK-3β, ERK, and c-Raf. Protein concentrations of the cell lysates were determined with a Bio-Rad protein assay kit. For Western blot analysis, 50 μg of the total protein from each sample was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 1% skim milk and incubated with specific antibodies for Akt (1: 1000; Cell Sig-

naling Technology), phospho-Akt (Ser473) (1: 1000; Cell Signaling), GSK-3β (1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-GSK-3β (Ser9) (1: 1000; Santa Cruz Biotechnology), ERK (1: 1000; Cell Signaling Technology), phospho-ERK (Thr202/Tyr204) (1: 500; Cell Signaling Technology), c-Raf (1: 1000; Cell Signaling Technology), and phospho-c-Raf (Ser338) (56A6) (1: 1000; Cell Signaling Technology). The membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST) and processed with an HRP-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The same membrane was reprobed to measure the ratio of phosphorylated protein to total protein. For example, membranes used in the evaluation of pAkt and pGSK-3β were reprobed to measure Akt and GSK-3β, respectively. The results of several Western blots were quantified on a Quantity One-4, 2,0 image analyzer (Bio-Rad, Hercules, CA) and were normalized to immunostained actin[14]. To investigate the importance of PKA in the inhibitory effect of cilostazol on the phosphorylation of Raf and ERK, VSMCs were treated simultaneously with 20 μM H89, a PKA inhibitor, and 100 μM cilostazol. Phosphorylation of Raf and ERK was then evaluated by Western blotting.

Statistical Analysis

All data are presented as the mean ± SEM from five or more independent experiments. Statistical comparisons of viabilities between different treatment groups were completed with Duncan's Multiple Range Test following one-way ANOVA. Statistical comparisons of results obtained from the evaluation of BrdU incorporation assay were performed among different treatment groups using Duncan's Multiple Range Test following two-way ANOVA. P-values less than 0.05 were considered significant.

Results

Anti-Proliferative Effects of Cilostazol on VSMCs

To evaluate the effects of cilostazol on VSMC proliferation, proliferating VSMCs were treated with various concentrations of cilostazol (0, 1, 10, 50, 100, or 200 μM) for 24 h, and their viability was then measured using an MTT assay. As shown in **Fig. 2**, cilostazol significantly decreased the viability of VSMCs treated with 10% FBS to induce proliferation in a dose-dependent manner ($p < 0.05$), but the viability of VSMCs that were not treated with 10% FBS, meaning that proliferation was not induced, was not affect-
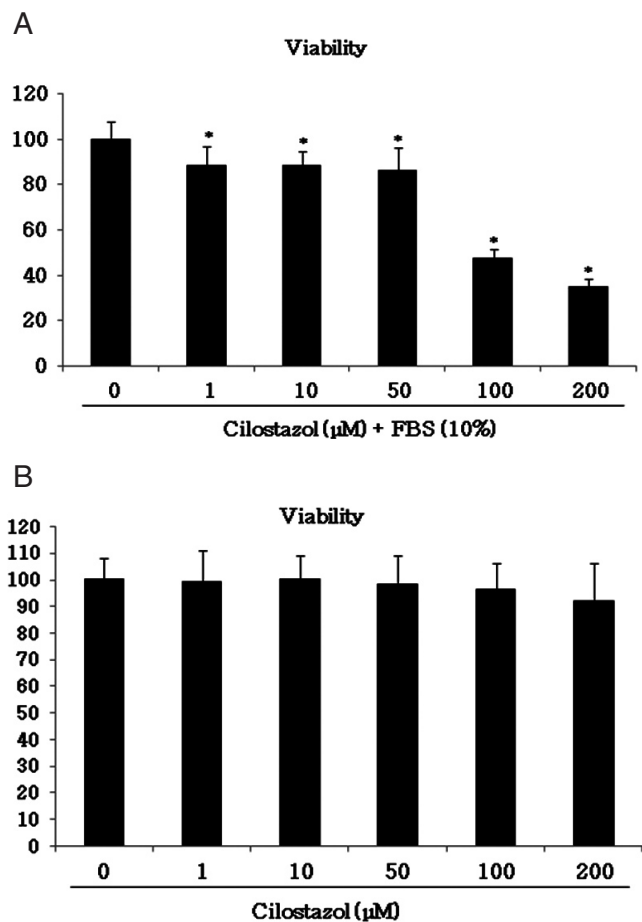
A

Viability

Cilostazol (μM) + FBS (10%)

B

Viability

Cilostazol (μM)

Fig. 2. Effect of cilostazol on the viability of proliferating VSMCs.

The data are represented as the mean (% of control) ± SEM. The treatment groups were compared using Duncan's Multiple Range Test following one-way ANOVA. (A) Viability of vascular smooth muscle cells (VSMCs) treated with 10% FBS to induce proliferation decreases with increasing cilostazol concentration (about 53.7 ± 6.3% at 100 μM cilostazol). *$p < 0.046$ (vs. control group). (B) Viability of VSMCs not treated with 10% FBS (proliferation was not induced) was not affected by treatment with cilostazol.

ed by treatment with cilostazol. These findings suggest that cilostazol inhibits the proliferation of VSMCs but does not induce the death of VSMCs. In addition, we determined whether cilostazol could affect the proliferation of VSMCs. The incorporation of BrdU was increased four-fold in FBS (10%)-stimulated VSMCs compared with unstimulated VSMCs; however, treatment of the cells with cilostazol (1, 10, 50, 100, or 200 μM) dose-dependently decreased the FBS-induced increase in the incorporation of BrdU ($p < 0.05$, **Fig. 3**).

Effects of Cilostazol on the ERK Pathway

To clarify whether ERK1/2, which are members of the MAPK family and are essential for VSMC proliferation, are influenced by cilostazol, we evaluated the expression of ERK1/2 and phosphorylated ERK1/2 depending on several conditions. When VSMCs were simultaneously treated with cilostazol and growth media for 24 h, cilostazol reduced the expression of phosphorylated ERK1/2 at Thr202/Tyr204 in a dose-dependent manner ($p < 0.05$, **Fig. 4A**).

Similarly, we confirmed that cilostazol decreased the activation of c-Raf, an immediate upstream activator of ERK1/2, in a dose-dependent manner (**Fig. 4B**). Consequently, our results suggest that the anti-proliferative mechanism of cilostazol involves inhibition of the ERK pathway in FBS-induced VSMC proliferation.

Effect of Cilostazol on the PI3K Pathway

To determine whether the anti-proliferative mechanism of cilostazol is related to inhibition of the PI3K pathway, we investigated whether treatment of cells with cilostazol affects the PI3K pathway. As shown in **Fig. 5A**, co-treatment with cilostazol and serum did not affect the level of phospho-Akt at Ser 473 in VSMCs. The intensity of phospho-Akt was normalized to that of Akt (**Fig. 5A**). Additionally, we ascertained that cilostazol had no effect on GSK-3β activity (**Fig. 5B**). This finding suggests that the anti-proliferative mechanism of cilostazol is not linked to inhibition of the PI3K pathway in VSMCs.

Importance of PKA in the Inhibitory Effect of Cilostazol on the ERK Pathway

In studies designed to investigate the importance of PKA in the inhibitory effect of cilostazol on the phosphorylation of Raf and ERK, 20 μM H89, a PKA inhibitor, was applied simultaneously with 100 μM cilostazol. Treatment with H89 significantly increased phophorylated ERK and Raf compared with VSMCs treated with only cilostazol (**Fig. 6**). This finding suggests that PKA plays a very important role in the inhibitory effect of cilostazol on the phosphorylation of Raf and ERK.

Discussion

The main purpose of the present study was to investigate the anti-proliferative mechanism of cilostazol in VSMCs, specifically as it relates to the ERK1/2 and PI3K pathways. In previous studies, it was confirmed that cyclic AMP (cAMP) and an adenylate cyclase activator, such as forskolin, which is also known as a cAMP stimulant, attenuated the apoptosis of endothelial cells and proliferation of VSMCs through various
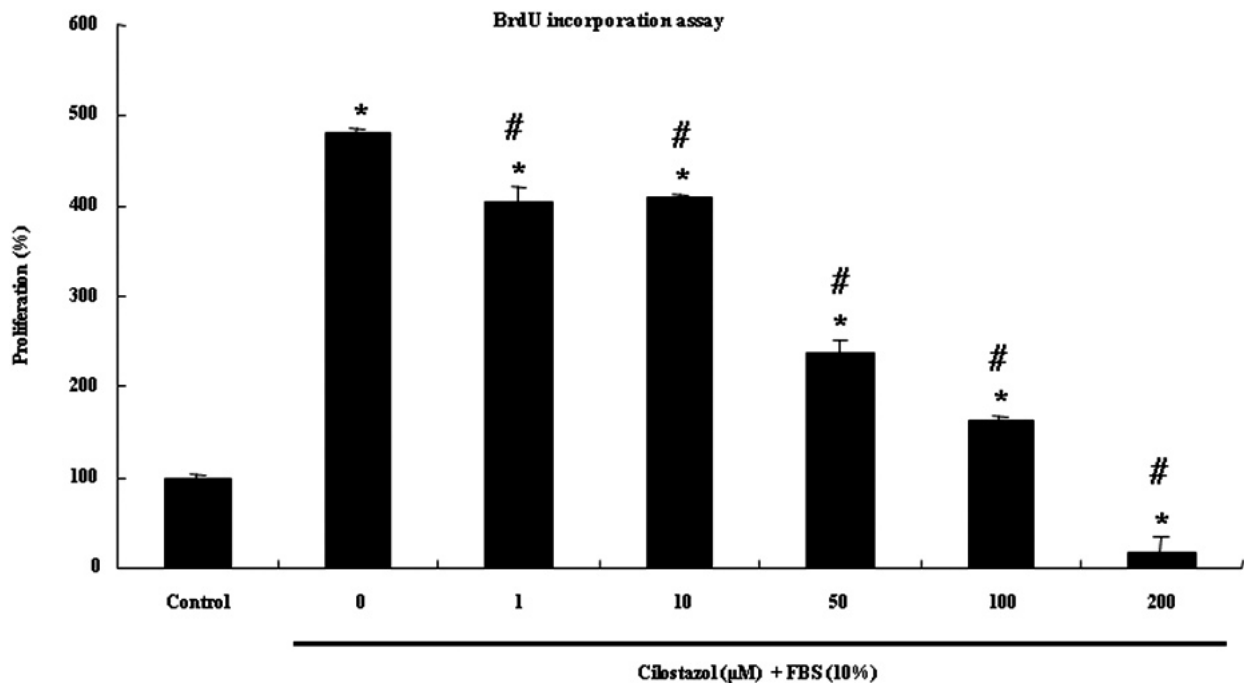
Fig. 3. Effects of cilostazol on VSMC proliferation.

The data are represented as the mean (% of control) ± SEM. Treatment groups were compared using Duncan's Multiple Range Test following two-way ANOVA. The amount of BrdU incorporation decreased in the group pretreated with cilostazol in a concentration-dependent manner compared with the group treated only with 10% FBS. *$p < 0.027$ (when the amount of BrdU-incorporated cells was compared with that of the control group); #$p < 0.023$ (when the amount of BrdU-incorporated cells was compared with that of VSMCs treated with 10% FBS).

mechanisms[5, 15, 16]. Recent studies have demonstrated that alterations of ERK, PI3K, and Akt are involved in the apoptotic process and dysfunction of endothelial cells, and that cilostazol reverses the dysfunction of endothelial cells through its influence on ERK, PI3K, and Akt[15]. In contrast, it has not yet been established whether cAMP is associated with VSMC proliferation. In the current study, we showed that increasing the intracellular cAMP level by treating cells with cilostazol[17, 18] inhibited FBS-induced VSMC proliferation in a dose-dependent manner (**Fig. 2**, **3**). Additionally, we found that the anti-proliferative effect of cilostazol was mediated by inhibition of the ERK pathway, whereas the PI3K pathway was not changed by cilostazol treatment (**Fig. 4**, **5**). Additionally, we found that PKA played a very important role in the inhibitory effect of cilostazol on the phosphorylation of Raf and ERK (**Fig. 6**).

Atherosclerosis types include spontaneous atherosclerosis, restenosis after percutaneous transluminal coronary angioplasty, autologous arterial or vein graft arteriosclerosis, and transplant arteriosclerosis. Atherosclerosis is regarded as a chronic inflammatory disease. The expression of adhesion molecules is one of the earliest events in atherosclerosis that plays a fundamental role in the pathogenesis of ischemic stroke. In all types of atherosclerosis, accumulation of VSMCs in the intima is a key event. A normal artery consists of quiescent arterial smooth muscle cells (SMCs) converted by a monolayer of endothelial cells that line the blood vessel. If the artery is injured by any kind of stress, the SMCs respond by proliferating and forming a neointima. The production of several growth factors, including PDGF-BB, TGF-β, EGF, and IGF-1, causes the proliferation of SMCs and is therefore a major event in the progression of atherosclerosis[19].

Cilostazol, a PDE3 inhibitor, is well known to increase intracellular cAMP levels and to decrease intracellular Ca^{2+} levels, inhibiting platelet aggregation and inducing vasodilatation. cAMP has been described as a mediator that participates in numerous physiological processes. A cMP is synthesized from ATP by adenylate cyclase and converted to 5'-AMP by PDE. Additionally, cAMP is one of the second messengers involved in intracellular signal transduction associated with various biological processes, including cell proliferation, differentiation, migration, and apoptosis. Many researchers have reported that cilostazol has an
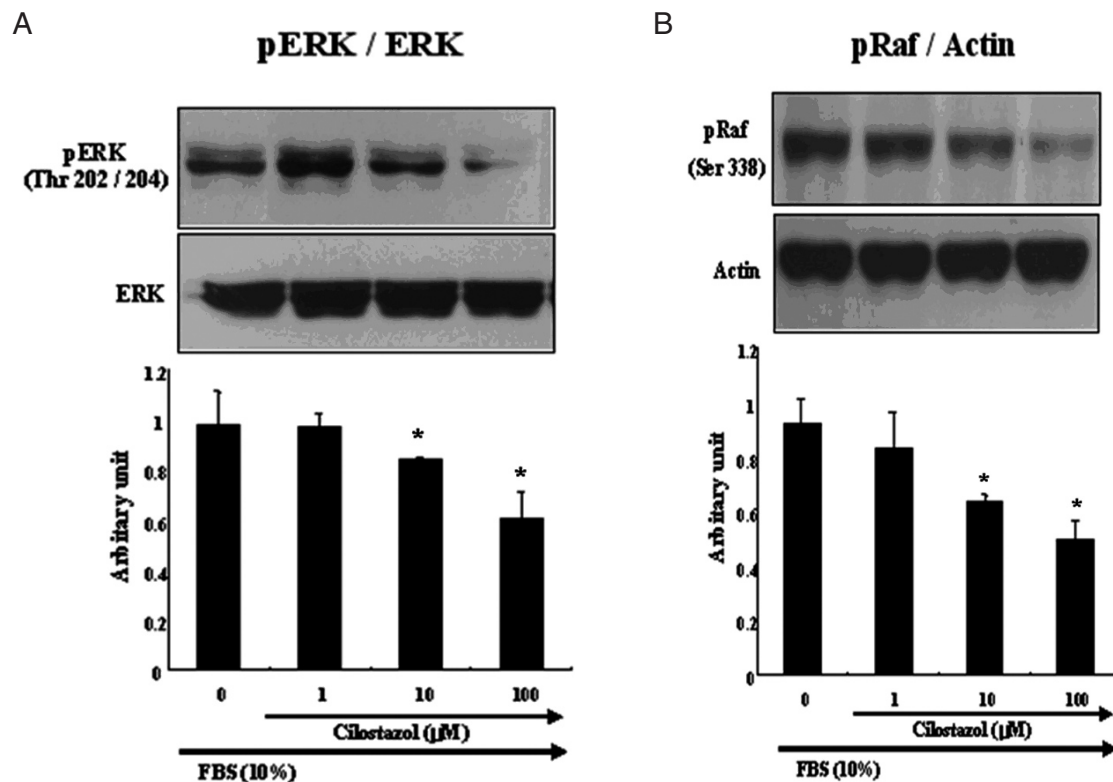
A

pERK / ERK

pERK
(Thr 202 / 204)

ERK

B

pRaf / Actin

pRaf
(Ser 338)

Actin

Fig. 4. Effects of cilostazol on the ERK pathway.

Immunoreactivities (IRs) were obtained by Western blotting (see Materials and Methods). Treatment groups were compared using Duncan's Multiple Range Test. Representative ECL radiographs of immunoblots demonstrate that treatment with cilostazol decreases the IR of phospho-ERK1/2 (Thr202/204) (A) and phospho-Raf (Ser338) (B) when compared with cells treated with 10% FBS. *$p < 0.039$ (compared to control group).

antiapoptotic effect on endothelial cells through cAMP/protein kinase A- and PI3K/Akt-dependent mechanisms[20]. In addition, cilostazol inhibits high glucose-mediated endothelial-neutrophil adhesion by decreasing adhesion molecule expression via NO production. It also prevents remnant lipoprotein particle-induced monocyte adhesion to endothelial cells by suppressing the expression of adhesion molecules and monocyte chemoattractant protein-1 via a lectin-like receptor for oxidized low-density lipoprotein receptor activation[21]. Furthermore, cilostazol suppresses superoxide production and the expression of adhesion molecules in human endothelial cells via the mediation of cAMP-dependent protein kinase-mediated Maxi-K channel activation[22, 23]. In summary, all of these findings show that cilostazol significantly reduces the apoptosis of endothelial cells. Nevertheless, the antiproliferative mechanism of cilostazol in VSMCs has not yet been clearly established.

VSMC proliferation and migration induced by various growth factors can develop in a variety of pathological processes, including atherosclerosis, hypertension, and restenosis after balloon angioplasty[24]. The inhibition of VSMC proliferation may be a potentially important therapeutic strategy for the treatment of atherosclerosis and restenosis[25]. The Raf/MEK/ERK signaling cascade, which can also be inhibited by protein kinase A[26-28], is the best-defined pathway involved in cell proliferation[29]. ERK plays a central role in this pathway[30] and is activated through phosphorylation by a single type of MEK1/2 (MAP kinase kinase), which is a dual-specific kinase[31]. ERK has been suggested to play a pivotal role in the control of VSMC proliferation[32]; moreover, specific inhibition of MAPK phosphorylation markedly inhibits VSMC growth *in vitro*[33]. The activation of ERK1/2 can increase the expression of downstream transcription genes, such as cyclin D1, resulting in protein synthesis and cell proliferation[34]. Therefore, confirmation that cilostazol inhibits the ERK pathway would allow us to explain an important portion of the antiproliferative mechanisms of cilostazol. In **Fig. 4** we showed that cilostazol significantly and efficiently reduced phosphorylated ERK (active form) and phos-
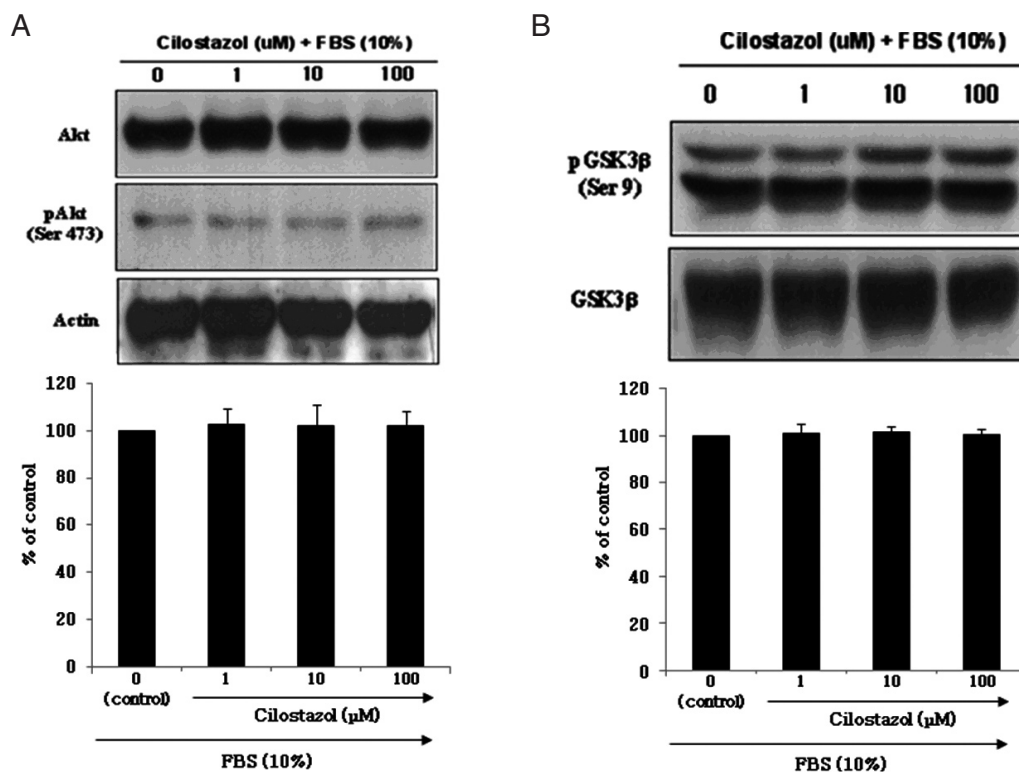
Fig. 5. Effects of cilostazol on the PI3K pathway.

Immunoreactivities (IRs) were obtained by Western blotting (see Materials and Methods). Treatment groups were compared using Duncan's Multiple Range Test. Representative ECL radiographs of immunoblots demonstrated that treatment with cilostazol did not affect the IR of phospho-Akt (Ser473) (A) and phospho-GSK-3β (Ser9) (B) compared with cells treated with 10% FBS.
There was no significant difference, so we did not add "*"

phorylated Raf (active form), suggesting that cilostazol effectively inhibits the ERK pathway. It is likely that this effect of cilostazol contributes to its anti-proliferative activity. These findings support the evidence that cAMP and cAMP stimulants, such as forskolin, suppress the phosphorylation of Raf and ERK[35-37], and that cilostazol increases cAMP[4]. We also found that the inhibitory effect of cilostazol on the phosphorylation of Raf and ERK significantly depends on PKA activity. This finding is supported by previous reports[38, 39].

To investigate whether suppression of the phosphorylation of ERK is associated with direct MEK inhibition induced by cilostazol or other mechanisms, VSMCs were treated simultaneously with 30 μM PD98059, a MEK inhibitor, and 100 μM cilostazol. Treatment with PD98059 seemed to slightly accentuate the effect of cilostazol that inhibits the phosphorylation of ERK, but the results were not statistically significant (data not shown); this result was not unexpected. Because cilostazol inhibited the phosphoryla-

tion of Raf, which is an upstream signal of MEK, and then inhibited MEK itself, the addition of an MEK inhibitor did not cause significant additional effects on the ERK pathway.

Our other interest in this study was the effect of cilostazol on the PI3K pathway in VSMCs. PI3K activates Akt (serine/threonine kinase) by generating specific inositol phospholipids, which recruit Akt to the cell membrane and enable Akt activation[40]. In its active state, Akt mediates cell survival and growth by phosphorylating and inactivating proapoptotic proteins; however, it has not yet been determined whether the PI3K pathway is affected by cilostazol. Therefore, we hypothesized that cilostazol could inhibit the PI3K pathway in VSMCs, and that this effect would also contribute to the anti-proliferative effect of cilostazol on VSMCs; however, we found that the PI3K pathway was not affected by cilostazol treatment, as shown in **Fig. 5**.

There were some limitations to the present study. First, the μM dose of cilostazol used in our experi-
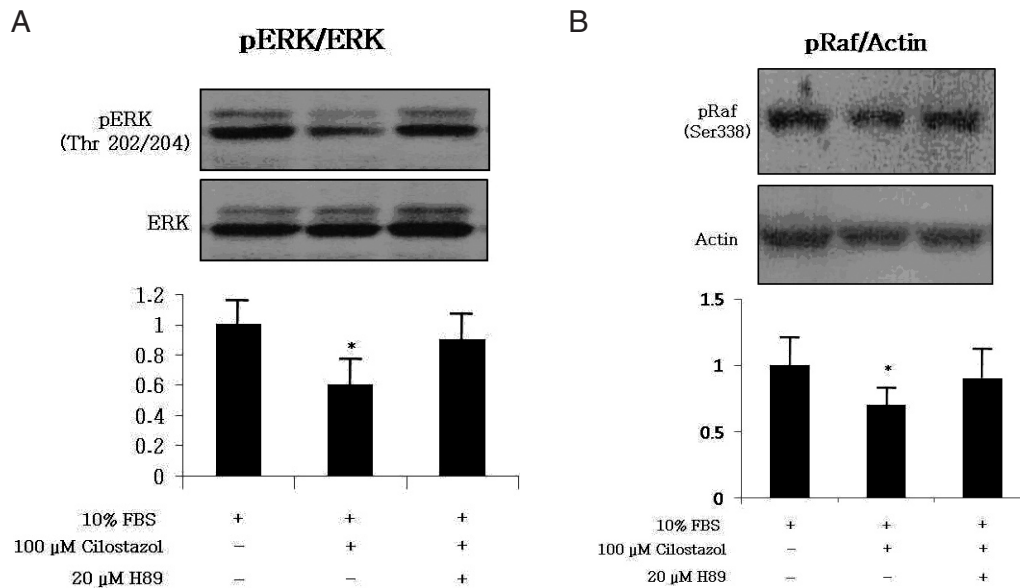
Fig. 6. Importance of PKA in the inhibitory effect of cilostazol on the ERK pathway.

H89, a PKA inhibitor, significantly increased the phosphorylation of ERK (A) and Raf (B) compared with VSMCs treated only with cilostazol. $*p < 0.014$ (compared to VSMCs treated with 10% FBS).

ments is not physiologically relevant *in vivo*. Because the present study was an *in vitro* study evaluating the anti-proliferative effects and mechanisms of cilostazol, long-term treatments with cilostazol at lower concentrations were not possible. As a result, cilostazol was used to treat cells at concentrations higher than would be physiologically possible, but for a relatively short duration. Higher concentrations were also chosen to facilitate the discovery of anti-proliferative properties and mechanisms. Accordingly, the major limitation of this study is that the biological significance of the findings was confined to an *in vitro* model and, accordingly, these data should be interpreted as an experimental model of the anti-proliferative properties of cilostazol.

Additionally, the effects of cilostazol on other signaling pathways involved in the proliferation of VSMCs, such as the p38 MAP kinase pathway, were not investigated because we were only interested in the effect of cilostazol on the signaling pathways investigated in the present study; however, it was reported that cilostazol inhibits the p38 MAP kinase pathway. This effect also plays an important role in the anti-proliferative action of cilostazol on VSMCs[41].

Finally, this study did not evaluate or confirm all of the precise mechanisms underlying the function of cilostazol. This information should be further investigated in future studies.

In conclusion, our findings suggest that the anti-proliferative mechanism of cilostazol is associated with inhibition of the ERK pathway but not the PI3K pathway. These data could provide a better understanding of the anti-proliferative effects of cilostazol, which are very important in preventing atherosclerosis and ischemic stroke.

Acknowledgements

References

1) Dzau VJ, Braun-Dullaeus RC, Sedding DG: Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med, 2002; 8: 1249-1256
2) Schwartz SM, Heimark RL, Majesky MW: Developmental mechanisms underlying pathology of arteries. Physiol Rev, 1990; 70: 1177-1209
3) Ishizaka N, Taguchi J, Kimura Y, Ikari Y, Aizawa T, Togo M, Miki K, Kurokawa K, Ohno M: Effects of a single local administration of cilostazol on neointimal formation in balloon-injured rat carotid artery. Atherosclerosis, 1999; 142: 41-46
4) Hidaka H, Hayashi H, Kohri H, Kimura Y, Hosokawa T, Igawa T, Saitoh Y: Selective inhibitor of platelet cyclic adenosine monophosphate phosphodiesterase, cilostamide, inhibits platelet aggregation. J Pharmacol Exp Ther, 1979; 211: 26-30
5) Kim MJ, Park KG, Lee KM, Kim HS, Kim SY, Kim CS,

Lee SL, Chang YC, Park JY, Lee KU, Lee IK: Cilostazol inhibits vascular smooth muscle cell growth by downregulation of the transcription factor E2F. Hypertension, 2005; 45: 552-556

6) Ito C, Kusano E, Akimoto T, Takeda S, Sasaki N, Umino T, Iimura O, Ando Y, Asano Y: Cilostazol enhances IL-1beta-induced NO production and apoptosis in rat vascular smooth muscle via PKA-dependent pathway. Cell Signal, 2002; 14: 625-632

7) Lee SY, Kang MJ, Cha JK: Cilostazol Reduces PAC-1 Expression on Platelets in Ischemic Stroke. J Clin Neurol, 2008; 4: 148-152

8) Robinson MJ, Cobb MH: Mitogen-activated protein kinase pathways. Curr Opin Cell Biol, 1997; 9: 180-186

9) Fisher M, Liu B, Glennon PE, Southgate KM, Sale EM, Sale GJ, Lewis MJ, Groves PH: Downregulation of the ERK 1 and 2 mitogen activated protein kinases using antisense oligonucleotides inhibits proliferation of porcine vascular smooth muscle cells. Atherosclerosis, 2001; 156: 289-295

10) Yang CM, Chien CS, Hsiao LD, Pan SL, Wang CC, Chiu CT, Lin CC: Mitogenic effect of oxidized low-density lipoprotein on vascular smooth muscle cells mediated by activation of Ras/Raf/MEK/MAPK pathway. Br J Pharmacol, 2001; 132: 1531-1541

11) Clemmons DR: Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer. Nat Rev Drug Discov, 2007; 6: 821-833

12) Hayashi K, Takahashi M, Kimura K, Nishida W, Saga H, Sobue K: Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells. J Cell Biol, 1999; 145: 727-740

13) Hayashi K, Shibata K, Morita T, Iwasaki K, Watanabe M, Sobue K: Insulin receptor substrate-1/SHP-2 interaction, a phenotype-dependent switching machinery of insulin-like growth factor-I signaling in vascular smooth muscle cells. J Biol Chem, 2004; 279: 40807-40818

14) Koh SH, Noh MY, Cho GW, Kim KS, Kim SH: Erythropoietin increases the motility of human bone marrow-multipotent stromal cells (hBM-MSCs) and enhances the production of neurotrophic factors from hBM-MSCs. Stem Cells Dev, 2009; 18: 411-421

15) Smith-Mungo L, Kagan HM: PKC-MEK-MAPK-dependent signal transduction pathway mediates the stimulation of lysyl oxidase expression by serum and PDGF in rat aortic smooth muscle cells. J Cell Biochem, 2002; 85: 775-784

16) Hayashi S, Morishita R, Matsushita H, Nakagami H, Taniyama Y, Nakamura T, Aoki M, Yamamoto K, Higaki J, Ogihara T: Cyclic AMP inhibited proliferation of human aortic vascular smooth muscle cells, accompanied by induction of p53 and p21. Hypertension, 2000; 35: 237-243

17) Mori D, Ishii H, Kojima C, Nitta N, Nakajima K, Yoshida M: Cilostazol inhibits monocytic cell adhesion to vascular endothelium via upregulation of cAMP. J Atheroscler Thromb, 2007; 14: 213-218

18) Zhao H, Quilley J, Montrose DC, Rajagopalan S, Guan Q, Smith CJ: Differential effects of phosphodiesterase PDE-3/PDE-4-specific inhibitors on vasoconstriction and cAMP-dependent vasorelaxation following balloon angioplasty. Am J Physiol Heart Circ Physiol, 2007, 292: H2973-2981

19) Qiao J, Huang F, Lum H: PKA inhibits RhoA activation: a protection mechanism against endothelial barrier dysfunction. Am J Physiol Lung Cell Mol Physiol, 2003; 284: L972-980

20) Fang Y, Olah ME: Cyclic AMP-dependent, protein kinase A-independent activation of extracellular signal-regulated kinase 1/2 following adenosine receptor stimulation in human umbilical vein endothelial cells: role of exchange protein activated by cAMP 1 (Epac1). J Pharmacol Exp Ther, 2007; 322: 1189-1200

21) Hong KW, Kim KY, Shin HK, Lee JH, Choi JM, Kwak YG, Kim CD, Lee WS, Rhim BY: Cilostazol prevents tumor necrosis factor-alpha-induced cell death by suppression of phosphatase and tensin homolog deleted from chromosome 10 phosphorylation and activation of Akt/cyclic AMP response element-binding protein phosphorylation. J Pharmacol Exp Ther, 2003; 306: 1182-1190

22) Park SY, Lee JH, Kim CD, Lee WS, Park WS, Han J, Kwak YG, Kim KY, Hong KW: Cilostazol suppresses superoxide production and expression of adhesion molecules in human endothelial cells via mediation of cAMP-dependent protein kinase-mediated maxi-K channel activation. J Pharmacol Exp Ther, 2006; 317: 1238-1245

23) Omi H, Okayama N, Shimizu M, Fukutomi T, Nakamura A, Imaeda K, Okouchi M, Itoh M: Cilostazol inhibits high glucose-mediated endothelial-neutrophil adhesion by decreasing adhesion molecule expression via NO production. Microvasc Res, 2004; 68: 119-125

24) Hashimoto A, Miyakoda G, Hirose Y, Mori T: Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. Atherosclerosis, 2006: 189: 350-357

25) Winkles JA, Gay CG: Serum, phorbol ester, and polypeptide mitogens increase class 1 and 2 heparin-binding (acidic and basic fibroblast) growth factor gene expression in human vascular smooth muscle cells. Cell Growth Differ, 1991; 2: 531-540

26) Dumaz N, Marais R: Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. J Biol Chem, 2003; 278: 29819-29823

27) Hafner S, Adler HS, Mischak H, Janosch P, Heidecker G, Wolfman A, Pippig S, Lohse M, Ueffing M, Kolch W: Mechanism of inhibition of Raf-1 by protein kinase A. Mol Cell Biol, 1994; 14: 6696-6703

28) Juliano RL, Reddig P, Alahari S, Edin M, Howe A, Aplin A: Integrin regulation of cell signalling and motility. Biochem Soc Trans, 2004; 32: 443-446

29) Ross R, Glomset JA: Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science, 1973; 180: 1332-1339

30) Roberts PJ, Der CJ: Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene, 2007; 26: 3291-3310

31) Lewis TS, Shapiro PS, Ahn NG: Signal transduction

through MAP kinase cascades. Adv Cancer Res, 1998; 74: 49-139

32) Lee JH, Johnson PR, Roth M, Hunt NH, Black JL: ERK activation and mitogenesis in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol, 2001; 280: L1019-1029

33) Koyama H, Olson NE, Dastvan FF, Reidy MA: Cell replication in the arterial wall: activation of signaling pathway following in vivo injury. Circ Res, 1998; 82: 713-721

34) Roovers K, Davey G, Zhu X, Bottazzi ME, Assoian RK: Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. Mol Biol Cell, 1999; 10: 3197-3204

35) Cospedal R, Lobo M, Zachary I: Differential regulation of extracellular signal-regulated protein kinases (ERKs) 1 and 2 by cAMP and dissociation of ERK inhibition from anti-mitogenic effects in rabbit vascular smooth muscle cells. Biochem J, 1999; 342 (Pt 2): 407-414

36) Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW: Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. Science, 1993; 262: 1065-1069

37) Cook SJ, McCormick F: Inhibition by cAMP of Ras-dependent activation of Raf. Science, 1993; 262: 1069-1072

38) Inada H, Shindo H, Tawata M, Onaya T: Cilostazol, a cyclic AMP phosphodiesterase inhibitor, stimulates nitric oxide production and sodium potassium adenosine triphosphatase activity in SH-SY5Y human neuroblastoma cells. Life Sci, 1999; 65: 1413-1422

39) Munoz B, Huerta M, Lopez-Bayghen E: Cilostazol reduces proliferation through c-Myc down-regulation in MDCK cells. Eur J Pharmacol, 2009; 616: 22-30

40) Lawlor MA, Alessi DR: PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J Cell Sci, 2001; 114: 2903-2910

41) Lee KM, Lee HJ, Kim MK, Kim HS, Jung GS, Hur SH, Kim HT, Cho WH, Kim JG, Kim BW, Lim JO, Choi HS, Lee KU, Park KG, Lee IK: Cilostazol inhibits high glucose- and angiotensin II-induced type 1 plasminogen activator inhibitor expression in artery wall and neointimal region after vascular injury. Atherosclerosis, 2009; 207: 391-398