Cilostazol Inhibits Vascular Smooth Muscle Cell Growth by Downregulation of the Transcription Factor E2F

Mi-Jung Kim, Keun-Gyu Park, Kyeong-Min Lee, Hye-Soon Kim, So-Yeon Kim, Chun-Soo Kim, Sang-Lak Lee, Young-Chae Chang, Joong-Yeol Park, Ki-Up Lee, In-Kyu Lee

Abstract—Neointimal formation, the leading cause of restenosis, is caused by proliferation of vascular smooth muscle cells (VSMCs). Patients with diabetes mellitus have higher restenosis rates after coronary angioplasty than nondiabetic patients. Cilostazol, a selective type 3 phosphodiesterase inhibitor, is currently used to treat patients with diabetic vascular complications. Cilostazol is a potent antiplatelet agent that inhibits VSMC proliferation. In the present study, we examine whether the antiproliferative effect of cilostazol on VSMCs is mediated by inhibition of an important cell cycle transcription factor, E2F. Cilostazol inhibited the proliferation of human VSMCs in response to high glucose in vitro and virtually abolished neointimal formation in rats subjected to carotid artery injury in vivo. Moreover, the compound suppressed high-glucose–induced E2F–DNA binding activity, and the expression of E2F1, E2F2, cyclin A, and PCNA proteins. These data suggest that the beneficial effects of cilostazol on high-glucose–stimulated proliferation of VSMCs are mediated by the downregulation of E2F activity and expression of its downstream target genes, including E2F1, E2F2, cyclin A, and PCNA. (Hypertension. 2005;45:552-556.)

Key Words: diabetes mellitus ■ hyperplasia ■ vascular smooth muscle cells

Neointimal hyperplasia and restenosis are major problems limiting the long-term efficacy of percutaneous transluminal coronary angioplasty. Common events in vascular response to arterial injury include proliferation and migration of vascular smooth muscle cells (VSMCs) within arterial intimal and neointimal formation in injured vessels. Patients with diabetes mellitus have higher restenosis rates after coronary angioplasty than nondiabetic patients. The transcription factor, E2F, has been implicated in the periodic regulation of cellular genes required for transition through G1 and entry into the S phase, including dihydrofolate reductase, c-myc, DNA polymerase, cdc2, and proliferating cell nuclear antigen (PCNA). E2F activity is regulated by interactions with RB family members. As cells progress toward S phase, RB family proteins are phosphorylated by G1 cyclin-complexes, resulting in the release of transcriptionally active E2F, which then leads to the activation of genes required for cell cycle progression. We recently showed that high glucose activates the DNA-binding activity of E2F, and decoy oligodeoxynucleotides against E2F inhibit the proliferation of VSMCs. These data suggest that downregulation of E2F could constitute a therapeutic target to prevent restenosis after angioplasty in patients with diabetes.

Cilostazol increases intracellular cAMP concentrations by selectively blocking phosphodiesterase type III. The clinical implications and pharmacokinetics with respect to the effects and safety of this drug have been well-established, especially in peripheral vascular disease. Cilostazol is a potent antiplatelet agent currently used in clinical practice to treat patients with diabetic vascular complications. Several lines of evidence indicate that cilostazol additionally inhibits the proliferation of VSMCs, reduces neointimal formation in balloon-injured rat carotid arteries, and inhibits restenosis after percutaneous transluminal coronary angioplasty. One mechanism by which cilostazol may inhibit VSMC proliferation is via an increase in intracellular cAMP, because cAMP inhibits the proliferation of VSMCs by induction of p53-mediated and p21-mediated apoptosis. However, Nadiri et al demonstrated that increased cAMP leads to inhibition of phosphorylation of pRB, which regulates the activity of the E2F family, and consequently leads to arrest of cells at G1 in human lymphocytes. Data from this investigation suggest another mechanism by which cilostazol inhibits VSMC proliferation, specifically through the suppression of E2F-mediated transcription. In the present study, we examine whether the antiproliferative effect of cilostazol on VSMCs is mediated by inhibition of E2F, which is regulated by pRB phosphorylation.
Materials and Methods

Animals
Nine- to 10-week-old male Sprague-Dawley rats (Hyochang, Daegu, Korea) weighing 280 to 320 grams were used in the experiments. All procedures were used in accordance with the institutional guidelines for animal research.

Rat Carotid Artery Balloon Injury
We examined the possible beneficial effects of cilostazol on neointimal formation by using a rat carotid artery balloon-injury model. Cilostazol (100, 30, and 10 mg/d per kg) was administered daily by gavage, and balloon injury was performed on day 4. Rats were anesthetized with 50 mg/kg of sodium pentobarbital (Entobar; Hanlim Pharmaceutical, Yong-In, Korea). Cilostazol treatment was continued until rats were euthanized at 2 weeks after balloon injury.

Cell Culture
Human VSMCs (HVSMCs) were isolated from the thoracic aorta of kidney transplantation donors by the explant method as described previously. Tissue collection was approved by the local Ethics Committee. HVSMCs were cultured in DMEM (Gibco BRL, Grand Island, NY) containing 20% fetal bovine serum (Gibco BRL). The indicated dose of cilostazol was added to the wells, and cells were incubated at 37°C for a further 5 hours. After 48 hours, indices of cell proliferation were determined with a WST cell counting kit (Wako, Osaka, Japan).

Growth Assay
HVSMCs were seeded into 96-well tissue culture plates. At 30% confluence, cells were rendered quiescent by incubation for 24 hours in defined serum-free medium. Cilostazol was donated by Otsuka Pharmaceutical Co (Osaka, Japan). Cilostazol treatment was administered daily by gavage, and balloon injury was performed on day 4. Rats were anesthetized with 50 mg/kg of sodium pentobarbital (Entobar; Hanlim Pharmaceutical, Yong-In, Korea). Cilostazol treatment was continued until rats were euthanized at 2 weeks after balloon injury.

Electrophoretic Mobility Shift Assay
Nuclear extracts were prepared from HVSMCs, as described previously. Briefly, the DNA probe for E2F was labeled with [γ-32P]ATP and T4 polynucleotide kinase. After end-labeling, the probe was purified with a NAP-5 column. Protein–DNA binding was visualized by autoradiography.

Histological Analysis
Immunohistochemistry and morphological analysis of neointima were performed as described previously.

Western Blot Analysis
Total protein extraction, Western blotting, and densitometric measurement of bands were performed as described previously.

Statistical Analysis
All results are expressed as means ± SEM. Analysis of variance was performed with Duncan test and used to determine significant differences in multiple comparisons. Values of P < 0.05 were taken as statistically significant. All experiments were performed at least 3 times.

Results

Effect of Cilostazol on Neointimal Formation in Balloon-Injured Rat Carotid Arteries
As shown in Figure 1, neointimal formation was detected in the vessels 2 weeks after injury. Treatment with cilostazol reduced neointimal formation in a dose-dependent manner.

Effects of Cilostazol and cAMP Stimulants on Inhibition of HVSMC Growth In Vitro
As expected, high glucose (22 mmol/L D-glucose) significantly increased the proliferation of cultured HVSMCs, compared with normal glucose (P < 0.05). Cilostazol (100 μmol/L, 10 μmol/L, and 1 μmol/L) inhibited this stimulation in a dose-dependent manner (P < 0.01, P < 0.05, and P < 0.05, respectively). Similarly, cAMP stimulants, such as forskolin (100 μmol/L) and 8-bromo-cAMP (3 mmol/L), also suppressed HVSMC growth stimulated by high glucose (P < 0.01) (Figure 2).
Effect of Cilostazol on the DNA-Binding Activity of E2F
In view of the finding that cilostazol inhibits the proliferation of HVSMCs and neointimal formation, we examined its effect on the cell cycle regulatory transcription factor, E2F. The increase in E2F–DNA binding activity under high glucose conditions was attenuated by cilostazol at a dose of 10 μmol/L (∗∗∗P<0.001 compared with NG; ∗P<0.05 compared with HG).

Effects of Cilostazol and cAMP Stimulants on Promoter Activity in HVSMCs
To further confirm the inhibition of E2F–DNA binding activity by cilostazol, we used E2F-responsive promoter constructs containing 4 E2F binding sites in the promoter. High glucose markedly stimulated E2F responsive promoter activity, compared with normal glucose (∗∗P<0.01). This increased luciferase activity was attenuated by cilostazol in a dose-dependent manner (100 μmol/L, 10 μmol/L, and 1 μmol/L) (∗∗∗P<0.001, ∗∗P<0.01, and ∗P<0.05, respectively). Similarly, forskolin (100 μmol/L) and 8-bromo-cAMP (3 mmol/L) inhibited luciferase gene expression (∗P<0.01) (Figure 3B).

Effects of Cilostazol and cAMP Stimulants on Expression of E2F and PCNA Proteins
We next examined the expression patterns of E2F downstream genes, such as cyclin A and PCNA. Treatment with high glucose increased the expression of E2F1, E2F2, cyclin A, and PCNA proteins. This glucose-induced stimulation of protein expression was inhibited by cilostazol and cAMP stimulants, such as forskolin and 8-bromo-cAMP (Figure 4).

To demonstrate the inhibitory effect of cilostazol on E2F downstream gene expression in vivo, we stained the neointimal region with PCNA. A marked increase in PCNA-positive cells in the neointimal region and dividing endothelial cells were noted 2 weeks after injury. The number of PCNA-positive cells in vessels treated with cilostazol was significantly lower than that in untreated vessels (Figure 5).

Discussion
Patients with diabetes mellitus have higher restenosis rates after coronary angioplasty than nondiabetic patients. Hyperglycemia is believed to play a pivotal role in this vascular response, the major pathogenesis being the proliferation of VSMCs. Multiple mechanisms involving protein kinase C and NF-κB have been implicated in the genesis of high-glucose–induced VSMC proliferation. Over the past decade, the transcription factor E2F has emerged as a key component of cellular proliferation, during which it controls the expression of genes required for cell cycle progression, especially in high-glucose–stimulated VSMCs. However, only a few investigators have examined the effects of pharmacological agents on E2F activity and VSMC proliferation. Here, we show that cilostazol effectively reduces high-glucose–stimulated E2F activity, as well as proliferation of HVSMCs in vitro and in vivo. In agreement with...
our previous data, high glucose increases E2F–DNA binding and luciferase activity of the E2F/H11003-luciferase construct, consequently stimulating VSMC proliferation. These results provide a theoretical background for the use of cilostazol in the treatment of vascular disease in diabetic patients.

In mammals, 6 members of the E2F family have been identified (E2F1 to E2F6), of which transcriptionally active E2F proteins are structurally and functionally divided into 2 groups. Expression of E2F1 to E2F3 is low in quiescent cells, and increases during growth stimulation, whereas E2F4 and E2F5 accumulate in quiescent cells or during differentiation. Several studies show that E2F proteins stimulate their own activity directly through the presence of binding sites in the promoters (E2F1 and E2F2), and E2F downstream transcription is mediated by the synthesis of new E2F1 and E2F2. Moreover, a previous study by our group demonstrated that high glucose increased the expression of endogenous targets of E2F, cyclin A, and PCNA. This expression was successfully inhibited by E2F decoy oligodeoxynucleotides. Accordingly, we investigated whether cilostazol could attenuate the increased expression of E2F1, E2F2, cyclin A, and PCNA mediated by high-glucose–stimulated E2F–DNA binding activity. Our data establish that cilostazol inhibits the high-glucose–stimulated increase in E2F1 and E2F2 proteins and their endogenous downstream targets, cyclin A, and PCNA. This study additionally shows that cilostazol attenuates the increase in PCNA-positive cells in the neointimal region and completely abolishes neointimal formation induced by balloon injury.

Recently, the mechanisms by which cAMP suppresses the proliferation and migration of VSMCs and prevents postangioplasty neointimal formation have been investigated. Newman et al reported that cAMP inhibits the production of IL-6 and migration of HVSMCs. Andolfi et al showed that the activation of cAMP-dependent protein kinase inhibits neointimal formation after vascular injury in a model of restenosis after angioplasty. Hayashi et al reported that cAMP directly inhibits abnormal VSMC growth and induces the expression of the anti-oncogenes, p53 and p21, and apoptosis. In addition, results from the present study strongly suggest that the inhibitory effect of cAMP on VSMC proliferation is mediated by downregulation of E2F activity.
Perspectives

The present study has clearly demonstrated that phosphodiesterase type 3 inhibitor, cilostazol, effectively reduces high-glucose–stimulated E2F activity and proliferation of HVSMDs in vitro and in vivo. The data presented herein are consistent with other reports showing a role for cAMP in VSMCs proliferation and neointimal formation. Taken together, these studies suggest that these agents increase cAMP in the vasculature, which thereby helps to prevent the development of restenosis after percutaneous transluminal coronary angioplasty, especially in patients with diabetes.

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