Cilostazol Promotes Vascular Smooth Muscles Cell Differentiation Through the cAMP Response Element-Binding Protein-Dependent Pathway

Wei-Jan Chen, Ying-Hwa Chen, Kwang-Huei Lin, Chiao Hsuan Ting, Yung-Hsin Yeh

Objective—Cilostazol, a potent type 3 phosphodiesterase inhibitor, has recently been found to reduce neointimal formation by inhibiting vascular smooth muscle cell (VSMC) proliferation. The aim of this study is to investigate whether cilostazol exerts an action on phenotypic modulation of VSMCs, another important process in the pathogenesis of neointimal formation.

Methods and Results—Cilostazol may convert VSMCs from a serum-induced dedifferentiation state to a differentiated state, as indicated by a spindle-shaped morphology and an increase in the expression of smooth muscle cell differentiation marker contractile proteins. The upregulation of contractile proteins by cilostazol involves the cAMP/protein kinase A (PKA) signaling pathway, because the cAMP analog mimicked and specific cAMP/PKA inhibitors opposed the effect of cilostazol. Furthermore, cilostazol-activated cAMP response element (CRE)-binding protein (CREB), including phosphorylation at Ser133 and its nuclear translocation. Deletion and mutational analysis of the contractile protein promoters along with chromatin immunoprecipitation using anti-CREB antibody showed that CRE is essential for cilostazol-induced contractile protein expression. Transfection of dominant-negative CREB (mutated Ser133) plasmid in VSMCs blocked cilostazol-stimulated contractile protein expression. In vivo, cilostazol upregulated contractile proteins and induced the activation of CREB in the neointima of balloon-injured arteries.

Conclusion—Cilostazol promotes VSMC differentiation through the cAMP/PKA/CREB signaling cascade. (Arterioscler Thromb Vasc Biol. 2011;31:2106-2113.)

Key Words: CREB ■ cilostazol ■ differentiation ■ neointimal formation ■ vascular smooth muscle cells

Phenotypic modulation of vascular smooth muscle cells (VSMCs) from a differentiated (contractile) to a dedifferentiated (synthetic, noncontractile) state plays a crucial role in the pathogenesis of neointimal formation and restenosis after angioplasty. In the neointima induced by balloon injury, VSMCs can perform noncontractile functions, including proliferation, migration, and elaboration, as well as degradation of extracellular matrix proteins. Proliferating VSMCs within the neointima express low levels of contractile proteins, such as smooth muscle α-actin (SM-α-actin), calponin, and smooth muscle myosin heavy chain (SM-MHC). The upregulation of these contractile proteins is considered to be characteristics of differentiated VSMCs. A similar change may occur when VSMCs are grown in conventional culture conditions. VSMCs, isolated from adult rat aorta and grown in the presence of serum, may undergo a rapid structural transition from a differentiated to a dedifferentiated state. Serum in the culture medium has been identified as a major contributor to induce VSMC dedifferentiation. Therefore, this study utilized a serum deprivation and restoration culture model to mimic phenotypic reversion in the neointima of balloon-injured arteries.

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Cilostazol is known as a selective type 3 phosphodiesterase (PDE3) inhibitor to increase intracellular cAMP concentrations. The increased cAMP levels in platelets contribute to the inhibitory effect of cilostazol on platelet aggregation and thrombosis, providing its clinical implication in treating peripheral vascular disease. Furthermore, some clinical and experimental studies have been directed toward its action in reducing neointimal formation in balloon-injured rat carotid arteries and restenosis after percutaneous transluminal coronary angioplasty by inhibiting VSMC proliferation. The mechanism by which cilostazol inhibits the proliferation of VSMCs is attributed to an increase in the intracellular cAMP levels. Because phenotypic modulation in VSMCs...
appears to be another critical process in the development of neointimal formation and restenosis,1 we hypothesize that cilostazol may have an effect on phenotypic modulation of VSMCs.

The aim of this study is, therefore, to determine whether cilostazol exerts an action on modulating VSMC differentiation, focusing on the expression of contractile proteins. The underlying signal transduction pathway responsible for the modulation was also investigated.

Materials and Methods

Cell Culture

Rat VSMCs were prepared by enzymatic digestion of the thoracic aortic media from adult Sprague-Dawley rats and cultured in Dulbecco’s modified Eagle’s medium and supplemented with 10% fetal bovine serum as described.7 Cells used in all experiments were between the fourth and seventh passage and were cultured on conventional uncoated dishes. Most chemicals were purchased from Sigma (St. Louis, MO). Cilostazol (kindly provided by Otsuka Pharmaceutical Co Ltd, Tokushima, Japan) was dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide in the culture medium was less than 0.1%, which had no effect on VSMC morphological changes.

Immunohisto- and Cytotoxic Chemical Analyses

Immunohisto- and cytotoxic chemical analyses were performed using specific antibodies (Dako, Carpinteria, CA), anti-SM-MHC (SM2, Seikagaku, Tokyo, Japan), antiphospho cAMP response element (CRE)-binding protein (p-CREB) (Cell Signaling, Beverly, MA), and antifos (Abcam, Cambridge, MA) antibodies as primary antibodies followed by FITC (green color) or Cy3 (red color, Chemicon, Temecula, CA)-conjugated secondary antibody and visualized by confocal immunofluorescent microscopy. Nuclei were visualized by 4,6-diamidino-2-phenylindole staining.

Western Blot Analysis

For Western blotting, immunoblotting was performed using anti-SM-a-actin, antitubulin (Santa Cruz, Delaware Ave, CA), anticalponin (Dako), anti-SM-MHC (SM2, Seikagaku), antiphospho-CREB, anti-CREB, antiphospho mitogen-activated protein kinases (MAPK), and anti-MAPK (Cell Signaling) antibodies as primary antibodies. Signals were detected using the enhanced chemiluminescence-detection method (Amersham, the Netherlands) and quantified by densitometry. The amount of chosen protein was expressed relative to tubulin.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was extracted using TRIzol reagent (Life Technologies, Rockville, MD) and real-time quantitative reverse transcription-polymerase chain reaction was performed as described previously.7 Oligonucleotide sequences are shown in the Supplemental Table, available online at http://atvb.ahajournals.org. GAPDH mRNA was used as the internal control.

Transient Transfection and Luciferase Assays

The transcriptional regulation of contractile protein genes was assessed with luciferase reporter genes under the control of rat SM-MHC (nucleotides −4200 to +11600) and rat calponin (−481 to +90) promoters within the pGL3-basic vector as described previously.7,14 The putative CREs located at the promoter regions of the calponin (TGAGATCA, −224 to −223) and SM-MHC (TGACCTCA, −153 to −146) genes were changed to AAGCTTCA and TCTGACCTCCTTC-3' and 5'-TGAGGTGCTGTATGAAACCC-3', respectively. The transcriptional activity was measured with a luminometer (Luminoskan TL FMS, Thermo Labsystems) and normalized by cellular protein concentrations.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed as described elsewhere.15 Briefly, serum-deprived VSMCs were treated with or without cilostazol (100 μmol/L) for 2 to 24 hours, cross-linked with 1% formaldehyde and sonicated. Soluble chromatin was then incubated with anti-CREBP-CREB, anti-CREB (Cell Signaling), anti-CREB-binding protein (CBP) antibody (Santa Cruz), or rabbit nonspecific IgG as a negative control. Fragments of the calponin and SM-MHC promoters were amplified using primer pairs (5'-AGGTGCTGTATGAAACCC-3' and 5'-TGAGGTTCTGACCTCCTTC-3') and 5'-GCCCTACCTGATCTGAAACCC-3', respectively.

Cell Proliferation Assay

The proliferative activities of VSMCs were determined by 5-bromo-2-deoxyuridine incorporation using an ELISA detecting kit (Roche, Mannheim, Germany) following the manufacturer’s instructions.

Small Interfering RNA

Chemically synthetic small interfering RNA (siRNA) for extracellular signal-regulated kinase 1 (ERK1) and its control siRNA were purchased from Dharmacon (Lafayette, CO) and transferred into VSMCs using DharmaFECT 1 (Dharmacon) according to the manufacturer’s instructions.

Rat Model of Balloon Injury

The animal study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. Adult male Wistar rats weighting 350 g to 400 g were anesthetized with an intraperitoneal injection of ketamine 100 mg/kg and xylazine 5 mg/kg. Angioplasty of the left external carotid artery was performed using an inflated 2F Fogarty embolectomy catheter. Cilostazol (100 mg/d/kg) was administered daily by gavage, and balloon injury was performed on day 4. Cilostazol treatment was continued until rats were euthanized at 14 days after balloon injury. The uninjured and injured carotid arteries were removed, fixed with 4% paraformaldehyde, and paraffin-embedded for hematoxylin-eosin staining or immunohistochemical analysis.

Statistical Analysis

Mean and SE were used to describe the data. Differences between 2 groups were determined by unpaired t-test. For multiple groups, one-way ANOVA with post-hoc Scheffe test was used to compare data among groups. Correlations between variables were calculated by Pearson coefficient. A value of P<0.05 was considered to be statistically significant.

Results

Morphological Changes Induced by Cilostazol in VSMCs

Adult rat VSMCs (70% to 80% confluence) cultured in 10% fetal bovine serum-containing medium rapidly dedifferentiate to a synthetic phenotype, characterized by a flattened morphology (Figure 1). After the addition of cilostazol to fetal bovine serum-containing medium, VSMCs exhibited a change to elongated and spindle-shaped cells (Figure 1). These changes induced by cilostazol were evident 8 hours after the addition of cilostazol and were completed by 24 hours.
Effect of Cilostazol on Contractile Protein Expression

To explore whether cilostazol induces the differentiated phenotype, the effect of cilostazol on the expression of contractile proteins (SM-α-actin, calponin, and SM-MHC) is examined. Western blot analysis showed that the expression of calponin and SM-MHC increased in the serum-deprived condition compared with cells cultured with serum only, which is consistent with previous reports showing that VSMCs in the serum-deprived media may display their differentiated characters (Figure 2A). Administration of cilostazol to serum-treated VSMCs induced the expression of calponin and SM-MHC in both dose- and time-dependent manners (Figure 2A and B). However, these changes could not be found in the expression of SM-α-actin, possibly because of its high expression constitutively (Figure 2A).1 The effect of cilostazol on contractile protein (SM-α-actin, calponin, and SM-MHC) mRNA expression in real-time reverse transcription-polymerase chain reaction was similar to that on protein expression (Figure 2C). Furthermore, treatment of VSMCs with cilostazol increased calponin and SM-MHC promoter activities in a dose-dependent manner (Figure 2D). Taken together, these data document that...
Cilostazol may modulate VSMCs from the serum-induced dedifferentiated state to the differentiated state as indicated by increased contractile protein expression.

Involvement of the cAMP/PKA Pathway in Cilostazol-Induced Contractile Protein Expression

Because the effect of cilostazol is typically achieved via the cAMP/PKA pathway, this study further investigates whether cilostazol induces VSMC differentiation through a cAMP/PKA-dependent mechanism. Treating VSMCs with the cAMP analog (8-bromo-cAMP) showed a dose-dependent increase in calponin expression, which was comparable with that found in cilostazol-treated cells (Supplemental Figure I, available online at http://atvb.ahajournals.org). Furthermore, specific cAMP/PKA inhibitors (Rp-cAMP and PAKi) blocked the effect of cilostazol on calponin expression (Supplemental Figure I). These data suggest that the cAMP/PKA pathway is associated with the promoting effect of cilostazol on VSMC differentiation.

Cilostazol-Induced CREB Activation

As its name indicates, CREB may be the downstream target of the cAMP/PKA pathway. Phosphorylation at the Ser133 region is necessary for the activation of CREB, whereby it regulates a number of genes that have the CRE sites in their promoter regions. Therefore, the next experiments are designed to determine whether cilostazol induces contractile protein expression via CREB activation. Cilostazol treatment produced a dose-dependent increase in the level of p-CREB without affecting total CREB level (Figure 3A). Time-dependent experiment showed that the maximal effect occurred at 6 hours, preceding the changes of contractile proteins (Figure 3B). As expected, the effect of 8-bromo-cAMP on CREB activation mimicked that of cilostazol, and specific cAMP/PKA inhibitors prevented CREB from activation by cilostazol (Supplemental Figure II, available online at http://atvb.ahajournals.org).

Immunocytochemistry was used to detect the nuclear translocation of p-CREB, another marker of CREB activation. Treatment of VSMCs with cilostazol and 8-bromo-cAMP for 30 minutes resulted in marked translocation of p-CREB into the nucleus compared with that of control cells (Figure 3C). As expected, specific cAMP/PKA inhibitors eliminated the nuclear translocation of p-CREB in response to cilostazol (Figure 3C). The cGMP analog (8-bromo-cGMP) and specific cGMP/PKG inhibitors did not alter basal and cilostazol-induced nuclear translocation of p-CREB, suggesting that the cGMP/PKG pathway is not involved in this process (Supplemental Figure III). Furthermore, the PDE4 inhibitor (rolipram) failed to stimulate the nuclear translocation of p-CREB, providing further evidence that this effect is PDE3/cAMP/PKA-dependent (Supplemental Figure III).

Cilostazol-Induced Contractile Protein Expression via CREB Activation

This study utilized a wild-type CREB plasmid to increase CREB level and inhibited CREB activation using a dominant-negative CREB plasmid in which serine 133 was changed to alanine (CREB-S133A). Western blot analysis showed that transfection of wild-type CREB in VSMCs augmented basal and cilostazol-induced calponin expression (Figure 4A). In contrast, cilostazol failed to upregulate calponin in CREB-S133A-expressing cells (Figure 4A). The transfection efficiency of CREB plasmids into VSMCs was confirmed by the
expression of GFP-CREB fusion proteins and/or an increase of total-form CREB expression in these cells (Figure 4A).

In cotransfection experiments, VSMCs were cotransfected with plasmids containing contractile protein (calponin and SM-MHC) promoter regions and CREB plasmids. The up-regulation of calponin and SM-MHC promoter activities in cilostazol-treated cells was unaffected, or even augmented, by cotransfection of wild-type CREB (Figure 4B). However, cotransfection of dominant-negative CREB in VSMCs blocked cilostazol-stimulated calponin and SM-MHC promoter activities (Figure 4B).

Using the computer program, only 1 putative CRE can be identified in their respective promoter regions of calponin and SM-MHC genes. In the promoter deletion constructs, cilostazol-increased transcriptional activity was only evident when the promoter constructs contained the CRE. Further, mutation of the CRE abolished the cilostazol effect on increasing transcriptional activities (Figure 5A). Taken together, these data demonstrate that CREB and CRE are essential for the regulation of contractile protein expression by cilostazol.

As is known, CREB constitutively binds to the CRE in the promoter region of responsive gene, and Ser133 phosphorylation promotes CREB activity via recruitment of the CBP.16,17 Chromatin immunoprecipitation assay revealed that the occupation of total-form CREB in the calponin and SM-MHC promoters was...
Role of CREB in Cilostazol-Inhibited VSMC Proliferation

The next experiment is designed to evaluate whether the effect of cilostazol on inhibiting VSMC proliferation is also mediated via CREB. In agreement with its effect on VSMC differentiation, transfection of wild-type CREB augmented and dominant-negative CREB attenuated the inhibitory effect of cilostazol on VSMC proliferation (Supplemental Figure IV).

Role of MAPKs in Cilostazol-Induced CREB Activation and Contractile Protein Expression

Because MAPKs are reported to phosphorylate and activate CREB, this study also investigates whether MAPKs are involved in cilostazol-induced CREB activation and contractile protein expression. Among 3 MAPKs, cilostazol only stimulated ERK1/2 phosphorylation with the maximal effect at 30 minutes, preceding the change of CREB phosphorylation (Supplemental Figure V). Knockdown of ERK1 by specific siRNA in cilostazol-treated VSMCs blocked its effect on VSMC proliferation (Supplemental Figure VI). Furthermore, transfection with ERK1 siRNA did inhibit cilostazol-induced calponin expression, implicating that this process is mediated by PKA/CREB and ERK/CREB pathways (Supplemental Figure VII).

Effect of Cilostazol on Contractile Protein Expression and CREB Activity in Balloon-Injured Rat Carotid Arteries

This study further evaluated whether the differentiation-promoting effect of cilostazol in vitro can be observed in an in vivo balloon injury model. In agreement with previous studies, the expression of VSMC differentiation markers, including calponin and SM-MHC, decreased in the balloon-injured arteries (Figure 6A–C). Cilostazol treatment opposed the downregulation of calponin and SM-MHC induced by balloon injury. Immunohistochemical analysis revealed that staining for calponin and SM-MHC was increased in the neointima of injured arteries of cilostazol-treated rat compared with control (Figure 6A–C). Although prior studies have reported that CREB can be activated by balloon injury, the number of p-CREB–labeled cells in the neointima of cilostazol-treated arteries was still higher than that of control (Figure 6D–G). These changes in the immunohistochemistry could be quantitatively documented by Western blot analysis (Figure 6A–G). Furthermore, we found that there was a positive correlation between p-CREB and c-fos, a transcription factor known to participate in cell proliferation and differentiation, in the neointima of either with or without cilostazol-treated arteries (Supplemental Figure VIII). These findings obtained from the balloon injury system are consistent with those from an in vitro condition.

Discussion

The present study provides evidence that cilostazol promotes VSMC differentiation as determined by cell morphology and high expression of contractile proteins. In addition to this differentiation-promoting effect, previous and our studies demonstrate that cilostazol also has an ability to suppress VSMC proliferation, both of which additionally contribute to its inhibitory effect on neointimal formation. Notably, VSMC proliferation rates and their modified differentiated pheno-
type are under distinct regulatory control. That is, cessation of proliferation is not always associated with differentiated phenotype of VSMC. Cilostazol induced the expression of differentiation marker contractile proteins and also inhibited the proliferation of VSMCs in a similar fashion, suggesting that cilostazol promotes a coordinated regulation of proliferation and contractile proteins expression to induce the differentiated VSMC phenotype.

Intracellular cyclic nucleotides (cAMP and cGMP) have been reported to be associated with VSMC proliferation. Increased intracellular cAMP levels and activation of the PKA pathway may inhibit VSMC proliferation in vitro and reduce neointimal formation after arterial injury in vivo. In contrast, the inhibitory effect of cGMP/PKG on VSMC proliferation and neointimal formation is less potent than that of cAMP/PKA. As is known, VSMC proliferation and differentiation may be regulated by independent mechanism.

The inference drawn from antiproliferative effect may not necessarily be applied to the differentiation-promoting effect. In this study, we have validated the role of cAMP/PKA-, but not cGMP/PKG-dependent pathway, in cilostazol-induced VSMC differentiation, providing further evidence that cilostazol inhibits VSMC proliferation and promotes differentiation through the same mechanism.

CREB is a widely expressed nuclear transcription factor, and it is a downstream target of multiple kinases including cAMP-dependent protein kinase/protein kinase A, ribosomal S6 kinase, MAPKs, phosphatidylinositol 3-kinase, and calmodulin-dependent protein kinase. Classicaly, CREB is activated by phosphorylation at Ser133 region, permitting its binding to the coactivator protein (CBP) at the CRE of target gene. In this study, we postulate that the cAMP/PKA/CREB signaling cascade mediates the effect of cilostazol on inhibiting VSMC proliferation and promoting VSMC differentiation. This conclusion is drawn on the basis of several experiments using molecular approaches. Firstly, the cAMP agonist mimics and specific PKA inhibitors oppose the effect of cilostazol on VSMC proliferation and differentiation (in combination with findings provided by Kim et al). Secondly, inhibition of CREB phosphorylation attenuates cilostazol-induced inhibition of VSMC proliferation and upregulation of differentiation marker contractile proteins, and vice versa. More importantly, stimulation of CREB phosphorylation by cilostazol leads to enhanced CBP-DNA interaction, which in turn induces the expression of contractile proteins. In addition, disruption of the CRE site by deletion or site-directed mutagenesis eliminates the responsiveness of contractile protein expression to cilostazol. Because cilostazol inhibits VSMC proliferation and induces VSMC differentiation through the same signaling cascade, we have identified an important mediator in regulating VSMC proliferation and differentiation simultaneously.

The involvement of CREB in the pathogenesis of neointimal formation after balloon injury has been the subject of intense investigation. However, most studies focused on its role in VSMC proliferation and migration, although the conclusion is still controversial. Because CREB is regulated by multiple pathways, it is conceivable that CREB may have pleiotropic effects on VSMC functions, depending on the source and duration of stimulation. Nevertheless, the consensus is that transient exposure to agonists results in phosphorylation of CREB, whereas long-term stimulation causes downregulation of CREB phosphorylation. The CREB content and phosphorylation are negatively correlated with the proliferation of VSMCs. Expression of constitutively active CREB decreases mitogen-stimulated VSMC proliferation and migration. This study provides the first demonstration of a drug that induces VSMC differentiation via the CREB-dependent pathway. We have documented a positive correlation between the expression of VSMC differentiation marker proteins and p-CREB either in vitro or in the neointima of cilostazol-treated arteries. Our findings are consistent with the quiescent role of CREB in the vessel wall, at least in the cilostazol-treated arteries. However, there is a negative correlation between the expression of contractile proteins and p-CREB in the neointima of balloon-injured arteries compared with that of intact arteries. One possible explanation for this contrary result is that the upregulation of CREB phosphorylation in balloon-injured arteries may reflect a protective response to insult induced by balloon injury. Furthermore, the fact that the activity of CREB in the neointima of control rats is still lower than that of cilostazol-treated arteries has raised this possibility.

Because ERK inhibition may alter the effect of cilostazol on VSMC proliferation and differentiation, it is possible that other upstream kinases of CREB also mediate this process. Some studies have linked ERK with VSMC proliferation and differentiation, although this is still debatable. The discrepancy may arise from differences in experimental design and cultural conditions. Furthermore, there is a positive correlation between p-CREB and the transcription factor, c-fos, in the neointima of balloon-injured arteries. Beyond the PKA/CREB pathway, the involvement of ERK/CREB or c-fos/CREB pathways in cilostazol-induced VSMC proliferation and differentiation merits further investigation.

Adenyl cyclases and cAMP PDEs coordinately regulate intracellular cAMP level by affecting its synthesis and hydrolysis, respectively. Among the PDE families, PDE3 and PDE4 family members constitute the major cAMP PDE activities in VSMCs. It has been reported that the PDE3/PDE4 ratio is associated with the phenotypic switch in VSMCs. That is, PDE3 activity and expression decrease in dedifferentiated VSMCs, which may make the PDE3 inhibitor ineffective in modulating phenotype of VSMC. However, we indeed demonstrate the ability of a selective PDE3 inhibitor, cilostazol, to convert VSMC phenotype in a cAMP-dependent manner. It is conceivable that the changes of PDE3 levels in cilostazol-treated VSMCs may not affect its effect on VSMC differentiation.

In conclusion, the present study demonstrates that cilostazol promotes VSMC differentiation through the cAMP/PKA/CREB-signaling cascade. Besides being known as an inhibitor of VSMC proliferation, cilostazol also has an ability to induce redifferentiation in dedifferentiated VSMCs within a short-term period. Identifying signaling intermediates and transcriptional mediators involved in VSMC differentiation.
provides an important insight into the pathogenesis of neointimal formation after balloon injury.

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**Disclosures**

None.

**References**


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Supplement Materials

Supplemental Figure I:

After 48 hours of serum deprivation, VSMCs at 50-60% confluence were treated with the indicated conditions for 36 hours. The expression of calponin and tubulin was evaluated by Western blot. The relative expression levels of each protein were quantified by densitometry and normalized to the levels under the control condition, which was set at 1.0. Each value represents the mean±SE of 4 independent experiments. P< 0.05; *: the different symbol represents the significant differences among groups.
Supplemental Figure II:

After 48 hours of serum deprivation, VSMCs at 50-60% confluence were treated with the indicated conditions for 6 hours. The expression of p-CREB, total-form CREB, and tubulin was evaluated by Western blot. The relative expression levels of each protein were quantified by densitometry and normalized to the levels under the control condition, which was set at 1.0. Each value represents the mean±SE of 4 independent experiments. P< 0.05; *: the different symbol represents the significant differences among groups.
Supplemental Figure III:

Confocal immunohistochemical analysis shows nuclear translocation of phospho-CREB in VSMCs treated with indicated conditions for 30 minutes. Fixed VSMCs were stained with an anti-phospho-CREB antibody.

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Supplemental Figure IV:

Growth-arrested VSMCs were transfected with indicated plasmids and treated with or without 100 μmol/L cilostazol for 36 hours. The BrdU incorporation into VSMCs was assayed by BrdU cell proliferation ELISA, as described in the Methods. The total amount of DNA per well was kept constant by adding the empty vector of CREB plasmids (C1). Each value (mean±SE [n=6]) is expressed as the fold of BrdU incorporation of control cells. P< 0.05; *, †, #: the different symbol represents the significant differences among groups.
Supplemental Figure V:

A. Growth-arrested VSMCs were treated with 100 μmol/L cilostazol for the indicated times. The expression of indicated proteins was evaluated by Western blot.

B. Growth-arrested VSMCs were treated with 10% FBS only as a control for the indicated times. The expression of indicated proteins was evaluated by Western blot.
Supplemental Figure VI:

After transfection with indicated siRNAs and/or treated with 100 μmol/L cilostazol for 36 hours, the BrdU incorporation into VSMCs was assayed by methods as described. P< 0.05; *: the different symbol represents the significant differences among groups.

![Graph showing fold induction with different conditions]
Supplemental Figure VII:

After transfection with indicated siRNAs and/or treated with 100 μmol/L cilostazol for 36 hours, the expression of indicated proteins was evaluated by Western blot.
Supplemental Figure VIII:

Left panels: confocal immunohistochemical analysis shows the relation between p-CREB and c-fos in the neointima induced by balloon injury. Right panels: relations between nuclear expression of p-CREB and c-fos using scatter plot were shown.