Induction of Angiogenesis by a Type III Phosphodiesterase Inhibitor, Cilostazol, Through Activation of Peroxisome Proliferator-Activated Receptor-γ and cAMP Pathways in Vascular Cells

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Objective—Peripheral arterial disease is highly prevalent in the elderly and in the subjects with cardiovascular risk factors such as diabetes. Approximately 2% to 4% of those affected with peripheral arterial disease commonly complain of intermittent claudication. Cilostazol, a type III phosphodiesterase inhibitor, is the only Food and Drug Administration–approved drug for the treatment of intermittent claudication. Cilostazol has been shown to be beneficial for the improvement of pain-free walking distance in patients with intermittent claudication in a series of randomized clinical trials. However, the underlying mechanism how cilostazol improved intermittent claudication symptoms is still unclear.

Approach and Results—In this study, the effect of cilostazol on ischemic leg was investigated in mouse ischemic hindlimb model. Administration of cilostazol significantly increased the expression of hepatocyte growth factor (HGF), vascular endothelial growth factor, angioptotin-1, and peroxisome proliferator-activated receptor-γ in vasculature. The capillary density in ischemic leg was also significantly increased in cilostazol treatment group when compared with control and aspirin treatment group. However, an increase in capillary density and the expression of growth factors was almost completely abolished by coadministration of HGF-neutralizing antibody, suggesting that cilostazol enhanced angiogenesis mainly through HGF. In vitro experiment revealed that cilostazol treatment increased HGF production in vascular smooth muscle cells via 2 major pathways: peroxisome proliferator-activated receptor-γ and cAMP pathways.

Conclusions—Our data suggest that the favorable effects of cilostazol on ischemic leg might be through the angiogenesis effect via peroxisome proliferator-activated receptor-γ and cAMP pathways.

Key Words: angiogenesis effect, cilostazol, intermittent claudication, hepatocyte growth factor, muscle development, peripheral-arterial disease

Despite the development of medical treatments for patients with peripheral artery disease (PAD), PAD is still a global health burden, affecting 20% of the people aged >70 years and 4% to 12% of the population aged 55 to 70 years.1,2 Approximately 2% to 4% of those affected with PAD complain of intermittent claudication (IC).3 IC is characterized by pain in the ischemic legs that occurs with physical activity, which subsides with rest. Along with limb-specific morbidity, patients with IC are at high risk of death caused by systemic atherosclerotic cardiovascular disease, principally stroke and myocardial infarction.4,5 When compared with age-matched controls, the patients with IC have 3- to 6-fold increase in the mortality as a result of cardiovascular disease. To prevent the cardiovascular events, systemic treatment, such as cholesterol-lowering drugs, antiplatelet drugs, and renin–angiotensin system inhibitors, is frequently used. These treatments are effective in the prevention of vascular events. However, none of these drugs or any other pharmacological agents that have been advocated for treating IC have gained acceptance, with the exception of cilostazol. Cilostazol is a phosphodiesterase type III inhibitor with pharmacological effects that include the inhibition of platelet activation and aggregation, induction of vasodilation, and increased blood flow to the limbs and has been approved for the treatment of IC.6,7 This drug has been shown to increase pain-free walking distance in series of randomized clinical trials.8,9 However, its precise mechanism to improve IC is not fully understood. Endothelium-dependent vasodilation by cilostazol has been
Role of HGF on Angiogenesis Induced by Cilostazol

Initially, we compared the effects of cilostazol or aspirin on the tissue perfusion in ischemic leg. As shown in Figure 1, perfusion ratio was significantly increased in the mice treated with cilostazol when compared with those treated with aspirin and control. As our previous report demonstrated that cilostazol stimulated vascular HGF expression, we also tested the effects of HGF-neutralizing antibody. Expectedly, the recovery of the perfusion by cilostazol was significantly attenuated by coadministration of HGF-neutralizing antibody (Figure 1A and 1B). The change in limb perfusion from day 1 to day 28 in aspirin-treated group was similar to that in control mice. To assess the angiogenic property of cilostazol, CD31-positive capillary arteries were counted in ischemic muscles at 28 days after surgery. Consistent with the data from laser Doppler imaging measurement, cilostazol treatment significantly increased the number of the capillary density when compared with other groups (Figure 1C and 1D), whereas coadministration of HGF-neutralizing antibody almost completely diminished the increase in the capillary density number in cilostazol treatment group. These data clearly demonstrated that the induction of angiogenesis with cilostazol was mediated by vascular HGF.

Interestingly, the level of HGF protein measured by ELISA was significantly higher in aorta of 0.3% cilostazol treatment group than in the control and aspirin group (Figure 2A). Sections from the tissues were then analyzed by immunohistochemistry to illuminate the distribution pattern of HGF protein. In aorta, HGF protein was mainly detected in middle layer of the vessel wall, suggesting their origin from VSMCs (Figure 2B). The expression level of HGF was clearly higher in cilostazol group than in the control and aspirin groups. In contrast, tissue from the adductor muscle showed that the protein expression was limited to in between skeletal muscles (Figure 2C). Using multicolor staining procedure, HGF was colocalized with CD31 (Figure 2D), α-smooth muscle actin (Figure 2E), and PAX7-positive cells (Figure 2F). These data suggest that HGF would be produced from SMC, endothelial cell (EC), and satellite cell in adductor muscle, consistent with our previous reports that cilostazol stimulated HGF production from VSMC12 and EC.16 In addition, other groups reported the role of HGF and its receptor cMet in satellite cells.17

Molecular Mechanisms of the Induction of HGF by Cilostazol

Using in vitro culture system, we further clarified the molecular mechanisms how cilostazol stimulated the expression of HGF in vascular cells. As shown in Figure 3, cilostazol at 10 μmol/L significantly increased the expression of HGF protein (Figure 3A and 3B) in human aortic VSMCs. Phosphorylation of its receptor, c-Met, was also induced by cilostazol (Figure 3A and 3C). In addition, conditioned medium from human VSMCs cultured with or without cilostazol in serum-free medium for 72 hours were transferred to the dish culturing human umbilical vein endothelial cell (HUVEC). Of importance, the conditioned medium from VSMCs cultured with cilostazol markedly induced the phosphorylation of c-Met and enhanced its downstream signals, phospho-Akt and phospho-endothelial NO synthase, in HUVEC (Figure 3D). Expectedly, conditioned medium from hVSMCs with cilostazol significantly accelerated the proliferation (Figure 3E) and tube formation (Figure 3F and 3G) of HUVEC when compared with conditioned medium without cilostazol. In contrast, the ability of conditioned medium from cilostazol treatment to promote proliferation and tube formation in vitro was abrogated by the addition of HGF-neutralizing Ab in medium. Taken together, these data revealed that cilostazol induced vascular HGF production in VSMCs, leading to the stimulation of the Akt/endothelial NO synthase pathway in HUVECs, which ameliorated angiogenesis.

Two Downstream Pathways of HGF Production Induced by Cilostazol

Finally, we explored how cilostazol induced vascular HGF production. We focused on the PPAR-γ transcription because recent study documented that cilostazol ameliorated metabolic abnormalities in db/db mouse model via PPAR-γ transcription.18 Tissue-specific activation of PPAR-γ signaling by
cilostazol was also reported in abdominal aorta. In addition, HGF is known as a downstream effector that mediates the anti-fibrotic action of PPAR-γ agonist. Thus, PPAR-γ transcription activity was initially measured. Addition of cilostazol significantly increased the activity of PPAR-γ transcription in a dose-dependent manner, whereas cilostazol did not activate PPAR-α and PPAR-δ (Figure 4A). Stimulatory effect of PPAR-γ on HGF production was confirmed by the observation that incubation of hVSMCs with endogenous PPAR-γ agonist, 15-deoxy-PGJ2, rapidly induced HGF mRNA expression in a dose-dependent manner (Figure 4B). 15-deoxy-PGJ2 was also able to increase HGF protein level and induce phosphorylation of HGF receptor, cMet, in hVSMCs, as demonstrated by Western blotting (Figure 4C). To reveal the link between PPAR-γ signaling and upregulation of HGF, the effect of cilostazol on the promoter activity of HGF gene was examined. A reporter construct, 0.2HGF-Luc, which lacks the PPAR-γ recognition element, was transfected into rat VSMCs, cilostazol failed to activate the promoter of HGF. These results indicated that cilostazol stimulated HGF promoter activity in a PPAR-γ recognition element–dependent manner. In contrast, our previous report demonstrated that cilostazol significantly enhanced HGF production in VSMCs via cAMP accumulation. This mechanism might be involved in the prevention of VSMC proliferation by cilostazol in an in-stent restenosis model. Then, hVSMCs were treated with cAMP analog, 8-Br-cAMP, with the PKA inhibitor, KT-5720, or the PPAR-γ antagonist, GW-9662, to explore potential contribution of cAMP and the PPAR-γ pathway in the upregulation of HGF. As shown in Figure 4F and 4G, addition of 8-Br-cAMP significantly stimulated HGF production in hVSMCs. KT-5720, but not GW-9662, significantly decreased the increase in HGF expression induced by 8-Br-cAMP. In addition, PPAR-γ knockout mouse VSMCs were stimulated with cilostazol (Figure 4H). As shown in Figure 4J, cilostazol treatment increased HGF expression in PPAR-γ knockout mouse VSMCs, which was
almost completely inhibited by PKA inhibitor, KT-5720. These data indicate the existence of 2 major pathways through which cilostazol increased HGF expression in VSMCs.

Finally, we compared the effects of cilostazol and aspirin on the expression level of several angiogenic growth factor genes in abdominal aorta. As shown in Figure 5, mice treated with 0.3% cilostazol for 1 week significantly increased the expression of angiopoietin-1, HGF, vascular endothelial growth factor (VEGF)-A, and PPAR-γ by 4- to 7-fold when compared with control group. In contrast, the increase in these angiogenic growth factor genes was not observed in the aspirin treatment group (Figure 5). Intriguingly, coadministration of HGF-neutralizing antibody almost completely abolished the increase in angiogenic growth factors including angiopoietin 1 (Ang 1), VEGF-A, and PPAR-γ by cilostazol. These data revealed that HGF is an essential organizer of angiogenesis, which mediated to the upregulation of angiogenic growth factors and angiogenesis-related genes.

Discussion

IC is the manifestation of PAD caused by inadequate blood supply to the lower extremity caused by the narrowing and hardening of the feeder artery with atherosclerotic change. Although IC is not a life-threatening disease, it interferes with mobility and daily-living activity, significantly impairing quality of life. Ideally, new collateral blood vessels form, allowing blood to circulate around the damaged area alleviating muscle pain. Despite significant advances in preventive medicine and interventional devices, patients with PAD with IC still have limited the therapeutic options, whereas the number of patients with IC is increasing with the aging world population.
Cilostazol has consistently shown to increase pain-free walking distance in patients with IC. On the basis of this evidence, recent guidelines from the American College of Chest Physicians and the American College of Cardiology/American Heart Association, TASC II, recommend the use of cilostazol for patients with refractory IC. In addition, Cilostazol for Restenosis (CREST) trial demonstrated that cilostazol reduced restenosis after coronary intervention by 36% over the standard therapy alone. These clinical results imply that cilostazol has proangiogenic and antiatherosclerotic actions under these pathological conditions. However, the underlying mechanism responsible for the improvement of IC is still unclear. Previously, we demonstrated that cilostazol increased local HGF production in VSMCs, resulting in the prevention of neointimal formation in rat balloon injury model. This favorable mechanism partly relies on the antiproliferation activity of HGF on VSMCs. In addition, cilostazol increased PPAR-γ transcription activity, leading to HGF production in VSMCs. In this study, cilostazol increased PPAR-γ mRNA level in abdominal aorta by 4- to 5-fold when compared with aspirin and control groups. These results and our data suggest that the activation of PPAR-γ and cAMP is tissue specific and may be differently regulated by cilostazol.

Unexpectedly, cilostazol increased the expression of not only HGF but also VEGF and Ang 1 in aorta, whereas the increase in expression was diminished by HGF-neutralizing antibody (HGF-Ab). Biscetti et al. nicely demonstrated that cilostazol administration resulted in upregulation of VEGF in the ischemic muscle of hindlimb ischemia model mice, contributing to cilostazol-induced angiogenesis. Furthermore, HGF and VEGF have been described as a nice duo in the stimulation of angiogenesis because several articles found cross talk between HGF and VEGF in various types of cells. These previous experiments and our result indicates that cilostazol upregulates VEGF through HGF, and both HGF and VEGF enhance angiogenesis in a coordinated manner. However, few studies have shown that HGF increased Ang 1 expression although HGF mediates Ang 1–induced smooth muscle cell recruitment. We confirmed that cilostazol induced angiogenesis via HGF in vivo and in vitro. Furthermore, we reported that 2 major pathways, PPAR-γ and cAMP, would mediate HGF production induced by cilostazol in VSMCs. The promoter region of HGF gene contains PPAR-γ responsible element at ≈−200 bp from transcription start and cAMP responsible element at ≈−650 and −960 from transcription start. As expected, cAMP analog increased HGF production, which was diminished by KT6720, the PKA inhibitor, but not the
Figure 4. Two downstream pathways of hepatocyte growth factor (HGF) production induced by cilostazol. A, human vascular smooth muscle cells (hVSMCs) were cultured in serum-free medium with or without cilostazol (CLZ) for 24 h. Transcriptional activity of peroxisome proliferator-activated receptors (PPARs) was measured by using PPAR-α/δ/γ Transcription Activity Assay Kit, n=4. *P<0.05 vs control (CTRL). B and C, The endogenous PPAR-γ agonist, 15-deoxy-PGJ2, increased HGF mRNA (B) and protein (C) expressions in time- and dose-dependent manner. *P<0.05 vs CTRL. D, Schematic presentation of the plasmids constructs 0.3HGF-luciferase (Luc) and 0.2HGF-Luc. The presence of novel PPAR-γ recognition element (PPRE, red letters) in HGF gene was denoted. E, rat VSMCs (rVSMCs) were transiently transfected with 0.3HGF-Luc or 0.2HGF-Luc together with control Renilla plasmid. Then, the cells were incubated with or without cilostazol (10 μmol/L) for 24 h. Ratio of Luc/Renilla activities are shown. *,**P<0.05 vs 0.2-Luc and 0.3-Luc control, respectively. n=4. F and G, hVSMCs were treated with cAMP analog, 8-Br-cAMP (100 μmol/L), with or without protein kinase A (PKA) inhibitor, KT-5720 (1 μmol/L), or PPAR-γ antagonist, GW-9662 (10 μmol/L). Expression level of HGF was detected by Western blotting (F) and relative expression of HGF/GAPDH is shown in the graph (G). *,**P<0.05 vs CTRL and cAMP, respectively. n=3. H and I, PPAR-γ knockout (KO) mouse VSMCs were treated with cilostazol (10 μmol/L) with or without PKA inhibitor, KT-5720 (1 μmol/L). Expression level of PPAR-γ mRNA [H] and HGF protein [I] was detected. *,**P<0.05 vs CTRL and CLZ, respectively. n=3. GW indicates GW-9662; and KT, KT-5720.
PPAR-γ inhibitor, GW9662, suggesting 2 distinct pathways that stimulated HGF production after cilostazol stimulation in VSMCs (Figure II in the online-only Data Supplement).

In this study, we demonstrated that cilostazol increased the expression of several angiogenic growth factors through HGF, which contributes, at least in part, to rescue limb ischemia. However, it is noteworthy that HGF inhibited the senescence of ECs and VSMCs by atherosclerogenic stimuli, such as angiotensin II, endothelin I, and transforming growth factor-β, via a ligand-dependent epidermal growth factor receptor degradation mechanism. This mechanism would explain the antifibrotic and anti-inflammatory action of HGF. The pleiotropic effects of HGF, other than angiogenesis, might also contribute to a better outcome because of cilostazol treatment when compared with aspirin treatment in an ischemic hindlimb mouse model.

In summary, cilostazol enhanced angiogenesis through HGF production in the ischemic hindlimb mouse model via 2 major pathways, PPAR-γ and cAMP, resulting in the improvement of blood flow.

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Disclosures
None.

References
5. Criqui MH, Langer RD, Fronen A, Feigelson HS, Klauber MR, McCann TJ, Browner D. Mortality over a period of 10 years in patients with...


**Significance**

Recently, cilostazol has been widely used and proved its benefit in a variety of vascular diseases, such as peripheral arterial disease with intermittent claudication and stroke. Although its use is sometimes limited because of drug tolerability (ie, tachycardia), cilostazol seems to be a promising agent in the management of these cerebralvascular disease. Therefore, in this study, the therapeutic effect of cilostazol beyond anticoagulation activity on ischemic leg was investigated in mouse ischemic hindlimb model. Administration of cilostazol, but not of aspirin, significantly increased capillary density in ischemic leg accompanied by the expression of several angiogenic growth factors. Coadministration of hepatocyte growth factor-neutralizing antibody revealed that cilostazol enhanced angiogenesis mainly through hepatocyte growth factor. In addition, we demonstrated that cilostazol increased hepatocyte growth factor production in vascular smooth muscle cells via 2 major pathways, peroxisome proliferator-activated receptor-γ and cAMP pathways. These data would explain the favorable effect of cilostazol when compared with aspirin on vascular bed in the peripheral arterial disease.
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Materials and Methods

Animal Preparation

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Committee of Osaka University School of Medicine. C57BL6 mice aged 8-10 weeks were fed throughout the experiment with normal chow (control), cilostazol (0.1 or 0.3 %), or aspirin (0.05 or 0.1 %). Two additional groups of animals were fed with normal chow or 0.3 %-cilostazol with intraperitoneally injection of HGF-neutralizing antibody (200 µg) once a week. One week later, mice were anesthetized with isoflurane for operative resection of one femoral artery, as described previously\(^1\). The ischemic/non-ischemic limb blood flow ratio was measured using laser Doppler imaging (LDI, Moor LDI-Mark 2, Moor Instruments, UK). Tissue sections from the adductor brevis muscles of ischemic limbs were harvested on day 28. The sections were stained with specific antibodies (supplemental table I). A total of 10 different fields (in 3 cross sections from 5 animals) were randomly selected, and CD31-positive capillary density was determined by confocal microscopy. Please see supplemental figure I for an illustrated protocol.

Cell Culture and Reagents
Human aortic VSMCs (hVSMCs, passage 4 to 9) and rat aortic VSMCs (rVSMCs, passage 4 to 9) purchased from Lonza Walkersville Inc. (Portsmouth, NH, USA) were cultured in DMEM with 10% fetal bovine serum (FBS). Mouse PPAR-γ knockout VSMCs were kindly donated from Dr. Koichi Yamamoto, Osaka University Graduate School of Medicine. Human umbilical vein cells (HUVECs, passage 4 to 9) purchased from Lonza Walkersville Inc. (Portsmouth, NH, USA) were cultured in endothelial basal medium-2 (EBM-2) (Clonetics, Walkersville, Maryland, USA) medium, supplemented with EGM and 5% FBS. All stimuli were performed after 24-hour serum starvation. Cilostazol was kindly donated from Otsuka Pharmaceutical Co Ltd (Tokushima, Japan). Aspirin (Sigma, St. Louis, Mo, USA), PPAR-γ antagonist GW-9662 (Cayman Chemical Co., Ann Arbor, MI, USA), 8-Bromoadenosine 3’-5’-cyclic monophosphate (8-Br-cAMP, Sigma, St. Louis, Mo, USA), and PKA inhibitor KT-5720 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were purchased from each company, as indicated.

**Isolation of Total RNA and RT-PCR**

Total RNA was isolated from the abdominal aorta and adductor muscle using RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's
instructions. DNase treated total RNA was reverse-transcribed with the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) to produce cDNA. Reverse transcription-generated cDNA encoding the target genes was amplified and quantified by real time PCR (RT-PCR) on the ViiA-7™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the primer set shown in Supplement Table II.

Western Blot Analysis

Western blotting was performed as previously described\(^1\). Information about the antibodies and reagents used in the study are available in the Supplement Table II.

PPAR $\alpha/\delta/\gamma$ Transcription Activity Assay

A PPAR $\alpha/\delta/\gamma$ transcription activity assay was performed according to the manufacturer's instruction (Abcam, Cambridge, UK).

HGF Promoter Activity

The reporter constructs 0.2HGF-Luc and 0.3HGF-Luc, which contain 0.2 and 0.3 kb of the 5’ flanking region of mouse HGF gene and the coding sequence for firefly
luciferase, were kindly gifted from Prof. Youhua Liu (University of Pittsburgh School of Medicine, Pittsburgh, PA, USA). Rat VSMCs were transfected with 0.2HGF-Luc or 0.3HGF-Luc plasmid (2.5 µg) using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA). Internal control reporter Renilla reniformis luciferase driven under the thymidine kinase (TK) promoter (pRL-TK 0.2 µg; Promega, Madison, WI, USA) was co-transfected for normalizing the transfection efficiency. Promoter activity was measured using the Dual Luciferase Assay System Kit, according to the manufacturer’s protocols (Promega).

**MTS Assay and Tube Formation Assay**

HUVECs were harvested and re-seeded in medium conditioned from hVSMCs treated with or without cilostazol (10 µM) for 72 hours. After 24 hours, mitogenic activity was assessed by an MTS assay (Promega). HUVECs (1×10⁵) were incubated at 37 °C for 24 hours on a growth factor-reduced Matrigel-coated 24-well dish as described previously¹. Tubular length per mm² was determined in 4 random fields.

**Statistical Analysis**

Values are expressed as the mean ± SD. ANOVA and t-test, followed by
Bonferroni adjustment for multiple comparisons, were used for evaluations of more than two groups. A probability (p) value less than <0.05 was considered to indicate significant mean differences.

Reference

Supplemental figure I. Protocol of the hind limb ischemia model. C57BL/6 mice aged 8-10 weeks were fed throughout the experiment with normal chow (control), cilostazol (0.1 or 0.3%), or aspirin (0.05 or 0.1%). Two additional groups of animals were fed with normal chow or 0.3% cilostazol with an intraperitoneal injection of HGF-neutralizing antibody (200 μg) once a week. One week later, mice were anesthetized for operative resection of one femoral artery. The ischemic/non-ischemic limb blood flow ratio was measured with laser Doppler imaging (LDI). Mice were sacrificed at day 28 for immunohistochemistry.
Supplemental figure II. Proposed mechanism of CLZ induced angiogenesis.
## Antibodies

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**Supplemental table I.** Antibodies used in this experiment.
### Primer sets

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**Supplemental table II.** Primer sets used in the experiment.