Cilostazol Inhibits Vascular Smooth Muscle Cell Proliferation and Reactive Oxygen Species Production through Activation of AMP-activated Protein Kinase Induced by Heme Oxygenase-1

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Cilostazol is a selective inhibitor of phosphodiesterase 3 that increases intracellular cAMP levels and activates protein kinase A, thereby inhibiting vascular smooth muscle cell (VSMC) proliferation. We investigated whether AMP-activated protein kinase (AMPK) activation induced by heme oxygenase-1 (HO-1) is a mediator of the beneficial effects of cilostazol and whether cilostazol may prevent cell proliferation and reactive oxygen species (ROS) production by activating AMPK in VSMC. In the present study, we investigated VSMC with various concentrations of cilostazol. Treatment with cilostazol increased HO-1 expression and phosphorylation of AMPK in a dose- and time-dependent manner. Cilostazol also significantly decreased platelet-derived growth factor (PDGF)-induced VSMC proliferation and ROS production by activating AMPK induced by HO-1. Pharmacological and genetic inhibition of HO-1 and AMPK blocked the cilostazol-induced inhibition of cell proliferation and ROS production. These data suggest that cilostazol-induced HO-1 expression and AMPK activation might attenuate PDGF-induced VSMC proliferation and ROS production.

Key Words: Cilostazol, Proliferation, ROS, AMPK, HO-1

INTRODUCTION

The proliferation of vascular smooth muscle cell (VSMC) plays a vital role in vascular diseases such as atherosclerosis and hypertension [1,2], which are most pronounced in coronary arteries, thereby producing acute coronary syndrome in affected subjects [3]. Consequently, anti-proliferation of VSMC is considered a marker of vascular health and can be used to gauge cardiovascular risk [4]. Therefore, strategies aimed at inhibiting VSMC proliferation might be of benefit in the prevention of atherosclerosis. This VSMC proliferation contributes to dominant cellular events in the re-narrowing of the vasculature, which causes the release of growth factors. Platelet-derived growth factor (PDGF) is a growth factor induced by VSMC, vascular endothelial cells, platelets or macrophages and plays an important role in intimal proliferation [5]. After stimulating cell growth, PDGF can induce proliferation of VSMC. Inhibition of PDGF-stimulated VSMC proliferation therefore represents an important point in the therapeutics for many vascular diseases.

ROS have been proposed to be important signaling molecules in many biological events such as cell proliferation which is important in the pathogenesis of intimal thickening in atherosclerosis [6]. One mechanism by which ROS provoked hypertension is via binding of nitric oxide (NO) and also increases intracellular free Ca²⁺ levels, which contribute to increased vascular tone [7]. ROS also has been shown to mediate the proliferative effects of many growth factors in VSMC. Several studies have demonstrated that PDGF can evoke intracellular oxidative stress and ROS may act as key mediator in the regulation of PDGF-induced VSMC proliferation [6,8].

AMP-activated protein kinase (AMPK) has an important function in cellular energy status. AMPK is activated by stress such as physical exercise and hypoxia, which increase the cellular AMP/ATP ratio. AMPK has also been implicated in the regulation of physiological signals, such as inhibition of cholesterol, fatty acid, and protein synthesis, and enhancement of glucose uptake and blood flow [9,10]. AMPK activation has several effects on vascular function and improves vascular abnormalities. Therefore, AMPK may serve as an important target to inhibit protein synthesis and cell growth and to treat vascular proliferative diseases.

Heme oxygenase-1 (HO-1) is known for its cytoprotective effects against oxidative damage [11]. Activation of HO-1

ABBREVIATIONS: AMPK, AMP-activated protein kinase; HO-1, hemeoxygenase-1; PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell; ROS, reactive oxygen species.
has therapeutically beneficial effects in vascular narrowing caused by VSMC proliferation [12]. An increase in intracellular cAMP-coupled protein kinase A (PKA) activation also has been noted to induce HO-1 gene expression in cultured VSMC [13].

Cilostazol increases intracellular cAMP concentrations by selectively blocking phosphodiesterase 3 (PDE-3), inhibiting platelet aggregation and inducing peripheral vasodilation [14]. Cilostazol is a potent antiplatelet drug used in clinical practice to treat patients with chronic vascular obstruction [15]. Recent clinical studies indicate that cilostazol inhibits the proliferation of VSMC [16]. One mechanism by which cilostazol may inhibit VSMC proliferation is via an increase in intracellular cAMP, because cAMP inhibits the proliferation of VSMC.

In this study, we investigated whether cilostazol suppressed proliferation of VSMC and ROS production via activation of AMPK and HO-1 expression. It is important to note how cilostazol inhibits VSMC proliferation. However, the effects of cilostazol mediated by activation of AMPK and HO-1 expression have not yet been clarified. These results may provide a better understanding of the anti-proliferation effect of cilostazol.

**METHODS**

**Materials**

Dulbecco’s modified eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (South Logan, UT, USA). RIPA protein extract buffer was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-AMPK, anti-phospho-AMPK and human platelet-derived growth factor BB (hPDGF-BB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cilostazol was donated by Otsuka Pharmaceuticals (Tokushima, Japan) and dissolved in DMSO.

**Cell culture**

Sprague-Dawley rats were anesthetized with pentobarbital (50 mg/kg). VSMCs were isolated from the thoracic aorta. The cells were processed using a 1 mm chop setting in 10 cm culture dish, and cultured with 5% fetal bovine serum (FBS-DMEM with 1% antibiotics-antimycotics (penicillin 10,000 U/ml, amphotericin B 25 μg/ml, streptomycin 10,000 μg/ml) in a CO₂ incubator. Aortic VSMCs were maintained in DMEM with 10% FBS and 1% antibiotic-antimycotics. We used VSMC from passages 4 to 8 at 70–90% confluence in 10 cm dishes, and cell growth was arrested by incubation of the cells in serum-free DMEM for 24 hr prior to use.

**Western blot analysis**

Whole cell extracts were prepared by lysing the cells in RIPA protein extract buffer. The protein concentration was quantified with protein assay reagent from Bio-Rad (Hercules, CA, USA). Equal amounts of protein were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated for 5 min at 100°C before loading. Total protein samples (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 2 hr at 100 V. The separated proteins were electrophoretically transferred onto a PVDF membrane for 1 hr 20 min at 100 V. The membranes were blocked with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 2 hr at room temperature. The membranes were then incubated with the primary antibodies (1: 1,000) in 5% skim milk in PBS overnight at 4°C. The membranes were then washed with four changes of wash buffer (0.05% Tween 20 in PBS) and incubated for 1 hr at room temperature in PBS-T containing anti-rabbit (Stressgen, Ann Arbor, MI, USA) or anti-mouse IgG (Santa Cruz, CA, USA) antibodies. Finally, after three more rinses with wash buffer, the membranes were exposed to ECL (Neuronex, Daegu, Korea) or ECL plus (GE Healthcare, Buckinghamshire, UK) western blot detection reagents.

**Cell proliferation assay**

Cell proliferation was analyzed using the MTT and bromodeoxyuridine (BrdU) assays. VSMCs were seeded on 24-well plates at 1×10⁵ cells per well in DMEM supplemented with 10% FBS. After different treatments, 50 μl of 1 mg/ml MTT solution was added to each well (0.1 mg/well) and incubated for 4 hr. The supernatants were aspirated, and the formazan crystals in each well were solubilized with 200 μl of dimethyl sulfoxide (DMSO). An aliquot of this solution (10 μl) was placed in each well of 96-well plates. Cell proliferation was assessed by measuring the absorbance at 570 nm using a microplate reader. The incorporation of BrdU was also analyzed using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany).

**Experiments using RNA interference**

Transfection of VSMCs with siRNA was performed using Lipofectamine 2,000, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Aliquots of 1×10⁶ cells were plated on 6-well plates the day before transfection and grown to about 70% confluence. The cells were then transfected with 10 μM AMPK or HO-1 siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) plus 100 pmol of Lipofectamine for 6 hr in Opti-MEM® I reduced serum medium (Invitrogen, Carlsbad, CA, USA). Following an incubation period of 48 hr, the protein level was measured using western blot analysis.

**Measurement of intracellular ROS**

Intracellular ROS production was measured by 2,7-di-chlorofluorescein fluorescence using live imaged laser scanning microscopy and fluorescence-activated cell sorting (FACS) analysis. Cells were grown in 60 mm dishes following treatment with or without PDGF. Subconfluent cells were incubated in the dark for 10 min in the presence of 10 μM DCF-DA. ROS production was detected from the oxidation of DCF-DA. ROS production was quantified with protein assay reagents from Bio-Rad (Hercules, CA, USA). Equal amounts of protein were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated for 5 min at 100°C before loading. Total protein samples (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 2 hr at 100 V. The separated proteins were electrophoretically transferred onto a PVDF membrane for 1 hr 20 min at 100 V. The membranes were blocked with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 2 hr at room temperature. The membranes were then incubated with the primary antibodies (1: 1,000) in 5% skim milk in PBS overnight at 4°C. The membranes were then washed with four changes of wash buffer (0.05% Tween 20 in PBS) and incubated for 1 hr at room temperature in PBS-T containing anti-rabbit (Stressgen, Ann Arbor, MI, USA) or anti-mouse IgG (Santa Cruz, CA, USA) antibodies. Finally, after three more rinses with wash buffer, the membranes were exposed to ECL (Neuronex, Daegu, Korea) or ECL plus (GE Healthcare, Buckinghamshire, UK) western blot detection reagents.
Cilostazol Activates AMPK via HO-1

Fig. 1. Cilostazol strongly induces HO-1 as well as AMPK activation in VSMC. (A) Cells were exposed to different concentrations (1 ∼ 200 μM) of cilostazol for 24 hr and (C) treated with 100 μM of cilostazol for the indicated times. Expression of HO-1, p-AMPK and AMPK were analyzed by western blot. Each graph represents the densitometry analysis of HO-1 expression and AMPK phosphorylation (Fig. 1B and D). Data are represented as the mean±S.E.M (n=3). *p-value < 0.01 compared with control.

**RESULTS**

**Cilostazol induces HO-1 expression and activates AMPK in VSMC**

To examine whether cilostazol induces HO-1 and activates AMPK in VSMC, we measured the protein level after treatment with cilostazol at the indicated dose and time. As shown in Fig. 1A, cilostazol dose-dependently increased the levels of both HO-1 expression and AMPK phosphorylation. Cilostazol 100 μM time-dependently increased HO-1 expression and AMPK phosphorylation (Fig. 1C). Each graph represents the densitometry analysis of HO-1 expression and AMPK phosphorylation (Fig. 1B and D).

**PDGF-stimulated proliferation is inhibited by cilostazol-induced HO-1 and AMPK activation**

Initially, we examined the effect of cilostazol on cell proliferation and BrdU incorporation of VSMC under PDGF-stimulated conditions. Treatment of VSMC with PDGF (10 ng/ml) significantly increased cell proliferation, as shown in Fig. 2A and B. The inhibitory effect of cilostazol, a well-known treatment for peripheral arterial disease, on VSMC proliferation was examined. As shown in each graph, cilostazol inhibited PDGF-stimulated VSMC proliferation in a dose-dependent manner. However, cilostazol did not change the basal proliferation of VSMC in the absence of PDGF. Next, we examined whether cilostazol induces HO-1 expression and AMPK activation in PDGF-stimulated condition. Cilostazol increased the levels of these molecules compared to those in the PDGF-treated group (Fig. 2C).

**Cilostazol attenuates PDGF-generated intracellular ROS production**

To examine whether cilostazol suppresses PDGF-generated...
Fig. 2. Cilostazol inhibits PDGF-stimulated VSMC proliferation via HO-1 and AMPK. Cell proliferation was determined by MTT assay (A) or BrdU incorporation assay (B) in the presence of indicated concentration of cilostazol for 24 hr. Dose-dependent inhibitory effect of cilostazol on percent change in VSMC proliferation is noted. (C) Protein expressions of HO-1 and p-AMPK were determined by western blot analysis. Data are represented as the mean±S.E.M (n=4). *p-value < 0.01 compared with control, †p-value < 0.05 compared with PDGF.

Fig. 3. Cilostazol suppresses ROS production generated by PDGF in VSMC. (A) ROS generated in viable cells produce a uniform bright green color in the cytoplasm and nuclei. For observation of intracellular ROS by fluorescence microscopy, cells were pretreated with cilostazol for 24 hr and then stimulated with PDGF (10 ng/ml) for 2 hr in the presence of 10 μM DCF-DA. (B) ROS production was assessed by FACS analysis. Data are represented as the mean±S.E.M (n=3). *p-value < 0.01 compared with control, †p-value < 0.01 compared with PDGF. Cil, cilostazol.
Cilostazol Activates AMPK via HO-1

Pharmacological inhibition of HO-1 and AMPK reverses the effect of cilostazol on proliferation and ROS production

To further demonstrate the interaction of cilostazol, HO-1, and AMPK, we examined the effects of HO-1 and AMPK inhibitors on VSMC proliferation. Cilostazol decreased the PDGF-induced proliferation of VSMC. Abolishing HO-1 and AMPK activity with SNPP IX and compound C restored the cilostazol-induced inhibition of VSMC proliferation (Fig. 4A and B). Interestingly, SNPP IX, an inhibitor of HO-1, suppressed activation of AMPK, but compound C, an inhibitor of AMPK, did not affect HO-1 expression (Fig. 4C). Thus, the HO-1/AMPK signaling pathway is responsible for the anti-proliferative activity of cilostazol, and HO-1 is suggested that possible upstream controller of AMPK as shown in Fig. 4C.

To examine whether cilostazol-induced HO-1 and AMPK activation suppress PDGF-generated ROS production, we observed morphological levels of ROS by fluorescence microscopy after treating with SNPP IX and compound C (Fig. 4D). After 2 hr from exposure to PDGF, cells showed morphological characteristics of ROS production. The PDGF-generated ROS production was decreased by treatment with cilostazol, but SNPP IX and compound C restored PDGF-generated ROS production.
Genetic inhibition of HO-1 and AMPK reverses the effect of cilostazol on proliferation and ROS production

In order to determine if HO-1 expression and AMPK activation by cilostazol is required to inhibit cell proliferation, we carried out experiments with siRNA against HO-1 and AMPK. As shown in Fig. 5A and B, suppression of HO-1 and AMPK by siRNA blocked the anti-proliferative effect of cilostazol. Interestingly, HO-1 siRNA transfection suppressed activation of AMPK, but AMPK siRNA did not affect HO-1 expression based on western blot analysis (Fig. 5C). ROS production was also reversed by HO-1 and AMPK siRNA transfection (Fig. 5D). Therefore, the HO-1/AMPK signaling pathway is responsible for the effects of cilostazol, and HO-1 expression and AMPK activation by cilostazol suppress PDGF-induced cell proliferation and ROS production.

**DISCUSSION**

Cilostazol has been developed as a PDE-3 inhibitor and is used to treat patients with peripheral vascular disease [16]. In addition, inhibition of VSMC proliferation by cilostazol has been reported [17]. Therefore, it is important to understand how cilostazol inhibits VSMC proliferation. The main purpose of the present study was to demonstrate the anti-proliferative mechanism of cilostazol in VSMC, specifically focused on the AMPK activation and HO-1 expression. We examined that cilostazol-induced AMPK phosphorylation and HO-1 expression.

In the previous study, HO-1 is recognized as a beneficial molecule for protecting against oxidative stresses [18] and vascular constriction as well as proliferation [19]. We first examined whether cilostazol induced HO-1 expression in VSMC, because HO-1 increases the intracellular cAMP levels [20]. HO-1 expression is associated with increases in adiponectin and AMPK activation [21,22]. Our results indicated that cilostazol increased HO-1 and p-AMPK expression with dose- and time-dependent manners (Fig. 1).
The present study also demonstrates that cilostazol plays a crucial role in the induction of HO-1 expression and HO-1 induces activation of AMPK in VSMC (Fig. 4C and Fig. 5C).

Weshowed that cilostazol caused phosphorylation of AMPK at Thr172 by inducing HO-1 in VSMC. The extent of AMPK phosphorylation at Thr172 strongly reflects its activity [23]. Cilostazol-induced AMPK activation has an attractive characteristic, because AMPK is reported to mediate beneficial and bio-protective effects of metformin [24] and adiponectin [25]. AMPK is a serine/threonine protein kinase, which serves as an energy sensor in eukaryotic cells. Several studies have revealed that AMPK activation strongly suppresses cell proliferation in normal cells as well as in tumor cells [26].

In the current study, we found that inhibition of PDGF-induced VSMC proliferation by cilostazol was mediated via AMPK phosphorylation and HO-1 expression. Compound C and AMPK siRNA strongly inhibited the cilostazol-induced AMPK phosphorylation, but did not affect expression of HO-1. Thus, inhibition of AMPK is irrelevant to HO-1 expression. However, AMPK phosphorylation and inhibition of VSMC proliferation were completely lost in the presence of the HO-1 inhibitor, SNPP IX, or HO-1 siRNA (Fig. 4A, B and Fig. 5A, B). These findings suggest that the anti-proliferative effect of cilostazol is mediated in part by an HO-1/AMPK signaling pathway. Compound C decreased AMPK expression (Fig. 4C). Although compound C is a well known inhibitor of AMPK and widely used to inhibit AMPK, Compound C also decreased AMPK expression in many experiments [27].

A previous study suggests that the anti-apoptotic effects of cilostazol via cAMP-dependent protein kinase are ascribed to its antioxidant function and its ability to reduce production of ROS [28]. Furthermore, AMPK activation independently regulates ROS production [29]. Therefore, we demonstrated that cilostazol suppresses ROS production in VSMC through AMPK activation. In addition, cilostazol significantly inhibits PDGF-induced ROS production. Thus, cilostazol inhibits PDGF-induced VSMC proliferation and ROS production by phosphorylation of AMPK through HO-1 expression (Fig. 4D and Fig. 5D). These findings suggest that the effect of cilostazol might be mediated by the HO-1/AMPK signaling pathway.

In conclusion, we have demonstrated that cilostazol-induced AMPK activation inhibits PDGF-induced VSMC proliferation and ROS production via phosphorylation of AMPK through HO-1/AMPK pathway. The ability of cilostazol to activate AMPK is promising, since AMPK activations mediate vascular protective effect. Our observations indicate that cilostazol-induced AMPK activation in VSMC may have beneficial effects on vascular proliferative disorders such as atherosclerosis, in addition to its selective inhibition of PDE-3.

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REFERENCES


