Cilostazol ameliorates metabolic abnormalities with suppression of pro-inflammatory markers in a db/db mouse model of type 2 diabetes via activation of peroxisome proliferator-activated receptorγ transcription

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Abbreviation: PPARγ, peroxisome proliferator-activated receptor γ; aP2, adipocyte fatty acid binding protein; FATP-1, fatty acid transport protein; GLUT4, glucose transport 4

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Abstract

In a previous study, cilostazol promoted differentiation of 3T3-L1 fibroblasts into adipocytes, and improved insulin sensitivity by stimulating PPARγ transcription. This study evaluated the in vivo efficacy of cilostazol to protect a db/db mouse model of Type 2 diabetes against altered metabolic abnormalities and pro-inflammatory markers via activation of PPARγ transcription. Eight-week old db/db mice were treated with cilostazol or rosiglitazone for 12 days. Cilostazol significantly decreased plasma glucose and triglyceride levels, as did rosiglitazone, a PPARγ agonist. Elevated plasma insulin and resistin levels were significantly decreased by cilostazol, and decreased adiponectin mRNA expression was elevated along with increased plasma adiponectin. Cilostazol significantly increased both adipocyte fatty acid binding protein (aP2) and fatty acid transport protein (FATP-1) mRNA expressions with increased glucose transport 4 in the adipose tissue. Cilostazol and rosiglitazone significantly suppressed pro-inflammatory markers (superoxide, TNF–α and vascular cell adhesion molecule-1) in the carotid artery of db/db mice. In in vitro study with 3T3-L1 fibroblasts, cilostazol significantly increased PPARγ transcription activity, as did rosiglitazone. The transcription activity stimulated by cilostazol was attenuated by KT5720, a cAMP-dependent protein kinase inhibitor, and GW9662, an antagonist of PPARγ activity, indicative of implication of PI3-k/Akt signal pathway. These results suggest that cilostazol may improve insulin sensitivity along with anti-inflammatory effects in Type 2 diabetic patients via activation of both cAMP-dependent protein kinase and PPARγ transcription.
Introduction

Insulin resistance is a major contributor to the pathogenesis of type 2 diabetes and plays a key role in associated metabolic abnormalities, such as dyslipidemia and hypertension (Goldstein, 2002). Recently, adipose tissue was recognized as a primary site of insulin resistance, rather than skeletal muscle (Hotamisligil, 2000). Arner (2003) emphasized that adipocytokines, such as tumor necrosis factor-α (TNF-α), adiponectin and resistin, modulate the underlying insulin resistance.

Peroxisome proliferator-activated receptor γ (PPARγ), as a receptor for the thiazolidinediones (TZDs), plays a key role in adipogenesis (Vidal-Puig et al., 1997). TZDs have been shown to ameliorate insulin resistance by binding to and activating PPARγ in adipose tissue, thereby promoting differentiation and increasing the number of small adipocytes (Rangwala and Lazar, 2004). Rosiglitazone has been reported to exert a potent anti-inflammatory effect with an increase in plasma adiponectin and a fall in resistin concentrations in the pathophysiology of metabolic syndrome, thus contributing to insulin sensitization (Ghanim et al., 2006).

Adiponectin expression that is regulated by PPARγ transcriptional activity (Iwaki et al., 2003) has been suggested to enhance insulin sensitivity by increasing oxidation of intracellular fatty acids and decreasing muscle and liver triglyceride levels (Yamauchi et al., 2002). In contrast, resistin, which is produced exclusively by adipocytes in mice, causes insulin resistance at high circulating levels (Steppan et al., 2001). Elevated resistin leads to insulin resistance associated with visceral adiposity (Rea and
Donnelly, 2004), thus playing a role for the link between obesity and type 2 diabetes. Resistin also promotes endothelial cell activation, thereby upregulating expressions of adhesion molecules and chemokines (Verma et al., 2003), which leads to inflammatory states associated with insulin resistance (Fernandez-Real and Ricart, 2003).

Cilostazol, (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone), a potent phosphodiesterase type III inhibitor, has been used as a vasodilating anti-platelet drug for the treatment of ischemic symptoms in chronic peripheral arterial obstruction or intermittent claudication and for preventing recurrence of cerebral infarction (Dawson et al., 1998; Kambayashi et al., 2003). Recently, it has drawn a great deal of interest due to its anti-inflammatory (Shin et al., 2004; Park et al., 2005) and anti-atherogenic actions (Lee et al., 2005).

Shin et al. (2004) showed that cilostazol exerts a cell protective effect by suppressing remnant lipoprotein particle-stimulated NAD(P)H oxidase–dependent superoxide formation and production of cytokines (TNF-α and IL-1β) in human umbilical vein endothelial cells. Park et al. (2005) recently addressed the anti-inflammatory actions of cilostazol by showing that cilostazol suppresses monocyte adhesion to human umbilical vein endothelial cells induced by remnant lipoprotein particle associated with suppression of VCAM-1, monocyte chemoattractant protein-1 expression and superoxide production. These results were confirmed by in vivo study with low-density lipoprotein receptor-null mice fed a high cholesterol diet (Lee et al., 2005). These cilostazol effects were ascribed to an increased cAMP-dependent
protein kinase-coupled maxi K channel opening. These results in suppressing superoxide generation, TNF-\(\alpha\) formation and vascular cell adhesion molecule (VCAM)-1 expression associated with decreased NF-\(\kappa\)B transcriptional activity.

In line with these findings, we recently showed for the first time that cilostazol stimulates PPAR\(\gamma\) transcriptional activity in human endothelial cells. Cilostazol downregulated resistin expression and upregulated adiponectin expression in 3T3-L1 adipocytes in conjunction with increased GLUT4 expression/translocation in adipocytes and glucose uptake in L6 myotube cell lines. These effects are similar to rosiglitazone, a PPAR\(\gamma\) agonist (Park et al., 2008). Given that cilostazol upregulates GLUT4 expression/translocation, along with increased expression of adiponectin via increased PPAR\(\gamma\) transcription in 3T3-L1 fibroblast cells, it is likely that cilostazol may improve insulin sensitivity in type 2 diabetic patients.

Thus, to explore this hypothesis in \textit{in vivo} study, we evaluated the efficacy of cilostazol to ameliorate the altered metabolic abnormalities and pro-inflammatory markers activated via PPAR\(\gamma\) transcription in the db/db mouse model of Type 2 diabetes.
Methods

Animals. All experimental procedures were conducted in accordance with the Animal Care Guidelines of the Animal Experimental Committee of the College of Medicine, Pusan National University. Studies used 8-week-old male lean C57BL/6 mice and diabetic leptin receptor-deficient C57BLKSJ-db/db mice purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were housed in a 12-h light-dark cycle. For the experiments, C57BLKSJ-db/db mice were orally administered vehicle (DMSO), 30 mg/kg/day cilostazol or 10 mg/kg/day rosiglitazone for 12 consecutive days. On the day of the experiment, the blood was removed under fast conditions. Blood glucose was measured with an Accu-Check advantage meter (Roche Diagnostics, Indianapolis, IN) and was expressed as mg per dl.

Analysis of Plasma Insulin, Resistin, Adiponectin and Triglyceride. After 12 days treatment, mice were given an overdose of thiopental sodium and decapitated. Trunk blood was collected in ice-chilled heparinized tubes. Serum triglycerides (TGs) levels were determined using a kit from AsanPharm (Asan Pharmaceutical Co., Seoul, Korea). Commercial ELISA kits were used for plasma insulin levels (Mercodia, Uppsala, Sweden), plasma adiponectin and resistin levels (R&D Systems, Minneapolis, MN) according to the manufacturers’ protocols.

Reverse Transcription Polymerase Chain Reaction (PCR). Total RNA was extracted from visceral
adipose tissue using TRIZOL reagent (Invitrogen). PCR primers for amplification of each gene were as follows: Adiponectin (sense, 5′-GACGTTACTACAACGTAGACGC-3′; antisense, 5′-CATTCTTTTCCTGACTGGTC-3′), aP2 (sense, 5′-ACATGATCATCAGCTAAATGGG-3′; antisense, 5′-TCATAAACACATCCACCACCAG-3′), FATP1 (sense, 5′-ACAGCCAGTTGGGCCCACTCAGA-3′; antisense, 5′-TGATTTTGGTGCTGCTTGTA-3′). Cycles comprised 95 °C for 1 min, following a hybridization step at 56 °C for 1 min, and an elongation step at 72 °C for 1 min. Relative abundance of mRNA was calculated after normalization to GAPDH.

Determination of GLUT4 in Plasma Membranes. To prepare membrane fractions, visceral adipose tissue was homogenized in buffer A (20 mM Tris-HCL, 150 mM NaCl, 10 mM EDTA, 50 mM NaF, 25 mM NaVO₄, proteinase inhibitor) and centrifuged at 12000 rpm for 2 min. Fat cakes were discarded, and the infranatant fat-free extract was centrifuged a second time at 100,000 rpm for 45 min to precipitate the plasma membrane fraction. The final pellet was homogenized in buffer B (20 mM Tris-HCL, 150 mM NaCl, 10 mM EDTA, 50 mM NaF, 25 mM NaVO₄, 1 % Triton X-100).

Protein (30 μg) was loaded into a 10% SDS-polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane (Amersham Bioscience, Inc.). The blocked membrane was incubated with an antibody to GLUT4 (Santa Cruz Biotechnology, Inc.). Immunoreactive bands were visualized with a chemiluminescent reagent from a SuperSignal West Dura Extended Duration Substrate kit (Pierce...
In situ detection of Superoxide in Arterial Walls. Carotid arterial segments near the bifurcation were cut into 5 μm thicknesses and prepared for subsequent in situ analyses. They were incubated with dihydroethidium (DHE) (5 μmol/l, Molecular probes, OR) in PBS for 30 min at 37 °C in a humidified chamber protected from light. DHE is oxidized on reaction with superoxide anion to ethidium bromide, which, in turn, binds to DNA in the nucleus and emits red fluorescence. The red fluorescence was detected through a 580-nm long pass filter using a fluorescence microscopy (Axiovert 200, Carl Zeiss, Oberkochem, Germany).

Immunohistochemistry for TNF-α and VCAM-1 Expressions in Carotid Arteries. Cross-sections (5-μm thickness) of the bifurcation segments of carotid arteries from db/db mice were made on a cryostat. Endogenous peroxidase activity was quenched by incubation with 0.3% H₂O₂ and non-specific antibody binding was blocked with CAS block (Zymed Laboratories, Inc., San Francisco, CA) for 10 min. The preparations were incubated overnight at 4 °C with anti-TNF-α antibody (diluted in 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and VCAM-1 antibody (diluted in 1:100; Santa Cruz). These were subsequently incubated with a biotinylated anti-goat, rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 1h, and then with an avidin-biotin peroxidase complex solution (Vectastain Elite
ABC kit, Vector Laboratories) for 1h. Finally, the immunoreaction products were visualized with a solution of 0.02% 3,3-diaminobenzidine tetrahydrochloride (diaminobenzidine substrate kit, Vector Laboratories).

**Cell Culture.** 3T3-L1 mouse fibroblasts (cell line of CL-173; American Type Culture Collection) were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and 1% antibiotics in a 5% CO₂ environment.

**Luciferase Reporter Assays.** 3T3-L1 fibroblast cells were transfected with pGL3-basic, peroxisome proliferator response element (PPRE)-pGL3, a renilla luciferase control reporter vector using Lipofectamine for promoter activity analysis (Kintsher et al., 2002). Luciferase activity in cell lysates was determined by a Dual-Luciferase reporter Assay System (Promega) using a Glomax 20/20 luminometer (Promega).

**Transfection of siRNA for PPARγ knockdown in 3T3-L1 cells.** The PPARγ siRNA oligonucleotide (Target Accession no. NM_011146) was synthesized by Bioneer (Daejeon, Korea). The siRNA negative control duplex was used as a control oligonucleotide. The siRNA molecules were transfected into 3T3-L1 cells using Lipofectamine 2000 (Invitrogen).
Drugs. Cilostazol (OPC-13013) [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone], generously donated from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan) and rosiglitazone (Alexis Biochemicals), were dissolved in dimethyl sulfoxide as a 10 mM stock solution.

GW9662 (2-chloro-5-nitrobenzanilide), PD98059, SP600125, SB239063 and LY294002 were from Sigma-Aldrich Chemicals. Others included SH5 (Calbiochem, San Diego, CA) and KT5720 (Alexis Biochemicals, San Diego, CA).

Statistical Analysis. The results are expressed as mean ± S.E.M. One-way ANOVA followed by Tukey’s multiple comparisons test was used for analyzing values between groups. Student’s t test was used for results from in vitro experiments. P < 0.05 was considered statistically significant.
Results

**Body Weight, Plasma Blood Glucose and Triglyceride in db/db Mice.** Treatment with rosiglitazone (10 mg/kg/day) for 12 days resulted in a significant weight gain for the db/db mice (47.9 ± 1.0 g, \( P < 0.05; n=7 \)) compared to the vehicle only group (43.6 ± 0.9 g, \( n=7 \)). Body weights for db/db diabetic mice were little altered by cilostazol treatment (30 and 50 mg/kg/day for 12 days) (Supplemental Table 1).

The blood glucose level for lean mice was 142.0 ± 4.3 mg/dl. For db/db mice, blood glucose was significantly higher in the vehicle group (510.0 ± 19.5 mg/dl). This was significantly reduced by treatment with 30 mg/kg cilostazol at day 8 (271.0 ± 30.7 mg/dl compared to 461.0 ± 38.1 mg/dl in vehicle group; \( P < 0.001 \)) and at day 12 (335.8 ± 15.9 mg/dl, \( P < 0.001 \)), but it was little affected by treatment with 20 mg/kg cilostazol. The blood glucose level in the rosiglitazone group was significantly decreased to 192.9 ± 11.7 mg/dl (\( P < 0.001 \)) at day 12 (Fig. 1A). The triglyceride levels in db/db mice (277.2 ± 42.6 mg/dl in vehicle group) were significantly reduced after treatment with cilostazol (30 mg/kg/day) to 145.9 ± 10.3 mg/dl (\( P < 0.05 \)) and rosiglitazone (10 mg/kg/day) to 110.7 ± 11.3 mg/dl (\( P < 0.01 \)) (Fig. 1B).

**Effects of Cilostazol on Plasma Insulin and Resistin.** Effects of cilostazol (30 mg/kg/day) on the plasma insulin and resistin levels were compared with those of rosiglitazone in the db/db mice. The mean plasma insulin level for lean mice was 1.48 ± 0.39 ng/ml. For db/db mice, this was significantly elevated
to 10.94 ±0.94 ng/ml in the vehicle group ($P < 0.001$). However, the plasma insulin level was markedly decreased in the cilostazol (30 mg/kg)-treated mice (8.22 ± 0.52 ng/ml, $P < 0.05$) and rosiglitazone (10 mg/kg)-treated mice (4.51 ± 0.50 ng/ml, $P < 0.001$) (Fig. 2A). Similarly, the plasma resistin level (lean mice; 32.6 ± 6.3 ng/ml) was significantly elevated in the vehicle only group to 80.9 ± 3.9 ng/ml ($P < 0.001$). The elevated resistin levels were significantly decreased by oral treatment with cilostazol ($P < 0.05$) and rosiglitazone ($P < 0.01$) (Fig. 2B).

Cilostazol Effects on Adiponectin Gene Expression in Adipose Tissue and Plasma Adiponectin Levels. We examined if expression of the adiponectin gene was regulated by cilostazol in the visceral adipose tissue using RT-PCR. Adiponectin mRNA expression in db/db mice was significantly lowered to 0.36 ± 0.03 when compared to lean mice. Cilostazol (30 mg/kg/day) significantly restored the adiponectin mRNA expression to 0.58 ± 0.04 ($P < 0.05$), as did rosiglitazone (10 mg/kg/day) (0.75 ± 0.09, $P < 0.001$) (Fig. 2C). Similarly, plasma adiponectin was 6473.6 ± 160.3 ng/ml in the lean mice, which was significantly decreased in the vehicle only db/db mice (2877.2 ± 216.5 ng/ml; $P < 0.001$). The decreased plasma adiponectin level was significantly recovered to 4128.6 ± 246.7 ng/ml ($P < 0.01$) by cilostazol (30 mg/kg/day), and to 6041.9 ± 150.5 ng/ml ($P < 0.001$) by rosiglitazone (10 mg/kg/day) in db/db mice (Fig. 2D).
Cilostazol Effects on aP2 and FATP1 Gene Expressions in Visceral Adipose Tissue. Expressions of aP2 (fatty acid-binding protein) and FATP1 (fatty acid transport protein1) mRNA were significantly lower in the db/db mice compared to lean mice. As shown in Fig. 3, aP2 mRNA was significantly increased in the cilostazol (30 mg/kg)-treated db/db mice to 0.81 ± 0.08 (P < 0.05) and in the rosiglitazone (10 mg/kg)-treated mice to 1.00 ± 0.13 (P < 0.01) when compared with vehicle-treated db/db mice (0.48 ± 0.03) (Fig. 3A). FATP1 gene expression showed similar results for aP2 mRNA levels after treatment with cilostazol and rosiglitazone (Fig. 3B).

Cilostazol Effects on GLUT4 Protein Expression in Adipose Tissue. The db/db mice showed significantly decreased GLUT4 protein expression that was translocated to the plasma membrane (0.59 ± 0.05 vs. lean mice =1.0, P < 0.001). GLUT4 expression was significantly increased by treatment with cilostazol (30 mg/kg/day) to 1.11 ± 0.09 (P < 0.05) and rosiglitazone (10 mg/kg/day) to 1.59 ± 0.21 (P < 0.001) (Fig. 4).

Effects on Pro-inflammatory Markers: Vascular Superoxide Formation, TNF-α and VCAM-1 Expression. An intimal change in the carotid artery is a widely accepted biomarker for atherosclerotic changes in type 2 diabetes (Koshiyama et al., 2001). The bifurcation area of the carotid artery was used as a surrogate for detecting pro-inflammatory processes. The intensity of red fluorescence that indicates a
dihydroethidium-positive area, a marker for superoxide anion, was markedly increased in the bifurcation area of the carotid artery of db/db mice (18.1 ± 2.2, \( P < 0.001 \)). This was markedly reduced to 6.0 ± 1.7 (\( P < 0.01 \)) and 7.8 ± 2.9 (\( P < 0.05 \)) by cilostazol and rosiglitazone, respectively.

TNF-\( \alpha \) and VCAM-1 were evaluated using immunohistochemical staining of arterial sections. A cross-section of the bifurcation area of the carotid artery from db/db mice showed markedly elevated TNF-\( \alpha \) (34.1 ± 5.5, \( P < 0.01 \)) and VCAM-1 (34.0 ± 3.3, \( P < 0.001 \)) expressions compared to lean mice. After treatment with cilostazol in the db/db mice, TNF-\( \alpha \) and VCAM-1 positive areas were significantly decreased to 16.4 ± 3.7 (\( P < 0.05 \)) and 9.1 ± 1.2 (\( P < 0.001 \)), respectively (Fig. 5).

**Cilostazol-stimulated PPAR-\( \gamma \) Transcription Activity.** After transfection of the PPRE-pGL3 vector into 3T3-L1 fibroblast cells, cilostazol (1 - 100 \( \mu \)M) and rosiglitazone (1 - 30 \( \mu \)M) significantly elevated PPAR\( \gamma \) transcriptional activity. The activity induced by 10 \( \mu \)M cilostazol increased to 12.4 ± 3.4 fold of the control (\( P < 0.05 \)), which was less by 2.5-fold that of 10 \( \mu \)M rosiglitazone (26.2±3.9 fold, \( P < 0.001 \)) (Fig. 6A). The transcription activity stimulated by cilostazol was attenuated by 1 \( \mu \)M KT5720, a cAMP-dependent protein kinase inhibitor, and 1 and 10 \( \mu \)M of GW9662, an antagonist of PPAR\( \gamma \) activity. By comparison, rosiglitazone effect was antagonized by GW9662 (1 and 10 \( \mu \)M), but marginally by KT5720 (1 \( \mu \)M) (Fig. 6 B).

We used PPAR\( \gamma \) siRNA to confirm that cilostazol specifically activates PPAR\( \gamma \) transcription 3T3-L1
fibroblast cells. Transfection of PPARγ siRNA to 3T3-L1 fibroblast cells resulted in the reduction of PPARγ protein and mRNA expression by approximately 80% (Fig. 6C). In line with these effects, cilostazol (10 μM) did not activate the PPARγ transcription activity in these 3T3-L1 fibroblast cells as did rosiglitazone (10 μM) as contrasted in the negative control cells. These results provide evidence that PPARγ transcription is, in part, required for the actions of cilostazol (Fig. 6D).

**Signal Pathways of Cilostazol-stimulated PPAR-γ Transcription Activity.** We identified the role of individual signaling pathways in the regulation of PPARγ transcriptional activity stimulated by cilostazol and rosiglitazone. Under treatment with inhibitors of PI3-K and Akt using LY294002 (10 μM) and SH5 (10 μM), cilostazol-induced PPARγ transcription was significantly suppressed (Fig. 6E), and rosiglitazone effect was suppressed under treatment with LY294002 (10 μM) and PD098059 [inhibitor of MEK1/2, the up-stream activator of extracellular signal-regulated kinases 1 and 2 (ERK1/2), 10 μM], respectively (Supplemental Figure1). Inhibitors of p38 MAP kinase (SB239063) and JNK (SP600125) showed little effect on the PPARγ transcription. These results indicate that stimulation of PPARγ transcriptional activity by cilostazol involves the PI3-K/Akt, and that by rosiglitazone implicates PI3K and MEK1/2 signal pathways in the 3T3-L1 cells.
Discussion

In the present study, treatment with cilostazol for 12 consecutive days in 8-week old db/db (leptin receptor-deficient mice, a model of Type 2 diabetes) was done to test its efficacy to suppress altered metabolic abnormalities and expressions of pro-inflammatory markers. These effects were compared to those of rosiglitazone. The results showed that cilostazol effectively lowered plasma glucose and triglyceride levels with increasing GLUT4 translocation to the plasma membrane. In conjunction with these results, cilostazol significantly ameliorated the elevated plasma insulin and resistin levels, and raised plasma adiponectin levels along with increased expression of adiponectin mRNA in the db/db mouse. These effects were comparable to those of rosiglitazone.

In previous experiments with COS-7 cells and human umbilical vein endothelial cells (Park et al., 2008), we observed that cilostazol increased PPARγ transcriptional activity, as did rosiglitazone. These increased activities are antagonized by GW9662 (Leesnitzer et al., 2002). These findings suggest that cilostazol exerts a pharmacological action similar to rosiglitazone for the activation of PPARγ transcription. Interestingly, it has been reported that protein kinase A activation enhanced PPARγ transcriptional activity (Lazennec et al., 2000). Watanabe et al. (2003) provided further evidence that 8-bromo-cAMP and forskolin increased PPARγ transcriptional activity. Lee et al. (2005) showed that cilostazol inhibition of phosphodiesterase type III significantly increased cAMP production in the aortic sinus of low density lipoprotein receptor-null mice that were fed a 0.2% w/w cilostazol-supplemented
high fat diet. In the present study using 3T3-L1 fibroblasts, cilostazol (1 - 100 μM) significantly increased PPARγ transcription, as did rosiglitazone (1 - 30 μM). The transcriptional activity induced by cilostazol was attenuated by KT5720, a cAMP-dependent protein kinase inhibitor, and GW9662, an antagonist of PPARγ activity. In contrast, rosiglitazone-induced activity was inhibited by GW9662, but not by KT5720. Thus, it seems likely that increased PPARγ transcriptional activity by cilostazol is mediated via activation of cyclic AMP-protein kinase.

Regarding the mechanism(s) by which cilostazol exerts anti-oxidative and anti-inflammatory actions, some reports showed that cilostazol protected against cell injury by suppressing NAD(P)H oxidase-dependent superoxide formation and cytokine production (TNF-α and IL-1β) in human umbilical vein endothelial cells (Shin et al., 2004). Park et al. (2005; 2006) showed that cilostazol reduced the expressions of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and a chemokine (MCP-1). In addition, it inhibited remnant lipoprotein particle-stimulated increased monocyte adhesion to human umbilical endothelial cells by suppressing NF-κB-dependent nuclear transcription via cyclic AMP-dependent protein kinase-activated maxi-K channel opening. Kim and Cheon (2006) showed that rosiglitazone activated the maxi-K channel via PPARγ activation. Based on these reports, it is suggested that maxi-K channel opening-coupled PPARγ activation by cilostazol may contribute to diminished expressions of adhesion molecules (VCAM-1) and a chemokine (MCP-1). Additional study is required to determine if the maxi-K channel opening is involved with the rosiglitazone-stimulated
PPARγ activation in 3T3-L1 fibroblasts.

Resistin plays a role in obesity-mediated insulin resistance by elevating blood glucose and insulin concentrations, and by interfering with insulin-mediated GLUT4 translocation to the cell surface in the hypoglycemic response of mice (Steppan et al., 2001; Nguyen et al., 2005). In contrast, adiponectin, a circulating protein produced by adipose tissue, has anti-inflammatory and anti-atherogenic properties that are modulated by PPARγ (Berg et al., 2001). Prolonged treatment of obese-diabetic mice with rosiglitazone increases plasma adiponectin levels and suppresses insulin resistance (Nguyen et al., 2005). These findings suggest that cilostazol may improve insulin sensitivity by upregulation of adiponectin with beneficial effects on glucose and lipid homeostasis via PPARγ activation.

Accumulating evidence shows that activation of PPARγ enhances the expressions of adipocyte-specific proteins, such as aP2 and FATP-1, and GLUT4 in the terminal differentiation stage. This leads to the accumulation of intracellular lipids and insulin-stimulated GLUT4 translocation that are coupled with glucose uptake (Rosen et al., 2000). Consistent with a report by Binnert et al. (2000), in which the FATP-1 mRNA level in human muscle with type 2 diabetes was low, the present results showed lower expressions of aP2 and FATP1 mRNA in the db/db mice, which were significantly reversed by cilostazol. In conjunction with these findings, cilostazol lowered plasma glucose levels accompanied by increased GLUT4 protein expression in the plasma membrane of visceral adipose tissue. It also decreased plasma insulin and resistin levels. These results are consistent with previous in vitro findings (Park et al., 2008), in
which the resistin-induced decreased GLUT4 expression was reversed in accordance with recovery of
glucose uptake after treatment with cilostazol. Again, this was similar to rosiglitazone. These findings
provide further evidence that cilostazol may improve insulin sensitivity via stimulation of PPARγ
transcriptional activity in the diabetic db/db mice.

Maeda et al. (2002) emphasized the importance of adiponectin for improving insulin resistance by
showing that adiponectin/ACRP30-knockout mice had delayed clearance of free fatty acids in plasma, low
levels of FATP-1 mRNA in muscle and high levels of TNF-α mRNA in adipose tissue along with high
plasma TNF-α. Maeda et al. (2002) also showed that reduced expressions of aP2 and FATP-1 mRNA in
the db/db mice corresponded to low adiponectin and high TNF-α levels in plasma. Thus, it is likely that
increased aP2 and FATP-1 mRNA expressions in the adipose tissue are related to the increased adiponectin
levels stimulated by cilostazol.

It has been shown that pro-inflammatory markers, such as reactive oxygen species, TNF-α,
interleukin-6 and C-reactive protein, are elevated in patients with type 2 diabetes (Kim et al., 2006).
Resistin and C-reactive protein upregulated adhesion molecules and chemokines, thereby providing a
mechanistic link to cardiovascular disease in the metabolic syndrome (Kawanami et al., 2006). In contrast,
evidence that adiponectin modulated endothelial inflammatory response was demonstrated by Ouchi et al.
(1999). They showed that adiponectin inhibited TNF-α-induced adhesion molecule expression on
endothelial cells, including VCAM-1, ICAM-1 and E-selectin, with suppressed monocyte adhesion to
endothelial cells.

Expressions of superoxide, TNF-α and VCAM-1 were markedly elevated in the bifurcation area of the carotid artery (Koshiyama et al., 2001) of db/db mice compared to lean mice. These pro-inflammatory markers were significantly reduced by treatment with cilostazol and rosiglitazone. Although the results are not shown, the plasma C-reactive protein level, which is known as the most sensitive marker and mediator of inflammation (Pasceri et al., 2000), was significantly suppressed by cilostazol in rats with non-genetic streptozotocin-induced type 1 diabetes. These results are consistent with the aforementioned reports of Shin et al. (2004) and Park et al. (2005; 2006).

Despite the beneficial effects of the thiazolidinediones, such as rosiglitazone and pioglitazone, for treating non-insulin dependent diabetes mellitus patients, the use of these thiazolidinediones is limited because of peripheral edema and weight gain due to increased plasma volume (Werner and Travaglini, 2001). Consistent with this report, rosiglitazone (10 mg/kg/day) treatment showed a significant weight gain at 12 days in db/db mice compared to a vehicle only group. This was in contrast to cilostazol-treated mice.

We showed that the transcriptional activity stimulated by cilostazol was attenuated by KT5720 (a cAMP-dependent protein kinase inhibitor) and GW9662 (an antagonist of PPARγ activity), whereas that by rosiglitazone was antagonized by GW9662, but only marginally by KT5720. This suggests implication of both cAMP-dependent protein kinase and PPARγ transcription activation in the actions of cilostazol.
Further, to define whether PPAR\(\gamma\) transcription is specifically involved in the actions of cilostazol, 3T3-L1 fibroblast cells were transfected with PPAR\(\gamma\) siRNA oligonucleotide. Cilostazol did not increase PPAR\(\gamma\) transcription activity in these PPAR\(\gamma\) knockdown 3T3-L1 fibroblast cells as did rosiglitazone, as contrasted to the negative control cells. Nevertheless, to provide a confirmative evidence to support implication of PPAR\(\gamma\) transcription in the action of cilostazol, transcription activity assay remains to be established by using co-transfection of vector encoding PPAR\(\alpha\) and PPAR\(\beta/\delta\) with a luciferase reporter construct under the control of PPAR-responsive elements in comparison with that of PPAR\(\gamma\).

The phosphoinositide-3-kinase (PI3K)/Akt pathway, downstream of insulin signaling, has previously been shown to be critically involved in adipocyte differentiation (Sakaue et al., 1998). This study also showed that PI3-K/Akt pathways were involved in the induction of cilostazol-induced PPAR\(\gamma\) transcription. On the other hand, PI3-K and MEK1/2 pathways were included in the rosiglitazone-induced transcription, consistent with the report of Prusty et al. (2002) demonstrating that activation of the MEK/ERK signaling pathway promotes adipogenesis by enhancing both C/EBP\(\alpha\) and PPAR\(\gamma\) gene expression during 3T3-L1 preadipocytes differentiation. Considering these results together, it is indicated that the signal pathways of both drugs are not identical.

Taken together, it is suggested that cilostazol, a non-thiazolidinedione, may offer an effective therapeutic window along with complementary effects for individuals at high risk for Type 2 diabetes by improving insulin sensitivity with anti-inflammatory effects via activation of both cAMP-dependent
protein kinase and PPARγ transcription.
References


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Footnotes

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Legends for Figures

Fig. 1. Effects of cilostazol and rosiglitazone on the plasma glucose (A) and triglyceride (B) levels from db/db mice. Animals were orally administered cilostazol (30 mg/kg/day) or rosiglitazone (10 mg/kg/day) once daily for 12 consecutive days. Results are mean ± SEM from 5 - 7 mice/group. **P < 0.01 vs. lean mice, * P < 0.05; ** P < 0.01; *** P < 0.001 vs. vehicle.

Fig. 2. Effects of cilostazol and rosiglitazone on the plasma insulin (A, N = 4 - 6 mice/group), resistin levels (B, N = 5 mice/group), adiponectin mRNA expression in visceral adipose tissue (C, N = 7 mice/group) and plasma adiponectin levels (D, N = 5 - 6 mice/group) from db/db mice. Animals were orally administered either vehicle, cilostazol (30 mg/kg/day) or rosiglitazone (10 mg/kg/day) once daily for 12 consecutive days. Results are mean ± SEM. ### P < 0.001 vs. lean mice, * P < 0.05; ** P < 0.01; *** P < 0.001 vs. vehicle.

Fig. 3. Effects of cilostazol on the aP2 mRNA (A) and FATP1 mRNA (B) expressions in the visceral adipose tissue from db/db mice. Animals were orally administered either vehicle, cilostazol (30 mg/kg/day) or rosiglitazone (10 mg/kg/day) once daily for 12 consecutive days. Results are mean ± SEM from 7 mice/group. ### P < 0.001 vs. lean mice, * P < 0.05; ** P < 0.01; *** P < 0.001 vs. vehicle.
Fig. 4. Effects of cilostazol and rosiglitazone on the GLUT4 protein expression in the membrane fraction of visceral adipose tissue from db/db mice. Results are mean ± SEM from 7 mice/group. ###P < 0.001 vs. lean mice, *P < 0.05; ***P < 0.001 vs. vehicle.

Fig. 5. A. In situ detection of superoxide anion, and immunohistochemical staining of TNF-α and VCAM-1 expression in the bifurcation region of carotid arteries from db/db mice. Animals were orally administered either vehicle, cilostazol (30 mg/kg/day) or rosiglitazone (10 mg/kg/ day) once daily for 12 consecutive days. B. Quantitative analyses. Results are mean ± SEM from 4 experiments. **P < 0.01; ###P < 0.001 vs. lean mice, *P < 0.05; **P < 0.01; ***P < 0.001 vs. vehicle.

Fig. 6. Transcriptional activation of PPARγ in 3T3-fibroblast cells. Cells were transfected with PPRE-pGL3, a renilla luciferase control reporter vector. A. Concentration-dependent increases in PPARγ transcriptional activity in 3T3-fibroblast cells that were treated with cilostazol (1 - 100 μM) and rosiglitazone (1 - 30 μM) for 24 h. Results are mean ± SEM from 4 experiments. #P < 0.05; ##P < 0.01; ###P < 0.001 vs. no treatment. B. Inhibitory effects of GW9662 and KT5720 on the cilostazol and rosiglitazone-stimulated PPARγ transcriptional activity. Results are mean ± SEM from 3 experiments. ###P < 0.001 vs. no treatment; *P < 0.05; **P < 0.01 vs. cilostazol alone; †††P < 0.001 vs. rosiglitazone alone. C. PPARγ siRNA transfection inhibits cilostazol-induced transcription. The ability of siRNA...
oligonucleotide to abrogate PPARγ expression was analyzed by RT-PCR and Western blot assay in the 3T3-L1 fibroblast cells. D. Cells were cotransfected with PPARγ siRNA and PPRE-pGL3 vector, and then PPARγ transcriptional activity in cilostazol- or rosiglitazone-stimulated cells was determined. Results are expressed as mean ± S.E.M. from 4 experiments. **P < 0.01; ###P < 0.001 versus no treatment; *** P < 0.001 versus negative control; $P < 0.05 versus cilostazol of negative control; ††P < 0.01 versus rosiglitazone of negative control. E. Effects of LY294002 (10 μM), and SH5 (10 μM) on the cilostazol-stimulated PPARγ transcription activity in 3T3-L1 cells. PD98059, SP600125 and SB239063 showed no effects, indicating stimulation of PPARγ transcription activity by cilostazol involves the signal pathways including PI3-K and Akt. Results are expressed as mean ± S.E.M. from 4 experiments. **P < 0.01 vs. no treatment; * P < 0.05; ** P < 0.01 vs. cilostazol alone.
Fig. 2
Fig. 3
Fig. 4
Fig. 5