Cilostazol Inhibits Monocytic Cell Adhesion to Vascular Endothelium Via Upregulation of cAMP

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Aim: Cilostazol is clinically used as an inhibitor of platelet aggregation. Although several reports have demonstrated its anti-inflammatory effect, its effect on monocytes and their adhesive interaction to vascular endothelium remains unclear. We thus examined the potential role of cilostazol towards monocyte endothelial interaction under physiological flow conditions.

Methods: THP-1 cells, a monocytic cell line, were pretreated with cilostazol (5 μM) for 48 hours. The cells were then perfused over TNF-alpha (5 μg/mL for 4 hours) -stimulated monolayers of human umbilical vein endothelial cells (HUVECs) at shear stress of 1.0 dyne/cm².

Results: TNF-α-activated HUVECs supported significantly more monocyte adhesion to HUVECs (7.32 ± 1.25/HPF) compared to inactivated HUVECs (0.74 ± 0.15/HPF), and the amount of adhesion to TNF-α-activated HUVECs was markedly reduced (3.63 ± 0.55/HPF) when THP-1 cells were incubated in the presence of cilostazol at 5 μM. Interestingly, surface expressions of integrins were not dramatically changed after cilostazol treatment. Intracellular concentration of cAMP was significantly increased after cilostazol treatment, and treatment with Forskolin and Dibutyryl-cAMP, potent inducers of cAMP, dramatically increased THP-1 adhesion to HUVECs.

Conclusion: These data suggest that cilostazol has a potential anti-inflammatory effect on monocyte-endothelial interactions via the upregulation of intracellular cAMP.


Key words: Atherosclerosis, Phosphodiesterase Type III (PDE3), Leukocyte-endothelial, THP-1 cells

Introduction

Atherosclerosis is a complex disease process associated with vascular wall dysfunction. The adhesion of circulating monocytes to the intimal endothelial cell monolayer is thought to be one of the earliest events in naturally occurring human and experimental animal models of atherosclerosis. Numerous adhesion receptors and counter receptors expressed on both endothelial cells and leukocytes appear to be involved in atherosclerosis, including selectins, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and β1- and β2- integrins.

Cilostazol [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone] is a phosphodiesterase of the Type III (PDE3) inhibitor and increases intracellular cyclic AMP (cAMP) levels via restraining hydrolysis of PDE3. The main actions of this compound are vasodilatation with restraint of platelet aggregation and a potential protective effect against atherosclerosis.

In vascular endothelial cells, the elevation of cAMP level leads to protein kinase A (PKA) activation followed by NO production, which may explain the anti-adhesive effect of cilostazol in endothelial cells. On the other hand, its effect on monocytes remains unclear. To gain more information about the cilostazol...
effect on atherosclerosis, we demonstrated the effects of cilostazol in monocytes and their adhesion to vascular endothelial cells.

**Materials and Methods**

**Reagents**

RPMI1640, fetal bovine serum (FBS), and forskolin were purchased from Sigma (Japan). Endothelial cell growth factor and recombinant human TNF-α were obtained from R&D Systems (Minneapolis, MN). Cilostazol was a gift from Otsuka Pharmaceutical (Japan). Dibutyryl-cAMP was purchased from Wako (Japan). HUTS21 was purchased from BD Biosciences Pharmingen (San Jose, CA). VLA4 was purchased from Upstate (Lake Placid, NY). CD18 was purchased from Southern Biotechnology Associates (Birmingham, AL). 7A10 was a gift from the Scripps Research Institute (La Jolla, CA).

**Cell Culture**

THP-1 cells were obtained from the American Type Culture Collection and grown in RPMI1640 medium containing 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine. Human umbilical vein endothelial cells (HUVECs) were isolated from normal term-umbilical veins and established in culture as previously described.9) Primary cultures were serially passaged (<1:3 split ratio) and maintained in medium 199 buffered with 25 mmol/L of RPMI1640 and supplemented with 20% FBS, endothelial cell growth factor (10 μg/mL), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and porcine intestinal heparin (50 μg/mL). For the experiments, HUVECs were sub-cultured to passage 2 or 3 and used.

**Viability Test**

To test THP-1 cell viability, we performed 6-diamidino-2-phenylindole (DAPI) staining. THP-1 cells were treated with cilostazol for 48 hours at the concentrations indicated in Table 1. DAPI staining was observed under a fluorescence microscope and the number of DAPI-positive cells was counted.

**Adhesion Assay under Laminar Flow**

The parallel-plate flow chamber used in the present study has been previously described in detail. Briefly, the chamber was composed of 2 aluminum steel plates separated by a 200-μm-thick silastic gasket, with a flow channel formed by removal of a 5 × 20-mm rectangular section of the gasket. Defined levels of flow were applied to the HUVEC monolayer by drawing the perfusion medium (D-PBS containing 0.2% human serum albumin) through the channel with a syringe pump (model 44 Harvard Apparatus). A plastic heating plate (Tokai Hit Co) was mounted on the stage of an inverted microscope (IX50, Olympus) to maintain the temperature at 37°C. Endothelial monolayers on the coverslips were stimulated with TNF-α for 4 hours and then positioned in the flow chamber, which was mounted on an inverted microscope. The monolayers were perfused for 5 minutes with perfusion medium and then examined carefully to verify them as confluent. Next, THP-1 cells were added to the perfusion medium to 10^6 cells/mL, and then drawn through the chamber for 10 minutes at controlled flow rates to generate calculated wall shear stresses of 1.0 and 2.0 dyne/cm². The entire period of perfusion was recorded on videotape with a digital video recorder that included a time generator. Captured images were then transferred to a PC for image analysis to determine the number of rolling and adherent cells in 5 to 10 randomly selected 20-times magnified microscopic fields for each experiment. Cells were considered to be adherent after 10 seconds of stable contact with the monolayer. Rolling leukocytes were easily recognized, because their velocity was much slower (up to 80 μm/s) than that of free-flowing cells. We then measured the rolling velocity using analysis software.

**Flow Cytometry**

THP-1 cells were first incubated with the indicated primary antibodies on ice for 40 minutes, washed twice with RPMI-1640 medium containing 5% FBS, and then incubated with fluorescein isothiocyanate (FITC)–labeled goat anti-mouse antibody (1:250 dilution). Fluorescence intensity was analyzed with a fluorescence-activated cell sorting system (FACSCaliber, Becton-Dickinson).

**cAMP Enzyme Immunoassay**

THP-1 cells were incubated in the presence of

**Table 1. Viable cell rate when processing THP-1 cells by cilostazol of various concentrations**

<table>
<thead>
<tr>
<th>Cilostazol (μM)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>96.3±0.4</td>
</tr>
<tr>
<td>1</td>
<td>94.7±1.0</td>
</tr>
<tr>
<td>5</td>
<td>92.7±1.0</td>
</tr>
<tr>
<td>10</td>
<td>91.3±1.0</td>
</tr>
</tbody>
</table>

Mean ± S.D.  
n = 3
cilostazol at 5 μM for 48 hours, and cAMP concentration in THP-1 cells was measured using a cAMP EIA Kit [Cayman Chemical Company: Ann Arbor, MI], following the manufacturer’s protocol. In brief, THP-1-associated cAMP (2×10⁶/cell) was extracted using 0.1 M HCl. AChE tracer and cAMP EIA antisera were added to 200 mL of extracted sample. cAMP activity was measured using a plate reader.

**Statistical Analysis**
Data are presented as the mean ± SEM as indicated. Statistical significance was calculated by one-way ANOVA with Tukey’s post-hoc analysis. P-values < 0.05 were considered significant.

**Results**

**Measurement of Viability**
First, we examined THP-1 cell viability by DAPI staining after cilostazol treatment. As shown in Table 1, cilostazol did not influence THP-1 cell viability at any concentration.

**Effect of Cilostazol on Adhesion of THP-1 Cells to Vascular Endothelium**
We examined the adhesion of THP-1 cells preincubated in the presence or absence of the indicated amount of cilostazol to activated vascular endothelium. As shown in Fig. 1A, treatment of HUVECs with TNF-α significantly induced THP-1 cell adhesion (7.32 ± 1.8 cells/HPF) as well as rolling (4.56 ± 1.3 cells/HPF). When THP-1 cells were incubated in the presence of cilostazol at 5 μM for 48 hrs, adhesion of THP-1 cells was significantly diminished (3.62 ± 0.6 cells/HPF, *p < 0.05 vs. no cilostazol). On the other hand, the number of rolling THP-1 cells was not significantly changed. Interestingly, incubation with cilostazol at 10 μM failed to reduce THP-1 cell adhesion. Next, we examined the time kinetics of the anti-adhesive effect of cilostazol at 5 μM. As shown in Fig. 1B, incubation with cilostazol gradually reduced THP-1 cell adhesion to activated HUVECs in a time-dependent manner. Representative microphotos of these interactions are shown in Fig. 2. Since preincubation with cilostazol for 48 hrs significantly inhibited THP-1 adhesion, we incubated THP-1 cells with cilostazol at 5 μM for 48 hrs.

**Effect of Cilostazol in Rolling Velocity of THP-1 Cells**
We next examined the potential effect of cilostazol on the rolling velocity of THP-1 cells over activated HUVECs. As shown in Fig. 3, the rolling velocity of THP-1 cells was significantly diminished after incubation with 1 and 5 μM of cilostazol (0 μM, 6.75 ± 1.25 μm/sec; 5 μM, 3.50 ± 0.15 μm/sec, *p < 0.05), although the number of rolling cells did not change among these conditions, as shown in Fig. 1A.

**Expression Levels of Integrin after Cilostazol Treatment**
Expression levels of integrin in THP-1 cells were
measured after cilostazol treatment and compared with control THP-1 cells. As far as we tested using antibodies against integrin-β1, VLA4, and integrin-β2, we did not detect any significant change by flow cytometry (data not shown).

cAMP Concentration is Increased in THP-1 Cells after Cilostazol Treatment

To further dissect the molecular mechanism of cilostazol-dependent modulation of THP-1 adhesion, we measured the cAMP concentration of THP-1 cells, which correlated with the amount of PDE inhibition by cilostazol. As shown in Fig. 4A, the concentration of cAMP was significantly increased after cilostazol treatment.

High-Concentration cAMP in THP-1 Cells by other Reagents Leads to Diminished Adhesion to Activated HUVEC

To evaluate the importance of cAMP concentration in cell adhesive interaction, we incubated THP-1 cells with forskolin and dibutyryl-cAMP, known inducers of cAMP. Treatment with forskolin and dibutyryl-cAMP inhibited THP-1 cell adhesion to activated HUVECs comparable to cilostazol (Fig. 4B).

Discussion

Leukocyte-endothelial interactions contribute to a variety of vascular disease processes, such as acute and chronic inflammation, and atherosclerosis.5,6,12 A number of soluble factors and cell surface adhesion
molecules, expressed by both endothelial cells and leukocytes, interact in a complex fashion to efficiently mediate leukocyte recruitment [13].

Cyclic nucleotide phosphodiesterase 3 (PDE3) belongs to the PDE family [14, 15], and is important in cyclic AMP (cAMP) -mediated signaling in vascular smooth muscle cells [15]. Intracellular cAMP level is regulated by their synthesis by adenylyl cyclases and hydrolysis by PDEs [16].

Cilostazol is a PDE3 inhibitor, and has been shown to affect endothelial cells to inhibit leukocyte and platelet adhesion [2, 17, 18]; however, the effect of cilostazol on monocytes was not extensively studied. Here we showed for the first time that cilostazol affects monocytes and reduces its adhesion to vascular endothelial cells under physiological flow conditions.

As shown in Fig. 1, inhibition of cell adhesion by cilostazol was prominent up to 5 μM, but diminished at 10 μM. We speculated that a high concentration of cilostazol (10 μM) lost its anti-adhesive effect by cell damage, the activation of various factors, or other factors, although this was not detected by DAPI staining (Table 1). When THP-1 cells were treated at 5 μM, we also observed the reduction of rolling velocity (Fig. 3). We thought that cilostazol inhibited not only stable adhesion but also the transition from rolling to stable adhesion that could be reflected in the change in rolling velocity. We would like to focus on the effect of cilostazol on rolling interaction in future projects.

In this study, we were able to demonstrate elevation of cAMP by cilostazol in monocytic cells. cAMP has been shown as a gating element in a number of different signaling pathways in monocytes [19] and it has been reported that elevation of cAMP in lymphocytes led to the suppression of immune functions by a protein kinase A-dependent mechanism [20]. Platelet aggregation was suppressed [21] by activation of PKA accompanying the rise of cAMP. cAMP enhanced NO production from endothelial cells [7, 8] by PKA and induced vasodilation. In addition, cAMP reduced intracellular Ca\(^{2+}\) concentration [22] and production of thromboxane A2 (TXA2) in platelets, thus leading to the inhibition of platelet aggregation [23]. Although we did not have direct evidence to link cAMP elevation and adhesive interaction in THP-1 cells, future studies will reveal this mechanism in detail.

It has been reported that cilostazol controlled VCAM-1 expression via inhibiting the activation of nuclear factor-kappaB [24]. In another report, it inhibited the expression of interleukin 1 (IL-1) beta, myeloperoxidase (MPO), and cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) [25] in neutrophils. In line with these previous observations, our findings in monocytic THP-1 cells confirmed the anti-inflammatory properties of cilostazol.

In conclusion, we have demonstrated that cilostazol inhibited monocytic THP-1 cell adhesion to activated endothelial cells via the elevation of cAMP in THP-1 cells. Our data may point to a new therapeutic potential of this compound.

Acknowledgments

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References

3) Schleser S, Ringseis R, and Eder K: Conjugated linoleic acids have no effect on TNFalpha-induced adhesion molecule expression, U937 monocyte adhesion, and chemokine release in human aortic endothelial cells. Atherosclerosis, 2006; 186:337-344


18) Park SY, Lee JH, Kim YK, Kim CD, Rhim BY, Lee WS, and Hong KW: Cilostazol prevents remnant lipoprotein particle-induced monocyte adhesion to endothelial cells by suppression of adhesion molecules and monocyte chemotactant protein-1 expression via lectin-like receptor for oxidized low-density lipoprotein receptor activation. J Pharmacol Exp Ther, 2005; 312:1241-1248


22) Corbin F, Blaise G, and Sauve R: Differential effect of halothane and forskolin on platelet cytosolic Ca<sup>2+</sup> mobilization and aggregation. Anesthesiology, 1998; 89:401-410

23) Kahn NN: Platelet-stimulated thrombin and PDGF are normalized by insulin and Ca<sup>2+</sup> channel blockers. Am J Physiol, 1999; 276:E856-E862
