Inhibition of Lipopolysaccharide-Induced Apoptosis by Cilostazol in Human Umbilical Vein Endothelial Cells

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Received July 2, 2001; accepted November 2, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT
This work describes the pharmacological inhibition by cilostazol and its metabolites, OPC-13015 and OPC-13213, of the apoptosis in the human umbilical vein endothelial cells (HUVECs) damaged by lipopolysaccharide (LPS) in comparison with its analog, cilostamide. Cilostazol and OPC-31213 caused a significant suppression of cell death induced by LPS (1 μg/ml) in a concentration-dependent manner but a modest suppression by cilostamide and OPC-13015. These compounds potently inhibited the 5,5-dimethyl-1-pyrroline-1-oxide (DMPO)/OH adduct formation and significantly reduced the increased intracellular reactive oxygen species (ROS) and tumor necrosis factor-α (TNF-α) production induced by LPS (1 μg/ml). An apoptotic death of HUVECs by 1 μg/ml LPS (DNA ladders on electrophoresis) was strongly suppressed by all these compounds. Incubation with LPS caused a marked decrease in Bcl-2 protein, which was significantly reversed by cilostazol and its analogs. The greatly increased Bax protein expression and cytochrome c release by LPS were, in contrast, suppressed by cilostazol and, to a lesser degree, by others. In conclusion, cilostazol and its analogs exert a strong protection against apoptotic cell death by scavenging hydroxyl radicals and intracellular ROS with reduction in TNF-α formation and by increasing Bcl-2 protein expression and decreasing Bax protein and cytochrome c release.

Cilostazol, a type III PDE inhibitor, has been introduced to increase the intracellular cyclic AMP level (Kimura et al., 1985). Its principal actions include inhibition of platelet aggregation, thrombosis, and vasorelaxation (Kimura et al., 1985; Kohda et al., 1999). The Food and Drug Administration currently approves cilostazol for treatment of intermittent claudication (Dawson et al., 1998). The cyclic AMP is a ubiquitous regulator of inflammatory and immune reactions. Katakami et al. (1988) showed that agents that increased intracellular cyclic AMP levels were able to inhibit LPS-induced TNF-α production in macrophages. TNF-α, a deleterious cytotoxic cytokine, is implicated in the ischemic brain damage through a variety of proinflammatory effects (Knoblaich et al., 1999). Koga et al. (1995) have demonstrated that TNF-α stimulates phosphodiesterase activity and decreases intracellular cyclic AMP in endothelial cells, and they suggested that intracellular cyclic AMP might modulate a signaling of TNF-α effect in the endothelial cells. On the other hand, LPS causes systemic release of TNF-α that is implicated as the primary mediator of the effects of LPS (Old, 1985), and both may mediate their deleterious effects by direct endothelial cell damage (Egido et al., 1993).

Oxidative stress is critically involved in apoptosis (Buttke and Sandstrom, 1994), and the antioxidants attenuate the apoptosis (Huang et al., 1998). Apoptosis is known as an energy-dependent molecular and biochemical process orchestrated by a genetic program (Hale et al., 1996). A growing number of studies have described that ROS, including H2O2 and its derived form hydroxyl radical, induce the apoptosis (Li et al., 1997), and the apoptotic processes of vascular smooth muscle cells play an important role in the genesis of atherosclerosis and restenosis (Kockx, 1998). Therefore, prevention of oxidative stress-mediated cell injury is an area of active investigation.

Bel-2 was originally identified as a human lymphoma oncogene (Tsujimoto and Croce, 1986) and suggested to suppress the apoptotic cell death in a variety of in vitro systems and cell lines, thereby promoting cell survival after injury (Chen et al., 1997). Bax was demonstrated, in contrast, to promote cell death (Davies et al., 1995).

Recently, we observed that cilostazol directly scavenged the hydroxyl and peroxyl radicals, but not superoxide. Furthermore, cilostazol significantly decreased the brain infarct/volume induced by occlusion of middle cerebral artery via

ABBREVIATIONS: PDE, phosphodiesterase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; ELISA, enzyme-linked immunosorbent assay; EPR, electron paramagnetic resonance; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; DCFH, 2′,7′-dichlorofluorescein; CREB, cyclic AMP-response element-binding protein.
mediation of increased cyclic AMP, scavenging ROS via a significant suppression of TNF-α formation in the cerebral cortex (J. M. Choi, H. K. Shin, and K. W. Hong, submitted for publication).

In the present study, we investigated how cilostazol and its metabolites suppress the DNA fragmentation and consequent cell death in HUVECs damaged by LPS in comparison with cilostamide, a selective PDE3 inhibitor (Sudo et al., 2000). To identify the mechanism(s) by which these compounds suppress cell death, we measured the abilities of the compounds to scavenge the ROS, including hydroxyl radicals, by EPR spin trapping and to inhibit the production of intracellular ROS. Finally, we elucidated the antiapoptotic effects of the compounds by assaying Bcl-2 and Bax protein expression.

**Materials and Methods**

**Cell Cultures.** HUVECs (CRL-1730, endothelial cell line derived from the vein of normal human umbilical cord (American Type Culture Collection, Manassas, VA)) were cultured in Kainig’s F-12K medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mg/ml heparin sodium, 0.03 to 0.05 mg/ml endothelial cell growth supplement, and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were grown to confluence at 37°C in 5% CO2 on 0.1% gelatin-coated culture dishes and used for experiments at no greater than passage 8.

**Cell Viability Assay.** Cell viability was assayed by the MTT conversion test. Briefly, HUVECs were seeded with 20,000 cells/well in 96-well gelatin-coated tissue culture plates. Confluent HUVECs received Kainig’s F-12K medium with 1% fetal bovine serum plus pharmacological reagent, 5 h prior to stimulation. Cells were exposed to LPS for 18 h. After incubation, 20 μl/well of MTT solution (5 mg/ml PBS) was added and incubated for 2 h. The medium was aspirated and replaced with 150 μl/well of ethanol/dimethyl sulfoxide solution (1:1). The plates were shaken for 20 min, and the optical density measured at 570 to 630 nm using ELISA (Bio-Tek Instruments, Inc., Winooski, VT).

**Electron Paramagnetic Resonance Spin Trapping.** The generation of hydroxyl radical characterized by EPR spin trapping techniques using DMPO was recorded in a flat-type quartz cell at room temperature using a Bruker EMX 300 X-band spectrometer (Bruker, Rheinstetten, Germany) with a TM110 cavity (a modulation frequency of 100 kHz, modulation amplitude of 1.00 G, microwave power of 2.002 mW, time constant of 81.9 ms, and center field of 3480 G). Results were expressed as arbitrary units. For hydroxyl radical, the reaction was initiated by addition of 5 μl of Fe2+ solution (10 mM FeSO4 in 10 mM HCl for Fenton reaction) to the buffer containing H2O2 (0.12 mM) and DMPO (1 mM) with each compound.

**Assay of Intracellular Reactive Oxygen Species.** Measurement of intracellular ROS was based on ROS-mediated conversion of nonfluorescent 2’,7’-dichlorofluorescin (DCFH) diacetate (Sigma Chemical Co., St. Louis, MO) into DCFH. The intensity of fluorescence reflects enhanced oxidative stress. To measure the intracellular ROS, HUVECs were preincubated for 5 h in the absence and presence of the compounds. Thereafter, cells were stimulated with 1 μg/ml LPS for 18 h, followed by incubation in the dark for 2 h in 50 mM phosphate buffer (pH 7.4) containing DCFH diacetate. This agent is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the fluorescent polar derivative DCFH and thereby trapped within the cells. The quantity of DCFH fluorescence was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm using a fluorescence plate reader (Bio-Tek Instruments, Inc.). All experiments were repeated at least three times. The background was from cell-free conditions. Results were expressed as percentage of control (nonstimulated HUVEC) fluorescence intensity.

**TNF-α Assay.** For analysis of TNF-α levels in the supernatants, confluent cells (1 × 106 HUVECs) were incubated in the 48-well plates in the absence and presence of the drug and stimulated with 1 μg/ml LPS for 18 h. TNF-α levels were assessed in supernatants using a commercially available Quantikine M human TNF-α immunoassay (R & D Systems, Minneapolis, MN), which is known to be cross-reactive with human TNF-α. TNF-α content was assessed by measuring absorbance at 450 nm using ELISA (Bio-Tek).

**DNA Fragmentation Assays.** Exponentially growing HUVECs were plated at 1 to 5 × 10⁶ cells in 100-mm culture dishes. After attachment, cells were pretreated with each compound for 5 h, followed by incubation in the medium containing 1 μg/ml LPS for 18 h. Oligonucleosomal fragmentation of genomic DNA was determined as described elsewhere. Cells were lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K). Digestion was continued for 1 to 3 h at 50°C, followed by addition of RNase A to 0.1 mg/ml and further incubation for 1 h. Running dye (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol) was added. Equivalent amounts of DNA (15–20 μg) were loaded into wells of 1.6% agarose gel and electrophoresed in the buffer (40 mM Tris-acetate and 1 mM EDTA) for 2 h at 6 V/cm. DNA was visualized by ethidium bromide staining. Quantification of bands was performed by Molecular Analyst Software using Bio-Rad’s Image Analysis System (Bio-Rad Laboratories, Hercules, CA).

**Western Blot Analyses for Expression of Bcl-2, Bax Protein, and Cytochrome c Release.** HUVECs were grown in 100-mm tissue culture dishes. The cells were washed with ice-cold PBS and lysed on ice in lysis buffer: 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100. Following centrifugation at 12,000 rpm, the protein concentration of the lysate was determined using Bio-Rad DC assay kit. For each sample, 50 μg of total protein was loaded into 12% SDS-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membrane (Amer sham Biosciences, Piscataway, NJ).

Mitochondrial cytochrome c was prepared via the following procedures. After washing cells (12 × 10⁶) with ice-cold PBS, cell pellets were resuspended in buffer A (20 mM HEPES-ROH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose. The cells were homogenized and then centrifuged twice at 750 g for 10 min at 4°C. The harvested supernatants were centrifuged at 10,000 g for 10 min at 4°C, and the resulting mitochondrial pellets were dissolved in 1× SDS sample buffer. Western blots were performed with the antibodies for Bcl-2, Bax, and cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The immunoreactive bands were visualized using chemiluminescent reagents as recommended by the Supersignal West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL). The signals of the bands were quantified using the Calibrated Imaging Densitometer (GS-710; Bio-Rad).

**Chemicals.** Cilostazol (OPC-13013) (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone) and its metabolites, OPC-13015 and OPC-13213, were generously donated by Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan) and dissolved in dimethyl sulfoxide as a 10 mM stock solution. Cilostamide was obtained from Sigma-Aldrich (Seoul, Korea) and dissolved in dimethyl sulfoxide as a 10 mM stock solution. Lipopolysaccharide (Escherichia coli, serotype 055:B5, Sigma-Aldrich) was dissolved in distilled water. MTT was purchased from Sigma-Aldrich.

**Statistical Analysis.** All results are expressed as mean ± S.E.M. Statistical differences between groups were determined by paired or unpaired Student’s t test or analysis of variance. P < 0.05 was considered to be significant.
Results

Cell Viability. The MTT conversion test showed that LPS-induced cell death was increased in a concentration-dependent manner. When HUVECs were incubated in the medium containing 1 µg/ml LPS for 18 h, cell death was 27.6 ± 5.4%. Cilostazol and OPC-31213 caused a significant suppression of cell death induced by LPS (1 µg/ml) in a concentration-dependent manner. However, cilostamide and OPC-13015 showed a modest suppression (Fig. 1). Following application of cilostazol (10⁻⁵ M) in the absence of LPS, the cell viability was 93.7 ± 4.8%.

EPR Spectra of Hydroxyl Radical Spin Adduct. EPR signals of the DMPO/OH spin adducts generated from the hydrogen peroxide-ferrous sulfate system were confirmed to be suppressed by coinubcation with catalase (0.5–10 U/ml). Cilostazol, cilostamide, OPC-13015, and OPC-13213 potently inhibited the DMPO/OH adduct formation in a concentration-dependent manner (Fig. 2). The signals were almost abolished by cilostamide, OPC-13015, and OPC-13213 at 5 × 10⁻⁷ M each, whereas these signals were suppressed by cilostazol at 10⁻⁵ M. The concentrations required to scavenge the hydroxyl radicals by 50% (IC₅₀) for cilostamide, OPC-13015, and OPC-13213 were 0.230 ± 0.005, 0.136 ± 0.04, and 0.097 ± 0.012 µM, respectively. Cilostazol has relatively low potency to scavenge the hydroxyl radical, with an IC₅₀ value of 2.58 ± 0.07 µM. When the reaction between xanthine and xanthine oxidase was used as a source of superoxide radical, those compounds including cilostazol were found not to inhibit theformation of DMPO/OOH (data not shown).

Scavenging of Intracellular ROS. The intracellularROS concentration was determined by measuring the intensity of fluorescence. Incubation of DCFH-loaded cells in the medium containing LPS (0.01 to 10 µg/ml) for 18 h showed a concentration-dependent increase in fluorescence intensity, and the intensity was over 132.7 ± 7.2% by LPS at 1 µg/ml. Pretreatment with cilostazol (10⁻⁸–10⁻⁵ M) significantly reduced the increased fluorescence stimulated by LPS (1 µg/ml) in a concentration-dependent manner. Cilostamide, OPC-13015, and OPC-13213 showed a similarity to the effect of cilostazol (Fig. 3).

Effect on TNF-α Levels. The level of TNF-α in the medium of cultured HUVECs was 22.4 ± 4.2 pg/mg protein. Upon application of LPS (0.1–100 µg/ml) for 18 h, the levels were concentration-dependently increased as shown in Fig. 4 (inset), and the level of TNF-α stimulated by 1 µg/ml LPS was 372.6 ± 15.8 pg/mg protein, which was markedly suppressed by treatment with cilostazol, cilostamide, OPC-13015, and OPC-13213 (10⁻⁵ M each) (Fig. 4).

Effect on DNA Fragmentation. Upon exposure to LPS for 18 h, cells showed morphological characteristics of apoptosis, including cell shrinkage and condensed chromatin.

![Fig. 1. Effect of cilostazol, cilostamide, OPC-13015, and OPC-13213 on cell viability measured by MTT assay in HUVECs. Cells were incubated in the medium containing 1 µg/ml LPS for 18 h. Cells were treated with each compound from 5 h before and during incubation with LPS. Inset, concentration-dependent increases in cell death by LPS. Results are expressed as mean ± S.E.M. of four experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle.](image)

![Fig. 2. Representative graphs showing effects of cilostazol, cilostamide, OPC-13015, and OPC-13213 on the EPR spectra of spin adduct of hydroxyl radicals. The signals of DMPO/OH spin adduct were generated by the reaction of H₂O₂-ferrous sulfate system in the presence of DMPO. Cilostamide, OPC-13015, and OPC-13213 potently and concentration-dependently inhibited the DMPO/OH spin adduct, whereas cilostazol showed less of a potency than other compounds.](image)

![Fig. 3. Inhibitory effects of cilostazol, cilostamide, OPC-13015, and OPC-13213 on the production of intracellular reactive oxygen species. Confluent HUVECs were preincubated for 5 h in the medium containing the compounds and stimulated with 1 µg/ml LPS for 18 h. Thereafter, the cells were incubated in Krebs-Ringer solution containing 5 mM 2′,7′-dichlorofluorescin diacetate for 2 h in the dark. Inset, concentration-dependent increases in fluorescence intensity stimulated by LPS. Results are expressed as mean ± S.E.M. of four experiments. ##, P < 0.01 and ###, P < 0.001 versus fluorescence intensity in the absence of LPS. **, P < 0.01; ***, P < 0.001 versus vehicle.](image)
Cilostazol, cilostamide, OPC-13015, and OPC-13213 (10⁻⁵ M each) strongly suppressed LPS-induced (1 µg/ml) DNA fragmentation manifested as DNA laddering (Fig. 5). The inhibition of DNA fragmentation by cilostazol was most prominent among four compounds, and OPC-13015 showed comparatively low efficacy.

**Effect on Bcl-2 Protein Expression.** Bcl-2 protein was present at a relatively high level in the control samples, which was expressed as relative density 1.0. It was concentration-dependently suppressed by LPS application. LPS (1 µg/ml) caused a significant decrease in Bcl-2 protein expression by 0.26 ± 0.04 relative density (26% of the control value). The suppressed Bcl-2 value was markedly recovered by pretreatment with cilostazol, cilostamide, and OPC-13213 (10⁻⁵ M each) to 0.80 ± 0.13, 0.68 ± 0.12, and 0.79 ± 0.05 relative density, respectively. OPC-13015 showed a modest recovery (Fig. 6).

**Effect on Bax Protein Expression.** Bax protein expression was low level in the control HUVECs obtained in the absence of LPS (expressed as relative density 1.0) but it was markedly and concentration-dependently elevated by application of 0.5, 1, and 5 µg/ml LPS to 11.1 ± 0.9, 17.5 ± 3.0, and 28.5 ± 1.4 relative density, respectively. Cilostazol (10⁻⁵ M) significantly inhibited Bax protein expression from 17.5 ± 3.0 to 3.49 ± 0.03 relative density (19.9% of the control value). However, cilostazol, OPC-13015, and OPC-13213 showed marginal inhibitory effects on the LPS-induced Bax protein (Fig. 7).

**Effect on Cytochrome c Release.** Cytochrome c release was not identified in the control samples. Thus, the cytochrome c release from mitochondria was concentration-dependently increased by LPS (0.1–5 µg/ml), and its level induced by LPS (1 µg/ml) was expressed as 100% (Fig. 8A). Cytochrome c release was significantly and concentration-dependently suppressed by cilostazol (10⁻⁶, 10⁻⁵, and 10⁻⁴ M) (Fig. 8B). Cilostazol and cilostamide (10⁻⁵ M each) inhibited more effectively LPS-induced cytochrome c release, whereas OPC-13015 and OPC-13213 (10⁻⁵ M each) showed low inhibition (Fig. 8C).

**Discussion**

In the present study, the major findings were that cilostazol and its analogs 1) strongly scavenged the hydroxyl radicals, 2) inhibited the production of intracellular ROS and TNF-α, 3) caused an increase in Bcl-2 protein and suppression of Bax protein expression and cytochrome c release, and 4) thereby exerted an inhibition of cell death induced by LPS in association with an antiapoptotic effect.

Recently, the Food and Drug Administration approved cilostazol for treatment of intermittent claudication (Dawson et al., 1998). Gotoh et al. (2000) have further reported that cilostazol treatment achieves a significant risk reduction in patients with recurrence of cerebral infarction with no clinically significant adverse reactions. The principal action mechanism of cilostazol, type III PDE inhibitor, was reported to include inhibition of platelet aggregation and vasorelaxation through activation of cyclic AMP (Umekawa et al., 1984). The importance of TNF-α and ROS generation after exposure to LPS was demonstrated to be associated with apoptosis and cell death (Böhler et al., 2000), and Polunovsky et al. (1994) reported that HUVECs hardly underwent programmed cell death in response to TNF-α alone. In the
present study, LPS was employed as an inducer of apoptosis instead of TNF-α.

An elevation of cyclic AMP was widely demonstrated to suppress superoxide and hydrogen peroxide generation in alveolar macrophages (Dent et al., 1994). Furthermore, cyclic AMP elevating agents such as Ro-201724, amrinone, milrinone, and pentoxyphylline reportedly inhibited TNF-α production in rat hearts (Katakami et al., 1988; Bergman and Holycross, 1996). TNF-α is a deleterious cytokine in stroke and mediates inflammatory, thrombogenic, and vascular changes associated with brain injury (Kochanek and Hallenbeck, 1992). TNF-α causes neuronal cell death via induction of nitric oxide or other free radicals in various cells and induces apoptosis (Kroemer et al., 1995; Li et al., 1997; Bohler et al., 2000). Incubation of HUVECs with LPS increased both intracellular ROS and TNF-α, and these variables were significantly suppressed by treatment with cilostazol and its analogs; these facts more strongly indicate that a significant inhibition of LPS-induced TNF-α production by cilostazol and its analogs may contribute to ameliorate the cell viability of HUVECs.

In the process of apoptosis, Bcl-2, a family of related genes encoding proteins that suppress programmed cell death, allows cells to adapt to an increased state of oxidative stress, fortifying the cellular antioxidant defenses and countering radical overproduction imposed by different cell death stimuli (Hockenbery, 1995). Bax, a family of genes encoding protein that renders cells more sensitive to apoptotic stimuli, is also involved in programmed cell death (Oltvai et al., 1993; Hockenbery, 1995). It has been demonstrated that both Bax protein and ROS enhance the permeability of the mitochondrial membrane and the release of cytochrome c (Marzo et al., 1998; Shimizu et al., 1999).


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