Cilostazol suppresses LPS-stimulated maturation of DC2.4 cells through inhibition of NF-κB pathway

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Summary
Cilostazol is a phosphodiesterase-3 inhibitor that functions as a platelet aggregation inhibitor and is used for treating peripheral artery diseases and ischemic stroke. Dendritic cells (DCs) play an active role in the immunological processes related to atherosclerosis. Cilostazol has anti-atherogenic and anti-inflammatory effects, but the effects of cilostazol on DC maturation remain unknown. The purpose of this study was to determine the effects of cilostazol on lipopolysaccharide (LPS)-induced maturation of DCs. DC2.4 cells were treated with cilostazol for 12 h and subsequently stimulated with LPS to induce maturation. Cilostazol reduced the expression of maturation-associated markers induced by LPS, such as CD40, CD86, and MHCII, improved the endocytotic function, and decreased production of the tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) of these cells. To further elucidate the mechanisms responsible for the inhibition of DC2.4 maturation by cilostazol, we investigated the effect of cilostazol on LPS-stimulated nuclear factor-kappa B (NF-κB) activation. Our results indicated that cilostazol treatment decreased IkBα degradation and inhibited NF-κB p65 translocation, and the inhibitory effects of cilostazol were cAMP-independent. Therefore, inhibition of NF-κB by cilostazol might result in the suppression of DC maturation. In conclusion, cilostazol suppressed LPS-stimulated DC maturation, which might contribute to its anti-atherosclerosis effect.

Key words: atherosclerosis; cilostazol; dendritic cells; lipopolysaccharide; nuclear factor-κB

INTRODUCTION
Atherosclerosis is a chronic inflammatory and immunological disease that accounts for most deaths in the majority of industrial countries. The development of atherosclerotic lesions is associated with a great variety of exogenous and endogenous risk factors and involves the inflammatory responses of the innate and adaptive immune systems (Ross 1999, Wick and Xu 1999, Hansson et al. 2002). Dendritic cells (DCs) are the most potent antigen-presenting cells in the body and have the unique ability to initiate a primary immune response to certain antigens by the activation of naive T cells (Banchereau and Steinman 1998). During recent years, the dominant role of DCs in the pathophysiological setting of atherosclerosis has become more and more apparent (Ludewig et al. 2002). DCs are present in their immature forms in
normal arteries and become mature DCs clustered with T cells during atherogenesis, suggesting that DC maturation is linked to the progression of atherosclerosis (Banchereau et al. 2000). Thus, inhibition of DC maturation might be beneficial for preventing the initiation and progression of atherosclerosis (Bobryshev 2005).

Cilostazol is a selective inhibitor of phosphodiesterase 3 (PDE3) and is known to increase intercellular cAMP content and activate protein kinase A (PKA), resulting in peripheral vasodilation and inhibition of platelet aggregation (Kimura et al. 1985). Cilostazol has been used clinically to treat peripheral artery diseases and ischemic stroke (Gotoh et al. 2000, Regensteiner and Hiatt 2002). In addition to its antiplatelet and vasodilator properties, studies have demonstrated the anti-atherogenic activity of cilostazol in different ways (Lee et al. 2005, Takase et al. 2007, Tsai et al. 2008). The effects of cilostazol on DC maturation remain unknown, so flow cytometry and cytokine assays were all used to detect DC maturation, and the possible mechanisms involved were preliminarily investigated.

**MATERIAL AND METHODS**

**Chemicals**

Cilostazol was kindly provided by Otsuka Pharmaceutical (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-labelled antibodies to mouse CD40 and CD86 and phycoerythrin (PE)-labeled antibody to mouse MHCII, and their corresponding isotope controls, were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Lipopolysaccharide (LPS) from *Escherichia coli* (055:B5), SQ22536, 3-isobutyl-1-methylxanthine (IBMX) and FITC-labeled dextran (40kd molecular mass) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies for mouse NF-κB p65 and IκB-α was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies for mouse β-actin and histone were purchased from Bioworld Technology (St. Louis Park, MN, USA).

**Cell culture**

DC2.4 cells, an immature cell line established from bone marrow progenitors from C57BL/6 mice (Shen et al. 1997), were kindly provided by Dr. Yan Wen (Cancer Research Institute, Southern Medical University, China). The cells were cultured in complete RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 μg streptomycin/ml, and 100U penicillin/ml. DC2.4 cells were maintained at 37 °C in a humidified incubator with 5% CO₂. Cells were passaged every three days and utilized for experimentation at 60–80% confluence.

**Flow cytometry**

DC2.4 cells were harvested and suspended in cold PBS containing 2% FBS and 0.1% NaN₃, blocked with rat IgG on ice for 15 min at 4 °C, and then washed. This was followed by incubation with an FITC-conjugated anti-mouse CD40 antibody, CD86 antibody, or PE-conjugated anti-mouse MHCII antibody for 40 min at 4 °C in the dark. Isotype-matched monoclonal antibodies were used as controls. After staining, the cells were analysed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were collected for 1.0×10⁴ cells per sample.

**Phagocytosis assays**

The phagocytes in mammals are reported to exhibit day-night variation in their phagocytic activity (Berger and Slapničková 2003), so to analyse the endocytosis of the DCs, 2×10⁵ cells were incubated at 37 °C for 1 h with 1 mg/ml dextran-FITC. Cells were then washed twice with cold PBS containing 5% FBS and analysed using a FACS Calibur flow cytometer. Control experiments were performed at 4 °C for 1 h to show that the uptake of dextran-FITC by the DCs was inhibited at low temperatures.

**Cytokine assays**

Media of cultured DCs were harvested and kept at –80 °C. Levels of IL-6 and tumor necrosis factor-α (TNF-α) in the supernatants from cultured cells were analysed using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, Franklin Lakes, NJ, USA). The cytokine concentration was evaluated according to the manufacturer’s instruction.
RNA isolation and quantitative real-time PCR (qRT-PCR)
DC2.4 cells were treated with various concentrations of cilostazol (10, 20, or 40 μM) for 12 h followed by 1 μg/ml LPS treatment for 6 h. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and then treated with DNase I. The extracted total RNA was then reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Paisley, UK) according to the manufacturer’s instructions. Real-time PCR reactions were performed in a 50-μL mixture containing 5 μl of the cDNA preparation, 5× PCR mix (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA, USA), and 10 pmol of each primer in a ABI 7500 real time PCR system (Applied Biosystems, Foster City, CA, USA) using the following PCR parameters: 93 °C for 3 min followed by 40 cycles at 93 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The fluorescence threshold (Ct) was calculated using the system software. GAPDH served as an internal standard for mRNA expression. Transcriptional activity of the TNF-α, IL-6, and GAPDH genes was evaluated using the software. The absorbance at 450 nm was determined using a microplate reader.

Intracellular cAMP measurements
DC2.4 cells were treated with LPS (1 μg/ml), cilostazol (40 μM) or IBMX (50 μM) for 10 min and then with lysis buffer. The intracellular cAMP was measured using a Parameter ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The absorbance at 450 nm was determined using a microplate reader.

Nuclear and cytoplasmic extracts and Western blot analysis
DC2.4 cells were treated with cilostazol (10, 20, or 40 μM) for 12 h prior to LPS (1 μg/ml) treatment for 30 min. DC nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic reagents (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Equal amounts of protein (20 μg) were loaded per lane, resolved by SDS-PAGE, and then electrotransferred to a nitrocellulose membrane. The membrane was incubated with the indicated antibodies to NF-κB p65 and IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed, further incubated with HRP-conjugated anti-rabbit IgG (1/1000 dilution), and detected using an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) following the manufacturer’s instructions.

Statistics
SPSS 13.00 for Windows statistic software was used. All statistical analyses are expressed as mean ± standard deviation (SD) of the indicated number of experiments. Data were compared using one-way analysis of variance as indicated, and this was followed by a post-hoc least significant difference (LSD) test. Data were evaluated at the significance level 2α=0.05.

RESULTS
Measure of viability
We first examined DC2.4 cell viability after incubation with various concentrations of cilostazol in the absence or presence of LPS. As shown in Fig. 1, the concentrations of cilostazol used (10, 20, and 40 μM) did not affect cell viability. Thus, 10, 20, and 40 μM of cilostazol were used in the subsequent experiments.

Fig. 1. Effect of cilostazol on the cell viability of DC2.4 cells. Cells were treated with the indicated concentrations of cilostazol (0, 10, 20, 40 and 80 μM) for 12 h followed by 1 μg/ml LPS treatment for 24 h. Cell viability was assessed by Cell Counting kit-8, and the results are expressed as the percentage of surviving cells over controls cells (no addition of cilostazol and LPS). Each value indicates the mean±SD and is representative of results obtained from three independent experiments.
Fig. 2. Cilostazol suppresses the expressions of typical surface molecules CD40, CD86 and MHCII during DC maturation. DC2.4 cells were pretreated with 40 μM cilostazol for 12 h followed by 1 μg/ml LPS treatment for 24 h. Cell surface molecules were analysed by flow cytometry. (A) The histograms consist of the expressions of DC2.4 cells treated with cilostazol, LPS and cilostazol+LPS. (B) The bar graphs show mean fluorescence intensity (MFI) results (mean±SD). (a) CD40, (b) CD86, (c) MHCII. Results are representative of three independent experiments. * Statistically significant versus cells treated with LPS in the absence of cilostazol.

Immunophenotypic expression of DC2.4 cells
The expression of typical surface molecules indicating DCs growth (from immature to maturation state) was detected (Fig. 2). DC2.4 cells were treated with cilostazol (40 μmol/L) for 12 h followed by LPS (1 μg/ml) for 24 h. Incubation with LPS alone markedly increased the expression of CD40, CD86, and MHCII. Unstimulated DC2.4 cells treated with cilostazol did not affect CD40, CD86, or MHCII expression. Pretreatment with cilostazol prevented the increased levels of CD40, CD86, and MHCII expression in LPS-stimulated DC2.4 cells.
Endocytosis in DC2.4 cells

FITC-dextran was used to determine the level of endocytosis in DCs. We analysed the antigen uptake of DC2.4 cells using a FACSCalibur flow cytometer (Fig. 3). Incubation with LPS alone decreased the endocytosis ability of DC2.4 cells. Unstimulated DC2.4 cells treated with cilostazol did not demonstrate any alteration in their endocytosis ability. Pretreatment with cilostazol partly reversed the decreased endocytosis in LPS-stimulated DC2.4 cells. The same experiments were also performed at 4 °C to show that the uptake of FITC-dextran by DC2.4 cells was inhibited at low temperature. These results are further evidence that cilostazol interferes with DC maturation.

Effect of cilostazol on TNF-α and IL-6 production in LPS-stimulated DC2.4 cells

Because cilostazol concentrations from 10 to 40 μM did not affect cell viability, 10, 20, and 40 μM cilostazol were chosen for analysing the effect of cilostazol on TNF-α and IL-6 production. DC2.4 cells were treated with the various concentrations of cilostazol for 12 h and then stimulated with LPS (1 μg/ml) for 24 h. The levels of TNF-α and IL-6 in the supernatants of cultured cells were analysed by ELISA. The production of TNF-α and IL-6 was increased in LPS-stimulated DC2.4 cells, and this was significantly attenuated by pretreatment with cilostazol (Fig. 4A). In a parallel experiment, qRT-PCR was performed to determine if cilostazol could inhibit the expression of these cytokines at the transcriptional level. The results indicated that treatment with cilostazol decreased TNF-α and IL-6 mRNA expression in DC2.4 cells stimulated with LPS (Fig. 4B).

Effect of cilostazol on cAMP production in LPS-stimulated DC2.4 cells

Cilostazol is a selective inhibitor of PDE3, which increases the level of intracellular cAMP. To evaluate if the inhibitory effect of cilostazol on DC maturation was dependent on the cAMP signalling pathway, we examined the effects of cilostazol, IBMX (a nonselective PDE inhibitor) on intracellular cAMP levels in DC2.4 cells. Intracellular cAMP levels were increased by IBMX, but not by cilostazol (Fig. 5A). In addition, in order to confirm the cAMP-dependent or -independent inhibitory effect of cilostazol on DC maturation, we examined the effects of SQ22536 (an adenylyl cyclase inhibitor and an irreversible and selective cAMP antagonist) on CD86 expression and TNF-α production. The results showed that SQ22536 did not reverse the inhibitory effect of cilostazol on CD86 expression (Fig. 5B) or TNF-α production.
Fig. 4. Effect of cilostazol on LPS-induced TNF-α and IL-6 production in DC 2.4 cells. Cells were treated with cilostazol (10, 20 and 40 μM) for 12 h prior to 1 μg/ml LPS treatment, supernatants and total RNA were isolated at 24 h or 6 h after LPS treatment, respectively. (A) The extracellular levels of TNF-α and IL-6 were measured in culture media using ELISA Kit. (a) TNF-α, (b) IL-6. (B) The levels of TNF-α and IL-6 mRNA were determined by quantitative real-time PCR after normalising to GAPDH and expressed as percentage relative to the untreated group. (a) TNF-α, (b) IL-6. Each value indicates the mean±SD and is representative of results obtained from three independent experiments. Symbols as in Fig. 2.

Inhibitory effect of cilostazol on NF-κB activation

NF-κB activation is an important event underlying DC maturation (Rescigno et al. 1998). Translocation of NF-κB from the cytoplasm to the nucleus is essential for LPS-stimulated maturation of DCs (An et al. 2002, Kaisho and Tanaka 2008). To identify whether the inhibitory effects of cilostazol on LPS-stimulated DC2.4 cells were regulated by the NF-κB, degradation of IκBα in the cytoplasm and nuclear translocation of the NF-κB p65 subunit in the nuclear extracts of DC2.4 cells were assessed by Western blot analysis (Fig. 6). LPS (1 μg/ml) strongly induced NF-κB p65 translocation in the nuclear extracts in association with markedly degraded IκBα in the cytoplasmic extracts of DC2.4 cells. Cilostazol inhibited LPS-induced IκBα degradation and NF-κB p65 translocation into the nuclei. These results indicated that cilostazol inhibited LPS-induced NF-κB activation.

DISCUSSION

In the present study, we describe how LPS-induced DC2.4 maturation was accompanied by phenotypic expression of costimulatory molecules and maturation markers in DC2.4 cells, such as CD40, CD86, and MHCII. Moreover, decreased endocytosis and increased expression of cytokines (i.e., IL-6 and TNF-α) in DC2.4 cells were observed. These effects could be reversed by treatment with cilostazol.

Cilostazol has been widely used to treat peripheral artery diseases and ischemic stroke. Recent studies have shown that cilostazol has anti-atherogenic and
Fig. 5. Effect of intracellular cAMP on CD86 expression and TNF-α production in DC2.4 cells. (A) Intracellular cAMP levels. Cells were treated with LPS (1 μg/ml), cilostazol (40 μM) or IBMX (50 μM) for 10 min, intracellular cAMP levels were measured using ELISA kit. Each value indicates the mean±SD and is representative of results obtained from three independent experiments. * Statistically significant versus cells treated with LPS and cilostazol.

(B) Effects of cilostazol on CD86 expression and TNF-α production by SQ22536 in LPS-stimulated DC2.4 cells. Cells were pretreated with SQ22536 (10 μM) for 30 min followed by cilostazol (40 μM) for 12 h, and then the cells were stimulated with LPS (1 μg/ml) for another 24 h. The CD86 expressions were analysed by flow cytometry and the extracellular levels of TNF-α in culture medium was measured using ELISA kit. (a) CD86 expression, (b) TNF-α production. Each value indicates the mean±SD and is representative of results obtained from three independent experiments. * Statistically significant versus cells treated with LPS in the absence of cilostazol.

anti-inflammatory effects. Cilostazol significantly decreases plaque lesion volume in low-density lipoprotein receptor-null mice (Lee et al. 2005) and suppresses the progression of atherosclerosis in ApoE KO mice (Takase et al. 2007). Furthermore, cilostazol reduces neointimal hyperplasia and macrophage infiltration in balloon-injured rabbit aortas and inhibits LPS-induced monocyte chemoattractant protein-1 (MCP-1) and MMP-9 expression in monocyctic THP-1 cells (Tsai et al. 2008). In addition, cilostazol significantly decreases the production of nitric oxide (NO), prostaglandin E2, interleukin-1 (IL-1), TNF-α, and MCP-1 in BV2 microglia following LPS stimulation (Jung et al. 2010) and attenuates LPS-induced cytokine expression (i.e., IL-6, TNF-α, and IL-1) in RAW 264.7 macrophages (Park et al. 2010). A recent study also observed that cilostazol can suppress TNF-α production in J774 murine macrophages through induction of heme oxygenase-1 expression (Park et al. 2011). Cilostazol can also suppress antigen-induced T-cell responses and Th17 cell differentiation in vitro, which correlates with enhanced Treg cell responses (Wang et al. 2010).

Inflammatory and immune injuries are the leading mechanisms underlying atherosclerotic vessel diseases (Ross 1999, Wick and Xu, 1999, Hansson et al. 2002, Ludewig et al. 2002). DCs were identified in human arteries in 1995 (Bobryshev and Lord 1995a). Since then, much has been reported on their effect on atherosclerosis. In healthy arteries, small numbers of DCs locate to the subendothelial layer of the intima, which act as a screen to recognize foreign and auto-antigen (Bobryshev 2005, Hansson 2005). In atherosclerotic arteries, the number of DCs increases and clusters, which indicates their potential role in the inflammatory-immune reactions related to atherosclerosis (Bobryshev and Lord 1995b, Bobryshev 2000, Soilleux et al. 2002, Cao et al. 2003, Bobryshev 2005). Corresponding to these pathological findings, studies have shown that DC maturation can be induced by different pro-atherogenic stimuli such as ox-LDL, advanced glycosylation end products, and nicotine, enabling these mature DCs to induce antigen-specific T-cell activation (Perrin-Cocon et al. 2001, Aicher et al. 2003, Ge et al. 2005). In atherosclerotic plaques, the colocalisation of DC and T cells indicates that the mature and activated DCs initiate an antigen-specific immune response, contributing to the progression of atherosclerosis (Bobryshev and Lord 1998). In LDLR−/− mice, hypercholesterolaemia-induced atherosclerotic lesions can be significantly reduced by depleting intimal CD11c+ DCs (Paulson et al. 2010). These findings suggest a close relationship between DCs and atherogenesis.
Fig. 6. Inhibitory effect of cilostazol on the NF-κB p65 translocation in DC2.4 cells. Cells were treated with Cilostazol (10, 20, 40 μM) for 12 h prior LPS (1 μg/ml) treatment for 30 min. DC nuclear and cytoplasmic extracts were prepared and subjected to Western blotting with indicated antibodies to IκBα and NF-κB p65. The results shown were representative of three independent experiments.

The maturation of DCs is the pivotal step for their function in the immune response. Activated DCs can arouse T-cell activation, further stimulating vascular inflammation and adhesion of monocytes in the atherosclerotic plaque (Bobryshev 2005). Our study was intended to investigate the effects of cilostazol on DCs maturation.

In this current study, we used LPS as a stimulus to induce DC2.4 cell maturation. The LPS-stimulated DC2.4 cells demonstrated increased phenotypic expression of costimulatory molecules and maturation markers of DCs (i.e., CD86, CD40 and MHCII), decreased endocytosis function, and increased cytokine secretion (i.e., IL-6 and TNF-α), which was consistent with the results of previous studies (Rhule et al. 2008, Fu et al. 2011). Cilostazol reduced the expression levels of CD86, CD40 and MHCII in LPS-stimulated DC2.4 cells. It is known that MHCII on DCs present the antigen peptide to helper T cells and CD86 is believed to be the most critical molecule for the amplification of T-cell responses. Ligation of CD40 on DCs acts as a maturation signal, increasing antigen presentation and the expression of other costimulatory molecules (Fujii et al. 2004). Reduced expression of costimulatory molecules is insufficient for DCs to activate naïve T cells, resulting in a reduced immune response mediated by T cells.

The upregulation of costimulatory molecules alone is insufficient for T-cell priming (Pasare and Medzhitov 2004). Mature DCs also secrete many cytokines, including IL-6 and TNF-α, which are two important pro-inflammatory cytokines that induce expression of costimulatory molecules and enhance DC interactions with T cells (Reis 2006). We observed that cilostazol decreased the IL-6 and TNF-α production in LPS-stimulated DC2.4 cells.

The ability to uptake antigens is efficient in immature DCs but is reduced upon maturation, and this is a characteristic and distinctive property differentiating immature and mature DCs (Reis 2006). In our study, the antigen uptake ability of DC2.4 cells was decreased after LPS stimulation. Treatment with cilostazol significantly prevented this downregulation of antigen uptake.

It is well known that cilostazol is a selective inhibitor of PDE3, which results in increased intracellular cAMP levels and subsequent suppression of inflammation (Tsai et al. 2008). Previous study has shown that cAMP-elevating agents such as IBMX suppress LPS-stimulated bone marrow-derived DC function via elevation of intracellular cAMP levels (Kambayashi et al. 2001). In our study, intracellular levels of cAMP in DC2.4 cells were not increased by cilostazol. In addition, we examined the effects of SQ22536 on the CD86 expression and TNF-α production in DC2.4 cells. Our results showed that SQ22536 did not reverse the effects of cilostazol on CD86 expression or TNF-α production. Thus, our study showed that the inhibitory effects of cilostazol on DC2.4 maturation were likely to be cAMP independent. Cilostazol inhibits LPS-stimulated DC2.4 maturation through other mechanisms.

Previous studies have shown that translocation of NF-κB from the cytoplasm to the nucleus is essential for LPS-stimulated maturation of DCs and that this controls the expression of pro-inflammatory cytokines (Kaisho and Tanaka 2008, Lu et al. 2008). Inactive NF-κB resides in the cytoplasm and is bound by its inhibitory subunit, IκB-α. Inflammatory stimuli, such as endotoxin, lead to degradation of IκB-α by promoting its phosphorylation, thus allowing the active NF-κB translocation (Brown et al. 1995). Studies have shown that treatment with cilostazol can inhibit LPS-induced NF-κB activation in vitro (Lee et al. 2005, Jung et al. 2010, Park et al. 2010). In order to identify the mechanisms by which cilostazol inhibited LPS-stimulated DC2.4 maturation, degradation of IκB-α in the cytoplasm and activation of NF-κB in the nuclear extracts of DC2.4 cells were assessed by Western blot analysis. In our study, LPS-induced IκB-α degradation and NF-κB p65 activation were reversed following cilostazol
treatment. Therefore inhibition of NF-κB by cilostazol might result in suppression of DC maturation.

Taken together, our study showed that cilostazol suppressed the phenotypic and functional maturation of DC2.4 cells through the inhibition of NF-κB, which maybe an important mechanism contributing to its anti-atherosclerosis effect.

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**COMPETING INTERESTS**

The authors declare they have no conflict of interest.

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