Journal of Cellular Biochemistry 103:54–66 (2008)

Cilostazol Attenuates MCP-1 and MMP-9 Expression In Vivo in LPS-Administrated Balloon-Injured Rabbit Aorta and In Vitro in LPS-Treated Monocytic THP-1 Cells

Chien-Sung Tsai,[1] Feng-Yen Lin,[2] Yung-Hsiang Chen,[3] Tung-Lin Yang,[1,4] Hsiao-Jung Wang,[5] Guo-Shine Huang,[6] Chih-Yuan Lin,[1] Yi-Tin Tsai,[1] Shing-Jong Lin,[5] and Chi-Yuan Li[6,7]*

[1]Division of Cardiovascular Surgery, Tri-service General Hospital, National Defense Medical Center, Taipei, Taiwan
[2]Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan
[3]Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan
[4]Department of Molecular and Cellular Biology, Graduate Institute of Basic Medical Science, Chang-Gung University, Taipei, Taiwan
[5]Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan
[6]Department of Anesthesiology, Tri-service General Hospital, National Defense Medical Center, Taipei, Taiwan
[7]Department of Medical Education and Research, Buddhist Tzu-Chi General Hospital, Taipei, Taiwan, Republic of China

Abstract Monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) are involved in vascular inflammation. We tested the hypothesis, and explored the underlining mechanisms that cilostazol, a phosphodiesterase 3 inhibitor with antiplatelet and antithrombotic properties, inhibits lipopolysaccharide (LPS)-induced MCP-1 and MMP-9 expression. In a rabbit aorta balloon-injury model, administration of LPS increased macrophage infiltration and MCP-1 and MMP-9 expression; cilostazol supplementation prevented this phenomenon and reduced intimal hyperplasia. In contrast, the reverse zymography showed that cilostazol did not affect TIMP-1 expression in serum. In monocytic THP-1 cells, cilostazol and N6,O2′-dibutyryl-cAMP (dioctanoyl-cAMP, a cAMP analog) dose-dependently inhibited LPS-induced MCP-1 protein expression and MMP-9 activation, but did not affect the tissue inhibitor of metalloproteinase-1. Quantitative real-time polymerase chain reaction (PCR) showed that cilostazol inhibited MCP-1 and MMP-9 mRNA expression. Cilostazol significantly inhibited LPS-induced activation of p38, JNK, and nuclear factor-κB, and the respective inhibitors of p38 and JNK greatly reduced the level of LPS-induced MCP-1 and MMP-9, suggesting the involvement of the p38 and JNK pathways. In conclusion, cilostazol administered with LPS in vivo reduced neointimal hyperplasia and macrophage infiltration in the balloon-injured rabbit aorta; in vitro, cilostazol inhibits LPS-induced MCP-1 and MMP-9 expression. These data suggest that cilostazol may play an important role in preventing endotoxin- and injured-mediated vascular inflammation. J. Cell. Biochem. 103: 54–66, 2008.

Key words: cilostazol; phosphodiesterase; monocyte chemoattractant protein-1; matrix metalloproteinase; lipopolysaccharide; inflammation

Atherosclerosis is a chronic vascular inflammatory response [Chait et al., 2005]. The cascade of leukocyte–endothelial cell interactions and leukocyte infiltration are crucial processes in atherogenesis. Leukocyte recruitment to inflammatory sites involves multiplex factors, such as the expression of cytokines, chemokines, and matrix metalloproteinases (MMPs) [Abusamieh and Ash, 2004]; these locally produced factors guide circulating leukocytes into the atherosclerotic site. The chemokines

Feng-Yen Lin, Yung-Hsiang Chen, and Tung-Lin Yang contributed equally and Shing-Jong Lin and Chi-Yuan Li contributed equally to this work.

*Correspondence to: Chi-Yuan Li, MD, MS, Department of Medical Education and Research, Buddhist Tzu-Chi General Hospital, No.289, Jian-Guo Rd., Xindian City, Taipei 231, Taiwan, ROC. E-mail: cyli@tzuchi.com.tw

Received 16 October 2006; Accepted 27 March 2007
DOI 10.1002/jcb.21388

bind to the activated cell surface, and generally behave as potent chemotactic factors for leukocytes and dendritic cells [Gadella and Jovin, 1995]. Monocyte chemoattractant protein-1 (MCP-1), a member of the CC chemokines, is characterized by its chemoattractant activity for monocytes, T cells, mast cells, and basophils [Rollins et al., 1991]. In addition to promoting the transmigration of circulating monocytes into tissues, MCP-1 exerts various other effects on monocytes, including superoxide anion induction and chemotaxis. [Yla-Herttuala et al., 1991]. Growing evidence suggests that MCP-1 is important in the inflammatory processes associated with the pathogenesis and progression of atherosclerosis [Aukrust et al., 2001], acute coronary syndrome [Matsumori et al., 1997], and congestive heart failure [Matsumori et al., 1997].

MMPs increase matrix degrading activity and accelerate leukocyte infiltration into vulnerable regions of human atherosclerotic plaques. The release of MMPs from leukocytes and the degeneration of extracellular matrix and soluble proteins by proteolysis are important factors contributing to inflammation. Clinically, the MMPs are implicated in morphogenesis, and tissue remodeling, in diseases such as arthritis, atherosclerosis, asthma, and tissue ulceration, and in the processes of tumor [Yassen et al., 2001]. MMP-9 degrades type IV collagen, the major constituent of basement membranes, and is released by macrophages, smooth muscle cells, and endothelial cells [Goetzl et al., 1996]. Elevated MMP-9 concentration in plasma has been shown in inflammatory illnesses such as rheumatoid arthritis, sepsis [Tomita et al., 2002], and acute respiratory distress syndrome, and in cardiovascular diseases [Rohde et al., 1999].

For more than a century, it has been demonstrated that infectious agents are responsible for vascular diseases [Nieto, 1998]. Systemic inflammation induced by LPS increases neoinitimal formation after balloon and stent injury in rabbits, which results from the increasing of serum IL-1β level and monocytes CD14 expression [Danenberg et al., 2002]. Although endotoxin has the potential role as a proinflammatory mediator of vascular diseases, the postulated mechanisms remain to be clarified.

Cilostazol is an inhibitor of cAMP phosphodiesterase III (PDE3), which elevates the intracellular cAMP level. Cilostazol is a platelet aggregation inhibitor and vasodilator and is useful for treating intermittent claudication [Dawson et al., 1998]. In addition to its antiplatelet and vasodilatation effects, many other anti-inflammatory effects have been described. Cilostazol suppresses the expression of heparin-binding epidermal growth factor-like growth factor in lipopolysaccharide (LPS)-stimulated smooth muscle cells and macrophages [Kayanoki et al., 1997] and reduces superoxide anion formation and nuclear factor-κB (NF-κB) activation in high cholesterol-fed mice [Lee et al., 2005]. Cilostazol also suppresses hyperplasia formation in balloon-injured rat arteries [Yoshimura et al., 1991], prevents restenosis after coronary stent implantation by inhibiting Mac-1-leukocytes [Husid, 2004], and inhibits platelet-derived growth factor-stimulated cell proliferation by increasing cAMP levels and inhibiting the mitogen-activated protein kinase (MAPK) signaling pathway [Matousovic et al., 1995]. Because PDE3 activity and expression increase significantly in the aorta of atherosclerosis-prone insulin-resistant rats [Thomas et al., 1990], inhibition of PDE3 may be therapeutically useful for treating and preventing atherosclerosis. This evidence suggests that cilostazol has the potential to prevent inflammatory responses during atherogenesis. However, little information is available about the effects and underlying molecular mechanisms of cilostazol on chemokines, MMP expression, or the activation of leukocytes. To elucidate the molecular mechanisms and identify the potential loci for therapeutic intervention, we employed a two-pronged approach with an in vivo system of simulating chronic inflammation by LPS-administration in endothelium-denuded rabbits and a more simplistic in vitro approach of eliciting inflammation, a key step in atherosclerosis, in cultured human monocytic cells (THP-1 cells) by the LPS. In addition, the effects of cilostazol treatment on these systems, focusing on MCP-1 and MMP-9 as well as relevant signal transduction pathways. Our study shows that cilostazol inhibits LPS-induced MCP-1 and MMP-9 expression by increasing cAMP and inhibiting the p38/JNK pathway, resulting in suppression of neointimal hyperplasia.

MATERIALS AND METHODS

Reagents for In Vitro and In Vivo Studies

Cilostazol was purchased from Otsuka Pharmaceutical Co Ltd.; LPS from *Escherichia*,

O55:B5, was purchased from Sigma; PD98059, SB230580, and SP600125 were purchased from Molecular Probes, Inc. The N6, O2′-dibutyryl-cAMP (dioctanoyl-cAMP) was purchased from Calbiochem.

Animal Balloon-Injury Experiment

An animal model that reflects the clinically relevant events of chronic inflammation and injury-induced atherogenesis was used. Thirty adult male New Zealand white rabbits (~2.5 kg) were provided a commercial normal chow diet of 60 g/kg/day and water ad libitum. The animals were randomly divided into five groups (six animals/group): group 1 served as the control; group 2 (LPS) received intravenous injections of LPS (220 ng/kg) through the ear vein; group 3 (endothelium–denudation, ED) received the balloon-injury treatment of the abdominal aorta; group 4 (ED+LPS) received the balloon-injury treatment and intravenous injections of LPS; and group 5 (cilostazol, STA-ZOL + ED + LPS) received 50 mg/day of cilostazol from weeks 1 through 5 in addition to receiving ED plus LPS. LPS was injected immediately and 1 week after balloon injury. Balloon injury of the abdominal artery was performed with a 3F Fogarty catheter through the femoral artery in anesthetized rabbits, passed to the abdominal aorta (~16 cm), inflated with normal saline, and withdrawn four times. Heparin (100 U/kg) was administered immediately after arteriotomy. Arterial blood was collected from the ear artery into sodium citrate-containing tubes. The animals were sacrificed at the end of 5 weeks, and the vessels were collected for immunohistochemical staining. All animals were treated under protocols approved by the Institutional Animal Care Committee of the National Defense Medical Center. The experimental procedures and animal care conformed to the "*Guide for the Care and Use of Laboratory Animals*" published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Immunohistochemical Staining and Intimal Hyperplasia Measurement

The harvested abdominal aortas were rinsed with ice-cold PBS, fixed in 4% buffered formaldehyde for 3 h at 4°C and subsequently dehydrated in sequential alcohol washes, cleared in xylene, and embedded in paraffin. Immunohistochemical staining was performed on serial 5-μm-thick paraffin-embedded sections from rabbit abdominal aortas using RAM-11, MCP-1, and MMP-9 antibodies (all working concentration of antibodies were 0.5 μg/mL). The antigen signal was developed with 3, 3′-diaminobenzidine or fluorescein isothiocyanate. Morphometry were used for intimal hyperplasia measurement as previous described [Lin et al., 2002]. One 5 mm thick cross-section was taken from each segment of the abdominal aorta and stained with hematoxylin and eosin. Morphometric analysis of six arterial cross-sections per animal was performed using an LV-2 Image Analyzer (Winhow Instruments, Taipei, Taiwan). The intima surface area /media surface area for each arterial cross section specimen was also determined.

Western Blot Analysis of Rabbit Serum MCP-1

Western blot analysis was used to determine the MCP-1 level in rabbit serum. Two hundred microliters of serum were pooled in each group (6 animals) and 100 ml of pooled serum was loaded to 15% SDS–polyacrylamide gels, subjected to SDS–PAGE, transferred to a PVDF membrane, and probed with antibodies (working concentration was 0.25 μg/ml) directed to MCP-1. Images were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies and chemiluminescence detection reagents (NEN Life Sciences).

Chemoinvasion Assay

The chemotactic activity of THP-1 cells in response to conditioned rabbit serum was measured in a 24-well Chemotaxis Transwell plate (Corning Costar, Cambridge, MA). THP-1 cells were resuspended in RPMI 1640 consisting of 0.5% bovine serum albumin at a density of 1.5×10^6/mL and transferred to the upper chamber of the Transwell plate. The conditioned rabbit serum was added to the lower chamber of the Transwell plate. The lower and upper chambers were separated by a 5-μm pore size polycarbonate membrane. THP-1 cells were left to transmigrate for 2 h. After incubation at 37°C in a humidified atmosphere with 5% CO_2, the number of cells that had migrated through the filter was determined by counting the cells under light microscopy.

Reverse Zymography for Rabbit Serum Tissue Inhibitor of Metalloproteinase-1 Activity

Reverse zymography was used to determine the TIMP-1 activity in rabbit serum. One microliter of serum was mixed with an equal volume of 2x SDS-Tris-glycine sample buffer, and then applied to a 12.5% SDS–PAGE containing gelatin and 10 μg/ml MMP-9. Serum containing TIMP-1 was electrophoresed, and the gels were washed with 2.5% Triton X-100. The gels were incubated in 50 mM Tris, pH 7.5, and 5 mM $CaCl_2$ at 37°C for 18 h, and then stained with Coomassie brilliant blue R-250 (0.2% Coomassie brilliant blue R-250, 45% methanol, 5% acetic acid). The blue gelatin staining was cleared by gelatinase action except where the TIMP-1 band blocked this activity.

Cell Culture

THP-1 cells, a human promyelomonocytic cell line, were obtained from ATCC (VA, USA) and grown in RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% antibiotic–antimycotic mixture. The cell density was maintained between 5×10^4 and 8×10^5 viable cells/ml, and the medium was refreshed every 2–3 days.

Quantitative Polymerase Chain Reaction

THP-1 cells were treated with cilostazol for 30 min only or 0–100 μM cilostazol followed by 100 ng/ml LPS treatment for 4 h. Total RNA was isolated from THP-1 using a TRIZOL reagent kit according to the manufacturer's instructions. cDNA was synthesized from total RNA using Superscript® II reverse transcriptase. Quantitative real time PCR was performed using a LightCycler and the FastStart DNA Master SYBR Green I kit (Roche, USA). The level of MCP-1 and MMP-9 mRNA expression was determined in arbitrary units by comparing with an external DNA standard that was amplified by the MCP-1 or MMP-9 primers. PCR primers were used as follows. MCP-1 forward primer, 5′-GGA GCA TCC ACG TGT TGG C-3′ and reverse primer, 5′-ACA GCT TCT TTG GGA CAC C-3′; MMP-9 forward primer, 5′-ACC TGG TTC AAC TCA CTC CG-3′ and reverse primer, 5′-AAG ATG CTG CTG TTC AGC G-3′; and GAPDH forward primer, 5′-TGC CCC CTC TGC TGA TGC C-3′ and reverse primer, 5′-CCT CCG ACG CCT GCT TCA CCA C-3′.

Enzyme-Linked Immunosorbent Assay

THP-1 cells seeded in 24-well plates at a density of 10^6 cells/ml/well, and were then pretreated with various concentrations of cilostazol for 20 min followed by LPS stimulation (100 ng/ml). After 24 h, the conditioned medium was collected to quantify MCP-1 using the DuoSet ELISA development kit (R&D Biosystems). In this assay, the solid-phase antibody captures the cytokines present in the standards or unknowns. Biotinylated goat anti-human MCP-1 antibody and streptavidin–HRP was added to each well in sequence. The optical density at 450 nm was determined using a microplate reader.

Gelatin Zymography

MMP-9 activity was determined by gelatin zymography as described previously [Kondo et al., 2002]. The THP-1 cells were cultured and treated as described; 10 μL of conditioned culture-medium was mixed with an equal volume of 2 × Tris-glycine-SDS sample buffer, and then applied to gelatin zymography gels. After electrophoresis, proteins were renatured in renaturing buffer and placed at 37°C for overnight developing in developing buffer. Gelatinase activity was revealed by negative staining with Coomassie brilliant blue R-250 (0.1% Coomassie brilliant blue R-250, 45.5% methanol, 9% acetic acid) and quantified by densitometry.

Western Blot Analysis of MAPKs

Western blot analysis was conducted to determine the activation of MAPKs in THP-1 cells stimulated by LPS. Total protein extracts were subjected to 10% SDS–PAGE and transferred to a PVDF membrane and probed with antibodies directed to p38, phospho-p38, SAPK/JNK, phospho-SAPK/JNK, p44/p42 (ERK) MAPK, or phospho-p44/p42 MAPK. Images were developed with HRP-conjugated secondary antibodies and chemiluminescence detection reagents (NEN). Protein expression levels were quantified according to optical density using ImageQuant software v.5.2 (Molecular Dynamics).

Electrophoretic Mobility Shift Assay

THP-1 cells were pretreated with cilostazol for 30 min followed by LPS stimulation for

45 min, and then the nuclear protein was extracted. Nuclear protein extracts were prepared as previously described [Santibanez et al., 2003]. The NF-κB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 3′-TCA ACT CCC CTG AAA GGG TCC G-5′) or AP-1 oligonucleotide (5′-CGC TTG ATG AGT CAG CCG GAA-3′ and 3′-GCG AAC TAC TCA GTC GGC CTT-5′) were used for EMSA, and the double-stranded DNA was end-labeled with digoxin-dUTP using terminal transferase. The DNA-binding reaction was performed with 6 μg of nuclear proteins and digoxin-labeled oligonucleotide at room temperature. Nuclear extract–oligonucleotide mixtures were separated from the unbound DNA probe by electrophoresis through a native polyacrylamide gel. The digoxin-labeled oligonucleotide was detected with anti-digoxin antibody conjugated with alkaline phosphatase. When the immunoblotting was completed, the signal was exposed to X-ray film. The supershift assays were performed with anti-p65 antibody specific to NF-κB and anti-c-jun antibody specific to AP-1. For a competition test, added 100-fold of "cold" competition probe to the binding reaction mixture before adding the labeled probe.

Statistical Analysis

Values are expressed as the mean ± SEM. Data were analyzed using Student's t test and one-way ANOVA followed by the Dunnett's test. $P < 0.05$ was considered significant.

RESULTS

Cilostazol Attenuates MCP-1 and MMP-9 Expression in Endothelium-Denuded Abdominal Aorta of Rabbits With Systemic Inflammation

The expression of MCP-1 and MMP-9 as well as the infiltration of monocytes play a crucial role during restenosis formation. We conducted the animal experiment to determine the influence of cilostazol on MCP-1 and MMP-9 expression and intimal hyperplasia in endothelium-denuded rabbit aorta with LPS-induced systemic inflammation. Figure 1A shows that the intima was slightly thickened in the LPS and ED groups and markedly thickened in the ED + LPS group compared with these areas in the control group. The ratio of intima surface area/media surface area was significantly lower in the STAZOL + ED + LPS group (0.37 ± 0.09) than in the ED + LPS group (2.32 ± 0.39, $P < 0.05$). Serial sections of abdominal aortas were stained immunohistochemically with antibodies against MCP-1, MMP-9, and RAM-11 (to identify macrophages). Compared with the control group, positive MCP-1 staining, but not MMP-9 staining, was seen on the luminal surface in the LPS-treated group. MCP-1 and MMP-9 expression was not significantly higher in the ED group than in the control group. Strong positive MCP-1 and MMP-9 staining was seen in the markedly thickened intima in the ED+LPS group. MCP-1 and MMP-9 expression in the intimal area was lower in the STAZOL + ED + LPS group than in the ED + LPS group. Monocytes will be activated and differentiated to macrophage in vessel wall after LPS and endothelium-denuded were administrated. We analyzed the expression of MMP-9 and MCP-1 in infiltrated/differentiated-monocytic cells, macrophages in vivo. Staining with antibody against RAM-11 to identify infiltrated macrophages showed that fewer macrophages infiltrated into the vessel wall. An exception was for the ED + LPS group. The infiltration of macrophages was significantly lower in the STAZOL + ED + LPS group than in the ED + LPS group. These results demonstrate that cilostazol significantly reduced macrophage infiltration and MCP-1 and MMP-9 expression in the neointima.

Cilostazol Reduces the MCP-1 Level in Rabbit Serum and Attenuates Rabbit Serum-Induced THP-1 Cell Chemotaxis

To determine the effect of cilostazol on MCP-1 level in rabbit serum, MCP-1 secretion was analyzed by Western blot and THP-1 cell chemotaxis was measured in the chemoinvasion assay using pooled serum. Western blot analysis (Fig. 1B) were repeated for three times and the representative data showed that the level of secreted MCP-1 was slightly higher in the ED and LPS groups than in the control group. A markedly stronger signal was observed in the ED + LPS group, and cilostazol treatment significantly reduced MCP-1 expression in the STAZOL + ED + LPS group. These results demonstrate that cilostazol significantly reduced the MCP-1 level in the serum of endothelium-denuded rabbit aorta in conjunction with systemic inflammation. The chemoinvasion assay showed that, compared with random migration ($0.5 \pm 0.1 \times 10^5$ cells) and the control ($1.0 \pm 0.3 \times 10^5$ cells) group, THP-1

cell migration was slightly higher in the ED group ($1.7 \pm 0.2 \times 10^5$ cells) and moderately higher in the LPS group ($2.1 \pm 0.6 \times 10^5$ cells). Cilostazol treatment (STAZOL + ED + LPS group, $3.1 \pm 0.7 \times 10^5$ cells) significantly inhibited the chemotactic activity ($9.1 \pm 1.4 \times 10^5$ cells) compared with the ED + LPS group (Fig. 1C). Because TIMP-1 reduces the activity of MMP-9, we also used reverse zymography to determine the influence of cilostazol on TIMP-1 activity in the serum. Figure 1D shows that TIMP-1 activity did not differ significantly between any of the groups, suggesting that the effect of cilostazol on decreased MMP-9

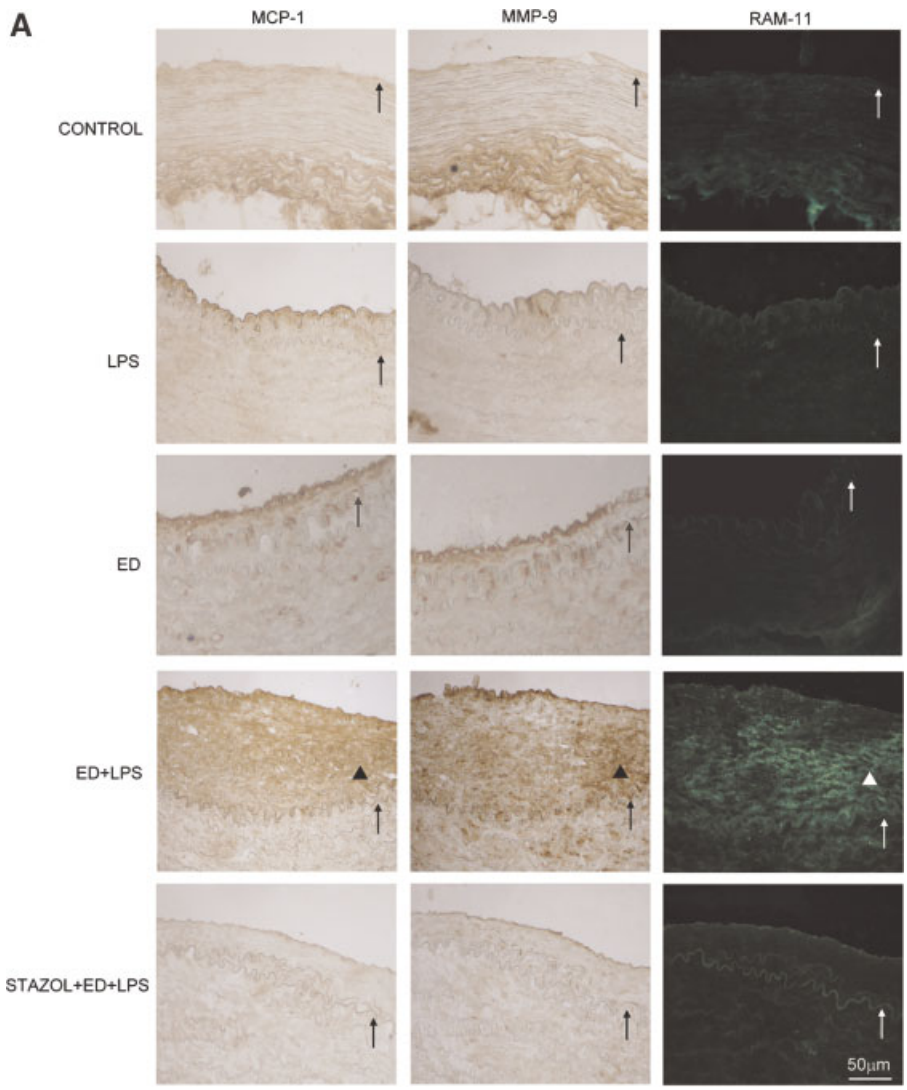

Fig. 1. MCP-1, MMP-9, and RAM-11 expression in rabbit abdominal aorta. The vessels were collected for immunohistochemical or immunofluorescent staining as described in the Materials and Methods. Antibody against RAM-11 was used for macrophages (indicated by arrowheads). The lumen is uppermost in all sections, and the internal elastic laminae are indicated by arrows. Scale bar indicates 50 μm (**A**). The level of plasma MCP-1 in the various groups (control, LPS, ED, ED + LPS, and STAZOL + ED + LPS) was analyzed by Western blot; the relative intensity of each band was measured by densitometry (**B**). The chemotactic activity of THP-1 cells in response to conditioned rabbit serum (**C**). THP-1 cells were incubated with RPMI 1640 medium with 1% BSA at 37°C for 2 h in the upper compartment of a chemotaxis chamber. The number of THP-1 cells that migrated to the lower compartment was counted under light microscopy. In random migration control experiments, RPMI 1640 medium was used in the lower compartment. The number of migrating cells is expressed as mean ± SEM. $^*P < 0.05$ compared with group 1 (control); $^\dagger P < 0.05$ compared with group 4 (ED + LPS). The effect of cilostazol on serum TIMP-1 activity in endothelium-denuded rabbit aortas with systemic inflammation (**D**). Bar graphs of the reverse zymography analysis data show relative intensity of each band measured by densitometry. Data are expressed as mean ± SEM and represent the results of three independent experiments.

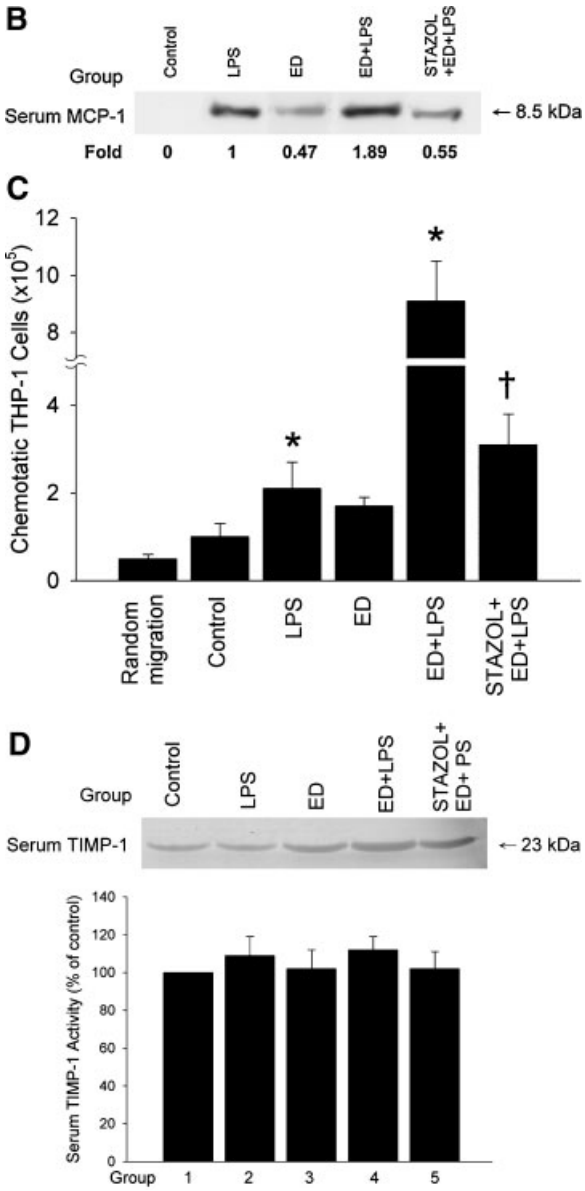

Fig. 1. (*Continued*)

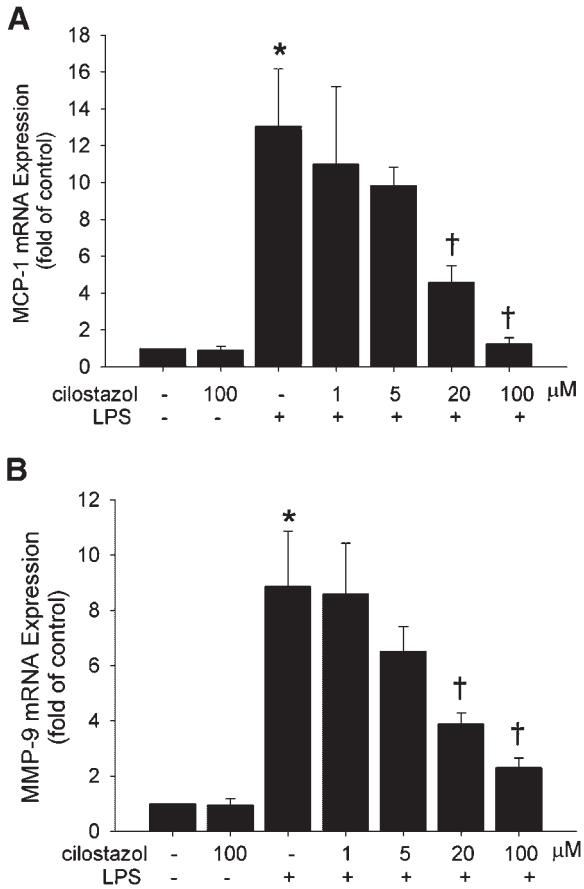

Fig. 2. Cilostazol reduces MCP-1 (**A**) and MMP-9 mRNA (**B**) expression. THP-1 cells were pretreated with 1–100 μM cilostazol for 30 min followed by 100 ng/ml LPS treatment for 4 h. MCP-1 and MMP-9 mRNA expression was analyzed by quantitative real-time PCR after normalizing to GAPDH. The results are from three separate experiments. Data are expressed as percentage (mean ± SEM) relative to the untreated group as a control. $^{*}P < 0.05$ compared with the control group; $^{\dagger}P < 0.05$ compared with the LPS-treated group.

expression in endothelium-denuded rabbit aorta in conjunction with systemic inflammation is not mediated by modulating TIMP-1 activity.

Cilostazol Suppresses LPS-Induced MCP-1 and MMP-9 mRNA Expression in THP-1 Cells

We next investigated the effects of cilostazol and the mechanisms underlying these effects on LPS-induced monocyte MCP-1 and MMP-9 activation in vitro using a monocytic THP-1 cell culture model. Q-PCR analysis showed that LPS significantly increased MCP-1 (Fig. 2A) and MMP-9 (Fig. 2B) mRNA expression. Pretreatment of THP-1 cells with cilostazol for 30 min reduced MCP-1 and MMP-9 mRNA expression in a dose-dependent manner.

Cilostazol Inhibits LPS-Induced MCP-1 Production and MMP-9 Activity in THP-1 Cells

To investigate whether cilostazol affects LPS-induced MCP-1 production and MMP activity, we used ELISA to quantify MCP-1 production and gelatin zymography analysis to measure MMP activity. Cells were pretreated with various concentrations of cilostazol for 30 min and were then stimulated with 100 ng/ml LPS for 24 h; the conditioned medium was collected to determine MCP-1 production and MMP

activity. ELISA showed a significantly higher MCP-1 level in the conditioned medium of the LPS-treated group (975.6 ± 99.9 pg/ml) than in the control group (447.6 ± 5.5 pg/ml, $P < 0.05$). Pretreatment with cilostazol suppressed LPS-induced MCP-1 expression in a concentration-dependent manner (Fig. 3A). Quantification of the band intensities with densitometry of the gelatin zymography analysis showed that $\geq$20 μM cilostazol significantly inhibited MMP-9 activity (Fig. 3B).

Cilostazol Inhibits MCP-1 and MMP-9 by Increasing cAMP and Inhibiting MAPKs

Because LPS activates MAPK signaling and cilostazol elevates the intracellular cAMP level, we investigated the underlying mechanisms involved in MCP-1 and MMP-9 regulation. Dioctanoyl-cAMP, a cell-permeable cyclic-AMP analog, decreased LPS-induced MCP-1 expression (Fig. 4A) and increased MMP-9 activity in a concentration-dependent manner (Fig. 4B), suggesting that increasing cAMP concentration inhibits MCP-1 and MMP-9. LPS caused a phosphorylation of p38 MAPK and SAPK/JNK but had no effect on ERK. SB203580 (a p38 MAPK inhibitor), SP600125 (an SAPK/JNK inhibitor), and PD98059 (an ERK inhibitor) inhibited LPS-induced MCP-1 (Fig. 4C) and MMP-9 (Fig. 4D). Pretreatment with cilostazol decreased LPS-induced p38 MAPK and SAPK/JNK phosphorylation (Fig. 4E) in a dose-dependent manner. These results suggest that cilostazol inhibits MCP-1 and MMP-9 by increasing the cAMP level and by inhibiting the p38 MAPK and SAPK/JNK pathways.

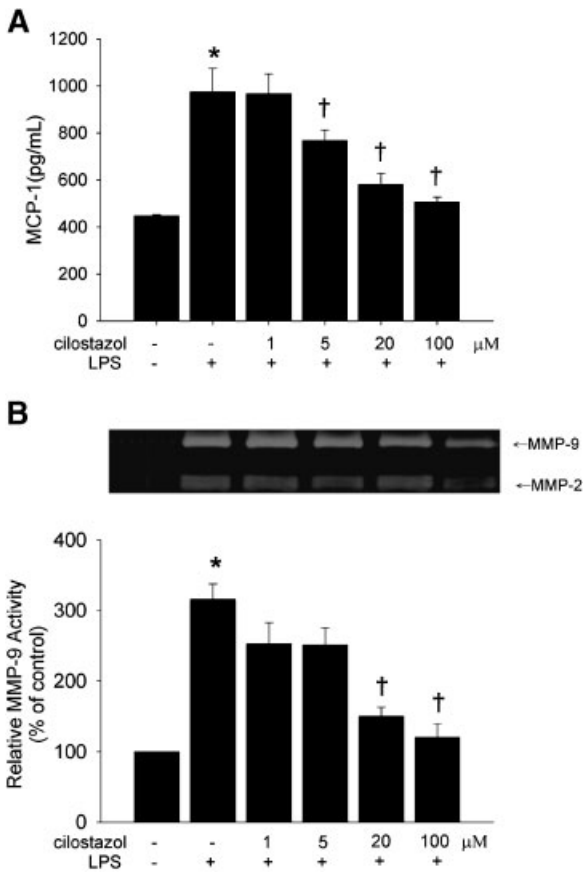

Fig. 3. Cilostazol reduces MCP-1 expression and MMP-9 activity in LPS-induced THP-1 cells. THP-1 cells were pretreated with cilostazol (1–100 μM) for 30 min followed by 100 ng/ml LPS treatment for 24 h. MCP-1 expression was quantified by ELISA (**A**), and secreted MMP-9 activity in the culture medium was measured by gelatin zymography (**B**). Bar graphs in the zymography analysis data show relative intensity of each band measured by densitometry. Data are presented as mean ± SEM and represent the results from three independent experiments. *$P < 0.05$ compared with control group; †$P < 0.05$ compared with the LPS-treated group.

Cilostazol Attenuates LPS-Induced NF-κB But Not AP-1 Activation

The activation of nuclear transcription factors such as NF-κB and AP-1 plays a key role in inflammation. EMSA showed that treatment of THP-1 cells with LPS activated both NF-κB (Fig. 5A) and AP-1 (Fig. 5B). Pretreatment with cilostazol (100 μM) significantly reduced the densities of the NF-κB-shifted bands in the LPS-stimulated groups. However, cilostazol has no significant inhibitory effect on LPS-induced AP-1 activation. The supershift assays were performed with anti-p65 antibody specific to NF-κB and anti-c-jun antibody specific to AP-1. The competition assays were used to check the specific for the probes of NF-kB and AP-1.

DISCUSSION

Our data demonstrate that MCP-1 and MMP-9 are upregulated in the rabbit neointima after arterial injury in a model of systemic inflammation. cilostazol significantly reduces the extent of neointimal hyperplasia; this reduction is associated with decreased MCP-1 and MMP-9 expression and macrophage infiltration. The in vitro study demonstrated that cilostazol inhibits the induction of MCP-1 and MM-9 by LPS in monocytes through the cAMP and p38/JNK signaling pathways.

We selected cilostazol as a compound with potential clinical use because of its potent antiplatelet and vasodilatory effects, and

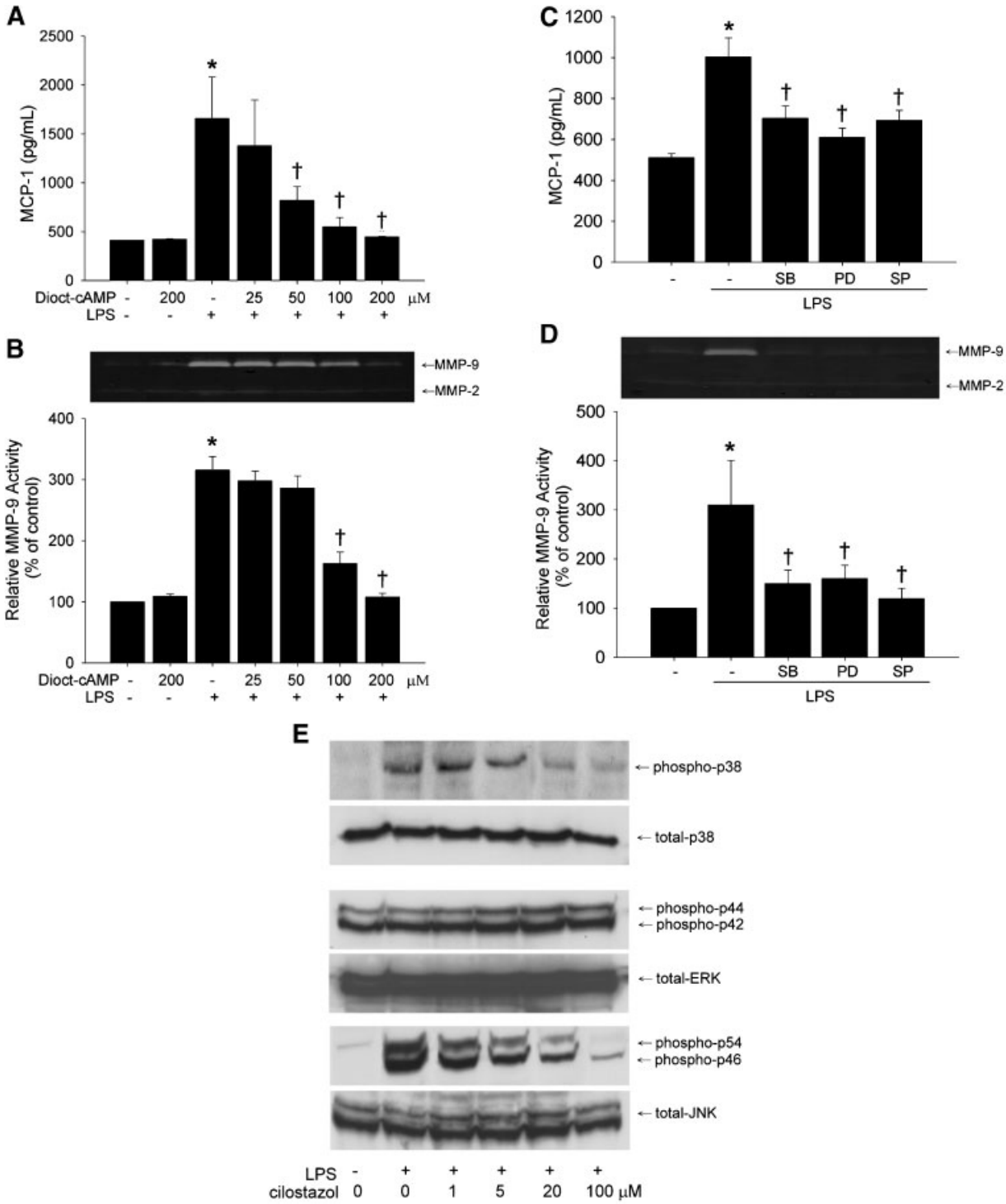

Fig. 4. Regulation of cAMP and MAPK signaling pathways on LPS-induced MCP-1 and MMP-9 expression. THP-1 cells were pretreated with dioct-cAMP (25–200 μM) for 30 min and then treated with 100 ng/mL LPS. Expression of MCP-1 and MMP-9 in the medium was verified by ELISA (**A**) and zymography analysis (**B**), respectively. THP-1 cells were pretreated with 10 μM of SB203580, SP600125, or PD98059 before LPS treatment, and the expression of MCP-1 and MMP-9 in the medium were verified by ELISA (**C**) and zymography analysis (**D**), respectively. THP-1 cells were pretreated with 0–100 μM of cilostazol for 30 min followed by LPS treatment for 5 min. p38, SAPK/JNK, and ERK activation were analyzed by Western blot (**E**). Bar graphs in the zymography data show relative intensity of each band measured by densitometry. Data represent the results of three independent experiments (mean ± SEM). $^{*}P < 0.05$ compared with control group; $^{\dagger}P < 0.05$ compared with the LPS-treated group.

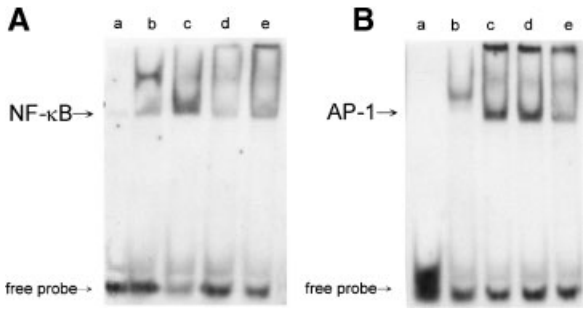

Fig. 5. The EMSA for activation of NF-κB (**A**) and AP-1 (**B**) in LPS-treated THP-1 cells. Nuclear proteins were extracted from untreated control cells (**lane a**), LPS-treated cells (**lane c**; 100 ng/ml for 45 min), cells treated with 100 μM cilostazol plus LPS (**lane d**). EMSA was performed as described in the Materials and Methods. **Lane b** represents cells treated in the supershift assay with anti-p65 antibody specific for NF-κB and anti-c-jun antibody specific for AP-1. **Lane e** represents cold competition assays with 100-fold of non-labeled probe to confirm the specific of NF-κB and AP-1.

minimal cardiac effects. Cilostazol also suppresses the expression and release of P-selectin and GPIIb/IIIa [Kariyazono et al., 2001], and inhibits the P-selectin-mediated interactions between platelets, leukocytes, and vascular endothelial cells [Kayanoki et al., 1997]. Our parallel ex vivo studies also demonstrated that cilostazol inhibits ADP-induced expression of P-selectin in platelets and the interaction of platelets and THP-1 cells (data not shown). The inhibitory effect of cilostazol on platelet function cannot be excluded as an explanation of the reduced hyperplasia and macrophage infiltration in our vascular damage model. Nevertheless, cilostazol in vitro inhibited MCP-1 secretion and MMP-9 activity in THP-1 cells in a platelet-free system, suggesting that the inhibitory in vivo effects of cilostazol on neointimal hyperplasia may act through both platelet-dependent and independent mechanisms.

The time course of changes in the plasma concentration of cilostazol followed a two-compartment model; we estimate that the peak concentration was 775 ng/ml in plasma (~2.09 μM) 3.65 h after oral administration of 100 mg cilostazol [Woo et al., 2002]. However, the pharmacokinetic and pharmacodynamic mechanisms of cilostazol are not understood completely. It is possible that some of its metabolites accumulate with prolonged treatment and that these may concentrate in different tissues. Our ex vivo studies demonstrated that ~5 μM cilostazol significantly inhibits ADP-induced expression of P-selectin in platelets and THP-1 cells–platelet aggregation (unpublished data). These data show that a lower concentration (~5 μM) of cilostazol is required to inhibit platelet function than the concentration (≥20 μM) needed to inhibit MCP-1 production and MMP-9 activity in THP-1 cells. In our animal study, the rabbits were provided commercial cilostazol at 50 mg/day by oral administration. Assuming a mean body weight of ~3 kg for each rabbit, we calculate that the plasma concentration of cilostazol could reach a peak value of ~15 μM. We also found that MCP-1 and MMP-9 expression were inhibited significantly by cilostazol in the in vivo ED+ LPS-treated group, which is similar to the results of the in vitro studies showing that ≥20 μM of cilostazol significantly inhibits MCP-1 expression and MMP-9 activity in THP-1 cells.

Monocyte infiltration and accumulation in the subendothelial space of the arterial wall is a prominent pathological change in atherogenesis [Gerszten et al., 2000]. Increasing evidence indicates that local overexpression of MCP-1 by infiltrating monocytes or vascular cells induces the accumulation of monocytes and the formation of form cells. Cilostazol inhibits MCP-1 expression in tumor necrosis factor-α (TNF-α) or remnant lipoprotein-induced vascular endothelial cells [Park et al., 2005], attenuates lumen renarrowing after coronary stent implantation [Douglas et al., 2005], and suppresses neointimal hyperplasia and smooth muscle cell proliferation in artery grafts [Fujinaga et al., 2004]. Our animal study provides the first evidence that systemic inflammation caused by LPS enhances MCP-1 expression in endothelium-denuded rabbit aorta. The data are also the first to show that administration of cilostazol inhibits the expression of MCP-1, resulting in decreased inflammatory response as evidenced by decreased macrophage infiltration and neointimal formation.

MMP-9 is secreted predominantly by macrophagesm, which plays an important role in tissue remodeling and provides the link to degenerative inflammation. Thus, regulation of MMP-9 is being actively pursued as a potential strategy to treat diseases using more selective approaches [Yassen et al., 2001]. Previous reports show that PDE inhibitors play a critical role in modulating MMP-9 expression [Oger et al., 2005]. Cilostazol also decreases the production of MMP-9 in tumor cells [Kim et al.,

2002]. We speculate that the salutary effects of cilostazol in preventing endotoxin- and injury-induced restenosis may arise from its antiplatelet properties [Dawson et al., 1998], and possibly by inhibition of MMP-9 activation. It is conceivable that some of the benefits of cilostazol result from a remodeling regulatory effect, which may serve to stabilize the atherosclerotic plaque from rupture and prevent macrophage infiltration.

MAPKs are activated in response to a wide variety of stimuli. Our results show that cilostazol inhibited LPS-stimulated signal transduction pathways (p38 and JNK) in THP-1 cells. The JNK inhibitor SP600125 markedly inhibited the expression of MCP-1 and the p38 inhibitor SB203580 markedly inhibited the activity of MMP-9 in LPS-treated THP-1 cells. Interestingly, the ERK inhibitor PD98059 also showed similar effects on MCP-1 and MMP-9 inhibition. Although all MAPK inhibitors can significantly inhibit LPS-activated MCP-1 and MMP-9 in THP-1 cells, cilostazol reduced the phosphorylation of p38 and JNK without affecting ERK, suggesting that the p38 and JNK pathways play a more important role in mediating the effect of cilostazol.

LPS stimulation activates two major transcription factors, NF-κB and AP-1, which in turn induce the expression of genes involved in chronic and acute inflammatory responses [Lin et al., 2005]. NF-κB and AP-1 activity can be modulated though phosphorylation by MAPKs. Cilostazol inhibits c-*Jun* and c-*Fos* phosphorylation as well as JNK upregulation, which is stimulated by TNF-α, and contributes substantially to suppress MCP-1 production by down-regulating AP-1 and NF-κB signaling [Lin et al., 2005]. We found that cilostazol significantly suppressed NF-κB activation but not AP-1 activation. Our findings raise the possibility that cilostazol reduces MCP-1 and/or MMP-9 expression by decreasing NF-κB activity.

Cilostazol increases the intracellular cAMP level by inhibiting PDE3. Previous reports indicate that activation of cAMP contributes to the anti-inflammatory effects of cilostazol, which inhibit the MAPK activation and NF-κB signaling pathways [Iwamoto et al., 2003]. The PDE3 antagonist-regulated cAMP pool is functionally related to PKA activation, phosphorylation, which ultimately decrease MAPK activity [Matousovic et al., 1995]. Cilostazol inhibits MCP-1 production by increasing cAMP/PKA signaling in vascular cells [Nishio et al., 1997; Matsumoto et al., 2005]. Our results support the notion that cAMP elevation plays a critical role in inhibiting the expression of MCP-1 and/or MMP-9, as previously described [Wuyts et al., 2003], although different mechanisms may be involved in the cAMP-regulation of MCP-1 and MMP-9 expression.

A recent study reported that cilostazol strongly inhibits TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1), reducing U937 cell adhesion to vascular endothelial cells [Ohtsuki et al., 2001]. Common events in the vascular response to arterial injury include proliferation and migration of vascular smooth muscle cells within the arterial intimal, and neointimal formation in injured vessels [Pauletto et al., 1994]. Because endothelial expression of adhesion molecules and smooth muscle cell proliferation are important events in atherogenic processes, cilostazol may prevent atherosclerosis through these antiplatelet-independent pleiotropic effects.

In conclusion, our data show that cilostazol effectively attenuates neointimal hyperplasia, inhibits macrophage infiltration, and decreases MCP-1 and MMP-9 expression in endothelium-denuded aortas of rabbits with LPS-induced systemic inflammation. We also demonstrated that cilostazol significantly decreases MCP-1 and MMP-9 in LPS-stimulated monocytic THP-1 cells by cAMP-, p38/JNK-, and NF-κB-related pathways. Our findings provide novel insights into the pleiotropic effects of cilostazol; these anti-inflammatory effects may provide a promising means of preventing atherogenesis and restenosis.

ACKNOWLEDGMENTS

This work was supported in part by the C.Y. Foundation for Advancement of Education, Sciences and Medicine; National Science Council of Taiwan (NSC 93-2314-B-016-062); Tri-service General Hospital, National Defense Medical Center, Taipei, Taiwan, ROC (TSGH-C93-75).

REFERENCES

Abusamieh M, Ash J. 2004. Atherosclerosis and systemic lupus erythematosus. Cardiol Rev 12:267–275.

Aukrust P, Berge RK, Ueland T, Aaser E, Damas JK, Wikeby L, Brunsvig A, Muller F, Forfang K, Froland SS, Gullestad L. 2001. Interaction between chemokines and

oxidative stress: Possible pathogenic role in acute coronary syndromes. J Am Coll Cardiol 37:485–491.

Chait A, Han CY, Oram JF, Heinecke JW. 2005. Thematic review series: The immune system and atherogenesis. Lipoprotein-associated inflammatory proteins: Markers or mediators of cardiovascular disease? J Lipid Res 46: 389–403.

Danenberg HD, Welt FG, Walker M 3rd, Seifert P, Toegel GS, Edelman ER. 2002. Systemic inflammation induced by lipopolysaccharide increases neointimal formation after balloon and stent injury in rabbits. Circulation 105:2917–2922.

Dawson DL, Cutler BS, Meissner MH, Strandness DE Jr. 1998. Cilostazol has beneficial effects in treatment of intermittent claudication: Results from a multicenter, randomized, prospective, double-blind trial. Circulation 98:678–686.

Douglas JS Jr, Holmes DR Jr, Kereiakes DJ, Grines CL, Block E, Ghazzal ZM, Morris DC, Liberman H, Parker K, Jurkovitz C, Murrah N, Foster J, Hyde P, Mancini GB, Weintraub WS. 2005. Coronary stent restenosis in patients treated with cilostazol. Circulation 112:2826–2832.

Fujinaga K, Onoda K, Yamamoto K, Imanaka-Yoshida K, Takao M, Shimono T, Shimpo H, Yoshida T, Yada I. 2004. Locally applied cilostazol suppresses neointimal hyperplasia by inhibiting tenascin-C synthesis and smooth muscle cell proliferation in free artery grafts. J Thorac Cardiovasc Surg 128:357–363.

Gadella TW Jr, Jovin TM. 1995. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. J Cell Biol 129:1543–1558.

Gerszten RE, Mach F, Sauty A, Rosenzweig A, Luster AD. 2000. Chemokines, leukocytes, and atherosclerosis. J Lab Clin Med 136:87–92.

Goetzl EJ, Banda MJ, Leppert D. 1996. Matrix metalloproteinases in immunity. J Immunol 156:1–4.

Husid MS. 2004. New onset migraine with aura following head injury: A case report. Headache 44:1048–1050.

Iwamoto M, Osajima A, Tamura M, Suda T, Ota T, Kanegae K, Watanabe Y, Kabashima N, Anai H, Nakashima Y. 2003. Adrenomedullin inhibits pressure-induced mesangial MCP-1 expression through activation of protein kinase A. J Nephrol 16:673–681.

Kariyazono H, Nakamura K, Shinkawa T, Yamaguchi T, Sakata R, Yamada K. 2001. Inhibition of platelet aggregation and the release of P-selectin from platelets by cilostazol. Thromb Res 101:445–453.

Kayanoki Y, Che W, Kawata S, Matsuzawa Y, Higashiyama S, Taniguchi N. 1997. The effect of cilostazol, a cyclic nucleotide phosphodiesterase III inhibitor, on heparin-binding EGF-like growth factor expression in macrophages and vascular smooth muscle cells. Biochem Biophys Res Commun 238:478–781.

Kim KY, Shin HK, Choi JM, Hong KW. 2002. Inhibition of lipopolysaccharide-induced apoptosis by cilostazol in human umbilical vein endothelial cells. J Pharmacol Exp Ther 300:709–715.

Kondo S, Kubota S, Shimo T, Nishida T, Yosimichi G, Eguchi T, Sugahara T, Takigawa M. 2002. Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. Carcinogenesis 23:769–776.

Lee JH, Oh GT, Park SY, Choi JH, Park JG, Kim CD, Lee WS, Rhim BY, Shin YW, Hong KW. 2005. Cilostazol reduces atherosclerosis by inhibition of superoxide and tumor necrosis factor-alpha formation in low-density lipoprotein receptor-null mice fed high cholesterol. J Pharmacol Exp Ther 313:502–509.

Lin SJ, Yang TH, Chen YH, Chen JW, Kwok CF, Shiao MS, Chen YL. 2002. Effects of Ginkgo biloba extract on the proliferation of vascular smooth muscle cells in vitro and on intimal thickening and interleukin-1beta expression after balloon injury in cholesterol-fed rabbits in vivo. J Cell Biochem 85:572–582.

Lin SJ, Shyue SK, Hung YY, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. 2005. Superoxide dismutase inhibits the expression of vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1 induced by tumor necrosis factor-alpha in human endothelial cells through the JNK/p38 pathways. Arterioscler Thromb Vasc Biol 25:334–340.

Matousovic K, Grande JP, Chini CC, Chini EN, Dousa TP. 1995. Inhibitors of cyclic nucleotide phosphodiesterase isozymes type-III and type-IV suppress mitogenesis of rat mesangial cells. J Clin Invest 96:401–410.

Matsumori A, Furukawa Y, Hashimoto T, Yoshida A, Ono K, Shioi T, Okada M, Iwasaki A, Nishio R, Matsushima K, Sasayama S. 1997. Plasma levels of the monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 are elevated in patients with acute myocardial infarction. J Mol Cell Cardiol 29:419–423.

Matsumoto T, Kobayashi T, Wakabayashi K, Kamata K. 2005. Cilostazol improves endothelium-derived hyperpolarizing factor-type relaxation in mesenteric arteries from diabetic rats. Am J Physiol Heart Circ Physiol 289:1933–1940.

Nieto FJ. 1998. Infections and atherosclerosis: New clues from an old hypothesis? Am J Epidemiol 148:937–948.

Nishio Y, Kashiwagi A, Takahara N, Hidaka H, Kikkawa R. 1997. Cilostazol, a cAMP phosphodiesterase inhibitor, attenuates the production of monocyte chemoattractant protein-1 in response to tumor necrosis factor-alpha in vascular endothelial cells. Horm Metab Res 29:491–495.

Oger S, Mehats C, Dallot E, Cabrol D, Leroy MJ. 2005. Evidence for a role of phosphodiesterase 4 in lipopolysaccharide-stimulated prostaglandin E2 production and matrix metalloproteinase-9 activity in human amniochorionic membranes. J Immunol 174:8082–8089.

Ohtsuki K, Hayase M, Akashi K, Kopiwoda S, Strauss HW. 2001. Detection of monocyte chemoattractant protein-1 receptor expression in experimental atherosclerotic lesions: An autoradiographic study. Circulation 104: 203–208.

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

Pauletto P, Sartore S, Pessina AC. 1994. Smooth-muscle-cell proliferation and differentiation in neointima formation and vascular restenosis. Clin Sci (Lond) 87:467–479.

Rohde LE, Ducharme A, Arroyo LH, Aikawa M, Sukhova GH, Lopez-Anaya A, McClure KF, Mitchell PG, Libby P, Lee RT. 1999. Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. Circulation 99:3063–3070.

Rollins BJ, Walz A, Baggiolini M. 1991. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. Blood 78:1112–1116.

Santibanez JF, Olivares D, Guerrero J, Martinez J. 2003. Cyclic AMP inhibits TGFbeta1-induced cell-scattering and invasiveness in murine-transformed keratinocytes. Int J Cancer 107:715–720.

Thomas MK, Francis SH, Corbin JD. 1990. Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. J Biol Chem 265:14964–14970.

Tomita T, Nakase T, Kaneko M, Shi K, Takahi K, Ochi T, Yoshikawa H. 2002. Expression of extracellular matrix metalloproteinase inducer and enhancement of the production of matrix metalloproteinases in rheumatoid arthritis. Arthritis Rheum 46:373–378.

Woo SK, Kang WK, Kwon KI. 2002. Pharmacokinetic and pharmacodynamic modeling of the antiplatelet and cardiovascular effects of cilostazol in healthy humans. Clin Pharmacol Ther 71:246–252.

Wuyts WA, Vanaudenaerde BM, Dupont LJ, Demedts MG, Verleden GM. 2003. Modulation by cAMP of IL-1beta-induced eotaxin and MCP-1 expression and release in human airway smooth muscle cells. Eur Respir J 22:220–226.

Yassen KA, Galley HF, Webster NR. 2001. Matrix metalloproteinase-9 concentrations in critically ill patients. Anaesthesia 56:729–732.

Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, Witztum JL, Steinberg D. 1991. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci USA 88:5252–5256.

Yoshimura T, Takeya M, Takahashi K. 1991. Molecular cloning of rat monocyte chemoattractant protein-1 (MCP-1) and its expression in rat spleen cells and tumor cell lines. Biochem Biophys Res Commun 174:504–509.