Cilostazol Protects Diabetic Rats from Vascular Inflammation via Nuclear Factor-κB-Dependent Down-Regulation of Vascular Cell Adhesion Molecule-1 Expression

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ABSTRACT

Vascular cell adhesion molecule (VCAM)-1 plays a critical role in the initiation and development of vascular inflammation and selective inhibition of adhesion molecules expressed by endothelial cells may present a new therapeutic strategy for the treatment of vascular complications associated with diabetes mellitus. Increasing evidence indicates that cilostazol, a cAMP phosphodiesterase inhibitor, reduces VCAM-1 expression on endothelial cells. In this study, we have tested the effect of cilostazol on the development of vascular inflammation in rats with streptozotocin-induced diabetes and determined the mechanism by which cilostazol prevents diabetes-induced vascular inflammation in the aorta. Diabetic rats were treated with different dose of cilostazol (27 or 9 mg/kg/day) for 8 weeks, and aortae were removed for the evaluation of vascular inflammation. The VCAM-1 protein expression and VCAM-1 mRNA transcripts were analyzed by immunohistochemical staining and in situ hybridization assay, respectively. Our results demonstrated that cilostazol treatment prevents the overexpression of VCAM-1 and protects diabetic rats from vascular inflammation. More importantly, our mechanistic studies suggested that cilostazol controls the VCAM-1 overexpression via inhibiting the activation of nuclear factor-κB.

Cardiovascular disease is one of the most common complications in diabetes patients and causes more than half of diabetes-related mortality (Jude et al., 2002; Erdmann, 2005), leading to a serious public health problem nowadays. Hyperglycemia increases the expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 in endothelial cells (Esposito et al., 2001; Zuccollo et al., 2005) and smooth muscle cells (SMCs) (Ribau et al., 1999), resulting in inflammation and vascular dysfunction (Renier et al., 2003; Altannavch et al., 2004). Thus, the prevention of the adhesion molecule expression in aortae may be an important therapeutic strategy for the treatment of cardiovascular disease in diabetic and nondiabetic patients as well (Jude et al., 2002).

In recent years, cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone), a potent phosphodiesterase III inhibitor, has drawn a great deal of interest because of its inhibitory effect on the overexpression of VCAM-1. Studies have shown that cilostazol suppresses adhesion molecule expression in human umbilical vein endothelial cells in vitro (Omi et al., 2004; Park et al., 2005) and the proximal ascending aorta in vivo in low-density lipoprotein receptor-null mice fed with cholesterol (Lee et al., 2005). However, the underlying mechanism of the protective effect of cilostazol on aortae remains controversial. VCAM-1 transcription is regulated by the binding of nuclear factor (NF)-κB to the NF-κB-binding sequence in VCAM-1 promoter (Aljada et al., 2001; Minhajuddin et al., 2005; Szasz et al., 2005). Exposure of human aortic endothelial cells to high glucose significantly induced the expression of VCAM-1 pro-
tein accompanied by increased activity of NF-κB (Kouroedov et al., 2004; Minhajuddin et al., 2005; Szaszti et al., 2005). Cilostazol-induced suppression of adhesion molecule expression, such as VCAM-1, resembled BAY 11-7085, a specific NF-κB inhibitor, in human umbilical vein endothelial cells (Park et al., 2005). In contrast, in other studies, cAMP was found to stimulate NF-κB activation in myeloid cells (Serkkola and Hurme, 1993). The conflicting results imply that cilostazol may have differential effects on different types of tissues or cells.

In the current study, we used Sprague-Dawley (SD) rats with streptozotocin (STZ)-induced diabetes to test whether cilostazol can protect diabetic animals from the development of hyperglycemia-induced cardiovascular disorders in vivo and the underlying mechanism by which cilostazol controls the overexpression of VCAM-1 in diabetic animals. We found that treatment of diabetic rats with cilostazol prevents the overexpression of VCAM-1 by aortic endothelial cells and, therefore, the development of vascular inflammation. Our in vitro studies have further demonstrated that cilostazol inhibits the activation of NF-κB, which, in turn, decreases the transcription of VCAM-1.

Materials and Methods

Induction of Cardiovascular Complications in Diabetic Rats. Six-week-old male SD rats with an initial body weight of 230 to 270 g were purchased (Sino-British Sippy-hk Lab Animal Ltd., Shanghai, China) and used in this study. Animals were housed with a 12-h light/dark cycle and provided with standard diet and water ad libitum in accordance with the guidelines established by the Animal Research Group’s Guide for the Care and Use of Laboratory Animals.

Diabetes was induced by a single i.p. injection of STZ (Sigma Chemical, St. Louis, MO) at 65 mg/kg prepared in 0.1 M citrate buffer, pH 4.2, as described previously (Rao et al., 1995; Bagrov et al., 2005). Animals injected with buffer alone were used as normal controls (n = 8). Seventy-two hours after STZ injection, blood glucose (BG) was measured by a One-Touch II Glucometer (LifeScan, Milpitas, CA), and animals were considered to be diabetic when the concentration of BG was equal to or higher than 16.7 mM. Diabetic rats were then treated with either high dose of cilostazol (n = 10, 27 mg/kg/day; Delerive et al., 2002) or low dose of cilostazol (n = 10, 9 mg/kg/day). Cilostazol was kindly provided by Otsuka Pharmaceutical (Tokushima, Japan). Diabetic animals without cilostazol treatment were used as diabetic controls (n = 12).

All the animals were monitored for BG levels and body weight once a week. Individual dose of cilostazol was adjusted to body weight during the treatment process. After cilostazol treatment for 8 weeks, all animals were sacrificed under 10% chloral hydrate (3 mg/kg i.p.) general anesthesia. HAAl, was examined immediately before sacrifice.

Vessels from aortal arch to iliac aorta were isolated, and adherent fat and connective tissues were removed. Part of the vessels were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline and connective tissues were removed. Part of the vessels were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline before sacrifice.

Immunohistochemical Staining and Histological Analysis. Four consecutive 5-μm sections were collected for each slide. Ten slides were made from each animal. Even-numbered sections were kept for immunohistochemical analysis (Dong et al., 1998). For immunohistochemical staining, sections were labeled with primary antibody of rabbit polyclonal antibodies against VCAM-1 or monoclonal mouse antibody against activated NF-κBp65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) after microwave antigen retrieval in citrate buffer. The binding of the primary antibodies was revealed by biotinylated anti-rabbit IgG or anti-mouse IgG and detected with the Vectastain ABC-streptavidin conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) and dianamidobenzidine tetrahydrochloride (Vector Laboratories) staining. Controls for immunospecificity were included in all experiments, and the primary antibody was replaced by phosphate-buffered saline or matching concentrations of normal rabbit or mouse serum (Bierhaus et al., 2001). The quantitative analysis of immunohistochemical staining was scored by two experienced operators who were blinded to the study protocol. Five unfolded continuous fields in each section were examined. The VCAM-1 expression was measured then by Image-Pro Plus 4.5 (Media Cybernetics, Inc., Silver Spring, MD) as described previously (Wada et al., 1996). In brief, the image analysis system was used to determine the integral optical density (IOD) of the labeled sections, which was defined as the integral sum of the surface area of single pixels of a digitized image of VCAM-1 multiplied by their corresponding OD values. IOD represents a value that takes into account both the intensity of the dianamidobenzidine tetrahydrochloride staining and the labeled surface areas. The threshold was chosen and kept constant throughout the analysis. The average IOD in the same slide was calculated. The number of activated NF-κBp65 (nuclear staining) was counted (×400) and expressed as percent positive cells. Sections from paraformaldehyde-fixed and paraffin-embedded aorta samples were stained with H&E and examined for vascular inflammation.

In Situ Hybridization (ISH). Sections were prepared as described above, and VCAM-1 mRNA was detected by in situ hybridization with digoxigenin-labeled oligonucleotide probes. The hybridization was performed at 37°C for 16 h, and signals were detected using an ISH detection kit according to the manufacturer’s instructions (Roche Diagnostics, Basel, Switzerland). Hybridization without the oligonucleotide probe or the anti-digoxigenin antibody served as controls. Quantification of VCAM-1 mRNA staining in the aorta was quantified as described above in immunohistochemical staining.

Electrophoresis Mobility Shift Assay. Aortae were homogenized in ice-cold lysis buffer A (10 mM HEPES/KOH, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.6% NP-40). The crude nuclei were collected after microcentrifugation at 5000 rpm for 10 min at 4°C and resuspended in 50 μl of ice-cold buffer B [20 mM HEPES, pH 7.9, 0.42 M KCl, 0.2 mM EDTA, 1.2 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol 10% (w/v), 0.5 mM dithiothreitol, and 0.5 μg/ml aprotinin]. Nuclei were incubated for 30 min at 4°C, vortexed vigorously every 10 min, and centrifuged at 14,000 rpm for 15 min. The concentration of resulting nuclear proteins was measured by Bradford assay (Bio-Rad, Hercules, CA).

To test the DNA-binding activity of NF-κB with VCAM-1, a double-stranded 22-mer oligonucleotide probe containing NF-κB-binding consensus sequence of human VCAM-1 promoter (5′-AGTTGAGGGGGACTTTCCCAGGC-3′; Promega, Madison, WI) was labeled with [γ-32P]ATP (5000 Ci/mmol at 10 μCi/ml), using T4 polynucleotide kinase. Binding reactions contained 10 μg of nuclear extract, 1 μg of [32P]-labeled DNA, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 50 mM NaCl, 0.5 mM dithiothreitol, 4% glycerol, 0.5 mM EDTA, and 0.5 μg of poly(dI/dC) (Promega). After incubation at 37 °C for 45 min, protein-DNA complexes were separated on 4% nondenaturing polyacrylamide gels and visualized by autoradiography. Binding reactions with a 200-fold excess of unlabeled double-stranded oligonucleotides (NF-κB or Sp1) were processed as described above and used as controls for binding specificity.

Statistical Analysis. Data were expressed as mean ± S.D. Statistical analysis was performed with SPSS 11.0 (SPSS Inc., Chicago, IL) between two groups using two-tailed Student’s t test for unpaired values, one-way analysis of variance for equal variance assumed populations, Dunnett’s t test for equal variance not assumed populations, when comparing groups of three or more, and Pearson analysis for correlation study. p < 0.05 was considered statistically significant.
Cilostazol Down-Regulates VCAM-1 in Aorta of Diabetic Rats

Results

No Effect of Cilostazol Treatment on Hyperglycemia in STZ-Induced Diabetes in SD Rats. STZ-induced diabetes in SD rats recapitulates many aspects of type 1 diabetes in humans and is the most commonly used animal model for the study of diabetes-induced complications (Bagrov et al., 2005; Brondum et al., 2005). To determine the effect of cilostazol treatment on STZ-induced diabetes, SD rats were injected with STZ (65 mg/kg i.p.) as described previously and treated with cilostazol for 8 weeks. Hyperglycemia was detected in STZ-treated animals and was not altered in cilostazol-treated groups compared with diabetic control animals (Fig. 1A). There was a steady gain of body weight in normal control rats treated with buffer alone, whereas diabetic rats treated with or without high or low dose of cilostazol had a significant loss of body weight (Fig. 1B). In addition, HbA1c levels were significantly higher in diabetic rats than in normal control rats and were not affected by either low or high doses of cilostazol (Fig. 1C). These results indicated that diabetes was successfully induced in SD rats, and the treatment of diabetic rats with cilostazol affected neither hyperglycemia nor body weight in diabetic animals.

Cilostazol Prevents Diabetes-Induced Overexpression of VCAM-1 in Aorta of Diabetic Animals. To determine the induction of vascular inflammation in diabetic animals, the aortas were examined by histological analysis. The wall of aorta was much thicker with overproliferation of vascular SMCs in diabetic animals than in cilostazol-treated or nondiabetic animals (Fig. 2). These results indicated that vascular inflammation induced in diabetic animals was inhibited by cilostazol treatment. To test whether cilostazol treatment prevents diabetes-induced vascular inflammation by inhibiting the overexpression of VCAM-1, the VCAM-1 expression in aortae was analyzed by immunohistochemical staining. As shown in Fig. 3A, there was a drastic increase of VCAM-1 expression in diabetic animals compared with normal control animals. However, the diabetes-induced overexpression of VCAM-1 was prevented in diabetic animals treated with either high or low dose of cilostazol (p < 0.01 or p < 0.05, respectively; Fig. 3A) consistent with previous observations (Omi et al., 2004; Park et al., 2005). These results indicated that cilostazol treatment protects diabetic animals from cardiovascular complications by inhibiting the overexpression of adhesion molecules, such as VCAM-1.

To test the possibility that the suppressive effect of cilostazol on the VCAM-1 protein expression is due to decreased VCAM-1 gene expression rather than the internalization of VCAM-1 protein, VCAM-1 gene transcription was measured by in situ hybridization. As shown in Fig. 3B, diabetic rats displayed a marked increase of VCAM-1 gene transcripts in aortic tissue compared with normal control rats (p < 0.01). The mRNA transcripts of VCAM-1 gene were significantly decreased in diabetic animals treated with cilostazol, regardless of the doses (p < 0.01 and p < 0.05).

The results presented in Fig. 3C further confirmed that cilostazol inhibits VCAM-1 gene transcription. It was positively correlated between mRNA of VCAM-1 and protein of VCAM-1 (Pearson correlation coefficient in diabetic control rats = 0.951, p = 0.001). Likewise, the reduced VCAM-1 protein expression in cilostazol treatment groups was positively correlated with low levels of VCAM-1 mRNA.

Cilostazol Inhibits the Activation of NF-κB in the Aorta of Diabetic Animals. After activation of NF-κB, the activated NF-κBp65 translocates into the nucleus and initiates the VCAM-1 transcription by binding to its target DNA sequences in VCAM-1 promoter (Ishibashi and Nishikawa, 2003). We hypothesized that cilostazol affects the NF-κB-mediated gene transcription and, as a result, prevents the overexpression of VCAM-1. To test this possibility, the activation of NF-κB in the aorta was analyzed by immunohisto-
chemical staining and electrophoresis mobility shift assay (EMSA). Strong staining of the activated NF-κBp65 subunit was detected in the aorta of diabetic rats (Fig. 4A), and the enhanced activation of nuclear NF-κBp65 is positively correlated to the increased mRNA transcripts of VCAM-1 gene in diabetic rats as shown in Fig. 4B (Pearson correlation coefficient = 0.885, p = 0.003). Interestingly, only weak staining of activated NF-κBp65 was observed in the aorta of diabetic animals treated with either high or low dose of cilostazol (Fig. 4A). Likewise, there was a positive correlation between weak staining of NF-κBp65 and the reduced amount of mRNA transcripts of VCAM-1 (Fig. 4B).

To determine the DNA-binding activity of NF-κBp65 specific for VCAM-1 gene, EMSA assay was performed. The results presented in Fig. 5A showed a strong NF-κBp65-specific gel-retarded band detected in samples derived from diabetic animals (Fig. 5A, lane 4). The binding of NF-κBp65 is DNA-specific because the band disappeared in the presence of excess of unlabeled NF-κB oligonucleotides (Fig. 5A, lane 5) but not Sp1 oligonucleotides (Fig. 5A, lane 6). In contrast, faint gel-retarded bands were found in samples from cilostazol-treated animals, and the reduced DNA-binding activity of NF-κBp65 was even more pronounced in animals treated with high dose of cilostazol (Fig. 5A, lanes 2 and 3). We have also performed Pearson correlation analysis to determine whether cilostazol-induced suppression of VCAM-1 mRNA transcripts was resulted from the reduced NF-κB activity. Our analysis shown in Fig. 5B indicated a positive correlation between DNA-binding activity of NF-κB and VCAM-1 mRNA level (correlation coefficient in diabetes controls = 0.897, p = 0.003).

Discussion

The elevated expression of VCAM-1 and other adhesion molecules on the surface of the endothelial cells promotes adhesion of leukocytes, particularly monocytes, and is the first step in the pathophysiological process leading to vascular lesions in diabetic individuals. We have tested the effect of cilostazol on the development of vascular inflammation in rats with STZ-induced diabetes. Our results demonstrated that cilostazol treatment inhibited the proliferation of SMCs and significantly down-regulated VCAM-1 expression by endothelial in diabetic rats in a dose-dependent manner and, as a result, protected diabetic animals from the development of vascular inflammation. These results confirmed our previous observation and those of others (Nishio et al., 1997; Otsuki et al., 2001; Wang et al., 2002) and provided evidence that cilostazol controls the overexpression of VCAM-1 in diabetic rats by inhibiting the activation of NF-κB.

How does cilostazol control the expression of VCAM-1 induced by hyperglycemia in diabetic animals? It has been reported recently that cilostazol may act directly on human endothelial cells to inhibit expression of adhesion molecules and neutrophil adhesion induced by high glucose (Omi et al., 2004). The expression of VCAM-1 on the surface of endothelial cells was significantly reduced in cilostazol-treated diabetic rats compared with control diabetic rats (Fig. 3A). Our ISH analysis showed that the VCAM-1 mRNA transcripts were also significantly reduced in diabetic rats treated with cilostazol, indicating that cilostazol altered the transcription of the VCAM-1 gene (Fig. 3B). This interpretation is consistent with the positive correlation between the reduced VCAM-1 mRNA content and the down-regulated expression of VCAM-1 protein. These results provide evidence that cilostazol prevents VCAM-1 overexpression in diabetic animals by inhibiting VCAM-1 gene transcription.

NF-κB is a nuclear transcription factor and initiates the transcription of genes associated with inflammatory responses. Advanced glycation end products induced by hyperglycemia stimulate NF-κB activation (Seki et al., 2003; Duan et al., 2005), which sustains the activation of NF-κB in diabetic rats (Bierhaus et al., 2001). The increased NF-κB activation results in the up-regulation of epithelial adhesion molecule expression (Ishibashi and Nishikawa, 2003), and the VCAM-1 expression is dependent, at least in part, on the activation of NF-κB and its binding activity (Ramana et al., 2004). We have tested the possibility that cilostazol controls the diabetes-induced overexpression of VCAM-1 via direct inhibition of NF-κB activation. Although strong staining of activated NF-κB in diabetic animals was detected by nuclear
staining, the cilostazol treatment markedly reduced the amount of activated NF-κB. Furthermore, the down-regulated VCAM-1 transcription was positively correlated with the weak nuclear staining of activated NF-κB in cilostazol-treated animals. Otsuki et al. (2001) have shown that cilostazol inhibits NF-κB binding to VCAM-1 recognition sequence but failed to prevent the translocation of NF-κBp65 with and without TNF-α in human umbilical vein endothelial cells.

Fig. 3. Cilostazol down-regulates VCAM-1 expression in the aorta of diabetic rats. After cilostazol treatment as described in Fig. 1, the aortae from controls and cilostazol-treated animals were removed for evaluating VCAM-1 protein expression by immunohistochemistry (A) and VCAM-1 mRNA by in situ hybridization (B). Scatter graph, correlation between VCAM-1 protein expression and VCAM-1 mRNA in the aorta (C). Black square, normal control group; white square, diabetes group; black triangle, high dose of cilostazol (27 mg/kg/day); white triangle, low dose of cilostazol (9 mg/kg/day). Values shown represent the mean ± S.D. of three independent experiments. Bar, S.D. **, p < 0.01 versus normal control group. #, p < 0.05; ##, p < 0.01 versus diabetes group.

Fig. 4. NF-κBp65 is reduced in the aorta of cilostazol-treated diabetic rats. A, paraffin sections of aortae from cilostazol-treated and control animals as previously indicated were incubated with antibody specific for NF-κBp65 subunit and biotinylated anti-mouse antibody. The nuclear staining of NF-κB was scored as described under Materials and Methods. Data were expressed as the mean ± S.D. of three independent experiments. Bar, S.D. B, data from Figs. 3B and 4A were analyzed. Scatter graph, correlation between VCAM-1 mRNA transcripts and the nuclear staining of NF-κBp65 in the aorta of cilostazol-treated and control animals. Black square, normal control group; white square, diabetes group; black triangle, high dose of cilostazol (27 mg/kg/day); white triangle, low dose of cilostazol (9 mg/kg/day). **, p < 0.01 versus normal control group. ##, p < 0.01 versus diabetes group.

However, the weak nuclear staining of NF-κBp65 detected in our study is more consistent with the recent finding that cilostazol inhibits NF-κB activation (Lee et al., 2005; Park et al., 2005), which is responsible for the down-regulation of VCAM-1 expression in cilostazol-treated low-density lipoprotein receptor mice and endothelial cells. In addition, cilostazol treatment prevents the overproliferation of aortic SMC in diabetic animals (Fig. 2) and human diabetic patients by decreasing carotid intima media thickness (Ahn et al., 2001; Shinoda-Tagawa et al., 2002; Mitsuhashi et al., 2004). Given that VCAM-1 is also expressed by aortic SMC, one would argue that cilostazol suppresses VCAM-1-mediated overexpression via the NF-κB pathway. Indeed, it has been found that the activation of NF-κB provokes accelerated proliferation of SMC (Seki et al., 2003; Duan et al., 2005). Taken together, we concluded that cilostazol protects diabetic ani-
DNA-binding activity in aorta of diabetic rats. Data shown were obtained with high dose of cilostazol (lane 2), or low dose of cilostazol (lane 3), normal control rats (lane 1), diabetic rats (lane 4), diabetic rats treated with nucleotide. Nuclear extracts were prepared from the aortic tissue of Ahn CW, Lee HC, Park SW, Song YD, Huh KB, Oh LS, Eikum BY, Shin YW, and Hong KW (2005) Cilostazol reduces atherosclerosis by inhibition of inflammation and tumor necrosis factor-α formation in low-density lipoprotein receptor-null mice fed high cholesterol. J Pharmacol Exp Ther 313:502–509.


