Cilostazol Protects Against Brain White Matter Damage and Cognitive Impairment in a Rat Model of Chronic Cerebral Hypoperfusion

Terubumi Watanabe, MD; Ning Zhang, MD; Meizi Liu, MD; Ryota Tanaka, MD, PhD; Yoshikuni Mizuno, MD, PhD; Takao Urabe, MD, PhD

Background and Purpose—White matter lesions contribute to cognitive impairment in poststroke patients. The present study was designed to assess the neuroprotective mechanisms of cilostazol, a potent inhibitor of type III phosphodiesterase, through signaling pathways that lead to activation of transcription factor cAMP-responsive element binding protein (CREB) phosphorylation using rat chronic cerebral hypoperfusion model.

Methods—Rats underwent bilateral common carotid artery ligation. They were divided into the cilostazol group (n=80) and the vehicle (control) group (n=80). Performance at the Morris water maze task and immunohistochemistry for 4-hydroxy-2-nonenal (HNE), glutathione-S-transferase-pi (GST-pi), ionized calcium-binding adaptor molecule 1, phosphorylated CREB (p-CREB), Bcl-2, and cyclooxygenase-2 (COX-2) were analyzed at baseline and at 3, 7, 14, 21, and 28 days after hypoperfusion.

Result—Cilostazol significantly improved spatial learning memory (6.8±2.3 seconds; P<0.05) at 7 days after hypoperfusion. Cilostazol markedly suppressed accumulation of HNE-modified protein and loss of GST-pi–positive oligodendrocytes in the cerebral white matter during the early period after hypoperfusion (P<0.05). Cilostazol upregulated p-CREB and Bcl-2 (P<0.05), increased COX-2 expression, and reduced microglial activation in the early period of hypoperfusion.

Conclusion—Our results indicate that cilostazol exerts a brain-protective effect through the CREB phosphorylation pathway leading to upregulation of Bcl-2 and COX-2 expressions and suggest that cilostazol is potentially useful for the treatment of cognitive impairment in poststroke patients. (Stroke. 2006;37:1539-1545.)

Key Words: apoptosis • neuroprotection • white matter

The number of patients afflicted with cerebral infarction is on the increase at present, and stroke is a leading cause of disability worldwide, with no effective clinical treatment that enhances recovery. Poststroke cognitive impairment and depressive disorders are recognized complications in such patients. White matter lesions (WMLs), such asBinswanger disease, are often observed in patients with ischemic cerebrovascular diseases and are thought to contribute to cognitive impairment. There is ample evidence to suggest that oxidative stress correlates with the inflammatory response to ischemia-induced WMLs.1 In addition, apoptosis of oligodendrocytes (OLGs), a major cellular component of the white matter, contributes directly to WMLs, as demonstrated in a rat cerebral ischemia model.2 Therefore, protection against the impact of oxidative stress, inflammatory damage, and apoptosis is important to reduce damage of the white matter.

Cilostazol, a potent inhibitor of type III phosphodiesterase, has been approved by the Japanese health authorities as an antiplatelet agent for the treatment of chronic cerebral infarction since 2001. Recently, few reports described other non-antiplatelet neuroprotective effects of cilostazol, such as anticytotoxic effect and antiapoptosis effect, in focal cerebral ischemic models.3–6 The main mechanism of this action is increased cAMP-responsive element binding protein (CREB) phosphorylation through intracellular signaling from type III phosphodiesterase–cAMP cascade, which could induce subsequent activation of antiapoptotic cascade.7 Although the neuroprotective mechanism of cilostazol has been investigated at an early period after ischemia,3 to our knowledge, there is no report that describes the brain-protective effects of cilostazol on WMLs in the chronic stage after ischemic insult.

WMLs can be induced in rat brains under chronic cerebral hypoperfusion by permanent occlusion of both common carotid arteries.8 In the present study, first we induced WMLs in a rat chronic cerebral hypoperfusion model by permanent occlusion of both common carotid arteries. We then tested the hypothesis that cilostazol has protective effects against WMLs caused by cerebral ischemia and that such effect is mediated through antioxidative and anti-inflammatory pathways, as well as antiapoptotic cascade by activating CREB phosphorylation.
Materials and Methods

Experimental Groups
All animal procedures were conducted after gaining the approval of the animal care committee of Juntendo University. Adult male Wistar rats (8 weeks old) weighing 250 to 270 g were obtained from Charles River Institute (Kanagawa Japan) and maintained on a 12-hour light/dark cycle with continuous access to food and water. Rats were divided at random into the following three groups: (1) the cilostazol group: rats of this group (n=80) were provided laboratory food mixed with 0.1% cilostazol (Otsuka Pharmaceutical) at 50 mg/kg per day after ligation of both common carotid arteries (LBCCA); (2) the vehicle group: rats of this group (n=80) underwent LBCCA but were provided normal animal food; and (3) the control sham-operated vehicle- and cilostazol-treated groups: these rats (n=5 each subgroup) underwent the same aforementioned protocol except for LBCCA. For occlusion of both common carotid arteries, anesthesia was induced with 1.0% to 2.0% isoflurane in 30% nitrogen and maintained during operation by administration of 70% nitrogen. Through a midline incision, the bilateral common carotid arteries were carefully separated from the cervical sympathetic and vagal nerves and ligated permanently. Rats of each group were euthanized at baseline (before LBCCA) or 3, 7, 14, 21, or 28 days after LBCCA, and the brain was dissected out immediately and sectioned for subsequent analyses (see below).

Measurement of Cerebral Blood Flow
Cerebral blood flow (CBF) was measured in a left temporal window by laser Doppler flowmetry (Laser Tissue Blood Flow Meter FLO-C1; Omega Wave, Inc). Measurements were made before, immediately after, and at 3, 7, 14, 21, and 28 days after LBCCA.

Water Maze Task
The water maze task was performed to evaluate LBCCA-related learning deficits using the method described previously by Gerlai. Briefly, aliquots containing 50 μg of protein were subjected to 10% SDS-PAGE. The protein bands were transferred onto polyvinylidene fluoride membrane (Bio-Rad) and probed for CREB by incubation with the primary antibody (3000:1; Santa Cruz Biotechnology) with rabbit polyclonal antibody against adenomatus polyposis coli (Ab-7; APC; 1:100, Calbiochem); mouse monoclonal antibody against Bcl-2 (1:100; Santa Cruz Biotechnology) with rabbit polyclonal antibody against GFAP (1:1000; Sigma Aldrich, Inc) or rabbit polyclonal antibody against GST-pi (1:100; Chemicon). Cy3-conjugated donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch Laboratories) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:100; Vector Laboratories) were used to demonstrate p-CREB and Bcl-2, respectively, in red fluorescence. On the other hand, fluorescein isothiocyanate–conjugated horse anti-mouse IgG (1:100; Vector Laboratories) and anti-rabbit IgG (1:100; Molecular Probes) were used to demonstrate GFAP, APC, and GST-pi immunoreactivity, respectively, in green fluorescence.

SDS-PAGE and Immunoblotting
Proteins were extracted from the corpus callosum area and measured as described previously. Briefly, aliquots containing 50 μg of protein were subjected to 10% SDS-PAGE. The protein bands were transferred onto polycrylamide membrane (Bio-Rad) and probed for CREB by incubation with the primary antibody (3000:1; Upstate Biotechnology), followed by incubation with horseradish peroxidase–conjugated secondary antibody (10 000:1; Santa Cruz Biotechnology). Blots were visualized using the ECL system (Amersham Biosciences) to assess the relationship among CREB, p-CREB, and Bcl-2. The membrane was washed and reprobed with p-CREB (2000:1; Cell Signaling Technology) or Bcl-2 (2000:1) antibody. Equal protein loading was confirmed by measuring α-tubulin.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling
For detection of in situ DNA fragmentation, TUNEL staining was performed using an In Situ Cell Death Detection Kit, TMR red

Immunohistochemistry
Immunohistochemistry was performed on 20-μm-thick free-floating coronal sections of the corpus callosum and the external capsule and internal capsule, which were prepared as described previously. After incubation in 3% H2O2, followed by 10% block ace in 0.1% PBS(−), the sections were immunostained overnight at 4°C using a mouse monoclonal antibody against 4-hydroxy-2-nonenal (HNE; 40:1; Japan Institute for the Control of Aging) to assess lipid peroxidation, a rabbit polyclonal antibody against ionized calcium-binding adaptor molecule 1 (Iba-1; 500:1; Wako Pure Chemicals; a marker of active microglia) to assess inflammation, a rabbit polyclonal antibody against glutathione-S-transferase-pi (GST-pi) (500:1; Chemicon International, Inc; as a marker of OLGs) to assess mature OLGs, and a rabbit polyclonal antibody against cyclooxygenase-2 (COX-2; 500:1; Santa Cruz Biotechnology) to assess cytotoxic oxygen radicals. The sections were then treated with secondary antibodies (500:1; Vectastain; Vector Laboratories). Immunoreactivity was visualized subsequently by the avidin-biotin complex method (Vectastain; Vector Laboratories).

Immunofluorescence
Double immunofluorescence staining and confocal laser scanning microscopy (Axiovert 100 mol/L; Carl Zeiss) were performed to determine the origin of phosphorylated CREB (p-CREB)– and Bcl-2–positive cells. The primary antibodies were rabbit polyclonal antibody against p-CREB (1:100; Upstate Biotechnology) with mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; 1:1000; Sigma Aldrich, Inc) or mouse monoclonal antibody against adenomatus polyposis coli (Ab-7; APC; 1:100, Calbiochem); mouse monoclonal antibody against Bcl-2 (1:100; Santa Cruz Biotechnology) with rabbit polyclonal antibody against GFAP (1:1000; Sigma Aldrich, Inc) or rabbit polyclonal antibody against GST-pi (1:100; Chemicon). Cy3-conjugated donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch Laboratories) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:100; Vector Laboratories) were used to demonstrate p-CREB and Bcl-2, respectively, in red fluorescence. On the other hand, fluorescein isothiocyanate–conjugated horse anti-mouse IgG (1:100; Vector Laboratories) and anti-rabbit IgG (1:100; Molecular Probes) were used to demonstrate GFAP, APC, and GST-pi immunoreactivity, respectively, in green fluorescence.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling
For detection of in situ DNA fragmentation, TUNEL staining was performed using an In Situ Cell Death Detection Kit, TMR red
(Roche). TUNEL staining was performed on 20 μm-thick free-floating coronal sections of coronal sections of the corpus callosum. After incubation in 0.1% sodium citrate in 0.1% Triton X-100 in 0.1%PBS(−), the sections were reacted with TUNEL reaction mixture for 60 minutes at 37°C in the dark.

**Cell Counts and Statistical Analysis**

In each HNE, p-CREB, Iba-1, COX-2, Bcl-2, and TUNEL section, the stained cells were counted in 3 predefined areas (0.25 mm²; n=8 each group) from axon-rich regions: the corpus callosum, the external capsule, and the internal capsule. Values presented in this

**Figure 2.** A, Immunostaining of HNE in the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Bar=50 μm. B, Number of HNE-positive cells in the corpus callosum. Values are expressed as mean±SEM. n=8 each group. *P<0.05; **P<0.01 vs vehicle group. C, Immunostaining of Iba-1 in the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Bar=50 μm. D, Number of Iba-1-positive cells in the corpus callosum. Values are expressed as mean±SEM. n=8 each group. *P<0.05; **P<0.01 vs vehicle group. E, Immunostaining of GST-pi in the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Bar=50 μm. F, Number of GST-pi-positive cells in the corpus callosum. Values are expressed as mean±SEM. n=8 each group. *P<0.05; **P<0.01 vs vehicle group.
study are expressed as mean±SEM. One-way ANOVA followed by post hoc Fisher protected least significant difference test was used to determine the significance of differences in various indexes among the different groups. A P value <0.05 denoted the presence of a statistically significant difference. The stained cells were counted in 3 predefined areas.

Results
Physiological Parameters
Hypoperfusion was confirmed by changes in CBF before and after LBCCA. There were no significant differences in CBF between the vehicle group and the cilostazol group throughout the entire period of the study (Figure 1A).

Improvement of Learning Memory
Before operation, all rats escaped in <10 seconds in the Morris water maze task. After LBCCA, the vehicle group showed a significantly longer escape latency than the sham group (P<0.05). Interestingly, the escape latency of the cilostazol group gradually decreased over the testing period compared with the vehicle group (P<0.05; Figure 1B).

Cilostazol Suppresses Lipid Peroxidation
Lipid peroxidation was assessed by immunostaining using anti-HNE antibody. In the sham operation group, HNE immunoreactivity was rarely observed in the corpus callosum. In the control group, HNE-positive cells were detected from 3 days after operation. The number of strained cells increased significantly, reaching a peak number at 14 days, and persisted during the whole investigation period. In the cilostazol group, the number and the intensity of immunoreactivity of HNE-positive cells were significantly decreased compared with the vehicle group (Figure 2A and 2B).

Effects of Cilostazol on Inflammatory Responses
In the vehicle group, ramified Iba-1–positive microglia were observed in the corpus callosum from 3 days after LBCCA, and their number reached a peak level at 7 days and stabilized thereafter. Such microglial activation was evidently reduced in the cilostazol group compared with the control group (Figure 2C and 2D). GST-pi–positive OLGs were observed in the corpus callosum area both in the vehicle and cilostazol groups after LBCCA. In the vehicle group, the number of OLGs decreased with time. However, administration of cilostazol prevented the loss of OLGs (Figure 2E and 2F).

Cilostazol Increases COX-2 Expression
COX-2 immunostaining was rarely observed in the vehicle group throughout the corpus callosum area after LBCCA. In contrast, COX-2 immunoreactivity was more prominent throughout the test period in the cilostazol group. Furthermore, the number and the intensity of immunoreactivity in COX-2–positive cells were significantly higher in the cilostazol group than the vehicle group (Figure 3A and 3B).

Cilostazol Augments p-CREB Expression
At all the investigated time points after LBCCA, p-CREB immunoreactivity was more prominent in the cilostazol group than the vehicle group, and it was most obviously observed at day 14 after LBCCA. Coexpression of p-CREB with GFAP was rarely observed (data not shown), and p-CREB–expressing cells were mainly identified as OLGs by colocalization with APC antibody (Figure 4A and 4B). Western blot analysis using anti–p-CREB antibody showed a specific single band in the corpus callosum with a molecular mass of 43 kDa. In the cilostazol group, the intensity of the band increased in a time-dependent manner compared with the control group (Figure 4E).

Cilostazol Increases Bcl-2 Expression
Bcl-2 immunoreactivity was rarely detected in the vehicle group, whereas in the cilostazol group, it was observed at all the investigated time points after LBCCA, and the immunoreactivity was most evident at day 14 after LBCCA. Bcl-2–expressing cells were identified as OLGs by colocalization with GST-pi antibody (Figure 4C and 4D). Western blot analysis using anti–Bcl-2 antibody showed a specific single band at 26 kDa. In the vehicle group, the intensity of the band increased in a time-dependent manner compared with the control group (Figure 4F).

Figure 3. A, Immunostaining of COX-2 in the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Bar=50 μm. B, Number of COX-2–positive cells in the corpus callosum. Values are expressed as mean±SEM, n=8 each group. *P<0.05; **P<0.01 vs vehicle group.
Figure 4. A, Double immunofluorescence staining of p-CREB and APC in the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Bar=50 μm. The white-surrounded representative part is shown at high-power magnification. Red indicates p-CREB immunoreactivity; green, APC immunoreactivity; yellow, p-CREB/APC immunoreactivity. B, Number of p-CREB–positive cells in the corpus callosum. Values are expressed as mean±SEM. n=8 each group. *P<0.05; **P<0.01 vs vehicle group. C, Double immunofluorescent staining of Bcl-2 and GST-pi in the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Bar=50 μm. Red indicates Bcl-2 immunoreactivity; green, GST-pi immunoreactivity; yellow, Bcl-2/GST-pi. D, Number of Bcl-2–positive cells in corpus callosum. Values are expressed as mean±SEM. n=8 each group. **P<0.01 vs vehicle group. E, Western blotting analysis of CREB, p-CREB, and Bcl-2. Samples were prepared from the corpus callosum of vehicle- and cilostazol-treated rats at days 3, 7, 14, 21, and 28 after LBCCA. Protein bands of 43, 43, and 28 kDa were detected by anti-CREB antibody, anti–p-CREB antibody and anti–Bcl-2 antibody, respectively. Equal protein loading was confirmed by measuring α-tubulin.
band in the corpus callosum with a molecular mass of 28 kDa. In the cilostazol group, the intensity of the band increased in a time-dependent manner compared with the control group (Figure 4E).

**Cilostazol Reduces Apoptotic Cell Death**

In the TUNEL staining, apoptotic cells represented TUNEL-positive cells containing apoptotic bodies and condensed nuclear fragments. The number of apoptotic cells in the corpus callosum was significantly lower in the cilostazol group than the vehicle group, especially at days 7 and 14 after LBCCA (Figure 5A and 5B).

**Discussion**

In the present study, we analyzed the mechanisms of action of cilostazol in a rat model of chronic cerebral hypoperfusion-induced WMLs. The major findings of the present study were that cilostazol significantly improved learning memory, suppressed the early accumulation of lipid peroxidation products, and eliminated the sequential inflammatory responses, as well as decreased the number of apoptotic cells.

After chronic cerebral hypoperfusion injury, oxygen free radicals contribute to chronic cerebral hypoperfusion-induced WMLs, which represent immediate direct cytotoxic damage of lipid peroxidation products and inflammatory injury.1 To our knowledge, this is the first report that describes the therapeutic effects of cilostazol on WMLs after ischemic insult and analyzes the neuroprotective mechanism of cilostazol on the CREB phosphorylation signaling pathway in OLGs.

We reported previously the usefulness of HNE-modified protein as a marker for oxidative neuronal damage after ischemia/reperfusion injury.10 Another study showed that the lipid peroxidation byproduct HNE had direct toxic effects not only to neurons but also to OLGs in the cerebral white matter after cerebral ischemia.11 The present study demonstrated that accumulation of HNE-modified protein was significantly suppressed by cilostazol from the early period after hypoperfusion, and these effects lasted until 28 days. Our results clearly indicate that the brain-protective actions of cilostazol are mediated via its antioxidant effects through the suppression of lipid peroxidation. Recently, Takei et al12 demonstrated that cilostazol significantly reduced $O_2^-$ generation through the intracellular signaling pathway with marked elevation of cAMP level. To our knowledge, the present study seems to provide the first evidence for the brain-protective role of the antioxidant cascade through the activation of cAMP and subsequent suppression of accumulation of HNE-modified protein by cilostazol in cerebral white matter injury.

In the present study, an interrelated mechanism that might account for the brain-protective effect of cilostazol is its anti-inflammatory effect mediated by inhibition of microglial activity, which tended to reduce apoptosis of OLGs in the cerebral white matter area. Microglial activation is considered a rapid cellular response to cerebral hypoperfusion, and it is the major source of inflammatory cytokines in the ischemic brain.8,13 Previous studies showed that activated microglia, acting as inflammatory elements, contribute to WMLs8,14 and suppression of activated microglia by immunosuppressants such as FK506 ameliorated white matter damage after chronic cerebral hypoperfusion.15 Taking our results of the distribution and number of activated microglia into consideration, it is possible that the low number of activated microglia was caused by cilostazol treatment, which subsequently provided some protective effect against OLG cell death and WMLs.

The present study demonstrated that cilostazol treatment resulted in overexpression of p-CREB and Bcl-2 after hypoperfusion. This finding suggests that the brain-protective role of cilostazol might be manifested through its antiapoptotic effect via the CREB phosphorylation signaling pathway and subsequent activation of Bcl-2. Recently, several reports demonstrated that cerebral ischemic stress activates the CREB phosphorylation pathway with pathological contribution from neurons and OLGs.2,16 Previous studies also showed that activation of CREB phosphorylation is mediated through protein kinase A, which regulates the transcription of target proteins such as Bcl-2,17 which is known to protect against both apoptotic18 and necrotic cell death.17 To our knowledge, the present study provides the first evidence for the brain-protective role of
cilostazol via the antiapoptotic cascade through cellular signaling pathway in WMLs of chronic cerebral hypoperfusion.

Our study also demonstrated that cilostazol treatment improved learning memory with increased COX-2 expression in the white matter. This result suggests that sustained expression of COX-2 in the white matter might play an important role in preservation of synaptic plasticity in chronic cerebral hypoperfusion. It has been reported that the CREB is present in the rat COX-2 promoter. Thus, the mechanism of cilostazol-induced improvement of cognitive impairment could be described as activation of CREB phosphorylation with subsequent sustained COX-2 expression in WMLs.

In conclusion, we have shown that cilostazol provides protection against cerebral hypoperfusion-induced cognitive impairment and white matter damage in a rat bilateral carotid artery occlusion model. The results suggest that cilostazol has potential therapeutic and brain-protective effects based on multitarget mechanism through cell signaling pathway of CREB phosphorylation, and may be helpful in the treatment of patients experiencing poststroke complications.

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