Cilostazol Attenuates Gray and White Matter Damage in a Rodent Model of Focal Cerebral Ischemia

Fumiaki Honda, MD; Hideaki Imai, MD, PhD; Makoto Ishikawa, PhD; Chisato Kubota; Tatsuya Shimizu, MD, PhD; Mari Fukunaga; Nobuhito Saito, MD, PhD

Background and Purpose—To evaluate whether delayed treatment with the antiplatelet agent cilostazol reduces the volume of infarction in the gray and white matter in a rodent model of permanent focal cerebral ischemia and to explore the mechanism of the neuroprotective effect in vivo.

Methods—Cilostazol (30 or 50 mg/kg) or vehicle was administered by gavage 30 minutes and 4 hours after the induction of cerebral ischemia by permanent occlusion of the left middle cerebral artery (MCA). Animals were euthanized 24 hours after MCA occlusion, and the volume of gray matter damage was evaluated by quantitative histopathology. Axonal damage was determined with amyloid precursor protein immunohistochemistry. Dynamic susceptibility contrast MRI was used to assess regional cerebral blood volume (CBV) and cerebral blood flow (CBF).

Results—Treatment with the higher dose of cilostazol (50 mg/kg) significantly reduced the volume of gray matter damage and axonal damage in the cerebral hemisphere by 45.0% (P<0.02) and 42.4% (P<0.002), respectively, compared with the control group. Relative CBV in the peri-infarct area after MCA occlusion was significantly increased in the cilostazol-treated group (50 mg/kg) compared with the control group (P<0.05). Relative CBF tended to be higher in the cilostazol-treated group compared with the control group.

Conclusions—Treatment with cilostazol significantly reduced the gray and white matter damage associated with permanent focal ischemia. Cilostazol improved CBV and CBF in the peri-infarct area. The major action of cilostazol is to increase perfusion in the ischemic penumbra. (Stroke. 2006;37:223-228.)

Key Words: cerebral ischemia, focal ▪ neuroprotection ▪ white matter

Brain ischemic damage is determined by the severity and the duration of the blood flow deficit. Middle cerebral artery (MCA) occlusion causes a core of severe cerebral ischemia surrounded by a region of oligemia, supported by the collateral blood supply, which is known as the penumbra. The blood flow in the penumbra via leptomeningeal anastomoses from the anterior or posterior cerebral artery is sufficient to keep cells viable for a limited time. Penumbral tissue has the potential for recovery and therefore is a target for acute intervention in ischemic stroke to reverse or minimize brain injury. Pharmacological strategies are based on antiplatelet agents, anticoagulants, and thrombolytics intended to preserve or restore cerebral blood flow (CBF).

Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone) increases the concentration of intracellular cAMP (AMP) by blocking its hydrolysis by phosphodiesterase type III and is approved for use for treating intermittent claudication by the Food and Drug Administration. Its principal actions include inhibition of platelet aggregation, antithrombosis in feline cerebral ischemia, and vasodilation via mediation of increased cAMP level. A recent clinical trial using a randomized, placebo-controlled, double-blind method also showed that long-term administration of cilostazol was safe and effective in preventing the recurrence of cerebral infarction, especially lacunar infarction. Recently, cilostazol was found to have neuroprotective effects against cerebral infarction in rats subjected to 2-hour occlusion of the MCA followed by 24-hour reperfusion.

This study evaluated the neuroprotective efficacy of delayed treatment with cilostazol in an established experimental model of permanent MCA occlusion using conventional histopathology to assess gray matter damage (perikarya) and quantify subcortical white matter damage (axons) and investigated the mechanism by which cilostazol exerts its neuroprotective effect in vivo using dynamic susceptibility contrast MRI to assess hypoperfused brain regions, relative CBF, and relative cerebral blood volume (CBV).

Induction of Focal Cerebral Ischemia

The protocol of this research project followed the guidelines for the care and use of laboratory animals of Gunma University Graduate...
School of Medicine. Male Sprague-Dawley rats (300 to 330 g) obtained from Charles River Japan (Tsukuba, Japan) were anesthetized with halothane in nitrous oxide–oxygen (70:30), then intubated and artificially ventilated. The femoral artery was cannulated for recording arterial pressure and blood gases. Rats were maintained normotensive, normocapnic, adequately oxygenated, and normothermic during anesthesia.

Focal cerebral ischemia was induced using a modification of the permanent MCA occlusion method. Briefly, a 2-cm skin incision was made, then a small subtemporal craniectomy was made. Cerebral ischemia was then induced by electrocoagulation of the MCA from a point proximal to the origin of the lenticulostriate artery to the point crossing the inferior cerebral vein. The MCA was then transected at the olfactory tract to ensure completeness of occlusion. The time of transection was taken as the exact time of MCA occlusion.

Drug Treatment
Rats were randomly divided into 3 groups and given cilostazol (30 or 50 mg/kg; n = 7) or vehicle (control group, n = 8). Cilostazol was dissolved in 25% dimethyl sulfoxide solution or vehicle was given orally by gavage as a bolus 30 minutes and 4 hours after MCA occlusion. During this period, physiological variables were continuously monitored and the animal’s behavior observed.

Tissue Processing
Rats were perfused fixed for neuropathological study. Briefly, the rats were deeply anesthetized with 5% halothane, placed in the supine position, and the thorax opened through bilateral incisions. A catheter was inserted into the left ventricle, the right atrium was incised, and heparinized saline was infused at a pressure equal to the mean arterial blood pressure (90 to 110 mm Hg) of the animal until the perfusate from the right atrium was bloodless. The saline was then followed by ∼300 mL of 4% paraformaldehyde in PBS. After removal of the brain, the forebrain was processed, embedded in paraffin wax, and cut into 6-μm sections at multiple levels.

Histology and Immunohistochemistry
Sections at 8 preselected coronal levels were stained with hematoxylin and eosin for assessment of ischemic damage to neuronal perikarya. Adjacent sections were processed for immunohistochemistry using amyloid precursor protein (APP) antibody to label ischemically damaged axons (disruption of axoplasmic flow leads to APP accumulation). Sections were mounted on amino-silane-coated slides, dried at 37°C overnight, placed in xylene to remove the wax, and dehydrated in absolute alcohol. Sections were microwaved for 10 minutes in 10 mmol/L citric acid, pH 6.0, and allowed to cool to room temperature for 60 minutes. Sections were incubated in 0.03% H2O2 in methanol for 30 minutes and for 1 hour in 50 mmol/L PBS, pH 7.6, containing 0.03% H2O2 in PBS, pH 7.2, containing 0.5% BSA and 10% normal horse serum. Monoclonal antibody to APP (Chemicon), diluted 1:2500 in PBS, was applied to the sections overnight at 4°C. Secondary antibody (biotinylated) horse anti-mouse (Vector Laboratories), diluted 1:100, was applied for 1 hour and the sections washed again (2×10 minutes). The avidin-biotinylated horseradish peroxidase complex (ABC kit; Vector Laboratories) was then applied for 1 hour. The sections were allowed to develop in 3,3′-diaminobenzidine solution, pH 7.6, containing 0.03% H2O2 for 5 minutes. Finally, the sections were dehydrated, cleared, and mounted for light microscopy analysis. Negative controls in which the primary antibody was omitted from the procedure were included in each protocol, and minimal staining was detected. Subsequent analysis was performed by an investigator unaware of the treatment in each animal.

Quantification of Gray Matter Damage
Hematoxylin and eosin–stained sections were examined by an investigator unaware of the treatment. Areas in which neuronal perikarya and parenchyma showed the morphological features of ischemic damage were delineated on scale diagrams (3.36 × actual size) of the forebrain on 8 predetermined coronal planes from anterior 10.50 mm to anterior 1.02 mm. The areas of brain damage were then measured with an image analyzer (Adobe Photoshop software; Adobe Systems) and integrated using the known distance between each coronal level to determine the total volume of ischemic damage in each specimen.

Quantification of Axonal Damage
Axonal damage was quantified by the previously reported method. Briefly, axon-rich structures were selected, including the corpus callosum, external capsule, internal capsule, anterior commissure, median forebrain bundle, fornix, optic tract, caudate putamen, and globus pallidus. Each region was assessed across the 8 stereotaxic coronal levels used to measure gray matter damage. This provided a total of 65 individual regions of white matter to be assessed in each animal. Axonal damage in each region was identified as intense APP immunoreactivity in swollen or bulbous axons, indicating APP accumulation as a result of disrupted axonal transport, and assigned a score of 1. If no APP accumulation was present, a score of 0 was assigned. Total APP score for the entire hemisphere (all 65 regions), for a neuroanatomical structure, or a specific stereotaxic coronal level could then be determined as the sum of individual components.

MRI Measurements
MRI used an Inova 300 Imaging System (7T; Varian) with the radio frequency excitation volume coil actively decoupled and used in combination with a fixed tuned and matched receiver surface coil (Rapid Biomedical GmbH) that was placed over the skull. Rats were placed in a stereotaxic head holder and 1.0% to 1.2% halothane in nitrous oxide–oxygen (70:30) delivered through a facemask for anesthesia. The fraction of inspired oxygen was adjusted to 30%. The rectal temperature was controlled at 37.5 ± 0.5°C by feedback using warm/cold water.

Suitable slices for perfusion analysis were identified on apparent diffusion coefficient (ADC) images obtained by multislice Stejskal-Tanner type pulsed gradient spin echo sequence using the following parameters: repetition time (TR) = 1500 ms; echo time (TE) = 40 ms; b-factors = 35, 400, 700, 1000, and 1300 s/mm²; number of scans = 1; slice thickness = 2 mm; and field of view = 40 × 40 mm. The matrix size was 256 × 128, and the images were 0-filled to 256 × 256. The slice for perfusion measurement was determined as the ADC image with the largest lesion area.

Relative CBF and relative CBV were studied with dynamic susceptibility contrast MRI. A series of 49 gradient-echo single-slice images with TR = 7.8 ms, TE = 3 ms, and flip angle 25°C was acquired. A bolus of the susceptibility contrast agent gadopentetate dimeglumine (0.3 mmol/kg; Nihon Schering K.K.) was injected intravenously into the tail vein 5 seconds after the start of image acquisition.

Assessment of Relative CBF and Relative CBV
Relative CBF and relative CBV were determined with commercially available image analysis software (MEDx; version 3.43; Medical Numerics, Inc.). The region of interest (ROI) was determined by manually tracing the ADC image. The perfusion analysis was performed using 5 ROIs determined by stereotaxic coordinates and corresponding to the cingulate, frontal, hindlimb, and forelimb cortex (ROI 1), parietal cortex (ROI 2), piriform and insular cortex (ROI 3), preoptic area (ROI 4), and caudate putamen and septal nuclei (ROI 5). The relative CBF and relative CBV in each ROI of the ischemic hemisphere were expressed as a ratio of that measured in the corresponding contralateral ROI.

Statistical Analysis
Parametric data were compared between multiple groups by 1-factor ANOVA followed by the Holm test and pairs of groups by Student’s unpaired 2-tailed t test. Data are presented as mean ± SD. Nonparametric data were compared between multiple groups by the Kruskal–Wallis test followed by the Mann–Whitney U test.
Results

The physiological variables are shown in the Table. There were no significant differences in physiological variables between the 3 experimental groups.

Gray Matter Damage

Neuronal perikarya in the ipsilateral cerebral cortex and caudate putamen clearly exhibited the characteristic morphological features of ischemic damage (ie, shrinkage and triangulation of the nucleus and cytoplasm and increased eosinophilia of the cytoplasm). The boundaries between the ischemic and nonischemic neuronal perikarya were identifiable in control and cilostazol-treated groups (Figure 1A through 1C). Rats treated with both doses of cilostazol (30 and 50 mg/kg) had significantly reduced volumes of infarction in the cerebral hemisphere and cerebral cortex (Figure 2A). Treatment with the higher dose of cilostazol (50 mg/kg) significantly reduced the volume of gray matter damage in the cerebral hemisphere and cerebral cortex by 45.0% and 48.5%, respectively, compared with the control group ($P<0.02$). Treatment with the lower dose of cilostazol (30 mg/kg) significantly reduced the volume of ischemic damage in the cerebral hemisphere by 43.2% ($P<0.05$) and cerebral cortex by 40.4% ($P<0.05$). Neither dose of cilostazol had a significant protective effect on the caudate nucleus in this model.

Axonal Damage

Axonal damage was recognized as intense APP immunoreactivity in swollen or bulbous axons within the ipsilateral subcortical white matter fiber tracts (Figure 1D through F). The anatomically defined zones of APP immunoreactivity allowed scoring for the presence or absence of APP axonal profiles in each region throughout the rostrocaudal extent of the MCA territory. Axonal damage was less extensive in cilostazol-treated animals. Total APP score in the cilostazol-treated groups (30 and 50 mg/kg) was significantly lower than in the control group ($P<0.002$; Figure 2B). Treatment

<table>
<thead>
<tr>
<th>Physiological Variables Before and After MCA Occlusion</th>
<th>MABP (mm Hg)</th>
<th>pH</th>
<th>Paco2 (mm Hg)</th>
<th>Paco2 (mm Hg)</th>
<th>Rectal Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>95±12</td>
<td>7.41±0.03</td>
<td>41±4</td>
<td>153±19</td>
<td>36.9±0.6</td>
</tr>
<tr>
<td>30 min after</td>
<td>98±11</td>
<td>7.41±0.05</td>
<td>43±8</td>
<td>141±19</td>
<td>36.8±0.6</td>
</tr>
<tr>
<td>4 h after</td>
<td>123±5</td>
<td>7.46±0.06</td>
<td>38±9</td>
<td>105±7</td>
<td>37.3±0.4</td>
</tr>
<tr>
<td>24 h after</td>
<td>122±9</td>
<td>7.50±0.01</td>
<td>35±2</td>
<td>100±15</td>
<td>37.7±0.1</td>
</tr>
<tr>
<td><strong>Cilostazol (30 mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>88±9</td>
<td>7.40±0.03</td>
<td>37±10</td>
<td>135±31</td>
<td>36.9±0.4</td>
</tr>
<tr>
<td>30 min after</td>
<td>83±10</td>
<td>7.42±0.03</td>
<td>43±6</td>
<td>141±38</td>
<td>36.9±0.4</td>
</tr>
<tr>
<td>4 h after</td>
<td>111±7</td>
<td>7.47±0.02</td>
<td>31±3</td>
<td>99±5</td>
<td>37.5±0.1</td>
</tr>
<tr>
<td>24 h after</td>
<td>114±14</td>
<td>7.49±0.01</td>
<td>30±4</td>
<td>106±18</td>
<td>38.0±0.2</td>
</tr>
<tr>
<td><strong>Cilostazol (50 mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>85±16</td>
<td>7.39±0.02</td>
<td>42±5</td>
<td>144±32</td>
<td>37.3±0.3</td>
</tr>
<tr>
<td>30 min after</td>
<td>90±13</td>
<td>7.40±0.05</td>
<td>43±6</td>
<td>146±40</td>
<td>36.8±0.3</td>
</tr>
<tr>
<td>4 h after</td>
<td>111±11</td>
<td>7.40±0.08</td>
<td>43±7</td>
<td>115±38</td>
<td>37.3±0.3</td>
</tr>
<tr>
<td>24 h after</td>
<td>117±7</td>
<td>7.48±0.01</td>
<td>34±4</td>
<td>107±22</td>
<td>37.5±0.2</td>
</tr>
</tbody>
</table>

Values are mean±SD. MABP indicates mean arterial blood pressure.
with the higher dose of cilostazol (50 mg/kg) significantly reduced the axonal damage in the corpus callosum, internal capsule, and caudate putamen, and treatment with the lower dose (30 mg/kg) in the caudate putamen (Figure 3B). APP scores in the external capsule, anterior commissure, globus pallidus, and ventral axon-rich structures such as the optic tract, median forebrain bundle, and fornix were not significantly different between the cilostazol-treated and control groups.

**CBF and CBV Study**

Representative ADC, CBV, and CBF images of control and cilostazol-treated groups are shown in Figure 4. The ADC image shows no remarkable difference in area of ischemic damage at 1 hour after MCA occlusion between the 2 groups. However, statistical analysis showed that CBV in ROI 1, located in the dorsomedial area where cerebral blood was supplied by leptomeningeal anastomosis from the anterior cerebral artery, was significantly increased in the cilostazol-treated group ($P<0.05$ versus control group; Figure 5). The area of ischemic damage had extended to the dorsomedial area between 1 hour and 24 hours after MCA occlusion in the control group. On the other hand, no such expansion of ischemia was seen in the cilostazol-treated group. Interestingly, the magnetic resonance perfusion study clearly demonstrated this phenomenon by revealing that the CBV in ROI 2 at 24 hours after MCA occlusion was significantly increased in the cilostazol-treated group ($P<0.05$ versus control group) and that the CBV in other areas at 1 and 24 hours after ischemia tended to be higher in the cilostazol-treated group compared with the control group. The CBF in all areas, but particularly in ROI 1, tended to be higher in the cilostazol-treated group compared with the control group.

**Discussion**

The major finding of this study is that the treatment with cilostazol provides significant protection against ischemic damage in gray and white matter. Quantitative methods demonstrated reduced ischemic damage to cortical neuronal perikarya and axons after administration of cilostazol. Evaluation of gray and white matter damage is equally important to assess the efficacy of potential protective agents. The neurological consequences of an ischemic insult reflect the effect of the stroke on gray and white matter. Despite this, gray matter damage has attracted much more attention than white matter damage, particularly in the experimental stroke model because of the lack of appropriate methodology for the assessment of white matter damage in vivo.

Over the last decade, >180 clinical trials of so-called neuroprotectants, including antagonists of glutamate release and receptor activation, Ca$^{2+}$ channel blockers, and antioxidants, have failed to demonstrate significant benefits on stroke outcome. One possible explanation of the poor
clinical performance of most neuroprotective compounds is that the extensive damage in the white matter is not reduced.\textsuperscript{14} The processes involved in white matter ischemia are only now beginning to be understood and recognized as equal in importance to gray matter ischemia.

The quantification of APP accumulation showed significant, cilostazol-induced reduction of axonal damage in the ischemic hemisphere. Interestingly, cilostazol was not equally effective in all white matter regions (Figure 3). The most marked reductions in white matter damage were found in peri-infarct regions such as the corpus callosum, internal capsule, and caudate putamen, whereas other white matter regions showed severe ischemia,\textsuperscript{15} such as the fiber tracts of the external capsule and anterior commissure. Cilostazol also produced significant attenuation of neuronal perikaryal damage (45% salvage of hemisphere), which was principally attributable to reduction in cortical damage (48.5%; Figure 2). Apparently, the white and gray matter contain zones that can and cannot be salvaged by pharmacological intervention.

Therefore, whether cilostazol elicits CBF increase in the peri-infarct zone (penumbra) in acute focal ischemia is important.

Dynamic susceptibility contrast, MRI can provide accurate relative CBF and CBV measurement even in the presence of pathological hemodynamics such as MCA occlusion.\textsuperscript{16} This bolus tracking MRI technique allows repeated measurements of blood flow reduction in the left hemisphere at 1 hour and 24 hours after MCA occlusion. The present study found that CBV was significantly increased in ROI 1 at 1 hour and ROI 2 at 24 hours after MCA occlusion in the cilostazol-treated group ($P<0.05$ versus control group), and that CBV in other areas at 1 and 24 hours tended to be higher in the cilostazol-treated group compared with the control group (Figure 5). CBF in all areas, particularly in ROI 1, tended to be higher in the cilostazol-treated group compared with the control group. The beneficial effect of cilostazol can be attributed to vasodilation\textsuperscript{17} as well as antiplatelet activity and antiapoptotic action.\textsuperscript{18} Cilostazol causes vasodilation of isolated, pressur-
ized rabbit spinal arterioles, which may be extrapolated to the cerebral microcirculation as the same arterioles feeding the central nervous system. Cilostazol also dilates the pial arterioles and inhibits formation of thrombosis during focal ischemia in the cat. Although the detailed mechanisms of action of cilostazol on vascular smooth muscle still remain unknown, presumably, phosphodiesterase 3 inhibition and corresponding cAMP increase in smooth muscles of arterioles are involved to some extent because phosphodiesterase 3 is strongly expressed in these cells. These observations suggest that cilostazol improves brain ischemic outcome in this model by restoring local CBF, resulting in minimization of the extent of brain damage and prevention of further brain injury attributable to long-term exposure to ischemic insult in the penumbra. This action of cilostazol is notable because aspirin, which is the most widely prescribed antiplatelet agent for stroke, was reported to acutely reduce CBF by ≈20% in a rabbit model. The aspirin irreversibly inhibits platelet aggregation by inhibiting cyclooxygenase. This, in turn, inhibits the conversion of arachidonic acid to prostacyclin or NO, a potent vasodilator. The future use of antiplatelet therapy for the treatment of acute cerebral ischemia depends on greater understanding of the activity of these agents on platelet function and on the cerebrovasculature within the brain parenchyma, especially as related to CBF. Treatment with cilostazol has the potential to minimize brain injury in acute stroke.

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References

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