Cilostazol, Not Aspirin, Reduces Ischemic Brain Injury via Endothelial Protection in Spontaneously Hypertensive Rats

Naoki Oyama, MD; Yoshiki Yagita, MD, PhD; Miki Kawamura, MD; Yukio Sugiyama, MD; Yasukazu Terasaki, MD, PhD; Emi Omura-Matsuoka, MD; Tsutomu Sasaki, MD, PhD; Kazuo Kitagawa, MD, PhD

Background and Purpose—It is well-established that hypertension leads to endothelial dysfunction in the cerebral artery. Recently, cilostazol has been used for the secondary prevention of ischemic stroke. Among antiplatelet drugs, phosphodiesterase inhibitors including cilostazol have been shown to have protective effects on endothelial cells. The aim of the present study is to investigate the effects of cilostazol and aspirin on endothelial nitric oxide synthase (eNOS) phosphorylation in the cerebral cortex, endothelial function, and infarct size after brain ischemia in spontaneously hypertensive rats (SHR).

Methods—Five-week-old male SHR received a 5-week regimen of chow containing 0.1% aspirin, 0.1% cilostazol, 0.3% cilostazol, or the vehicle control. The levels of total and Ser1177-phosphorylated eNOS protein in the cerebral cortex were evaluated by Western blot. To assess the contribution of eNOS in maintaining cerebral blood flow, we monitored cerebral blood flow by laser-Doppler flowmetry after L-N5'(1-iminoethyl)ornithine infusion. Additionally, we evaluated residual microperfusion using fluorescence-labeled serum protein and infarct size after transient focal brain ischemia.

Results—In SHR, the blood pressure and heart rate were similar among the groups. Cilostazol-treated SHR had a significantly higher ratio of phospho-eNOS/total eNOS protein than vehicle-treated and aspirin-treated SHR. Treating with cilostazol, but not aspirin, significantly improved cerebral blood flow response to L-N5'(1-iminoethyl)ornithine. Cilostazol also increased residual perfusion of the microcirculation and reduced brain damage after ischemia compared to vehicle control and aspirin.

Conclusions—These findings indicate that cilostazol, but not aspirin, can attenuate ischemic brain injury by maintaining endothelial function in the cerebral cortex of SHR. (Stroke. 2011;42:2571-2577.)

Key Words: brain ischemia • endothelial function • hypertension • phosphodiesterase-3 inhibitor

Hypertension is one of the most important risk factors for cerebrovascular disease and is closely associated with endothelial dysfunction. Some studies observed endothelial dysfunction in patients with hypertension or cerebrovascular disease.1-2 In hypertensive animal models, hypertension impairs endothelium-dependent vasodilatation, cerebrovascular autoregulation, and cerebral blood flow (CBF) responses, and it exacerbates ischemic brain damage.3-6 Endothelial dysfunction is often characterized by a decrease in the bioavailability of endothelium-derived nitric oxide (NO). In endothelial cells, NO is produced by endothelial nitric oxide synthase (eNOS), and eNOS activity is regulated primarily by calcium-calmodulin activation and multisite phosphorylation of specific serine or threonine residues. Most importantly, phosphorylation of eNOS-Ser1177 is thought to play a crucial role in eNOS activation. In a cerebral ischemia model, modification of the eNOS-Ser1177 phosphorylation state modulated CBF and the outcome of ischemic injury.7 These findings are further supported by our previous observations that hypertension impaired cerebrovascular function by decreasing eNOS-Ser1177 phosphorylation and exacerbated ischemic brain damage.8

Cilostazol, a phosphodiesterase-3 inhibitor, is used as an antiplatelet drug for the secondary prevention of ischemic stroke as well as a treatment for intermittent claudication with peripheral artery disease in Asian countries. In particular, cilostazol appears to be more effective in patients with hypertension, diabetes, or lacunar infarction.9 Recently, an aspirin-controlled, double-blind, randomized trial (CSPS 2) showed that cilostazol was superior to aspirin in secondary stroke prevention.10 These beneficial effects of cilostazol have been attributed not only to its antiplatelet functions but also to other actions on the cerebrovascular endothelium.11,12 Several studies showed that short-term pretreatment and

Received November 26, 2010; accepted April 4, 2011.
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The online-only Data Supplement is available at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.111.609834/-/DC1.
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© 2011 American Heart Association, Inc.
Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.111.609834

2571
post-treatment with cilostazol attenuated ischemic brain injury by increasing NO production and eNOS activation. However, the comparative effects of cilostazol and aspirin on cerebrovascular function have not been investigated.

We hypothesized that chronic cilostazol treatment, in contrast to aspirin treatment, (1) can prevent hypertension-induced cerebrovascular dysfunction through increased eNOS-Ser1177 phosphorylation and the eNOS-dependent CBF

Figure 1. Temporal profiles of systolic blood pressure (A) and heart rate (B) in spontaneously hypertensive rats that were treated with the vehicle control, aspirin, 0.1% cilostazol, or 0.3% cilostazol. Data are expressed as the mean ± standard deviation.

Figure 2. Representative Western blots and effects of antiplatelet drugs on the expression levels of the nitric oxide synthase (NOS) isoforms: (A) phospho-eNOS, total eNOS and β-actin, and (B) phospho-neuronal NOS, total neuronal NOS, and β-actin. All values were normalized by setting the densitometry of the control sample to 1.0. Data are expressed as the mean ± standard deviation. WKY indicates Wistar Kyoto rats.
response, and (2) can improve the infarct volume in a focal brain ischemia model.

**Materials and Methods**

**Preparation of Animals**

All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine. Male spontaneously hypertensive rats (SHR; Charles River, Yokohama, Japan) and Wistar Kyoto rats (Charles River) were used for experiments. For SHR, 5-week-old prehypertensive rats were divided into the following 4 groups: (1) vehicle-treated SHR that received a standard rat chow for 5 weeks; (2) aspirin-treated SHR that received a chow containing 0.1% aspirin (Otsuka Pharmaceutical, Tokyo, Japan) for 5 weeks; (3) normal-dose cilostazol-treated SHR that received a chow containing 0.1% cilostazol (Otsuka Pharmaceutical) for 5 weeks; and (4) high-dose cilostazol-treated SHR that received a chow containing 0.3% cilostazol for 5 weeks. To assess the influences of cilostazol on physiological parameters, body weight, blood pressure, and heart rate were measured weekly throughout the study using the tail-cuff method (5–10 weeks of age). The average daily food intake was measured for all treatment groups. Daily food intake was estimated by measuring the weight of food consumed by each cage. The changes in body weight and food consumption are shown in Supplemental Table I (http://stroke.ahajournals.org). The daily drug dosage in 0.1% aspirin-treated SHR and 0.1% cilostazol-treated SHR was estimated to be ~80 mg/kg. These doses of aspirin and cilostazol have antiplatelet or antithrombotic effects in rats and have often been used as the relevant dose range to humans.12,16–19

Detailed methods of Western blot, evaluation of CBF response, middle cerebral artery (MCA) occlusion, evaluation of residual CBF during MCA occlusion, evaluation of infarct size, and statistical analysis are available in the online Data Supplement.

**Results**

**Effects of Aspirin and Cilostazol on eNOS Phosphorylation**

As shown in Figure 1, aspirin and cilostazol did not significantly affect the systolic blood pressure and heart rate in SHR. Compared to Wistar Kyoto rats, the ratio of phospho-eNOS/total eNOS protein was clearly reduced in vehicle-treated and aspirin-treated SHR (Figure 2A). In SHR treated with 0.1% and 0.3% cilostazol, the ratio of phospho-eNOS/total eNOS protein was significantly higher than that in vehicle-treated and aspirin-treated SHR and similar to the ratio in Wistar Kyoto rats. There were no differences among the groups in total eNOS expression and phosphorylation of neuronal NOS, another NOS isoform (Figure 2A, B).

**Effects of Aspirin and Cilostazol on the eNOS-Dependent Regional CBF Response**

There were no significant differences among the groups in mean arterial pressure response to L-N5-(1-iminoethyl)ornithine (L-NIO; a relatively selective neuronal NOS inhibitor; Figure 3A). All blood gas parameters remained within the normal limits before and 30 minutes after L-NIO infusion and were similar among all of the groups (Supplemental Table II). The influences of L-NIO on eNOS expression were not observed (Supplemental Figure III).

L-NIO administration decreased regional CBF (rCBF) and reached a minimum 10 to 20 minutes after starting the infusion in all of the groups (Figure 3B). The maximum and the total rCBF response to L-NIO in the vehicle-treated SHR were 10.9%±2.3% and 260.4%±63.9%×min, respectively (Figure 3C, D). Treating with 0.1% and 0.3% cilostazol (0.1% cilostazol, 16.3%±1.9% and 421.9%±65.9%×min; 0.3% cilostazol, 16.9%±5.6% and 449.2%±177.2%×min), but not aspirin (13.4%±3.7% and 343.9%±101.0%×min), significantly improved both the maximum and the total rCBF response (Figure 3C, D). In contrast, there were no differences in the maximum and the total rCBF response to 7-nitroindazole (a relatively selective neuronal NOS inhibitor) between vehicle-treated and cilostazol-treated SHR (Supplemental Figure IV).
Effects of Aspirin and Cilostazol on Residual Perfusion and Infarct Size After Transient Focal Brain Ischemia

There were no significant differences in blood gas parameters among the groups or before MCA occlusion, before reperfusion, and after reperfusion (Supplemental Table V). Mean arterial pressure was elevated during MCA occlusion in all groups, and there were no differences among the groups in the rate of arterial pressure changes (Figure 4A).

In the laser Doppler study, MCA occlusion decreased the ipsilateral rCBF to 15% to 25% of baseline, and reperfusion recovered rCBF to baseline under anesthesia in all of the groups. Compared with the vehicle-treated SHR, the rCBF reduction in cilostazol-treated SHR was slightly reversed from 0 to 10 minutes and significantly reversed from 20 to 80 minutes after MCA occlusion, whereas aspirin did not attenuate the rCBF reduction (Figure 4B). In addition, the residual perfusion of the microcirculation after MCA occlusion was assessed using dichlorotriazinyl amino fluorescein-labeled serum. In the peripheral region, capillary fluorescence was minimally detected in vehicle-treated and aspirin-treated SHR, whereas it was relatively preserved in cilostazol-treated SHR (Supplemental Figure VI and Figure 5A). In the quantitative assessment, cilostazol clearly preserved the residual microperfusion in the peripheral region compared to vehicle control and aspirin (Figure 5B). There were no effects of cilostazol in the striatum, the center region of the MCA territory in the cortex, and the anterior cerebral artery territory (Figure 5B). Treating with cilostazol, but not aspirin, significantly decreased the infarct size 48 hours after transient MCA occlusion compared to the vehicle control, and these beneficial effects of cilostazol were also observed 7 days after ischemia (Figure 6A–C). In contrast, this neuroprotective effect of cilostazol was abolished in the presence of L-NIO (Figure 6D).

Discussion

The present study provides novel data that cilostazol, but not aspirin, preserved eNOS-Ser1177 phosphorylation and the eNOS-dependent CBF response in the cerebral cortex of SHR. Additionally, we showed that these cilostazol-mediated effects could reduce the infarct volume after transient focal brain ischemia. These observations suggest that cilostazol has a protective effect against hypertension-induced endothelial dysfunction as well as antiplatelet effects and can effectively prevent infarct enlargement.

The eNOS-Ser1177 phosphorylation is frequently used to evaluate cerebrovascular function because this phosphorylation state plays pivotal roles in modulating eNOS activity and subsequently regulating CBF and the outcome of ischemic injury, although other eNOS sites, such as serine 633 and threonine 495, can also contribute to eNOS activity. Several previous studies indicated that cilostazol positively affects eNOS-Ser1177 phosphorylation. In apolipoprotein E-deficient mice, an animal model of atherosclerosis, a 2-week cilostazol treatment regimen normalized NO-dependent cerebrovascular reactivity by restoring eNOS-Ser1177. In cultured endothelial cells, it has been shown that cilostazol enhanced eNOS activity and NO production through AMP-activated protein kinase-dependent, cAMP/protein kinase A-dependent, and phosphatidylinositol 3-kinase/Akt-dependent phosphorylation of eNOS-Ser1177. In cultured endothelial cells, it has been shown that cilostazol enhanced eNOS activity and NO production through AMP-activated protein kinase-dependent, cAMP/protein kinase A-dependent, and phosphatidylinositol 3-kinase/Akt-dependent phosphorylation of eNOS-Ser1177. In cultured endothelial cells, it has been shown that cilostazol enhanced eNOS activity and NO production through AMP-activated protein kinase-dependent, cAMP/protein kinase A-dependent, and phosphatidylinositol 3-kinase/Akt-dependent phosphorylation of eNOS-Ser1177.

We also evaluated the contribution of eNOS activity to cerebrovascular function. It was previously reported that physiological cerebrovascular function could be evaluated by measuring the decrease in CBF induced by NOS inhibitors. The eNOS activity was evaluated by measuring the CBF response to L-NIO. In fact, our previous study showed...
that SHR had a significantly lower rCBF response to L-NIO than normotensive rats, and these findings suggested that hypertension decreased the eNOS activity in the cerebral artery.8 In the present study, cilostazol-treated SHR had a significantly higher rCBF response to L-NIO than vehicle-treated SHR. Furthermore, we assessed the rCBF response to 7-nitroindazole, a relatively selective neuronal NOS inhibitor, because L-NIO may affect neuronal NOS as well as eNOS, depending on the dose. We found no differences in the rCBF response to 7-nitroindazole between vehicle-treated and cilostazol-treated SHR. These results imply that cilostazol can protect physiological cerebrovascular function through the maintenance of eNOS function. However, aspirin treatment failed to preserve eNOS-Ser1177 phosphorylation or to improve the eNOS-dependent CBF response in SHR. Previous studies indicated that aspirin could induce endothelium-derived NO release and provide vascular protection against hypertension by reducing superoxide production.17,27 However, other studies reported that aspirin could not enhance NO availability in endothelial cells or prevent endothelial dysfunction.12,28,29 Therefore, it has remained unclear whether aspirin has beneficial effects on endothelial function, particularly cerebrovascular function.

The current study showed that cilostazol could maintain eNOS phosphorylation and reduce infarct size in SHR. Dipyridamole, a phosphodiesterase-5 inhibitor, has been shown to decrease the cerebral infarct size, and the combination of subtherapeutic doses of statin and dipyridamole increased eNOS activity and cerebral blood flow and reduced infarct volume.30 In addition to these phosphodiesterase inhibitors, it has been reported that several modalities, such as statin, angiotensin II type 1 receptor blocker, Rho kinase inhibitor, and L-arginine, upregulated eNOS expression, increased eNOS activity, and inhibited oxidative stress.3,6,8,31–33 All of these modalities increase endothelium-derived NO availability and have been shown to enhance CBF, resulting in neuroprotection after cerebral ischemia. Together with our data, these findings indicate that preserving endothelial function before ischemic insult is extremely critical to lessen ischemic brain injury. Furthermore, we showed that early chronic treatment has beneficial effects on ischemic brain damage in hypertensive rats. Our results are consistent with the previous clinical evidence that cilostazol was more useful in preventing secondary stroke in patients at high risk with hypertension and lacunar infarction, and may suggest that we should start cerebrovascular protection as
early as possible in hypertensive patients with ischemic stroke.\textsuperscript{9}

A previous study reported that the plasma cilostazol concentration in 0.1% cilostazol-treated rats was \(\sim1\ \text{mol/L}.\)\textsuperscript{16} Based on the in vitro IC\textsubscript{50} value, this plasma cilostazol concentration is estimated to be sufficient to inhibit phosphodiesterase-3. However, the most appropriate dose of cilostazol for preservation of endothelial function is still unclear. Therefore, we used high-dose cilostazol (0.3% cilostazol) as well as normal-dose cilostazol (0.1% cilostazol). As a result, we did not find any significant differences in eNOS phosphorylation and the eNOS-dependent rCBF response among the different cilostazol doses. It is likely that the plasma concentration of cilostazol in rats receiving chow containing 0.1% cilostazol is sufficiently high to protect endothelial function.

This study analyzed the effects of aspirin and cilostazol on the eNOS protein in the cerebral cortex. As we reported previously, double immunohistochemical staining with eNOS and von Willebrand factor, an endothelial cells marker, revealed that the eNOS signals colocalized with von Willebrand factor.\textsuperscript{8} These results indicated that eNOS was predominantly expressed in brain blood vessels. Therefore, we considered that our Western blot results using cerebral cortex homogenates mostly represent eNOS expression in the intraparenchymatous cerebral vessels.

**Conclusions**

In summary, chronic treatment with cilostazol can prevent cerebrovascular dysfunction in SHR by maintaining eNOS-Ser\textsuperscript{1177} phosphorylation, resulting in reduced ischemic brain damage. Our work provides important evidence that clarifies the underlying mechanisms by which cilostazol is protective against ischemic brain injury.

**Acknowledgments**

Food mixed with aspirin or cilostazol was kindly provided by Otsuka Pharmaceutical, Tokyo, Japan. The authors thank K. Nishiyama for technical assistance and C. Kurano for secretarial assistance.

**Sources of Funding**

This work was supported by a grant-in-aid from the Ministry of Education, Science, and Culture in Japan.

**Disclosure**

None.
References


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Stroke. 2011;42:2571-2577; originally published online July 28, 2011; doi: 10.1161/STROKEAHA.110.609834

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Supplemental Methods

Western Blot

Total protein was prepared from the cerebral cortex in the rats after 5 weeks of treatment, and Western blot analysis was performed as previously described. Also, to assess the influences of L-N\textsuperscript{5}-(1-iminoethyl)ornithine (L-NIO, a relatively selective eNOS inhibitor; Calbiochem, San Diego, CA, USA) on endothelial nitric oxide synthase (eNOS) expression, the brains were quickly removed at 15 minutes after L-NIO administration in vehicle-treated spontaneously hypertensive rats (SHR). A piece of cerebral cortex tissue was rapidly removed and homogenized as soon as possible in lysis buffer containing a protease inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland). Homogenates were centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatants were electrophoresed on 0.1% sodium dodecyl sulfate (SDS)-containing polyacrylamide gels, and the separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membrane was blocked with 5% skim milk in phosphate buffered saline with 0.1% Tween-20 (T-PBS) for 30 minutes and then incubated with the primary antibodies overnight at 4°C. The membranes were washed in T-PBS and incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (1:5000; GE Healthcare, Buckinghamshire, UK) for 1 hour. The blots were developed by enhanced chemiluminescence using ECL Western blotting detection reagents (GE Healthcare). Digital images were produced by densitometric scans of autoradiographs and quantified using ImageJ 1.40 software. The following primary antibodies were used: mouse anti-eNOS monoclonal antibody (1:1000; BD Transduction Laboratories, San Diego, CA), rabbit anti-phospho-eNOS (Ser\textsuperscript{1177}) polyclonal antibody (1:1000; Cell Signal Technology, Beverly, MA), rabbit anti-neuronal nitric oxide synthase (nNOS) polyclonal antibody (1:2000; Invitrogen, Carlsbad, CA), rabbit anti-phospho-nNOS (Ser\textsuperscript{1417}) polyclonal antibody (1:2000; Abcam Limited, Cambridge, UK), and mouse anti-β-actin monoclonal antibody (1:250000; Sigma, St Louis, MO).

Evaluation of the CBF Response to L-NIO

To assess the contribution of eNOS in maintaining cerebral blood flow (CBF), we continuously monitored regional CBF (rCBF) in the cerebral cortex by laser-Doppler flowmetry (Laser Doppler Blood Flow Meter, model TBF-LNIT; Unique Medical Co., Ltd., Tokyo, Japan) after administrating L-NIO, and
evaluated the regional CBF (rCBF) response. General anesthesia was induced with 4.0% halothane and maintained with 0.5% halothane using a face mask. The skull overlying the parietal cortex was thinned with a drill until translucent at 1 mm posterior and 4.5 mm lateral to the bregma. A polyacrylamide column with an inner diameter of 0.8 mm was attached with dental cement, and the laser-Doppler flow probe was placed across the column to monitor cortical microperfusion. After achieving a stable baseline rCBF, L-NIO (20 mg/kg in saline) was administered for 2.5 minutes via the left femoral vein. rCBF values were recorded in 5- to 10-minute intervals during the study. The rCBF measurements are expressed as a percentage of the baseline. Furthermore, to assess the possible involvement of nNOS in this examination, we compared the rCBF response to 7-nitroindazole (7-NI), a relatively selective nNOS inhibitor (Sigma, St Louis, MO), in SHR treated with the vehicle control or 0.1% cilostazol. 7-NI (25 mg/kg) was suspended in peanut oil and intraperitoneally injected for 10 seconds. The rCBF values were recorded as described above. The rCBF response was assessed using two methods: (1) the maximum rCBF response that was defined as the magnitude of the maximum decrease during the 30-minute recording period (arrows in Figure 3B and Supplemental Figure S4B) and (2) the total rCBF response that was defined as the integral of the rCBF response curve from 0 to 30 minutes after drug infusion (shaded areas in Figure 3B and Supplemental Figure S4B). The left femoral artery was catheterized to continuously monitor the arterial blood pressure (BP), and the arterial blood gas parameters were analyzed at baseline and 30 minutes after drug administration. The rectal temperature was maintained at 37.0°C ± 1.0°C during the procedure.

**Middle Cerebral Artery (MCA) Occlusion**

MCA was occluded for 80 minutes by an intraluminal filament technique as previously described. The tip of a 4-0 nylon monofilament was heated, and a round ball was formed. Each animal was placed in a supine position, and the right common carotid artery (CCA) was exposed by a midline incision under halothane anesthesia. The external carotid artery and proximal CCA were occluded by a 5-0 silk suture. The distal CCA was occluded loosely with a 5-0 silk suture, and the round tip filament was introduced into the CCA. The filament was advanced until the tip occluded the origin of the middle cerebral artery. At 80 minutes after MCA occlusion, the filament was withdrawn to allow reperfusion.

**Evaluation of the Residual CBF During MCA Occlusion**

To evaluate the residual CBF during MCA occlusion, a polyacrylamide column was attached to the
skull that was thinned with a drill at 1 mm posterior and 4.5 mm lateral to the bregma. The laser-Doppler flowmetry probe was placed across the column, and the regional cerebral blood flow values were recorded in 5- to 10-minute intervals during the study. The rCBF measurements are expressed as a percentage of the values before CCA occlusion. Arterial BP was monitored continuously via the left femoral artery, and arterial blood gas parameters were analyzed before MCA occlusion, before reperfusion, and after reperfusion. The rectal temperature was maintained at 37.0°C ± 1.0°C during the procedure.

Additionally, to visualize the residual perfusion of the microcirculation after MCA occlusion, we labeled plasma with dichlorotriazinyl amino fluorescein (DTAF; excitation 489 nm, emission 515 nm; Sigma) as previously described. One hundred milligrams of DTAF was dissolved in a solution containing 20 mL of normal rat serum and 1 mL of 0.5 mol/L Tris-HCl, pH 9.0. DTAF was conjugated to serum proteins at room temperature for 1 hour while maintaining the pH at 9.0 with the addition of 1 N NaOH. At the end of the reaction, the pH was readjusted to 7.4 by adding 1 N HCl, and the solution was stored in aliquots at −30°C. At 80 minutes after MCA occlusion, 0.1 mL of DTAF-conjugated rat serum was injected into the saphenous vein for 10 seconds. Fifteen seconds after the injection, each rat was decapitated, and the brain was fixed in 80% ethanol for 24 hours. For measurement, three 50-µm-thick coronal brain slices were obtained every 0.5 mm beginning at a section 1.0 mm rostral to bregma using a vibratome. They were examined with the same magnification and resolution under a fluorescence microscope (Eclipse 80i; Nikon Inc).

To quantitatively assess the residual perfusion of the microcirculation, the DTAF-labeled microvessels were evaluated in four regions: the cortical region of the anterior cerebral artery territory, the peripheral cortical region of the MCA territory, the core region of the MCA territory in the cortex, and the dorsolateral region of the striatum (regions 1, 2, 3, and 4, respectively, in Supplemental Figure 6). The residual perfusion levels were calculated as the mean areas of vessels within regions of interest (ROI; 0.8 mm × 0.8 mm) that were obtained by counting the number of pixels in the fluorescence images after the background was subtracted using ImageJ software. The residual perfusion index was defined as the rate of the mean residual perfusion area of the ROI on the ipsilateral side (regions 1, 2, 3, and 4 in Supplemental Figure 6) compared to that of the same region on the contralateral side (regions 5, 6, 7, and 8, respectively, in Supplemental Figure 6). These evaluations were performed in SHR treated with the vehicle control, aspirin, or 0.1% cilostazol (respectively, n=5).
Evaluation of Infarct Size

Infarct volume was evaluated at 48 hours and 7 days after inducing ischemia. Additionally, to assess the contributions of eNOS to the neuroprotective effects of cilostazol, the MCA was occluded for 80 minutes at 15 minutes after L-NIO was administered to the vehicle- and cilostazol-treated SHR, and the infarct volume was evaluated 48 hours after ischemia. The brains were removed and sectioned coronally into six 2-mm-thick slices. The sections were placed in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 30 minutes at 37°C and fixed in 10% formalin. The area with ischemic lesions and the area of both hemispheres (mm$^2$) were calculated on TTC-stained coronal sections by tracing these areas on the computer screen using ImageJ software. To reduce errors associated with tissue processing for histological evaluation, the lesion area was corrected with the indirect method, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere. Infarct volume (mm$^3$) was determined by integrating the appropriate area and section thickness.

Statistical Analysis

All data are shown as the mean ± standard deviation. Differences between two means were examined by the Mann-Whitney U test. One-way analysis of variance and Scheffe’s post-hoc test were used for multiple comparisons, and the Wilcoxon rank test was used to compare paired sample observations. Software SPSS 11.5 was used for statistical analysis. $P$-values < 0.05 were considered statistically significant.
### Supplemental Tables, Figures, and Figure Legends

#### S1: Body Weight and Food Intake

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<tr>
<th>Treatment</th>
<th>Pre-administration body weight (g)</th>
<th>Post-administration body weight (g)</th>
<th>Daily food intake (g/kg)</th>
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<td>110 ± 5</td>
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Values are the mean ± standard deviation. N=6 per group. SHR, spontaneously hypertensive rats.

Post-administration body weight indicates the body weight after 5 weeks of treatment.
## S2: Arterial Blood Gas Values Before and After L-NIO Infusion

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<td>Before infusion</td>
<td>7.38 ± 0.02</td>
<td>44.0 ± 1.6</td>
<td>80.4 ± 5.1</td>
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<td>30 min after infusion</td>
<td>7.38 ± 0.02</td>
<td>44.2 ± 2.2</td>
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<td><strong>Aspirin-treated SHR</strong></td>
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<td>30 min after infusion</td>
<td>7.37 ± 0.02</td>
<td>44.1 ± 1.2</td>
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<td><strong>Cilostazol-treated SHR (0.1%)</strong></td>
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<td>7.39 ± 0.03</td>
<td>43.8 ± 2.2</td>
<td>84.3 ± 5.6</td>
</tr>
<tr>
<td>30 min after infusion</td>
<td>7.38 ± 0.03</td>
<td>42.9 ± 1.7</td>
<td>87.1 ± 4.4</td>
</tr>
<tr>
<td><strong>Cilostazol-treated SHR (0.3%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before infusion</td>
<td>7.38 ± 0.02</td>
<td>43.7 ± 1.5</td>
<td>82.2 ± 4.1</td>
</tr>
<tr>
<td>30 min after infusion</td>
<td>7.38 ± 0.03</td>
<td>44.5 ± 2.3</td>
<td>83.6 ± 5.0</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation. N=9 per group. L-NIO, L-$$N^5$$-(1-iminoethyl)ornithine; SHR, spontaneously hypertensive rats.
S3. Effects of L-NIO on the expression levels of the phospho-eNOS and total eNOS in vehicle-treated SHR. Top panels show representative Western blots. All values were normalized by setting the densitometry of the control sample to 1.0. Data are expressed as the mean ± standard deviation. There were no significant effects of L-NIO on total and phosphorylated eNOS protein.
S4. Time-course of the mean arterial pressure (A) and rCBF (B) after 7-NI administration. Measurements are expressed as a percentage of the baseline. In (B), the maximum rCBF response was defined as the magnitude of the maximum decrease during the 30-minute recording period (arrow), and the total rCBF response was defined as the integral of the rCBF response curve from 0 to 30 minutes after drug administration (shaded area). C, The maximum rCBF response to 7-NI. D, The total rCBF response to 7-NI. Data are expressed as the mean ± standard deviation.
### S5. Arterial Blood Gas Values Before MCA Occlusion, Before Reperfusion, and After Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>( \text{PaCO}_2 ), mmHg</th>
<th>( \text{PaO}_2 ), mmHg</th>
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</thead>
<tbody>
<tr>
<td><strong>Vehicle-treated SHR</strong></td>
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<td></td>
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<tr>
<td>Before MCA occlusion</td>
<td>7.38 ± 0.03</td>
<td>43.3 ± 2.0</td>
<td>92.4 ± 11.4</td>
</tr>
<tr>
<td>Before reperfusion</td>
<td>7.39 ± 0.03</td>
<td>42.8 ± 1.9</td>
<td>93.7 ± 6.2</td>
</tr>
<tr>
<td>After reperfusion</td>
<td>7.38 ± 0.03</td>
<td>43.1 ± 2.0</td>
<td>95.3 ± 9.7</td>
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<tr>
<td><strong>Aspirin-treated SHR</strong></td>
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</tr>
<tr>
<td>Before MCA occlusion</td>
<td>7.38 ± 0.03</td>
<td>43.5 ± 1.6</td>
<td>92.9 ± 13.6</td>
</tr>
<tr>
<td>Before reperfusion</td>
<td>7.37 ± 0.03</td>
<td>42.8 ± 2.6</td>
<td>87.5 ± 4.5</td>
</tr>
<tr>
<td>After reperfusion</td>
<td>7.37 ± 0.03</td>
<td>42.1 ± 3.5</td>
<td>86.8 ± 3.9</td>
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<tr>
<td><strong>Cilostazol-treated SHR (0.1%)</strong></td>
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<td></td>
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</tr>
<tr>
<td>Before MCA occlusion</td>
<td>7.38 ± 0.02</td>
<td>42.6 ± 3.4</td>
<td>95.2 ± 4.3</td>
</tr>
<tr>
<td>Before reperfusion</td>
<td>7.38 ± 0.02</td>
<td>42.7 ± 2.7</td>
<td>88.4 ± 7.1</td>
</tr>
<tr>
<td>After reperfusion</td>
<td>7.39 ± 0.02</td>
<td>42.3 ± 1.9</td>
<td>90.2 ± 4.8</td>
</tr>
<tr>
<td><strong>Cilostazol-treated SHR (0.3%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before MCA occlusion</td>
<td>7.38 ± 0.02</td>
<td>43.1 ± 2.6</td>
<td>92.2 ± 5.5</td>
</tr>
<tr>
<td>Before reperfusion</td>
<td>7.37 ± 0.02</td>
<td>43.4 ± 2.3</td>
<td>91.5 ± 4.4</td>
</tr>
<tr>
<td>After reperfusion</td>
<td>7.38 ± 0.02</td>
<td>43.2 ± 2.1</td>
<td>90.5 ± 6.6</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation. N=7 per group. SHR, spontaneously hypertensive rats; MCA, middle cerebral artery.
S6. Schematic representation of the regions of interest for measuring the residual perfusion of the microcirculation after MCA occlusion. The dark area is defined as the ischemic core, and light shaded area is defined as the ischemic penumbra. The areas of interest were chosen to represent brain areas in the following four regions: the cortical region of the anterior cerebral artery territory (region 1), the peripheral cortical region of the MCA territory (region 2), the center region of MCA territory in the cortex (region 3), and the dorsolateral part of the striatum (region 4). Regions 5, 6, 7, and 8 indicate the same region in the contralateral side of regions 1, 2, 3, and 4, respectively.
Supplemental References


Cilostazol, Not Aspirin, Reduces Ischemic Brain Injury via Endothelial Protection in Spontaneously Hypertensive Rats

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自然発症高血圧ラットにおいてシロスタゾールは血管内皮細胞を保護することで虚血性脳損傷を抑制するが、アスピリンには同様の効果はない

Stroke 2011; 42: 2571-2577