Protective Effect of Endothelin Type A Receptor Antagonist on Brain Edema and Injury After Transient Middle Cerebral Artery Occlusion in Rats

Yoshiyuki Matsuo, PhD; Shin-ichi Mihara, PhD; Mitsuyoshi Ninomiya, PhD; Masafumi Fujimoto, PhD

Background and Purpose—Recent evidence strongly suggests that endothelins (ETs) play an important role in the regulation of blood-brain barrier (BBB) functions. The aim of the present study was to evaluate the role of ETs on edema formation and BBB permeability change after cerebral ischemia/reperfusion.

Methods—We examined the brain tissue ET-1 content and evaluated the time and dose response of the therapeutic effects of the specific ET type A receptor (ETₐ) antagonist, S-0139, on brain edema formation, development of infarction, and disruption of BBB after 1 hour of middle cerebral artery occlusion (MCAO) in rats.

Results—After 1-hour MCAO and reperfusion, the brain ET-1 content did not change during the first 3 hours, increased at 6 hours, and rose almost continuously over 48 hours in the ischemic region as well as in the ischemic rim. Rats infused with S-0139 (0.03 to 1.0 mg/kg per hour) during reperfusion showed dose-dependent and significant attenuation of the increase in brain water content 24 hours after reperfusion. When the infusion of S-0139 was begun after 10 minutes and 1 hour of reperfusion, the brain edema formation and infarct size were significantly attenuated. Furthermore, posttreatment with S-0139 significantly attenuated the increased Evans blue dye–quantified albumin extravasation and improved the mortality of animals after cerebral ischemia/reperfusion.

Conclusions—Our data demonstrate that infusion with S-0139, an ETₐ antagonist, results in significant reduction of brain injury and plasma extravasation after transient MCAO. Thus, ETs may contribute to cerebral ischemia/reperfusion injury at least partly by increasing the BBB permeability via ETₐs. (Stroke. 2001;32:2143-2148.)

Key Words: blood-brain barrier ■ brain edema ■ brain injuries ■ cerebral ischemia, focal ■ endothelins ■ rats

The endothelin (ET) isopeptides (ET-1, ET-2, and ET-3) are potent vasoconstrictors of vascular smooth muscle. The vascular effects of ETs are mediated by 2 receptor subtypes, ETₐ, which is a vasoconstrictor, and ETₐ, which is a vasodilator. In the brain, ETs and their receptors that localize in neurons, glial cells, smooth muscle cells, and microvessel endothelial cells have been implicated in physiological and pathophysiological roles by modulating neuronal functions and regulation of cerebral blood flow and metabolism.

A marked elevation in plasma or brain ET-1 levels has been reported in patients with ischemic stroke and in animals with cerebral ischemia. Topical ET-1 can reduce cerebral blood flow and induce neuronal damage. Moreover, ET receptor antagonists have been demonstrated to decrease the neuronal damage in experimental models. Thus, the roles of ETs on the pathogenesis of cerebral ischemic injury have been implicated, but the precise mechanisms are unclear.

Disruption of blood-brain barrier (BBB) and edema formation are induced by ischemia and have been implicated in the progression of ischemic brain injury. Recently, it has been suggested that ETs regulate BBB functions. Stanimirovic et al have demonstrated that ET-1 induces Cr release from human brain endothelial cells, which suggests that ET-1 enhances the permeability of BBB. These findings may implicate ET-1 in the pathogenesis of brain edema accompanying cerebrovascular disorders. However, few studies have analyzed the role of ETs on BBB dysfunction and edema formation in experimental cerebral ischemia. Thus, the aims of the present study were to evaluate the time course of brain ET-1 content and to analyze the roles of ETs on edema formation and the change of BBB permeability after cerebral ischemia/reperfusion.

Materials and Methods
All procedures performed on the animals in the present study were conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals and were approved by the Shionogi Animal Care and Use Committee.

Ischemia/Reperfusion Model
We induced transient focal ischemia by means of right middle cerebral artery (MCA) occlusion (MCAO) in male Wistar rats.
Briefly, rats (270 to 320 g) were anesthetized and maintained with a gas mixture of 98.5% air and 1.5% halothane. After median incision of the neck skin, the right external carotid artery (ECA) was dissected, and an 18-mm-long 4-0 nylon thread (coated with silicon) was inserted from the ECA to the right internal carotid artery to occlude the origin of the right MCA. After surgery, anesthesia was discontinued, and the rats were allowed free access to food and water until the next procedure. Neurological deficits characterized by severe left-sided hemiparesis and right Horner’s syndrome were used as criteria for ischemic insult. After 1 hour of MCAO, the thread was removed to allow reperfusion of the ischemic area via the right common carotid artery.

**Measurement of Tissue ET-1 Content**

To measure brain immunoreactive (ir)-ET-1 content, rats were decapitated under halothane anesthesia at 1, 3, 6, 12, 24, and 48 hours after reperfusion. Brain samples of the ischemic/reperfused hemisphere were taken on ice from the cerebral cortex perfused by the anterior cerebral artery (ACA), i.e., the ACA area, the cerebral cortex perfused by the MCA, i.e., the MCA area, and the caudate putamen. MCA and ACA areas were defined as reported previously. Brain samples were immediately frozen in liquid nitrogen, weighed, and stored at −80°C until ET extraction. Tissues were homogenized with an Ultra-Turrax (Julke & Kunkel) for 60 seconds (wet weight/dry weight). These samples was then measured by the wet and dry method as recently described. Brain ET-1 content was also measured in complete sham-operated rats immediately after the operation.

**Water Content**

To measure brain water content, rats were decapitated under halothane anesthesia 24 hours after reperfusion. The brain was dissected into the ACA area, the MCA area, and the caudate putamen. MCA and ACA areas were defined as reported previously. The brain samples were dried in an oven at 110°C for 24 hours. The water content of these samples was then measured by the wet and dry method as follows: water content (%) = (wet weight−dry weight)/100/wet weight. Brain water content was also measured in complete sham-operated rats 24 hours after the operation.

**Infarct Size**

Rats were anesthetized with sodium pentobarbital and perfused with physiological saline containing 0.2% heparin 24 hours after reperfusion. Brains were removed, cut into 1-mm coronal sections, and immersed in 2% triphenyltetrazolium chloride solution at 37°C for 30 minutes. The areas that remained colorless to triphenyltetrazolium chloride staining, which reflects mitochondrial damage, were quantified as infarct areas by an image analysis system (NIH image).

**BBB Permeability for EB**

To evaluate the effect of the specific ET type A receptor (ET₄) antagonist, 27-O-3-(2-(3-carboxy-acryloylamino)-5-hydroxyphenyl)-acryloyloxy myricerone sodium salt (S-0139), on the BBB disruption that developed after transient MCAO, we measured the extravasation of Evans blue dye (EB) as a marker of albumin extravasation. The method used to measure brain tissue PO₂ was similar to that recently described. Briefly, the rats were anesthetized with pentobarbital and placed in a stereotaxic apparatus with the incisor bar 3.3 mm below the interaural line. The skull was exposed, and a small hole was drilled for implantation of a carbon fiber microelectrode. The working electrode was prepared from a carbon fiber 7 μm in diameter that was sealed in a fine-tipped glass capillary, and an Ag/AgCl electrode was used as a reference electrode, with a silver wire serving as an auxiliary electrode. After puncture of the dura, the working electrode was carefully inserted into the right frontoparietal cortex (1.4 mm posterior and 6 mm lateral to the bregma and 4.0 mm ventral to the dura) and fixed in place with dental cement and screws to the skull of the rat. This area becomes the ischemic core during MCAO. The animals were then left to recover for 24 hours. The electrodes were connected to the recording circuit and were polarized at −750 mV.

**ET₄ Antagonist**

S-0139, a selective ET₄ antagonist, was synthesized by the chemical division of Shionogi Research Laboratories. To examine the effect of S-0139 on postischemic brain damage, rats were implanted subcutaneously with osmotic minipumps (Alzet 2001D, Alza Corp) filled with S-0139 dissolved in sterile saline under halothane anesthesia for 10 minutes and 1 and 3 hours after reperfusion following MCAO. The output of the pump was fed to a cannula inserted in the femoral vein, and rats were treated for 24 hours with varying doses of S-0139 of ~0.03 to 1.0 mg/kg per hour.

**Statistical Analyses**

All data are expressed as mean±SE. Data were analyzed by 1-way ANOVA followed by post hoc comparison with the use of the Fisher protected least significant difference (PLSD) test, the Dunnett test, or an unpaired t test when appropriate. Mortality was analyzed by using the Fisher exact probability test.

**Results**

In the sham-operated rats, the ir-ET-1 levels in the ACA area, the MCA area, and the caudate putamen were 41.9±8.8, 45.2±8.2, and 39.0±9.2 fmol/g tissue, respectively. Brain Absorbance of the extracted solution was measured with a spectrophotometer. The quantitative calculation of the dye content in the brain was based on external standards in the brain-soaked solution. BBB permeability was also measured in complete sham-operated rats immediately after surgery.
ir-ET-1 content did not change during the first 3 hours of reperfusion. In the cerebral cortex, ir-ET-1 content significantly increased at 6 hours after reperfusion, and the increase of ir-ET-1 tended to last up to 48 hours after reperfusion (Figure 1A and 1B). In the caudate putamen, ir-ET-1 content began to increase at 6 hours and significantly increased at 48 hours after reperfusion (Figure 1C).

The infusion of S-0139 (0.03 to 1.0 mg/kg per hour) that began after 10 minutes of reperfusion showed significant attenuation of the increase in brain water content in the ACA area, the MCA area, and the caudate putamen in a dose-dependent manner 24 hours after reperfusion (Figure 2). The infusion of S-0139 that began after 10 minutes and 1 hour of reperfusion significantly attenuated the infarct size as well as the brain edema formation determined at 24 hours after reperfusion (Figure 3). The infusion of S-0139 that began after 3 hours of reperfusion did not modify the infarct size, although there was a slight but significant reduction of water content in the ACA area only (Figure 3). There is a possibility that infarct volume is influenced by brain swelling. Therefore, we have confirmed that the infusion of S-0139 that began after 10 minutes of reperfusion reduced the infarct volume (mm³, corrected to eliminate swelling influences13) at 24 hours after reperfusion in another series of experiments (232.0±19.5 mm³ for vehicle [n=13] versus 124.6±20.0 mm³ for S-0139 [n=12], P=0.0008).

To clarify the possible mechanism for ETs on ischemic brain injury, we examined the effects of S-0139 on the postischemic BBB disruption, neutrophil infiltration, and

**Figure 1.** Temporal profile of ir-ET-1 content in ACA areas (A), MCA areas (B), and caudate putamen (C) after 24-hour reperfusion following 1-hour MCAO. Sham op indicates sham operation. Each point is mean±SEM of 5 animals. *P<0.05 vs sham-operated rats (Fisher PLSD test).

**Figure 2.** Dose-dependent effect of posttreatment with S-0139 (0.3 mg/kg per hour IV) on brain water content in the ACA area, MCA area, and caudate putamen (A) after 24-hour reperfusion following 1-hour MCAO. Data are presented as mean±SEM. *P<0.05 and **P<0.01 vs vehicle (Dunnett test).

**Figure 3.** Therapeutic time window of posttreatment with S-0139 (0.3 mg/kg per hour IV) on brain water content in the ACA area, MCA area, and caudate putamen (A) and infarct size of cerebrum (mm from bregma) (B) after 24-hour reperfusion following 1-hour MCAO. Data are presented as mean±SEM. *P<0.05 and **P<0.01 vs vehicle (Dunnett test).
The brain EB content was markedly increased in the ischemic hemisphere by 3 hours and was further increased between 3 and 6 hours after reperfusion (Table 1). Subsequently, EB extravasation disappeared until 24 hours after reperfusion (data not shown). Infusion of S-0139 during reperfusion significantly reduced EB accumulation in the ischemic cortex by 6 hours after reperfusion (Figure 4). S-0139 did not affect the changes in tissue PO₂ in the basal level during MCAO (Figure 4). After reperfusion, tissue PO₂ increased to 135.2±13.7% of the basal level at 30 minutes and continued to rise until 6 hours after reperfusion (Figure 4). S-0139 did not affect the changes in tissue PO₂ in the ischemic cortex.

Almost all animals survived up to 2 weeks after 1-hour MCAO. Therefore, the effect of S-0139 on the mortality was evaluated in rats with 2-hour MCAO. At 24 hours after reperfusion following 2-hour MCAO, the mortality for control animals, treated with S-0139 was 71.4% (15 of 21 rats) and 33.3% (7 of 21 rats), respectively. The mortality of the animals was significantly improved in S-0139–treated animals by using the Fisher exact probability test (P=0.029 versus control rats, Table 3).

### Discussion

The present study indicates that (1) a 1-hour MCAO in the rat provides sufficient stimulation to elevate the ET-1 level in the damaged brain and (2) brain edema formation, the size of infarction, the mortality, and albumin extravasation could be reduced by the ET₃ antagonist S-0139. These results strongly suggest that pathways elicited by ETs via ET₃ are involved in cerebral ischemia/reperfusion injury.

Marked elevation in plasma ET-1 levels has been reported in patients with ischemic stroke and in cerebral ischemia in animals, and there is a tendency of higher ET-1 levels being associated with more severe neurological deficits. In the present study, the increase in ET-1 level in the ischemic brain occurred between 3 and 6 hours and lasted up to 48 hours after reperfusion, and treatment with S-0139 significantly attenuated the brain edema formation. In our previous study, brain water content increased slightly but significantly at the early reperfusion and reached a plateau during 1 and 6 hours of reperfusion, when the brain ET-1 content remained unchanged. After 6 hours of reperfusion, when we noted a significant increase of ET-1, water content began to increase again and continued to rise significantly until 72 hours after reperfusion. These results suggest that an increase in brain ET-1 content contributes to brain edema.

The source of ET-1 remains to be clarified during cerebral ischemia and reperfusion. It is possible that ET-1 is released from microvessels, because the presence of ETs has been demonstrated in the endothelial cells. ET is also localized in neurons and glial cells. During the development of cerebral infarction, neural ischemic injury progresses to necrosis with

### Table 1. EB Leakage in Brain After 1-h MCAO

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ACA Area (m/g Wet Tissue)</th>
<th>MCA Area (m/g Wet Tissue)</th>
<th>Caudate Putamen (m/g Wet Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated animals</td>
<td>10</td>
<td>5.10±0.91</td>
<td>4.98±0.87</td>
<td>7.97±0.72</td>
</tr>
<tr>
<td>0–3 h after reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control animals</td>
<td>10</td>
<td>11.82±2.84</td>
<td>26.80±7.07*</td>
<td>36.33±9.30</td>
</tr>
<tr>
<td>S-0139–treated animals</td>
<td>10</td>
<td>8.02±1.49</td>
<td>11.01±2.14†</td>
<td>26.56±5.70</td>
</tr>
<tr>
<td>3–6 h after reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control animals</td>
<td>10</td>
<td>18.78±5.09*</td>
<td>36.06±8.84*</td>
<td>70.68±26.29*</td>
</tr>
<tr>
<td>S-0139–treated animals</td>
<td>10</td>
<td>8.59±0.83†</td>
<td>15.36±2.98†</td>
<td>28.97±3.77†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Treatment with S-0139 (0.3 mg/kg per h IV) was begun after 10 min of reperfusion.

### Table 2. MPO Activity in Brain 24 h After 1-h MCAO

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ACA Area (U/g Wet Tissue)</th>
<th>MCA Area (U/g Wet Tissue)</th>
<th>Caudate Putamen (U/g Wet Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated animals</td>
<td>6</td>
<td>0.006±0.002</td>
<td>0.010±0.003</td>
<td>0.003±0.001</td>
</tr>
<tr>
<td>MCAO/R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control animals</td>
<td>6</td>
<td>0.054±0.014*</td>
<td>0.159±0.027†</td>
<td>0.065±0.016*</td>
</tr>
<tr>
<td>S-0139–treated animals</td>
<td>6</td>
<td>0.046±0.012</td>
<td>0.151±0.041*</td>
<td>0.047±0.008*</td>
</tr>
</tbody>
</table>

MCAO/R indicates 1-h MCAO and reperfusion. Values are mean±SEM. Treatment with S-0139 (0.3 mg/kg per h IV) was begun after 10 min of reperfusion.

*P<0.05 vs sham-operated animals (Fisher PLSD test).
an activation of microglia and astrocyte proliferation. Glial cells synthesize ET during active gliosis. Therefore, the reactive astroglia seems to be a good candidate for the production of ET-1 during ischemia/reperfusion.

Protective effects of ET receptor antagonists have been reported in experimental cerebral ischemia. However, the mechanism of action of the ET receptor antagonists in cerebral ischemia is unclear. Published reports suggest that brain edema after cerebral ischemia accentuates tissue necrosis and that therapy with hyperosmolar agents reduces the extent of brain injury. The mechanisms of stroke-induced brain edema are only partially understood. A simple explanation would be that it is related to net water uptake because of BBB disruption. Recently, Stanimirovic et al demonstrated that ET-1 induced release from human brain endothelial cells, which suggests that ET-1 enhances the permeability of BBB via the ETA. It has also been suggested that brain edema could occur as a result of energy and ion pump failure with an intact BBB during ischemia and reperfusion. Kawai et al reported that ETs stimulate sodium uptake into rat brain endothelial cells via ET_{A}, which suggests that ETs play a role in regulating ion transport across the BBB. In our present study, S-0139 attenuates BBB disruption and edema formation after cerebral ischemia/reperfusion. The neuroprotective effect of ET receptor antagonists may result from the inhibition of BBB dysfunction via the ETA.

Important roles of ET_{A}s and their antagonism in brain injury have been suggested. On the other hand, the lack of a prominent role for ET_{A}/ET_{B} antagonism in brain injury has been reported. Recently, Barone et al reported that the selective ET_{A} antagonist provided neuroprotection in both head trauma and focal stroke, whereas the mixed ET_{A}/ET_{B} antagonist did not provide protection. It has been suggested that ET_{B} may be a clearance receptor. Willette et al have demonstrated that mixed ET_{A}/ET_{B} antagonists increase ET-1 in plasma and brain. These results may suggest that the ET_{B} antagonist–induced increases in ET-1 counteract the protective effects of receptor blockade.

Polymorphonuclear leukocytes (PMNLs) play an important role in the development of ischemia/reperfusion injury by reducing microvascular blood flow, initiating thrombosis, and releasing free-oxygen radicals. It has been reported that anti-leukocyte interventions can attenuate brain injury after ischemia/reperfusion. Recently, it has been reported that ETs enhance the expression of intercellular adhesion molecule-1 and interleukin-8 on brain capillary endothelial cells via the ETA. These findings suggest the possibility of PMNLs being implicated in ET-induced brain injury. However, against our expectation, S-0139 could not attenuate the PMNL infiltration into the ischemic brain.

Postischemic cerebral blood flow disturbances (hyperemia and hypoperfusion) have been implicated in the pathogenesis of brain injury. Recently, Spatz et al reported that the ETA antagonist BQ123 prevents hypoperfusion after transient forebrain ischemia in mongolian gerbils. There is a possibility that S-0139 alters local blood flow and tissue oxygenation, which may account for the reduction in ischemic brain injury. Therefore, we evaluated the effect of S-0139 on tissue PO2, which is the result of tissue perfusion and tissue oxygen utilization. S-0139 did not affect the changes in tissue PO2 in the ischemic cortex. Further work is required to clarify the effect of S-0139 on local blood flow.

In the present study, we demonstrated that the infusion of S-0139 that began 1 hour after reperfusion significantly attenuated brain injury. However, against our expectation, the infusion of S-0139 that began 3 hours after reperfusion did not modify infarct size. Although we could not detect the increase in ET-1 content until 6 hours after reperfusion, brain ET-1 content may have begun to increase in local regions early in the reperfusion, when we noted a significant increase of EB extravasation. The anatomic localization and expression of ETs and their receptors after cerebral ischemia/reperfusion should be analyzed in future studies. Plasma S-0139 concentration reached a plateau within 30 minutes after starting the infusion (data not shown). However, we could not exclude the possibility that a rise in brain S-0139 concentration was delayed.

Although important roles of ET-1 in ischemic brain injury have been suggested, there are recent publications arguing against the role of ET-1 in stroke. Haapaniemi et al have reported that plasma ET-1 is not increased in patients with ischemic stroke. Although their report is in disagreement with several previous studies, there is the possibility that plasma ET-1 may not always reflect the increase in local production of ET-1. Further work is required to clarify the correlation between plasma and brain ET-1 levels. Bhardwaj et al reported that the ET_{A} antagonist Ro 61-1790 failed to improve the outcome in focal ischemia in cats. However, no information is available regarding plasma or brain ET-1.

### TABLE 3. Mortality of Animals at 24 h After 2-h MCAO

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rats</th>
<th>Mortality Rate, % P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control animals</td>
<td>21</td>
<td>15 6 71.4</td>
</tr>
<tr>
<td>S-0139–treated animals</td>
<td>21</td>
<td>7 14 33.3 0.029*</td>
</tr>
</tbody>
</table>

Treatment with S-0139 (0.3 mg/kg per h IV) was begun after 10 min of reperfusion.

*P<0.05 vs control animals (Fisher exact probability test).
levels in cat MCAO models. Differences in the effects of ET₄ antagonists may reflect differences in methodology and ischemic models.

We conclude that ETs might contribute to cerebral ischemia/reperfusion injury by increasing the BBB permeability via ET₄s and that ET₄ antagonists may in the future provide an effective therapeutic intervention for reducing ischemic brain damage and be of benefit when used in conjunction with thrombolytic therapies.

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References

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