Activation of Sphingosine 1-Phosphate Receptor-1 by FTY720 Is Neuroprotective After Ischemic Stroke in Rats

Yu Hasegawa, MD, PhD; Hidenori Suzuki, MD, PhD; Takumi Sozen, MD; William Rolland, BS; John H. Zhang, MD, PhD

Background and Purpose—FTY720 is a known sphingosine 1–phosphate receptor agonist. In the present study, we investigated the neuroprotective effect of postischemic administration of FTY720 in rats with 2 hours transient middle cerebral artery occlusion (MCAO).

Methods—One hundred eleven male rats were randomly assigned to sham-operated and MCAO treated with vehicle, 0.25 mg/kg and 1 mg/kg of FTY720, another selective sphingosine 1–phosphate receptor-1 agonist SEW2871 (5 mg/kg), or 0.25 mg/kg of FTY720 plus a sphingosine 1–phosphate antagonist, VPC23019 (0.5 mg/kg). Drugs were injected intraperitoneally immediately after reperfusion. Neurological score and infarct volume were assessed at 24 and 72 hours after MCAO. Western blotting, immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end-labeling were conducted at 24 hours after MCAO.

Results—FTY720 significantly reduced infarct volume and improved neurological score at 24 and 72 hours after MCAO compared with the vehicle group. SEW2871 showed similar neuroprotective effects to FTY720, whereas VPC 20319 abolished the neuroprotective effects of FTY720. FTY720 significantly retained Akt and extracellular signal-regulated kinase phosphorylation and Bcl-2 expression and decreased cleaved caspase-3 expression and terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end-labeling-positive neurons at 24 hours after MCAO. VPC23019 blocked the antiapoptotic effects of FTY720.

Conclusions—These data suggest that activation of sphingosine 1–phosphate-1 by FTY720 reduces neuronal death after transient MCAO. (*Stroke* 2010;41:00-00.)

Key Words: apoptosis ■ cerebral ischemia ■ FTY720 ■ sphingosine 1-phosphate receptor-1

Sphingosine 1-phosphate (S1P) is a bioactive metabolic product of sphingolipids generated by sphingosine kinase. Recent studies have shown that S1P binds to the S1P family of G protein-coupled receptors and regulates multiple cellular events. FTY720 is a known agonist of S1P receptor-1 (S1P1), -3 (S1P3), -4 (S1P4), and -5 receptors (S1P5). FTY720 exerts immunomodulatory actions by affecting lymphocyte production, trafficking, and apoptosis through S1P receptors. FTY720 has been demonstrated to reduce ischemia reperfusion injury in the kidney and liver through S1P1 modulation causing a transient lymphopenia by a reversible redistribution of lymphocytes. However, it is not clear whether FTY720 possesses neuroprotective effect through S1P1 in ischemic stroke.

In this study, we hypothesized that FTY720 may exert neuroprotection through S1P1-mediated antiapoptotic mechanisms in an experimental transient middle cerebral artery occlusion (MCAO) model in rats. We administered FTY720 intraperitoneally 2 hours after MCAO (immediately after reperfusion) and evaluated the extent of ischemic injury and apoptosis.

Materials and Methods

Experimental Animals

All experiments were approved by the institutional Animal Care and Use Committee of Loma Linda University. One hundred eleven male Sprague-Dawley rats (Harlan, Indianapolis, Ind) weighing 330 to 400 g were randomly divided into the following groups: preoperation (n=10), sham-operated (n=10), MCAO treated with vehicle (dimethyl sulfoxide [DMSO]; n=30), MCAO with low (n=30) and high (n=8) doses of FTY720, MCAO with SEW2871 (n=9), and MCAO with low dose of FTY720+VPC23019 (n=19).

MCAO Model

Anesthesia was induced with 4% isoflurane and maintained with 2.5% isoflurane, 30% oxygen, and 70% medical air with a face mask. Arterial blood gas analysis, mean arterial blood pressure, heart rate, and blood glucose before, during, and after MCAO were analyzed through the left femoral artery. The rectal temperature was monitored and kept at 37.0±0.5°C by using a feedback-regulated heating system during surgery.

Transient focal ischemia was induced by occluding the middle cerebral artery using the intraluminal technique. Briefly, the left common carotid artery was exposed and 3-0 nylon suture coated with poly-L-lysine was introduced into the left internal carotid artery.
through the common carotid artery. After 2 hours, the suture was withdrawn to allow middle cerebral artery reperfusion. Neurological score was measured 10 minutes before reperfusion using a modification of the neurological score of Bederson et al. Accordingly, Grade 0 was recorded in the absence of observable deficits, Grade 1 in the presence of forelimb flexion, Grades 2 and 3 when there was decreased resistance to lateral push in the absence or presence of circling, respectively, and Grade 4 was assigned to comatose animals. Rats with Grades 0 and 4 were excluded from further experiments.

Administration of Drugs
Five groups of rats with transient MCAO received DMSO, low-dose (0.25 mg/kg; FTY group) and high-dose (1 mg/kg; FTY-high group) FTY720 in DMSO, a selective S1P1 agonist, SEW2871 (5 mg/kg; SEW group) in DMSO, or low-dose FTY720+SEW1P1, S1P3, S1P4 antagonist VPC23019 (0.5 mg/kg; VPC+FTY group) in DMSO/1N HCL (95:5) intraperitoneally immediately after reperfusion. The dissolving manner and dosage were followed as previously described. DMSO (1.1 g/mL/kg) was used at a concentration ≤0.4 mL.

Measurement of the Area of Early Ischemic Brain Injury
Animals were decapitated 24 (n=8) and 72 (n=9) hours after MCAO. Their brains were cut into 2-mm-thick coronal slices using a rodent brain matrix. Six selected sections (±2.5, ±3.3, and ±1 mm from the bregma) were stained for 10 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C. The area of ischemic brain injury was measured by a blinded observer with ImageJ software (Version 1.40; National Institutes of Health, Bethesda, Md). Infarct areas were corrected to compensate for edema formation by subtracting the area of the intact ipsilateral hemisphere from the area of the intact contralateral hemisphere. Then the infarct areas on each slice were added together and multiplied by slice thickness to give the infarct volume.

Neurological Scoring
A 25-point scoring system was used to evaluate the neurological deficit at 24 and 72 hours after MCAO in a blind fashion using a modification of the method described by Garcia et al.

Western Blot Analysis
The left ischemic cortex in the middle cerebral artery region (middle cerebral artery cortex) was obtained from preoperation (healthy control) and 24 hours after MCAO (n=5, respectively). Protein concentration was determined using DC protein assay (Bio-Rad, Hercules, Calif). Individual samples (50 µg each) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane for 80 minutes at 70 V (Bio-Rad). Blotting membranes were incubated for 2 hours with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated overnight at 4°C with the following primary antibody: antiphosphoextracellular signal-regulated kinase (ERK) 1/2, antiphospho-Akt antibody (Ser473), anti-akt antibody (Ser21), antiphospho-Akt (Ser473), and anti-Bcl-2 (1:1000; Cell Signaling Technology). Beta-actin (Santa Cruz Biotechnology) was used as an internal control for every experiment. Blotting membranes were incubated for 1 hour with secondary antibodies (1:2000; Santa Cruz Biotechnology) and processed with an enhanced chemiluminescence reagent kit (ECL plus kit; Amersham Bioscience, Arlington Heights, Ill). The images were scanned and analyzed semiquantitatively in a blind fashion using the ImageJ software. Changes in phosphorylation of Akt-Ser-473 and ERK-1 and expression of Bcl-2 and 17 kDa (cleaved) caspase-3 were expressed as a percentage of the preoperative level. Beta-actin (Santa Cruz Biotechnology) was used as an internal control for every experiment.

Histology
Samples from sham-operated, vehicle, FTY, and VPC+FTY groups (n=5, respectively) were used for experiments. At 24 hours after MCAO, the brains were fixed by cardiovascular perfusion with phosphate-buffered saline and 10% paraformaldehyde. The brains were quickly removed and postfixed in 10% paraformaldehyde followed by 30% sucrose (weight/volume) for 3 days. Ten-micron-thick coronal sections at the level of bregma +1 mm (rostroly) were cut on a cryostat (Leica Microsystems LM3050S) and mounted on poly-l-lysine-coated slides.

Immunofluorescence Staining
Double-fluorescence labeling was performed at 24 hours after MCAO as described previously. The following primary antibodies were used: (1) anti-S1P1 antibody (1:100; Cayman Chemical, Ann Arbor, Mich); (2) antiphospho-Akt (Ser473) antibody immunohistochemistry-specific (1:100; Cell Signaling Technology); and (3) antineuronal nuclei antibody (1:200; Chemicon International, Temecula, Calif). For negative controls, the primary antibodies were omitted and the same staining procedures were followed.

Terminal Deoxynucleotidyl Transferase-Mediated Uridine 5’-Triphosphate-Biotin Nick End-Labeling Staining
We immunostained brain sections with antineuronal nuclei antibodies and then subjected the sections to terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining with an in situ cell death detection kit (Roche Inc, Mannheim, Germany). A mixture of fluorescein isothiocyanate-labeled nucleotides and terminal deoxynucleotidyl transferase was applied onto brain sections for 60 minutes at 37°C in a dark humidified chamber as previously described. Incubation with labeling solution without the enzyme served as a negative labeling control. The apoptotic neurons were counted in the peri-infarct cortex (3 fields were counted in each case at ×400 magnification) in a blinded manner. The number of cells was expressed as cells/mm².

Statistical Analysis
All values are expressed as the mean±SD. Statistical differences among the various groups were assessed with one-way analysis of variance followed by a Tukey-Kramer post hoc analysis. The differences between the 2 groups were compared using unpaired t test. Differences of P<0.05 were considered significant.

Results

Mortality
The mortality rate was as follows: 10% (3 of 30 rats) in the vehicle group and 10% (3 of 30 rats) in the FTY group within 72 hours; and 11% (1 of 9 rats) in the SEW group and 5% (1 of 19 rats) in the VPC+FTY group within 24 hours. No rats in the sham-operated and FTY-high groups died.

Physiological Parameters
No statistical differences were observed among the groups with regard to physiological parameters (Table).

Infarct Volume and Neurological Score
The neurological score before reperfusion was significantly different among the groups (data not shown). The total infarct volume in the FTY group was significantly smaller than in the vehicle group at both 24 (105±61 mm³ versus 225±94 mm³; Figure 1A) and 72 (119±84 mm³ versus 198±68 mm³; Figure 2A) hours after MCAO. This reduction of infarct size was derived from reduced infarct volume in the cortex (63±62 mm³ versus 141±60 mm³; Figure 2B), but not in the subcortex (57±27 mm³ versus 57±18 mm³; Figure 2C) at 72 hours after MCAO. A significant improvement in neurological score was observed between the FTY and vehicle
groups at both 24 (14±4 versus 10±2; Figure 1B) and 72 (17±4 versus 11±4; Figure 2D) hours after MCAO. The FTY-high group had a similar neuroprotective effect compared with the FTY group at 24 hours after MCAO (108±62 mm³ and 14±4, respectively; Figure 1A–B).

Table. Arterial Blood Gas Analysis, Mean Arterial Blood Pressure (BP), Heart Rate (HR), and Blood Glucose (BG) Before, During, and After MCAO

<table>
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<tr>
<th>Group</th>
<th>pH</th>
<th>PO₂, mm Hg</th>
<th>PCO₂, mm Hg</th>
<th>BP, mm Hg</th>
<th>HR, per min</th>
<th>BG, mg/dL</th>
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<tr>
<td>DMSO</td>
<td>7.41±0.04</td>
<td>209±8</td>
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<td>136±10</td>
<td>317±16</td>
<td>266±32</td>
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<td>FTY</td>
<td>7.41±0.03</td>
<td>213±7</td>
<td>41±4</td>
<td>139±9</td>
<td>311±11</td>
<td>260±21</td>
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<tr>
<td>FTY-high</td>
<td>7.40±0.04</td>
<td>200±26</td>
<td>41±3</td>
<td>139±7</td>
<td>322±16</td>
<td>261±20</td>
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<tr>
<td>SEW</td>
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<td>315±16</td>
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<tr>
<td>VPC</td>
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<td>324±20</td>
<td>269±30</td>
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<td>326±24</td>
<td>208±37</td>
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</table>

Values are the mean±SD. There are no significant differences among the groups.

To examine whether FTY has a neuroprotective effect through S1P1, we evaluated the effects of S1P1-related drugs. A selective S1P1 agonist, SEW2871, showed similar neuroprotective effects as FTY720 in terms of infarct volume and neurological score (109±65 mm³ and 17±2, respectively; Figure 1A–B). On the other hand, a S1P antagonist, VPC23019, abolished these protective effects by FTY720 (254±107 mm³ and 10±3, respectively; Figure 1A–B).

Phosphorylation of Akt and ERK and Expression of Bcl-2 and Caspase-3 After Transient MCAO

To examine whether Akt and ERK underlie neuroprotection through S1P1 in the MCAO model, we measured phosphorylated Akt and ERK in the middle cerebral artery cortex (Figure 3A). Although phosphorylation of Akt (Figure 3C) significantly decreased in the vehicle and VPC+FTY groups (24.5%±18.7% and 31.5%±13.8%, respectively), it was retained in the FTY group (130%±63.2%).

Because the phosphorylation-related alterations were similar in ERK-1 (44 kDa) and ERK-2 (42 kDa) after ischemia, we focused on phosphorylation of ERK-1 in this study. Phosphorylation of ERK-1 (Figure 3D) significantly decreased among all groups (vehicle: 28.6%±4%, FTY: 70.2%±10.4%, VPC+FTY: 32.7%±4.6%), but the level in the FTY group was significantly preserved.

The Bcl-2 expression (Figure 3E) significantly decreased in the vehicle and VPC+FTY groups (56.3%±24.8% and 62.4%±21.9%, respectively), whereas the administration of FTY retained the Bcl-2 level (99.7%±16.3%).

On the other hand, the cleaved caspase-3 expression (Figure 3F) significantly increased in the vehicle and VPC+FTY groups (260.2%±47.4% and 269%±66.4%, respectively), but FTY decreased the cleaved caspase-3 level (168%±21.5%).
Immunohistochemical Evaluations of S1P1, Phosphorylated Akt, and TUNEL in Neurons

Immunofluorescence showed that S1P1 was expressed in neurons in the peri-infarct cortex at 24 hours after MCAO (Figure 4A–B). In the peri-infarct cortex, phosphorylated Akt in the FTY group was preserved better than in other groups (Figure 4C). TUNEL-positive neurons were observed in the peri-infarct cortex at 24 hours after MCAO (Figure 5A) and a significant decrease in TUNEL-positive neurons was observed in the FTY group (425 ± 194/mm²) compared with the vehicle (932 ± 180 mm²) and VPC+FTY (936 ± 228 mm²) groups (Figure 5B). In the sham group, there were many

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**Figure 2.** Quantitative analysis of infarct volume (A, total; B, cortex; C, subcortex) and neurological score (D) in groups treated with vehicle (n=9), 0.25 mg/kg of FTY720 (FTY; n=9), and/or sham-operation (sham; n=5) at 72 hours after MCAO. Values are the mean±SD; †P<0.05 versus sham, *P<0.05 versus vehicle, unpaired t test or analysis of variance.

**Figure 3.** Changes in Akt and ERK phosphorylation, and Bcl-2 and cleaved caspase-3 expression in the infarct middle cerebral artery region at 24 hours after MCAO (n=5). Representative ischemic brain (A) and Western blots (B) and quantitative analysis of Akt-Ser-473 (phosphor-Akt; C) and ERK-1 (phospho-ERK-1; D) phosphorylation and Bcl-2 (E) and 17 kDa caspase-3 (cleaved caspase-3; F) expression in groups treated with vehicle, 0.25 mg/kg FTY720 (FTY), 0.5 mg/kg VPC23019+0.25 mg/kg FTY720 (VPC+FTY), and preoperation (pre-ope). The band density values were calculated as a ratio of that of β-actin, and the values from the preoperation were used as 100%. Values are the mean±SD; †P<0.05 versus sham, *P<0.05 versus vehicle, #P<0.05 versus VPC+FTY, analysis of variance.
phosphorylated Akt- and no TUNEL-positive neurons (data not shown).

Discussion

The present study demonstrated that activation of S1P1 is neuroprotective against cerebral ischemia. This conclusion is drawn from studies using FTY720 and SEW2871, two S1P1 agonists, and VPC23019, a S1P1 receptor antagonist. Moreover, this study showed that the neuroprotective effect of FTY720 is associated with deactivation of caspase-3 and activation of Akt and ERK.

FTY720 is rapidly phosphorylated in vivo by sphingosine kinase type 2 to form FTY720 phosphate, which behaves as a full agonist on S1P1, S1P4, and S1P5 at low nanomolar concentrations. Neurons express mainly S1P1 and S1P3 with lower levels of S1P receptor-2 and no or negligible amounts of S1P5. Therefore, it can be speculated that FTY720 mainly acts as a S1P1 agonist in neurons. S1P1 is expressed on neuronal cell bodies and shows a widespread expression in the brain with higher expression in the gray matter compared with the white matter. These findings correspond with our results that FTY720 significantly reduced infarct volume in the cortex but not in the subcortex.

Signal through S1P1 has been associated with activation of Akt and ERK. Akt activation is a principal factor in the prevention of apoptosis in animal models of cerebral ischemia and neuronal injury was correlated with Akt activity. In this study, FTY720 decreased cleaved caspase-3 expression and TUNEL-positive cells and phosphorylated Akt in neurons. So it is likely that Akt activation (phosphorylation) with FTY720 is a main factor to induce antiapoptotic effect. On the other hand, whether activation of ERK is protective or detrimental to neurons is controversial. It is believed that elevated ERK phosphorylation plays a role in cell survival in the penumbra and ERK activity may block apoptosis by enhancing the level of the antiapoptotic protein Bcl-2 through cAMP responsive element binding protein activation. In this study, ERK was dephosphorylated after MCAO but FTY720 maintained ERK phosphorylation and Bcl-2 expression compared with the vehicle and VPC+FTY groups. Therefore, we speculate that FTY720 activates Akt and ERK through S1P1 activation, rescuing neurons from cell death in the peri-infarct cortex affected by apoptotic mechanisms.

In contrast to its protective effects, FTY720 was shown to cause apoptosis in T-cell lines at micromolar concentrations. However, it is reported that the blood concentration range of FTY720 is <100 ng/mL when given to rats at 0.1 to 1 mg/kg. In fact, it is impossible for FTY720 to induce apoptotic cell death in lymphocytes at a dose range of 0.1 to 1 mg/kg in vivo. In our study, 0.25 mg/kg and 1 mg/kg of FTY720 showed similar neuroprotective effects. Therefore, it is safe to administer FTY720 <1 mg/kg intraperitoneally.

S1P is considered to be a prosurvival lipid because of its involvement in cellular differentiation, proliferation, migra-
tion, cytoskeletal reorganization, cellular proliferation, and survival. S1P has been reported to decrease 3 days after cerebral ischemia, and therefore deceased S1P may contribute to apoptosis after cerebral ischemia. FTY720 crosses the blood–brain barrier and remains a long time in the brain and activates S1P1. Thus, FTY720 may compensate for the decreased endogenous S1P and induce antiapoptotic mechanisms against cerebral ischemia.

In this study, we demonstrated that FTY720 has an antiapoptotic effect against cerebral ischemia. On the other hand, FTY720 was reported to inhibit harmful lymphocytes infiltration and act protectively against ischemia–reperfusion injury in the kidney and liver. In addition, the effects of FTY720 on cerebral blood flow during reperfusion has not been studied. In this regard, further studies in cerebral ischemia are needed.

It has been suggested that FTY720 downregulated S1P1 and inhibited lymphocyte egress from secondary lymphoid tissues, being considered as the basis for the therapeutic effect of FTY720. However, in some other cell types, for example, endothelial cells, sustained treatment is suggested not to induce receptor downregulation, and the consequent continued signaling persists. We showed that S1P1 were sufficiently expressed in neurons in the peri-infarct cortex after MCAO in this study.

In conclusion, FTY720 reduced neuronal injury and improved neurobehavior after cerebral ischemia by activation of Akt and ERK through S1P1, which prevented apoptosis. Clinical trials for treatment of multiple sclerosis and renal transplantation have demonstrated the efficacy of FTY720, which could be a novel compound available for treatment of patients with stroke.

Sources of Funding
This study was partially supported by grants (NS053407) from the National Institutes of Health to J.H.Z.

Disclosures
None.

References


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Stroke. published online November 25, 2009;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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