Synergistic Effect of an Endothelin Type A Receptor Antagonist, S-0139, With rtPA on the Neuroprotection After Embolic Stroke

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Background and Purpose—Using a model of embolic stroke, the present study tested the hypothesis that blockage of endothelin-1 with S-0139, a specific endothelin type A receptor (ET\textsubscript{A}) antagonist, enhances the neuroprotective effect of recombinant tissue plasminogen activator (rtPA) by suppressing molecules that mediate thrombosis and blood brain barrier (BBB) disruption induced by ischemia and rtPA.

Methods—Rats (n=104) subjected to embolic middle cerebral artery (MCA) occlusion were randomly divided into 1 of 4 infusion groups with 26 rats per group: (1) the control group in which rats were administered saline, (2) the monotherapy rtPA group in which rtPA was intravenously administered at a dose of 10 mg/kg 4 hours after MCA occlusion, (3) the monotherapy S-0139 group in which S-0139 was intravenously given 2 hours after MCA occlusion, and (4) the combination of rtPA + S-0139 group in which S-0139 and rtPA were given 2 and 4 hours after MCA occlusion, respectively. Measurements of infarct volume and parenchymal hemorrhage, behavioral outcome, and immunostaining were performed on rats euthanized 1 and 7 days after stroke.

Results—The combination therapy of S-0139 and rtPA significantly (P < 0.01) reduced infarct volume (24.8 ± 0.9% versus 33.8 ± 1.5% in control) and hemorrhagic area (7.1 ± 6.1 \(\mu\)m\(^2\) versus 36.5 ± 19.2 \(\mu\)m\(^2\) in control) and improved functional recovery compared with control saline-treated animals. Immunostaining analysis revealed that the combination therapy had the synergistically suppressed ischemia- and rtPA-induced ICAM-1, protease-activated receptor 1 (PAR-1), as well as accumulation of platelets in cerebral microvessels. Furthermore, the combination treatment synergistically reduced loss of laminin, ZO1, and occludin in cerebral vessels.

Conclusions—These data suggest that S-0139 provides the neuroprotection by suppressing ischemia- and rtPA-triggered molecules that evoke thrombosis and BBB disruption. (Stroke. 2008;39:2830-2836.)

Key Words: antagonist ■ embolic ■ integrity ■ patency ■ rat ■ stroke

The endothelins (ETs) are potent vasoactive peptides and ET-1 to ET-3 induce vasoconstriction through the ET receptor subtype, ET\textsubscript{A}.\textsuperscript{1} ET\textsubscript{A} is expressed in cerebral endothelial cells and regulates blood brain barrier (BBB) function.\textsuperscript{1,3-4} Acute stroke patients exhibit significantly elevated ET-1 levels in plasma and cerebrospinal fluid.\textsuperscript{5,6} In experimental stroke, increased ETs exacerbate BBB leakage, whereas blockage of ET\textsubscript{A} with S-0139, a specific ET\textsubscript{A} antagonist, suppresses disruption of BBB permeability and brain edema.\textsuperscript{3,4,7}

Although the neuroprotective effects of S-0139 have been demonstrated,\textsuperscript{3,4} it has not been examined whether blockage of ET\textsubscript{A} with S-0139 enhances thrombolysis. As disruption of the BBB and an increase risk of hemorrhage limit the therapeutic window for thrombolysis of stroke with tissue plasminogen activator (tPA) to 3 hours,\textsuperscript{5,9} there remains a need for agents that reduce the incidence and severity of bleeding and are effective in extending the therapeutic window for rt-PA. Using a model of embolic stroke, the present study tested the hypothesis that a combination treatment of S-0139 with tPA significantly reduce infarct volume when tPA is given 4 hours after stroke. We further hypothesized that the combination treatment reduces expression of molecules that mediate thrombosis, leukocyte adhesion, and BBB disruption, and thereby reduces ischemic cell damage.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.
General Procedures
Male Wistar rats were anesthetized with isoflurane (1% to 2.5% in a mixture of 70% N2O and 30% O2) with the use of a face mask. The rectal temperature was maintained at 37±0.5°C throughout the surgical procedure by means of a feedback-regulated water heating system.

Animal Models
Rats weighing 375 to 400 g were subjected to embolic MCA occlusion. Briefly, a single intact, fibrin-rich, 24-hour-old homologous clot was placed at the origin of the MCA via a 15-mm length of modified polyethylene catheter (PE-50).

Experimental Protocols
To examine the effect of S-0139 on extending the therapeutic window of recombinant tPA (rTPA), rats were subjected to the embolic MCA occlusion and randomized into 1 of 4 infusion groups with 26 rats per group: (1) the control group, in which rats were administered saline, (2) the monotherapy rTPA (Genetics) group, in which rTPA was infused intravenously at a dose of 10 mg/kg 4 hours after MCA occlusion (10% bolus and the remainder at a continuous infusion over a 30 minutes interval via a syringe infusion pump, Harvard Apparatus), (3) the monotherapy S-0139 group, in which S-0139 was infused intravenously at a dose of 3 mg/kg/h for 22 hours (n=6) or 72 hours (n=20) infusion starting 2 hours after MCA occlusion, and (4) the combination of rTPA + S-0139 group in which S-0139 and rTPA (10 mg/kg) were given 2 hours and 4 hours after MCA occlusion, respectively. S-0139 was infused intravenously at a dose of 3 mg/kg/h for 22 hours (n=6) or 72 hours (n=20). Six rats per group were euthanized 24 hours after MCA occlusion for measurements of infarct volume and the incidence of hemorrhage.

Neurological and Behavioral Assessment
To detect sensorimotor impairments, an array of behavioral tests including foot-fault, modified neurological severity score (mNSS), and adhesive-removal test were performed before MCA occlusion and at 1 and 7 days after MCA occlusion by an investigator blinded to the experimental groups. These tests are sensitive and reliable indices of sensorimotor impairments after ischemic stroke and have been applied extensively in our laboratory to assess neurological outcome after MCA occlusion in rats.

Measurements of Infarct Volume and Hemorrhage
Infarct volume and petechial hemorrhage were measured on hematoxylin and eosin (H&E) stained 7 coronal sections from rats euthanized 7 days after MCA occlusion for immunostaining analysis, whereas 20 rats from each group were euthanized 24 hours after MCA occlusion for measurements of infarct volume and the incidence of hemorrhage.

Immunohistochemistry
Immunostaining was performed on frozen brain coronal sections from rats euthanized 24 hours after MCA occlusion, according to our published methods. To detect adhesive molecule expression in the cerebral microvessels, a monoclonal anti-intercellular adhesion molecule-1 (ICAM-1) antibody (BD Bioscience) was used at a titer of 1:50. To examine the integrity of cerebral microvessels, a rabbit polyclonal antitype IV collagen antibody (Abcam) and a rabbit polyclonal anti-ZO1 antibody (Zymed) were used at a titer of 1:200 and 1:100, respectively.

Quantification
Frozen coronal sections used in the present study were located at the center of the territory supplied by the MCA (between anterior-posterior [AP]+10.6 mm-genu corpus callosum and AP+8.74 mm-anterior commissure crossing). A total of 4 sections (each one at 400 μm apart) per rat were used for immunostaining. The numbers of ICAM, and PAR-1 immunoreactive vessels were counted throughout the entire ipsilateral hemisphere because normal cerebral vessels do not express these genes. The numbers of thrombocyte immunoreactive vessels were measured from 6 0.55-mm² fields located in the ischemic boundary (Figure 1A). The numbers of immunoreactive vessels in the 4 sections per rat were averaged to obtain a mean number for each brain according to published methods. Data are presented as the number of vessels.

Measurements of occludin, ZO-1, collagen type IV, and laminin positive vessels were performed in the ischemic boundary (Figure 1B, 0.55 mm²×7 fields) and homologous areas of the contralateral hemisphere. Briefly, occludin, ZO-1, collagen type IV, and laminin immunoreactive vessels were digitzed under a 40× objective (Olympus) via the MCID system. Density of vessels in each field of view was determined by the MCID system. Data are presented as a percent of vessels in the ipsilateral hemisphere versus the contralateral hemisphere.

Statistical Analysis
Analysis of variance (ANOVA) for repeated measures, with fixed factors rTPA and S-0139 and repeated factor sample, was used to test the synergy of combination treatments rTPA and S-0139. Analysis began with testing for rTPA by S-0139 interaction, followed by a subgroup analysis. A significant treatment interaction between rTPA and S0139 at the critical level of 0.05 was further evaluated for superadditive effect (synergy) or subadditive effect. All data are presented as mean±SE, and a P<0.05 was considered as statistically significant.

Results
The Combination Therapy Is Neuroprotective
In rats subjected to embolic MCAo, monotherapy of S-0139 2 hours after MCA occlusion significantly reduced ischemic lesion volume (27.9±1.4% of the contralateral hemisphere, n=20) compared with the controls (34.3±2.8%, n=20), which was associated with significant improvement of functional outcome 7 days after MCA occlusion (Figure 1C to 1E). In contrast, monotherapy of rTPA 4 hours after MCA occlusion neither reduced infarct volume (34.9±1.4%, n=20 versus 33.8±1.5%, n=20 in control), nor improved functional recovery (Figure 1C to 1E), but significantly (P<0.05) increased hemorrhagic area (116.8±33.8 μm² versus 36.5±19.2 μm² in control). Combination of S-0139 at 2 hours and rTPA at 4 hours after MCA occlusion significantly (P<0.01) reduced infarct volume (24.8±0.9%, n=20 versus 33.8±1.5%, n=20 in control) and hemorrhagic area (7.1±6.1 μm² versus 36.5±19.2 μm² in control) and improved functional recovery compared with the rats treated with saline (Figure 1C to 1E).

The Combination Therapy of rTPA and S-0139 Reduces ICAM-1 Expression
Uptregulation of ICAM-1 facilitates adhesion of neutrophils to endothelial cells, which contributes to secondary thrombosis in cerebral microvessels. Endothelin-1 increases ICAM-1 expression on cultured rat aortic endothelial cells.
To examine whether ETA antagonist, S-0139, blocks ICAM-1 expression on cerebral endothelial cells, immunostaining for ICAM-1 was performed. Occlusion of the MCA resulted in induction of ICAM-1 in cerebral vessels (Figure 2G), which is consistent with our previous findings. The monoclonal antibody to ICAM-1 given 2 hours after stroke and combination therapy of S-0139 given 2 hours and rtPA administered 4 hours after stroke improve neurological function determined by adhesive removal test (C), foot-fault test (D), and mNSS (E) 7 days after MCA occlusion. \( * P<0.05 \) vs the saline group and \( # P<0.05 \) vs monotherapy of S-0139 group. \( n=20 \) rats/group. CC indicates corpus callosum; lv, lateral ventricle.

**Figure 1.** A schematic of coronal sections shows 6 (A) and 7 (B) areas where images were acquired for analysis of thrombocytes and vascular integrity, respectively. The effects of S-0139 on behavioral tests (C to E). Monotherapy of S-0139 given 2 hours after stroke and combination therapy of S-0139 given 2 hours and rtPA administered 4 hours after stroke improve neurological function measured by adhesive removal test (C), foot-fault test (D), and mNSS (E) 7 days after MCA occlusion. \( * P<0.05 \) vs the saline group and \( # P<0.05 \) vs monotherapy of S-0139 group. \( n=20 \) rats/group. CC indicates corpus callosum; lv, lateral ventricle.

**Figure 2.** Immunostaining shows ICAM-1 immunoreactive vessels in the ischemic hemisphere from representative rats treated with rtPA alone (A through C) and S-0139 in combination with rtPA (D through F). C and F indicate the cortex. B and E represent the striatum. G, Quantitative data of ICAM-1 immunoreactive vessels (\( n=6 \) per group, \( # \) vs saline and \( * \) vs rtPA). Bar=25 \( \mu \)m (B to F). Monotherapy of rtPA was administered 4 hours after stroke, whereas combination therapy of S-0139 and rtPA was given 2 and 4 hours after stroke, respectively.
monotherapy of rtPA given 4 hours after MCA occlusion further increased ($P<0.05$) the number of ICAM-1–positive vessels (Figure 2A to 2C and 2G). However, the combination therapy of rtPA (4 hours) and S-0139 (2 hours) had the synergistic effect of suppressing ICAM-1 in microvessels (Figure 2D to 2F and 2G), suggesting that S-0139 suppresses ischemia- and rtPA-upregulated ICAM-1 expression in cerebral endothelial cells.

The Combination Therapy of rtPA and S-0139 Reduces PAR-1 and Thrombosis

PAR-1, a receptor for thrombin, induces thrombosis. To examine the effect of S-0139 on formation of secondary thrombosis, we measured PAR-1 and thromboctye immunoreactive vessels. The monotherapy of S-0139 administered 2 hours after stroke significantly reduced the number of PAR-1 (Figure 3E) and thromboctye (Figure 3F) positive vessels, whereas the monotherapy of rtPA given 4 hours after stroke significantly augmented the number of PAR-1 (Figure 3A and 3E) and thromboctye (Figure 3C and 3F) immunoreactive vessels compared to saline-treated animals. The combination therapy of rtPA and S-0139 substantially decreased the number of PAR-1 (Figure 3B and 3E) and thromboctye (Figure 3D and 3F) positive vessels compared to saline control, and statistical analysis revealed that the combination therapy has a synergistic effect, indicating that blockage of ETA by S-0139 abolishes stroke- and rtPA-induced secondary thrombosis.

The Combination Therapy of rtPA and S-0139 Augments Vascular Integrity

To examine the effects of S-0139 on cerebral microvascular integrity, we measured collagen type IV and laminin immunoreactivity, 2 of the major basal lamina components of cerebral microvessels, and the tight junction proteins, occludin and ZO1. Consistent with previous findings, embolic stroke dramatically reduced collagen type IV (Figure 4D), laminin (Figure 4H), ZO1 (Figure 5B and 5E), and occludin (Figure 5G and 5J) immunoreactive vessels in the ipsilateral hemispheres compared with the homologous area of the contralateral hemisphere. Treatment with S-0139 2 hours after stroke significantly blocked loss of laminin (Figure 4H), ZO1 (Figure 5C and 5E), and occludin (Figure 5G and 5J), but not collagen IV immunoreactive vessels (Figure 4H). However, the monotherapy of rtPA administered 4 hours after stroke exacerbated reduction of collagen type IV (Figure 4B and 4E).

Figure 3. Photomicrographs show PAR-1 (A and B) and platelet (C and D) immunoreactive vessels in the ischemic boundary of the cortex (area 2 of Figure 1A) from representative rats treated with rtPA alone (A and C) or S-0139 in combination with rtPA (B and D). E and F, Quantitative data of PAR-1 and thromboctye immunoreactive vessels (n=6 per group), respectively. Please see methods for details of quantification of PAR-1 and thromboctye immunoreactive vessels. Bar=25 μm (A to D). Monotherapy of rtPA was administered 4 hours after stroke, whereas combination therapy of S-0139 and rtPA was given 2 and 4 hours after stroke, respectively.

Figure 4. Photomicrographs show collagen IV (A to C) and laminin (E to G) immunoreactive vessels in the cortex of homologous areas of the contralateral hemisphere (A and E) and in the ischemic boundary of the cortex (areas 1 and 2 of Figure 1B) from representative rats treated with rtPA alone (B and F) or S-0139 in combination with rtPA (C and G). D and H, Quantitative data of percentage of collagen IV and laminin, respectively, positive vessels in the ipsilateral hemisphere vs the contralateral hemisphere (n=6 per group). Bar=20 μm (A to G). Monotherapy of rtPA was administered 4 hours after stroke, whereas combination therapy of S-0139 and rtPA was given 2 and 4 hours after stroke, respectively.
and 4D), laminin (Figure 4F and 4H), ZO1 (Figure 5D and 5E), and occludin (Figure 5H and 5J) immunoreactive vessels. The combination therapy of rtPA and S-0139 abolished exacerbation of these immunoreactive vessels triggered by ischemia and rtPA (Figures 4C, 4D, 5D, 5E, 5I, and 5J).

**Discussion**

The present study demonstrates that combination treatment of rtPA (4 hours) and S-0139 (2 hours) significantly reduced infarct volume and improved neurological outcome without increasing the incidence of hemorrhagic transformation compared with animals treated with saline and rtPA alone (4 hours). Immunostaining analysis revealed that the combination therapy synergistically blocked ischemia- and rtPA-induced ICAM-1 and PAR-1 expression as well as accumulation of platelets in cerebral microvessels. Furthermore, the combination treatment synergistically reduced loss of laminin, ZO1, and occludin in cerebral vessels. These data indicate that S-0139, an ET$_A$ antagonist, in combination with rtPA at 4 hours after stroke has a neuroprotective effect, whereas S-0139 suppresses ischemia- and rtPA-triggered molecules that evoke thrombosis and BBB disruption and likely contributes to the beneficial effects of the combination therapy.

S-0139 is a potent ET$_A$ antagonist and has neuroprotective effects in experimental models of stroke and BBB disruption. In a filament model of transient MCA occlusion, S-0139 given 1 hour after transient MCA occlusion has been shown to reduce infarct volume, which was associated with decreased BBB leakage and neutrophil infiltration. However, S-0139 does not have a neuroprotective effect when it is given to the rat subjected to permanent MCA occlusion. Administration of rtPA 2 hours after embolic MCA occlusion reduces infarct volume and improves neurological outcome. The present study shows that monotherapy of rtPA given 4 hours after MCA occlusion did not have the neuroprotective effects and exacerbated hemorrhage, which is consistent with previous findings in this model of embolic stroke. However, blockage of ET-1 with S-0139 substantially reduced the infarct volume and hemorrhage induced by rtPA. Moreover, the combination therapy 4 hours after stroke has a synergistic effect on improvement of functional outcome. These data suggest that S-0139 is effective to reduce the adverse side effects of thrombolysis with rtPA.

Molecular mechanisms underlying the neuroprotective effects of S-0139 on stroke and on thrombolysis have not been investigated. Using immunohistochemistry, the present study shows that S-0139 significantly reduced ICAM-1 and PAR-1 immunoreactive vessels in ischemic boundary regions, when S-0139 was administered 2 hours after embolic stroke. More importantly, we found that S-0139 (2 hours) in combination with rtPA (4 hours) has a synergistic effect on decreasing these immunoreactive vessels. Stroke induces adhesion molecule expression including ICAM-1 in cerebral vessels,
which facilitates leukocyte adhesion.26 When neutrophils adhere to injured endothelium, they interact with platelets and form clots that contribute to secondary thrombosis in cerebral microvessels and disruption of the BBB.26 Delayed fibrinolysis with rt-PA further increased in ICAM-1 expression and neutrophil accumulation in ipsilateral cerebral vessels.27 The present study suggests that endothelin-1 could mediate stroke- and thrombolysis-upregulated ICAM-1 expression, which is consistent with previous findings showing that endothelin-1 induces ICAM-1 expression on cultured endothelial cells.28 Moreover, our data suggest that ET 

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may be involved in PAR-1 expression. PAR-1 is the prototype thrombin receptor, and plasmin, which is generated from the cleavage of plasminogen by rt-PA, activates PAR-1 that also promotes thrombosis formation.29 Blockage of ET 

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with BQ123 inhibits the effect of PAR-1 activated by thrombin on NMDA-mediated nociceptive activity.29 Collectively, these data suggest that suppressing ICAM-1 and PAR-1 expression by S-0139 likely contributes to the beneficial effect of this ET 

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antagonist on cerebral vascular patency. Indeed, the present study demonstrates that monotherapy of S-0139 and combination therapy of S-1039 with rtPA significantly reduced platelet aggregation in cerebral vessels.

The present study shows that either monotherapy of S-0139 or combination therapy of S-0139 with rtPA significantly reduced degradation of collagen IV and laminin immunoreactivity, two of the major basal lamina components of cerebral microvessels.9 Concurrently, both therapies significantly reduced loss of endothelial tight junction proteins ZO1 and occludin. These data suggest that S-0139 could benefit cerebral vascular integrity after stroke and thrombosis with rtPA.

In the present study, we used a model of embolic MCAo that mimics the malignant MCA infarction of human stroke.10,30,31 In this model, we demonstrate that development of secondary thrombosis contributes to impairment of cerebral microvascular patency and integrity.9 Stroke elevates endothelin-1 levels in cerebral endothelium and astrocytes and astrocytes are integral to composite the BBB.32 ET 

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is expressed in cerebral endothelial cells.1–4 Consistently, the present study suggests that S-0139 has multi-targeted effects on cerebral microvascular thrombosis and microvascular integrity. However, future studies are warranted to delineate signaling pathways that mediate interaction between rtPA and ET-1 receptor activation, which extend the therapeutic window of rtPA to 4 hours after stroke.

In summary, our data indicate that the combination therapy of S-0139 and rtPA 4 hours after stroke is neuroprotective and that S-0139 augments cerebral vascular patency and integrity by suppressing ischemia- and rtPA-triggered molecules that mediate thrombosis and BBB disruption.

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Disclosures

None.

References


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