Dissociation of Vasospasm and Secondary Effects of Experimental Subarachnoid Hemorrhage by Clazosentan

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Background and Purpose—Endothelin receptor antagonists such as clazosentan decrease large-artery vasospasm after experimental and clinical subarachnoid hemorrhage. We used clazosentan to gain insight into the pathophysiology of subarachnoid hemorrhage by determining if decreasing vasospasm is associated with alleviation of other secondary complications of subarachnoid hemorrhage such as oxidative stress, endothelial nitric oxide synthase dysfunction, microthromboembolism, and neuronal injury.

Methods—Mice were subjected to subarachnoid hemorrhage by injection of blood into the chiasmatic cistern. They were treated with clazosentan or vehicle by continuous intraperitoneal infusion for 48 hours. Middle cerebral artery vasospasm, superoxide anion radical, peroxynitrite, microthromboemboli, endothelial nitric oxide synthase uncoupling, cerebral blood flow, neuronal injury, and mortality were assessed.

Results—Clazosentan preserved cerebral blood flow, alleviated vasospasm, and decreased mortality but did not affect superoxide anion radical, peroxynitrite, or microthromboemboli in the brain. Endothelial nitric oxide synthase uncoupling and neuronal injury also were not reduced by clazosentan.

Conclusions—This study shows large-artery vasospasm is pathophysiologically independent of some other effects of subarachnoid hemorrhage. The findings have implications for development of treatments for this disease. (Stroke. 2011; 42:1454-1460.)

Key Words: clazosentan ■ endothelin ■ mice ■ subarachnoid hemorrhage ■ vasospasm

Subarachnoid hemorrhage (SAH) may be spontaneous or traumatic. Spontaneous SAH accounts for approximately 5% of all cases of stroke. Although the initial mortality is high, it has been declining. Furthermore, those who survive to be treated in the hospital still can have morbidity and mortality from delayed complications such as delayed cerebral ischemia.

Delayed neurological deterioration usually is assumed to be due to ischemia, hence the term delayed cerebral ischemia. Some investigators, however, think other processes contribute to and/or cause delayed neurological deterioration. These include delayed neuronal apoptosis, early brain injury, reactive oxygen species and other free radicals, inflammation, microcirculatory vasospasm, microthromboembolism, and cortical spreading ischemia. Evidence for these additional mechanisms includes the report that endothelin antagonists reduce angiographic vasospasm in humans but have no effect on clinical outcome. On the other hand, the lack of effect on clinical outcome could be due to inadequate sample size, insensitivity of the dichotomous outcome measure, or drug side effects. One way to test the hypothesis that other secondary complications could independently contribute to poor outcome would be to determine the effects of clazosentan in animal models. Clazosentan is a putative specific antagonist of endothelin A receptors with no documented neuroprotective, anti-inflammatory, anticonvulsant, or antithrombotic effects and thus may be a useful tool to address this question.

We developed a mouse model of SAH that causes arterial vasospasm, microthromboemboli, and neuronal injury. Here we use clazosentan to specifically antagonize endothelin A receptors and alleviate cerebral vasospasm and determine if other secondary complications including endothelial nitric oxide synthase (eNOS) uncoupling, microthromboemboli, and neuronal cell death/degeneration also are prevented, thus indicating that these phenomena are secondary to vasospasm.

Materials and Methods

Animals

Experimental protocols were approved by the Institutional Animal Care Committee. We used 30 endothelial nitric oxide synthase (NOS)-lacZ transgenic mice (Marsden Lab) weighing 19 to 25 g. eNOS-lacZ mice were generated using a murine eNOS promoter with the eNOS ATG site mutated to prevent translation. The original founder mice were hybrids of C57BL×SJL F2.13 Mice were genotyped using Southern blot analysis of genomic tail DNA, and
both sexes were used at an age of 2 to 6 months. We previously used these mice to assess eNOS expression after SAH and used them in this study so the animal model was not changed.12

**SAH and Clazosentan Treatment**

Mice were anesthetized with ketamine (10 mg/kg) and xylazine (4 mg/kg) intraperitoneally. SAH was created by injection of autologous blood into the prechiasmatic cistern.11 Blood (nonheparinized, 60 μL) was withdrawn from the heart of a donor mouse (same background strain) and injected through a spinal needle over 15 seconds. Mice were randomly allocated to treatment with clazosentan or vehicle. For clazosentan-treated animals, clazosentan was administered by an intraperitoneal injection of 1 mg/kg before SAH and then constantly infused at 1 mg/kg/hr with an osmotic pump (1003D; Alzet, Cupertino, CA) that was implanted in the peritoneal cavity. For the vehicle group, SAH was created as described previously and 0.9% NaCl was injected and infused instead of clazosentan. In some experiments, we also included naïve animals as untreated controls. Mice were euthanized 48 hours after surgery. They were either perfused with phosphate-buffered saline and fixed with 4% paraformaldehyde or brains were removed without fixation.

**Hematoxylin and Eosin Staining and Vasospasm Measurement**

Paraffinized sections of the brain obtained as previously described were deparaffinized with xylene and rehydrated through a decreasing gradient of ethanol solutions.11 Slides were stained with hematoxylin and eosin, coverslipped with mounting medium, and viewed under a light microscope. Slides were scanned with a digital scanner (MIRAX; Carl Zeiss, Göttingen, Germany) and viewed with MIRAX software (Carl Zeiss). The lumen area and thickness of the middle cerebral artery were quantified by a blinded observer using Image J (National Institutes of Health, Bethesda, MD).

**Peroxy nitrite Assay and Antimitrotyrosine**

After deparaffinization and rehydration, antigen retrieval was performed by heating the sections for 25 minutes in 0.01 mmol/L sodium citrate (pH 6.0) at 96°C. Endogenous peroxidase activity was quenched by incubating the sections for 30 minutes in 0.3% H2O2 in water. Sections were blocked with 10% normal goat serum in phosphate-buffered saline for 20 minutes. Polyclonal antimitrotyrosine antibody (rabbit polyclonal 1:200) was used as a marker for peroxynitrite and incubated for 60 minutes. A secondary biotinylated antibody (goat antirabbit) was added to specimens for 30 minutes. The VIP reaction was then performed using the VECTASTAIN ABC Kit.

**Nitric Oxide, Super oxide Anion Radical Detection**

Superoxide anion radical and nitric oxide were detected in homogenized fresh or deep frozen brain tissue using spectrophotometric methods. The cell-permeable fluorophore 4,5-diaminofluorescein-2-diacetate (DAF-2DA; Alexis Biochemicals, Gruenberg, Germany) was used to detect nitric oxide and a chemiluminescence probe, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-β]pyrazin-3-one, for superoxide anion radical detection. Tissues were incubated with either 10 μmol/L DAF-2DA for 30 minutes or 4 μmol/L 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-β]pyrazin-3-one at room temperature in the dark. DAF-2DA was excited at 495 nm and emission read at 515 nm in a spectrofluorometer. 2-Methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-β]-pyrazin-3-one luminescence was read directly at 495 nm. All experiments were repeated 3 times. Because 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-β]-pyrazin-3-one crosses cell membranes, the superoxide anion radical detected is from both intra- and extracellular sources.

**Western Blots for eNOS and Inducible Nitric Oxide Synthase**

Brain tissue was excised and stored at −80°C. Tissue was homogenized in 300 μL 1% RIPA buffer with 0.1% protease inhibitor and centrifuged at 13 000 rpm for 12 minutes at 4°C. Protein was quantified using the Bradford method. Thirty micrograms of protein was loaded and separated by electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membrane. We used Ponceau S and Gel Code to stain the membrane and gel, respectively. Blots were incubated with 5% milk for 60 minutes followed by incubation with primary monoclonal antibodies (1:1000 dilution) against phosphorylated S1177-eNOS (BD Biosciences, San Jose, CA), eNOS (Cell Signaling, Danvers, MA) and inducible nitric oxide synthase (Abcam, Cambridge, MA). After washing in phosphate-buffered saline, membranes were incubated in horseradish peroxidase conjugated antigen polyclonal antibody (Abcam) at a dilution of 1:1000 for 50 minutes at room temperature. Reactions were developed with ECL reagent mix (Amersham Biosciences). Protein intensities were quantified by densitometric analysis using Image J (National Institutes of Health).

For detection of eNOS monomer and dimer, we used low-temperature sodium dodecyl sulfate–polyacrylamide gels techniques as described.14,15 Samples were subjected to sodium dodecyl sulfate–polyacrylamide on 8% gels at 4°C.

**Immunohistological Staining for Fibrinogen**

After deparaffinization and rehydration, antigen retrieval, and endogenous peroxidase quenching, sections were blocked with 10% normal goat blocking serum in phosphate-buffered saline for 20 minutes and incubated with fibrinogen first antibody (rabbit antirat 1:200; Immunology Consultants Laboratory, Newberg, OR) for 60 minutes. They were washed with phosphate-buffered saline and incubated with secondary biotinylated antibody (goat antichicken; Millipore) for 30 minutes. Staining was visualized with VIP using the VECTASTAIN 7 ABC Kit (Vector) and counterstained with 0.5% methyl green.

**Fluoro-Jade Staining**

Fluoro-jade B (Histo-Chem, Jefferson, AR) staining was performed according to a previously published protocol.11 After deparaffinization and rehydration, the slides were incubated in 0.06% potassium permanganate (VWR International, Strasbourg, France) for 15 minutes. Slides were then rinsed in deionized water and immersed in 0.001% Fluoro-jade B in 0.1% acetic acid for 30 minutes. Then they were washed and dried at 60°C for 15 minutes. Sections were cleared in xylene and coverslipped with a nonaqueous, low-fluorescence, styrene-based mounting medium (Sigma). Slides were viewed under a confocal microscope and images taken using constant parameters (laser power, exposure time, and pinhole size).

**Statistical Analysis and Data Quantification**

All data are presented as means±SD except cerebral blood flow data are mean±SEM. Data were compared within groups over time and between groups at each time by analysis of variance or Student t test for continuous variables. P<0.05 was considered significant. To keep the quantification on staining consistent, we preselected 5 fixed symmetrical areas for cortex and 3 for hippocampus on a proper coronal section of mouse brain.16 For fibrinogen staining, we took 1 image from each fixed area (10 images from the cortex, 6 images from the hippocampus) and counted all the microthromboemboli in the section. To determine Fluoro-jade B staining in the cortex, we used higher magnification (400×) and took 3 images from each of the preselected areas (30 images for both sides of the cortex). For the hippocampus, we counted all positive cells in all regions of the hippocampus (dentate gyrus, CA3 and CA1). All counting was done blinded to treatment group.

**Results**

Clazosentan Ameliorated Vasospasm, Reduced Mortality, and Improved Cerebral Blood Flow

Clazosentan decreased middle cerebral artery vasospasm after SAH (Figure 1A). The lumen area/wall thickness ratio was significantly higher in clazosentan-treated animals compared with the vehicle group. This is consistent with other reports that clazosentan can ameliorate vasospasm in experimental SAH.17,20,21 Clazosentan also significantly reduced mortality compared with clazosentan-free controls (Figure 1B). This is in line with data from previous studies demonstrating that clazosentan significantly reduces mortality in experimental SAH.17,22,23 Finally, we also investigated the effects of clazosentan on cerebral blood flow, a key parameter of neuroprotection in SAH.24 Our data demonstrates that clazosentan significantly improved cerebral blood flow compared with the vehicle group (Figure 1C). This is consistent with previous studies demonstrating that clazosentan can improve cerebral blood flow in experimental SAH.25,26
pared with vehicle-treated animals (24.5 ± 4.3 for naive control, 23 ± 9 for clazosentan, 22.2 ± 0.9 for vehicle; P < 0.01; Figure 1A). In comparison with naive controls, lumen area was decreased to 49% ± 12% in SAH animals treated with vehicle and to 81% ± 18% by clazosentan, which was signifi-
cantly larger than in the vehicle group (P < 0.01).

Eighty-eight percent of clazosentan-treated animals survived in comparison to 67% of vehicle-treated mice (Figure 1B; P = 0.57, Fisher exact test). Clazosentan also significantly improved cerebral blood flow (CBF) acutely after SAH compared with vehicle (Figure 1C). CBF recovered to baseline in the clazosentan-treated group but remained decreased in vehicle-treated animals (100% ± 6% of baseline versus 75% ± 7%, respectively, at 30 minutes post-SAH; P < 0.001; Figure 1C).

Clazosentan Did Not Prevent eNOS Uncoupling After SAH

We previously demonstrated that eNOS is uncoupled after SAH, as shown by an increase in eNOS monomer and decrease in functional dimer.12 In this study, clazosentan did not affect eNOS dimer/monomer ratio (Figure 2A). However, the ratio in both groups was significantly reduced in comparison to the ratio in untreated animals levels (2.2 ± 0.5, 2.3 ± 0.7, and 20.3 ± 0.2 for vehicle, clazosentan, and untreated; P < 0.001; Figure 2A).

To assess eNOS function, we measured phosphorylation of eNOS at Ser1177, which is required for activation and nitric oxide synthesis, as well as nitric oxide production. SAH increased eNOS phosphorylation as compared with untreated mice and clazosentan did not affect this (10 ± 1 AU for vehicle, 12 ± 2 AU for clazosentan and 5.6 ± 0.4 for untreated; P < 0.001; Figure 3A). Clazosentan- and vehicle-treated animals demonstrated significantly reduced nitric oxide in comparison to that in the untreated animals (P < 0.001; Figure 3B). Nitric oxide levels were 8.6 ± 2.4 relative fluorescence units (RFUs) for clazosentan and 7.5 ± 0.9 RFUs for vehicle. They were significantly reduced in comparison to the value obtained from the untreated mice (58 ± 17 RFUs; P < 0.001; Figure 3B).

Clazosentan Had No Effect on Nitrosative/Oxidative Stress or Inducible Nitric Oxide Synthase

Clazosentan treatment also did not reduce oxidative stress or inducible nitric oxide synthase induction and was quantitatively and qualitatively similar to the vehicle-treated animals. Oxida-
tive stress, assessed by superoxide anion radical, was increased by SAH but not affected by treatment with clazosentan (33 ± 10 RFUs for clazosentan and 34 ± 4 RFUs for vehicle; P > 0.05; Figure 4C). These values were significantly higher than observed in untreated mice (5 ± 2 RFUs; P < 0.01; Figure 4C).

To evaluate nitrosative stress, peroxynitrite was measured indirectly through detection of nitrotyrosine deposits perivas-
cularly in brain parenchyma. Qualitative assessment of the nitrotyrosine staining suggested that both clazosentan- and
vehicle-treated animals had similar levels, which were substantially higher than seen in untreated mice (Figure 4A).

SAH was associated with marked elevation of inducible nitric oxide synthase protein in comparison to saline-injected animals. Clazosentan- and vehicle-treated animals showed similar levels of inducible nitric oxide synthase (33±7 AU and 33±13 AU, respectively).

Clazosentan Had No Effect on Microthromboemboli or Apoptotic Cell Death
Further findings in keeping with a specific effect of clazosentan on vasospasm but not other phenomena associated with SAH, and with the phenomena being independent of vasospasm, clazosentan did not decrease microthromboemboli as compared with vehicle in the cortex and hippocampus after

Figure 2. A, Analysis of endothelial nitric oxide synthase (eNOS) dimer/monomer ratio demonstrating that both clazosentan and vehicle had similarly low ratios indicating the predominance of the monomeric form, thus suggesting uncoupling of eNOS in comparison to untreated mouse levels. B, Clazosentan- and vehicle-treated animals showed similar levels of inducible nitric oxide synthase (33±1006 11006 AU and 33±7 AU, respectively).

D, Western blots demonstrating an increase in the functional dimeric and dysfunctional monomeric form of eNOS in brain tissues from both clazosentan- and vehicle-treated groups, suggesting that clazosentan did not recouple dysfunctional eNOS after subarachnoid hemorrhage (SAH). Values are means±SD; n=5 for all groups.

Figure 3. A, Bar graph shows a similar level of phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser1177 from both clazosentan- and vehicle-treated animals in comparison to lower expression in untreated mice (P<0.001); lower panel shows representative Western blot for the 3 groups. B, Clazosentan- and vehicle-treated animals had a statistically significant decrease in nitric oxide (NO) in comparison to the untreated animals. Values are means±SD; n=5 for all groups; t test, P<0.001.
Microthromboemboli counts were 11 ± 4 and 11 ± 4 in the hippocampus and 36 ± 8 and 38 ± 7 in the cortex for clazosentan and vehicle, respectively (Figure 5B). Also, neuronal cell death and degeneration were detected using Fluoro-jade B staining and both clazosentan- and vehicle-treated animals had increased numbers of Fluoro-jade B-positive cells in comparison to the untreated mice (Figures 5A and 5C). Counts of Fluoro-jade B cells were 6.7 ± 2.4 and 7.3 ± 0.5 in the hippocampus and 15 ± 5 and 15 ± 6 in the cortex for clazosentan and vehicle, respectively (Figure 5B). These cells had the appearance of neurons.

**Discussion**

The important new findings of this study are that clazosentan prevents large-artery vasospasm, improves CBF, and reduces mortality after SAH in mice. In addition to these positive effects, however, clazosentan did not prevent neuronal injury, microthromboembolism, increased superoxide anion radical and peroxynitrite, and eNOS uncoupling. These results are in agreement with those found in a double-blind, vehicle-controlled Phase II study (clazosentan to overcome neurological ischemia and infarction occurring after subarachnoid hemorrhage [CONSCIOUS-1]) that investigated the role of clazosentan in patients with aneurysmal SAH.7 In that study, patients treated with clazosentan, 15 mg/hr, demonstrated a 65% relative risk reduction in angiographic vasospasm. However, ameliorating angiographic vasospasm did not reduce mortality or improve clinical outcome in that study. Our findings are consistent with another study showing that clazosentan prevented reduced CBF compared with vehicle in a single-injection rat model of SAH.17

A question about CONSCIOUS-1 was why the reduction in angiographic vasospasm was not associated with improved clinical outcome. This could be because angiographic vasospasm does not contribute to outcome, although multiple lines of experimental and clinical evidence do not support this notion.6,18 For example, in this study, clazosentan reduced mortality and large-artery vasospasm. Reduced mortality, however, could be due to the ability of clazosentan to preserve CBF acutely after experimental SAH. This was suggested to be due to prevention of acute vasospasm, which may not be clinically important or may not be prevented because clazosentan is not administered immediately after clinical SAH.17

On the other hand, neuronal injury, microthromboembolism, oxidative stress, and eNOS dysfunction were not prevented by clazosentan. These observations indicate that restoration of CBF and prevention of large-artery vasospasm may not be sufficient to reverse the injurious effects of SAH. The dissociation of these effects of SAH has important implications for pathophysiology and treatment of SAH. They are consistent with emerging evidence suggesting that SAH leads to adverse effects such as inflammation, nuclear factor κ B and astrocyte activation, edema, oxidative stress, and apoptosis and that these cause brain injury by ischemic and nonischemic mechanisms.19 Although it seems obvious that neuronal injury would cause poor outcome, the contributions of microthromboembolism, oxidative stress, and eNOS dysfunction require confirmation. In clinical studies, treatments directed at microthromboembolism and oxidative stress have not been beneficial.20,21

Meta-analysis of the effect of endothelin receptor antagonists in patients with SAH showed these drugs significantly
reduced angiographic or transcranial Doppler vasospasm and delayed cerebral ischemia but did not reduce mortality or improve outcome.8 The authors raised other possible explanations such as that drug side effects offset the benefits, sample sizes were too small, or the clinical outcome measures were insensitive.

Some of the limitations of this work are that we used a mouse model of SAH. Only 1 dose of clazosentan was used and it is possible that other doses would alleviate the other secondary complications of SAH. Measurements of eNOS and nitric oxide were from whole brain tissue so the location of the changes is unknown. Further studies are necessary to define the role of microthromboembolism and oxidative stress in SAH.

Sources of Funding
The Physicians services incorporated foundation (PSI) fund.

Disclosures
R.L.M. received research support from The Physicians Services Incorporated Foundation. R.L.M. is a consultant for Actelion Pharmaceuticals and Chief Scientific Officer of Edge Therapeutics, Inc. Actelion provided clazosentan for this study.

References
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Stroke. 2011;42:1454-1460; originally published online March 31, 2011;
doi: 10.1161/STROKEAHA.110.604728

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/42/5/1454

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