Role of SCH79797 in Maintaining Vascular Integrity in Rat Model of Subarachnoid Hemorrhage

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Background and Purpose—Plasma thrombin concentration is increased after subarachnoid hemorrhage (SAH). However, the role of thrombin receptor (protease-activated receptor-1 [PAR-1]) in endothelial barrier disruption has not been studied. The aims of this study were to investigate the role of PAR-1 in orchestrating vascular permeability and to assess the potential therapeutics of a PAR-1 antagonist, SCH79797, through maintaining vascular integrity.

Methods—SCH79797 was injected intraperitoneally into male Sprague-Dawley rats undergoing SAH by endovascular perforation. Assessment was conducted at 24 hours after SAH for brain water content, Evans blue content, and neurobehavioral testing. To explore the role of PAR-1 activation and the specific mechanism of SCH79797’s effect after SAH, Western blot, immunoprecipitation, and immunofluorescence of hippocampus tissue were performed. A p21-activated kinase-1 (PAK1) inhibitor, IPA-3, was used to explore the underlying protective mechanism of SCH79797.

Results—At 24 hours after SAH, animals treated with SCH79797 demonstrated a reduction in brain water content, Evans blue content, and neurobehavioral deficits. SCH79797 also attenuated PAR-1 expression and maintained the level of vascular endothelial-cadherin, an important component of adherens junctions. Downstream to PAR-1, c-Src–dependent activation of p21-activated kinase-1 led to an increased serine/threonine phosphorylation of vascular endothelial-cadherin; immunoprecipitation results revealed an enhanced binding of phosphorylated vascular endothelial-cadherin with endocytosis orchestrator β-arrestin-2. These pathological states were suppressed after SCH79797 treatment.

Conclusions—PAR-1 activation after SAH increases microvascular permeability, at least, partly through a PAR-1-c-Src-p21-activated kinase-1–vascular endothelial-cadherin phosphorylation pathway. Through suppressing PAR-1 activity, SCH79797 plays a protective role in maintaining microvascular integrity after SAH. (Stroke. 2013;44:1410-1417.)

Key Words: microvascular permeability • protease activated receptor-1 • rat • subarachnoid hemorrhage • VE-cadherin

Brain edema is one of the fatal pathologies after subarachnoid hemorrhage (SAH).1 Previous research suggested that increased microvascular permeability might be a primary contributor to brain edema.2 Normally, the vascular endothelial barrier is maintained by 2 key junctions between endothelial cells, tight junctions (TJs), and adherens junctions (AJs).3 In the past 2 decades, much attention has been paid to the involvement of TJs in the increased vascular permeability after SAH.4 However, the integrity of AJs is also critical in maintaining microvascular permeability, and disruption of AJs can lead to interstitial edema.5 AJs comprise transmembrane protein vascular endothelial (VE)-cadherin and α-, β-, γ-, and δ-catenins in the cytoplasm.6 Inhibition of VE-cadherin by homophilic binding resulted in increased microvascular permeability.6

Protease-activated receptor-1, -3, and -4 (PAR-1, 3, and 4, but not PAR-2) are thrombin receptors, which are a subfamily of G-protein–coupled receptors.7 PAR-1 is a major receptor on the endothelial cells.8 PAR-1 activation increases the activity of downstream protein kinases, such as c-Src and p21-activated kinase-1 (PAK1), which leads to phosphorylation of key target proteins.9 Before PAR-1 orchestrates its actions, it must be activated by thrombin, which increased in plasma after SAH.10–12 Although PAR-1 activation could lead to cerebral vasospasm,13 its role in increasing microvascular permeability after SAH is not clear yet.
In this study, we demonstrated that PAR-1 activation mediated an increase in microvascular permeability after SAH. In addition, the protective effect of SCH79797, a specific PAR-1 antagonist, was also evaluated.

**Materials and Methods**

All procedures were approved by the Loma Linda University Animal Care Committee.

**SAH Model and Study Protocol**

The endovascular perforation model of SAH was established in male Sprague-Dawley rats (300–320g; Harlan, Indianapolis, IN) as previously described. With 2% to 3% isoflurane anesthesia, a sharpened 4-0 monofilament nylon suture was inserted rostrally into the right internal carotid artery from the external carotid artery stump and perforated the bifurcation of the anterior and middle cerebral arteries. Blood pressure and blood gas were recorded via the right femoral artery. Rectal temperature was maintained at 37°C during surgery. Sham-operated rats underwent the same procedures except the suture was withdrawn without puncture.

First, 34 rats were randomly divided into 4 groups. Finally, 27 rats (described as 27/34, same below) were used after excluding the dead and unqualified animals according to the inclusion criteria (see below). The roles of a PAR-1–specific agonist, SFLRN (Tocris, Ellisville, MO; 1 mg/kg as reported by the others), and a thrombin-specific inhibitor, argatroban (Sigma-Aldrich, St. Louis, MO), were evaluated (Figure 1A). Next, 53 rats were randomly divided into 6 groups (42 rats were used, 42/53). The dose-dependent effects of PAR-1–specific antagonist, SCH79797 (Tocris, Ellisville, MO), were evaluated (Figure 1B). Subsequently, 91 rats were randomly divided into 4 groups (28 rats were used, 28/35) to explore the mechanism of SCH79797’s effects (Figure 35). Rats were then euthanized by intracardiac perfusion with 0.01 mol/L phosphate-buffered saline, and brains were divided into the same regions as the water content study. The amount of extravasated Evans blue dye was measured by spectrophotofluorometry.

**Western Blot Analyses**

Protein samples (30 µg) from the hippocampus were loaded on a Tris glycine gel, electrophoresed, and transferred to a nitrocellulose membrane. Membranes were blocked with a blocking solution, followed by incubation overnight at 4°C with the following primary antibodies: (1:1000; all antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA until specifically indicated): mouse anti-PAR-1, goat anti–VE-cadherin, goat anti–phosphorylated-c-Src and c-Src, rabbit anti–phosphorylated-PAK1 and PAK1, rabbit anti–β-actin, and mouse anti–phosphorylated-VE-cadherin/thrombine. Immunoblots were processed with appropriate secondary antibodies (1:2000) for 1 hour at 21°C. The bands were detected with a chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL) and quantified by densitometry with Image J software (National Institutes of Health, Bethesda, MD). β-Actin was blotted on the same membrane as a loading control.

**Immunoprecipitation**

The samples were mixed and rotated for 2 hours at 4°C with corresponding primary antibody and incubated overnight with protein G-agarose. The immunoprecipitated proteins were collected by centrifugation, the pellets were washed and boiled in loading buffer and analyzed as described above for immunoblotting.

**Immunofluorescence Staining**

The coronal sections (10 µm thickness) containing the bilateral hippocampus were cut on a cryostat (Leica Microsystems, Bannockburn, IL) and mounted on poly–lysine–coated slides. Sections were incubated overnight at 4°C with goat anti–VE-cadherin, rabbit anti–fibronectin primary antibodies, and DAPI. Appropriate fluorescence dye–conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) were applied in the dark for 1 hour at 21°C. For negative controls, the primary antibodies were omitted and the same staining procedures were performed. The sections were visualized with a fluorescence microscope, and the photomicrographs were saved and merged with Image Pro Plus software (Olympus, Melville, NY).

**Statistical Analyses**

The data of neurological scores and tail bleeding time were expressed as median±SD, and 1-way ANOVA with the Tukey–Kramer post hoc tests was used. A P value of < 0.05 was considered significant.

**Results**

**PAR-1 Agonist Neutralized the Protective Effects of Argatroban**

After SAH, PAR-1 expression in the hippocampus was significantly increased and peaked at 2 to 6 hours and 24
hours (Figure 2A). Argatroban, a thrombin-specific inhibitor, significantly decreased brain water content (left hemisphere: SAH+argatroban 79.23±0.25% versus SAH+normal saline 79.63±0.24%, \( P < 0.05 \); right hemisphere: SAH+argatroban 79.21±0.09% versus SAH+normal saline 79.79%±0.14%, \( P < 0.01 \)) and reduced neurological deficits (\( P < 0.05 \)) at 24 hours after SAH. These protective effects were neutralized by simultaneously applying a PAR-1 agonist, SFLLRN (brain water content: left hemisphere, SAH+argatroban+SFLLRN 79.93±0.69% versus SAH+argatroban 79.23±0.25%, \( P < 0.05 \); right hemisphere: SAH+argatroban+SFLLRN 80.29±0.75% versus SAH+argatroban 79.21±0.09%, \( P < 0.01 \); Figure 2B–2D). These results indicated that, as a major thrombin receptor on endothelial cells, PAR-1 activation could lead to increased microvascular permeability and neurological impairments after SAH.

**Dose-Dependent Effect of SCH79797**

At 24 hours post injury, the dose of 25 μg/kg SCH79797 (PAR-1 antagonist) was most effective at decreasing brain water content (left hemisphere: SAH+25 μg/kg SCH 79.34±0.15% versus SAH+dimethyl sulfoxide [DMSO] 79.65±0.23%, \( P < 0.05 \); right hemisphere: SAH+25 μg/kg SCH 79.25±0.19% versus SAH+DMSO 79.62±0.36%, \( P < 0.05 \); Figure 3A) and neurological deficits (\( P < 0.05 \); Figure 3B and 3C). In addition, SAH grades were not significantly different among the groups (Figure I in the online-only Data Supplement).

**SCH79797 Provided Significant Neuroprotection After SAH**

At 24 hours after SAH, in addition to reducing brain edema and neurological impairments (Figure 3), SCH79797 also decreased Evans blue extravasations in the brain (μg/g; left hemisphere: SAH+25 μg/kg SCH 1.21±0.24 versus SAH+DMSO 2.08±0.68, \( P < 0.05 \); right hemisphere: SAH+25 μg/kg SCH 1.87±0.08 versus SAH+DMSO 3.25±0.96, \( P < 0.05 \); Figure 4A). Mortality occurred within 6 hours after surgery, and the mortality of the SAH+DMSO group (26.47%, 9 of 34 rats) was not significantly different from the SAH+25 μg/kg SCH79797 group (20.00%, 7 of 35 rats; \( P = 0.52 \), \( \chi^2 \) tests). In addition, the tail bleeding time was not significantly altered after SCH79797 treatment (\( P > 0.05 \); Figure 4B).

SCH79797 significantly decreased PAR-1 expression (Figure 4C; Figure II in the online-only Data Supplement) and maintained the level of VE-cadherin by reducing the cleavage of VE-cadherin (≈80 kDa fragment; \( P < 0.05 \); Figure 4D).
At 24 hours after SAH, immunofluorescence staining of microvessels in the hippocampus was performed. In the sham group, VE-cadherin was abundantly distributed in the borders of endothelial cells (Figure 5A1-a). Fibronectin, a well-established indicator for evaluating microvascular integrity, is a high-molecular weight glycoprotein normally distributed in the vessel walls and blood. In the sham group, fibronectin was exclusively distributed in the vessel walls.

Figure 2. The effects of the protease-activated receptor-1 (PAR-1) agonist, SFLLRN. There were 2 peak levels of PAR-1 expression at 2 to 6 hours and 24 hours after subarachnoid hemorrhage (SAH; A, n=6 each group). SFLLRN neutralized the protective effects of the thrombin inhibitor, argatroban, on attenuating brain edema (B) and neurological deficits (C and D) at 24 hours after SAH. B to D, n=5, 7, 7, 8 in sham, SAH+normal saline (NS), SAH+argatroban (ARG), and SAH+ ARG+SFLLRN groups. *P<0.05, **P<0.01 compared with the sham group; #P<0.05, ##P<0.01 compared with the SAH+NS group; §P<0.05, §§P<0.01 compared with the SAH+ARG group. The Western blot results were representative of 3 independent experiments. BS indicates brain stem; C, cerebellum; LH, left hemisphere; and RH, right hemisphere.

Figure 3. The dose-dependent effects of SCH79797. Compared with other doses, the dose of 25 μg/kg SCH79797 was more effective on decreasing brain water content (A) and neurological deficits (B and C) at 24 hours after SAH. n=5, 6, 7, 8, 7 in sham, subarachnoid hemorrhage (SAH)+dimethyl sulfoxide (DMSO), SAH+10, 25, 100, 250 μg/kg SCH79797 groups. *P<0.05 compared with the sham group; #P<0.05 compared with the SAH+DMSO group. BS indicates brain stem; C, cerebellum; LH, left hemisphere; RH, right hemisphere.
walls (Figure 5A2). After SAH, the level of VE-cadherin was significantly decreased with numerous fibronectin extravasations scattered around the microvessels (Figure 5B1-b and B2). After SCH79797 treatment, these pathological findings were markedly attenuated (Figure 5C1-c and C2).

**SCH79797 Maintained VE-cadherin Level Through Blocking PAR-1-c-Src-PAK1 Pathway**

At 24 hours after SAH, the levels of phosphorylated c-Src and PAK1 were significantly increased, which were reversed by SCH79797 treatment ($P < 0.05$; Figure 6A and 6B). A PAK1 inhibitor, IPA-3, decreased the level of phosphorylated PAK1 (Figure 6B) but not phosphorylated c-Src (Figure 6A); however, a c-Src inhibitor, PP2 (1 mg/kg, IP), significantly decreased the level of phosphorylated-PAK1 ($P < 0.05$, Figure III in the online-only Data Supplement), which indicated c-Src is an upstream regulator of PAK1 in the PAR-1 signaling pathway.

The immunoprecipitation results revealed that the phosphorylation of VE-cadherin on its serine/threonine residues facilitates its binding with endocytosis orchestrator β-arrestin-2, a cytoplasmic adaptor protein that orchestrates the endocytosis of membrane proteins. This binding was attenuated by SCH79797 and IPA-3 ($P < 0.05$; Figure 6C and 6D). These results indicated that activated PAK1 modulates phosphorylation and consequent endocytosis (degradation) of VE-cadherin after SAH, and SCH79797 decreases VE-cadherin endocytosis by suppressing c-Src–dependent PAK1 activation in the PAR-1 signaling pathway.

**Discussion**

In this study, we investigated the role of PAR-1 in orchestrating increased microvascular permeability after SAH. We also found the PAR-1 antagonist, SCH79797, preserved microvascular integrity and provided neurobehavioral protection, which was partly mediated via suppression of VE-cadherin endocytosis induced by c-Src–dependent PAK1 activation.

Thrombin plays roles in both coagulation processes and receptor-dependent inflammation, some of which are mediated via PAR activation. Thrombin leads to brain edema after forebrain ischemia and intracerebral hemorrhage. The increased plasma thrombin after SAH is partly attributed to the disruption of arteries, endogenous thrombin production, and transfer from CSF to veins. As a major thrombin receptor on endothelial cells, the role of PAR-1 in inflammation after SAH was not clear. In this study, we found the expression of
PAR-1 was significantly increased after SAH (Figure 2A), which indicated that increased thrombin in plasma and PAR-1 on endothelial cells leads to some pronounced pathologies in the vessels, such as vasospasm as reported previously.13 In addition, we explored the role of PAR-1 in the formation of brain edema. We found the protective role of the thrombin inhibitor, argatroban, was reversed by the PAR-1 agonist, SFLLRN (Figure 2B–2D). In addition, the PAR-1 antagonist, SCH79797, significantly reduced brain water content (Figure 3A). These results indicated PAR-1 activation did play a vital role in increasing microvessel permeability, regardless of the roles of other thrombin receptors (eg, PAR-4).

After determining the role of PAR-1 in increasing vessel permeability, we examined the effects of PAR-1 inhibition after SAH. In fact, the protective role of PAR-1 antagonists in other cardiovascular disease models have been reported.24 We found 25 μg/kg SCH79797 (a potent, specifically selective PAR-1 receptor antagonist)25 was the most effective in attenuating brain edema, Evans blue extravasations, and neurobehavioral deficits. In addition, PAR-1 expression was decreased after SCH79797 treatment, although the mechanism was not clear. The unactivated PAR-1 may be delivered to the cell surface and cycles constitutively between the plasma membrane and an early endosomal recycling compartment,26 forming a cytoplasmic pool. This PAR-1 internalization is dependent on clathrin and dynamin.27 SCH79797 inhibited PAR-1 activation after SAH, which may indirectly promote the internalization and degradation of PAR-1 in lysosomes. Therefore, the level of PAR-1 was reduced after SCH79797 treatment. Furthermore, tail bleeding time, a well-established parameter for evaluating coagulation function,28 was not significantly prolonged after SCH79797 treatment, which was similar to the results reported by the others.29 These results suggested SCH79797 plays a protective role in maintaining microvascular integrity, clinically, without interfering with coagulation function and increasing the risk of further hemorrhage in SAH patients.

The permeability between endothelial cells is mainly determined by TJs and AJs.3 In fact, TJs and AJs are sometimes intermingled.30 AJs form earlier than TJs during embryogenesis and are also established before TJs.31 The integrity of AJs is critical in regulating microvascular permeability, and the disruption of AJs leads to interstitial edema.5 AJs in microvessels are mainly composed of VE-cadherin, β-catenin, α-catenin, and p120.7 The extracellular domain of VE-cadherin mediates homophilic interactions,32 and the inhibition of VE-cadherin homophilic binding results in the

Figure 5. Immunofluorescence staining of microvessels in the hippocampus. In the sham group, vascular endothelial (VE)-cadherin was abundantly distributed in the borders of endothelial cells (A1-a), whereas fibronectin was distributed exclusively in the vessel walls (A2). At 24 hours after SAH, the level of VE-cadherin was significantly decreased (B1-b), and numerous fibronectin positive extravasations were scattered around the microvessels (B2). These pathologies were attenuated by SCH79797 treatment (C1-c and C2). DAPI staining showed the nuclei of endothelial cells. Star indicates microvessel lumen; arrowhead, endothelial cell; and arrow, VE-cadherin (a and c). a to c, Magnification of the dashed area in the (A1 to C1), respectively. A to C, scale bar, 20 μm; a to c, scale bar, 5 μm (n=6 each group).

DMSO indicates dimethyl sulfoxide; and SAH, subarachnoid hemorrhage.
increased microvascular permeability. In this study, at 24 hours after SAH, with increased expression of PAR-1, the level of VE-cadherin was significantly decreased because of cleavage (Figure 4D). In addition, VE-cadherin was absent from the borders of endothelial cells, while microvascular permeability was enhanced (Figure 5). These pathological changes were alleviated by SCH79797 treatment. These results demonstrated SCH79797 preserved microvascular integrity by maintaining the level of VE-cadherin via attenuating its cleavage induced by PAR-1 activation.

Finally, we explored the mechanism of VE-cadherin cleavage and the neuroprotective roles of SCH79797. It had been reported that the phosphorylation of VE-cadherin, induced by c-Src and PAK1, resulted in VE-cadherin undergoing endocytosis and degradation by binding with β-arrestin-2 in vitro. In this study, both c-Src and PAK1 were activated after SAH (Figure 6A and 6B). Meanwhile, the serine/threonine phosphorylation and endocytosis of VE-cadherin, orchestrated by β-arrestin-2, were also markedly increased (Figure 6C and 6D), which was decreased by the PAK1 inhibitor, IPA-3. SCH79797 alleviated the phosphorylation and endocytosis of VE-cadherin by decreasing the levels of phosphorylated c-Src and PAK1. These results indicated that PAR-1 induced phosphorylation and endocytosis of VE-cadherin via c-Src–dependent PAK1 activation, which partly mediated VE-cadherin loss after SAH. Through blocking the PAR-1-c-Src-PAK1 pathway, SCH79797 maintained VE-cadherin levels by suppressing the endocytosis of VE-cadherin after SAH.

In conclusion, we showed that the increased endocytosis of VE-cadherin, induced by the PAR-1-c-Src-PAK1 pathway, might be partly responsible for increased microvascular permeability after SAH. The PAR-1 antagonist, SCH79797, played a neuroprotective role by maintaining microvascular integrity via blocking the PAR-1-c-Src-PAK1 signaling pathway.

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Disclosures
None.

References


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Supplemental Figure 1 (S1)

The average SAH grading scores among various doses of SCH79797 groups. The average SAH grading scores were similar among the operated-groups ($P>0.05$, ANOVA, sham group=0). n=5,6,7,9,8,7 in sham, SAH+DMSO, SAH+10,25,100,250μg/kg SCH79797 groups, respectively.

Supplemental Figure 2 (S2)

Immunofluorescence staining of microvessels for PAR-1 in the hippocampus. At 24 hours following SAH, PAR-1 expression in endothelial cells (membrane and cytoplasm) and vessel wall of the hippocampus were significantly increased (Figure B), which could be attenuated by the 25μg/kg SCH79797 treatment.
(Figure C). The “star” meant microvessel lumen; the “arrowhead” indicated endothelial cell. In figures A, B and C, scale bar=20μm, n=6 each group.

**Supplemental Figure 3(S3)**

**Supplemental Figure 3** c-Src inhibitor PP2 decreased the level of phosphorylated-PAK1 at 24 hours following SAH. The level of phosphorylated-PAK1 in hippocampus was significantly increased at 24 hours after SAH, which could be attenuated by PP2 treatment (1mg/kg, IP) \( (P<0.05, \text{ANOVA, } n=6 \text{ in each group).} \) ※\( P<0.05 \text{ compared with sham group; } \) #\( P<0.05 \text{ compared with SAH+DMSO group.} \)