Recombinant Osteopontin Stabilizes Smooth Muscle Cell Phenotype via Integrin Receptor/Integrin-Linked Kinase/Rac-1 Pathway After Subarachnoid Hemorrhage in Rats

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Background and Purpose—Recombinant osteopontin (rOPN) has been reported to be neuroprotective in stroke animal models. The purpose of this study is to investigate a potential role and mechanism of nasal administration of rOPN on preserving the vascular smooth muscle phenotype in early brain injury after subarachnoid hemorrhage (SAH).

Methods—One hundred and ninety-two male adult Sprague-Dawley rats were used. The SAH model was induced by endovascular perforation. Integrin-linked kinase small interfering RNA was intracerebroventricularly injected 48 hours before SAH. The integrin receptor antagonist fibronectin-derived peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), focal adhesion kinase inhibitor Fib-14, and Rac-1 inhibitor NSC23766 were administered 1 hour before SAH induction. rOPN was administered via the intracerebroventricular and nasal route after SAH. SAH grade, neurological scores, brain water content, brain swelling, hematoxylin and eosin staining, India ink angiography, Western blots, and immunofluorescence were used to study the mechanisms of rOPN on the vascular smooth muscle phenotypic transformation.

Results—The marker proteins of vascular smooth muscle phenotypic transformation α-smooth muscle actin decreased and embryonic smooth muscle myosin heavy chain (SMem) increased significantly at 24 and 72 hours in the cerebral arteries after SAH. rOPN prevented the changes of α-smooth muscle actin and SMem and significantly alleviated neurobehavioral dysfunction, increased the cross-sectional area and the lumen diameter of the cerebral arteries, reduced the brain water content and brain swelling, and improved the wall thickness of cerebral arteries. These effects of rOPN were abolished by GRGDSP, integrin-linked kinase small interfering RNA, and NSC23766. Intranasal application of rOPN at 3 hours after SAH also reduced neurological deficits.

Conclusions—rOPN prevented the vascular smooth muscle phenotypic transformation and improved the neurological outcome, which was possibly mediated by the integrin receptor/integrin-linked kinase/Rac-1 pathway. (Stroke. 2016;47:1319-1327. DOI: 10.1161/STROKEAHA.115.011552.)

Key Words: blotting, Western ■ cerebral arteries ■ muscle, smooth, vascular ■ rats, Sprague-Dawley ■ subarachnoid hemorrhage

It is likely that neuroprotection will not occur without the proper control of blood circulation in the brain after subarachnoid hemorrhage (SAH). The cerebral vascular neural network, which includes vascular smooth muscle cells (VSMCs), is involved in the pathophysiology of SAH. A recent study indicated that the phenotypic transformation of the vascular smooth muscle may play an important role in vascular dysfunction associated with strokes.  
The vascular smooth muscle of cerebral arteries typically switch from contractile to synthetic type and functionally from contraction to repair and migration after injury or hemorrhagic stroke. The vascular smooth muscle can be stained and visualized with α-smooth muscle actin (α-SMA), and an accurate marker for synthetic VSMCs is embryonic smooth muscle myosin heavy chain (SMem)/nonmuscle myosin heavy chain isoform. Osteopontin is a multifunctional extracellular matrix glycoprotein, and by activating its cell surface integrin receptors, it has been implicated in the regulation of smooth muscle cell phenotype. The integrin-linked kinase (ILK)
Materials and Methods

Animals

One hundred and ninety-two male adult Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were used in this study. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Experimental Design

The experiment was designed as follows (Figure II in the online-only Data Supplement).

Experiment I

To determine the time course of the vascular smooth muscle phenotypic transformation after SAH, 36 rats were randomly assigned into 5 groups: sham (n=6), 6 hours after SAH (n=6), 12 hours after SAH (n=6), 24 hours after SAH (n=6), and 72 hours after SAH (n=6). Western blots were used to detect the protein expression of α-SMA and SMemb in the cerebral vessels of each group. Double immunohistochemistry staining of SMemb and von Willebrand factor were also performed in sham (n=2) and 24 hours (n=2) and 72 hours (n=2) after SAH.

Experiment II

In the outcome evaluation, 30 rats were randomly divided into 4 groups: sham (n=6), SAH (n=6), SAH+vehicle (5 μL of sterile phosphate-buffered saline [PBS]) (n=6), and SAH+0.02 ng/μL rOPN (0.1 ng in 5 μL of sterile PBS; n=6). Vehicle or rOPN was injected intracerebroventricularly 3 hours after SAH onset. Neurological scores, hematoxylin, and eosin staining were assessed at 24 hours after SAH. Double immunohistochemistry staining of SMemb and von Willebrand factor were also performed in sham (n=2), SAH (n=2), and SAH+0.02 ng/μL rOPN (n=2).

Experiment III

Forty-eight rats were randomly assigned into 8 groups for the mechanism study: sham (n=6), SAH+vehicle (5 μL of sterile PBS; n=6), SAH+0.02 ng/μL rOPN (0.1 ng in 5 μL of sterile PBS; n=6), SAH+0.02 ng/μL rOPN (0.1 ng in 5 μL of sterile PBS)+Fib-14 (0.2 mg in 5 μL of PBS; Sigma-Aldrich, St. Louis, MO), SAH+0.02 ng/μL rOPN (0.1 ng in 5 μL of sterile PBS)+Fib-14 (Ellisville, MI; 0.2 mg in 5 μL of PBS; Tocris Bioscience), or SAH+0.02 ng/μL rOPN (50 μg/5 μL; Santa Cruz Biotechnology) was administered 3 hours after SAH induction by a pump at a rate of 0.5 μL/min, respectively. GRGDSP (100 pmol in 1 μL; Sigma-Aldrich, St. Louis, MO), Fib-14 (Ellisville, MI; 0.2 mg in 5 μL of PBS; Tocris Bioscience), or NSC23766 (30 μg/5 μL; Santa Cruz Biotechnology) was administered 1 hour before SAH induction. ILK siRNA (500 pmol/5 μL) or scrambled siRNA (Life Technologies) was infused at the same rate 48 hours before SAH modeling.

Severity of SAH

The severity of SAH was blindly assessed at each euthanization as previously described. Animals that received a score of <7 were excluded from the study.

Neurological Outcome Assessment

Neurological impairments were blindly evaluated using an 18-point score system known as the modified Garcia scale and another 4-point score system known as the beam balance test.

Brain Volume and Cerebral Blood Volume

Brain volume was calculated as previously described. Cerebral blood volume was calculated as previously described. Parallel samples of the brain and arterial blood were harvested for hemoglobin assay according to an established protocol. Cerebral blood volume was calculated based on measured brain hemoglobin and was compared with the measured volume of the entire brain.

Brain Water Content

The brains were removed 24 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight) and once more after drying for 72 hours at 105°C. The percentage of water content was calculated as follows: (wet weight−dry weight)/wet weight.

India Ink Angiography

The lumen diameter was determined by India ink angiography 24 hours after SAH as previously described. The smallest lumen diameter within each vascular segment of intracranial cerebral arteries
morphometric analysis

The brain sections encompassing the BA were stained with hematoxylin and eosin according to the routine protocol. Histological photographs were serially captured with a microscope camera. At the predetermined anatomic locations, the cross-sectional areas and the thickness of BA were measured using ImageJ software (National Institutes of Health).

Western Blot Analysis

Western blot tests were performed as reported previously. The circle of Willis blood vessels and BAs were harvested under a microscope and homogenized. Primary antibodies used were anti-SMemb (Abcam), the anti-α-SMA, the anti-ILK, the anti-phospho-FAK, the anti–total FAK, the anti–β-actin, and the antitubulin (Santa Cruz Biotechnology).

Immunofluorescence Staining

Immunofluorescence staining was performed on the fixed frozen brain sections as previously described. Sections were incubated overnight at 4°C with the anti-SMemb (Abcam) or the anti-α-SMA (Santa Cruz Biotechnology) with the anti–von Willebrand factor (Millipore, Temecula, CA), followed by appropriate fluorescence dye–conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) for 2 hours at room temperature. The sections were visualized with a fluorescence microscope (Olympus OX51, Tokyo, Japan).

Rac-1–GTP–Binding Assay

Rac-1 activation assay was performed using PAK1-PBD color agarose beads according to the manufacturer’s protocol (Cell Biolabs). Proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to Western blot analysis using anti–Rac–1–specific antibody.

Statistical Analysis

Neurological scores were expressed as medians 25th to 75th percentiles and were analyzed using Mann–Whitney U tests or Kruskal–Wallis tests, followed by Steel–Dwass multiple comparisons. Other values were expressed as mean±SD were analyzed by 1-way ANOVA, followed by a Tukey multiple comparison test. P<0.05 was considered a statistical difference.

Results

Mortality and SAH Severity Scores

The total mortality of SAH in this study was 14.06% (27 of 192 rats). The mortality was not significantly different among

Figure 1. Expression profile of embryonic smooth muscle myosin heavy chain (SMemb) and α-smooth muscle actin (α-SMA) in cerebral vessels after subarachnoid hemorrhage (SAH). A, Expression time curve of SMemb and α-SMA in the cerebral vessels within 72 hours after SAH. B and C, Quantitative analyses of SMemb and α-SMA time course after SAH. D, Immunofluorescence staining for SMemb (green), vascular endothelial cell (von Willebrand factor) (red), and DAPI (4',6-diamidino-2-phenylindole; blue) in the walls of middle cerebral artery (MCA) and basilar artery (BA). Scale bars, 50 μm (top) and 25 μm (bottom). Relative densities have been normalized against the sham group; n=6 rat per group per time point. *P<0.05 vs sham.
the groups, respectively (data not shown). In addition, there was no significant difference in average SAH grading score among the groups in each experiment (Figure III in the online-only Data Supplement).

Expression Profile of the Marker Proteins of Vascular Smooth Muscle Phenotype in Cerebral Vessels After SAH

We investigated whether the marker proteins of vascular smooth muscle phenotype would respond to early brain injury after SAH. Western blot analysis was performed to determine the protein expression of α-SMA and SMemb at 6 hours, 12 hours, 24 hours, and 72 hours after SAH (Figure 1A). Results showed that the α-SMA level decreased as early as 6 hours after SAH and had its significant depression at 24 hours. Its level continued to decrease at 72 hours, whereas it had no significant difference at 24 hours (Figure 1C). In addition, the expression of SMemb was significantly increased in the cerebral vessels at 24 hours (Figure 1B) compared with sham animals.

To further identify the result, immunofluorescence analysis was performed for SMemb expression of the middle cerebral artery and the BA. The double immunofluorescence staining revealed that the SMemb immunoreactivity increased on the wall of cerebral vessels at 24 and 72 hours after SAH (Figure 1D).

Exogenous rOPN Treatment Improved the Neurological Deficits and Phenotypic Transformation of VSMCs 24 Hours After SAH

A further experiment was conducted to test whether rOPN attenuates the vascular smooth muscle phenotypic transformation after SAH. As shown in Figure 2B, SAH animals demonstrated severe behavior deficits compared with sham animals. The rOPN group had a significant improvement in neurological functions compared with the SAH group.

Consistent with neurological improvement, we performed immunofluorescence analysis (Figure 2A) and hematoxylin and eosin staining (Figure 2C) for the BA, the SMemb immunoreactivity increased at 24 hours after SAH, and the rOPN can reduce the immunoreactivity of the SMemb. In addition, the cross-sectional area of the BA in the rOPN group was also significantly increased, and the wall thickness was significantly reduced compared with SAH (Figure 2D).

GRGDSP Inhibitors Abolished the rOPN-Induced Effect of the Vascular Smooth Muscle Phenotype

Then, we determined which receptor was involved in the beneficial effects observed by rOPN. The integrin receptor antagonist GRGDSP (100 pmol in 1 μL) was administered. GRGDSP abolished the rOPN-induced improvement of neurological scores (Figure 3A). To further evaluate the vascular smooth muscle phenotypic transformation, Western blot
for ILK and p-FAK was conducted. The results revealed that rOPN treatment stabilized vascular smooth muscle phenotype by significantly improving the expression of ILK and p-FAK (Figure 3B–3D) and GRGDSP reversed the expression of rOPN on ILK and p-FAK.

Inhibitor of FAK Failed to Attenuate the Effects of rOPN on Vascular Smooth Muscle Phenotypic Transformation 24 Hours After SAH

We determined whether the integrin signaling mediators, FAK and ILK, were able to transduce the integrin-dependent regulation mechanisms. The inhibition of FAK (Fib-14; 0.2 mg dissolved in 5 μL of PBS) administered 1 hour before SAH induction neither significantly improved the neurological defect (Figure IVA in the online-only Data Supplement) nor modified the marker proteins of phenotype (Figure IVB–IVD in the online-only Data Supplement).

ILK In Vivo Knockdown and the Inhibitor of Rac-1 Abolished the Effects on Vascular Smooth Muscle Phenotypic Transformation by rOPN 24 Hours After SAH

A set of siRNAs directed against ILK were mixed and administered, and the knockdown efficiency was validated by Western blot analysis. ILK levels were significantly reduced compared with the negative control siRNA groups (Figure V in the online-only Data Supplement). rOPN in vivo knockdown sufficiently abolished the protective effect of rOPN treatment as shown in modified Garcia test (Figure 4A).

To further identify downstream pathways of ILK, the levels of active Rac-1 were determined. rOPN protects the vascular smooth muscle phenotype by upregulating the levels of active Rac-1, and ILK siRNA pretreatment reserved the upregulated level of active Rac-1 compared with the rOPN group, whereas scrambled siRNA did not show those effects (Figure 4B).

To strengthen the role of Rac-1 in the pathway, the inhibitor of Rac-1 (NSC23766; 30 μg dissolved in 5 μL of PBS) was administered. NSC23766 and ILK in vivo siRNA administration both decreased the levels of α-SMA and increased SMemb compared with the rOPN treatment group, whereas scrambled siRNA did not show those effects (Figure 4C and 4D).

Intranasal Delivery of rOPN Ameliorated Neurological Deficits and Improved the Brain Swelling, Brain Edema, and Lumen Diameter 24 Hours After SAH

To investigate the clinical translational treatment with rOPN, the nasal administration of rOPN (rOPN dissolved in PBS was administered alternately into the left and right nares. rOPN (5 μg) in a total volume of 50 μL was administered intranasally to each animal.) was performed at 3 different time points (1 hour, 3 hours, and 6 hours) after SAH induction. There was a significant improvement in the neurological score at 1 hour and 3 hours in the treatment group (Figure 5A and 5B).

Brain swelling is represented by wet brain weight, brain volume (size), and cerebral blood volume (hemoglobin content). Both brain wet weight and brain volume were significantly increased 24 hours after SAH. The SAH group showed increased brain water content 24 hours after SAH in both hemispheres. rOPN treatment at 1 hour and 3 hours significantly reduced the brain water content in bilateral cerebral hemispheres, whereas treatment at 6 hour did not have the effect (Figure 5C). rOPN treatment at 1 hour and 3 hours both attenuated increases in wet brain weight and brain volume (Figure 5D) compared with the SAH group. A marked increase in cerebral blood volume was noted after SAH, and
cerebral blood volume in rOPN treatment at 1 hour and 3 hours were both significantly lower (Figure 5D).

India ink angiography was performed to measure the lumen diameter within each vascular segment of intracranial cerebral arteries. The 3-hour treatment group of rOPN significantly increased the lumen diameter in the left internal carotid artery, left middle cerebral artery, left anterior cerebral artery, and BA 24 hours post SAH compared with the SAH group (Figure 6A and 6B).

Discussion
This study demonstrates that as the marker proteins of vascular smooth muscle phenotype, the α-SMA decreased and SMemb increased significantly at 24 hours after SAH. rOPN alleviated neurological impairment and the vascular smooth muscle phenotypic transformation associated with an increase of ILK and the activation of Rac-1. An RGD-dependent integrin receptor antagonist GRGDSP reduced rOPN-induced ILK upregulation and Rac-1 activation. Knockdown of ILK by siRNA and selective Rac-1 inhibition using NSC23766 abolished the rOPN-induced preservation of the vascular smooth muscle phenotypic transformation. The nasal administration of rOPN 3 hours after SAH was also effective for preventing vascular smooth muscle phenotype. Taken together, these findings suggested that the phenotypic transformation of vascular smooth muscle was involved in the early brain injury in experimental SAH, and rOPN prevented the phenotypic transformation of vascular smooth muscle after SAH in rats via the integrin receptor/ILK/Rac-1 pathway.

The vascular neural network, which is an extended classical neurovascular unit, represents a new physiological unit to consider for therapeutic development in stroke. The vascular smooth muscle, as an important part of vascular neural network, might be an alternative therapy for early brain injury after SAH. Previous studies suggested that vascular smooth muscle phenotypic transformation may be caused by local tissue pressure or stretch increases and by blood metabolic products after SAH. Contractile vascular smooth muscle contributed to vascular tone and regulation of blood vessel diameter and blood flow distribution. Vascular smooth muscle phenotypic transformation from contractile to synthetic phenotype may result in decreasing autoregulatory capacity and regional cerebral blood flow to enhance brain swelling and brain edema. One of the mechanisms of the blood–brain barrier breakdown after SAH is the proteolysis of tight junction proteins by matrix metalloproteinases. A previous study indicated that matrix metalloproteinase-9 was expressed in the vascular wall, and the costaining for VSMCs showed that the matrix metalloproteinase-9 expression localized to the VSMCs after SAH. The switch of vascular smooth muscle phenotype may not markedly affect the size or function of the large arteries in the previous study, but some studies reported that unbalanced contractile/synthetic vascular smooth muscle phenotype affected the size of the cerebral arteries and aggravated brain swelling and brain edema. It is presumed that
because of the limited 2 or 3 layers of smooth muscle cells in smaller arteries, vascular smooth muscle phenotype change causes the loss of small artery vascular tone and affects blood–brain barrier and autoregulation. The main observations of this study reports that vascular smooth muscle phenotypic transformation may be involved in the pathophysiology of early brain injury after SAH.

Osteopontin binds with RGD-dependent integrins and certain variant forms of CD44, which activate intracellular signaling pathways and mediate osteopontin’s variable biological functions. Endogenous osteopontin induction has protective effects after ischemic injuries in the various organs, including the kidneys, heart, lungs, and brain. Studies on osteopontin knockout mice further support the beneficial role of osteopontin in both adult and neonatal stroke models. Osteopontin knockout mice had greater thalamic neurodegeneration after ischemia. FAK, a cytoplasmic tyrosine kinase, and ILK, a serine/threonine protein kinase, are both key signaling components downstream of integrin engagement by its ligand. Binding of a ligand to the integrin receptors initiates its signaling, which leads to the phosphorylation of FAK and the activation of ILK, thereby modulating downstream signaling. In this study, we investigated the role of the 2 well-known mediators of integrin pathways, ILK and FAK. rOPN stabilized vascular smooth muscle phenotype via phosphorylation of FAK and the activation of ILK. Inhibiting the RGD-dependent integrin receptor with GRGDSP lead to a reduction of ILK and p-FAK. The inhibition of FAK did not affect the expression of α-SMA and SMemb, whereas knockdown ILK by siRNA exacerbated the outcomes and regulated the expression of α-SMA and SMemb. The present results indicated that rOPN regulates the α-SMA and SMemb, and therefore, the phenotype switches pathway through ILK but not FAK. ILK-binding protein interacted with Rac-specific guanine nucleotide exchange factor, α-Pix, activating Rac-1, which

Figure 5. Recombinant osteopontin (rOPN) by intranasal ameliorated neurological deficits and improved the brain swelling and brain edema 24 hours after subarachnoid hemorrhage (SAH). rOPN by intranasal ameliorated neurological deficits of modified Garcia test (A) and beam balance (B) in indicated groups 24 hours after SAH. rOPN treatment attenuated brain water content assessment (C) and brain swelling (D) of wet brain weight, blood volume, and brain volume at 24 hours after SAH; n=6 rats per group. *P<0.05 vs sham and #P<0.05 vs vehicle. BS indicates brain stem; C, cerebellum; LH, left hemisphere; and RH, right hemisphere.
has an important role in maintaining endothelial barrier integrity. The observations of this study are consistent with previous reports that the activation of ILK was linked with the levels of active Rac-1, and Rac-1 inhibitor NSC23766 attenuated the vascular protection of rOPN in this study. These observations suggested that Rac-1 activation is a downstream of ILK and is critical for rOPN to stabilize vascular smooth muscle phenotypes. In addition, Rac-1 could inhibit the vascular smooth muscle phenotypic transformation through upregulation of serum response factor–dependent gene transcription.

Intranasal administration provides a direct route to the brain by endocytosis and transport along olfactory nerves or by extracellular flow through intercellular clefts in the olfactory epithelium to diffuse into the subarachnoid space. It is a safe, efficient, noninvasive, and clinically translational route to deliver various neuroprotectants. Our previous studies have demonstrated that nasal administration of 5 μg rOPN for 30 minutes after SAH attenuated neuronal apoptosis and the reduction of brain water content in rats. In this study, we gave the rOPN at 3 different time points after SAH to evaluate the most appropriate time of administration. Our data showed that intranasal administration of rOPN 3 hours after SAH significantly stabilized vascular smooth muscle phenotype. This therapeutic window is feasible for clinical translation for SAH patients.

This study has limitations that it is focused on the vascular smooth muscle phenotypic transformation of large cerebral arteries but is not designed to study small arteries or arterioles. Apparently, more evidence from small arteries and arterioles is needed to verify the role of osteopontin in smooth muscle phenotype switch. In addition, the time of clearance of rOPN from the cerebrospinal fluid circulation and the long-term effects of osteopontin on neurological and neurobehavioral outcomes need to be investigated.

Conclusions
This study demonstrated for the first time that the phenotypic transformation of vascular smooth muscle was involved in the pathophysiology of early brain injury in experimental SAH. Extraneous rOPN protected the phenotypic transformation and improved neurological outcome, mediated possibly by the integrin receptor/ILK/Rac-1 pathway. Nasal administration of rOPN 3 hours after SAH improved functional outcomes by stabilizing the phenotype of vascular smooth muscle. Our results have potentials to lead to a new target for SAH treatment and clinical translation.

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Disclosures
None.

References


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Supplemental Material

Supplemental Figure I. Proposed pathway in the present study.

Supplemental Figure II. Experimental design and animal groups’ classification.
Supplemental Figure III. SAH grading of each group after SAH.

Supplemental Figure IV. Modified Garcia test in indicated gro24 hours following SAH (A). Expression change of alpha-SMA, SMemb in the cerebral vessels 24 hours following SAH (B). Quantitative analysis of SMemb (C) and...
alpha-SMA (D). Relative densities have been normalized against the sham group. n=6 rats per group, *P<0.05 vs. Sham; #P<0.05 vs vehicle. &P<0.05 vs rOPN.

**Supplemental Figure V.** Inhibition effect of ILK siRNA. Expression change of ILK in the cerebral vessels 24 hours following SAH (A). Quantitative analysis of ILK (B). Relative densities have been normalized against the sham group. n=6 rats per group, *P<0.05 vs SiILK.