Preservation of Tropomyosin-Related Kinase B (TrkB) Signaling by Sodium Orthovanadate Attenuates Early Brain Injury After Subarachnoid Hemorrhage in Rats

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Background and Purpose—Recent studies reported that apoptosis was involved in the pathogenesis of early brain injury after subarachnoid hemorrhage (SAH). The aim of this study was to examine whether sodium orthovanadate (SOV) prevents post-SAH apoptosis by modulating growth factors and its downstream receptor tyrosine kinases.

Method—Rats were operated on with the endovascular perforation model. SAH animals were treated with vehicle, 3 mg/kg and 10 mg/kg SOV, and evaluated regarding neurofunction and brain edema. The expression of growth factors such as mature brain-derived neurotrophic factor, insulin-like growth factor-1, and vascular endothelial growth factor and phosphorylation of tropomyosin-related kinase B, which is a receptor tyrosine kinase for brain-derived neurotrophic factor and the downstream pathway in antiapoptosis, was examined by Western blot analysis. Neuronal cell death was measured with terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end-labeling staining. We also administered K252a, a tropomyosin-related kinase B antagonist, to examine the mechanisms for neuroprotective effects by SOV.

Results—SOV significantly improved neurofunction and reduced brain edema after SAH. SOV increased mature brain-derived neurotrophic factor and prevented post-SAH tropomyosin-related kinase B inactivation and caspase-3 activation, resulting in attenuation of neuronal cell death in the cortex and hippocampal CA1 region. Preinjection of K252a abolished the beneficial effects of SOV.

Conclusions—The current study showed that brain-derived neurotrophic factor-induced tropomyosin-related kinase B activation by SOV was necessary for protection against early brain injury after SAH. (Stroke. 2011;42:477-483.)

Key Words: apoptosis • brain-derived neurotrophic factor • early brain injury • subarachnoid hemorrhage • sodium orthovanadate • tropomyosin-related kinase B

A neursmal subarachnoid hemorrhage (SAH) is one of the most life-threatening diseases with a high mortality and disability rate.1 Although post-SAH cerebral vasospasm has been long studied and treated, the outcome is not improved even if angiographic vasospasm is reversed.2,3 Early brain injury (EBI), which occurs within 48 hours after cerebral aneurysm rupture, has been considered as a new target for improving outcome after SAH.2 Recent studies reported that apoptosis was involved in the pathogenesis of EBI after experimental SAH or in a clinical setting.5,6 Therefore, it is considered that an antiapoptotic treatment can be 1 of the therapeutic candidates for EBI after SAH.

Some growth factors, each of which is a ligand for the specific receptor tyrosine kinase (RTKs), can prevent neuronal apoptosis in ischemic stroke.7,8 Because RTKs are activated by phosphorylation of an intracellular tyrosine residue after binding to the ligands, it is possible that inactivation of RTKs may be associated with neuronal injury after SAH. Sodium orthovanadate (SOV) behaves as a tyrosine phosphatase inhibitor and can phosphorylate RTKs.9 We demonstrated that SOV ameliorated ischemic neuronal injury after transient middle cerebral artery occlusion through antiapoptotic mechanisms.10 Therefore, it is possible that SOV has an antiapoptotic effect through RTK activation and attenuates EBI after SAH.

In this study, we hypothesized that the activation of a specific RTK signaling by SOV might attenuate EBI after experimental SAH through antiapoptosis. We examined expression changes of some growth factors after SAH and SOV administration and focused on the role of brain-derived neurotrophic factor (BDNF) and the RTK, tropomyosin-related kinase B (TrkB), in neuroprotective effects of SOV.

Materials and Methods

Experimental Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University. One hundred seventy-two Sprague-Dawley rats (Harlan, Indianapolis, IN) weighting...
280 to 350 g were divided randomly into the following groups: sham-operated (sham group: n=22), SAH+saline (vehicle group: n=45), SAH+3 mg/kg (low-SOV group: n=7) or 10 mg/kg (SOV group: n=45) of SOV in saline, dimethyl sulfoxide (DMSO; as a vehicle)+sham-operated (V+sham group: n=11). DMSO+SAH treated with 10 mg/kg SOV in saline (V+SOV group: n=21), and 0.5 mg/mL K252a in DMSO+SAH treated with 10 mg/kg SOV in saline (K+SOV group: n=21).

**Induction of SAH**
Anesthesia was maintained with 2.5% isoflurane, 30% oxygen, and 70% medical air through a face mask. Blood pressure and blood gas were measured through the left femoral artery. The rectal temperature was kept at 36.5°C±0.5°C by using a feedback-regulated heating system during surgery.

The SAH model was produced as described previously. Briefly, the left common carotid artery was exposed and 4-0 sharpened nylon sutures were advanced to the left internal carotid artery until resistance was felt and perforated at the place. Immediately after surgery, saline, 3 mg/kg or 10 mg/kg of SOV was injected intraperitoneally.

**SAH Severity**
The severity of SAH was blindly evaluated by using the SAH grading scale at the time of euthanasia. Rats with SAH grading scores ≤7, which had no significant brain injury, were excluded from this study (vehicle: 6, SOV: 4, K+SOV: 3, and D+SOV: 2).

**Measurement of Brain Water Content**
Animals were decapitated at 24 and 72 hours after SAH induction (n=6). The brains were quickly removed and divided into the right and left cerebral hemispheres, cerebellum, and brain stem. After measuring the weights (wet weight), they were kept in an oven at 105°C for 72 hours and weighed again (dry weight). The following formula was used to calculate the percentage of brain water content (BWC): [(wet weight–dry weight)/wet weight]×100%.

**Neurological Scoring and Mortality**
Using a 22-point scoring system with a modification of the method described by Garcia et al., neurological deficits were blindly assessed 1 hour before euthanasia in each animal.

**Intracerebroventricular Infusion**
Rats were placed in a head holder under 2.5% isoflurane. The needle of a 10-μL Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was inserted through a burr hole into the right lateral ventricles using the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of bregma. Ten microliters of DMSO (as a vehicle; 1.1 mg/μL) or K252a in DMSO, which is a TrkB antagonist, was automatically infused at a rate of 1 μL/min from 50 to 60 minutes before the induction of SAH or sham operation. The needle was removed 10 minutes after the infusion and the burr hole was plugged with bone wax.

**Western Blot Analysis**
The left cerebral hemisphere (perforation side) was isolated and collected at 4 and 24 hours after SAH induction (n=5). Protein concentration was determined using a DC protein assay (Bio-Rad, Hercules, CA). Individual samples (50 μg each) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane for 100 minutes at 120 V (Bio-Rad). Blotting membranes were incubated for 2 hours with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated overnight at 4°C with the following primary antibody: anti-insulin-like growth factor-1 (IGF-1; 1:1000; Chemicon International, Temecula, CA) antibodies. The membranes were incubated for 1 hour with appropriate secondary antibodies (1:2000; Santa Cruz Biotechnology) and processed with an enhanced chemiluminescence reagent kit (ECL plus kit; Amersham Bioscience, Arlington Heights, IL). The images were analyzed semiquantitatively in a blind fashion using the Image J software (National Institutes of Health, Bethesda, MD). Beta-actin (Santa Cruz Biotechnology) was used as an internal control for every experiment. Changes in phosphorylation of TrkB and Akt and expression of 14 kDa (cleaved) caspase-3 were expressed as a percentage of the values in sham-operated rats at each time point after SAH. Values are mean±SD; *P<0.05 versus sham; †P<0.05 versus vehicle, analysis of variance.
Terminal Deoxynucleotidyl Transferase-Mediated Uridine 5'-Triphosphate-BiotinNick End-Labeling Staining

Samples from sham-operated, saline- and SOV-treated rats were used \( n = 5 \). At 24 hours after SAH, the rat brains were fixed by cardiovascular perfusion with phosphate-buffered saline and 10% paraformaldehyde and postfixed in 10% paraformaldehyde followed by 30% sucrose (weight/volume) for 3 days. Ten-micron-thick coronal sections at the level of bregma-2 mm were cut on a cryostat (LM3050S; Leica Microsystems Bannockburn, IL) and mounted on poly-L-lysine-coated slides.

We immunostained brain sections with antineuronal nuclei antibody \( (1:200; \text{Chemicon International}) \) and then subjected the sections to terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining with an in situ cell death detection kit \( (\text{Roche Inc, Mannheim, Germany}) \). Then the sections were mounted with 4'-6-diamidino-2-phenylindole. Incubation with labeling solution without the enzyme served as a negative labeling control. The TUNEL-positive neurons were measured in the left pyriform cortex (they were counted in 3 fields per case at \( \times 400 \) magnification and expressed as the mean number of TUNEL-positive neurons/mm\(^2\)) and the left CA1 region in hippocampus (TUNEL-positive neurons/ neurons, percent) in a blind manner.

Statistical Analysis

All values were expressed as mean±SD and were analyzed using unpaired \( t \)-tests, or 1-way analysis of variance with Scheffe correction as appropriate. \( P < 0.05 \) was considered significant.

Results

Mortality and SAH Grade

No statistical differences were observed among the groups with regard to physiological parameters (data not shown). The mortality rate was not significantly different between the vehicle group \( (15.4\% \ [6 \text{ of } 39 \text{ rats}]) \) and the low-SOV \( (14.3\% \ [1 \text{ of } 7 \text{ rats}]); P = 0.94 \) or the SOV \( (19.5\% \ [8 \text{ of } 41 \text{ rats}]; P = 0.63) \) group within 72 hours. No sham-operated rats died.
The SAH grading scores (SAH severity) were similar among the groups at each time point (Figure 1A; \( P \geq 0.08, 0.79, \) and 0.74, vehicle versus SOV at 4, 24, and 72 hours, respectively; \( P \geq 0.64, \) vehicle versus low-SOV at 24 hours).

**Neurological Score and BWC**

Although neurological scores were significantly deteriorated at 24 (14.8 ± 3.9) and 72 (15.2 ± 3.2) hours post-SAH, SOV significantly improved the scores at both time points (Figure 1B; 18.7 ± 1.4, 18.7 ± 0.8, respectively). The BWC in the left hemisphere (perforation side) also significantly improved in the SOV group compared with the vehicle group at 24 (79.1% ± 0.3% versus 79.7% ± 0.3%) and 72 (79.2% ± 0.3% versus 79.6% ± 0.1%) hours after SAH (Figure 1C). There was no significant difference in the BWC in the right hemisphere, cerebellum, or brain stem among the groups (data not shown). Because the low-SOV group showed no significant improvements in neurological scores and BWC compared with the vehicle group, the low-SOV group was excluded from further analysis.

**Growth Factors and the RTK-Related Signaling**

To examine whether growth factors–RTKs are modulated in SAH, we first evaluated the m-BDNF, IGF-1, and VEGF levels. The m-BDNF levels were similar in the sham and vehicle groups at 4 and 24 hours, but significantly increased in the SOV (177% ± 56%) group compared with the sham and vehicle (87% ± 13%) groups at 4 hours after SAH (Figure 2A). The IGF-1 levels in the vehicle and SOV groups were not significantly changed compared with the sham group at 4 and 24 hours post-SAH, although the IGF-1 levels in the SOV group (112% ± 14%) were significantly higher than in the
vehicle group (82%±12%) at 4 hours (Figure 2B). Neither SAH nor SOV affected VEGF levels at 4 and 24 hours post-SAH (Figure 2C).

Next, we focused on the BDNF-related signal transduction. Phosphorylation of TrkB, a RTK for BDNF, was significantly decreased after SAH (50%±15%) but preserved in the SOV (85%±21%) group compared with the sham group at 4 hours after SAH, although the expression levels of total TrkB were unchanged through the observation period (Figure 3A–B). Phosphorylation of Akt was not significantly changed in the vehicle group but significantly increased in the SOV (750%±500%) group compared with the sham group at 4 hours after SAH (Figure 3C).

On the other hand, the cleaved caspase-3 expression level was significantly higher in the vehicle group (274%±112%) than in the sham group, and SOV significantly decreased the level (138%±61%) at 24 hours post-SAH (Figure 3D).

TUNEL-Positive Neurons
There were many TUNEL-positive neurons seen in the left pyriform cortex and hippocampus at 24 hours after SAH (data not shown). A significant decrease in TUNEL-positive neurons was observed in the SOV group (75%±23/mm²) compared with the vehicle (366±68/mm²) groups in the cortex (Figure 4A). In the left hippocampal CA1 region, a significant decrease in TUNEL-positive neurons was also seen in the SOV group (5.2%±2.3%) compared with the vehicle (11.0%±3.8%) groups (Figure 4B), whereas the cells with 4’-6-diamidino-2-phenylindole were not disarranged in both groups (data not shown).

Effect of K252a on SOV-Induced Neuroprotection
To address whether TrkB activation preserved by SOV-induced BDNF upregulation was neuroprotective, K252a, a specific TrkB antagonist, was administered to SOV-treated SAH rats. There were no significant differences in the mortality rate between the V+SOV (15.8% [3 of 19 rats]) and K+SOV (11.1% [2 of 18 rats]) groups (P=0.95). No rats in the V+sham group died.

The SAH grading scores were similar between the V+SOV and K+SOV groups at 4 (P=0.93) and 24 (P=0.22) hours post-SAH (Figure 5A).

A significant deterioration in neurological scores was observed in the K+SOV group (13.8±3.6) compared with the V+SOV group (11.8±3.7) at 24 hours post-SAH (Figure 5B).
the V+SOV (17.6 ± 1.9) and V+sham groups (20.6 ± 1.2) at 24 hours after SAH (Figure 5B). The BWC in the left hemisphere was also significantly deteriorated in the K+SOV group (79.9% ± 0.5%) but not in the V+SOV group (79.4% ± 0.4%) compared with the V+sham group (78.8% ± 0.1%; Figure 5C).

K252a abolished SOV-induced preservation of TrkB phosphorylation (66% ± 14%; Figure 6A) and suppression of cleaved caspase-3 expression (191% ± 67%; Figure 6B).

**Discussion**

The current study showed that SOV was protective for EBI after SAH at least partly through the induction of BDNF and the preservation of TrkB activation. This conclusion came from the following results; SOV preserved TrkB activation associated with BDNF induction and suppressed neuronal cell apoptosis, leading to the attenuation of brain edema and neurological impairments after SAH, whereas K252a, a specific TrkB antagonist, abolished the beneficial effects of SOV.

BDNF is one of the growth factors and the effect on neuronal survival is associated with the activation of m-BDNF–TrkB signaling. In neuron, N-methyl-D-aspartic acid induces a Ca²⁺ signal and enhances a BDNF release and TrkB activation, which results in Akt activation and neuronal survival. Because it is reported that SOV activated tyrosine kinase-dependent Ca²⁺ responses, which in turn resulted in a Ca²⁺ store-dependent enhancement of N-methyl-D-aspartic acid responses, we speculate that SOV upregulated BDNF expression through N-methyl-D-aspartic acid–Ca signaling in the brain after SAH in this study. Further studies will clarify if N-methyl-D-aspartic acid antagonists block BDNF expression and make post-SAH EBI worse.

TrkB has two isoforms, a full-length TrkB that has a tyrosine residue and a truncated TrkB that lacks intrinsic tyrosine kinase activity. Whereas a full-length TrkB exerts prosurvival function in neuron, it is reported that a truncated TrkB inhibits a full-length TrkB signaling through dominant-negative mechanisms. In our study, BDNF might bind to both receptors, and truncated TrkB receptors might dominantly deactivate the tyrosine kinase signaling of full-length TrkB after SAH. SOV might recover the tyrosine kinase activity of full-length TrkB by upregulating m-BDNF and/or by directly acting to a tyrosine residue of full-length TrkB, preserving the phosphorylation level of TrkB.

The expression levels of growth factors after SAH have been poorly investigated and reported to be diverse according to reports, possibly because of limited injury of an irregular pattern in the post-SAH brain. Serum IGF-1 concentrations were reported to decrease in the acute phase of human SAH and to be a predictive factor for poor outcome. On the other hand, VEGF was upregulated and associated with blood–brain barrier disruption in the brain after SAH. In this study, there were no significant changes in expression levels of m-BDNF, IGF-1, or VEGF after SAH. One possible reason for this discrepancy is that post-SAH brain injuries in this study were less severe and not enough for changing the expression levels of growth factors compared with the previous study. Preserved IGF-1 levels in SOV-treated SAH animals at 4 hours may also be explained by the fact that SOV prevented post-SAH brain injuries.

Akt is a well-known principal factor in antiapoptotic signaling. Previously, we demonstrated that SOV had a neuroprotective effect through the Akt activation in cerebral ischemia. The Akt activation by SOV was also shown in this study, accounting for the neuroprotection against post-SAH EBI. Because a specific TrkB antagonist abolished SOV-induced Akt activation and neuroprotection, the BDNF–TrkB pathway was necessary for the SOV-induced neuroprotection in this study. However, we cannot exclude the possibility that many other kinases also mediated the protection, because SOV can inhibit both protein tyrosine phosphatases and protein serine–threonine phosphatases. Even the mechanisms by which SOV activated Akt remain unclear.

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**Figure 6.** Changes in phosphorylation of TrkB at 4 hours (A) and expression of cleaved caspase-3 at 24 hours (B) after SAH (n=5 each). The band density values are calculated as a ratio of that of β-actin, and the values from the sham operation are used as 100%. Values are mean ± SD; *P < 0.05 versus V+sham; †P < 0.05 versus K+SOV, analysis of variance.
SOV might directly activate Akt (Ser473) in addition to the activation through a BDNF–TrkB pathway. Moreover, SOV preserved IGF-1 expression that also acts as a ligand for Akt signaling in this study. Thus, SOV might activate Akt through multiple pathways. Further studies are needed to clarify the neuroprotective mechanisms by SOV against post-SAH EBI.

In conclusion, this study suggested that activation of BDNF–TrkB signaling by SOV attenuated EBI after SAH through antiapoptotic mechanisms. Because SOV has been used to treat patients with diabetes mellitus, it may be a good candidate treatment for EBI after human SAH.

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

Disclosures
None.

References
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Stroke. 2011;42:477-483; originally published online December 30, 2010;
doi: 10.1161/STROKEAHA.110.597344
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
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