Aneurysmal subarachnoid hemorrhage (SAH) is one of the most life-threatening diseases with high mortality and disability rates. Past research has focused primarily on SAH-induced vasospasm, which was considered to be the main reason for the occurrence of delayed neurological deficits. However, failure of antivasospastic agents to improve the outcome in SAH patients in clinical trials has shifted the research interest to SAH-induced early brain injury (EBI).

Neuronal apoptosis, along with the development of brain edema, are 2 main pathological processes in EBI. Several apoptotic pathways have been described in preclinical SAH models.

Osteopontin (OPN) is a extracellular matrix protein, which, by interacting with its cell surface integrin receptors, has been implicated in cell proliferation and in reduction of apoptotic cell death. When administered intracerebroventriculartly, recombinant OPN (rOPN) has been demonstrated to be neuroprotective in various preclinical stroke models; however, the translatability of rOPN into clinical trials is limited by the invasive nature of its administration route. To overcome this difficulty, nasal drug delivery has been suggested as a noninvasive treatment modality for central nervous system (CNS) therapeutics that cannot pass through the blood–brain barrier.

In this study, we hypothesized that nasal administration of rOPN attenuates EBI after SAH. Furthermore, we hypothesized that the antiapoptotic effect of rOPN is mediated through FAK–phosphatidylinositol 3-kinase–Akt–induced inhibition of caspase-3 cleavage.

**Key Words:** administration, intranasal ◼ apoptosis ◼ early brain injury ◼ recombinant osteopontin ◼ subarachnoid hemorrhage

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Material and Methods

Experimental Design

All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Adult male Sprague–Dawley rats (n=144, weighing 280–320 g; Harlan, Indianapolis, IN) were housed in a light-and-temperature–controlled environment and fed ad libitum. Animals were divided randomly into the following groups while still under anesthesia: sham-operated (sham; n=23), SAH+PBS (vehicle; n=38), SAH+1 µg rOPN (rOPN-1; n=9; low dose), SAH+5 µg rOPN (rOPN-5; n=36; high dose), SAH+5 µg rOPN+FAK inhibitor 14 (Fib-14; n=9), SAH+5 µg rOPN+Wortmannin (Wor; n=9), naive+PBS (n=10), and naive+5 µg rOPN (n=10). For the descriptive part of the study (neurological scores and brain water content), we used the sham, vehicle, high-dose, and low-dose groups, and for the mechanistic part of the study (Western blot), we added the Wor and Fib-14 groups.

Endovascular Perforation Model of SAH

The endovascular perforation model was performed for the induction of SAH as previously described. Briefly, rats were anesthetized, intubated, and kept on artificial ventilation with 3% isoflurane in a 60%/40% medical air/oxygen gas mixture. A sharpened 4-0 nylon suture was inserted into the left internal carotid artery and advanced until resistance was felt at the bifurcation of the anterior and middle cerebral artery. The suture was then further advanced to puncture the vessel, before being withdrawn. For sham operations, a suture was inserted into the left carotid artery, but no vessel perforation was performed.

Nasal Administration of rOPN

Nasal administration of rOPN (mouse rOPN, His-tagged; EMD Bioscience, Ellisville, MI; 0.2 mg dissolved in 5 µL PBS) was administered 1 hour before SAH induction. This was achieved by inserting a 26-gauge Hamilton syringe through a small burr hole into the nasal cavity. The suture was then further advanced to puncture the vessel, while still under anesthesia: sham-operated (sham; n=23), SAH+PBS (n=10) or PBS alone (n=10). Briefly, rats were anesthetized, intubated, and were kept on artificial ventilation during the procedure with 3% isoflurane in 60%/40% medical air/oxygen gas mixture. CSF was collected by puncturing the cisterna magna at 1, 2, 3, 4, and 24 hours after rOPN administration. The concentration of OPN in the CSF was calculated using an ELISA kit for rodent OPN (Enzo Life Sciences, Ann Arbor, MI) as indicated by the manufacturer. Commercially available ELISA kits can only detect full-length, intact OPN, and because its cleavage by thrombin may occur after SAH, we used naïve animals for this part of the experiment.

Intracerebroventricular and Intravenous Drug Administration

Anesthetized rats were secured onto a stereotactic head frame, while receiving continuous 2.5% isoflurane anesthesia. Fib-14 (Tocris Bioscience, Ellisville, MI; 0.2 mg dissolved in 5 µL PBS) was administered 1 hour before SAH induction. This was achieved by inserting a 26-gauge Hamilton syringe through a small burr hole into the left lateral ventricle at the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of the skull. The PI3K inhibitor Wortmannin (Sigma-Aldrich) was dissolved in PBS and injected intravenously at a dose of 15 µg/kg at 1 hour before SAH induction.

SAH Grade

The severity of SAH was blindly evaluated using the SAH grading scale at the time of euthanasia as previously reported. Rats with mild SAH (SAH grades ≤7) were excluded from the study. A total of 9 animals were excluded due to mild SAH (vehicle, 3; rOPN-5, 4; rOPN-1, 1; Fib-14, 1).

Neurological Score

Neurological scores were evaluated at 1 hour before euthanization by a blinded observer according to the 18-point scoring system described by Garcia et al. Brain Water Content

Brains were removed at 24 or 72 hours after surgery and separated into left hemisphere, right hemisphere, and cerebellum. Each part was weighed immediately after removal (wet weight) and once more after drying in 105°C for 72 hours. The percentage of water content was calculated as [(wet weight–dry weight)/wet weight]×100%.

Western Blot

Rats were euthanized at 24 hours after surgery and ipsilateral (left) brain hemispheres were processed for Western blot analysis as previously described. Equal amounts of protein (50 µg) were loaded onto polyacrylamide SDS gels and separated by electrophoreses, before being transferred to a nitrocellulose membrane. Membranes were then blocked and probed overnight at 4°C with the following primary antibodies: antiphospho-FAK (1:1000), antiphospho-Akt (Ser 473; 1:1000) purchased from Cell Signaling Technology (Danvers, MA); anticleaved caspase-3 (CC3; 1:200) and antif–actin (1:500) purchased from Santa Cruz Biotechnology (Dallas, TX). Next, immunoblots were processed with the appropriate secondary antibodies (1:2000; Santa Cruz) and bands were visualized using the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL). Quantification was performed by optical density methods using the ImageJ software (National Institutes of Health). Results are expressed as relative density to f–actin, normalized to the mean value of the sham group.

Detection of OPN in the Brain After Nasal Administration

ELISA detection of OPN in the cerebrospinal fluid (CSF) was performed in naive rats that received nasal administration of 5 µg rOPN in 50 µL PBS (n=10) or PBS alone (n=10). Briefly, rats were anesthetized, intubated, and were kept on artificial ventilation during the procedure with 3% isoflurane in 60%/40% medical air/oxygen gas mixture. CSF was collected by puncturing the cisterna magna at 1, 2, 3, 4, and 24 hours after rOPN administration. The concentration of OPN in the CSF was calculated using an ELISA kit for rodent OPN (Enzo Life Sciences, Ann Arbor, MI) as indicated by the manufacturer. Commercially available ELISA kits can only detect full-length, intact OPN, and because its cleavage by thrombin may occur after SAH, we used naïve animals for this part of the experiment.

Quantification of Neuronal Cell Death

Immunohistochemistry was performed in sham-operated, rOPN-treated, and vehicle animals (n=5 per group). At 24 hours after SAH induction, rats underwent transcardial perfusion with PBS and 10% paraformaldehyde. Next, brains were collected and dehydrated using 30% sucrose (weight/volume) for 3 days. Ten-micrometer-thick coronal brain sections, at the level of bregma ±2 mm anterior and posterior, were cut on a cryostat (LM3050S; Leica Microsystems, Bannockburn, IL) and mounted onto poly-l-lysine–coated glass slides. These brain sections were immunostained with anti-NeuN (1:100; Millipore, Billerica, MA) and then subjected to terminal deoxynucleotidyl transferase-deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) using an in situ cell death detection kit (Roche, Germany). TUNEL-positive neurons were counted in the left piriform cortex (4 different areas in 500×500 µm grids) by a blinded investigator.

Statistical Analysis

Data are expressed as a mean±SEM. Mortality data were analyzed by Fisher exact test. SAH grading and ELISA data were analyzed using Student t test. All other data were analyzed by one-way ANOVA followed by Tukey post hoc test. A P value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism for Windows.

Results

Mortality and SAH Grade

No significantly different SAH grades were observed among all SAH groups (data not shown). Similarly, mortality rates
were not significantly different between the SAH groups (\(P>0.05\)). Mortality rates were as follows: sham, 0% (0 of 23); vehicle, 34% (12 of 35); rOPN-5, 28% (9 of 32); rOPN-1, 25% (2 of 8); Fib-14, 25% (2 of 8); Wor, 33% (3 of 9). No mortality was observed in naive animals subjected to CSF collection. Operated but excluded animals were not considered.

**Neurological Scores and Brain Water Content**

Experimental SAH evoked a significant increase in water content within the left hemisphere and right brain hemisphere as well as within the cerebellum at 24 hours after surgery (\(P<0.05\), vehicle versus sham; Figure 1A). The water content in both brain hemispheres was significantly reduced by 5 µg of nasal rOPN administration (rOPN-5; \(P<0.05\) compared with vehicle). Furthermore, both rOPN treatment regimes significantly reduced the water content in the cerebellum (\(P<0.05\) compared with vehicle).

Neurological scores were significantly worse (lower scores) in the vehicle group (\(P<0.05\) compared with sham); however, SAH animals that received 5 µg of rOPN demonstrated significantly improved neurological performances at 24 hours after SAH induction (\(P<0.05\) compared with vehicle; Figure 1B). Because the 1 µg rOPN treatment group failed to reduce brain water content and neurological deficits at 24 hours after SAH induction, further experiments were conducted using only the higher dose of rOPN (rOPN-5).

Brain water content at 72 hours was similar between all experimental groups (\(P>0.05\); Figure 2A); however, significantly worse neurological scores were observed in vehicle animals (\(P<0.05\) compared with sham; Figure 2B). This was reversed by nasal administration of 5 µg rOPN (\(P<0.05\) compared with vehicle).

**Quantification of OPN in the CSF**

Intranasally administered rOPN resulted in tendentially increased OPN levels within the CSF at 3 hours after treatment (Figure 3). Furthermore, the concentration of OPN significantly increased at 4 and 24 hours after rOPN administration in naive rats (\(P<0.05\) compared with PBS).

**Expressions of Phosphorylated FAK, Phosphorylated Akt, and CC3 at 24 Hours After SAH Induction**

Western blot analysis was used to quantify the expressions of phosphorylated FAK, phosphorylated Akt, and CC3 in the ipsilateral (left) brain hemisphere at 24 hours after surgery (\(n=6\) in each group). Nasal administration of 5 µg rOPN (rOPN-5) significantly increased the expression of phosphorylated FAK compared with sham-operated and vehicle animals (\(P<0.05\); Figure 4A). However, administration of Fib-14 prior to SAH induction and nasal rOPN administration reversed this treatment effect at 24 hours after surgery (\(P<0.05\) compared with rOPN-5). Administration of Wor resulted in a significantly increased phosphorylated FAK expression when compared with the Fib-14 group (\(P<0.05\)).

Hemispheric levels of phosphorylated Akt were significantly increased in rOPN-treated animals, when compared with sham-operated and vehicle animals (\(P<0.05\); Figure 4B). This treatment effect was reversed by both Fib-14 and Wor (\(P<0.05\) compared with rOPN-5).

Experimental SAH and vehicle administration resulted in a significant increase in CC3 expression at 24 hours after surgery (\(P<0.05\) compared with sham; Figure 4C), which was significantly reduced by rOPN treatment (\(P<0.05\) compared with vehicle). This treatment effect was reversed in animals of Fib-14 and Wor groups (\(P<0.05\) compared with rOPN-5).

**Quantification of Neuronal Cell Death**

Consistent with the Western blot results, immunohistochemical analysis demonstrated an increased number of TUNEL and NeuN double-stained cells (TUNEL+neurons) in the piriform cortex and hippocampus (Figure 5A and 5B). rOPN treatment...
reduced the number of TUNEL+ neurons when compared with vehicle administration at 24 hours after SAH induction in the piriform cortex (Figure 5C).

Discussion

Recent studies have demonstrated that rOPN, when given intracerebroventricularly, decreased brain edema and attenuated cerebral vasospasm following experimental SAH. However, the invasiveness of the administration route and the pre-SAH treatment regime are substantial limitations of these investigations. Keeping in mind that a lot of drugs failed to improve outcome in clinical trials, which were otherwise shown to have beneficial effects in animal studies, we aimed in this current study to provide the basis for a possible clinical translation using noninvasive nasal application for rOPN as a promising therapeutic strategy to protect against SAH-induced EBI.

Our main results suggested that intranasal rOPN post-treatment attenuated EBI (brain edema and neuronal apoptosis), which ultimately improved neurological scores in rodents after SAH induction.

The blood–brain barrier limits the distribution of systemically administered therapeutics to the CNS; however, intranasal administration has emerged as an alternative strategy for drug delivery into the CNS and clinical trials show promising outcomes when using this method in humans. Specifically, nasal administration of drugs provides rapid delivery of molecules to the CNS via bulk flow along olfactory and trigeminal perivascular channels and slower delivery via olfactory bulb axonal transport. Dendritic processes of the olfactory neurons are directly exposed in the upper nasal passage and their axons project to the olfactory bulb. Moreover, the uptake of drugs into the CNS occurs either by endocytosis and transport along olfactory nerves or by extracellular flow through intercellular clefts in the olfactory epithelium to diffuse into the subarachnoid space.

The duration between drug administration and its detection in the brain varies based on the molecular structure of the compound. It also varies in different animal species. For instance, intranasally administered insulin in rodents binds to receptors in the hippocampus and the frontal cortex within 60 minutes after completion of the treatment. The concentration of rOPN, however, has been shown to increase by 50% at 2 hours after nasal administration in a mouse model of ischemic stroke. In our study, rOPN was detectable in CSF at 3 hours after treatment and the difference between vehicle and rOPN administration was found to be significant after 4 hours. We also detected high levels of rOPN at 24 hours after its nasal administration.

In our study, we have measured the rOPN in naive animals because rOPN gets easily cleaved by thrombin derived from the hemorrhage in the CSF and there is no available kit for measuring thrombin-cleaved rOPN. We think that this unique...
feature of rOPN is very beneficial in SAH because thrombin-cleaved rOPN has been proven to be more effective in vitro and in vivo studies.10,23

The sudden intracerebral pressure increase causes a short global ischemia resulting in neuronal apoptosis. This leads to cytotoxic edema followed and exacerbated by the vasogenic edema. The neuronal apoptosis occurs in the cerebral cortex as well as in the hippocampus and is linked to late cognitive and memory dysfunctions after SAH.4,5,24 In agreement with previous work, this current study showed increased apoptotic neuronal cells after experimental SAH; however, nasal rOPN treatment reduced the number of cells undergoing DNA fragmentation in the final phase of apoptotic cell death, resulting in attenuation of brain edema.

The antiapoptotic role of the PI3K–Akt pathway in neurons after SAH has been previously reported after experimental SAH.4,6 FAK, a cytoplasmic tyrosine kinase, is a critical downstream effector that mediates signal transduction pathways triggered via integrin receptors.25 Binding of a ligand to the integrin receptors initiates its signaling, which then leads to the phosphorylation of FAK at tyrosine 397 (Y397), thereby activating the kinase.26 Activation of FAK results in stimulation of PI3K, which is directly associated with decreased cellular apoptosis.27 Consistent with these previous reports, we found in our study that rOPN treatment increased the levels of phosphorylated FAK and phosphorylated Akt, resulting in a decrease of proapoptotic CC3. To manipulate this proposed pathway, we used Fib-14, a small-molecule inhibitor, which specifically decreases Y397 phosphorylation,15 thereby decreasing FAK activation. We also used Wor, which is an irreversible inhibitor of PI3K. The fact that these 2 inhibitors abolished the protective effects of rOPN supported our hypothesis that rOPN mediates its antiapoptotic effects over FAK–PI3K–Akt–induced inhibition of caspase-3 cleavage.

We have proven in our study that intranasal administration of 5 µg rOPN has beneficial effects in attenuating EBI, but our study has limitations. In order to be able to move forward to clinical trials, more studies have to be conducted to determine the behavior of rOPN in vivo, such as the clearance from the CSF circulation, the most effective and toxic doses, and the most appropriate way and time of the administration.

Conclusions
This study found that nasal administration of rOPN improved functional outcomes after experimental SAH in rats, by attenuating neuronal apoptosis and the reduction of brain water content. rOPN can be detected in the CSF within 3 hours after its intranasal administration. The FAK–PI3K–Akt pathway is possibly involved in the antiapoptotic effects of rOPN.

Disclosures
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References


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