Isoflurane Attenuates Blood–Brain Barrier Disruption in Ipsilateral Hemisphere After Subarachnoid Hemorrhage in Mice

Orhan Altay, MD; Hidenori Suzuki, MD, PhD; Yu Hasegawa, MD, PhD; Basak Caner, MD; Paul R. Krafft, MD; Mutsumi Fujii, MD; Jiping Tang, MD; John H. Zhang, MD, PhD

Background and Purpose—We examined effects of isoflurane, volatile anesthetics, on blood–brain barrier disruption in the endovascular perforation model of subarachnoid hemorrhage (SAH) in mice.

Methods—Animals were assigned to sham-operated, SAH+vehicle–air, SAH+1%, or 2% isoflurane groups. Neurobehavioral function, brain water content, Evans blue dye extravasation, and Western blotting for sphingosine kinases, occludin, claudin-5, junctional adhesion molecule, and vascular endothelial cadherin were evaluated at 24 hours post-SAH. Effects of sphingosine kinase (N,N-dimethylsphingosine) or sphingosine-1-phosphate receptor-1/3 (S1P1/3) inhibitors (VPC23019) on isoflurane’s action were also examined.

Results—SAH aggravated neurological scores, brain edema, and blood–brain barrier permeability, which were prevented by 2% but not 1% isoflurane posttreatment. Two percent isoflurane increased sphingosine kinase-1 expression and prevented a post-SAH decrease in expressions of the blood–brain barrier-related proteins. Both N,N-dimethylsphingosine and VPC23019 abolished the beneficial effects of isoflurane.

Conclusions—Two percent isoflurane can suppress post-SAH blood–brain barrier disruption, which may be mediated by sphingosine kinase 1 expression and sphingosine-1-phosphate receptor-1/3 activation. (Stroke. 2012;43:2513-2516.)

Key Words: blood–brain barrier ▪ early brain injury ▪ isoflurane ▪ sphingosine kinase-1 ▪ sphingosine-1-phosphate receptor ▪ subarachnoid hemorrhage

Blood–brain barrier (BBB) disruption has been an important prognostic factor after aneurysmal subarachnoid hemorrhage (SAH). The BBB is critical for brain homeostasis and is located at the cerebral microvessel endothelial cells, which maintain their barrier characteristics through cell–cell contacts made up of tight and adherens junctions. Stabilization of tight junctions involves a complex network of occludin, claudin-5, and junctional adhesion molecule. Adherens junctions consist of vascular endothelial (VE) cadherins.

Recently, we reported that 2% isoflurane, a volatile anesthetic, prevented post-SAH neuronal apoptosis through sphingosine-related pathway activation. Sphingosine-1-phosphate (S1P) is generated from sphingomyelin by sphingosine kinase-1 (SphK1) and SphK2 and was reported to enhance endothelial barrier integrity. However, it remains underdetermined whether isoflurane prevents BBB disruption. This study is the first to demonstrate that isoflurane posttreatment prevents BBB disruption after SAH in mice and that the mechanism involves SphK1 expression and S1P receptor-1/3 (S1P1/3) activation.

Methods

For expanded methods, see the online-only Data Supplement. The Loma Linda University animal care committee approved all protocols.

In Study 1, male CD-1 mice (30–38 g; Charles River, Wilmington, MA) were randomly divided into sham-operated+vehicle–air (n=17), SAH+vehicle–air (n=25), SAH+1% isoflurane (n=9), and SAH+2% isoflurane (n=22) groups. A SAH endovascular perforation model was produced and sham-operated mice underwent identical procedures except that the suture was withdrawn without puncture. One hour post-SAH, 1% or 2% isoflurane (Baxter, Deerfield, IL) was continuously administered for 1 hour with vehicle air (30% O2, and 70% medical air). All evaluations were blindly performed at 24 hours postsurgery. Eighteen-point SAH grading and 18-point neurological scores were evaluated in all surviving animals as previously described. Brain water content (n=6 per group) and Evans blue dye extravasation (n=5 per group) were measured as previously described. Western blot (n=6 per group) was performed on the left cerebral hemisphere (perforation side) using anti-SphK1 (Abgent, San Diego, CA), anti-SphK2 (Lifespan Biosciences, Seattle, CA), antioccludin, anticlaudin-5, antijunctional adhesion molecule-A, and anti-VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies as previously described.
Figure 1. SAH grade (A), neurological score (B), brain water content (C), and Evans blue dye extravasation (D) at 24 hours post-SAH (Study 1). Vehicle-air, SAH + vehicle-air group; 1% or 2% ISO, SAH +1%, or 2% isoflurane group; left or right, left or right cerebral hemisphere; values, median (25th to 75th percentiles (A–B)) or mean ± SD (C–D); *P < 0.05, Kruskal-Wallis test (A–B) or analysis of variance (C–D). SAH indicates subarachnoid hemorrhage.

Figure 2. Representative Western blots and quantitative analysis of occludin (A), JAM-A (B), claudin-5 (C), and VE-cadherin (D) expressions in the left cerebral hemisphere at 24 hours post-SAH (Study 1). The protein band density values are calculated as a ratio of that of β-actin. Vehicle-air, SAH + vehicle-air group; 2% isoflurane, SAH +2% isoflurane group; values, mean ± SD; *P < 0.05, analysis of variance. JAM indicates junctional adhesion molecule; VE, vascular endothelial; SAH, subarachnoid hemorrhage.
In Study 2, animals were randomly divided into dimethyl sulfoxide (a vehicle)/sham-operated/vehicle–air (n=11), dimethyl sulfoxide/SAH 2% isoflurane (n=11), N,N-dimethylsphingosine (a SphK antagonist; Enzo LifeSciences Inc, Plymouth Meeting, PA)/SAH 2% isoflurane (n=18), and VPC23019 (a S1P1/3-receptor antagonist; Avanti Polar Lipids Inc, Alabaster, AL)/SAH 2% isoflurane (n=18) groups. N,N-dimethylsphingosine (0.17 g/0.5 L) or VPC23019 (0.26 g/0.5 L) was infused into the right lateral ventricle at a rate of 0.1 L/min 1 hour before surgery. The vehicle groups were given the same volume (0.5 L) of dimethyl sulfoxide (1.1 g/mL/kg) diluted in phosphate-buffered saline. Isoflurane was administered like Study 1. SAH grading, neurological scores (all surviving animals), brain water content (n=6 per group), and Western blotting for SphK1, claudin-5, and VE-cadherin (n=5 per group) were performed at 24 hours postsurgery as described previously. Data were expressed as median±25th to 75th percentiles or mean±SD and were analyzed using Kruskal-Wallis test followed by Steel-Dwass multiple comparisons, one-way analysis of variance with Tukey-Kramer post hoc tests, Fisher exact, or χ² tests as appropriate. P<0.05 was considered statistically significant.

Results

Isoflurane Prevents Post-SAH BBB Disruption (Study 1)

The mortality was not different among the SAH groups (vehicle–air, 32.0% [8 of 25 mice]; 1% isoflurane, 33.3% [3 of 9]; and 2% isoflurane, 22.7% [5 of 22]) at 24 hours. No sham-operated mice died. SAH grade was similar among the groups (Figure 1A). Although 1% isoflurane had no significant effects, 2% isoflurane improved post-SAH neurological impairments (P=0.010), brain water content (P=0.003), and Evans blue dye extravasation (P=0.005) in the left cerebral hemisphere compared with the vehicle group (Figure 1B–D). Western blots showed that 2% isoflurane significantly increased SphK1, but not SphK2, in the left cerebral hemisphere compared with the sham (P=0.002) and vehicle (P<0.001) groups (online-only Data Supplement Figure I). In addition, 2% isoflurane increased expressions of
occludin, junctional adhesion molecule-A, and VE-cadherin compared with the sham \(P=0.001\), respectively) and vehicle \((P<0.001, \text{ respectively})\) groups and claudin-5 expression compared with the vehicle group \((P=0.010; \text{ Figure 2})\).

**SphK and S1P1/3-Receptor Antagonists Inhibit Isoflurane’s Effects (Study 2)**

No sham-operated mice died. The mortality was not different among the dimethyl sulfoxide + SAH+2% isoflurane (26.7%, 4 of 15 mice), N,N-dimethylsphingosine + SAH+2% isoflurane (38.9%, 7 of 18), and VPC23019 + SAH+2% isoflurane (38.9%, 7 of 18) groups. SAH grade was similar among the groups (online-only Data Supplement Figure IIA).

Both N,N-dimethylsphingosine and VPC23019 significantly aggravated neurological scores \((P<0.001 \text{ and } P=0.003, \text{ respectively}; \text{ online-only Data Supplement Figure IIB})\) and brain edema in the left cerebral hemisphere \((P<0.002 \text{ and } P=0.05, \text{ respectively}; \text{ Figure 3A})\) and decreased expressions of SphK1, claudin-5, and VE-cadherin in 2% isoflurane-treated SAH mice (Figure 3B–D).

**Discussion**

A key pathological manifestation of post-SAH early brain injury is BBB disruption.\(^1\) BBB dysfunction may allow greater influx of bloodborne cells and substances into brain parenchyma, thus amplifying inflammation, leading to further parenchymal damage and edema formation. In this study, 1-hour 2% isoflurane administration at 1 hour post-SAH improved neurological score, brain edema, and BBB permeability associated with increased SphK1 expression and S1P1/3 activation. Isoflurane also prevented a post-SAH decrease in expressions of tight junction (occludin, junctional adhesion molecule-A, and claudin-5) and adherens junction (VE-cadherin) proteins.

S1P is well known to decrease endothelial permeability.\(^5\)-\(^7\) Vascular endothelial cells produce S1P, whereas they express S1P1, S1P2, and S1P3 with S1P1 > S1P2 > S1P3.\(^7\) S1P was reported to induce the formation of tight junctions through S1P1.\(^5\) FTY720, a S1P-receptor modulator, accompanied by an increase in S1P1/5 and a decrease in S1P3/4, reversed BBB disruption in a rat model of encephalomyelitis.\(^8\) This study suggested that isoflurane induced SphK1 to synthesize S1P and that the activation of S1P1/3 was required for maintenance of post-SAH BBB function in mice. However, nothing is known on effects of S1P3 on endothelial permeability.\(^7\) Taken together, SphK1 and S1P1 activation may be key factors for isoflurane to induce S1P-mediated protection of post-SAH BBB.

Isoflurane anesthesia dose-dependently increases cerebral blood flow at the same time as decreasing metabolism\(^8\) and significantly increased BBB permeability associated with capillary dilatation at 3% in normal animals.\(^9\) Isoflurane inhibited neuronal injury dose-dependently, which was maximal at 2%.\(^10\) In this study, we tested 2 concentrations (1% and 2%) of isoflurane treatment because it is clinically relevant and demonstrated that only 2% isoflurane is protective against post-SAH BBB disruption in mice. Our data suggest that 2% isoflurane may work not only for anesthesia, but also for prevention of post-SAH BBB disruption through the sphingosine-related pathway.

This study has some limitations, including no studies of effects of isoflurane or the inhibitors on cerebral blood flow and sham-operated animals, comparisons of isoflurane’s neuroprotective effects with other anesthetics as well as the detailed mechanisms how isoflurane protects or enhances BBB-related proteins. Thus, further studies are needed.

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**Disclosures**

None.

**References**

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Supplemental Methods

Experimental Design and Animal Groups

The animal and ethics review committee at Loma Linda University evaluated and approved all protocols. Eight-week-old male CD-1 mice (weight 30 to 38g; Charles River, Wilmington, MA) were used for the study (n=143).

To examine whether isoflurane prevented blood-brain barrier (BBB) disruption after subarachnoid hemorrhage (SAH) in mice (study 1), animals were randomly divided into 4 groups and evaluated at 24 hours: sham-operated+vehicle-air (n=17), SAH+vehicle-air (n=25), SAH+1% isoflurane (n=9), and SAH+2% isoflurane (n=22).

To determine whether the protective effects by 2% isoflurane involved sphingosine kinase (SphK) expression and sphingosine 1-phosphate (S1P) receptor activation (study 2), we used a SphK antagonist, N, N-dimethylsphingosine (DMS), and a S1P receptor-1 (S1P1)- and S1P3-receptor antagonist, VPC23019. Animals were randomly divided into 4 groups and evaluated at 24 hours post-SAH: dimethyl sulfoxide (DMSO, a vehicle)+sham-operated+vehicle-air (n=11), DMSO+SAH+2% isoflurane (n=15), DMS+SAH+2% isoflurane (n=18), and VPC23019+SAH+2% isoflurane (n=18).

Mouse SAH Model

SAH endovascular perforation model was produced as described previously.1 Briefly, animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg). A sharpened 4-0 monofilament nylon suture was advanced through the left internal carotid artery (ICA) to perforate the anterior cerebral artery. In the sham surgery the filament was advanced 5 mm through the ICA without perforating the artery. Body temperature was kept constant (37.5±0.5°C) during the operation.

Drug Administration

One hour after SAH induction, 1% or 2% isoflurane (Baxter, Deerfield, IL) was continuously administered for 1 hour with vehicle air (30% O2 and 70% medical air).

For pharmacological interventions, the following inhibitors were used: 1) a potent and specific SphK inhibitor DMS2 (Enzo Life Sciences Inc., Plymouth Meeting, PA) dissolved in DMSO (final concentration, 0.17µg/0.5µL); and 2) a S1P1- and S1P3-receptor antagonist VPC230193 (Avanti Polar Lipids Inc., Alabaster, Alabama) dissolved in DMSO/1N hydrogen chloride (95:5; final concentration, 0.26µg/0.5µL). These drugs were further diluted in phosphate-buffered saline (PBS) to a total volume of 0.5µL, and administered intracerebroventricularly 1 hour before surgery. The vehicle groups were given the same volume (0.5µL) of DMSO (1.1g/mL/kg) diluted in PBS.

The dose for intracerebroventricular injections was calculated by determining the conversion coefficient by taking the ratio between blood volume (84.7±1.2mL/kg; 2.3mL per mouse)4 and cerebrospinal fluid (CSF) volume (0.04mL per mouse).5 The dose for systemic administration (DMS, 0.33mg/kg=0.01mg/mouse; VPC23019, 0.5mg/kg=0.015mg/mouse) that was used in previous studies2,6 was divided by the conversion coefficient (0.01mg/57.5=0.17µg for DMS; 0.015mg/57.5=0.26µg for VPC23019) to achieve an equivalent CSF concentration of the inhibitors.
Intracerebroventricular Infusion

Mice were placed in a head holder (Stoelting Stereotactic Instrument, Wood Dale, IL) and a 26g-gauge needle of a 10µL Hamilton syringe (Microliter #701; Hamilton Company, Reno, NV) was inserted through a burr hole perforated on the skull into the right lateral ventricle using the following coordinates relative to bregma: 0.1mm posterior; 0.9mm lateral; and 3.1mm below the horizontal plane of bregma. Sterile DMSO or drug (0.5µL) was automatically infused at a rate of 0.1µL/minute 60 minutes before the sham surgery or SAH induction. The needle was removed 10 minutes after completion of the infusion, and the burr hole was quickly plugged with bone wax.

Severity of SAH

The severity of SAH was blindly evaluated using the SAH grading scale at sacrifice. The SAH grading system was as follows: the basal cistern was divided into six segments, and each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood clot in the segment: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood clot with recognizable arteries; and grade 3, blood clot obliterating all arteries within the segment. The animals received a total score ranging from 0 to 18 after adding the scores from all six segments. Eight mice with SAH grading scores ≤7, which had no significant brain injury, were excluded.

Mortality and Neurological Scores

Mortality was calculated at 24 hours after SAH. Neurological score was blindly evaluated at 24 hours after SAH as previously described. The evaluation consists of six tests that can be scored 0 to 3 or 1 to 3. These six tests include: spontaneous activity; symmetry in the movement of all four limbs; forelimbs outstretching; climbing; side stroking; and response to vibrissae (whisker stimulation). Animals were given a score of 3 to 18 in 1-number steps (higher scores indicate greater function).

Brain Water Content (Brain Edema)

Brains were quickly removed and separated into the left and right cerebral hemispheres, cerebellum, and brain stem, and weighed (wet weight) at 24 hours after surgery (n=6 per group). Next, brain specimens were dried in an oven at 105°C for 72 hours and weighed again (dry weight). The percentage of water content was calculated as ([wet weight-dry weight]/wet weight)X100%.

BBB Disruption

At 24 hours after operation, a 2% solution of Evans blue dye (4mL/kg of body weight) was injected intraperitoneally, and allowed to circulate for 3 hours. Under deep anesthesia, mice (n=5 per group) were sacrificed by intracardial perfusion with PBS, and brains were removed and divided into the same regions as the brain water content study. Brain specimens were weighed, homogenized in PBS, and centrifuged at 15,000g for 30 minutes. Then, 0.5mL of the resultant supernatant was added to an equal volume of 50% trichloroacetic acid. After overnight incubation and centrifugation at 15,000g for 30 minutes at 4°C, the supernatant was taken for spectrophotometric quantification of extravasated Evans blue dye at 610nm as described previously.
Western Blotting
The left cerebral hemisphere (perforation side) was isolated and collected at 24 hours after SAH (n=6 per group in study 1; n=5 per group in study 2). Western blotting was performed as previously described using the following primary antibodies: anti-SphK1 (1:250, Abgent, San Diego, CA), anti-SphK2 (1:250, Lifespan Biosciences, Seattle, CA), anti-occludin, anti-claudin-5, anti-JAM-A, and anti-VE-cadherin (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

Statistics
Neurologic scores and SAH grade were expressed as median±25th to 75th percentiles, and other data were expressed as mean±SD. After confirming that each population being compared followed a normal distribution using Shapiro-Wilk W tests, neurologic scores and SAH grade were analyzed using Kruskal-Wallis test, followed by Steel-Dwass multiple comparisons. Other statistical differences were analyzed using one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc tests. Differences in mortality were tested using Fisher’s exact tests or chi-square tests as appropriate. P<0.05 was considered statistically significant.

In the statistical analysis, we calculated the power of the tests. The number of animals per group necessary to reach the desired power of 0.800 was in the range of 4 to 6.
Supplemental Figures

Figure S1. Representative Western blots and quantitative analysis of SphK1 (A) and SphK2 (B) expressions in the left cerebral hemisphere at 24 hours after SAH (study 1). The protein band density values are calculated as a ratio of that of β-actin. Vehicle-air, SAH+vehicle-air group; 2% isoflurane, SAH+2% isoflurane group; values, mean±SD; *P<0.05, ANOVA.

Figure S2. SAH grade (A) and neurological score (B) at 24 hours after SAH (study 2). Values, median±25th to 75th percentiles; *P<0.05, Kruskal-Wallis test.
Supplemental References


