Isoflurane Post-Treatment Ameliorates GMH-Induced Brain Injury in Neonatal Rats

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Background and Purpose—This study investigated whether isoflurane ameliorates neurological sequelae after germinal matrix hemorrhage (GMH) through activation of the cytoprotective sphingosine kinase/sphingosine-1-phosphate receptor/Akt pathway.

Methods—GMH was induced in P7 rat pups by intraparenchymal infusion of bacterial collagenase (0.3 U) into the right hemispheric germinal matrix. GMH animals received 2% isoflurane either once 1 hour after surgery or every 12 hours for 3 days. Isoflurane treatment was then combined with sphingosine-1-phosphate receptor-1/2 antagonist VPC23019 or sphingosine kinase 1/2 antagonist N,N-dimethylsphingosine.

Results—Brain protein expression of sphingosine kinase-1 and phosphorylated Akt were significantly increased after isoflurane post-treatment, and cleaved caspase-3 was decreased at 24 hours after surgery, which was reversed by the antagonists. Isoflurane significantly reduced posthemorrhagic ventricular dilation and improved motor, but not cognitive, functions in GMH animals 3 weeks after surgery; no improvements were observed after VPC23019 administration.

Conclusions—Isoflurane post-treatment improved the neurological sequelae after GMH possibly by activation of the sphingosine kinase/Akt pathway.

Key Words: apoptosis ■ caspase-3 ■ isoflurane ■ sphingosine kinase
Data are expressed as mean±SEM. Western blot and ventricular dilation data were analyzed by 1-way ANOVA with the Tukey test. Behavior data were analyzed by 1-way ANOVA on ranks, with the Student–Newman–Keuls test. A P value of <0.05 was considered statistically significant.

Results
Isoflurane Activated Cerebral SphK1/Akt Signaling and Reduced CC3 Expression After GMH

Western blot analyses were conducted at 24 hours after surgery (n=6 per group). Decreased brain protein levels of SphK1 were found in all GMH groups compared with sham-operated animals (P<0.05; Figure 1A). Isoflurane treatment significantly increased brain levels of SphK1 (P<0.05 compared with vehicle), which was reversed in Iso1h+VPC and Iso1h+DMS (both P<0.05 compared with Iso1h).

Significantly decreased levels of SphK2 were measured in brain specimens of GMH animals (P<0.05 compared with sham; Figure 1B); however, SphK2 was similarly expressed in the brain of treated and untreated GMH animals (P>0.05). Changes in the expression of phosphorylated and, therefore, activated Akt (p-Akt, Ser473) were evaluated as a ratio to total Akt (Figure 1C). Akt phosphorylation was significantly reduced within the brain of vehicle animals (P<0.05 compared with sham); however, isoflurane treatment significantly increased the p-Akt/Akt ratio. This treatment effect was

![Figure 1](https://example.com/figure1.png)

Figure 1. Western blot analysis of (A) sphingosine kinase-1 (Sphk1), (B) Sphk2, (C) p-Akt/Akt, and (D) cleaved caspase-3 (CC3) at 24 hours after germinal matrix hemorrhage induction. Values are expressed as mean±SEM, normalized to sham. *P<0.05 compared with sham; †P<0.05 compared with vehicle, and ‡P<0.05 compared with Iso1h. n=6 per group.
marginally reduced by VPC ($P>0.05$ compared with Iso1h) and reversed by DMS ($P<0.05$ compared with Iso1h). CC3 expression was significantly increased in the vehicle group ($P<0.05$ compared with sham; Figure 1D); however, isoflurane treatment significantly reduced CC3 expression ($P<0.05$ compared with vehicle), which was reversed by VPC and DMS ($P<0.05$ compared with Iso1h).

**Isoflurane Improved Long-Term Motor Function After GMH**

Motor function and coordination were evaluated using foot fault and rotarod assessments. Cognitive function (spatial memory) was evaluated via the Morris water maze. Neurofunctional testing was conducted between days 21 and 28 after surgery. GMH animals demonstrated significantly more foot faults than sham ($P<0.05$; Figure 2A). Iso1h and Iso3d reduced the number of foot faults compared with vehicle administration ($P<0.05$), which was reversed by VPC ($P<0.05$ compared with Iso1h and Iso3d). Similarly, Iso3d demonstrated significantly better rotarod performances compared with vehicle and Iso1h animals ($P<0.05$; Figure 2B). Vehicle and Iso3d+VPC animals spent less time in the target quadrant during probe water maze trials ($P<0.05$ compared with sham; Figure 2C); however, no significant differences were found between treated and untreated GMH animals ($P>0.05$).

**Isoflurane Reduced Ventricular Volume After GMH**

At 28 days after GMH induction, histological brain samples were used for the analysis of ventricular volume (Figure 3A and 3B). GMH surgery resulted in significant ventricular dilation ($P<0.05$ compared with sham); however, Iso3d showed significantly reduced ventricular volumes when compared with those that received vehicle or single isoflurane administration ($P<0.05$).

**Discussion**

This study evaluated the efficacy of isoflurane post-treatment as a potential therapeutic modality for GMH-induced brain injury. We found that 60 minutes of isoflurane exposure, starting at 1 hour after GMH induction, increased SphK1 and p-Akt, resulting in decreased CC3. This antiapoptotic effect was reversed by the S1PR1/2 antagonist VPC or the pan-SphK antagonist DMS. On the basis of these findings, we suggest that isoflurane exerts its antiapoptotic properties, at least to some extent, through activation of the SphK/S1PR/Akt pathway. Isoflurane-mediated activation of S1P anabolic enzymes, SphK1 and 2, followed by subsequent stimulation of S1PR and Akt, has been demonstrated in preclinical studies of adult hemorrhagic stroke and hypoxic-ischemic brain injury; herein, we report this molecular association in neonatal GMH. Although isoflurane post-treatment did not cause...
Sphk2 upregulation, pretreatment has been shown to be neuroprotective in a mouse ischemic stroke model. In this study, knockout mice for the SPHK2 gene did not benefit from the neuroprotective effects of isoflurane before middle cerebral artery occlusion. In addition, the expression of Sphk2 in wild-type mice was significantly increased, whereas Sphk1 expression did not change from pretreatment with isoflurane. In our study, only Sphk1 showed a significant increase because of anesthetic post-treatment. The differences between our findings compared the above reference could be because of pre versus post exposure and the nature of ischemic versus hemorrhagic stroke pathologies. Apoptotic cell death, reaching a maximum at 24 hours after injury induction, was shown in a rabbit model of GMH, suggesting that apoptotic processes begin shortly after brain hemorrhage. Thus, treatments targeting apoptotic cell death must be given in a timely manner. For this reason we chose to administer our first treatment at 1 hour after GMH. Apoptotic cell death after GMH may be of multifactorial pathogenesis, mediated by local inflammation, proteases, and free radicals. Expression of the Sphk1/S1PR/Akt pathway beyond 24 hours has not been performed in the GMH model; however, in adult experimental subarachnoid hemorrhage, the rise in pAkt levels are found to occur in the delayed phase.

Long-term evaluations of functional recovery were conducted to demonstrate a lasting protection of isoflurane post-treatment. We found that multiple exposures to isoflurane improved delayed motor deficits, yet only a marginal cognitive improvement was seen after GMH. The underlying reason for this outcome remains to be elucidated.

The improvement of long-term behavior deficits paralleled reduced ventricular volume 28 days after GMH. Our long-term results suggest that isoflurane-mediated neuroprotection after GMH was at least partially achieved via S1PR signaling. Neurotoxic effects of isoflurane have been observed by an increase of CC3 in the intact brain. However, our results indicate that this proapoptotic signal is inhibited by isoflurane post-treatment during neonatal hemorrhagic stroke. Furthermore, studies have shown that isoflurane toxicity can affect the developing brain and should be the subject of additional investigation. We suggest that the balance of apoptosis and brain development during the healthy neonatal period is delicate and therefore susceptible to pharmacological interventions that may be toxic, such as anesthetic administration. However, after cerebral hemorrhage, proapoptotic factors are overwhelmingly elevated, an environment that is well documented to be amenable to antia apoptotic agents.

In conclusion, we have demonstrated that isoflurane post-treatment provides significant long-term protection from GMH-induced brain injury. Its protective effects may be partially mediated by stimulation of Sphk1/S1PR/Akt signaling, with consequent reduction of apoptotic cell death.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL

Operative Procedure

Time pregnant Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN); and P7 rat pups of both genders were subjected to germinal matrix hemorrhage (GMH) induction. General anesthesia was achieved with isoflurane (3% in 30/70% Oxygen/Medical Air). The anesthetized rat pup was placed prone with its head secured onto a stereotaxic frame. Next, the scalp was sterilized with betadine and a small midline incision was made to expose the skull sutures and bregma. The following coordinates were measured relative to bregma: 1.8 mm rostral and 1.5 mm lateral to the right. At these coordinates a small cranial burr hole was made, using a standard dental drill (1 mm), through which a 26-gauge needle was lowered 2.8 mm into the brain parenchyma. Next, clostridial collagenase VII-S (0.3 U, Sigma, St. Louis, MO) was infused through the needle at a rate of 0.25 µl/minute. The needle was left in place for 10 minutes after completed collagenase infusion, to prevent its back-leakage along the needle tract. The needle was then slowly withdrawn, the burr hole was sealed with bone wax, and the skin was sutured closed. All animals were allowed to fully recover on a 37°C warm heating blanket before being returned to their dams. Sham procedure consisted of needle insertion only.

Western Blotting

Whole brain samples were collected at 24 hours after surgery, and processed according to previously published protocols. Briefly, individual protein concentrations of each specimen were determined via DC protein assays (Bio-Rad, Hercules, CA). Equal amounts of protein (50 µg per sample) were separated by SDS-PAGE and transferred onto nitrocellulose membranes, which then were blocked and incubated with the following antibodies: anti-sphingosine-kinase-1 (1:250, Abgent, San Diego, CA), and anti-sphingosine-kinase-2 (1:250, Lifespan Biosciences, Seattle, WA). The following primary antibodies were obtained from Cell Signaling Technology (Denver, MA): anti-phosphorylated-Akt (Ser473, 1:1000), anti-Akt (1:1000), and anti-cleaved-caspase-3 (1:1000). Anti-β-actin and all appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (1:500, Santa Cruz, CA). After incubation with the primary and secondary antibodies, immunoblots were visualized with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL). Images were semi-quantitatively analyzed, using Image J (NIH) and results were expressed as mean±SEM normalized to sham.

Long-term Behavioral Testing

Rodent motor and cognitive functions were evaluated between day 21 and 28 after surgery, as previously described. Foot-fault testing assessed the number of limb missteps through the openings of a horizontally elevated wire-grid (20 cm x 40 cm), onto which rats were placed for 2 minutes. The total number of foot faults was counted for each animal.
The rotarod test assessed motor and coordination function in animals subjected to GMH or sham surgery. The apparatus (Columbus Instruments, Columbus, OH) consists of a horizontally rotating cylinder (7 cm in diameter, 9.5 cm in width) that was programmed to maintain a constant velocity or to accelerate 2 rpm every 5 seconds, thus requiring continuous walking in order to avoid falling. The latency to fall was recorded by a photobeam circuit for each animal.

Cognitive function and memory ability was assessed in rodents via the Morris water maze, as previously described. This test required each animal to localize a slightly submerged platform in a round pool (diameter: 110 cm) filled with water. Following the cued trails (visible platform), the platform was submerged for 10 successive trials and then removed from the pool (probe trial). The time each animal spent in the target quadrant (containing the previous location of the platform) was recorded for 60 seconds by an overhead camera, and analyzed via a computerized tracking system (Noldus Ethovision, Tacoma, WA).

**Histopathological analysis**

At day 28 after surgery rats were terminally anesthetized with isoflurane (>5%) and transcardially perfused with ice-cold PBS and 10% paraformaldehyde. Next, brains were removed and post-fixed in paraformaldehyde (at 4°C for 3 days) and dehydrated in 30% sucrose for the same amount of time. Frozen coronal brain sections of 10 µm were cut every 600 µm on a cryostat (CM3050S, Leica Microsystems). All sections were stained with cresyl violet and morphometric analysis was conducted via computer-assisted (Image J, NIH) hand delineation of the cerebroventricular system. The borders of the cerebroventricular system were based on the criteria derived from stereologic studies using optical dissector principles. Ventricular volume was calculated as average ventricular area multiplied by the depth (µm) between first and last brain slice, showing cerebroventricular structures.
Supplemental References


