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Isoflurane post-treatment ameliorates GMH-induced brain injury in neonatal rats

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Abstract

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 (0.3U) 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-phoshate 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 capase-3 was decreased at 24 hours after surgery; which was reversed by the antagonists. Isoflurane significantly reduced post-hemorrhagic ventricular dilation and improved motor, but not cognitive, functions in GMH animals 3 weeks after surgery; no improvements were observed following VPC23019 administration.

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

Keywords

Isoflurane; Germinal Matrix Hemorrhage; sphingosine kinase; Akt; caspase; apoptosis; neonatal rat

Introduction

Rupture of immature blood vessels within subventricular tissue, termed germinal matrix hemorrhage (GMH), occurs in approximately 35 live births per 10,000 presenting increasing socio-economic burdens. GMH often causes developmental delays, mental retardation, cerebral palsy, and post-hemorrhagic hydrocephalus.[1] Clinical management of GMH is

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Disclosures None

limited, invasive, and insufficient; therefore investigative studies are needed to assess novel therapeutic modalities.[2]

Volatile anesthetics such as isoflurane have demonstrated neuroprotection in experimental models of adult hemorrhagic stroke.[3–5] Yet the efficacy of isoflurane has not been evaluated following neonatal GMH.

We hypothesized isoflurane post-treatment ameliorates GMH-induced apoptotic cell death by increasing sphingosine kinase expression and sphingosine-1-phoshate receptor (S1PR)-induced activation of cytoprotective Akt. Additionally, isoflurane administration may ameliorate long-term neurological deficits and post-hemorrhagic ventricular dilation in a dose dependant manner.

Methods

Loma Linda University Animal Care Committee approved all protocols and procedures. Detailed methods are available in the online-only Supplemental Materials. GMH was induced in P7 rat pups by stereotaxic-assisted infusion of 0.3U collagenase, as described.[6] Thirty rat pups were utilized to determine brain expression of sphingosine kinases 1 and 2, phosphorylated Akt, total Akt, and cleaved caspase-3 by Western blot. GMH animals received either 30/70% oxygen/medical air (Vehicle); 60 minutes 2% isoflurane exposure given 1 hour after surgery (Iso1h); isoflurane combined with sphingosine-1-phosphate receptor antagonist VPC23019 (0.5mg/kg given IP at 30 minutes after surgery, Iso1h+VPC), or isoflurane combined with the pan-sphingosine kinase inhibitor N,N-dimethylsphingosine, (0.4mg/kg given IP at 30 minutes after surgery, Iso1h+DMS).

Forty additional rat pups were utilized to determine isoflurane effects on long-term behavioral performances (tested between day 21 and 28 post-GMH) and hemorrhage-induced ventricular dilation, as described.[6, 7] For this, GMH animals received either 30/70% oxygen/medical air (Vehicle); 60 minutes 2% isoflurane exposure given at 1 hour after surgery (Iso1h); or 60 minutes 2% isoflurane exposure given every 12 hours for 3 days (Iso3d). The Iso3d treatment was then combined with VPC23019 (0.5mg/kg given IP 30 minutes prior to each isoflurane treatment, Iso3d+VPC). (n=8 for each group)

Data are expressed as mean±SEM. Western blot and ventricular dilation data were analyzed by One-way ANOVA with the Tukey test. Behavior data was analyzed by One-way ANOVA on ranks, with the Student-Newman-Keuls test. A P value of <0.05 was considered statistically significant.

Results

Isoflurane Activated Cerebral Sphingosine Kinase-1/Akt Signaling and Reduced Cleaved-Caspase-3 Expression after GMH

Western blot analyses were conducted at 24 hours after surgery (n=6 per group). Decreased brain protein levels of sphingosine kinase-1 (SphK1) were found in all GMH groups compared to sham operated animals ($p<0.05$; Fig 1A). Isoflurane treatment significantly increased brain levels of SphK1 ($p<0.05$ compared to vehicle), which was reversed in Iso1h +VPC and Iso1h+DMS (both $p<0.05$ compared to Iso1h). Significantly decreased levels of sphingosine kinase-2 (SphK2) were measured in brain specimens of GMH animals ($p<0.05$ compared to sham; Fig 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 (Fig 1C). Akt phosphorylation was significantly reduced within the brain of vehicle animals ($p<0.05$

compared to sham); however, isoflurane treatment significantly increased the p-Akt/Akt ratio. This treatment effect was marginally reduced by VPC ($p>0.05$ compared to Iso1h) and reversed by DMS ($p<0.05$ compared to Iso1h). Cleaved caspase-3 (CC3) expression was significantly increased in the vehicle group ($p<0.05$ compared to sham; Fig 1D); however, isoflurane treatment significantly reduced CC3 expression ($p<0.05$ compared to vehicle), which was reversed by VPC and DMS ($p<0.05$ compared to 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 day 21 and 28 after surgery. GMH animals demonstrated significantly more foot faults than sham ($p<0.05$; Fig 2A). Iso1h and Iso3d reduced the number of foot faults compared to vehicle administration ($p<0.05$), which was reversed by VPC ($p<0.05$ compared to Iso1h and Iso3d). Similarly, Iso3d demonstrated significantly better rotarod performances compared to vehicle and Iso1h animals ($p<0.05$; Fig 2B). Vehicle and Iso3d+VPC animals spent less time in the target quadrant during probe water maze trials ($p<0.05$ compared to sham; Fig 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 utilized for analysis of ventricular volume (Fig 3A-B). GMH surgery resulted in significant ventricular dilation ($p<0.05$ compared to sham); however Iso3d showed significantly reduced ventricular volumes when compared to 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-sphingosine kinase antagonist DMS. Based on 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,[3] and hypoxic-ischemic brain injury;[8] herein we report this molecular association in neonatal GMH. Even though isoflurane post-treatment did not cause Sphk2 upregulation, pretreatment has been shown to be neuroprotective in a mouse ischemic stroke model.[9] In this study, knockout mice for the SPHK2 gene did not benefit from the neuroprotective effects of isoflurane before middle cerebral artery occlusion. Additionally, 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 due to anesthetic post-treatment. The differences between our findings compared the above reference could be due to 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,[10] suggesting 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 etiology, mediated by local inflammation, proteases and free radicals.[10, 11] “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[12].

Long-term evaluations of functional recovery were conducted to demonstrate a lasting protection of isoflurane post-treatment.[13] 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-conferred 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[14]. However, our results indicate that this pro-apoptotic signal is inhibited by isoflurane post-treatment during neonatal hemorrhagic stroke. Furthermore, studies have shown that isoflurane toxicity can affect the developing brain[15] 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. On the other hand, after cerebral hemorrhage pro-apoptotic factors are overwhelmingly elevated, an environment that is well documented to be amenable to anti-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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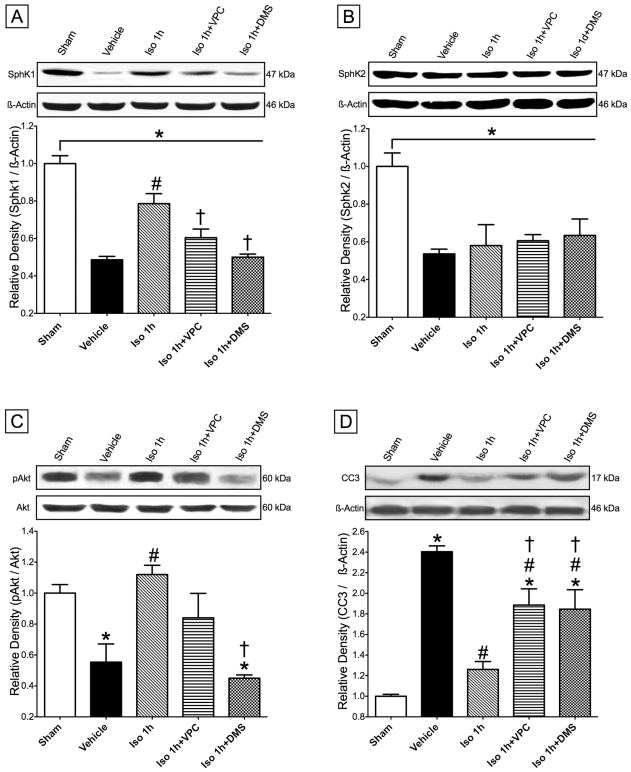

Figure 1.

Western blot analysis of (**A**) sphingosine kinase-1 (Sphk1), (**B**) sphingosine kinase-2 (Sphk2), (**C**) p-Akt/Akt, and (D) cleaved caspase-3 (CC3) at 24 hours post GMH-induction. Values are expressed as mean±SEM, normalized to sham. * $p<0.05$ compared to sham, # $p<0.05$ compared to vehicle, † $p<0.05$ compared to Iso1h. N=6 per group.

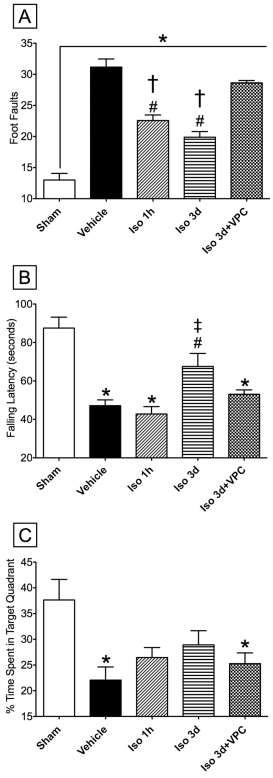

Figure 2.

(**A**) Foot fault, (**B**) Rotarod, and (**C**) Morris water maze-probe trial testing at 21 to 28 days post GMH-induction. Values are expressed as mean±SEM. * $p<0.05$ compared to sham, # $p<0.05$ compared to vehicle, † $p<0.05$ compared to Iso3d+VPC, ‡ $p<0.05$ compared to Iso1h. N=8 per group.

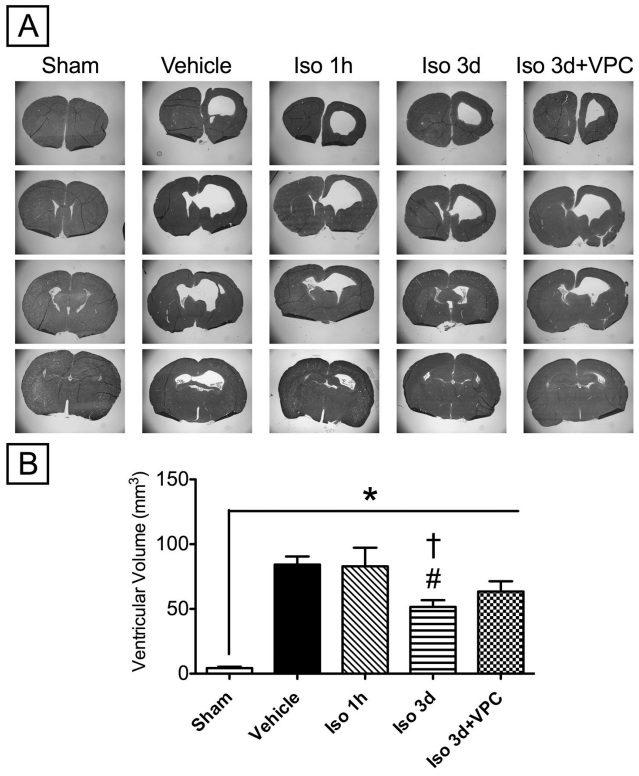

Figure 3.

(**A**) Representative microphotographs of cresyl-violet stained brain sections illustrating ventriculomegaly at 28 days post GMH-induction. (**B**) Quantification of ventricular volume at 28 days post GMH-induction. Values are expressed as mean±SEM. * $p<0.05$ compared to sham, # $p<0.05$ compared to vehicle, † $p<0.05$ compared to Iso1h. N=8 per group.