Isoflurane Posttreatment Reduces Neonatal Hypoxic–Ischemic Brain Injury in Rats by the Sphingosine-1-Phosphate/Phosphatidylinositol-3-Kinase/Akt Pathway

Yilin Zhou, MD; Tim Lekic, BS; Nancy Fathali, BS; Robert P. Ostrowski, PhD; Robert D. Martin, MD; Jiping Tang, MD; John H. Zhang, MD, PhD

Background and Purpose—Isoflurane, administered before or during cerebral ischemia, has been shown to exhibit neuroprotection in animal models of ischemic stroke. However, the underlying mechanism remains to be elucidated. In the present study, we determined whether isoflurane posttreatment provides neuroprotection after neonatal hypoxia–ischemia (HI) in rats and evaluated the role of the sphingosine-1-phosphate/phosphatidylinositol-3-kinase/Akt pathway in this volatile anesthetic-mediated neuroprotection.

Methods—HI was induced in postnatal day 10 (P10) rat pups by unilateral carotid ligation and 2 hours of hypoxia. For treatment, 2% isoflurane was administered immediately after HI for 1 hour. As pharmacological interventions, the sphingosine-1-phosphate antagonist VPC23019, phosphatidylinositol-3-kinase inhibitor wortmannin, or opioid antagonist naloxone was administered before HI. Isoflurane posttreatment was evaluated for effects on infarct volume at 48 hours after HI and brain atrophy and neurological outcomes at 4 weeks after HI. The expression of phosphorylated Akt and cleaved caspase-3 was determined by Western blotting and immunofluorescence analysis.

Results—Isoflurane posttreatment significantly reduced infarct volume at 48 hours after HI. VPC23019 or wortmannin abrogated the neuroprotective effect of isoflurane, whereas naloxone did not inhibit the isoflurane-induced neuroprotection. Isoflurane posttreatment significantly preserved phosphorylated Akt expression and decreased cleaved caspase-3 levels. These effects were reversed by VPC23019 and wortmannin, respectively. Isoflurane also confers long-term neuroprotective effects against brain atrophy and neurological deficits at 4 weeks after HI.

Conclusions—Isoflurane posttreatment provides lasting neuroprotection against hypoxic–ischemic brain injury in neonatal rats. Activation of the sphingosine-1-phosphate/phosphatidylinositol-3-kinase/Akt pathway may play a key role in isoflurane posttreatment-induced neuroprotection. (Stroke. 2010;41:1521-1527.)

Key Words: Akt • apoptosis • isoflurane • neonatal hypoxia–ischemia • sphingosine 1-phosphate

Perinatal hypoxia–ischemia (HI) continues to be a major contributor to mortality and lifelong neurological impairments in infants and children.1,2 The incidence is as high as 1 in 4000 live births.2 Although the underlying pathophysiology of neonatal HI, including oxidative stress, excitotoxicity, inflammation, and apoptosis,2 has been intensively studied, successful treatments for neonatal HI are still lacking.

Isoflurane, a volatile anesthetic, has been commonly and safely used in surgical procedures for decades. Accumulating evidence indicates that isoflurane, administered before or during experimental cerebral ischemia, provides neuroprotection in both in vivo and in vitro models.3,4 Isoflurane has also been reported to reduce ischemia–reperfusion injury in the myocardium5 and kidney.6 However, whether isoflurane, administered after hypoxic–ischemic insult, confers neuroprotection in neonates has not been studied yet. The molecular pathways underlying isoflurane-induced protection have been incompletely mapped and require to be further elucidated.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite and has been shown to bind to specific G protein-coupled receptors (the S1P receptors) and regulate multiple cellular events, including promoting cell proliferation, survival, migration, and inhibiting apoptosis.7 The prosurvival phosphatidylinositol-3-kinase (PI3K)/Akt have been shown to be downstream molecules regulated by the S1P1 receptor signaling.7 A recent study reported that isoflurane ameliorates renal ischemia–reperfusion injury by initiating the S1P/S1P

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From the Departments of Anesthesiology (Y.Z., R.D.M., J.H.Z.), Physiology and Pharmacology (T.L., N.F., R.P.O., J.T., J.H.Z.), and Neurosurgery (J.H.Z.), Loma Linda University School of Medicine, Loma Linda, Calif.
Correspondence to John H. Zhang, MD, PhD, Department of Physiology, Loma Linda University School of Medicine, Risley Hall, Room 223, 11041 Campus Street, Loma Linda, CA 92354. E-mail johnzhang3910@yahoo.com
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receptor signaling pathway. However, it is unknown whether the activation of S1P/S1P receptor signaling transduction is vital for isoflurane-mediated neuroprotection in the setting of experimental cerebral hypoxia–ischemia. In the present study, we tested the effect of isoflurane as posttreatment in a rat neonatal HI model. We hypothesized that isoflurane posttreatment will result in decreased brain injury and improved neurobehavioral outcomes by activating the S1P/PI3K/Akt signaling pathway.

Materials and Methods
Animals
All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Timed pregnant Sprague-Dawley rat pups were purchased from Harlan Laboratories, Indianapolis, Ind. One hundred seventy-seven P10 rats were used in this study and randomly divided into the following groups: sham-operated (n = 18), HI group (n = 31), HI groups treated with isoflurane (n = 30), VPC23019 + isoflurane (n = 29), VPC23019 alone (n = 10), wortmannin + isoflurane (n = 29), wortmannin alone (n = 10), naxolone + isoflurane (n = 10), and naxolone alone (n = 10). Rat pups of both genders were subjected to right common carotid artery ligation under isoflurane anesthesia. Surgery time for each pup did not exceed 5 minutes. After recovery for 1 hour, the pups were placed in a hypoxia chamber, which was submerged in a water bath at a stable temperature of 37°C, and subjected to 8%O2 in N2 for 2 hours. Sham-operated animals underwent anesthesia and incision only.

Isoflurane Posttreatment and Pharmacological Interventions
In the treatment group, rat pups received isoflurane posttreatment for 1 hour immediately after hypoxia by transferring them to 60-mL syringes continually flushed with 2% isoflurane carried by 30%O2 and 70% medical air. In the HI group, pups were placed in the syringes flushed with 30% O2 and 70% medical air for 1 hour.

For pharmacological interventions, the specific S1P1/S1P3 antagonist VPC23019 (0.5 mg/kg, intracerebroventricularly; VPC+HI+Iso group) or the PI3K inhibitor wortmannin (86 ng/pup, intracerebroventricularly; WM+HI+Iso group) was administered just before the HI surgery. The nonselective opioid antagonist naxolone (5 mg/kg, intraperitoneally; Nax+HI+Iso group) was given immediately before and after the hypoxia.

Infarct Volume and Brain Atrophy Quantification
As studied previously, 2.3,5-triphenyltetrazolium chloride monohydrate staining was performed to determine the infarct volume at 48 hours after HI. The infarct volume was traced and analyzed by Image J software (Version 1.40; National Institutes of Health, Bethesda, Md). Brain atrophy was assessed at 4 weeks after HI. Brain tissue loss was expressed as the mass ratio of the ipsilateral hemisphere compared with the contralateral hemisphere.

Neurobehavioral Tests
All neurobehavioral tests were performed in a blinded setup. At 4 weeks after HI, animals underwent the following 3 neurobehavioral tests.

T-maze test for spontaneous alternation has been used to examine exploratory behavior and working memory by hippocampus dysfunction. As studied previously, rats were placed in the stem of a T-shaped maze and allowed to freely explore the 2 arms of the maze throughout a 10-trial continuous alternation session. The spontaneous alternation rate was expressed as the ratio of the alternating choices to the total number of the choice.

In the foot-fault test, the rats were placed on a horizontal grid floor for 2 minutes. The foot-fault was defined as when the rat inaccurately placed a fore- or hindlimb and it fell through 1 of the openings in the grid. Number of foot-faults was recorded. The left/right side difference of foot-faults was used for the statistical analysis.

The modified grip-traction test has been used to test the muscle strength of the rat by hanging the rat to a horizontal rope by its forepaws. Time to falling (maximum 60 seconds) was recorded.

Western Blotting
The brain samples were collected at 24 hours after HI (n = 6, respectively). Proteins of the ipsilateral hemisphere were extracted by homogenizing in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, Calif). Western blotting was performed as described previously using antiphospho-Akt (Ser473) and anticleaved caspase-3 (Cell Signaling Technology, Danvers, Mass) antibodies.

Immunofluorescence Staining
Triple-fluorescence staining of the ipsilateral hippocampus and peri-infarct region was performed at 24 hours after HI as described previously. The following primary antibodies were used: (1) anti-Akt1,2,3 (phospho-Ser473) antibody (Assay designs; Ann Arbor, Mich); (2) anticleaved caspase-3 antibody (Cell Signaling Technology); and (3) anti-MAP2 antibody (Santa Cruz Biotechnology).

Statistical Analysis
Data were expressed as the mean±SEM. Statistical differences among groups were analyzed by using 1-way analysis of variance followed by the Holm-Sidak method. P<0.05 was considered statistically significant.

Results
Mortality
The mortality rate in the HI group and isoflurane posttreatment group was as follows: 9.8% (3 of 31 pups) in the HI group and 6.7% (2 of 30 rats) in the isoflurane-treated group.

Isoflurane-Mediated Reduction in Infarct Volume Depends on the S1P/PI3K/Akt Signaling Pathway
Forty-eight hours after neonatal HI brain injury, we determined the effect of isoflurane posttreatment on infarct volume. Isoflurane posttreatment resulted in a significant reduction in infarct volume compared with the HI group (21.9±3.4% and 31.4±2.1%, respectively; Figure 1). There was no difference in isoflurane posttreatment-mediated reduction in infarct volume between genders (data not shown). To test if this isoflurane-induced neuroprotection depends on the S1P/PI3K/Akt signaling pathway or is through opioid receptor signal transduction, pups in the pharmacological intervention groups were pretreated with the S1P receptor antagonist VPC23019, the PI3K inhibitor wortmannin, or the nonselective opioid antagonist naxolone, respectively. Pretreatment with VPC23019 and wortmannin completely abrogated the reduction in infarct volume provided by isoflurane posttreatment (30.9±2.1% and 30.4±1.7%, respectively; Figure 1), whereas naxolone did not inhibit the isoflurane-mediated neuroprotection (23.1±2.4%; Figure 1). The compounds did not affect the infarct volume when administered alone (n = 10, data not shown). These findings are consistent with the hypothesis that the isoflurane-induced neuroprotection requires activation of the S1P/PI3K/Akt signaling pathway.
Isoflurane Confers Long-Term Neuroprotection Against Brain Atrophy and Neurological Deficits

Isoflurane posttreatment provided short-term protection against neonatal HI brain injury; we then sought to determine whether this beneficial effect is long-lasting. At 4 weeks after HI, extensive atrophy of ipsilateral brain tissue was observed in the HI group. Isoflurane posttreatment prevented the brain tissue loss (Figure 2). Administration of VPC23019 or wortmannin abolished the isoflurane-induced protection. Values are the mean ± SEM; *P < 0.05 versus HI group; #P < 0.05 versus sham-operated group.

We also tested the effect of isoflurane posttreatment on the functional outcomes. Animals in the sham-operated group performed normally in all 3 neurobehavioral tests at 4 weeks after HI. T-maze testing for spontaneous alternation demonstrated a significantly worse performance in the HI group. T-maze testing for spontaneous alternation demonstrated a significantly worse performance in the HI group (Figure 3A). Animals in the HI group had significantly more foot-faults with their left-sided fore- and hind-limbs, which is contralateral to the brain injury site, compared with the sham-operated and isoflurane-treated groups (Figure 3B). In the modified grip-traction test, rats in the HI group hung onto the rope approximately 15.5 ± 2.8 seconds compared with 37.6 ± 0.9 seconds in the sham-operated group and 26.8 ± 2.2 seconds in the treatment group (Figure 3C). Likewise, pretreatment with VPC23019 or wortmannin reversed the isoflurane-mediated improvement in functional outcomes (Figure 3A–C). Taken together, isoflurane posttreatment significantly improved the neurobehavioral outcomes at 4 weeks after HI. This long-lasting isoflurane-induced neuroprotection also depends on the S1P/PI3K/Akt signaling pathway.

Isoflurane Increases Phosphorylation of Akt and Decreases Expression of Cleaved Caspase-3 After HI

To further confirm that the S1P/PI3K/Akt signaling pathway underlies isoflurane-induced neuroprotection in the neonatal HI model, we measured phosphorylated Akt and cleaved caspase-3 levels in the ipsilateral hemisphere (Figure 4). The phosphorylation of Akt significantly increased after isoflurane posttreatment (Figure 4A–B), whereas cleaved caspase-3 levels drastically decreased (Figure 4C–D). These effects were reversed on the pretreatment with VPC23019 or wortmannin, respectively (Figure 4A–D).

Consistent with the Western blotting results, the immunofluorescence analysis revealed that the expression of phosphorylated Akt was decreased in neurons in ipsilateral hippocampus and peri-infarct regions of the HI group (Figures 5G and 6F), which was restored by isoflurane posttreatment (Figures 5L and 6J). VPC23019 or wortmannin prevented the isoflurane-induced increase in phosphorylated Akt level, respectively (Figures 5Q, 5V, 6N, and 6R). On the other hand, the cleaved caspase-3 level was increased in neurons in the HI group (Figures 5H and 6G) but was reduced by isoflurane posttreatment (Figures 5M and 6K). Once more, VPC23019 and wortmannin blocked the isoflurane-induced decrease in cleaved caspase-3 level, respectively (Figures 5R, 5W, 6O, and 6S). These results support the hypothesis that the activation of the S1P/PI3K/Akt pathway contributes to the isoflurane-induced protection against HI brain injury.
Isoflurane is used in clinical practice as a volatile anesthetic in the United States. In this study, we tested 2 hypotheses: (1) isoflurane posttreatment protects against HI brain injury in neonatal rats; and (2) this protective effect is through the S1P/PI3K/Akt signaling pathway. We found that 2% isoflurane, given immediately after HI, reduced infarct volume in the short-term as well as brain atrophy and neurobehavioral deficits in the long-term. Moreover, blocking the S1P receptor with VCP23019 or inhibiting PI3K by wortmannin abolished the isoflurane-mediated beneficial effects. In addition, we showed that administration of VCP23019 and wortmannin also blocked the isoflurane-induced recovery of Akt activity and decrease in cleaved caspase-3 expression, a marker of apoptotic cell death. These findings suggest that isoflurane-mediated neuroprotection against neonatal HI depends on S1P/PI3K/Akt signaling.

Volatile anesthetics, including isoflurane, have been demonstrated to provide protection against ischemic injury in various organs. Different mechanisms underlying this beneficial effect have been proposed. Isoflurane preconditioning has been shown to exhibit neuroprotection by upregulating inducible nitric oxide synthase in a neonatal HI model. Chiari et al reported the PI3K pathway was involved in isoflurane-mediated protection against myocardial infarction. However, the upstream signaling pathway involved in volatile anesthetics-mediated protection is unclear. Weihrauch et al reported that isoflurane mediates cardio-

Discussion

Evaluation of neurological outcomes in groups of sham operation (sham), HI or treated with isoflurane (HI+iso), VPC23019+isoflurane (VPC+HI+iso), wortmannin+isoflurane (WM+HI+iso); n=7 to 10 in each group. Animals in the HI group exhibited severe neurobehavioral impairments in the T-maze test (A), foot-fault test (B), and modified grip-traction test (C). Isoflurane posttreatment significantly improved neurological outcomes at 4 weeks after HI. These beneficial effects were abrogated by administration of VPC23019 or wortmannin. Values are the mean ± SEM; *P<0.05 versus HI group; #P<0.05 versus sham-operated group.

Figure 3. Representative Western blots (A and C) and quantitative analysis of phosphorylated Akt (pAkt) and cleaved caspase-3 expression (B and D) in groups of sham operation (sham; n=6), HI (HI; n=6) or treated with isoflurane (HI+iso; n=6), VPC23019+isoflurane (VPC+HI+iso; n=6), and wortmannin+isoflurane (WM+HI+iso; n=6) at 24 hours after HI. The HI-induced decrease in pAkt level was prevented by isoflurane posttreatment. Pretreatment with VCP23019 or wortmannin reversed the isoflurane-mediated increase in pAkt expression (A–B). The cleaved caspase-3 level was increased in the HI group, which was decreased by isoflurane posttreatment. This isoflurane-induced reduction in the cleaved caspase-3 expression was blocked by administration of VPC23019 or wortmannin, respectively (C–D). Values are the mean ± SEM; *P<0.05 versus HI group; #P<0.05 versus sham-operated group.

Figure 4. Evaluation of neurological outcomes in groups of sham operation (sham), HI or treated with isoflurane (HI+iso), VPC23019+isoflurane (VPC+HI+iso), wortmannin+isoflurane (WM+HI+iso); n=7 to 10 in each group. Animals in the HI group exhibited severe neurobehavioral impairments in the T-maze test (A), foot-fault test (B), and modified grip-traction test (C). Isoflurane posttreatment significantly improved neurological outcomes at 4 weeks after HI. These beneficial effects were abrogated by administration of VPC23019 or wortmannin. Values are the mean ± SEM; *P<0.05 versus HI group; #P<0.05 versus sham-operated group.
tection in a rabbit myocardial ischemia model through the opioid receptor. However, we found that naloxone, a nonselective opioid antagonist, could not abolish the isoflurane posttreatment-induced neuroprotection in the neonatal HI model. The discrepancy between our finding and that of Weihrauch et al is probably due to different species and ischemic models (organs) studied in these 2 experiments. Most volatile anesthetics are lipophilic and have been reported to activate sphingomyelin hydrolysis in brain tissue by increasing membrane fluidity and allowing the sphingomyelinase to increase its hydrolytic effect. Sphingosine is the sphingomyelin breakdown product. It is then phosphorylated by the sphingosine kinase to form S1P. Likewise, isoflurane has been shown to enhance the production of S1P, the sphingolipid metabolite in the renal cortex in vivo, and in human proximal tubule cells. In addition, a recent study showed that isoflurane activates sphingosine kinase activity and synthesis of S1P in renal tubule cells to afford renal protection through the S1P signaling pathway in the setting of renal ischemia–reperfusion injury. Thereby, we sought to examine whether there is a role for the S1P signaling pathway in mediating isoflurane posttreatment-induced neuroprotection after neonatal HI. Because neurons and astrocytes express mainly S1P1 and S1P3 receptors, we used VPC23019, a competitive antagonist at both the S1P1 and S1P3 receptors, as a pharmacological intervention. We found that administration of VPC23019 blocked the isoflurane-mediated reduction in infarct volume and apoptotic cell death as indicated by the reversal of isoflurane-induced decrease in the cleaved caspase-3 levels in the ipsilateral hemisphere. Furthermore, VCP23019 reversed isoflurane-induced activation of Akt. These data implicate that the activation of the S1P/PI3K/Akt signaling contributes to the isoflurane-mediated neuroprotective effects in neonatal HI.

S1P is an important lipid mediator and ligand for a family of 5 G-protein-coupled receptors (S1P1 to S1P5). On binding to its specific receptors, S1P has been implicated in diverse biological processes, including cell growth, differentiation, survival, and migration. The PI3K/Akt signaling cascade has been shown to play a key role in preventing apoptosis under hypoxic or ischemic conditions. Morales-Ruiz et al demonstrated that S1P activates PI3K/Akt signaling through the pertussis toxin-sensitive G-protein in endothelial cells. In our current study, we first confirmed that wortmannin, a PI3K

Figure 5. Representative immunofluorescence images showing the colocalization of phosphorylated Akt (pAkt; green) and cleaved caspase-3 (red) with MAP2 (blue)–positive cells in the ipsilateral hippocampus in groups of sham operation (sham; A–D), HI (HI; F–I) or treated with isoflurane (HI+Iso; K–N), wortmannin–isoflurane (WM+HI+Iso; P–S), and VPC23019–isoflurane (VPC+HI+Iso; U–X) at 24 hours after HI. Cells in the ipsilateral hippocampus were fixed and stained with 4',6-diamidino-2-phenylindole (E, J, O, T, Y). Arrows indicate apoptotic cells with pyknotic nuclei. The number of nuclei with an apoptotic morphology was much lower in the isoflurane-treated group compared with the HI group and the groups pretreated with VPC23019 or wortmannin. Scale bar of images A–D, F–I, K–N, P–S, and U–X: 50 μm; scale bar of images E, J, O, T, and Y: 20 μm.
inhibitor, given intracerebroventricularly, inhibited the increase in phosphorylated Akt levels seen after isoflurane posttreatment. We then demonstrated that pretreatment with wortmannin abolished the isoflurane-induced neuroprotection, as shown by a reversal of the reduction in infarct volume and cleaved caspase-3 expression provided by isoflurane posttreatment. Taken together, our findings indicate that the S1P/PI3K/Akt signaling pathway underlies isoflurane-mediated neuroprotection in neonatal HI.

Despite our findings, the reversal of isoflurane-mediated neuroprotection with the nonspecific inhibitor wortmannin cannot completely exclude involvement of other pathways. Studies using Akt small interfering RNA would be more specific to confirm the contribution of the PI3K/Akt signaling cascades. In this study, we used VCP23019, a competitive antagonist at the S1P1 and S1P3 receptors, as the pharmacological approach. Involvement of other S1P receptor subtypes cannot be ruled out. In this regard, further studies are needed. In contrast to its beneficial effect, isoflurane has been shown to induce apoptotic neurodegeneration in P7 rat pups when administered for 6 hours. However, Zhao et al showed that isoflurane pretreatment (1.5% for 30 minutes) does not induce neuronal loss in P7 rat pups after HI. Also, Wise-Faberowski et al reported that isoflurane treatment (30, 60, and 90 minutes) reduces oxygen and glucose deprivation-induced neuronal apoptosis in a concentration-dependent manner (1.13%, 2.3%, or 3.3%) in neuronal cell culture prepared from newborn rats. Therefore, we believe that it is safe to administer isoflurane for 1 hour as a posttreatment after neonatal HI. Isoflurane appears to confer a dose-dependent dual action. Excessive exposure causes neurodegeneration, possibly through alteration of N-methyl-D-aspartate and γ-aminobutyric acid type A receptors, which overwhelms the isoflurane-mediated protective pathways. Further experiments are warranted with the goal to enhance isoflurane neuroprotection and avoid its neurotoxicity.

Overall, we conclude that isoflurane posttreatment reduced infarct volume, brain atrophy, and improved neurobehavioral

![Figure 6](image-url). Representative immunofluorescence colocalization of phosphorylated Akt (pAkt; green) and cleaved caspase-3 (red) with MAP2 (blue)-positive cells in the ipsilateral peri-infarct region in groups of sham operation (sham; A–D), HI (HI; E–H) or treated with isoflurane (HI+Iso, I–L), wortmannin+isoflurane (WM+HI+Iso; M–P), and VPC23019+isoflurane (VPC+HI+Iso; Q–T) at 24 hours after HI. Scale bar: 50 μm.
outcomes in neonatal HI in rats. The isoflurane-induced neuroprotection is S1P/PI3K/Akt signaling pathway-dependent. Because isoflurane has been introduced into clinical practice over 3 decades, it may represent a new and effective treatment against neonatal HI brain injury.

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Disclosures
None.

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