Isoflurane cerebral preconditioning in a spontaneous hypertension rat model is associated with sphingosine kinases

*Jun Ge^{1,2,B–F}, *Keyu Kong^{1,2,B–F}, Xiaoqiang Cheng^{1,2,B,C,E,F}, Tianyi Wu^{2,B,D,F}, Dingge Liu^{2,B,D,F}, Lingling Peng^{2,B,D,F}, Zhijia Shen^{2,B,D,F}, Guoyuan Lu^{3,A,F}, Yanping Shen^{3,A,C,E,F}

¹ Department of Surgery, First Affiliated Hospital of Soochow University, Suzhou, China

² Medical College, Soochow University, Suzhou, China

³ Department of Nephrology, First Affiliated Hospital of Soochow University, Suzhou, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;

D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online)

Adv Clin Exp Med. 2020;29(6):695–700

Address for correspondence

Yanping Shen

E-mail: shenyanping@suda.edu.cn

Funding sources

None declared

Conflict of interest

None declared

* These authors contributed equally to this work.

Received on June 16, 2019

Reviewed on January 23, 2020

Accepted on May 1, 2020

Published online on June 29, 2020

Abstract

Background. Isoflurane preconditioning could reduce different kinds of brain injury via sphingosine kinase (SPK). Both sphingosine kinase 1 and sphingosine kinase 2 play important roles in brain protection. However, the effects of isoflurane preconditioning on SPK expression in hypertension have not been investigated before.

Objectives. To verify whether the neuroprotective effects of the anesthetic isoflurane after an ischemic injury are altered in hypertension and to identify its possible mechanisms involving SPK.

Material and methods. Wistar rats (control) and spontaneous hypertension rats (SHR) were exposed to isoflurane preconditioning before transient middle cerebral artery occlusion. The infarct volumes of cortical and subcortical brain areas were measured. The expression levels of SPK1 and SPK2 were measured before and after isoflurane preconditioning.

Results. In the SHR group, isoflurane preconditioning significantly reduced only the infarct volumes of the subcortical brain ($p < 0.05$), not of the cortical brain. After 3 h of isoflurane exposure and preconditioning, SPK2 levels in the SHR group increased in the cortical brain ($p < 0.05$), but not in the subcortical brain area. Unlike in the control group, isoflurane exposure and preconditioning could significantly increase SPK2 levels in both cortical and subcortical brain area.

Conclusions. The brain protection effects induced by isoflurane preconditioning after an ischemic injury are mainly mediated by the SPK2 isoform and are somewhat impaired in hypertension. Attention should be paid to ischemic injury patients with hypertension.

Key words: cerebral preconditioning, isoflurane, hypertension, brain ischemic injury

Cite as

Ge J, Kong K, Cheng X, et al. Isoflurane cerebral preconditioning in a spontaneous hypertension rat model is associated with sphingosine kinases. *Adv Clin Exp Med*. 2020;29(6):695–700. doi:10.17219/acem/121944

DOI

10.17219/acem/121944

Copyright

Copyright by Author(s)

This is an article distributed under the terms of the Creative Commons Attribution 3.0 Unported (CC BY 3.0) (<https://creativecommons.org/licenses/by/3.0/>)

Introduction

Cerebral preconditioning is a procedure by which a noxious stimulus below the threshold of damage, such as hypoxia, isoflurane and pro-inflammatory cytokines, is applied to the brain in order to induce tolerance to it or to a different noxious stimulus.^{1,2} Isoflurane, one of the anesthetics which is typically inhaled, is widely used in various surgical procedures. Pre-exposure to isoflurane increases tolerance to hypoxic ischemic cerebral damage, including that incurred during a stroke.³ The sphingosine-1-phosphate (S1P) receptor is widely distributed throughout the nervous system. It mediates multiple cellular processes, including proliferation, apoptosis and migration, and is regulated by the expression of sphingosine kinase (SPK).⁴ Studies have found that isoflurane could reduce different kinds of brain injury, including both hypoxic ischemia and hemorrhage, in rats through S1P-related pathway.^{5,6} Moreover, a recent study has indicated that fingolimod (FTY720), a novel protective agent for ischemic brain injury, can be phosphorylated by SPK and that it can then activate the S1P receptor, suggesting that SPK may be an important effector in neuroprotective mechanisms.^{7,8} Altray et al.⁶ illustrated that in hemorrhage damage, isoflurane could attenuate neuronal cell death in the cortex, associated with an increase in SPK1. In hypoxic ischemic cerebral damage, Lai et al. found a similar result – that isoflurane preconditioning could elevate tolerance levels, mediated by the overexpression of SPK2.⁹ Both SPK1 and SPK2 play important roles in brain protection.

Currently, with the aging of society, hypertension is becoming increasingly common worldwide. It is well known that hypertension is a risk factor for hypoxic ischemic cerebral damage such as stroke. However, the effects of isoflurane preconditioning on SPK expression during hypertension have not been investigated before. With a spontaneous hypertension rat (SHR) model, we evaluated the protective effects of isoflurane preconditioning on hypoxic ischemic cerebral damage, as well as the biological roles of SPK.

Material and methods

Reagents and instruments

Isoflurane, Tween 20, and 2,3,5-trihenylterzolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, USA). Coated monofilament was purchased from Doccol Corporation (Sharon, USA). Chloral hydrate was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Protein marker was purchased from Fermentas (Waltham, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Merck Millipore (Burlington, USA). Primary antibodies of SPK1 (sc-22702) and SPK2 (sc-22704) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). β -actin and IgG secondary antibody

were purchased from Multi Sciences (Hangzhou, China). EZ-ECL kit was purchased from Biological Industries (Beit HaEmek, Israel); PH Indicator was purchased from Leici Device Works (Shanghai, China). A 5417R High-speed Freezing Centrifuge was purchased from Eppendorf (Hamburg, Germany). A 752 Ultraviolet Spectrophotometer was purchased from Puxi General Instruments (Beijing, China). A DYY-4C electrophoresis system was purchased from Tanon Science & Technology (Shanghai, China). A UVP BioSpectrum Imaging System was purchased from UVP (Upland, USA).

Model establishment

Wistar and SHR rats (male, 300–350 g), provided by the Animal Experiment Center of Soochow University, were housed with a 12-hour light–dark cycle with food and water provided ad libitum. All experimental procedures were carried out in strict accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978) and Laboratory Animal Guidelines for ethical review of animal welfare (GB/T 35892-2018, China). The experimental procedures have been approved by the Ethics Committee of the First Affiliated Hospital of Soochow University, Suzhou, China.

Cerebral preconditioning with isoflurane

For the experimental group, the Wistar and SHR rats were exposed to 1% isoflurane for 3 h in a chamber ($N_2:O_2 = 7:3$). They recovered in an incubator at 28°C for 30 min, and were then returned to their cages for 24 h. The control rats were placed in a chamber without isoflurane. Three hours after isoflurane pre-treatment, samples were collected for detection of SPK expression.

Middle cerebral artery assay

The rats were anesthetized with 2% isoflurane, and sustained with 1.5% isoflurane ($N_2:O_2 = 7:3$). The rectal temperature was measured and maintained within the normal physiological range during the surgical procedure. The left middle cerebral artery (MCA) was carefully isolated and occluded with a coated monofilament for 60 min, following the surgical approach described by Izumi et al.¹⁰ The rats were allowed to recover and awaken back in the chambers following the operation.

Measurement of cerebral infarct volume

Twenty-four hours after the operation, the rats were anesthetized with chloral hydrate (1 g/kg). Infarct volume was marked with TTC staining. The rats were perfused with 2% TTC in ddH₂O for 10 min using a catheter placed into the ascending aorta via the apex of the left

ventricle. The brain was carefully removed 45 min later and frozen in isopentane for cryomicrotome. The brain tissues were sliced into 1-mm-thick sections. The volume of MCA occlusion was measured with an image analysis system (ImageJ; National Institutes of Health, Bethesda, USA), as described by O'Donnell et al.¹¹

Western blot

The cerebral cortex and subcortex were homogenized in lysis buffer, and the supernatants were extracted after 30 min of centrifugation at 14,000 rpm. Protein concentrations were determined with a spectrophotometer at 595 nm. A total of 70 µg of proteins was subjected to SDS-PAGE and then transferred onto a PVDF membrane. Thereafter, non-specific binding was blocked with 5% bovine serum albumin for 2 h at room temperature. The membranes were then incubated overnight at 4°C with antibodies against SPK1 (sc-22702) and SPK2 (sc-22704). After rinsing in phosphate-buffered saline-Tween (PBST; 0.1% Tween 20), the membranes were incubated with the corresponding secondary antibody for 1–2 h at room temperature. The bands were visualized using chemiluminescence (Rockford, USA). The expression levels were normalized to β-actin.

Statistical analysis

The data is presented as means ± standard error (SE) and analyzed using SPSS v. 13.0 software (SPSS Inc., Chicago, USA). Statistical analysis was carried out with one-way analysis of variance (ANOVA). A p-value ≤0.05 was considered to be statistically significant.

Results

Determination of cerebral infarct volume in rats pretreated with isoflurane

The TTC staining analysis showed that isoflurane preconditioning significantly reduced the infarct volumes of both the cortical ($129.9 \pm 7.3 \text{ mm}^3$ vs $109.2 \pm 5.9 \text{ mm}^3$; $p < 0.05$; Fig. 1A) and subcortical ($59.2 \pm 5.1 \text{ mm}^3$ vs $41.6 \pm 5.9 \text{ mm}^3$; $p < 0.05$; Fig. 1A) brain areas.

Determination of cerebral infarct volume in SHR rats pretreated with isoflurane

In the SHR group, TTC staining analysis revealed that isoflurane preconditioning significantly reduced the infarct volumes of the subcortical brain areas ($62.9 \pm 5.3 \text{ mm}^3$ vs $43.1 \pm 5.6 \text{ mm}^3$; $p < 0.05$; Fig. 1B) but not the cortical brain areas ($131.8 \pm 7.2 \text{ mm}^3$ vs $129.9 \pm 6.1 \text{ mm}^3$; Fig. 1B).

SPK1/2 expression in cortical and subcortical brain areas of Wistar and SHR rats pretreated with isoflurane

Before isoflurane preconditioning, the SPK1 and SPK2 expression levels in both the cortical and subcortical brain areas of the Wistar rats and the SHR rats did not significantly differ from those of the control group (Fig. 2,3). After isoflurane preconditioning, the SPK1 expression levels were not altered in either the cortical or subcortical brain areas of the Wistar and SHR rats (Fig. 2). However, SPK2 expression levels increased in the cortical and subcortical brain areas of the Wistar rats ($p < 0.05$; Fig. 3A,C). In the SHR rats, isoflurane preconditioning clearly increased SPK2 expression in the subcortical brain areas ($p < 0.05$; Fig. 3), but not in the cortical region.

Fig. 1. A. Effects of isoflurane preconditioning on infarct volume of Wistar rats after cerebral hypoxia/ischemia. Both the infarct volume of brain cortical areas and subcortical areas were significantly reduced after isoflurane preconditioning (* $p < 0.05$). B. Effects of isoflurane preconditioning on infarct volume of SHR rats after cerebral hypoxia/ischemia. Only the infarct volume of brain subcortical areas was significantly reduced after isoflurane preconditioning (* $p < 0.05$).

Fig. 2. A and C. Effects of isoflurane preconditioning on SPK1 expression in cerebral cortical areas: isoflurane preconditioning had no effect on SPK1 expression in the cortical brain areas of either Wistar rats or SHR rats. B and D. Effects of isoflurane preconditioning on SPK1 expression in sub-cerebral cortical areas: isoflurane preconditioning had no effect on SPK1 expression in subcortical brain areas of either Wistar rats or SHR rats

Fig. 3. A and C. Effects of isoflurane preconditioning on SPK2 expression in cerebral cortical areas: isoflurane preconditioning significantly increased SPK2 expression in cortical brain areas in Wistar rats but not in SHR rats (* $p < 0.05$). B and D. Effects of isoflurane preconditioning on SPK2 expression in sub-cerebral cortical areas: isoflurane preconditioning significantly increased SPK2 expression in subcortical brain areas in Wistar rats and SHR rats (* $p < 0.05$)

Discussion

Cerebral preconditioning has a neuroprotective effect, to a certain extent, and it particularly enhances tolerance to cerebral hypoxia-/ischemia-induced injury. A recent study has suggested that the neuroprotective role of isoflurane preconditioning may be mediated by the upregulation of SPK.⁹ Our study demonstrated that, after cerebral hypoxia-/ischemia-induced injury, isoflurane preconditioning reduced the infarct volumes of cortical and subcortical brain areas, which was associated with the upregulation of SPK2. However, isoflurane preconditioning of SHR rats had a neuroprotective effect on the cortical brain, but not on the subcortical brain areas. In addition, the protective effects of isoflurane on brain damage tissue caused by hypoxia-/ischemia-induced injury could be mainly associated with upregulated SPK2. However, in hypertension, the connection between SPK2 and isoflurane in the brain cortex might be weaker.

The SPK1 and SPK2 are the 2 isoforms of SPK. Their amino acid sequences are 80% homologous and differ only in the central domain and the amino terminal. They differ in their cellular distribution, subcellular localization, and enzymatic characteristics. The SPK1 is mainly distributed in the renal proximal tubules and cardiocytes, while SPK2 is primarily expressed in the brain tissue.¹² Consistent with a previous study, SPK1 was detected in both the cortical and subcortical brain areas of Wistar rats, and its expression was not altered after isoflurane preconditioning.⁹ In SHR rats, SPK1 expression in cortical and subcortical brain areas did not differ from that in Wistar rats before and after isoflurane preconditioning. This data suggests that SPK1 might not be related to the hypertension and neuroprotective effects induced by isoflurane preconditioning. A recent study has indicated that cerebral microvascular SPK2 may play an important role in the protective effects induced by cerebral preconditioning with hypoxia.^{9,13} In addition, upregulated expression of SPK2 in cortical brain areas was shown to be associated with the neuroprotective role of isoflurane preconditioning.⁹ Our study demonstrated that isoflurane preconditioning enhanced the SPK2 expression levels in both the cortical and subcortical brain areas, which was closely related to the neuroprotective effects induced by isoflurane preconditioning. However, a neuroprotective effect was only induced in the subcortical brain areas in SHR rats, which was related to the upregulation of SPK2, which suggests that isoflurane preconditioning mainly protects the subcortical brain areas from cerebral hypoxia-/ischemia-induced injury – probably through the upregulation of SPK2.

Numerous studies have demonstrated that SPK can be activated by various stimuli, such as activation of G-protein-coupled receptors^{14,15} and tyrosine kinase receptors.^{16,17} The SPK can also mediate phosphorylation and proliferation targeting on cell membranes.¹⁸ These studies suggest that the change in activity or expression of SPK may be the common signal involved in the neuroprotective role

induced by cytokines and drugs. Moreover, SPK-related sphingomyelin metabolites could regulate hypoxia-induced factor (HIF) and other signaling pathways. Combined with previous studies, our data suggests that SPK2 could be one of the signals involved in the neuroprotective effect induced by cerebral preconditioning. Song et al.¹⁹ have found that SPK2 could play its role through interacting with Bcl-2. In our study, prior to isoflurane preconditioning, SPK2 expression (in the cortical and subcortical brain areas) in SHR rats did not differ from that in Wistar rats, suggesting that SPK2 may not be involved in hypertension and its pathophysiological changes. However, isoflurane preconditioning attenuated the neuroprotective effect in the cerebral cortex without SPK2 changes, suggesting that the cerebral SPK2-related signaling pathways (supposedly Bcl-2) are altered in hypertension. Bcl-2 is closely related to pregnancy hypertension, with a downregulated expression of Bcl-2.²⁰ We assume there is also a special relationship between Bcl-2 and hypoxic/ischemic brain damage with hypertension.

Furthermore, our study provides experimental support for the treatment of patients with hypertension using cerebral preconditioning with isoflurane. It is worth mentioning that fingolimod (FTY720), an analogue of sphingosine, is applied in the treatment of multiple sclerosis; after phosphorylation by SPK, particularly SPK2, fingolimod can be transformed into its active state and further activate 4 S1P receptors to exhibit its therapeutic action.²¹ The present study suggests that decreased expression of SPK2 in a hypertension-related cerebral cortex could lead to altered efficacy of fingolimod in patients with multiple sclerosis and hypertension. This data provides an experimental basis for evaluating the pharmacodynamics of fingolimod or for designing novel SPK2-related drugs.

Conclusions

The present study demonstrates that isoflurane preconditioning reduces the infarct volume not only in MCA-occluded Wistar rats, but also in SHR rats with MCA occlusion. More importantly, the protective effects against hypoxia/ischemic damage gained from isoflurane preconditioning were associated with an upregulated expression of cerebral SPK2. In hypertension patients, we propose that the brain protection effects induced by isoflurane preconditioning would be somewhat impaired after an ischemic injury.

ORCID iDs

Jun Ge <https://orcid.org/0000-0002-3823-5409>
 Keyu Kong <https://orcid.org/0000-0002-5839-6074>
 Xiaoqiang Cheng <https://orcid.org/0000-0002-6853-9083>
 Tianyi Wu <https://orcid.org/0000-0002-5549-6272>
 Dingge Liu <https://orcid.org/0000-0001-7936-3127>
 Lingling Peng <https://orcid.org/0000-0002-6711-6695>
 Zhijia Shen <https://orcid.org/0000-0003-4535-6767>
 Guoyuan Lu <https://orcid.org/0000-0003-0389-3935>
 Yanping Shen <https://orcid.org/0000-0002-4546-850X>

References

1. Dirnagl U, Becker K, Meisel A. Preconditioning and tolerance against cerebral ischaemia: From experimental strategies to clinical use. *Lancet Neurol.* 2009;8(4):398–412.
2. Gidday JM. Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci.* 2006;7(6):437–448.
3. Zheng S, Zuo Z. Isoflurane preconditioning induces neuroprotection against ischemia via activation of P38 mitogen-activated protein kinases. *Mol Pharmacol.* 2004;65(5):1172–1180.
4. Harada J, Foley M, Michael M, Waeber C. Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells. *J Neurochem.* 2004;88(4):1026–1039.
5. Zhou Y, Lekic T, Fathali N, et al. Isoflurane posttreatment reduces neonatal hypoxic-ischemic brain injury in rats by the sphingosine-1-phosphate/phosphatidylinositol-3-kinase/Akt pathway. *Stroke.* 2010;41(7):1521–1527.
6. Altray O, Yu H, Sherchan P, et al. Isoflurane delays the development of early brain injury after subarachnoid hemorrhage through sphingosine-related pathway activation in mice. *Crit Care Med.* 2012;40(6):1908–1913.
7. Yu H, Suzuki H, Takumi S, Rolland W, Zhang JH. Activation of sphingosine 1-phosphate receptor-1 by FTY720 is neuroprotective after ischemic stroke in rats. *Stroke.* 2010;41(2):368–374.
8. Wei Y, Yemisci M, Kim HH, et al. Fingolimod provides long-term protection in rodent models of cerebral ischemia. *Ann Neurol.* 2011;69(1):119–129.
9. Ming YL, Ying W, Tao Q, Wang Y, Smith C, Waeber C. Sphingosine kinase 2 mediates cerebral preconditioning and protects the mouse brain against ischemic injury. *Stroke.* 2012;43(1):199–204.
10. Izumi Y, Roussel S, Pinard E, Seylaz J. Reduction of infarct volume by magnesium after middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab.* 1991;11(6):1025–1030.
11. O'Donnell ME, Lien T, Lam T, Liu X, Anderson SE. Bumetanide inhibition of the blood-brain barrier Na-K-Cl cotransporter reduces edema formation in the rat middle cerebral artery occlusion model of stroke. *J Cereb Blood Flow Metab.* 2004;24(9):1046–1056.
12. Bryan L, Kordula T, Spiegel S, Milstein S. Regulation and functions of sphingosine kinases in the brain. *Biochim Biophys Acta.* 2008;1781(9):459–466.
13. Wacker BK, Park TS, Gidday JM. Hypoxic preconditioning-induced cerebral ischemic tolerance: Role of microvascular sphingosine kinase 2. *Stroke.* 2009;40(10):3342–3348.
14. Graler MH, Goetzl EJ. The immunosuppressant FTY720 downregulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* 2004;18(3):551–553.
15. Kluk MJ, Hla T. Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim Biophys Acta.* 2002;1582(1–3):72–80.
16. Wang FH, Nobes C, Hall A, Spiegel S. Sphingosine 1-phosphate stimulates rho-mediated tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 fibroblasts. *Biochem J.* 1997;324(Pt 2):481–488.
17. Yogi A, Glaucia EC, Anna BA, et al. Sphingosine-1-phosphate-induced inflammation involves receptor tyrosine kinase transactivation in vascular cells: Upregulation in hypertension. *Hypertension.* 2011;57(4):809–818.
18. Grey A, Xu X, Hill B, et al. Osteoblastic cells express phospholipid receptors and phosphatases and proliferate in response to sphingosine-1-phosphate. *Calcif Tissue Int.* 2004;74(6):542–550.
19. Song DD, Zhang TT, Chen JL, et al. Sphingosine kinase 2 activates autophagy and protects neurons against ischemic injury through interaction with Bcl-2 via its putative BH3 domain. *Cell Death Dis.* 2017;8(7):e2912.
20. Varol F, Uzunoglu R, Erbas H, Süt N, Sayin C. VEGFR-1, Bcl-2, and HO-1 ratios in pregnant women with hypertension. *Clin Appl Thromb Hemost.* 2015;21(3):285–288.
21. Hla T, Brinkmann V. Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation. *Neurology.* 2011;76(8 Suppl 3):S3–S8.