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Isoflurane preconditioning improves short-term and long-term neurological outcome after focal brain ischemia in adult rats

Liaoliao Li and Zhiyi Zuo

Department of Anesthesiology, University of Virginia, Charlottesville, VA22908, USA

Abstract

Isoflurane preconditioning improved short-term neurological outcome after focal brain ischemia in adult rats. It is not known whether desflurane induces a delayed phase of preconditioning in the brain and whether isoflurane preconditioning-induced neuroprotection is long-lasting. Two-month-old Sprague-Dawley male rats were exposed to or were not exposed to isoflurane or desflurane for 30 min and then subjected to a 90-min middle cerebral arterial occlusion (MCAO) at 24 hr after the anesthetic exposure. Neurological outcome was evaluated at 24 hr or 4 weeks after the MCAO. The density of the terminal deoxynucleotidyl transferase biotinylated UTP nick end labeling (TUNEL) positive cells in the penumbral cerebral cortex were assessed 4 weeks after the MCAO. Also, rats were pretreated with isoflurane or desflurane for 30 min. Their cerebral cortices were harvested for quantifying B-cell lymphoma-2 expression 24 hr later. Here, we showed that pretreatment with 1.1% or 2.2% isoflurane, but not with 6% or 12% desflurane, increased B-cell lymphoma-2 expression in the cerebral cortex, improved neurological functions and reduced infarct volumes evaluated at 24 hr after the MCAO. Isoflurane preconditioning also improved neurological functions and reduced brain infarct volumes in rats evaluated 4 weeks after the MCAO. Isoflurane preconditioning also decreased the density of TUNEL-positive cells in the penumbral cerebral cortex. We conclude that isoflurane preconditioning improves short-term and long-term neurological outcome and reduces delayed cell death after transient focal brain ischemia in adult rats. Bcl-2 may be involved in the isoflurane preconditioning effect. Desflurane pretreatment did not induce a delayed phase of neuroprotection.

Keywords

Bcl-2; desflurane; isoflurane; preconditioning; neuroprotection

Introduction

Stroke is the third leading cause of death in the United States. The underlying pathophysiology for stroke is ischemic brain injury. Studying how to reduce ischemic brain injury has been a major focus of medical research in recent years. However, very few treatments to reduce ischemic brain injury in clinical practice have been established.

Address correspondence to: Dr. Zhiyi Zuo, Department of Anesthesiology, University of Virginia Health System, 1 Hospital Drive, PO Box 800710, Charlottesville, Virginia 22908-0710. Tel: 434-924-2283, Fax: 434-924-2105, zz3c@virginia.edu.

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One promising approach for limiting ischemic injury is the phenomenon of preconditioning. Preconditioning has received substantial recent attention and was initially described in the brain and heart whose injury after an episode of prolonged anoxia and ischemia was attenuated by a prior exposure of the brain and heart to episodes of short hypoxia or ischemia (Murry et al., 1986, Schurr et al., 1986). We and others have shown that isoflurane, a commonly used volatile anesthetic, can also induce a preconditioning effect in the brains of neonatal and adult rats (Kapinya et al., 2002, Zheng and Zuo, 2004, Kitano et al., 2007, Li et al., 2008). This anesthetic preconditioning may have significant implications because perioperative stroke is a serious complication that is not uncommon during and following procedures, such as carotid endarterectomy, cardiac pulmonary bypass and intracranial vascular surgery and even noncardiac and nonvascular surgery (Dacey et al., 2005, Wilson and Ammar, 2005, Bateman et al., 2009). Many of these patients develop stroke within 4 days after these procedures (Wilson and Ammar, 2005). Intraoperative use of volatile anesthetics may precondition these patients whose brain ischemia occurred within a short period of time after surgery if volatile anesthetic preconditioning can indeed induce neuroprotection in humans. In this regard, it is important to determine whether other currently used volatile anesthetics, such as desflurane, can induce a delayed preconditioning effect in the brain.

It has been reported that brain infarction is matured at 2 days after focal brain ischemia in rats (Garcia et al., 1993, Lin et al., 1993). However, it is now known that ischemic brain injury is a dynamic process characterized with ongoing cell death lasting for at least 2 weeks after brain ischemia in rats (Li et al., 1995). Numerous strategies are neuroprotective in animal studies. However, very few of them are effective to improve neurological outcome in clinical trials. One of the reasons for this phenomenon is that the majority of the animal studies evaluated neurological outcome within a few days after brain ischemia and clinical trials examined the outcome many months later. Indeed, it has been shown that some of the neuroprotective methods may just delay cell death after brain ischemia (Dietrich et al., 1993, Trescher et al., 1997, Kawaguchi et al., 2000). Thus, it is necessary to evaluate the effectiveness of a strategy to improve the long-term neurological outcome in animal studies. This requirement has been recommended as one of the criteria for determining the usefulness of a neuroprotective strategy by leading experts in stroke-related research (StrokeTherapy, 1999).

We and others have shown that isoflurane preconditioning can improve the long-term neurological outcome in neonatal rats suffering from brain ischemia and hypoxia (McAuliffe et al., 2007, Zhao et al., 2007). It is not known whether isoflurane preconditioning can also improve the long-term neurological outcome in adult rats after focal brain ischemia. Neonatal brains are significantly different from adult brains in many aspects including structure, sensitivity to ischemia and mechanisms to cause ischemic brain injury (Dobbing and Sands, 1979, McDonald and Johnston, 1990, Johnston, 2002). Thus, we designed this study to determine whether, in addition to isoflurane, desflurane can induce a delayed preconditioning effect in the brain and whether isoflurane preconditioning improves the long-term neurological outcome after brain ischemia in adult rats. Also, we determined the role of B-cell lymphoma-2 (Bcl-2), a protective protein (Yang et al., 1997), in the anesthetic preconditioning-induced neuroprotection.

Materials and Methods

The animal protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80-23) revised in 1996.

Animal Groups

Two-month-old male Sprague-Dawley rats weighing 280 to 300 g were randomly assigned to be exposed to or not exposed to 1.1% or 2.2% isoflurane or 6% or 12% desflurane for 30 min at 24 hr before a 90-min right middle cerebral arterial occlusion (MCAO). Their motor coordination, neurological deficit scores and infarct volumes were evaluated 24 hr after the MCAO.

In the second set of experiments, male Sprague-Dawley rats weighing 280 to 300 g were randomly assigned into two groups for studying: rats that were subjected to the 90-min MCAO only and rats that were pretreated with 2% isoflurane for 30 min at 24 hr before the MCAO. In addition to these two experimental groups, rats of the last two purchases (6 to 10 rats were purchased each time) were randomly assigned to the third group. This group of rats was used as control in the study to determine the density of terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) positive cells in the cerebral cortex. These control rats were not subjected to MCAO or isoflurane exposure. The motor coordination, neurological deficit scores, infarct volumes and density of TUNEL positive cells of animals in this set of experiments were evaluated at 4 weeks after the MCAO.

In the third set of experiments, male Sprague-Dawley rats weighing 280 to 300 g were randomly assigned to be exposed to or not exposed to 1.1% or 2.2% isoflurane or 6% or 12% desflurane for 30 min. The frontal brain cortex area 1 of these rats was harvested at 24 hr after the exposure to volatile anesthetics for Western analysis of Bcl-2 expression. These rats were not subjected to the MCAO.

Our previous studies have consistently shown that exposure to 2% isoflurane for 30 min induces preconditioning effects in rat brains (Zheng and Zuo, 2004, Li et al., 2008). One minimum alveolar concentration (the concentration required to suppress movement to a noxious stimulus in 50% of subjects) for isoflurane and desflurane in rats is about 1.1% and 6%, respectively (Orliaguet et al., 2001). Thus, we chose 1.1% and 2.2% isoflurane and 6% and 12% desflurane for dose-response study here.

Volatile Anesthetic Preconditioning/Pretreatment

Isoflurane preconditioning was performed as we described before (Zheng and Zuo, 2004, Li et al., 2008). Briefly, anesthesia was induced with isoflurane and then maintained with 1.1% or 2.2% isoflurane via an endotracheal tube for 30 min. Similarly, desflurane pretreatment was performed by inducing anesthesia with desflurane and then maintaining with 6% or 12% desflurane via an endotracheal tube for 30 min. Respiration was controlled by a ventilator to maintain normal end-tidal O₂ and CO₂ concentrations. The inhaled and exhaled gases were monitored with a Datex infrared analyzer (Capnomac, Helsinki, Finland). Temporalis muscle temperature was maintained at 37 ± 0.2°C during the anesthesia.

While the animals in the experimental groups were exposed to isoflurane or desflurane, animals in the control group were placed in an air-tight chamber gassed with the carrier gases (60% O₂-40% N₂) for anesthetics for 30 min.

Middle Cerebral Arterial Occlusion

The person who performed MCAO was blind to the group assignment of rats. Rats were anesthetized with isoflurane or desflurane and then intubated and mechanically ventilated with 60% O₂-40% N₂ containing 2% isoflurane or 11% desflurane. The same anesthetic was used in this procedure and for preconditioning/pretreatment in a particular rat. The rats in the MCAO only group received the same anesthetics here as for the corresponding rats in the same set of experiments for comparison. During anesthesia, temporalis muscle temperature was strictly

maintained at $37 \pm 0.2^{\circ}\text{C}$ by warming blanket and lamps. The inhaled and exhaled gases were also monitored to maintain normal end-tidal CO_2 concentrations. The tail artery was cannulated for monitoring arterial blood pressure and blood sampling for measuring blood gases and glucose. This arterial catheter was removed at 10 min into MCAO. As we described previously (Zheng and Zuo, 2004), the MCAO was achieved by advancing a 3-0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd., Beijing, China) with a rounded tip to the right internal carotid artery via the external carotid artery until slight resistance was felt. These sutures were specially prepared by the company for the purpose of MCAO and were not coated with poly-L-lysine per the company. The ipsilateral common carotid artery was occluded temporarily during the placement of the suture. Isoflurane or desflurane anesthesia was stopped and endotracheal tube was removed once the tail arterial catheter was removed (10 min after the suture was in place). Rats were reanesthetized by isoflurane or desflurane at 90 min after the onset of MCAO to remove the suture. This reanesthesia lasted for about 2 min. After recovery from anesthesia, rats were placed back in their cages with *ad libitum* access to food and water.

Evaluation of Motor Coordination, Neurological Deficit Scores, and Infarct Volumes

Neurological deficit scores were evaluated based on an eight-point scale by a person blinded to the group assignment. Rats were scored as follows: 0, no apparent deficits; 1, failure to extend left forepaw fully; 2, decreased grip of the left forelimb; 3, spontaneous movement in all directions, contralateral circling only if pulled by the tail; 4, circling or walking to the left; 5, walking only if stimulated; 6, unresponsiveness to stimulation and with depressed level of consciousness; 7, dead (Rogers et al., 1997).

Motor coordination was evaluated 24 hr before the transient MCAO and immediately before they were euthanized for brain harvest as we described before (Zhao et al., 2007, Li et al., 2008). The rats were placed on an accelerating rotarod. The speed of the rotarod was increased from 4 rpm to 40 rpm in 5 min. The latency and the speed of rat's falling off the rotarod were recorded. Each rat was tested three times. The speed-latency index (latency in seconds $\times$ speed in rpm) of each of the three tests was calculated and the mean index of the three trials was used to reflect the motor coordination function of each rat before or after the MCAO. All rats were trained for 3 continuous days before the formal tests. They were placed on the rotarod three times each day and this training occurred just before they were subjected to the preconditioning/pre-treatment protocol.

The assessment of infarct volumes at 24 hr after the MCAO was performed after 2,3,5-triphenyltetrazolium chloride staining as we described before (Zheng and Zuo, 2004, Zhao et al., 2006, Li et al., 2008). The evaluation of infarct volumes at 4 weeks after the transient MCAO was performed after hematoxylin and eosin staining as described previously (Zheng and Zuo, 2004, Sakai et al., 2007) because 2,3,5-triphenyltetrazolium chloride staining will no longer adequately differentiate the infarcted area from the non-infarcted brain regions at this time point. The assessments of infarct volumes were performed by a person blinded to the group assignment. Briefly, rats were euthanized by 5% isoflurane and transcardiacally perfused by saline and then 4% phosphate-buffered paraformaldehyde. Brains were removed and stored in the same fixative solution for 7 days. Eight-micrometer-thick paraffin coronal sections were taken by using a microtome at 2-mm intervals (usually total 6 slices) over the entire brain. Three sections were taken at the beginning and at the end of the 2-mm interval. The sections were stained with hematoxylin and eosin. The infarct areas in each brain section (4-week follow-up study) or in the rostral and caudal sides of each brain slice (24-hr follow-up study) were quantified using the NIH Image 1.60. The infarct volumes were calculated as follows. In the 24-hr follow-up study, the sum of the infarct areas in the rostral and caudal sides of each brain slice was divided by 2 to get the average infarct area of the brain slice. The infarct volume of the brain slice was calculated by multiplying the average infarct area of the slice by the

thickness of the slice (2 mm). The total infarct volume in the brain was the sum of infarct volume of each brain slice. The infarct volume of rats in the 4-week follow-up study was calculated in a similar way by averaging the infarct areas in the 6 brain sections taken from the 2-mm interval. To account for the cerebral edema and differential shrinkage resulting from brain ischemia and tissue processing and to correct for the individual difference in brain volumes, the percentage of infarct volume in the ipsilateral hemisphere volume was calculated (Swanson et al., 1990).

TUNEL and TUNEL Positive Cell Density Counting

To determine the density of TUNEL positive cells in the penumbral regions, sections at bregma + 1.5 mm were stained using a TUNEL kit (catalog number 17-141) from Millipore (Temecula, CA). The staining was performed according to the protocol provided by the manufacturer. The positive staining was revealed by FITC-conjugated avidin and visualized under a fluorescent microscope. The density of TUNEL-positive cells was determined as follows by a person blinded to the group assignment. A reticle ($\sim 0.15 \text{ mm}^2$) was used to count cells in the same size area. Seven determinations, each on different locations in the penumbral cerebral cortex that is immediately adjacent to the infarct areas under microscope, were performed and averaged to yield a single number (density of the TUNEL positive cells) for the individual rat.

Western Analysis

Total lysates of the frontal cortex area 1 (50 μg protein per lane) were subjected to Western analysis as we described before (Li et al., 2008). The primary antibodies used were the mouse monoclonal anti-Bcl-2 antibody (1:200 dilution; catalog number: sc-509; Santa Cruz Biotechnology, Santa Cruz, CA) and the rabbit polyclonal anti-actin antibody (1:1000 dilution; catalog number: A2066; Sigma Chemical). The protein bands were visualized with the enhanced chemiluminescence methods. Quantitative analysis of the protein bands was performed using an ImageQuant 5.0 GE Healthcare Densitometer (GE Healthcare, Sunnyvale, CA). The densities of Bcl-2 protein bands were normalized to those of actin to control for errors in protein sample loading and transferring during Western analysis. The results from the samples of rats pretreated with isoflurane or desflurane were normalized to the control rats on the same film to control for the error caused by different exposure times of films.

Statistical Analysis

For the 24-hr observation experiments, the results of physiological parameters, speed-latency index ratio, infarct size and Western blotting results were analyzed by one way analysis of variance followed by the Tukey test after confirmation of normal distribution of the data (data are presented as means $\pm$ S.D.) or by Kruskal-Wallis analysis of variance on ranks followed by the Dunn's test when the data are not normally distributed. Neurological deficit scores were analyzed by Kruskal-Wallis analysis of variance on ranks followed by the Dunn's test (data are presented as medians with 95% interval).

For the 4-week observation experiments, statistical analysis of the results of physiological parameters, speed-latency index ratio and infarct size between the MCAO group and isoflurane preconditioning plus MCAO group was performed by Student's *t* test. Neurological deficit scores were analyzed by Mann-Whitney rank sum test. TUNEL positive cell density was analyzed by one way analysis of variance followed by the Tukey test (data are presented as means $\pm$ S.D.).

A $P \leq 0.05$ was accepted as significant. All statistical analyses were performed with the SigmaStat (Systat Software, Inc., Point Richmond, CA).

Results

Pretreatment with isoflurane, but not desflurane, improved short-term neurological outcome

In the 24-hr observation experiments, one rat died during the 24-hr observation period. This rat was from the MCAO only group for the set of isoflurane preconditioning experiment. Its brain had severe edema and infarction. This rat contributed data to the neurological deficit scores only. Pretreatment with 1.1% and 2.2% isoflurane significantly improved neurological deficit scores and reduced brain infarct volumes after the MCAO (Fig. 1). In contrast, pretreatment with 6% or 12% desflurane did not change the neurological outcome as measured by neurological deficit scores, performance on rotarod and infarct volumes (Fig. 2).

Isoflurane preconditioning improved long-term neurological outcome

In the 4-week observation experiments, total 27 rats were in the MCAO only group and 24 rats in the isoflurane preconditioning plus MCAO group. Five rats in the MCAO group and two rats in the isoflurane preconditioning plus MCAO group died during the period from 6 hr to 7 days after the MCAO. Necropsy showed that all 7 rats had severe brain edema and infarction. The mortality rates were 18.5% and 8.3%, respectively, for the MCAO only group and the isoflurane preconditioning plus MCAO group ($P = 0.517$ by z-test for the comparison). Rats died during the 4-week observation contributed data to the neurological deficit scores only. Thus, data from 22 rats that survived till the end of the 4-week observation period from each study group were used in the analysis of brain infarct volumes, performance on rotarod and TUNEL positive cell density.

Compared with rats subjected to MCAO alone, rats pre-exposed to isoflurane had smaller brain infarct volumes, better performance on rotarod and improved neurological deficient scores at 4 weeks after the 90-min MCAO (Fig. 3). Further analysis showed that isoflurane preconditioning significantly reduced the total brain infarct volume, cerebral cortical infarct volume and subcortical infarct volume. Interestingly, there were more TUNEL positive cells in the penumbral cerebral cortex of rats with MCAO only than in the cerebral cortex of normal control rats, suggesting that more cells are dying even at this delayed time point after MCAO. This delayed cell death was also attenuated by isoflurane preconditioning (Fig. 4).

To reduce confounding factors on neurological outcomes, physiological parameters, such as blood pressure, arterial blood gases and glucose, and temperature, were monitored and controlled during the surgery to achieve the MCAO. There were no differences between rats in MCAO only and isoflurane preconditioning plus MCAO groups in the 4-week observation experiments (Table 1). Rats pretreated with various concentrations of isoflurane or desflurane in the 24-hr observation experiments had physiological values similar to those in the 4-week observation experiments and, thus, are not reported here.

Pretreatment with isoflurane, but not desflurane, increased the Bcl-2 expression

Compared with control rats, rats pretreated with 1.1% or 2.2% isoflurane had a significantly increased expression of Bcl-2 proteins in the frontal cerebral cortex area 1, an ischemic penumbral region after MCAO. However, pretreatment with 6% or 12% desflurane did not significantly alter the expression of Bcl-2 proteins (Fig. 5).

Discussion

We have shown that preconditioning with volatile anesthetics including desflurane can induce an acute phase of neuroprotection whose effective window is from a few minute to 2 - 3 hours after the preconditioning stimulus and usually involves modulation of the functions of existing proteins (Nandagopal et al., 2001, Zheng and Zuo, 2003, Wang et al., 2007). The potency of

this anesthetic preconditioning-induced neuroprotection was linearly correlated with their anesthetic potency and the EC50 for isoflurane to induce the acute phase of preconditioning effect was about 1.1% (Zheng and Zuo, 2003, Wang et al., 2007). Our current study showed that preconditioning with isoflurane at 1.1% and 2.2% (about 1 and 2 minimum alveolar concentrations, respectively (Orliaguet et al., 2001)) induced a delayed phase of neuroprotection. These results are consistent with previous studies (Kapinya et al., 2002, Zheng and Zuo, 2004, Li et al., 2008). However, pretreatment with desflurane at 6% and 12% (also about 1 and 2 minimum alveolar concentrations, respectively (Orliaguet et al., 2001)) did not induce a delayed phase of neuroprotection. Whether desflurane can induce a delayed phase of preconditioning effects in the brain has not been reported previously. Our findings suggest that, unlike the situation with the acute phase of neuroprotection, anesthetic preconditioning-induced delayed phase of neuroprotection is an agent-specific effect. Consistent with the idea that different volatile anesthetics may have different effects, it has been shown that desflurane was less potent than isoflurane to provide protection against ischemia-reperfusion in rat kidney when the anesthetics were used during the ischemia and reperfusion (Lee et al., 2004). In addition, unlike isoflurane, desflurane failed to induce Bcl-2 expression in the brain in our current study, a possible mechanism for its inability to induce a delayed phase of neuroprotection.

We then determined whether isoflurane preconditioning-induced delayed phase of neuroprotection can be long-lasting. The importance of determining whether a neuroprotective strategy can improve long-term neurological outcome in animal studies has been recognized in recent years. Two lines of evidence contribute to this recognition: 1) ischemic brain injury is a dynamic process that has ongoing brain cell death lasting for sometime (at least for 2 weeks in rodents) after the brain ischemia (Li et al., 1995), and 2) some neuroprotective methods, such as brief hypothermia applied after brain ischemia, may improve short-term but not improve the long-term neurological outcome (Dietrich et al., 1993, Trescher et al., 1997). To determine whether isoflurane preconditioning can improve long-term neurological outcome in adult brain, we evaluated the brain infarct volumes and neurological functions at 4 weeks after brain ischemia in adult rats. Our results showed that animals preconditioned with isoflurane had smaller infarct volumes and better neurological deficit scores and motor coordination functions than did MCAO only rats. These results indicate that isoflurane preconditioning improves long-term neurological outcome after focal brain ischemia in adult rats.

One of the significant findings in our study is that there are more dying cells in the penumbral region in rats at 4 weeks after an episode of transient focal brain ischemia than in control rats, suggesting that cell death is still ongoing even at this delayed time point. Interestingly, this delayed cell death after brain ischemia is attenuated by isoflurane preconditioning. These results suggest the neuroprotective effects of isoflurane preconditioning on ischemia-induced delayed cell death.

We used a commonly used transient focal brain ischemia model produced by filament MCAO. The ipsilateral common carotid artery was temporarily occluded during the filament insertion and was not permanently ligated. Our practice may avoid the chronic hypoperfusion stress to brain cells after the filament removal. Such a chronic hypoperfusion or ischemic status may occur if the ipsilateral common carotid artery is permanently ligated during the MCAO procedure. To support this possibility, it has been shown that MCAO plus permanent ligation of the ipsilateral common carotid artery produced much bigger brain infarct volumes than MCAO without the ligation of the artery at 14 days after brain ischemia (Sakai et al., 2007). Since we did not ligate the common carotid artery, reasons other than chronic hypoperfusion are needed to explain the cell death at 4 weeks after the acute episode of brain ischemia as shown in our study. It is known that post-ischemia inflammation can last for weeks (Clark et al., 1993, Zhang et al., 1994, Barone et al., 2002, Dirnagl et al., 2003). Inflammation and the

associated oxidative stress can cause cell death. It is also possible that the acute brain ischemia may have programmed some cells for apoptosis that takes a long time to finally manifest. Consistent with the inflammation theory, our previous studies have shown that isoflurane preconditioning reduces the activation and injury of macrophages and microglia after the application of lipopolysaccharide and interferon γ (Xu et al., 2008a, Xu et al., 2008b). These effects may contribute to the isoflurane preconditioning-reduced delayed cell death after brain ischemia as shown in this study.

Preconditioning-induced delayed phase of neuroprotection that occurs from a few hours to many days after the application of preconditioning stimuli often requires synthesis of protective proteins (Dirnagl et al., 2003). Bcl-2, a well-known antiapoptotic protein, has been implicated in the isoflurane preconditioning-induced long-term improvement of neurological outcome in the neonatal rat brain in our previous study (Zhao et al., 2007). Our study has also shown that isoflurane preconditioning increases Bcl-2 expression, decreases cytochrome c release from mitochondria and reduces cell apoptosis in the ischemic penumbra of adult rats after MCAO (Li et al., 2008). Thus, Bcl-2 may have contributed to the neuroprotection observed in this study. Consistent with this idea, our current study showed that isoflurane pretreatment significantly increased Bcl-2 expression and induced a preconditioning effect and that desflurane pretreatment did not change the Bcl-2 expression and also did not induce a preconditioning effect in the brain. Of note, it has been shown that isoflurane or sevoflurane (another commonly used volatile anesthetic) preconditioning-induced neuroprotection may be mediated by mitochondrial K_{ATP} channels (Xiong et al., 2003, Kehl et al., 2004). Further studies are needed to understand the relationship between Bcl-2 expression and mitochondrial K_{ATP} channel activation after volatile anesthetic exposure.

Two previous studies involving neonatal rats (McAuliffe et al., 2007, Zhao et al., 2007) and our current study using adult rats have consistently shown that isoflurane preconditioning can improve long-term neurological outcome after brain ischemia. In contrast, isoflurane applied during brain ischemia has not been consistently shown to improve long-term neurological outcome. An early study showed that isoflurane application during a 70-min MCAO did not improve neurological outcome evaluated at 14 days after the MCAO (Kawaguchi et al., 2000). However, a recent study showed that isoflurane application during a 50-min MCAO or an 80-min MCAO improved neurological outcome evaluated at 14 days after the MCAO and that this improved neurological outcome was evident even at 8 weeks after the 50-min MCAO (Sakai et al., 2007). The reasons for these different findings from these two studies are not clear. However, the former study permanently ligated the ipsilateral common carotid artery during the insertion of intraarterial suture, which may cause chronic hypoperfusion to the brain even after the relief of the MCAO. The later study only temporarily occluded the ipsilateral common carotid artery. This difference in the animal model may have contributed to the conflicting findings/conclusions from those two studies.

In conclusion, we have shown that volatile anesthetic preconditioning-induced delayed phase of neuroprotection may be agent-specific because pretreatment with isoflurane, but not desflurane, induced this type of neuroprotection. We have also shown that isoflurane preconditioning can improve the long-term neuropathological and neurological function outcome after brain ischemia. Isoflurane preconditioning also reduced the delayed cell death in the ischemic penumbral region. This isoflurane preconditioning-induced neuroprotection may involve increased Bcl-2 expression in the brain.

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Abbreviations

Bcl-2

B-cell lymphoma-2

MCAO

middle cerebral arterial occlusion

TUNEL

terminal deoxynucleotidyl transferase biotinylated UTP nick end labeling

Fig. 1. Isoflurane preconditioning improved short-term histological and neurological function outcome after focal brain ischemia in adult rats

A: Brain slices stained with 2,3,5-triphenyltetrazolium chloride from representative rats subjected to MCAO only or isoflurane preconditioning plus MCAO. **B:** Percentage of infarct volume in ipsilateral hemisphere volume. Results are the means $\pm$ S.D. (n = 12 - 14). **C:** Neurological deficit scores evaluated immediately before the animals were euthanized for the assessment of infarct sizes (data are presented in panel B) or assigned 7 to the animals that died before this end time point for observation. Results are presented in a box plot format (n = 13 - 14). •: lowest or highest score (the score will not show up if it falls in the 95% interval); between lines: 95% interval of the data; inside boxes: 25-75% interval including the median of the data. **D:** The performance on rotarod. Rats were tested before and 24 hr after the MCAO and the speed-latency index ratio of these two tests are presented. Results are the means $\pm$ S.D.

(n = 12 - 14). * $P < 0.05$ compared with the corresponding MCAO only group. Iso: isoflurane preconditioning.

Fig. 2. Desflurane pretreatment did not affect short-term histological and neurological function outcome after focal brain ischemia in adult rats

A: Brain sections stained with 2,3,5-triphenyltetrazolium chloride from representative rats subjected to MCAO only or desflurane pretreatment plus MCAO. **B:** Percentage of infarct volume in ipsilateral hemisphere volume. Results are the means $\pm$ S.D. ($n = 11 - 12$). **C:** Neurological deficit scores evaluated immediately before the animals were euthanized for the assessment of infarct sizes (data are presented in panel B). Results are presented in a box plot format ($n = 11 - 12$). $\bullet$: lowest or highest score (the score will not show up if it falls in the 95% interval); between lines: 95% interval of the data; inside boxes: 25-75% interval including the median of the data. **D:** The performance on rotarod. Rats were tested before and 24 hr after the MCAO and the speed-latency index ratio of these two tests are presented. Results are the means $\pm$ S.D. ($n = 11 - 12$). Des: desflurane preconditioning.

Fig. 3. Isoflurane preconditioning improved long-term histological and neurological function outcome after focal brain ischemia in adult rats

A: Brain sections stained with hematoxylin and eosin from representative rats subjected to MCAO only or isoflurane preconditioning plus MCAO. The infarct areas are traced out with black line. **B:** Percentage of the total brain infarct volume (Total), cortical infarct volume (Cortical) and subcortical infarct volume (Subcortical) in ipsilateral hemisphere volume. Results are the means $\pm$ S.D. ($n = 22$). **C:** Neurological deficit scores evaluated immediately before the animals were euthanized for the assessment of infarct sizes (data are presented in panel B) or assigned 7 to the animals that died before this end time point for observation. Results are presented in a box plot format ($n = 24 - 27$). $\bullet$: lowest or highest score (the score

will not show up if it falls in the 95% interval); between lines: 95% interval of the data; inside boxes: 25-75% interval including the median of the data. **D:** The performance on rotarod. Rats were tested before and 4 weeks after the MCAO and the speed-latency index ratio of these two tests are presented. Results are the means $\pm$ S.D. (n = 22). * P < 0.05 compared with the corresponding MCAO only group. Iso: isoflurane preconditioning.

Fig. 4. Isoflurane preconditioning reduced dying cells evaluated at 4 weeks after the 90-min right middle cerebral arterial occlusion (MCAO)

Cells in the penumbral cerebral cortex were examined after terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL). Quantitative data are presented as the means $\pm$ S.D. ($n = 4$ for the control group, and $n = 22$ for MCAO and iso + MCAO groups). * $P < 0.05$ compared with the control group. ^ $P < 0.05$ compared with the MCAO only group. Iso: isoflurane preconditioning.

Fig. 5. Isoflurane, but not desflurane, increased the Bcl-2 protein expression in the frontal cerebral cortex area 1

Rats were exposed to or not exposed to isoflurane or desflurane for 30 min and the frontal cerebral cortex area 1 was harvested at 24 hr after the exposure for Western blotting. **A:** exposure to 1.1% or 2.2% isoflurane. **B:** exposure to 6% or 12% desflurane. A representative Western blot is shown on the top panel and the graphic presentation of the Bcl-2 protein abundance quantified by integrating the volume of autoradiograms from 8 to 12 rats for each experimental condition is shown in the bottom panel. Values in graphs are expressed as fold change over the control rats and presented as the means $\pm$ S.D. * $P < 0.05$ compared with the control.

Table 1

Physiological parameters in rats pretreated with 2% isoflurane and then subjected to transient middle cerebral arterial occlusion (MCAO) in the 4-week observation experiment.

	MCAO		Isoflurane + MCAO	
	Before MCAO	During MCAO	Before MCAO	During MCAO
MABP (mm Hg)	110 ± 8	109 ± 9	107 ± 6	108 ± 6
Heart rate (beat/min)	337 ± 17	336 ± 16	343 ± 15	342 ± 16
Temperature (°C)	37.0 ± 0.1	37.0 ± 0.1	37.0 ± 0.2	36.9 ± 0.1
Arterial blood pH	7.44 ± 0.24	7.46 ± 0.22	7.46 ± 0.25	7.45 ± 0.24
PaCO ₂ (mm Hg)	34 ± 18	34 ± 17	35 ± 19	34 ± 19
PaO ₂ (mm Hg)	253 ± 103	290 ± 183	324 ± 153	316 ± 161
Glucose (mg/dl)	130 ± 14	121 ± 23	142 ± 52	150 ± 38

Arterial samples were taken 10 min before and after the onset of MCAO. Data are means ± S.D. (n = 7 - 27). There were no statistical differences in these physiological parameters among the rats pretreated with or without 2% isoflurane for 30 min at 24 hr before the MCAO. MABP: mean arterial blood pressure.