

# Isoflurane preconditioning increases B-cell lymphoma-2 expression and reduces cytochrome c release from the mitochondria in the ischemic penumbra of rat brain

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## Abstract

We and others have shown that prior exposure to the volatile anesthetic isoflurane induces ischemic tolerance in the brain. Our results also suggest that isoflurane preconditioning reduces cell apoptosis in the penumbral region of rat brain. We designed this study to determine whether isoflurane preconditioning decreased mitochondria-dependent cell apoptosis. Adult male Sprague–Dawley rats were exposed to or not exposed to 2% isoflurane for 30 min at 24 h before the permanent middle cerebral arterial occlusion. Western blotting was used to quantify protein expression in the cytosolic and mitochondrial fractions of non-ischemic brain cortex and brain cortex in the ischemic core and penumbra. Isoflurane preconditioning significantly decreased the infarct volume of cerebral cortex and improved neurological outcome. Isoflurane increased the expression of the antiapoptotic B-cell lymphoma-2 (Bcl-2) proteins in the cerebral cortex of rats without brain ischemia. Rats preconditioned with isoflurane before brain ischemia had increased Bcl-2 expression in the penumbra. Isoflurane preconditioning reduced the release of cytochrome c from the mitochondria and the activation of caspase 3 in the penumbra. However, isoflurane preconditioning did not alter the translocation of Bid and Bax from the cytosol to the mitochondria, identified mechanisms for Bcl-2 to block the release of cytochrome c from the mitochondria. Our results suggest that isoflurane preconditioning increases Bcl-2 expression to block the release of cytochrome c from the mitochondria to decrease the cell apoptosis in the penumbra.

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**Keywords:** Isoflurane; Preconditioning; Neuroprotection; Cytochrome c; Bcl-2; Mitochondria

## 1. Introduction

Ischemic brain injury is the underlying pathophysiology contributing to the mortality and morbidity in patients with stroke or head trauma. Although intensive research has been devoted to developing methods to reduce ischemic brain injury, clinically effective methods have not yet been established.

Ischemic preconditioning is a well-known phenomenon in which short episodes of ischemia induce ischemic tolerance (Murry et al., 1986). We and others have shown that clinically commonly used and relatively safe volatile anesthetics, such as isoflurane, can also induce ischemic tolerance (Kapinya et al.,

2002; Zhao and Zuo, 2004; Zheng and Zuo, 2003; Zheng and Zuo, 2004). Our study showed that rats pretreated with 2% isoflurane at 24 h before the permanent middle cerebral arterial occlusion (MCAO) reduced terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling positive cell density in the penumbra (Zheng and Zuo, 2004), suggesting that isoflurane preconditioning can reduce cell apoptosis after brain ischemia. However, the mechanisms for this effect are not known.

The release of cytochrome c from the mitochondria to the cytosol is an important mechanism to induce cell apoptosis (Jiang and Wang, 2004). The cytosolic cytochrome c can bind with the protease-activating factor-1 to form the apoptosome that will activate caspase 9 (Zou et al., 1997). The activated caspase 9 will activate caspase 3, which will ultimately cause cell apoptosis (Jiang and Wang, 2004). B-cell lymphoma-2 (Bcl-2) is an antiapoptotic protein. One of the important

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mechanisms for Bcl-2 to block apoptosis is to prevent the release of cytochrome c from the mitochondria (Kluck et al., 1997). Although it is not entirely clear how Bcl-2 can block this release, the proposed mechanisms include the inhibition of Bid and Bax (Cahill et al., 2006; Ferrer and Planas, 2003; Gwag et al., 2001) translocation from cytosol to the mitochondria. Both Bid and Bax are predominantly in the cytosol of healthy cells and will translocate to the mitochondria to enhance the cytochrome c release after apoptotic stimulation (Cahill et al., 2006; Ferrer and Planas, 2003; Gwag et al., 2001).

We have shown that isoflurane preconditioning can induce Bcl-2 expression in the human neuroblastoma SH-SY5Y cells (Zuo et al., 2006). Thus, we hypothesize that isoflurane preconditioning can induce the Bcl-2 expression to reduce the release of cytochrome c from the mitochondria in the penumbra to provide neuroprotection against brain ischemia.

## 2. 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.

### 2.1. Materials

A mouse monoclonal anti-Bax antibody (catalog number sc-23959), a rabbit polyclonal anti-Bid antibody (catalog number sc-11423), a mouse monoclonal anti-Bcl-2 antibody (catalog number sc-509), a goat polyclonal anti-caspase-3 p20 antibody (catalog number sc-1225), a horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG secondary antibody (catalog number sc-2020), a HRP-conjugated goat anti-rabbit IgG secondary antibody (catalog number sc-2004) and a HRP-conjugated goat anti-mouse IgG secondary antibody (catalog number sc-2005) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal anti-cytochrome c antibody (catalog number 556433) was purchased from BD Pharmingen™ (San Jose, CA). A rabbit polyclonal anti-actin antibody (Catalog number A2066) and the protease inhibitor cocktail (catalog number P2714) were obtained from Sigma Chemical (Saint Louis, MI). A rabbit polyclonal anti-mitochondrial voltage-dependent anion channel (VDAC) antibody (catalog number 4866) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Polyvinylidene difluoride membranes for Western blotting and reagents for measuring protein concentrations were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents were obtained from Pharmacia-Amersham Biotech (Piscataway, NJ). Other chemicals were purchased from Sigma.

### 2.2. Animal groups

Two groups of 2-month-old male Sprague–Dawley rats were studied: rats that were subjected to the permanent right MCAO

only and rats that were pretreated with 2% isoflurane for 30 min at 24 h before the MCAO. Two other groups of 2-month-old male Sprague–Dawley rats were exposed or not exposed to 2% isoflurane for 30 min. Their parietal brain tissues were harvested 24 h after the isoflurane exposure for Western analysis of Bcl-2 expression.

### 2.3. Isoflurane preconditioning

Anesthesia was induced with isoflurane and then maintained with 2% isoflurane via an endotracheal tube for 30 min. Respiration was controlled by a ventilator to maintain normal end-tidal O<sub>2</sub> and CO<sub>2</sub> concentrations. The inhaled and exhaled gases were monitored with a Datex infrared analyzer (Capnomac, Helsinki, Finland).

### 2.4. Permanent middle cerebral artery occlusion

The rats were anesthetized with isoflurane and then intubated and mechanically ventilated with 60% O<sub>2</sub>–40% N<sub>2</sub> containing 2% isoflurane. The tail artery was cannulated for monitoring arterial blood pressure and blood sampling for measuring blood gases and glucose. Permanent MCAO was accomplished as we described previously (Zheng and Zuo, 2004). The MCAO was achieved by advancing a 3–0 monofilament nylon suture with a rounded tip to the right internal carotid artery via the external carotid artery until slight resistance was felt. Isoflurane anesthesia was stopped once the suture was in place. After recovery from anesthesia, rats were placed back in their cages with *ad libitum* access to food and water. During anesthesia, temporalis muscle temperature was strictly maintained at 37±0.2 °C by warming blanket and lamps. The inhaled and exhaled gases were also monitored to maintain normal end-tidal CO<sub>2</sub> concentrations.

### 2.5. Evaluation of infarct volume, neurological deficit scores, and motor coordination

The evaluation of infarct volume at 24 h after the permanent MCAO was performed after 2,3,5-triphenyleterazolium chloride staining as described previously (Alessandrini et al., 1999). Briefly, brain hemispheres were cut into 2 mm thick coronal brain slices (usually total 6 slices) in the rostral–caudal direction. The slices were incubated with 2,3,5-triphenyleterazolium chloride for 30 min at 37 °C. The infarct areas were quantified using the NIH Image 1.60. 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 volume, the percentage of infarct volume in the ipsilateral hemisphere volume was calculated (Swanson et al., 1990).

Neurological deficit scores were evaluated 24 h after the permanent MCAO based on an eight-point scale. 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 h before and 24 h after the permanent MCAO. 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.

## 2.6. Brain tissue sampling and the fractionation of cytosol and mitochondria

The rats were killed by deep isoflurane anesthesia and transcardially perfused by cold phosphate buffered saline (PBS) at 24 h after the permanent MCAO. The frontal cortex area 1 and parietal cortex area 1 from bregma +2 to 0 mm in the right cerebral hemisphere and the corresponding frontal cortex area 1 in the left hemisphere were removed. The frontal cortex area 1 has decreased blood flow and are classified as penumbral areas after MCAO (Memezawa et al., 1992; Nagasawa and Kogure, 1989). The parietal cortex area 1 has a very significant decrease of blood flow, is almost always infarct after MCAO and is considered as ischemic core in this brain ischemic model (Sakai et al., 2007).

The total lysates and the cytosolic and mitochondrial fractions of the harvested brain tissues were prepared as previously described (Hirai et al., 2004). Each brain tissue sample was homogenized in ice-cold lysis buffer [200 mM mannitol, 80 mM HEPES-KOH (pH 7.4), and the protease inhibitor cocktail] by 35 strokes of gentle pounding in a glass tissue grinder. Homogenates were centrifuged at  $750 \times g$  for 10 min at 4 °C. After removing some of the supernatants that were used as total lysates, the rest of the supernatants were centrifuged at  $8000 \times g$  for 20 min at 4 °C. The resulting pellets were washed 3 times and suspended in the lysis buffer as the mitochondrial fraction. Supernatants of  $8000 \times g$  centrifugation were centrifuged at  $100,000 \times g$  for 1 h at 4 °C. The resulting supernatants were cytosolic fraction. The protein concentration of each sample was determined using the Bradford assay.

## 2.7. Western analysis

Proteins of 50  $\mu g$ , 20  $\mu g$  and 40  $\mu g$  per lane of the total lysates, mitochondrial and cytosolic fractions, respectively, were subjected to 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene fluoride membrane. After incubation with PBS containing 5% nonfat milk and 0.1% Tween-20 for 1 h at room temperature, the membranes were probed with various primary antibodies in PBS containing 0.1% Tween-20 (PBST). The primary antibodies used include the anti-Bcl-2 antibody (1:200 dilution), anti-Bax antibody (1:200 dilution), anti-Bid antibody

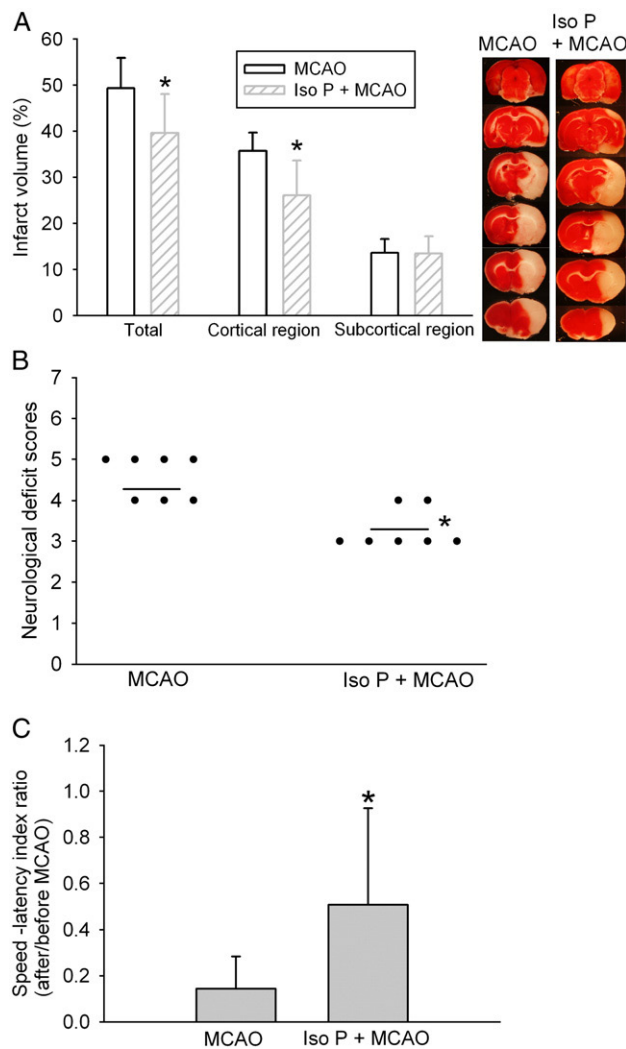


Fig. 1. Isoflurane preconditioning improves neurological outcome after permanent right middle cerebral arterial occlusion (MCAO). Panel A: Percentage of infarct volume in ipsilateral hemisphere volume is shown in the left panel. Results are the means  $\pm$  S.D. ( $n=7$ ). Brain slices stained with 2,3,5-triphenyleterazolium chloride from representative rats subjected to MCAO only or isoflurane preconditioning plus MCAO are shown in the right panel. Panel B: Neurological deficit scores that were evaluated immediately before the animals were sacrificed for the assessment of infarct sizes (data are presented in Fig. 1A) are shown. Each circle represents the score for a single rat. A horizontal bar indicates the mean value for each group. Panel C: The performance on rotarod. Rats were tested before and 24 h after the MCAO. The speed–latency index ratio of the tests at these two time points is presented. Results are the means  $\pm$  S.D. ( $n=7$ ). \* $P \leq 0.05$  compared with the corresponding MCAO group. Iso P: isoflurane preconditioning.

(1:200 dilution), anti-cytochrome c antibody (1:1000 dilution), anti-caspase-3 p20 antibody (1:200 dilution), anti-VDAC antibody (1:1000 dilution) and anti-actin antibody (1:1000 dilution) overnight at 4 °C. After being rinsed with PBST, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies for 1 h at room temperature. 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

Table 1

Physiological parameters in rats subjected to permanent middle cerebral arterial occlusion (MCAO)

|                           | MCAO        |             | Isoflurane + MCAO |             |
|---------------------------|-------------|-------------|-------------------|-------------|
|                           | Before MCAO | During MCAO | Before MCAO       | During MCAO |
| MABP (mm Hg)              | 97±14       | 98±13       | 99±16             | 101±17      |
| Heart rate (beat/min)     | 332±18      | 336±15      | 322±34            | 331±12      |
| Temperature (°C)          | 37.1±0.1    | 37.1±0.1    | 37.0±0.2          | 37.0±0.2    |
| pH                        | 7.29±0.07   | 7.37±0.1    | 7.32±0.1          | 7.27±0.06   |
| PaCO <sub>2</sub> (mm Hg) | 50±6        | 45±8        | 46±10             | 53±1        |
| PaO <sub>2</sub> (mm Hg)  | 216±35      | 196±31      | 223±50            | 341±137     |
| Glucose (mg/dl)           | 237±34      | 221±56      | 197±66            | 184±20      |

Arterial samples were taken 10 min before and after the onset of MCAO. Data are mean±S.D. ( $n=3-10$ ). There were no statistical differences in these physiological parameters among the rats pretreated with or without 2% isoflurane for 30 min before the MCAO. MABP: mean arterial blood pressure.

densities of Bcl-2, Bax, Bid, cytochrome c and caspase-3 p20 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 treated with the isoflurane preconditioning and then the MCAO were normalized to the corresponding data of rats with MCAO only on the same film to control for the error caused by different exposure times of films.

### 2.8. Statistical analysis

Parametric data are presented as means±S.D. Statistical analysis of the results of physiological parameters, speed–latency index ratio, infarct size, and the Western blotting between the MCAO groups and isoflurane preconditioning plus MCAO group was performed by Student's *t* test after confirmation of a normal distribution of the data or Mann–Whitney rank sum test when the data are not normally distributed. Neurological deficit scores were analyzed by Mann–Whitney rank sum test. A  $P \leq 0.05$  was accepted as significant.

### 3. Results

As we have shown previously (Zheng and Zuo, 2004), rats preconditioned with 2% isoflurane for 30 min had significantly smaller brain infarct volumes than rats without isoflurane pretreatment after the MCAO (Fig. 1). This reduction of brain infarct volume was due to the decreased infarct volume of the cerebral cortex. Isoflurane preconditioning did not affect the infarct volumes of the subcortical areas. Consistent with the neuropathological data, isoflurane preconditioning also improved the neurological deficit scores and the motor coordination (Fig. 1). However, isoflurane preconditioning did not change the hemodynamics, blood gases and glucose levels during the procedure of MCAO (Table 1).

As shown in the Fig. 2, the mitochondrial fractions contained abundant VDAC proteins, a mitochondrial marker. The VDAC proteins were not detected in the cytosolic fractions. These

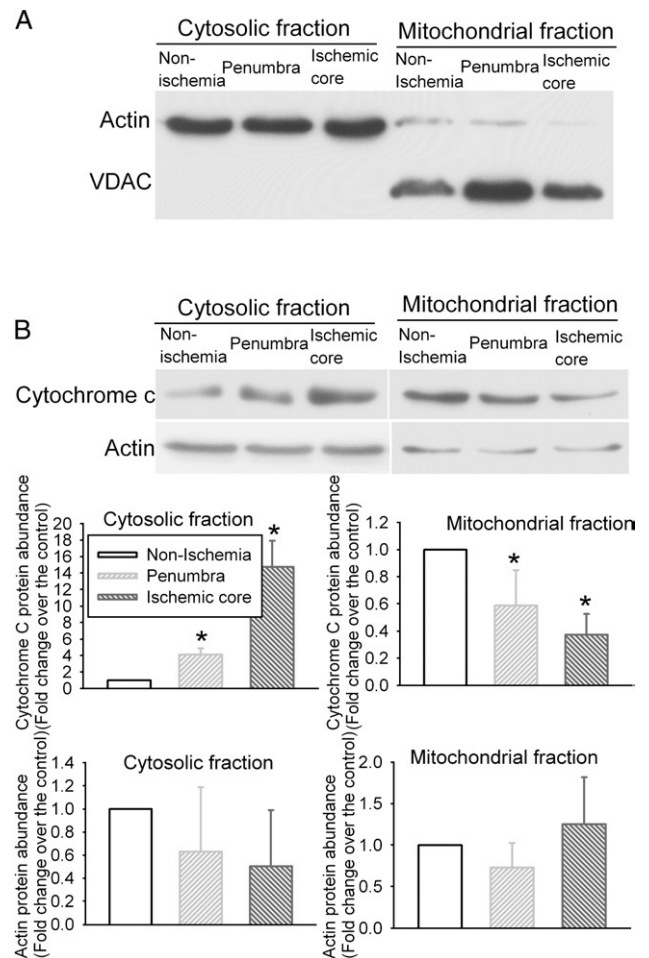


Fig. 2. Brain ischemia induces release of cytochrome c from the mitochondria. Rats were subjected to the permanent right middle cerebral arterial occlusion (MCAO). The left frontal cortex area 1 (non-ischemia), right frontal cortex area 1 (penumbra) and right parietal cortex area 1 (ischemic core) were harvested. The cytosolic and mitochondrial fractions were prepared for Western blotting. Panel A is a representative Western blot to show the excellent separation of mitochondrial fractions from the cytosolic fractions. Panel B: a representative Western blot is shown on the top panel and the graphic presentation of the cytochrome c and actin protein abundance quantified by integrating the volume of autoradiograms from 5 rats is shown in the bottom panel. Values in graphs are expressed as fold change over non-ischemic brain cortex and presented as the means±S.D. \*  $P < 0.05$  compared with the corresponding value from the non-ischemic brain cortex. VDAC, voltage-dependent anion channel.

results suggest that we have excellent separation of mitochondrial fractions from cytosolic fractions with our method. Although the amount of actin, the protein used as internal control, in the cytosolic and mitochondrial fractions was not affected by brain ischemia (Fig. 2), cytochrome c in the cytosolic fraction of the ischemic penumbra (right frontal cortex area 1) and core (right parietal cortex area 1) was significantly higher than that in the frontal cortex area 1 contralateral to the ischemic side. Cytochrome c in the mitochondrial fraction of the ischemic penumbra and core was significantly lower than that in the frontal cortex area 1 contralateral to the ischemic side (Fig. 2). These results suggest that ischemia induced cytochrome c release from the mitochondria. This cytochrome c release in the



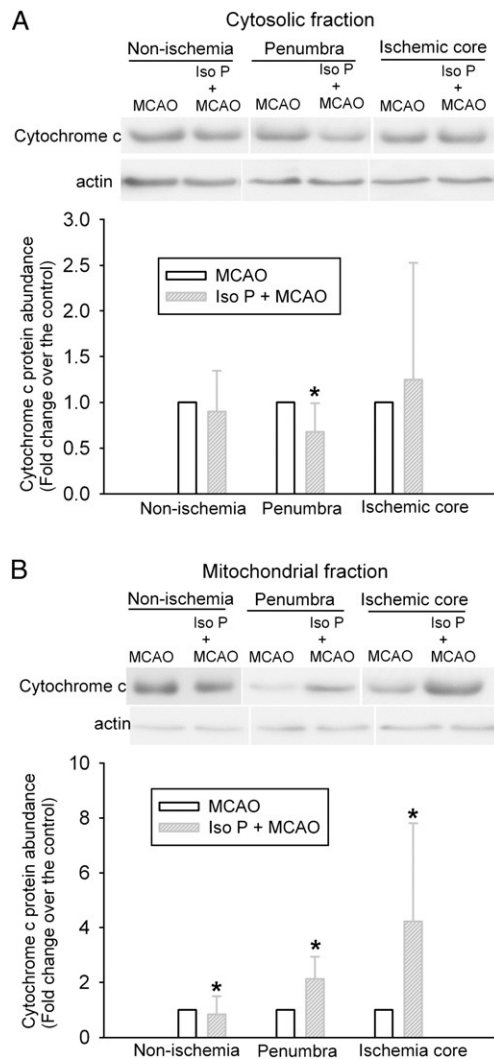


Fig. 3. Isoflurane decreases release of cytochrome c from the mitochondria in the ischemic penumbra after the permanent right middle cerebral arterial occlusion (MCAO). Rats were exposed to or not exposed to 2% isoflurane for 30 min before the MCAO. The left frontal cortex area 1 (non-ischemia), right frontal cortex area 1 (penumbra) and right parietal cortex area 1 (ischemic core) were harvested and the cytosolic and mitochondrial fractions were prepared for Western blotting. Panel A: Cytosolic fraction; Panel B: Mitochondrial fraction. A representative Western blot is shown on the top panel and the graphic presentation of the cytochrome c protein abundance quantified by integrating the volume of autoradiograms from 5 to 7 rats is shown in the bottom panel. Values in graphs are expressed as fold change over the rats subjected to the MCAO only and presented as the means  $\pm$  S.D. \*  $P < 0.05$  compared with the MCAO only. Iso P: isoflurane preconditioning.

penumbra was significantly reduced by isoflurane preconditioning because more cytochrome c proteins were in the mitochondria and less cytochrome c proteins were in the cytosol in the penumbra of rats preconditioned with isoflurane and then MCAO than rats with MCAO only (Fig. 3). Consistent with this result, the active caspase 3 (20 KDa form) in the ischemic penumbra was significantly reduced by isoflurane preconditioning (Fig. 4).

Isoflurane exposure significantly increased the expression of Bcl-2 proteins in the cerebral cortex of rats without brain

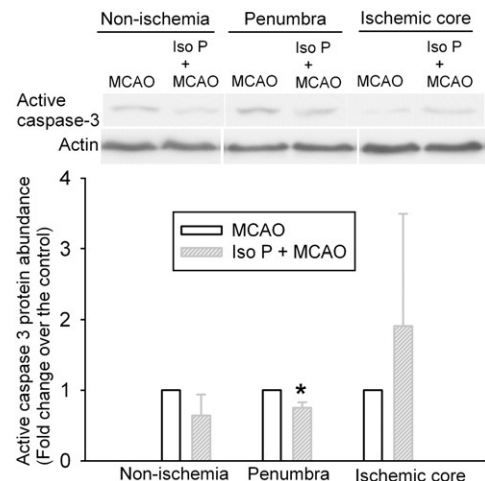


Fig. 4. Isoflurane decreases the activation of caspase 3 in the ischemic penumbra after the permanent right middle cerebral arterial occlusion (MCAO). Rats were exposed to or not exposed to 2% isoflurane for 30 min before the MCAO. The left frontal cortex area 1 (non-ischemia), right frontal cortex area 1 (penumbra) and right parietal cortex area 1 (ischemic core) were harvested for Western blotting of the active caspase 3 (the 20 KDa form). A representative Western blot is shown on the top panel and the graphic presentation of the active caspase 3 protein abundance quantified by integrating the volume of autoradiograms from 5 to 7 rats is shown in the bottom panel. Values in graphs are expressed as fold change over the rats subjected to the MCAO only and presented as the means  $\pm$  S.D. \*  $P < 0.05$  compared with the MCAO only. Iso P: isoflurane preconditioning.

ischemia (Fig. 5). The expression of Bcl-2 proteins in the penumbral region of rats pretreated with isoflurane was significantly higher than that in rats with MCAO only (Fig. 5). These results suggest that the increased Bcl-2 expression contributes to the reduced cytochrome c release from the mitochondria in the penumbra. We then determined whether this Bcl-2 protective effect was via blocking the translocation of Bid and Bax from the cytosol to the mitochondria. There was no significant difference in the expression of Bid and Bax proteins in the cytosolic and mitochondrial fractions prepared from the penumbra of rats pretreated with or without isoflurane before the MCAO (Figs. 6 and 7), suggesting that Bcl-2 does not affect the translocation of Bid and Bax to block the cytochrome c release from the mitochondria.

#### 4. Discussion

Our study showed that isoflurane preconditioning reduced brain infarct volumes and improved neurological functions after the MCAO. This protective effect is mainly from the reduction of the infarct volume in the cerebral cortex. Since we used permanent MCAO and the collateral circulation to the striatum is very poor in rats after MCAO, it is not surprising to observe that isoflurane preconditioning did not reduce the subcortical infarct volume. Similarly, the parietal cortex area 1 has a significant decrease of blood flow and is considered as ischemic core after MCAO (Sakai et al., 2007). However, frontal cortex area 1 has decreased blood flow and are classified as penumbral areas after MCAO (Memezawa et al., 1992; Nagasawa and

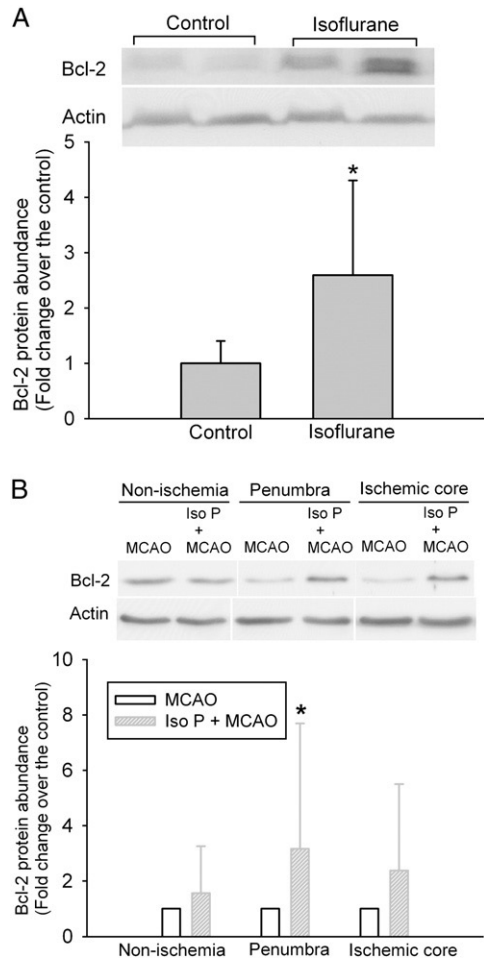


Fig. 5. Isoflurane increases the expression of Bcl-2 in the cerebral cortex. Panel A: Rats were exposed to or not exposed to 2% isoflurane for 30 min and the cerebral cortex was harvested for Western blotting of Bcl-2. A representative Western blot is shown on the top panel and the graphic presentation of the Bcl-2 protein abundance quantified by integrating the volume of autoradiograms from 8 rats is shown at 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 only. Panel B: Rats were exposed to or not exposed to 2% isoflurane for 30 min at 24 h before the MCAO. The left frontal cortex area 1 (non-ischemia), right frontal cortex area 1 (penumbra) and right parietal cortex area 1 (ischemic core) were harvested for Western blotting. 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 5–7 rats is shown at the bottom panel. Values in graphs are expressed as fold change over the rats subjected to the MCAO only and presented as the means  $\pm$  S.D. \* $P$  < 0.05 compared with the MCAO only. Iso P: isoflurane preconditioning.

Kogure, 1989). Thus, our findings are consistent with the idea that isoflurane preconditioning reduces the infarction/cell death in the penumbral regions. We observed about 20–25% reduction in infarct brain volumes by isoflurane preconditioning. This relatively small reduction may be related to the use of a severe brain ischemia model, permanent MCAO, in this study. Nevertheless, we still observed improvement of neurological functions by isoflurane preconditioning in this model.

Our previously published data suggest that isoflurane preconditioning reduced cell apoptosis in the penumbral region

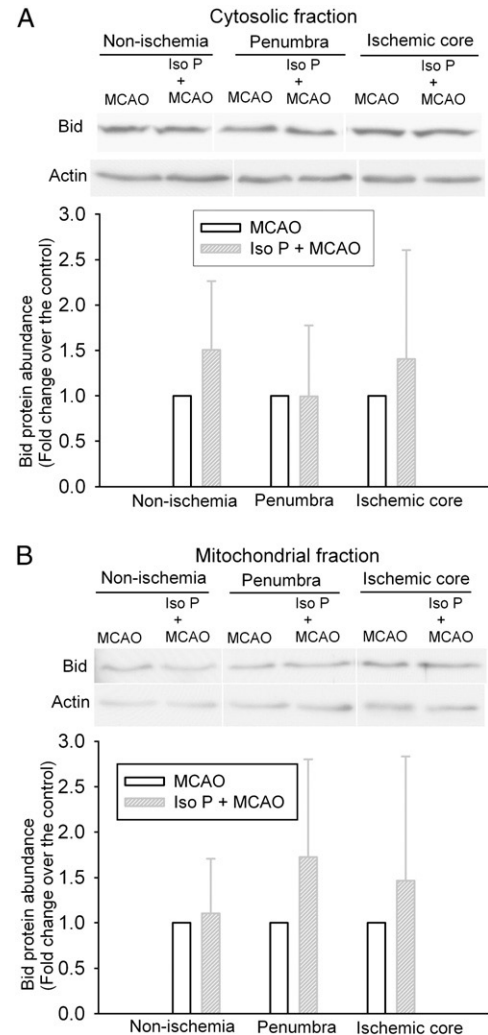


Fig. 6. Isoflurane did not affect the translocation of Bid in the ischemic penumbra after the permanent right middle cerebral arterial occlusion (MCAO). Rats were exposed to or not exposed to 2% isoflurane for 30 min before the MCAO. The left frontal cortex area 1 (non-ischemia), right frontal cortex area 1 (penumbra) and right parietal cortex area 1 (ischemic core) were harvested and the cytosolic and mitochondrial fractions were prepared for Western blotting. Panel A: Cytosolic fraction; Panel B: Mitochondrial fraction. A representative Western blot is shown on the top panel and the graphic presentation of the Bid protein abundance quantified by integrating the volume of autoradiograms from 5–7 rats is shown in the bottom panel. Values in graphs are expressed as fold change over the rats subjected to the MCAO only and presented as the means  $\pm$  S.D. Iso P: isoflurane preconditioning.

(Zheng and Zuo, 2004). We have also shown that isoflurane induced Bcl-2 expression in the human neuroblastoma SH-SY5Y cells (Zuo et al., 2006). Thus, it is possible that isoflurane preconditioning increases Bcl-2 in the penumbral region to decrease cell apoptosis. Our results showed that a 30-min isoflurane exposure increased Bcl-2 expression in the rat cerebral cortex at 24 h after the isoflurane exposure. Isoflurane preconditioning also increased the Bcl-2 protein expression in the penumbral region at 24 h after the MCAO. Since Bcl-2 is a well-known antiapoptotic molecule (Kluck et al., 1997; Yang et al., 1997), our results suggest a role of Bcl-2 in the isoflurane preconditioning-induced decrease of cell apoptosis.

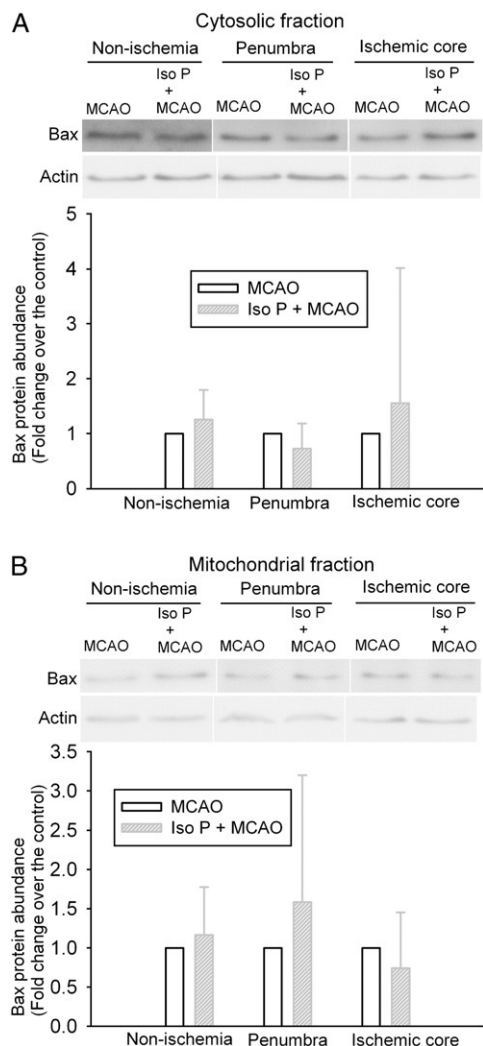


Fig. 7. Isoflurane did not affect the translocation of Bax in the ischemic penumbra after the permanent right middle cerebral arterial occlusion (MCAO). Rats were exposed to or not exposed to 2% isoflurane for 30 min before the MCAO. The left frontal cortex area 1 (non-ischemia), right frontal cortex area 1 (penumbra) and right parietal cortex area 1 (ischemic core) were harvested and the cytosolic and mitochondrial fractions were prepared for Western blotting. Panel A: Cytosolic fraction; Panel B: Mitochondrial fraction. A representative Western blot is shown on the top panel and the graphic presentation of the Bax protein abundance quantified by integrating the volume of autoradiograms from 5–7 rats is shown in the bottom panel. Values in graphs are expressed as fold change over the rats subjected to the MCAO only and presented as the means  $\pm$  S.D. Iso P: isoflurane preconditioning.

One of the important mechanisms for Bcl-2 to block cell apoptosis is to decrease cytochrome c release from the mitochondria (Kluck et al., 1997; Yang et al., 1997). Consistent with this idea, our results showed that the release of cytochrome c from the mitochondria in the penumbra was inhibited by isoflurane preconditioning. Once cytochrome c is released into cytosol, it will form the apoptosome to activate caspase 9 and then caspase 3 to induce cell apoptosis (Jiang and Wang, 2004). Our results also showed that the active caspase 3 in the penumbra was decreased by isoflurane preconditioning. These results suggest that isoflurane preconditioning provides protection in the penumbra by increasing

Bcl-2 expression to block the activation of caspase 3. Consistent with our findings, a recent study has shown that ischemic preconditioning reduced the number of apoptotic neurons, cytochrome c release from the mitochondria and the activation of caspase 9 in the hippocampal CA1 region of rats after global brain ischemia (Liu et al., 2007).

It has been suggested that Bcl-2 can block the translocation of Bid and Bax from cytosol to the mitochondria to decrease the release of cytochrome c from the mitochondria (Cahill et al., 2006; Ferrer and Planas, 2003; Gwag et al., 2001). However, our results suggest that isoflurane preconditioning does not change the translocation of Bid or Bax in the penumbra. In addition to affecting the translocation of Bid and Bax to block the release of cytochrome c, it has been proposed that Bcl-2, by binding to the mitochondrial membrane, can directly block the release of cytochrome c from the mitochondria (Kluck et al., 1997).

It is not known yet how isoflurane can induce the expression of Bcl-2. We have shown that isoflurane preconditioning-induced neuroprotection may be p38 activation-dependent (Zheng and Zuo, 2004). Signal transducer and activator of transcription 3 (STAT3) increases Bcl-2 expression (Alas et al., 2001). Inhibition of p38 has been shown to inhibit STAT3-increased Bcl-2 expression (Vega et al., 2004). We and others have shown that isoflurane can induce the expression of inducible nitric oxide synthase (Kapinya et al., 2002; Zhao and Zuo, 2004). Nitric oxide produced by inducible nitric oxide synthase can activate STAT3 (Hierholzer et al., 2004). Future studies will be designed to determine whether p38, STAT3 and inducible nitric oxide synthase are involved in the isoflurane-induced Bcl-2 expression.

In summary, our results showed that isoflurane preconditioning reduced cell death in the penumbral region. This protection may involve Bcl-2 to block the cytochrome c release from the mitochondria and, therefore, to decrease cell apoptosis in the penumbra.

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