Isoflurane preconditioning protects rat brain from ischemia reperfusion injury via up-regulating the HIF-1α expression through Akt/mTOR/s6K activation

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Abstract: The volatile anesthetic isoflurane (ISO) has been widely used in ischemia reperfusion (IR) injury because its abilities to induce and trigger recovery are faster and smoother than other agents. However, the underlying molecular mechanisms for preconditioning the ISO in protecting the brain against IR injury are still largely unclear. In this paper, we investigated the neuroprotective effect of the ISO in the in vivo and in vitro models of IR injury and evaluated the possible correlation with Akt/mTOR/s6K signaling pathway. From the in vivo studies, we demonstrated that ISO preconditioning alleviated the IR-induced neurological deficits, infarct volume, brain edema and cell apoptosis, which were mainly due to the up-regulation of the p-Akt, p-mTOR and p-s6K proteins by the histopathological detections and Western blotting assay. The in vitro studies, demonstrated that ISO preconditioning reduced the release of OGD-induced lactate dehydrogenase (LDH) and enhanced the OGD-inhibited cell viability. It has also been observed that the hypoxia inducible factor-1α (HIF-1α) was increased under ISO preconditioning. Utilization of the BEZ235, a PI3K/mTOR dual inhibitor, halted the ISO-induced up-regulation of the HIF-1α, and inhibited the phosphorylation of the Akt, mTOR and s6K proteins. Besides, the ISO reduced the OGD-induced cell apoptosis, which was blocked by the BEZ235. In fact, these results thus suggest that the ISO preconditioning may provide potential neuroprotection against IR injury via up-regulating the HIF-1α expression through the Akt/mTOR/s6K activation.

Key words: Isoflurane, ischemia reperfusion, Akt/mTOR/s6K, HIF-1α, OGD.

Introduction

Cerebral ischemia reperfusion (IR), a pathophysiological phenomenon commonly encountered from various neurological and cardiovascular procedures, is regarded as one of the serious perioperative procedural complications (1). It has been invariably associated with oxidative stress, inflammatory response, and cell apoptosis (2). It is well recognized that statins and some volatile anesthetics are widely used to treat IR injury, although the therapies have not been very satisfactory, involving some side effects. Several researches have been conducted to identify effective strategies and agents to ameliorate brain IR injury.

ISO is an inhaled anesthetic broadly used in clinical surgical operations because of its excellent quality and minimal systemic toxicity (3). The action targets of the ISO are present throughout the central nervous system (CNS), such as the spinal cord, brain stem and cortex. The original ISO was applied only as an analgesic in the CNS, such as the spinal cord, brain stem and cortex. However, the HIF-1α is stabilized via reducing the activity of the prolyl hydroxylases that target the protein for degradation under hypoxia (14). There is increasing evidence which confirms that the HIF-1α activation may reduce the brain injury post IR, but the possible potential mechanisms have not been fully elucidated.

Research has found that the PI3K/Akt pathway is involved in tumor angiogenesis by an HIF-1α-dependent mechanism (15). The Akt/mTOR/s6K signaling pathway plays a vital role in normal cellular functions including cell proliferation, cell apoptosis, cell migration, energy metabolism and protein synthesis (16-18). Also, several studies have lately focused on the new function of the Akt/mTOR/s6K signaling pathway, and showed that the pathway is necessary and sufficient to protect against IR injury (19-22). But only a few studies have been conducted to investigate into whether the activation of the Akt/mTOR/s6K signaling pathway has a role to play in the neuroprotection of the ISO preconditioning.

In this study, we investigated the neuroprotective
effect of the ISO in rats with middle cerebral artery occlusion (MCAO) in vivo, and further, tried to clarify the contribution of the ISO and the Akt/mTOR/s6K signaling pathway in IR injury of the rat brain. We also used the mouse brain endothelial cell line (bEnd.3) and oxygen glucose deprivation (OGD) model to mimic the IR injury in vitro. In summary, we tested the hypothesis that the neuroprotective effect of the ISO preconditioning is associated with the inhibition of apoptosis and mediated by up-regulating the HIF-1α expression through the activation of the Akt/mTOR/s6K signaling pathway in vivo and in vitro.

Materials and Methods

Experimental animals and groups

Seventy-two adult male Sprague-Dawley rats weighing between 250 to 300 g were used. The animals were maintained on a 12-h light/dark cycle with free access to food and water. They were adapted to these conditions for at least seven days prior to the experiments. All the animal experiments involving animal use were approved by the Animal Ethics Committee of Ningbo No.2 Hospital. The animals were randomly assigned into sham group (n=8), IR group (n=8) and IR+ISO group (n=8). The rats in the sham group underwent the IR surgical procedure, except that the thread was not inserted into the common carotid artery. The rats in the IR group were used to establish the MCAO model. In the IR+ISO group, the animals were pre-treated with 1.5% ISO (Baxter Healthcare, Deerfield, USA) dissolved in dimethyl sulfoxide (DMSO; Sigma, NY, USA), and then were subjected to the IR surgical procedure. All the drugs were intraperitoneally administered.

Cell culture and exposure to oxygen glucose deprivation (OGD)

Mouse brain endothelial cells bEnd.3 (ATCC, Manassas, USA) were cultured in a media composed of Dulbecco’s Modified Eagle’s Medium (DMEM/F; HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 4.5 g/L glucose (Sigma, NY, USA) and antibiotics (100 U/mL penicillin G, 100 mg/mL streptomycin) (Gibco, Grand Island and NY, USA) in a humidified atmosphere with 5% CO₂ at 37°C and subcultured every three days.

The OGD induction used to mimic ischemic injury was based on the method described prior (23). The groups were randomly assigned into the following categories: the control group (n=8), OGD group (n=8) and OGD+ISO group (n=8). After 12 days in vitro, the inserts were transferred to a sterilized 6-well plate and incubated with 1 mL of OGD medium (glucose-free) for 10 min to deplete glucose (BSS) from intracellular stores and the extracellular space. The OGD medium included a combination of 150 mM NaCl, 6.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, and 5.0 mM HEPES at pH 7.6. Next, the medium was exchanged for an OGD medium filled with 95% N₂/5% CO₂ atmosphere at 37°C for 60 min. The oxygen level was controlled at less than 0.02% O₂, where they were maintained for 60 min. Then the cultures were restored and maintained in an incubator with a 5% CO₂/95% O₂ atmosphere at 37°C in the culture medium for 6h. After the OGD period, the cultures were carefully washed twice with BSS₅₋. Cell density was maintained at 80% or less confluence and then treated with 2% ISO (Baxter Healthcare, Deerfield, USA) dissolved in DMSO. BEZ235 was dissolved in DMSO at concentration of 20 μmol/L and stored at -20°C until further use.

Experimental rat model of cerebral IR

The methods employed have been described earlier in detail (24). In brief, the rats from all the groups were initially anesthetized with 10% choral hydrate in a mixture of O₂ and NO₂ during the surgery. A 1.5-cm longitudinal incision was made in the midline of the ventral cervical skin. The right common carotid artery (CCA), right internal carotid artery (ICA) and right external carotid artery (ECA) were isolated. A silicon-coated fishing line (diameter 0.32 mm) was introduced into the right CCA and advanced 17 min into the ICA until its tip was 1–3 mm away from the origin of the MCA. After 60 min of occlusion, the suture was removed to allow reperfusion. Rectal temperature was strictly maintained at 37±0.5°C using a warming blanket and lamps from the commencement of the surgery until the animal recovered from anesthesia.

Quantification of the brain perfusion deficits, infarct volume, brain edema

Assessments regarding the neurological deficits were made on the animals in all the groups. The rats that succumbed within 24 h post MCAO were excluded. Assessments were made 24 h after reperfusion just before the brain tissue sampling was done. The degrees of neurological deficit were graded from grades 0–4, in which 0 = no deficit; 1 = failure to extend the left forepaw fully; 2 = circling to the left; 3 = falling to the left; 4 = unable to walk spontaneously.

Briefly, 24 h post MCAO, the anesthetized rats were decapitated and the brains were removed. For the morphometric study, 2-mm-thick coronal sections were cut using the rat brain matrix. The sections were stained using a 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution for 20 min at 37°C and fixed by immersion in 10% formalin. Normal brain tissues were stained red, while the unstained (white) area was considered to be the infarct area. Pictures were taken of the brain slices with a digital camera and the volume of the infarct was analyzed using a computer-assisted image system with ACT-2U software (Nikon, Xi’an, China). The degree of infarction was calculated by employing the equation: %infarct area=infarct area/total area of slice×100.

The rats (n=8, in each group) were anesthetized and the brains were removed at 24 h after reperfusion. The rat brains were then sectioned in the coronal plane at 2-mm intervals. After weighing for wet weight, the brain slices were oven dried at 70°C for 72 h, and weighed again for dry weight. The brain edema was calculated as follows: (wet weight−dry weight)/wet weight×100%.

MTT assay

Cell viability was investigated by measuring the metabolism of MTT. The MTT solution (5mg/mL) was added to the culture medium and, the pretreated bEnd.3 cells were sustained for 4 h at 37°C, after which the MTT solution was removed. The solubilization solution
containing 20% sodium dodecyl sulfate (SDS) at pH 4.8 and 50% dimethyl formamide were added and absorptions at 490 nm were measured. Cell viability was determined as the percentage of living cells.

**Lactated hydrogenase (LDH) analysis**

Cell viability was quantified via measuring the LDH release at 6 h after the OGD restoration using a colorimetric LDH cytotoxicity detection kit (Clontech, Mountain View, CA, USA) according to the manufacturer’s manual. The LDH activity was determined via a colorimetric assay using an absorbance wave length of 492 nm and a reference wave length of 655 nm in a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The background absorbance was subtracted and the percentage of LDH release was calculated based on the LDH standard curve.

**Flow cytometry analysis**

The floating cells were collected post treatment. The adherent cells were incubated in 2 mM EDTA–PBS at 37°C for 10 minute. They were then centrifuged and stained with FITC-A (BD Biosciences, San Jose, California, USA) and PE-Texas Red-A (BD Biosciences, San Jose, California, USA) according to the manufacturer’s instructions. In brief, the cells were washed twice with PBS and incubated in 100 μL of binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; 140 mM sodium chloride (NaCl); and 2.5 mM calcium chloride) containing FITC-A (1:20) and 5 mg/mL for 15 min at room temperature (RT) in the dark. Next 400 mL of binding buffer was added to each sample. Apoptosis was analyzed by flow cytometry within 1h.

**Western blotting**

The Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify protein concentrations. All proteins from tissues were separated by the 10% SDS-PAGE, blotted and probed with the primary antibodies, including anti-HIF-1α antibodies (Novus Biologicals Inc., Littleton, CO) and HIF-1β antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, USA); anti-Akt, anti-mTOR, anti-s6K antibodies and their phosphorylated antibodies anti-pAkt (Ser473), anti-p-mTOR (Ser2448), anti-p-s6K (Ser371) (Cell Signaling, Danvers, MA, USA) and anti-β-actin antibody (Santa Cruz, CA, USA); GAPDH (Abcam, Cambridge, MA, USA); anti-Cleaved caspase-3, anti-Caspase-3, anti-Bcl-2 and anti-Bax antibodies (Sigma, NY, USA). The blots were visualized using a chemiluminescence system (Amersham Bioscience, Buchinghamshire, UK).

**Statistical analysis**

Data are expressed as the mean±SD. Statistical significance was determined using the student’s t-test when there were two experimental groups. When more than two groups were compared, a statistical evaluation of the data was performed employing the one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

**Results**

ISO pre-conditioning alleviated the IR-induced brain perfusion neurological deficits, infarct volume and brain edema in rats

A provisional 60 min of the focal cerebral ischemia followed by reperfusion for 6h induced brain infarct, brain edema and neurological deficits. The ISO pre-treatment exhibited significant protection accompanied by reductions in the neurological deficits (Figs. 1A&B), infarct volume (Fig. 1C), and brain edema (Fig. 1D) compared with the IR group.

ISO pre-conditioning inhibited IR-induced cell apoptosis in vivo

To further investigate the effect of ISO preconditioning on cell apoptosis, the expression of the apoptosis-related proteins including Caspase-3, pro-apoptotic proteins Cleaved caspase-3, Bax and Bcl-2 were detected by Western blot analysis (Fig. 2A). We found that the levels of Cleaved caspase-3 and Bax were significantly up-regulated in the IR group compared with the sham group (Figs. 2C&D). The Bcl-2 expression was lower in the IR group than in the sham group (Fig. 2E). However, the ISO pre-conditioning remarkably inhibited the IR-induced cell apoptosis in all the tests conducted (Figs. 2C-E). In short, the data suggest that the ISO preconditioning decreased the increased cell apoptosis after the cerebral IR injury, which may contribute to the ISO-mediated neuroprotection.

ISO preconditioning further activated the Akt/mTOR/s6K signaling pathway in vivo

To investigate whether the Akt/mTOR/s6K was involved in the neuroprotective effect of the preconditioning with ISO, we evaluated the expression of the p-Akt, p-mTOR and p-s6K proteins (Fig. 3A). Western blot analysis demonstrated that the Akt/mTOR/s6K phosphorylation was significantly increased after cerebral IR injury in vivo (Figs. 3B-D). Preconditioning...
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with the ISO further activated the phosphorylation of the Akt/mTOR/s6K signaling pathway (Figs. 3B-D). These data suggest that recovery from the cerebral IR injury with the ISO preconditioning may occur in part through the activation of the Akt/mTOR/s6K signaling pathway.

ISO preconditioning attenuated the IR-induced cell viability injury in vitro

The LDH activity rate was significantly elevated in the OGD group compared with the control group. However, the ISO preconditioning significantly inhibited the increased LDH release in a dose-dependent manner. It was note worth that the 2% ISO exerted the strongest inhibitory effect among the different concentration groups (Fig. 4A). We also found that the 2% ISO preconditioning distinctly improved cell survival post OGD-induced injury (Fig. 4B). These results confirm the specific protective effects of the 2% ISO against OGD-induced injury in vitro.

The BEZ235 treatment blocked the activation of the HIF-1α expression and Akt/mTOR/s6K phosphorylation induced by the ISO in vitro

We examined the effect of the BEZ235 treatment on the HIF-1α expression and Akt/mTOR/s6K phosphorylation (Figs. 5A-E). We observed that the expression of the Akt, mTOR and s6K shows no significant difference under different conditions. Consistent with the in vivo results, the ISO remarkably increased the phosphorylation of the Akt, mTOR and s6K (Figs. 5A, C-E). The administration of the BEZ235 effectively decreased the ISO-induced Akt/mTOR/s6K phosphorylation (Figs. 5A, C-E). However, the protein expression involved in the Akt/mTOR/s6K pathway showed no significant difference among the OGD groups without any treatment and the one treated with BEZ235 alone. Besides, the treatment with BEZ235 also inhibited the mTOR downstream HIF-1α expression. In brief, our results indicated that the HIF-1α expression and Akt/mTOR/s6K signaling could be primarily involved in the ISO-mediated neuroprotection against the OGD-induced injury.

The BEZ235 inhibited the anti-apoptotic effects of the ISO against OGD injury in vitro

The results showed that the BEZ235 significantly weakened the anti-apoptotic effect of the ISO against the OGD-induced injury. Similar to the data in vivo, flow cytometry analysis indicated that the ISO preconditioning inhibited the OGD-induced cell apoptosis in...
These results suggest that the ISO preconditioning inhibited the ISO-mediated activation in the bEnd.3 cells. (A) The bEnd.3 cells were collected, stained with FITC-A/PE-Texas Red-A detected by flow cytometry. The right panel indicates the apoptotic cells. (B) A bar diagram of apoptotic cells rate from four separate groups. *P<0.05 vs. the control group, †P<0.05 vs. the OGD+ISO group.

Figure 6. BEZ235 inhibited the ISO-mediated anti-apoptotic effect in vitro. (A) The bEnd.3 cells were collected, stained with FITC-A/PE-Texas Red-A detected by flow cytometry. The right panel indicates the apoptotic cells. (B) A bar diagram of apoptotic cells rate from four separate groups. *P<0.05 vs. the control group, †P<0.05 vs. the OGD group, △P<0.05 vs. the OGD+ISO group.

bEnd.3 cells, which was blocked by the BEZ235 administration (Figs. 6A&B). The results fully suggest that the Akt/mTOR/s6K signaling pathway played a crucial role in the ISO-induced anti-apoptotic effect on neuroprotection.

Discussion

IR injury is a common complication resulting from a variety of clinical diseases for multiple organizations, certain organs. Under the central nervous system, the brain is an ischemia hypoxia sensitive organ, and any occurrence of IR brain injury may cause catastrophic consequences, directly affecting the prognosis of the disease, surgical success rate and patient survival rate. Therefore, the identification of a safe and reliable method to prevent brain IR injury has always been an effective therapy for cerebral protection in the field of medicine and neurobiology (25).

Several studies have demonstrated that the volatile anesthetic preconditioning mediated neuroprotection very well during the IR injury (26, 27). As a typical general anesthetic, due to its fast and effective anesthesia induction, the ISO was widely applied in the research of IR injury (7). Several studies have demonstrated the protective effects of the ISO on the IR injury in multiple organizations, certain organs and different animals (28, 29). However, despite the extensive research, the precise mechanisms responsible for ISO induced protection against the IR injury continue to remain unclear. We aimed at a better and comprehensive exploration regarding whether the ISO preconditioning protects against brain IR injury via up-regulating the HIF-1α expression through the Akt/mTOR/s6K activation.

In our study, we successfully established the rat model with the middle cerebral IR and discovered that the ISO pretreatment obviously improved the neurological deficits, brain infarction, and degree of brain edema in vivo. The outcomes concur with the earlier findings that the ISO plays a significant role as a neuroprotective agent post MCAO (5). Besides, cell apoptosis is an important cause of nerve injury and the mechanism of cerebral IR injury is mainly focused on neural cell apoptosis (30). Studies have confirmed that the volatile anesthetics may inhibit the cerebral IR injury through different anti-apoptotic mechanisms (31, 32). Our results also confirmed that the ISO preconditioning increased the expression of the anti-apoptotic protein Bcl-2 and inhibited the expression of the Bax and the Cleaved caspase-3 in vivo. These results suggest that the ISO preconditioning decreased the activation of cell apoptosis after cerebral IR injury and the anti-apoptotic effects may contribute to the ISO-mediated neuroprotection.

The Akt/mTOR/s6K signaling pathway is believed to be the central mediator in the signal transduction pathways involved in cell growth and cell survival. The neuroprotective role of the Akt/mTOR/s6K signaling pathway in cerebral IR has been extensively studied (33, 34). Early neuro-protective ISO therapy could increase the chances of survival and faster recovery from IR (14). The neuro-protective effect of sevoflurane post-conditioning may be partly due to the activation of the PI3K/Akt pathway and the inhibition of neuronal apoptosis (35). It is well demonstrated that ISO protects the heart against ischemia and decreases myocardial infarction by activation of PI3K (36). Thus, we hypothesized that the ISO-induced neuroprotective effects are mediated by activation of the Akt/mTOR kinase signaling pathway. In this context, the results revealed that the phosphorylation of the Akt/mTOR/s6K was significantly further activated in the bEnd.3 cells post ISO against OGD injury, which strongly suggests that the recovery from cerebral IR injury with the ISO preconditioning is possibly due to the activation of the Akt/mTOR/s6K signaling pathway.

In vitro studies demonstrated that the ISO reduced the OGD-induced cell injury using the MTT and the...
LDH release assays in the bEnd.3 cells. Consistent with the in vivo results, our studies also confirmed that the ISO activated the Akt/mTOR/s6K pathway and diminished the cell apoptosis in vitro. Therefore, it is possible that the anti-apoptotic effect of the Akt/mTOR/s6K pathway in the ISO preconditioning in rat may be responsible for protecting the brain from ischemia injury. To evaluate this hypothesis, we administered a PI3K/mTOR dual inhibitor to further investigate the direct relationship between the Akt/mTOR/s6K signaling pathway and the ISO-induced brain neuro-protective effect (37). By administering BEZ235, we confirmed that the BEZ235 abrogated the up-regulation of the p-Akt, p-mTOR, p-s6K and HIF-1α, and increased the cell apoptosis. The HIF-1α as the primary factor has been involved in ISO-mediated neuroprotection. The ISO-induced up-regulation of the HIF-1α during hypoxia in vitro has already been reported (38). The HIF-1α levels demonstrated are believed to play a crucial role in the recovery from hypoxia-ischemia and stroke in the neonatal brain (39). Data obtained from the study support that the ISO protects the cardiovascular system from ischemia injury via up-regulating the HIF-1α expression (40). Besides, our study demonstrated a direct link between the Akt/mTOR/s6K pathway activation and attenuation of the cerebral endothelial cells apoptosis in ISO-induced neuroprotection. Interestingly, the expression of the proteins involved in the Akt/mTOR/s6K signaling pathway showed no significant difference between the OGD groups without any treatment as well as when treated with the BEZ235 alone. The results suggest that the protective effect of the ISO preconditioning in the bEnd.3 cells was prevented by the BEZ235, possibly by blocking the phosphorylation of the Akt/mTOR signaling pathway and down-regulating the HIF-1α.

Based on these studies, the results point to the possibility that the ISO preconditioning offers protective effects against the IR-induced brain nerve injury via up-regulating the HIF-1α expression through the activation of Akt/mTOR/s6K. These findings clearly highlight a basic mechanism for new therapeutic strategies against IR injury and confirm that the ISO is a potential therapy agent in the treatment of brain IR injury.

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