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Research Report

Isoflurane preconditioning activates HIF-1α, iNOS and Erk1/2 and protects against oxygen–glucose deprivation neuronal injury

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ARTICLE INFO

Article history:
Accepted 23 September 2008
Available online 7 October 2008

Keywords:
Isoflurane preconditioning
Hypoxia inducible factor-1α
Ischemia
Primary hippocampal neuron

ABSTRACT

Preconditioning neurons with isoflurane, a commonly used volatile anesthetic in clinical practice, improves tolerance of subsequent ischemia in both intact animal models and *in vitro* preparations. To investigate the mechanisms of this protection, we primarily cultured rat hippocampal neurons and simulated ischemia *in vitro* by oxygen–glucose deprivation (OGD). Neuron viability was measured. Neuron injury was observed by inverted phase contrast microscope and assessed by lactate dehydrogenase (LDH) release. Gene expression was examined by Western blot and reverse transcription-polymerase chain reaction (RT-PCR). Isoflurane exposure for 2 h at 24 h before a 2 h OGD dose-dependently reduced cell injury. Isoflurane accumulated phosphorylation/activation of extracellular signal-related kinases 1 and 2 (Erk1/2) and hypoxia inducible factor (HIF)-1α, a transcription factor involved in cell survival. Inhibition of the phospho-Erk1/2 partially abolished the isoflurane preconditioning-induced HIF-1α protein content accumulation and neuroprotection. Isoflurane also increased inducible nitric oxide synthase (iNOS) mRNA levels, a downstream gene of HIF-1α. Thus, the current results indicate that isoflurane preconditioning activates HIF-1α during protection against OGD neuronal injury and the activation might be partly mediated by the Erk1/2 pathway.

1. Introduction

It is documented that the fluorinated volatile anesthetic isoflurane protects the brain when administered during or immediated before an ischemic insult to the brain similar to that achieved by ischemic preconditioning (Miura et al., 1998; Sakai et al., 2007). Although the mechanisms involved in isoflurane preconditioning are poorly understood, this conditioning has potential clinical prospects. Identifying the mechanisms by which isoflurane and other volatile anesthetic agents mediate their anti-ischemic actions may be of special clinical significance in protection against the ischemic events that frequently occur in neurosurgical patients in the perioperative period.

Recently, we and others demonstrated that isoflurane modulates the expression of several genes concerned with cell survival including hypoxia inducible factor (HIF)-1, an important DNA-binding complex (Semenza and Wang, 1992)

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0006-8993/$ – see front matter.
doi:10.1016/j.brainres.2008.09.069

whose activity is influenced by intracellular oxygen *in vitro* (Li et al., 2006b) and *in vivo* (Wang et al., 2006). This heterodimeric transcriptional factor consists of two subunits, HIF-1α and HIF-1β, HIF-1α is the specific and oxygen-regulated subunit of the HIF-1 complex and determines the level of HIF-1 activity, whereas HIF-1β is constitutively expressed. The functional HIF-1 promotes transcriptions of a wide range of genes that may help cells to adapt to hypoxic/ischemic circumstances, such as inducible nitric oxide synthase (iNOS) gene (Jung et al., 2000). Additionally, iNOS has been shown to play a central role in neuroprotection against cerebral ischemic injury induced by isoflurane (Kapinya et al., 2002; Zhao and Zuo, 2004). In both *in vivo* and *in vitro* studies, extracellular signal-related kinases 1 and 2 (Erk1/2) has been demonstrated to be crucially involved in modulating brain cell death and survival after ischemia (Irving and Bamford, 2002; Nozaki et al., 2001). It has also been shown that phosphorylation of Erk1/2 is required for HIF-1α activation during the ischemic preconditioning-induced neuroprotection (Das et al., 2005; Li et al., 2008). Since the signal transduction pathways that are involved in anesthetic-induced preconditioning share many common steps with the pathways that are activated by ischemic preconditioning (Clarkson, 2007; Kitano et al., 2007), we speculated that isoflurane preconditioning might activate HIF-1α, iNOS and Erk1/2 during neuroprotection against oxygen–glucose deprivation (OGD) injury in primary hippocampal neurons. In this study, we therefore investigated the effects of isoflurane on the expression of these three genes as well as the cell viability and lactate dehydrogenase (LDH) release in the OGD hippocampal neurons, the possible signaling pathway was also investigated.

2. Results

2.1. *Cell viabilities were reduced with an increase in the periods of OGD*

We first investigated the effects of OGD in different periods (0, 0.5, 1, 2 or 4 h) on cell viability by exposing the hippocampal neurons in OGD conditions at 37 °C. The MTT assay results, as shown in Fig. 1, indicated that the cell viability was reduced with an increase in the periods of OGD. A steep drop of the cell viability was found at 1, 2 and 4 h of ischemia. The differences in the neuron viabilities between 1 and 2 h of OGD are greater than that between 2 and 4 h. Therefore, 2 h of OGD was used in the following experiment.

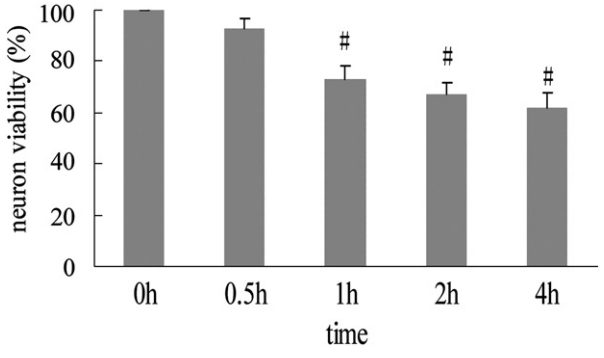

Fig. 1 – Effects of different periods of OGD on neuron viability. The hippocampal neurons were treated with OGD for 0, 0.5, 1, 2 or 4 h and the cell viabilities were then assayed as described in Experimental procedures. Results are presented as the percentage of control neuron viability (0 h). The data were presented as mean ± S.D. ($n=6$). #$P<0.05$ versus 0 h.

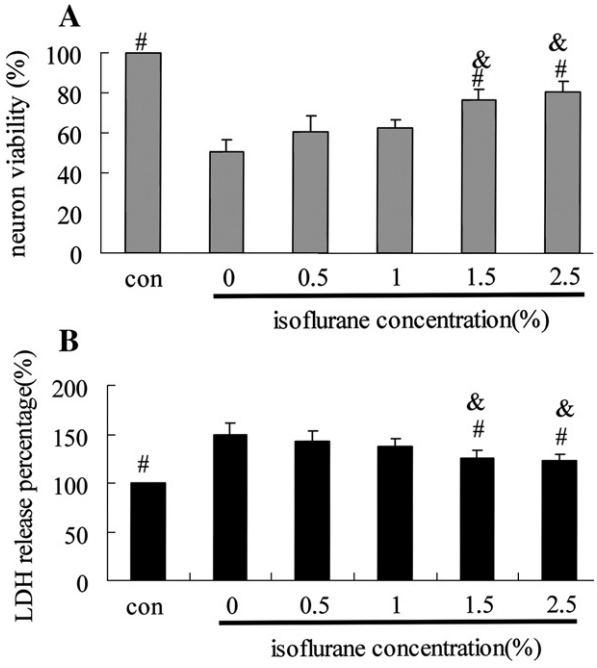

Fig. 2 – Effects of various concentrations of isoflurane preconditioning on the viabilities (A) and LDH release (B) of OGD neurons. The hippocampal neurons were incubated with different concentrations of isoflurane for 2 h, rinsed with PBS 3 times and returned to control conditions for 24 h, then treated with OGD for 2 h. Results are presented as the percentage of control neurons. Con = neurons without isoflurane or OGD treatment. The data were mean ± S.D. ($n=6$). #$P<0.05$ versus 0% isoflurane, and $P<0.05$ versus control.

2.2. *1.5% isoflurane induced the highest increase in the viability of OGD neurons*

Then we investigated the effects of isoflurane preconditioning in different concentrations on the viabilities of OGD neurons. The hippocampal neurons were incubated with 0, 0.5%, 1%, 1.5% or 2.5% of isoflurane for 2 h, rinsed with phosphate buffered saline (PBS) 3 times to remove isoflurane after isoflurane pretreatment, and returned to control conditions for 24 h, then treated with OGD for 2 h. It was found that the neuron viability increased with the concentrations of isoflurane added, although 1% isoflurane was not effective compared with OGD neurons without isoflurane incubation. It reached the highest value at 1.5%, and then maintained with the concentrations of isoflurane (Fig. 2A). LDH assay also showed that pretreatment with 1.5% isoflurane induced a significant decrease in LDH release in the OGD neurons (Fig. 2B). Therefore, 1.5% isoflurane was used in the following experiments.

2.3. *Isoflurane preconditioning time-dependently protected hippocampal neurons against OGD injury*

To find out the protective effects of isoflurane preconditioning duration against OGD injury, the hippocampal neurons were incubated with 1.5% isoflurane for 0, 1, 2, 4 or 8 h, rinsed with PBS 3 times and returned to control conditions for 24 h before the neurons were treated with OGD for 2 h. It was found that the viability of the OGD neurons pretreated with isoflurane (2, 4 or 8 h) was significantly higher than that in the OGD neurons without isoflurane preconditioning (all $P<0.05$ versus '0h+2h') (Fig. 3A). These data were in agreement with the corresponding morphological changes (Fig. 3B) and implied that 1.5% isoflurane preconditioning for 2, 4 and 8 h could protect against OGD neuronal injury.

2.4. *Isoflurane preconditioning induced a significant increase in expression of HIF-1α protein and iNOS mRNA*

To understand the potential mechanisms involved in the protective effects of isoflurane preconditioning against OGD injury, we investigated the effects of isoflurane preconditioning on expression of HIF-1α. The expression of HIF-1α protein and mRNA was then determined by Western blot and RT-PCR. The findings showed that incubation with OGD or isoflurane alone for 2 h significantly induced the levels of the HIF-1α protein in the neurons and a further accumulation was achieved by ISO+OGD (neurons treated with 1.5% isoflurane for 2 h, then rinsed with PBS 3 times and returned to control conditions for 24 h before treated with OGD for 2 h). In addition, expression of HIF-1α was higher after OGD than that

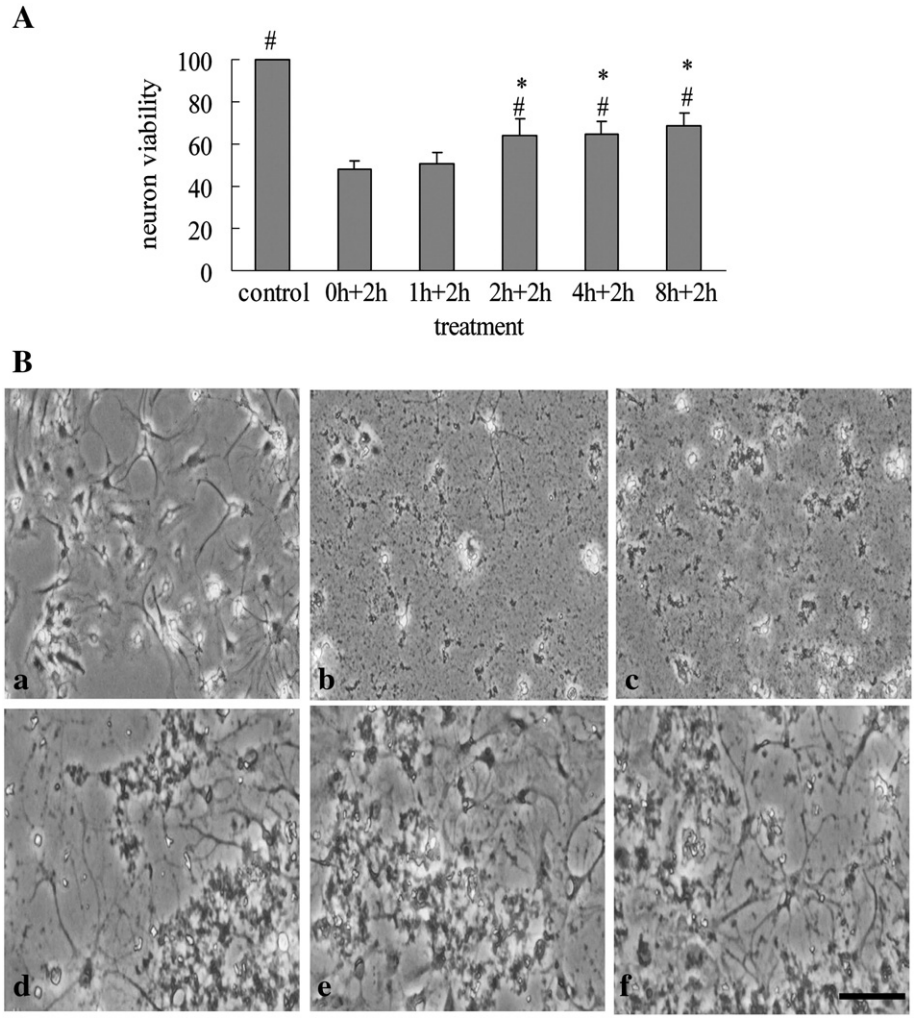

Fig. 3 – Effects of isoflurane preconditioning duration and OGD on the viability and morphology of hippocampal neurons. The hippocampal neurons were pretreated with 1.5% isoflurane for 0, 1, 2, 4 or 8 h, rinsed with PBS 3 times and returned to control conditions for 24 h, then treated with OGD for 2 h. The neuron viabilities were then assayed and morphological changes were observed. (A) The neuron viabilities (control = 0 h hypoxia+24 h normoxia; 0 h+2 h=0 h isoflurane+24 h normoxia+2 h OGD; 1 h+2 h=1 h isoflurane+24 h normoxia+2 h OGD; 2 h+2 h=2 h isoflurane+24 h normoxia+2 h OGD; 4 h+2 h=4 h isoflurane+24 h normoxia+2 h OGD and 8 h+2 h=8 h isoflurane+24 h normoxia+2 h OGD), and (B) morphological changes (a, control; b, 0 h+2 h; c, 1 h+2 h; d, 2 h+2 h; e, 4 h+2 h; and f, 8 h+2 h). The data presented were mean±S.D. ($n=6$). #$P<0.05$ versus 0 h+2 h; *$P<0.05$ versus control. Scale bar=100 μm.

after isoflurane (Fig. 4A). RT-PCR results showed that levels of HIF-1α mRNA in the hippocampal neuronal cultures were unaltered in OGD, isoflurane neurons or OGD neurons pretreated with isoflurane (Fig. 4B). These results indicated that isoflurane preconditioning modulates HIF-1α via a post-transcriptional pathway and implied that the protective effect induced by isoflurane pretreatment in hippocampal neurons might be associated with the increased content of HIF-1α protein.

iNOS has been shown to play an important role in neuroprotection induced by isoflurane preconditioning, and which is known to be regulated by HIF-1α activation under hypoxic/ischemic conditions (Jung et al., 2000). Fig. 4C showed a remarkable iNOS mRNA increase after OGD incubation and a

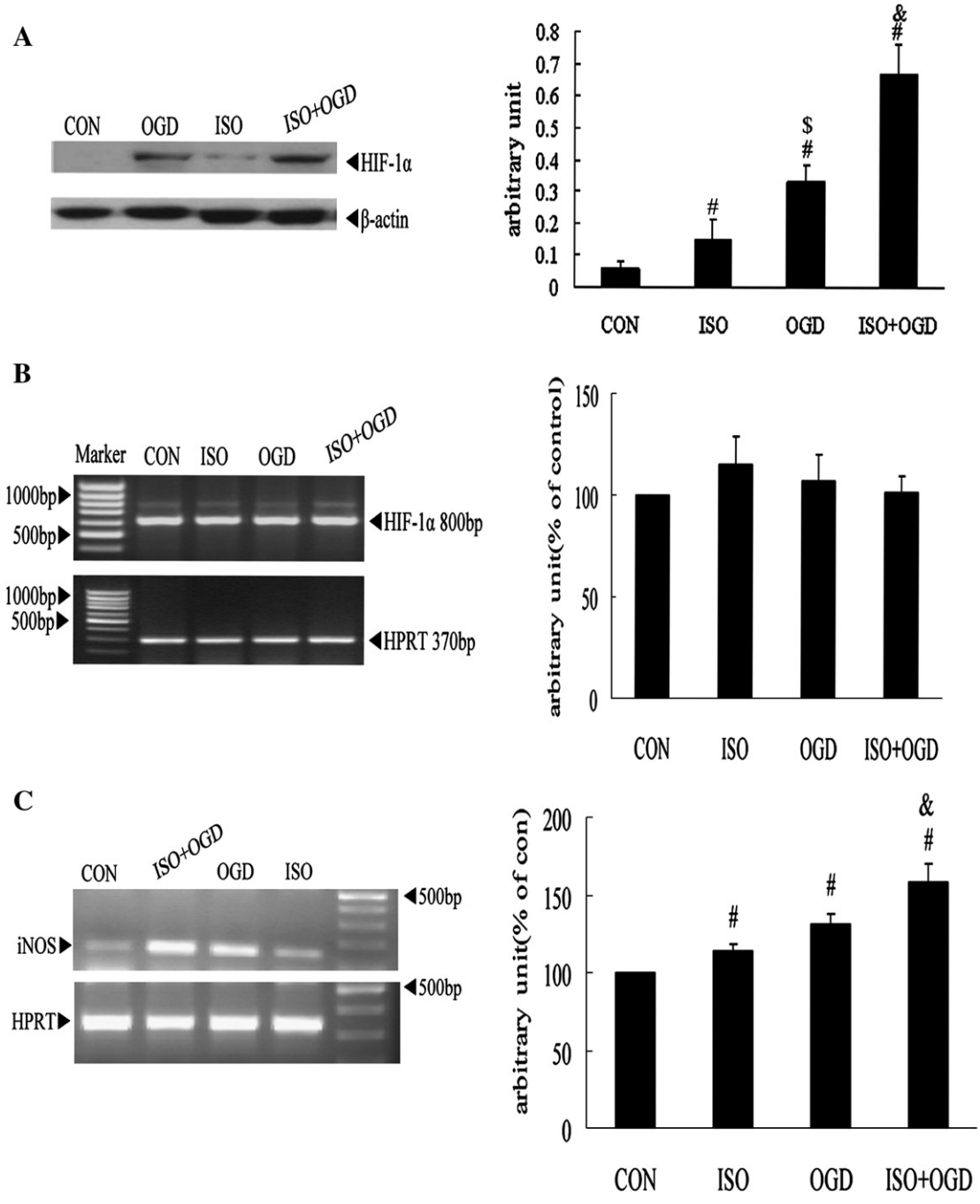

Fig. 4 – Effects of isoflurane preconditioning on HIF-1α mRNA and protein expression and iNOS mRNA in hippocampal neurons. (A) Western blot analysis of HIF-1α protein from neurons treated with isoflurane, OGD or OGD pretreated with isoflurane. RT-PCR analysis of HIF-1α mRNA (B) and iNOS mRNA(C) from neurons treated with isoflurane, OGD or OGD pretreated with isoflurane. The left panel is densitometric analysis of the bands. Values were presented as mean ± S.D. ($n=3$). CON = neurons without isoflurane or OGD treatment, ISO = neurons incubated with isoflurane for 2 h, OGD = neurons incubated with OGD for 2 h. ISO + OGD = neurons incubated with isoflurane for 2 h, rinsed with PBS 3 times and returned to control conditions for 24 h, then treated with OGD for 2 h. #$P<0.05$ versus CON, & $P<0.05$ versus OGD, \$$P<0.05$ versus ISO.

further accumulation in ISO+OGD neurons. Densitometric analysis indicated that HIF-1α protein and iNOS mRNA further increased in ISO+OGD group than that in OGD-only group (1.5- to 2-fold compared with the OGD-only group). Furthermore, dose–response analysis revealed that 1.5% and 1% isoflurane incubation for 2 h caused the maximal induction of HIF-1α protein and iNOS mRNA respectively (Figs. 5A and B).

2.5. *Isoflurane induces HIF-1α expression and neuroprotection via Erk1/2 phosphorylation*

Previous studies have shown isoflurane leads to phosphorylation of Erk1/2 in slice cultures and rat heart during protection against hypoxic/ischemic injury (Gray et al., 2005; Wang et al., 2006), and this signaling cascade is involved in the induction of HIF-1α protein by hypoxic or nonhypoxic stimuli (Sutton et al., 2007; Zhang et al., 2006). Then, we investigated the inhibition of Erk1/2 on expression of p-Erk (phosphorylated Erk, active Erk)/t-Erk (total Erk, phosphorylated and dephosphorylated Erk) and HIF-1α in the neurons treated with isoflurane. PD98059 (the selective Erk1/2 inhibitor) was added to the culture medium at a final concentration of 30 μM for 1 h prior to 1.5% isoflurane treatment (2 h) as a previously described application in hippocampal neurons (Rapoport and Ferreira, 2000). Western blot analysis found that PD98059 significantly attenuated the level of p-ERK/t-ERK in the isoflurane neurons (Figs. 6A and B, samples were collected immediately after isoflurane exposure). In the case of HIF-1α, isoflurane incubation increased HIF-1α protein content, and PD98059 alleviated the induction of HIF-1α protein by isoflurane (Figs. 6C and D). This implied that isoflurane induced the contents of HIF-1α protein via Erk1/2 pathway. However, the contents of HIF-1α in the neurons preincubated with PD98059 were still higher than those in the control group, indicating that the inhibition of Erk1/2 could not completely inhibit the positive effects of isoflurane on the contents of HIF-1α. To exclude the potential effects of PD98059 on the expression of p-ERK/t-ERK and HIF-1α, we also examined whether PD98059 can affect expression of these proteins in the absence of isoflurane. Incubation with PD98059 did not induce any significant change in the expression of these proteins as compared to the neurons without PD98059 treatment (data not shown).

We then asked whether blockade of Erk1/2 phosphorylation prevented isoflurane preconditioning in neuron cultures. The neurons were preincubated with or without PD98059 for 1 h and then treated with 1.5% isoflurane for 2 h, rinsed with PBS 3 times and returned to control conditions for 24 h, then treated with OGD for 2 h. The LDH release assay revealed that PD98059 prevented neuroprotection afforded by isoflurane preconditioning (138±7% versus 151±7% of control LDH release, $n=10$, $P<0.05$; Fig. 6E). MTT assay also ascertained this conclusion (75±6% versus 64±7% of control neuron viability, $n=10$, $P<0.05$; Fig. 6F). PD98059 alone did not alter LDH release or neuron viability.

3. Discussion

The major findings of our study were as follows: (1) isoflurane preconditioning protects against OGD neuronal injury. (2) A maximum of protection is seen a concentration of 1.5% for isoflurane. (3) isoflurane preconditioning activates HIF-1α protein content and subsequent iNOS mRNA expression in hippocampal neurons.(4) The increased content of HIF-1α protein and decreases in OGD neuronal injury, which were induced by isoflurane preconditioning, could be significantly but not completely inhibited by PD98059.

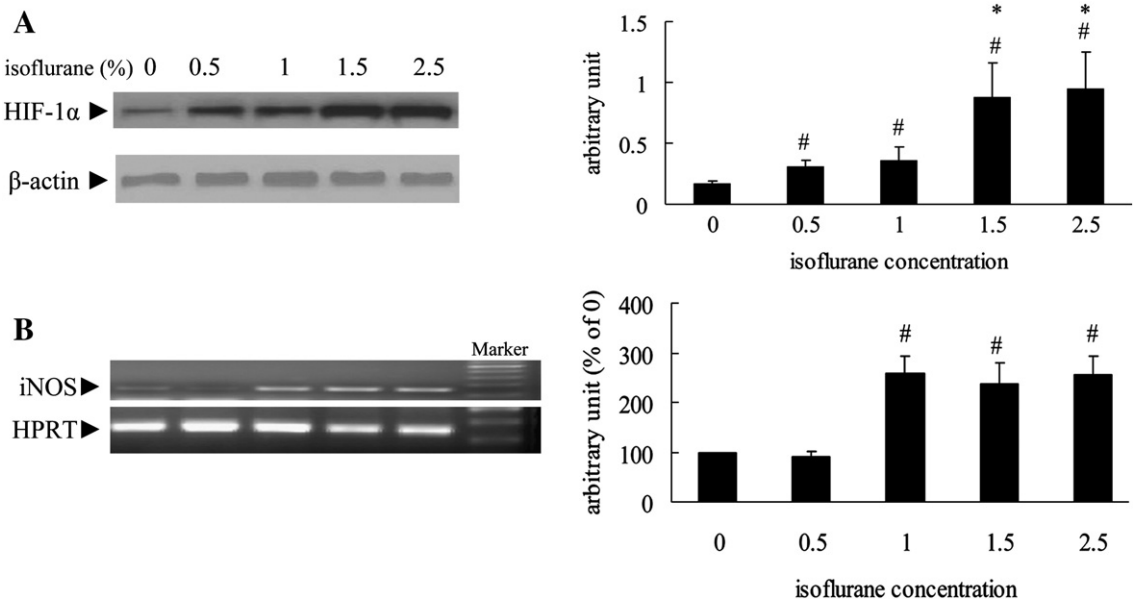

Fig. 5 – Dose–response relationship for isoflurane and HIF-1α protein, iNOS mRNA expression. (A) Western blot of HIF-1α protein and (B) RT-PCR of iNOS mRNA from neurons treated with various concentrations of isoflurane for 2 h. The left panel is densitometric analysis of the bands. Values were presented as mean±S.D. ($n=3$). #$P<0.05$ versus 0% isoflurane, *$P<0.05$ versus 1% isoflurane.

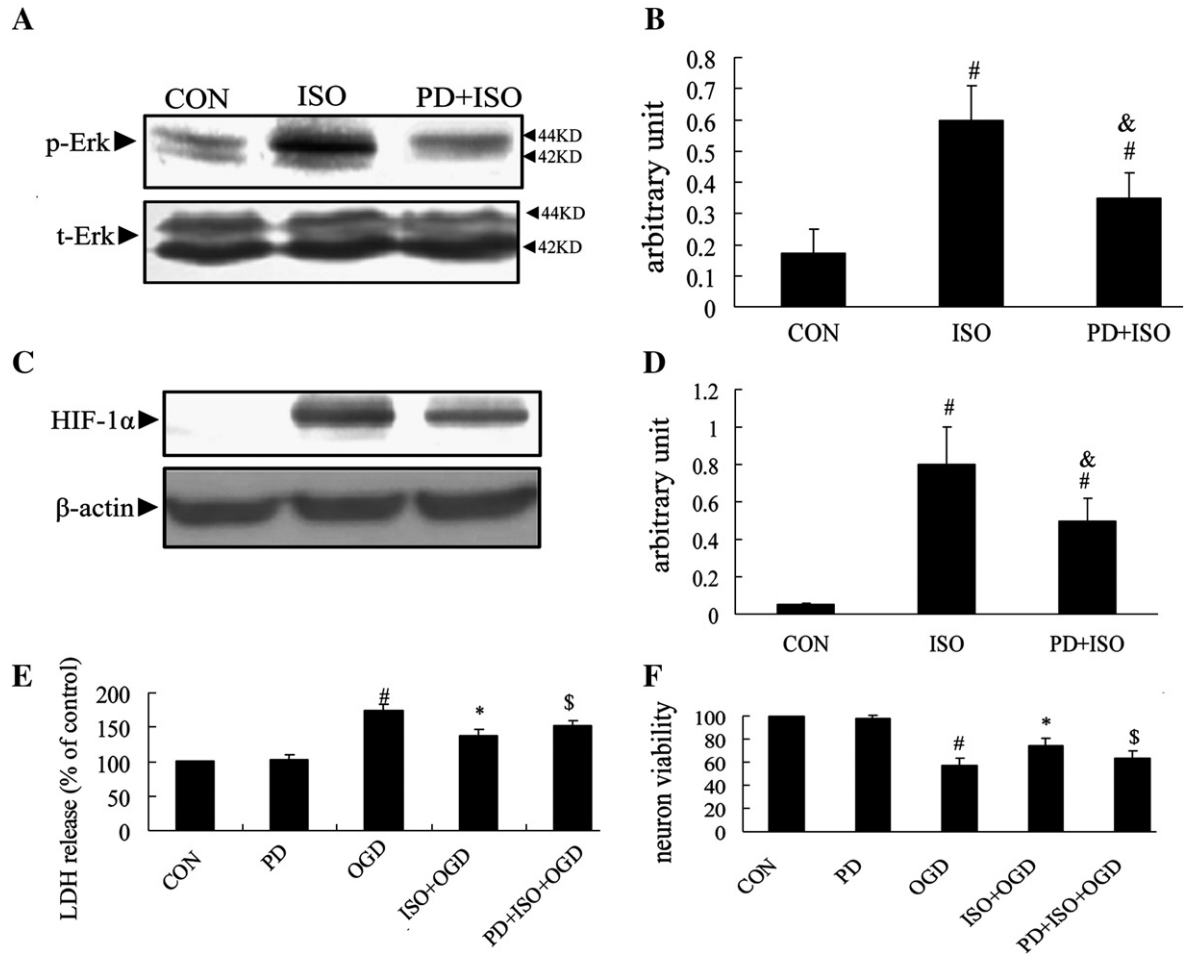

Fig. 6 – Inhibition of Erk1/2 phosphorylation reduced isoflurane induced neuroprotection and HIF-1α expression in hippocampal neurons. Representative Western blots of Erk1/2 (A) and HIF-1α (C) protein in neurons treated with indicated stimuli. Histogram representing neuron injury, measured by LDH release (E) or MTT assay (F) ($n=10$). (B and D) Quantification of expression of p-Erk1/2 and HIF-1α. Data are the mean ± S.D. of normalized densitometry measurements from Western blots ($n=4$ for p-Erk1/2 and $n=5$ for HIF-1α). CON, ISO, OGD, and ISO + OGD denotation were described previously. PD = neurons incubated with PD98059 for 1 h, PD + ISO = neurons incubated with PD98059 for 1 h before isoflurane treatment (2 h), PD + ISO + OGD = neurons incubated with PD98059 for 1 h before isoflurane treatment for 2 h, rinsed with PBS 3 times and returned to control conditions for 24 h, then treated with OGD for 2 h. #$P<0.05$ versus CON, & $P<0.05$ versus ISO, *$P<0.05$ versus OGD, and \$$P<0.05$ versus ISO + OGD.

It has long been known that the brain is very sensitive to hypoxia and ischemia. In our *in vitro* study, neuron viabilities decreased to 60–70% of control after 1–4 h incubation with OGD. Nevertheless, there is still oxygen storage in neuronal culture, even after 90–180 min of hypoxic incubation as indicated previously (Meloni et al., 2002). The brain, like other organs, is capable of inducing protective mechanisms when challenged by stressors or substrate deprivation. It has been generally accepted that the development of delayed phase of neuroprotection requires new protein synthesis (Nandagopal et al., 2001). Numerous mediators and mechanisms have been proposed to explain the phenomenon. Very recently, we and others demonstrated that isoflurane activates HIF-1α *in vitro* and *in vivo* (Li et al., 2006b; Wang et al., 2006). Regarding isoflurane as a preconditioning stimulus, it is straightforward to propose that the neuroprotective effect, at least partially, is achieved via HIF-1-dependent signaling. Intracellular hypoxia is a major stimulus for the production and enhanced activity of HIF-1α. In addition to hypoxia, nonhypoxic mediators (e.g., reactive oxygen species, nitric oxide, growth factors) can increase HIF-1α mRNA transcription and protein translation, resulting in an accumulation of the HIF-1α protein under normoxic conditions (Dery et al., 2005; Scharte et al., 2003). Our laboratory has demonstrated that isoflurane induces HIF-1α protein accumulation independent of hypoxia via protein translation (Li et al., 2006b). The current study also showed that isoflurane, as a nonhypoxic mediators, induced HIF-1α protein in hippocampal neurons. The increase in HIF-1α protein in hippocampal neurons occurred without a corresponding change in HIF-1α mRNA level, suggesting that the increase was modulated at posttranscriptional pathway. Nextly, we explored the potential role of iNOS in

the mechanisms of isoflurane neuroprotection. This notion was derived from literature reporting that iNOS plays an important role in both ischemic and isoflurane preconditioning in rat brain (Kapinya et al., 2002; Luo et al., 2007; Zhao and Zuo, 2004; Zhao et al., 2007). Our finding of simultaneous peaks in HIF-1α protein and iNOS mRNA offers evidence implying that isoflurane preconditioning induces iNOS via HIF-1α dependent pathway during protection against OGD injury in hippocampal neurons. Nevertheless, additional work needs to be done to ascertain this conclusion. Although a big increase of iNOS expression may be harmful (Anggard, 1994), multiple studies have shown that a relatively small increase of iNOS expression may be beneficial and plays a critical role in neuroprotection induced by isoflurane preconditioning (Kapinya et al., 2002; Zhao and Zuo, 2004). The downstream events of iNOS to induce protection as shown in the previous studies include activation of protein kinase C, heat shock proteins and manganese superoxide dismutase (Nandagopal et al., 2001; Zaugg et al., 2003). Furthermore, protein kinase C can also activate adenosine triphosphate sensitive potassium channels, an important effector for cardioprotection induced by many preconditioning stimuli (Zaugg et al., 2003).

It is well documented that activation of the Erk1/2 pathway leads to increased transcriptional activity of the HIF-1 complex and down-stream gene activation(Lee et al., 2002; Sutton et al., 2007). Our findings demonstrated that isoflurane preconditioning could significantly increase not only HIF-1α but also p-ERK/t-ERK. PD98059 significant reduced the expression of p-ERK/t-ERK and HIF-1α content as well as cell injury in the OGD neurons with isoflurane preconditioning. These suggested that the increased HIF-1α as well as the neuroprotective role induced by isoflurane might be partly mediated by the activation of the Erk1/2 pathway. However, it should be pointed out that further studies are absolutely needed to further understand whether the pathway has a major or direct role in isoflurane induction of HIF-1α and iNOS. In addition, it was noticed that although the increased contents of HIF-1α induced by isoflurane could be significantly inhibited by PD98059, the contents of HIF-1α in the PD98059 neurons were still higher than those in the control group. These facts might indicate that some other pathways might also be involved in the isoflurane-induced neuroprotection by increasing HIF-1α contents apart from Erk1/2 pathways.

Collectively, isoflurane is capable of preconditioning neurons against OGD injury. The activation of HIF-1α, iNOS expression and the increased cell viability found in the OGD neurons pretreated with isoflurane suggest that HIF-1α may play a role in the neuroprotection processes. Isoflurane preconditioning induced HIF-1α protein content might be partly via the Erk1/2 pathways. Nevertheless, our study has limitations that may restrict extrapolation to clinical conditions. One significant limitation is that *in vitro* models do not replicate the temporal loss of anesthetic neuroprotection observed in intact rodents (Sullivan et al., 2002). Another possible limitation is that, at least in our study, it seemed weakly neuroprotective compared with conditions when isoflurane is present during the simulated ischemia. Further studies are needed to clarify the durability and power of isoflurane preconditioning.

4. Experimental procedures

4.1. *Animals*

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation and was conducted according to the Guidelines for Animal Experimentation of Shanghai Jiao Tong University School of Medicine (Shanghai, China). The animals were studied at Shanghai Jiao Tong University School of Medicine (Shanghai, China). Newborn Wistar rats within 24 h, purchased from the Laboratory Animal Center, Shanghai Jiao Tong University School of Medicine. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless specified in the text.

4.2. *Neuronal cell culture*

Pure hippocampal cultures were prepared from Newborn Wistar rats within 24 h and plated at a density of 4×10^5 cells per well in 24-well plates coated with poly-D-lysine in Neurobasal medium, serum-free B-27 supplement (Life Technologies/BRL), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Glutamate (25 μmol/L) was added during the initial 3 days *in vitro* (DIV). Cultures were kept at 37 °C and 5% CO_2 in a humidified incubator and fed beginning at 4 DIV with cultivating medium (as described above, but without glutamate) by replacing half of the medium twice a week. The percentage of neuronal and astrocytic cells was determined as follows: neuronal cultures were fixed for 30 min at 4 °C with 4% paraformaldehyde, 4% sucrose, and 0.1 M PBS, pH 7.4; cells were washed, permeabilized with 0.3% Triton X-100, blocked with 10% serum, and incubated overnight at 4 °C in anti-NeuN monoclonal antibody (1:1000; Chemicon, Temecula, CA) and anti-GFAP polyclonal antibody (1:4000; Dako, Carpinteria, CA) with 3% serum; cells were then washed, and incubated with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 546 secondary fluorescent antibodies (Molecular Probes, Eugene, OR). The number of NeuN- and GFAP-positive cells was counted in eight separate fields over two wells, and the percentage of NeuN-positive neurons and GFAP-positive astrocytes was determined. Purity of neuronal cultures was 94% neurons and 5% astrocytes.

4.3. *Isoflurane preconditioning*

Isoflurane preconditioning was performed as described previously (Kapinya et al., 2002). Briefly, the neuron-enriched cultures were kept after 8 days *in vitro* at 37 °C in a closed chamber in an atmosphere of 1.4% isoflurane, 5% CO_2, 20% O_2, and N_2 (remainder) for a 2-hour period. Cells treated identically but without the addition of isoflurane served as controls.

4.4. *OGD treatment*

To mimic cerebral ischemia *in vitro*, OGD was performed with the pretreated or control cells 24 h later. For this purpose, we used a custom-made temperature-controlled anaerobic glove box as previously described (Chavez et al., 2006), the glove-box system was set up at 37 °C with an atmosphere of 5% CO_2 and

95% N_2 (oxygen deprivation). All solutions were equilibrated for at least 12 h. Cells were transferred into the chamber, washed with PBS, and incubated with a preequilibrated glucose-free balanced salt solution for up to 2 h. At the end of the procedure, cells were removed from the chamber, fresh Neurobasal media were added (reoxygenation), and the cultures were returned to a regular incubator. Cell death was assayed at 24 h after OGD measuring LDH released into the media. For experimental controls, we used cultures that were subjected to the same procedures but maintained with glucose-containing media at normoxia in a standard cell-culture incubator. The glove-box system used in this study was equipped with an inverted microscope that allowed us to visually inspect cell viability before terminating each experiment. All manipulations, including cell harvesting and cell lysis, were performed within the chamber.

4.5. *Assessment of cell survival*

Neurons were maintained for a period of 24 h, and their survival was assessed by phase-contrast microscopy with Trypan Blue exclusion assay and quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (Wu et al., 2006). Briefly, after OGD in each group, MTT was added to all those assays at a final concentration of 0.5 mg/mL for 4 h at 37 °C. The amount of MTT formazan was dissolved by dimethyl sulfoxide (DMSO), quantified by determining calorimetrically its absorbance at 570 nm using a microplate reader. Experiments were performed in triplicate, and repeated with at least three separate batches of cultures.

4.6. *Morphological observation*

Morphological changes in the cells were observed using the inverted research microscope (Nikon) as described previously (Zhu et al., 1997).

4.7. *Neuronal injury assay*

Neuronal injury was quantitatively assessed by the measurement of LDH activity in the medium 24 h after the injury (Koh and Choi, 1987). In each experiment, the results of the LDH measurements from the controls (neurons without isoflurane or OGD treatment) were set as 100%. Isoflurane preconditioning alone did not change the LDH release compared with the control. The results from the sister cultures subjected to both isoflurane preconditioning and/or OGD were then calculated as a percentage of the control.

4.8. *Western blot analysis*

Western blot analysis was performed as we described previously (Li et al., 2006a). Primary polyclonal rabbit anti-p44/42 MAP kinase (Thr202/Tyr204) antibody, 1:1000; phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, 1:1000; primary monoclonal mouse anti-HIF-1α, 1:500 dilution and β-actin, 1:5000; were obtained from Cell Signaling Technology (Beverly, MA) or Sigma.

4.9. *Reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from hippocampal neurons with the use of Trizol reagent (Gibco-BRL, Life Technologies, MD). Single-stranded cDNA was synthesized from 1 μg total RNA by the use of the first-strand cDNA Synthesis Kit (Promega Corp). The synthesized cDNA as a template in the PCRs was stored at −20 °C until use. The PCRs were performed in 25 μL volume, which included 1 μL cDNA sample, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 1× PCR buffer, 1 μL of each specific primer, and 0.4 μL (2 U) Taq DNA polymerase (Gibco-BRL). The relative purity of the isolated RNA was assessed spectrophotometrically. The total RNA was then quantified and the integrity was tested by gel electrophoresis. The total RNA (2 μg) from each sample was retrotranscripted to cDNA by using the reverse transcription kit (GIBCO). The primers used for HIF-1a, iNOS and hypoxanthine-guanine phosphoribosyl transferase (HPRT) are shown in Table 1. Amplification of DNA was performed with an initial denaturation at 94 °C for 5 min, followed by 35 reaction cycles (94 °C for 1 min, 60 °C for 2 min, 72 °C for 1 min) and by a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1.5% agarose gel. The optical density of the bands was determined by revising the Gel Image Analysis System. Optical density measurements were normalized to the optical density of the HPRT band used as an internal standard.

4.10. *Statistical analysis*

All values are presented as means ± S.D. Comparisons among multiple groups involved one-way ANOVA followed by the

Table 1 – Nucleotide sequences of PCR primers used in RT-PCR

Primer	Orientation	Sequence(5′–3′)	PCR product size (base pairs)	Gene Bank accession no.
HIF-1α	Sense	GTCTCGAGATGCAGCCAGATCTCG	800	NM024359
	Antisense	GGTCAGATGATCAGAGTCCAAAGC		
iNOS	Sense	GGTGGAAGCAGTAACAAAGGA	231	NM000625
	Antisense	GACCTGATGTTGCCGTTGTTG		
HPRT	Sense	CCTGCTGGATTACATTAAAGCACTG	370	AF009656
	Antisense	CCTGAAGTACTCTTATAGTCAAAG		

RT-PCR = reverse transcription-polymerase chain reaction; HIF-1α = hypoxia inducible factor-1α; iNOS = inducible nitric oxide synthase; HPRT = hypoxanthine-guanine phosphoribosyl transferase.

Student–Newman–Keuls test and Dunnett's test (for comparison of multiple experimental treatments to a common control value). All analyses were performed by using software SPSS 11.0. A value of $P<0.05$ was considered significant.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (No. 30801079), Science Foundation of Shanghai Education Committee (No. 08YZ35), Youth Science Foundation of Shanghai Health Bureau (No. 2007Y34) and Shanghai Rising-Star Program (No. 08QA14044).

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