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Effects of Conditioning with Sevoflurane before Reperfusion on Hippocampal Ischemic Injury and Insulin-Like Growth Factor-1 Expression in Rats

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Abstract

Sevoflurane pre-conditioning before ischemia can reduce ischemia-reperfusion injuries in cardiac, pulmonary and cerebral tissues. It is uncertain whether sevoflurane conditioning before reperfusion has similar protective effects on neuronal injuries. In this study, we explored the effect of sevoflurane conditioning (at concentrations of 1.5%, 2.4% or 3.0%) on the morphology and molecular mechanisms of the hippocampal CA_1 region in male Sprague-Dawley rats subjected to global cerebral ischemia. We determined the pathological results by hematoxylin and eosin (H&E) staining and examined the mRNA levels of insulin-like growth factor-1 (IGF-1) and protein levels of p-JNK1/2 and p-Akt1 in the hippocampus at 24 h, 48 h and 72 h after global cerebral ischemia-reperfusion. Our data showed that O_2 post-conditioning and lower dose (1.5%) of sevoflurane did not ameliorate ischemia-induced CA_1 injury. However, higher doses of 2.4% and 3.0% sevoflurane post-conditioning alleviated the CA_1 injury and enhanced the expression levels of IGF-1 mRNA. Furthermore, sevoflurane post-conditioning inhibited the activations of p-JNK1/2 and enhanced activation of p-Akt1. In conclusion, these results suggest that post-conditioning with sevoflurane at 2.4% and 3.0% ameliorates global cerebral ischemia induced hippocampal CA_1 injury by up-regulating the expression of IGF-1 mRNA followed by the activation of p-Akt1 and inhibition of the activation of p-JNK1/2.

Key Words: insulin-like growth factor-1, post-conditioning, reperfusion injury, sevoflurane

Introduction

Sevoflurane is a widely used inhalational anesthetic with desirable clinical properties. One previous study demonstrated that sevoflurane had a protective effect on the ischemia-reperfusion injury in cardiac, pulmonary and cerebral tissues (14). It has been implicated that pre-conditioning with sevoflurane has a protective effect on ischemic injury *via* the possible action of ischemia pre-conditioning (1). However, it is uncertain whether conditioning with sevoflurane just before reperfusion can also alleviate ischemia-reperfusion induced neuronal injury.

Accumulating evidences suggest that insulin-like growth factor-1 (IGF-1) plays important roles in mediating neuronal apoptosis in brain ischemia (7).

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The survival effect for up-regulating IGF-1 gene expression was primarily due to the activation of PI3K/Akt or pMAPK pathway in cultured hippocampal CA_1 neurons (19). Akt, also known as protein kinase B, is one of the downstream kinases of phosphoinositide 3-kinase (PI-3K) that is involved in survival signaling in the IGF-1-mediated signaling cascades (10). On the other hand, c-Jun N-terminal kinases (JNK) signaling pathway contributes to apoptosis. In neuronal cells, JNK activation induces, whereas JNK inhibition prevents, apoptotic cell death (3, 4). We, therefore, thought that the IGF-1/Akt/JNK signaling pathways may play a role in sevoflurane conditioning before ischemia in the global cerebral ischemia.

In this study, we used a global cerebral ischemia rat model to investigate the conditioning effects of sevoflurane before reperfusion on the morphology of ischemic hippocampal CA_1 region by hematoxylin & eosin (H&E) staining. We further investigated the neuroprotective mechanisms by analyzing the expression levels of IGF-1 mRNA, and p-Akt1 and p-JNK1/2 proteins in the rats.

Materials and Methods

Animal Surgical Procedures and Experimental Groups

Adult male Sprague-Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai 201615, PRC) weighing 250 ± 10 g were given free access to food and water before surgery. All experimental protocols were approved by the Animal Care and Use Committee of the college and in accordance with the declaration of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, People's Republic of China (Publication No. 80-23, revised 1996). Transient brain ischemia (15 min) was induced by the vessel occlusion method as described previously (8). In brief, under chloral hydrate (300-350 mg/kg, i.p.) anesthesia, common carotid arteries were exposed. Fifteen minutes of ischemia was induced by occluding these arteries with aneurysm clips. After removal of clips for inducing reperfusion injury, these rats were allowed to recover for 24, 48 or 72 h. Animals meeting the criteria of a completely flat bitemporal eletroencephalograph, maintenance of dilated pupils and the absence of a corneal reflex during ischemia were selected for the present experiments. Carotid artery blood flow was restored by releasing the clips. During ischemia and reperfusion, rectal temperature was maintained at about 37°C. The sham operation was performed using the same surgical exposure procedures except for occlusion of these vessels.

A total of 108 animals were divided into 6 groups (n = 18 in each group): sham-operation group (S), ischemia without conditioning group (C), conditioning with oxygen group (O_2) and conditioning with three concentrations of sevoflurane groups (Sevo1, Sevo2 and Sevo3). Those in conditioning with oxygen group were treated with 100 % oxygen 30 min before the reperfusion. Those in Sevo1, Sevo2 and Sevo3 groups were respectively administered with 1.5% (0.65 MAC), 2.4% (1.0 MAC) and 3.0% (1.3 MAC) sevoflurane, respectively, 30 min before the reperfusion. The samples of hippocampus of the animals were examined at 24, 48 and 72 h after the onset of cerebral ischemia and reperfusion (I/R).

Sevoflurane Conditioning

The sevoflurane (Abbott Laboratories, Queenborough, UK) was mainly preserved in a self-made organic glass box (35 cm × 20 cm × 15 cm) which was connected with the anesthesia machine at one side and with an end tidal anesthetics monitoring instrument at the opposite side. The sevoflurane in an evaporation jar was transported into the organic glass box with the oxygen (oxygen flow: 3 l/min). When the end tidal concentration of sevoflurane was stabilized to the target value, the rats were placed into the organic glass box to perform conditioning. The sevoflurane concentration inside the box and the pulse oxygen saturation (ranged from 99% to 100%) at the forepaws of the animals were monitored during the post-conditioning. A CO_2 absorption equipment was set in the box.

Tissue Preparation

Rats were decapitated at specified time points of reperfusion after 15 min of ischemia. Some hippocampal sections were immediately fixed in 10% formaldehyde solution for H&E staining, and other hippocampal sections were separated into CA_1 and CA_3/DG from hippocampal fissure and CA_1 and were rapidly frozen in liquid nitrogen. Frozen tissue samples were homogenized in 1:10 (w/v) ice-cold homogenization buffer containing 50 mM MOPS [3-(N-morpholino) ropanesulfonic acid, pH 7.4], 100 mM KCl, 320 mM sucrose, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol, phosphatase and protease inhibitors (20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 50 mM NaF, and 1 mM each of EGTA, EDTA, sodium orthovanadate, p-nitrodomains phenyl phosphate, phenylmethylsulfonyl fluoride and benzamidine, and 5 mg/mL each of aprotinin, leupeptin and pepstatin A). The homogenates were centrifuged at 800 $\times g$ for 10 min at 4°C. Supernatants as cytosol part were collected and protein concentrations were determined by the method of Lowry *et al*. (11). The samples were stored at -70°C.

Microscopic Examination

The samples of rat hippocampus were made into 5-μm sections. The section was dewaxed, hydrated, dehydrated and embedded for H&E staining. The pathological changes of region CA_1 of the hippocampus was photographed under a microscope.

Reverse Transcription (RT)-PCR and Western Blotting

Total RNA from hippocampal samples was extracted by using the Trizol method and was subject to agarose gel electrophoresis. The integrity of the RNA was determined by the ratio of 260 nm/280 nm using a spectrophotometer. Reverse transcription of 1 μg total RNA was performed using the reverse transcription reaction system supplied in AMV primary strand synthesis kit (Sangon Co., Shanghai, PRC). The synthesized cDNA, previously preserved at -20°C, was used as the template to perform PCR. The primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, People's Republic of China). The sequence, structure and function of the IGF-1 gene were derived from the GenBank database in the National Center for Biotechnology Information (NCBI). The designed primer sequences were as follows: IGF-1: upstream primer (5'-TTCTTTCCG TGCTGGGTC-3'), downstream primer (5'-CATGGC TGGGGTGTTGAAGGTCTCA-3'); β-actin: upstream primer (5'-TGGTGGGTATGGGTCAGAAGGACTC-3'), downstream primer (5'-CATGGCTGGGGTGTT GAAGGTCTCA-3'). The amplified products were subject to 2% agarose gel electrophoresis, stained in ethidium bromide and visualized by the Tanon gel system (Shanghai Tanon Science & Technology Co., Ltd., Shanghai, PRC). The density of the band with the appropriate molecular mass was determined semi-quantitatively by densitometry using an image analyzing system (Alpha Innotech, San Leandro, CA, USA).

Western blotting was performed as described previously (3, 10). In brief, 50 μg protein of each sample was heated at 100°C for 5 min with a loading buffer containing 0.125 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% mercaptoethanol and 0.002% bromphenol blue, then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. The proteins were electro-transferred onto a nitrocellulose filter (NC, pore size 0.45 μm) as described previously (20). The blotted filters were incubated with 3% BSA in TBST (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20) at 4°C for 6 h, and probed with an anti-JNK1/2 antibody (Promega, Madison, WI, USA; 1:5000), anti-active (diphosphorylated) JNK1/2 antibody (1:5,000 dilution; Promega Madison, WI, USA), anti-Akt1 antibody (1:2,000; Upstate Biotechnology, Lake Placid, NY, USA), or anti-active (diphosphorylated) Akt1 (1:1,000; Upstate Biotechnology) at 4°C overnight. Detection was carried out using alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000; Sigma, Saint Louis, MO, USA), goat anti-mouse IgG (1:20,000; Sigma), donkey anti-rabbit IgG (1:5,000; Promega), and anti-β-actin IgG (Sigma) and developed using BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitroblue tetrazolium) color substrate (Promega). Each band was scanned and analyzed with an image analyzer (LabWorks Software, UVP. Inc., Upland, CA, USA). The opitical density of the band in each lane was expressed as fold versus that in the sham control on the same filter.

Statistics

All the values are expressed as means ± standard deviation (SD) in RT-PCR from 6 independent rats and in western blotting from 4 independent rats. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range method or Newman-Keuls tests. $P < 0.05$ was considered significant.

Results

Microscopic Analysis

In group S (Fig. 1A), the neurons in the CA_1 region of the hippocampus were well and tightly arranged. The top dentrites in the CA_1 region were in parallel with each other and formed a clear layer; the pyramidal cells were in a polygonal or elliptic shape; the round nucleus with a visible nucleolus was situated in the center; the cytoplasm was in abundance and stained uniformly. In group C (Fig. 1B), the neurons in the CA_1 region of the hippocampus at each time point arranged badly (indicated by star); the cells were swollen, the edge of cell membrane was blurred and the nuclei shrank and became darker (indicated by arrowheads). The morphology was similar to a previous finding in a rat model with cerebral ischemia/reperfusion injury (2). In group Sevo1 (Fig. 1D), the morphological changes resembled to those in groups O_2 (Fig. 1C) and C (Fig. 1A). In groups Sevo2 (Fig. 1E) and Sevo3 (Fig. 1F), the neurons in the CA_1 region of the hippocampus at each time point arranged were still arranged well and tightly like in Group S; a majority of the cells were normal with visible nucleolus and some cells were swollen.

Expression of IGF-1 mRNA in the Hippocampal CA_1 Region

RT-PCR was used to investigate the expression levels of IGF-1 mRNA. The expression levels of

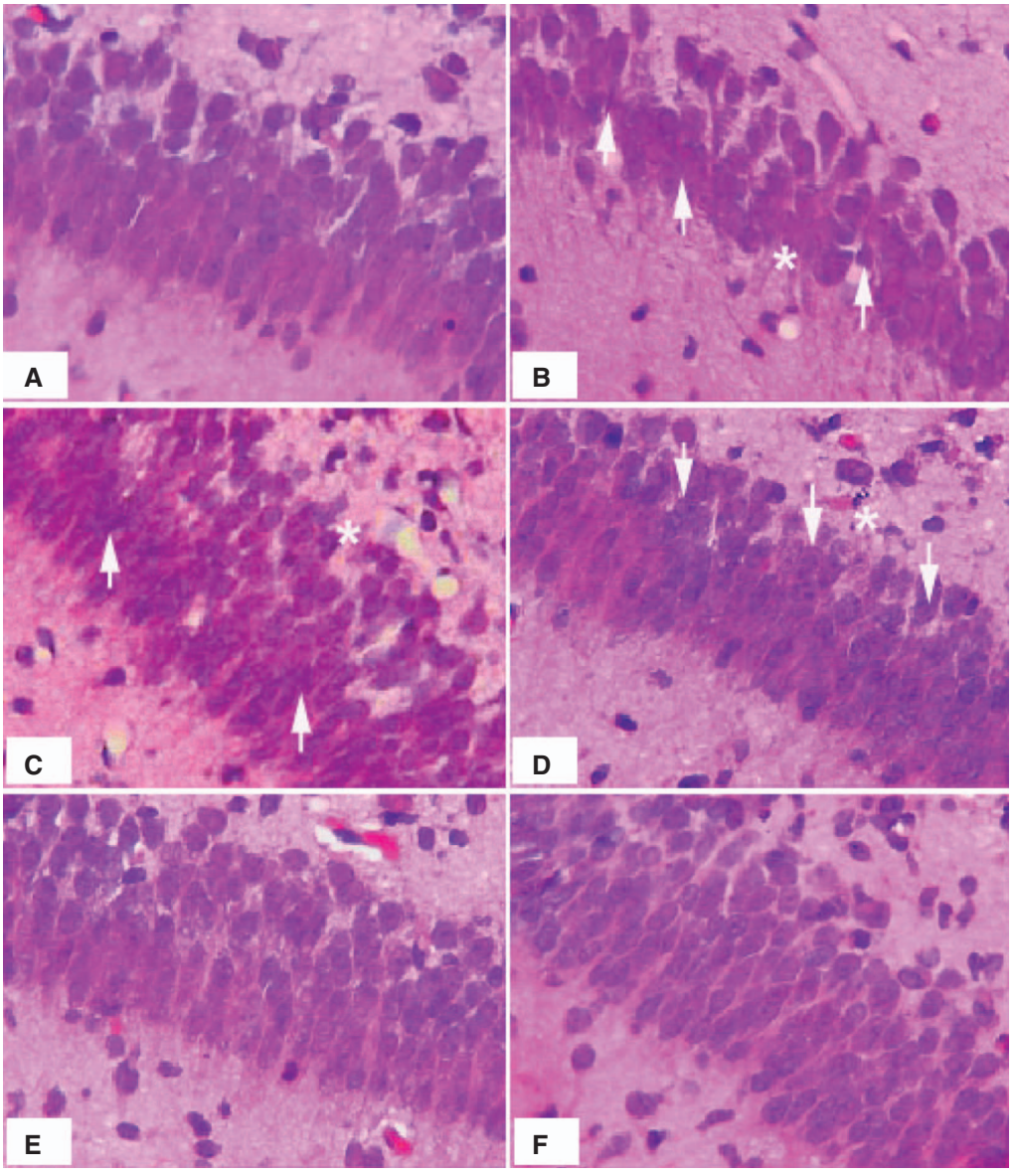

Fig. 1. Pathological findings of hippocampal CA_1 neurons in the six experimental groups (H&E, 40 × 10). A: 72 h in group S; B: 72 h in group C; C: 72 h in group O_2; D: 72 h in group Sevo1; E: 72 h in group Sevo2; F: 72 h in group Sevo3. The damaged neurons in the CA_1 region of the hippocampus arranged irregularly is indicated by asterisks and the nuclei that are shrank and darker are indicated by arrowheads.

IGF-1 mRNA at each time point in group C increased significantly when compared with those in group S. Expression levels of IGF-1 mRNA in group Sevo1 resembled those in group C, but the IGF-1 expression levels in groups Sevo2 and Sevo3 increased significantly compared with those in group C. IGF-1 mRNA expression in group O_2 was not different from that in group C (Table 1).

Activation of p-JNK1/2 and p-Akt1 in Hippocampal CA_1 Subfield

To further detect the protective effects of sevoflurane post-conditioning, activation of p-JNK1/2 and p-Akt1 in hippocampal CA_1 subfield was examined. Some previous studies have indicated that activation of JNK1/2 in hippocampal CA_1 subfield reaches two peaks at 30 min and 3 days after lethal ischemia injury (3). The latter was considered to account for delayed neuronal death. Thus, we investigated the effects of sevoflurane post-conditioning on the activation of p-JNK1/2 at the 72 h time point. Furthermore, some experiments have demonstrated enhanced p-Akt1 activation at 10 min reperfusion (20). Hence, we selected the 10-min time point to investigate p-Akt1 activation. To test our hypothesis, we first

Table 1. Ratio of the optical density between IGF-1 and β-actin in rat hippocampus in each group (%, $\bar{x} \pm s$, n = 6)

Time Post-I/P	Group S	Group C	Group O_2	Groups Sevo		
				Sevo1	Sevo2	Sevo3
24 h	55.8 ± 2.8	68.7 ± 5.7*	67.9 ± 4.7	69.3 ± 3.4	72.3 ± 4.1#	74.6 ± 6.1#
48 h	54.3 ± 3.9	69.3 ± 4.5*	66.2 ± 5.2	68.5 ± 4.9	74.5 ± 6.9#	75.2 ± 5.5#
72 h	56.6 ± 3.0	69.9 ± 6.9*	65.6 ± 5.8	68.2 ± 5.1	75.4 ± 5.7#	76.1 ± 3.6#

*$P < 0.05$ compared with group S; #$P < 0.05$ compared with group C.

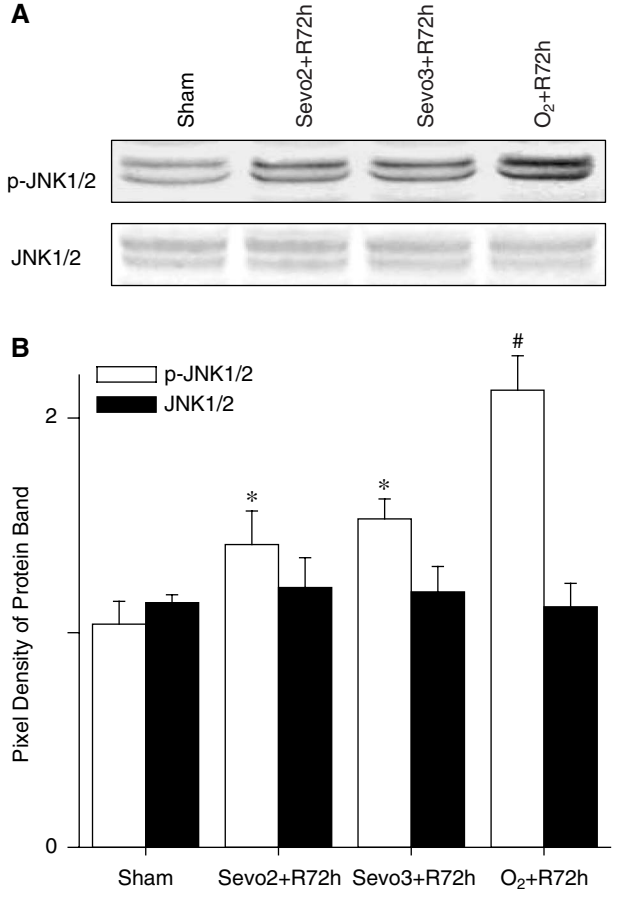

Fig. 2. Western blotting analysis of JNK1/2 activation and expression in hippocampal CA_1 subfields in ischemic brains with or without sevoflurane post-conditioning. The activations of p-JNK1/2 were markedly increased in the groups with O_2 post-conditioning and higher dose (2.4% and 3.0%) of sevoflurane post-conditioning (A). However, the enhanced p-JNK1/2 levels are significantly depressed in the rats with sevoflurane post-conditioning when compared to the group with O_2 post-conditioning at hippocampal CA_1 region. The total protein expression levels of JNK1/2 in CA_1 were kept at a similar level in all four groups. Semiquantitative analysis of the levels of p-JNK1/2 and JNK1/2 in the hippocampal CA_1 regions is demonstrated in (B). Data are expressed as means ± SD (n = 4). *$P < 0.05$ indicates statistical difference versus the sham group. #$P < 0.05$ indicates statistical difference versus sevoflurane post-conditioning (ANOVA followed by Newman-Keuls test).

examined whether the activation of p-JNK 1/2 was changed by sevoflurane-conditioning treatment. The activation of p-JNK1/2 was markedly decreased in the rats with sevoflurane conditioning in contrast with the same control with O_2 conditioning at the hippocampal CA_1 region (Fig. 2A). But the total protein expression levels of JNK 1/2 were kept at the same levels as in the O_2-conditioning group (Fig. 2B). We further explored the mechanism of sevoflurane conditioning by the activation of p-Akt1. Akt1 increased markedly in the rats with sevoflurane conditioning at 10 min of reperfusion compared with the same control with O_2 conditioning. Coinciding with other studies, there were no significant changes in CA_1 in the total protein levels of Akt1 (Fig. 3).

Discussion

Sevoflurane is becoming more important as an inhalant anesthetic in clinical applications. Previous studies showed that the drug had a protective effect on the ischemic or hypoxic injury in vital organs (9, 12, 17). The mechanism of this function of sevoflurane was unknown. Toner *et al*. considered that sevoflurane could reduce the release of catecholamine and excitatory neurotransmitter such as glutamine induced by cerebral ischemic injury, thus, alleviating harmful stimulations to neurons (7, 18). Twenty-four hours after oxygen-glucose deprivation to cultured rat cortical neurons, sevoflurane could reduce neurons to release lactate dehydrogenase in a dose-dependent manner to improve the survival of cells. Sevoflurane could also reduce the release of free radicals showing an anti-oxidation characteristic (1). In addition, sevoflurane could inhibit the voltage-gated calcium channel of neuronal presynaptic membrane and protein kinase C (15), up-regulate the hippocampal anti-apoptosis proteins Bcl-2 and Mdm-2 and suppress ischemia-mediated expression of the apoptosis activation protein Bax (16), thus, serving to protect the central nerve system. Whether sevoflurane conditioning has impacts on IGF-1 and its receptor *via* the above-mentioned mechanisms needs to be further studied.

The expression levels of IGF-1 at the global cerebral ischemia position were correlated to the

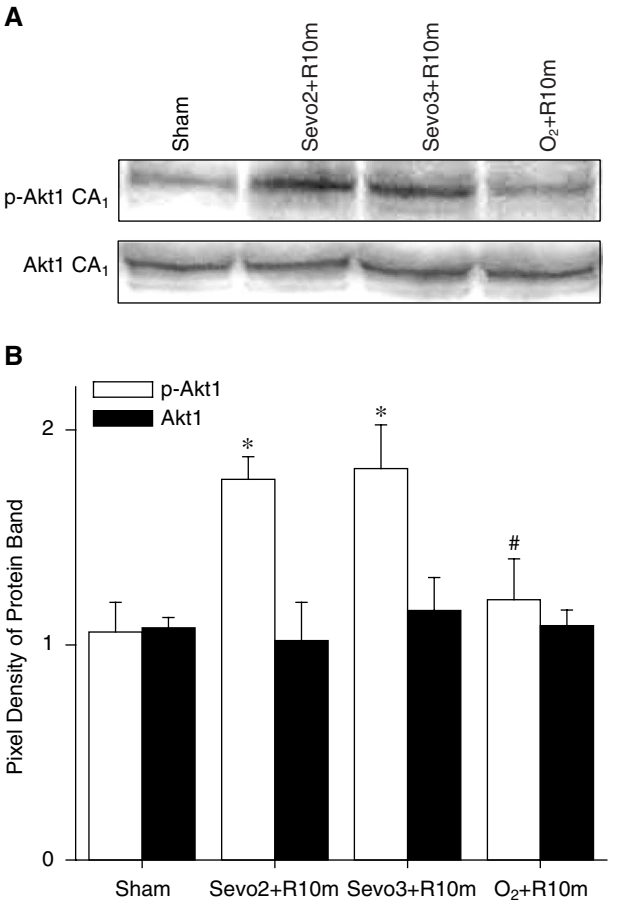

Fig. 3. Western blotting analysis of Akt1 activation and expression in hippocampal CA_1 subfields in ischemic brains with or without sevoflurane post-conditioning. The activation levels of p-Akt1 were markedly decreased in the rats with sevoflurane (2.4% and 3.0%) post-conditioning in contrast with the sham group or the group with O_2 post-conditioning at the hippocampal CA_1 region (A). The total protein expression levels in Akt1 were similar in all four groups. Semi-quantitative analysis of the levels of p-Akt1 and Akt1 in hippocampal CA_1 regions is displayed in (B). Data are expressed as means ± SD (n = 4). $^*P < 0.05$ indicates statistical difference versus the sham group. $^{\#}P < 0.05$ indicates statistical difference versus sevoflurane post-conditioning (ANOVA followed by Newman-Keuls test).

injury of neuron. Both of the endogenous and exogenous IGF-1 could inhibit apoptosis induced by a diverse range of factors *via* multiple mechanisms. In the present study, it was showed that over-expression of IGF-1 after cerebral ischemia and reperfusion injury was synchronized with the pathological manifestation, indicating that the synthesis of IGF-1 and its receptor accompanied by the injury might be a protective factor for a body to fight against ischemic injury. The protective properties of IGF-1 have been demonstrated in multiple experimental systems (6, 10, 19). IGF-1 protects neurons from apoptosis and offers neuroprotective support to hippocampal CA_1 subfield (10, 19). In our study, we employed a global ischemia model to investigate the effect of conditioning with different concentrations of sevoflurane on the expression levels of hippocampal IGF-1 mRNA during cerebral ischemic injury in rats. In this study, it was showed that, 24, 48 and 72 h after global cerebral ischemic injury, the expression levels of hippocampal IGF-1 mRNA were higher in group C than in group S, indicating that cerebral ischemia and reperfusion could enhance the expression of hippocampal IGF-1 mRNA. Furthermore, conditioning with different concentrations of sevoflurane, the expression levels of hippocampal IGF-1 mRNA in groups Sevo2 and Sevo3 increased significantly when compared with that in group C; the expression of hippocampal IGF-1 mRNA in group O_2 was not different from that in group C. These results indicated that sevoflurane could up-regulate the expression of IGF-1 in the hippocampus.

Previous studies indicated that IGF-1 can activate the Akt and JNK pathways. Some researchers had revealed that over-expression of IGF-1 and its receptor could activate the PI-3K/Akt and MAPK signal pathways, and inhibit the apoptosis caused by ischemia and hypoxia (14). JNK, an important signaling protein in cell apoptosis, is activated significantly in I/R, and is also involved in ischemic neuronal death (4). In this study, we found that activated p-Akt and inhibited p-JNK were found by sevoflurane conditioning. On the other hand, in the IGF-1-mediated signaling cascades, Akt is activated by PI-3K through phosphorylation of Akt-S473 (20). Therefore, PI-3K pathways participate in the mechanism of protective effects induced by sevoflurane conditioning. Consistent with the above facts, the activation of Akt is induced by sevoflurane conditioning in our results. However, O_2 conditioning did not activate Akt activation. Therefore, the data suggest that sevoflurane conditioning plays a role associating closely with neuroprotective properties of IGF-1-mediated Akt/JNK pathways in ischemic injury.

On the other hand, in the two reperfusion signaling pathways, like the reperfusion injury salvage kinase (RISK) pathway and the survivor activating factor enhancement (SAFE) pathway, two apparently distinct signal transduction pathways been reported to convey pre-conditioning and post-conditioning protection (5, 13). For example, the mitogen extra-regulated kinase 1/2 (MEK1/2)–extra-regulated kinase 1/2 (Erk1/2) prosurvival kinase pathway contributes to the RISK pathway, whereas JAK/STAT3 plays a role in the SAFE pathway. We suggest that RISK and SAFE pathways may also contribute to sevoflurane

conditioning-induced neuroprotection.

In the present study, sevoflurane used in group Sevo1 was a clinical sub-anesthesia dosage and the sevoflurane dosages used in groups Sevo2 and Sevo3 were the MAC value of rats and 99% of effective dosage (ED99) in clinical anesthesia. Compared with those in group C, the conditioning with a lower dose of sevoflurane in group Sevo1 had not obvious effects on the hippocampal ischemic injury, suggesting the protective effect of sevoflurane was correlated to its concentration. However, with the increase of the dosage, sevoflurane would result in more respiratory and circulatory adverse events. Therefore, it remains to be determined whether sevoflurane serves to protect the central nerve system in a dose-dependent manner, or the dosage by which sevoflurane has a maximal efficacy but minimal adverse effects.

In summary, we showed that sevoflurane conditioning before reperfusion injury enhanced the expression levels of IGF-1 mRNA, inhibited the activations of JNK1/2 and enhanced activation of Akt1. These findings may provide some clues to understanding the mechanism underlying sevoflurane conditioning, and to find an alternative way for clinical therapy in brain diseases such as stroke in the future.

Acknowledgments

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