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Protective Effect of Isoflurane and Sevoflurane on Ischemic Neurons and Expression of Bcl-2 and ICE Genes in Rat Brain[1]

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Objective To study the protective effect of volatile anesthetics, isoflurane and sevoflurane, on ischemic neurons after cerebral ischemia-reperfusion in rats and its possible molecular mechanism. **Methods** Rat cerebral ischemia-reperfusion model was developed by occlusion of the middle cerebral artery (MCA) and bilateral common carotid arteries (CCAs) 1 h after reperfusion. Using flow cytometry (FCM) and Northern blot hybridization, we calculated the number of apoptotic bodies and detected the expression of bcl-2 mRNA and interleukin-1β converting enzyme (ICE) mRNA. **Results** The apoptotic bodies in hippocampus analyzed by FCM peaked at appeared 24 h after reperfusion, and decreased about 54% and 40%, respectively, after treatment with isoflurane and sevoflurane, as compared with ischemic group. There was no significant difference in the expression of bcl-2 mRNA and ICE mRNA between the inhaled anesthetic groups and ischemic group in hippocampus 24 h after MCA/CCAs occlusion. **Conclusion** Isoflurane and sevoflurane partially inhibit apoptosis but have no significant effect on the expression of bcl-2 and ICE genes.

Key words: Isoflurane; Sevoflurane; Apoptosis; bcl-2; ICE

INTRODUCTION

Apoptosis, a type of programmed cell death, plays an important role in the embryogenesis, normal development and maintenance of many adult tissues. It is strictly regulated by the expression or activation of several genes and proteins. The proto-oncogene bcl-2 is one of such genes that were first cloned in a B cell lymphoma line. Bcl-2 is a member of a family of related genes encoding proteins that either promote (e.g., bax, bcl-xS) or suppress (e.g., bcl-2, bcl-xL) programmed cell death. Bcl-2 is expressed in cells that survive fetal development, and inhibits programmed cell death in many *in vitro* systems. In the nervous system, bcl-2 protects against various stimuli that induce apoptotic neuronal death.

Sevoflurane and isoflurane are volatile anesthetics suitable for both induction and maintenance of anesthesia[1]. Clinically, they produce a pattern of rapid recovery with little excitation on emergence[2]. Their capability of increasing cerebral blood flow[3] and preserve cerebral autoregulation[1] makes them an attractive agent for the preservation of neuronal function. There is some evidence that sevoflurane and isoflurane are neuroprotective agents[4]. However, the protection afforded by volatile anesthetics in later neuronal death, i.e. apoptosis, caused by local ischemia has not been investigated. In this study, the influence of isoflurane and sevoflurane on the apoptosis of ischemic neurons and the possible molecular mechanism were investigated.

MATERIALS AND METHODS

Materials

Adult male Wistar rats weighing 320-350 g were purchased from Animal Center, Chinese Academy of Medical Sciences. The materials used were as follows: isoflurane (Abbott Laboratories), sevoflurane (Maruishi Pharmaceutical Co, Osaka, Japan), propidium iodide (Sigma-Aldrich Co, USA), DNAase-free RNAase and RNA isolation kit (Promega Co., USA), nylon membrane (Hybond-N, Amersham International, UK), oligodeoxynucleotide probe to bcl-2 and ICE (Gibco Co., USA), α-^{32}P-dATP (DuPont Co., USA), terminal deoxynucleotidyl-transferase (TdT) (Life Technologies, Gaithersburg, MD), operating microscope (×16, ×10) (Shanghai Medical Instruments Co. Ltd., China), microchip

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(Beijing Meidike Company, China), flow cytometer (FACScalibur, BD Company, USA) and centrifuge (Beijing Centrifuge Factory, China).

Methods

Animal models One hundred and ten male Wistar rats weighing 250-350 g were purchased from Experimental Animal Institute, Chinese Academy of Medical Sciences. The rats were of class II and raised in a clean environment at 20℃-26℃ with humidity of 40%-70% in a 12 light-dark cycle. The rats had free accesss to food and water, fasted for 18 hours before surgery. The animals were anesthetized with chloral hydrate (300 mg/kg, ip). Rectal temperature was continuously monitored during surgery and body temperature was maintained at 37℃-38℃ with a thermostatically controlled warming blanket. The rats were fixed in supine position and the bilateral common carotid arteries (CCAs) were exposed. An 1-cm incision perpendicular to and bisecting a line between the lateral canthus of the right eye and the external auditory canal was made. The underlying temporal muscle was excised. With the aid of an operating microscope, the right middle cerebral artery (MCA) was exposed through a 2 mm hole burr-drilled 2-3mm rostral to the fusion of the zygomatic arch with the squamous bone. The MCA and bilateral CCAs were occluded using microclips. One hour later the microclips were removed. Blood re-flow in MCA and CCAs was visually verified. Wounds were sutured and the rats were returned to their cages. The rats were randomly divided into 4 experimental groups, 5 rats per group. Group 1, sham-operated group without occlusion of CCAs and MCA; group 2, ischemia group with occlusion of CCAs and MCA for 1 h; group 3, isoflurane treated group (isoflurane was inhaled for 1 h) with occlusion of CCAs and MCA; group 4, sevoflurane treated group (sevoflurane was inhaled for 1 h) with occlusion of CCAs and MCA. The isoflurane and sevoflurane were used at the concentration of 1.0 minimal alveolus concentration (MAC).

Flow cytometry At 8 h, 16 h, 24 h, and 72 h after reperfusion, the rats were killed and the hippocampus in ischemic hemisphere was dissected and gently triturated in 0.01 mol/L phosphate buffer solution (PBS, pH7.4). The PBS solution containing dissociated cells was passed through a copper wire gauze (53 μm in diameter) to remove residues and was centrifuged (1000 r/min) for 10 min. The pellets were resuspended and fixed in ice-cold 70% ethanol for 12 h. Cells were washed twice with PBS and the concentration of cells in PBS was adjusted to 2×10^6 cells/mL. The DNAase-free RNAase (1 mg/mL) was added into 1 mL suspension and then the mixture was incubated at 37℃ for 30 min. After being washed twice with PBS, cells were stained with propidium iodide (PI) at the concentration of 50 μg/mL. PI was excited with an argon laser (351 nm), and emission was detected at 600±20 nm. Distribution of DNA contents in 10 000 cells of each group was measured by flow cytometry and the data were analyzed by multicycle DNA content and cell cycle analysis software Cell Quest and ModF.

Northern blot hybridization Rats were killed at 8 h, 16 h and 24 h after reperfusion and the hippocampus in the ischemic hemisphere was dissected. The brain tissue samples were rapidly frozen in liquid nitrogen and kept at -80℃. The samples were homogenized and total RNA was extracted using RNA isolation kit according to the manufacturer's instructions, and subjected to electrophoresis on 1% agarose gel. RNA was electroferred on a positively charged nylon membrane, which was prehybridized for 1 h and then hybridized by incubation with radiolabeled DNA probes for 18 h. A 40-mer antisense oligodeoxynucleotide probe complementary to *bcl-2* mRNA was constructed, the sequence being 5′-CACTGAATGCTCTCCGGTACC GCAG TTCAA ACTCATCGCC-3′. The sequence 5′-ATGGCCGACAAGGTCCTGAAGGAGAAGAG AAAG GTG TTT-3′ was used as ICE mRNA probe[6-7]. The oligodeoxynucleotide probes were labeled with α-[^{32}P] dATP (specific activity>10^6Ci/mol) by using terminal deoxynucleotidyl-transferase (TdT). Unincorporated nucleotides were separated by sepharose G-15 columns. The membrane was washed and exposed to Kodak film at 0℃ for 24 h. The blots in the films were quantified by optical scanning densitometry.

TUNEL staining To analyze quantitatively the change of apoptosis in the hippocampal CA1, detection of DNA fragmentation was conducted using TUNEL kits (Progmega). The rats were perfused transcardially with 4% paraformaldehyde under anesthesia 72 h after operation or ischemic insult. The number of TUNEL-positive cells was determined by counting the number of neurons possessing dark-brown stainied nuclei along 1mm linear length of the hippocampal CA1.

Statistical Analysis

Data were presented as $\bar{x} \pm s$. Statistical analyses were performed by Student's two sample *t*-test using SPSS 10.0 software. $P<0.05$ was considered statistically significant.

RESULTS

FCM Measurement

The number of apoptotic bodies reached the maximum 24 h post-reperfusion; the majority of cells were at G1/G2 of the cycle. As the time of reperfusion prolonged, the residual dead cells gradually increased and became the main content. It indicated that cells mainly died via necrosis thereafter. In groups 3 and 4, the number of apoptotic bodies decreased about 54% and 40%, respectively, compared with group 1 ($P<0.01$) (Fig.1).

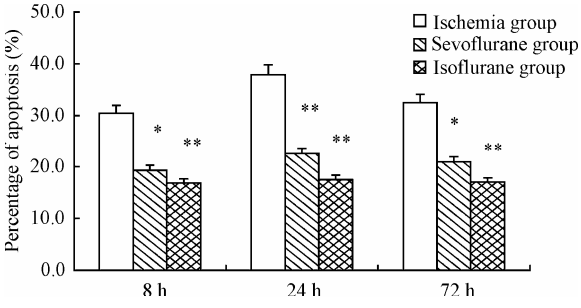

FIG. 1. Percent of apoptosis in hippocampus at different reperfusion time points after 1 h ischemia.*P<0.05; **P<0.01 vs ischemia group ($\bar{x} \pm s$, n=5).

Northern Blot Hybridization Analysis

The total RNA of bcl-2 mRNA in group 1 was shown at a low level. Compared with sham-ischemia group, its expression after 1 h ischemia increased to 1.73 folds at 8 h, 1.91 folds at 16 h, and 2.10 folds at 24 h post-reperfusion, respectively (Fig. 2). Compared with group 2, the bcl-2 mRNA expressions in groups 3 and 4 were 1.32 folds and 1.30 folds higher, respectively. There was no difference between anesthetic- inhaled groups and ischemic group. The ICE mRNA expression after 1 h ischemia increased 1.23 folds at 8 h, 1.35 folds at 16 h, and 1.47 folds at 24 h post-reperfusion, respectively, compared with sham-ischemia group (Fig. 2). The ICE mRNA expressions at 24 h post-reperfusion after 1 h ischemia in groups 3 and 4 were 0.87 fold and 0.91 fold higher than that in group 2. There was no difference between anesthetic-inhaled groups and ischemic group.

TUNEL Staining

There were almost no TUNEL-postitive cells in the hippocampus in sham-operated group. Clear TUNEL-potitive cells were detected in the CA1 field in the other groups. As noted in Fig. 3, the number of TUNEL-positive cells significantly decreased in anesthetic-inhaled groups compared with that in ischemic group.

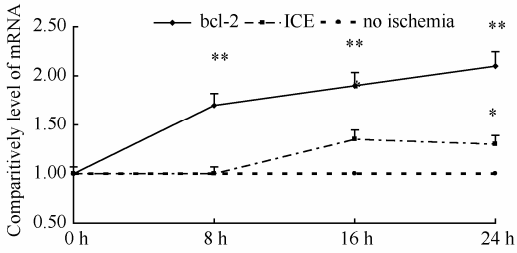

FIG. 2. Expression of bcl-2 mRNA and ICE mRNA at different reperfusion time points after 1 h ischemia in rat hippocampus. *P<0.05; **P<0.01 vs ischemia group. ($\bar{x} \pm s$, n=5)

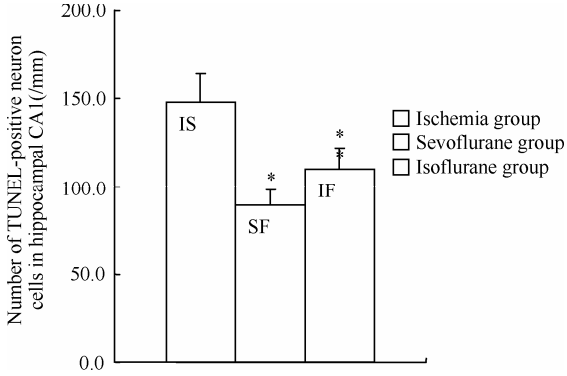

FIG. 3. Influence of volatile anesthetics on apoptosis of neurons in hyppocampla CAI *, P<0.05 ± s, n=5)

DISSCUSSION

The present data show that incomplete cerebral ischemia could enhance the expressions of bcl-2 mRNA and ICE mRNA in rat hippocampus at 16 h and 24 h after 1 h cerebral ischemia and reperfusion, which could not be suppressed by volatile anesthetics, isoflurane and sevoflurane. The peak of apoptotic bodies in the FCM analysis appeared 24 h after reperfusion, and the peak of apoptosis treated with isoflurane or sevoflurane was lowered by about 54% and 40%, respectively, compared with ischemic group. These data indicate that the neuroprotective effects of volatile anesthetics, isoflurane and sevoflurane, may be related to the reduction of apoptosis neuron.

Sevoflurane has been reported to be an effective neuroprotective agent in cerebral ischemia[8-9] in our study; it has been confirmed that the apoptosis of neurons could be inhibited by isoflurane and sevoflurane after ischemia-reperfusion. However,

compared with ischemic group, there was no difference between anesthetic-inhaled groups. Bcl-2 plays a key role in preventing the entry of cytochrome C into cytoplasm. It also plays a role in stabilizing mitochondria and maintaining its membrane potential, thus preventing the generation of free radicals. Bcl-2 also regulates calcium flux into the mitochondria. Thus, the expression of bcl-2 in neurons induced by ischemia might be an important event that ensures neuronal survival[10-11]. This indicates that sevoflurane and isoflurane protect ischemic neurons not by inducing overexpression of bcl-2, but by some other mechanisms.

ICE shares homology with the gene *ced-3*, one of the death genes in the nematode *Caenorhabditis elegans,* and is the first identified member of the caspase family which is believed to execute apoptosis[12]. ICE and related members of the caspase family are involved in apoptosis; several recent studies support the involvement of ICE itself (caspase 1) in ischemic brain damage[13-14]. In this study, the expression of ICE mRNA did not change significantly after reperfusion and ischemia, suggesting that there is no relationship between the expression of ICE and the protection of sevoflurane and isoflurane against ischemic neurons.

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