Neuroscience 159 (2009) 1309–1315

HYPERBARIC OXYGEN PRECONDITIONING REDUCES ISCHEMIA–REPERFUSION INJURY BY INHIBITION OF APOPTOSIS VIA MITOCHONDRIAL PATHWAY IN RAT BRAIN

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Abstract—This study examined the hypothesis that apoptotic inhibition via mitochondrial pathway was involved in hyperbaric oxygen preconditioning (HBO-PC)–induced neuroprotection on ischemia–reperfusion injury in rat brain. Male Sprague–Dawley rats (250∼280 g, *n*=144) were divided into control, middle cerebral artery occlusion (MCAO) for 90 min, and HBO-PC plus MCAO groups. HBO-PC was conducted four times by giving 100% oxygen at 2.5 atm absolute (ATA), for 1 h at 12 h intervals for 2 days. At 24 h after the last HBO-PC, MCAO was performed and at 24 h after MCAO, neurological function, brain water content, infarct volume, and cell death were evaluated. Enzymatic activity of capase-3 and −9, and expression of cytochrome *c*, Bcl-2 and Bax proteins were performed in the samples from hippocampus, ischemic penumbra and core of the brain cortex, respectively. HBO-PC reduced brain edema, decreased infarction volume, and improved neurological recovery. HBO-PC reduced cytoplasm cytochrome c levels, decreased caspase enzyme activity, upregulated the ratio of Bcl-2 and Bax expression, and abated the apoptosis of ischemic tissue. HBO-PC protects brain tissues from ischemia–reperfusion injury by suppressing mitochondrial apoptotic pathways.

Key words: hyperbaric oxygenation, prevention, middle cerebral artery occlusion, apoptosis, ischemic penumbra.

Hypoxic or ischemic preconditioning may have clinical potentials for neuroprotection for neurosurgery patients who undergo temporary clipping of major intracranial vessels during aneurismal or cerebral bypass surgery procedures. The neuroprotective effect of preconditioning has been established in animal models (Rehman et al., 2008; Yu et al., 2008; Shimoda et al., 2007) and observed in clinical cases after multiple transient ischemic attacks (Sitzer et al., 2004). However, the safety concerns and practical feasibility have limited the application of preconditioning in practice.

Hyperbaric oxygen (HBO) has been used for multiple neurological diseases (Lou et al., 2004; Al-Waili et al., 2005; Rosenthal et al., 2003; Ostrowski et al., 2006) and proved a safe treatment modality in all age and gender groups, including neonates (Calvert et al., 2004) and pregnant mothers (Xiao et al., 2006). Hyperbaric oxygen preconditioning (HBO-PC) has been reported to increase ischemic tolerance against neuronal injury in animals (Wada et al., 2001; Nie et al., 2006; Speit et al., 2000; Dong et al., 2002) Recently, we have observed that a single dose of HBO-PC reduced hypoxic–ischemic brain injury in neonatal rats (Freiberger et al., 2006), However, the dosage, the timing of application, and the mechanisms of HBO-PC remain to be determined (Wada et al., 2001; Zhang et al., 2004). Therefore, we examined the neuroprotective effect and mechanisms of a short term HBO-PC in an established middle cerebral artery occlusion (MCAO) rat model.

EXPERIMENTAL PROCEDURES

Experimental groups

The Experimentation Ethics Committee of the Second Military Medical University in Shanghai, China approved the animal protocols of this study. All methods and animal procedures were met or exceeded all federal guidelines for the humane use of animals in research. All efforts were made to minimize the number of animals used and their suffering. A total of 144 male Sprague–Dawley rats weighing 250∼280 g (Slaccas, Shanghai, China) were used. They were housed at a temperature of 22–24 °C and 12 h light/dark cycle controlled environment with free access to food and water prior to and following surgery. Rats were randomly assigned to one of the following three groups: control group (*n*=24), MCAO group (*n*=60), and HBO-PC plus MCAO group (*n*=60). All animals were sacrificed at 24 h after MCAO.

HBO-PC

HBO-PC was administered by using 100% oxygen at 2.5 atmosphere absolute for 1 h at 12 h intervals four times in 2 days. The last HBO-PC was performed at 24 h before MCAO. Compression was performed at 1 kg/cm^2/min and decompression was performed at 0.2 kg/cm^2/min. None of the animals had seizures during or after HBO-PC. Chamber temperature was maintained between 22 and 25 °C. Accumulation of CO_2 was prevented by using a small container with calcium carbonate crystals. To minimize the effects of diurnal variation, all exposures were started at 8:00 AM. MCAO rats were placed in the same rodent chamber for 1 h at 12 h interval for 2 days in room air.

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Abbreviations: CCA, common carotid artery; ECA, external carotid artery; HBO, hyperbaric oxygen; HBO-PC, hyperbaric oxygen preconditioning; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; TTC, 5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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doi:10.1016/j.neuroscience.2009.01.011

MCAO procedures

MCAO was produced by the filament model initially reported by Zea-Longa et al. (1989) with some modifications. Briefly, the rats were anesthetized with an i.p. injection of 2% pentobarbital sodium (40 mg/kg) and were allowed to breathe spontaneously. A supplemental anesthetic dose was added if necessary. Via a midline neck incision, the submandibular glands were separated to allow access to the right carotid artery. The common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were isolated from connective tissues. The ECA and the proximal end of the CCA stump were cut and a suture loop was put around the distal end of the CCA. A microvascular clip was temporarily put on the ICA. A 0.24 mm diameter carbon fishing-line with a pretreated rounded tip was introduced via the CCA stump into the ICA. After removal of the clip, the filament was advanced further in the ICA until a resistance was felt at approximately 18.0±0.5 mm from the carotid bifurcation. The filament was fastened by tightening the loop around the distal CCA stump and the neck incision was closed. During surgery, a heating blanket was used to maintain the rectal temperature at 37.0±0.5 °C. After 90 min MCAO, blood flow was restored by the withdrawal of the intraluminal suture and then the awaking rats were returned to their cages.

Neurobehavioral functional scoring

The neurological scoring systems proposed by Dean et al. (2003) and Ohlsson et al. (1995) were adopted with modifications as shown in Table 1. All neurological evaluations were done blinded by a researcher without knowledge of the animal groups. The neurological testing was composed by thee sets: Zea-Longa score, beam-walking test, and prehensile traction test. The higher score represents severe deficits.

Table 1. Neurological deficit score for rats

Zea-longa score	
0	The rat has no neurological defect
1	The rat is unable to extend affected forward limb
2	The rat circles while walking
3	The rat tumbles to its side because of hemiplegia while walking
4	The rat is unable to walk and unconsciousness is present
5	The rat is dead
Beam-walking test	
0	The rat crosses the beam with no foot slips
1	The rat crosses the beam with a few foot slips
2	The rat traverses the beam with more than 50% foot slips
3	The rat can traverse the beam, but the affected hind limb does not aid in forward locomotion
4	The rat falls down while walking
5	The rat is unable to traverse the beam but remains sitting across the beam
6	The rat falls down from the beam
Prehensile traction test	
0	The rat hangs on 5 seconds and brings rear limb up to rope
1	The rat hangs on 5 seconds, no third limb up to rope
2	The rat hangs on 3 to 4 seconds
3	The rat hangs on 0 to 2 seconds

Minimum score: 0, namely healthy rats; maximum score: 14, namely death.

TTC staining

Infarct volume was determined by staining with 2,3,5-triphenyltetrazolium chloride (TTC). Briefly, the brains were quickly removed and placed at −20 °C for 15 min, and then cut into five 2 mm coronal slices starting at 1 mm from the frontal pole. After incubation in 1% TTC in 0.2 mol/L phosphate-buffered saline (PBS) at 37 °C for 30 min, the slices were fixed in 4% paraformaldehyde in 0.1 mol/L PBS. After 24 h, the sections were digitally photographed and infarction volumes were analyzed using image analysis system (Image J software (http://www.quickvol.com), a public domain image analysis program developed at the National Institutes of Health). The percentage of infarction (infarct ratio) was calculated by dividing the infarct volume by the total volume of the slices.

Brain water content

The brains were harvested and quickly separated to the left and right hemispheres, cerebellum and the brain stem. Brain samples were weighed on a precise electronic balance and placed in an oven at 100 °C for 48 h. After 48 h, the samples were weighed again and the water content was calculated according to the following formula:

$$[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%.$$

In situ labeling of DNA fragmentation

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed on paraffin-embedded sections according to the manufacturer's instructions (Roche Molecular Biochemicals, Inc., Mannheim, Germany). The sections were dewaxed and rehydrated according to standard protocols, pretreated with proteinase K (20 μg/mL in 0.01 mol/L PBS) for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1 h at 37 °C. We dried the area around the sample and added converter-AP to samples for 1 h at 37 °C. After rinsing with PBS (5 min, three times), sections were stained in the dark with Nitroblue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). For negative controls, the sections were incubated without terminal deoxynucleotidyl transferase. There were two distinct patterns of TUNEL staining. Some cells were densely labeled and showed clear apoptotic characteristics. Other cells were weakly labeled and considered to be necrotic cells. Only the densely labeled cells were counted as TUNEL positive cells. The ischemic core and penumbra of the cerebral cortex and CA1 of hippocampus were photographed (Leica, Germany) in each section. The data were represented as the number of cells per mm^2.

Ischemic core and penumbra dissections

Ischemic core and penumbra were dissected according to well-established protocols in rodent models of unilateral proximal MCAO (Ashwal et al., 1998; Lei et al., 2004). Briefly, each hemisphere was cut longitudinally, from dorsal to ventral at 1.5 mm from the midline to exclude medial brain structures that were supplied primarily by the anterior cerebral artery. A transverse diagonal incision at approximately the 2 o'clock position separated the core from the penumbra.

Caspase activity assay

The activity of caspase-3 and −9 was measured with caspase-3 and caspase-9/CPP32 fluorometric Assay Kit (BioVision, Inc.,