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EFFECTS OF PRECONDITIONING WITH NORMOBARIC HYPEROXIA ON NA^+/CA^{2+} EXCHANGER IN THE RAT BRAIN

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Abstract—*Background* Recent studies suggest that normobaric hyperoxia (HO) reduces hypoxia-reoxygenation injury in the rat brain. We have attempted to determine the effect of HO on Na^+–Ca^{2+} exchangers (NCX) in the rat stroke model.

***Methods:* Rats were divided into two experimental groups. The first group was exposed to 95% inspired HO for 4 h/day for 6 consecutive days (HO). The second group acted as the control, and was exposed to 21% oxygen in the same chamber. Each main group was subdivided to middle cerebral artery occlusion (MCAO-operated) and intact (without any surgery) subgroups. After 48 h from pretreatment, MCAO-operated subgroups were subjected to 60 min of right MCAO. After 24 h reperfusion, neurologic deficit score (NDS) and infarct volume were measured in MCAO-operated subgroups. The NCXs expression levels of the core, penumbra and subcortical regions were assessed in sham-operated and intact subgroups.**

***Result:* Preconditioning with HO decreased NDS and infarct volume, and increased the expression of NCX1, NCX2 and NCX3 in the penumbra, NCX2, NCX3 in the core and NCX1 and NCX3 in the subcortex.**

***Conclusion:* Although further studies are needed to clarify the mechanisms of ischemic tolerance, HO partly is associated with the expression of NCX1, 2, 3 consistent with an active role in the genesis of ischemic protection.**

Key words: Na^+–Ca^{2+} exchanger, normobaric hyperoxia, brain ischemia, reperfusion, stroke, neuroprotection.

INTRODUCTION

Noxious stimuli applied at doses close to but below the threshold of cell injury induce adaptive responses that protect the brain against additional stress from the same (tolerance) or other (cross-tolerance) stimuli. Ischemic tolerance (IT) is an endogenous phenomenon that can result in subsequent resistance to severe ischemic injury. This phenomenon has been reported in several organs including the brain (Kitagawa et al., 1990). Among different stresses, hypoxia (Gidday et al., 1994; Gill et al., 1996), ischemia (Kitagawa et al., 1990; Toyoda et al., 1997), anoxia (Péerez-pinzn et al., 1996), oxidative stress (Ohtsuki et al., 1992) and inhibitors of oxidative phosphorylation (Riepe et al., 1997; Ruscher et al., 2002) induce tolerance to subsequent cerebral (focal or global) ischemia. Most of such stimuli, however, lack potential for clinical translation because of associated toxicity. For this reason, safe nonpharmacologic stimuli have been sought. Previously, similar protection has been shown to be conferred by normobaric hyperoxia (HO) (Bigdeli et al., 2007), perhaps (as in other situations Ravati et al., 2001) through the generation of oxygen-free radicals and hydroxyl radicals (Wada et al., 2001).

The induction and maintenance of cerebral IT may be mediated through changes in expression availability of a variety of mediators, including NMDA (*N*-methyl-D-asparticacid) receptors (Gonzalez-Zulueta et al., 2000), antiapoptotic factors (Shimazaki et al., 1994), interleukin1 (Ohtsuki et al., 1996), superoxide dismutase (Gidday et al., 1994; Gill et al., 1996; Toyoda et al., 1997), reactive oxygen species (Ravati et al., 2001), nitric oxide-dependent p21 ras (monomeric G proteins) activation (Gill et al., 1996), metallothioneins (Trendelenburg et al., 2002), activation of vascular endothelial growth factor receptor and Akt (protein kinases B) (Wick et al., 2002), erythropoietin (Swanson et al., 1990), caspase-3 (McLaughlin et al., 2003), NF-κB (nuclear factor-Kappa B), and proinflammatory cytokines (Mattson et al., 2000) (Bigdeli et al., 2008), excitatory amino acid transporters (EAAT) or glutamate transporters (Romera et al., 2004; Pradillo et al., 2006; Bigdeli et al., 2008). It is relevant to mention that the Na^+–Ca^{2+} exchanger (NCX) gene was upregulated after transient global ischemia in rats (Majda et al., 2001). NCX family have three mammalian genes (*NCX1*, *NCX2*, *NCX3*) (Blaustein and Lederer, 1999) and their three proteins are differentially expressed in distinct regions of the central nervous system where they might underlie different physiological and pathophysiological functions (Yu and Colvin, 1997; Canitano et al., 2002; Papa et al., 2003b). NCX is a nine transmembrane segment protein widely distributed in the brain (Papa et al., 2003a) that couples, in a bidirectional way, the movement of Ca^{2+} and Na^+ ions across the cell membrane in the central nervous system. Thus, NCX plays a relevant role in the maintenance of the intracellular balance of these two ions. A great number of conflicting reports on the effects of NCX modulation on cell damage, induced by anoxic

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Abbreviations: EAAT, excitatory amino acid transporters; HO, normobaric hyperoxia; I-HO, HO in intact animals; I-RA, intact RA; IT, ischemic tolerance; MCAO, middle cerebral artery occlusion; NCX, Na^+–Ca^{2+} exchangers; NDS, neurologic deficit score; NF-κB, nuclear factor-kappa B; RA, room air; S-RA, sham-operated groups.

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conditions, have been published (Andreeva et al., 1991; Amoroso et al., 1997, 2000; Masada et al., 2001; Takahashi et al., 2003). The results of studies provided evidence *in vivo* regarding the ability of NCX activation to reduce the extent of brain infarct volume after permanent middle cerebral artery occlusion (MCAO) and its selective pharmacological blockade produced a worsening of the brain lesion, thus suggesting a protective role played by the antiporter during the events leading to brain ischemia (Pignataro et al., 2004). We recently showed that preconditioning with HO increased the expression of EAAT1, EAAT2, EAAT3, TNF-α converting enzyme, and serum TNF-α (Bigdeli et al., 2009). Here, in the first part we studied HO pretreatment as an IT-inducing factor and operated MCAO on Rats (estimated IV and neurologic deficit score – NDS). In the second part we sought to identify whether such effects might be associated with changes in the expression of three sodium-calcium exchangers (NCX1, NCX2, and NCX3) and used intact rats (control and HO groups without MCAO operation).

EXPERIMENTAL PROCEDURES

Animals and group assignment

Thirty-six adult male Sprague–Dawley rats, 10–12 weeks old, weighing 250–350 g, were divided randomly into two groups of 15 animals and sham group (S) with six animals. One of these groups was placed in an environmental chamber and exposed to a hyperoxic atmosphere (95% oxygen: normobaric hyperoxic groups, or HO) intermittently (for 4 continuous hours of each day for each of 6 consecutive days, yielding a total hyperoxic exposure of 24 h). The other groups were similarly placed in the environmental chamber and exposed to room-air equivalent (21% oxygen: normobaric normoxic groups, RA (room air)) for similar time periods. All experimental animal procedures were conducted with the approval of the Ethics Committee of the Shahid Beheshti University of Iran. Every effort was made to minimize the number of animals used and their suffering. The rats were housed individually at 24°C with food and water provided ad libitum with lights on from 07:00 to 19:00 (light cycle) and off from 19:00 to 07:00 (dark cycle).

Each main group was subdivided to MCAO-operated (MCAO; $n = 9$) for NDS, infarct volume evaluations and intact subgroups ($n = 6$) for the assessment of NCX1, NCX2, and NCX3 expression levels). Animals were then placed in ordinary RA for a further 48 h, after which MCAO-operated (O) subgroups were subjected to 60 min of MCAO. Twenty-four hours later, neurobehavioral studies and then infarct volume measurement were performed. In the sham-operated (S) subgroup, all steps were similar to the RA group, except MCAO. In each intact subgroup, all steps were similar to the RA and HO groups without any surgery procedure (I-RA, and I-HO). Finally 48 h after pretreatment, sham-operated and intact subgroup animals were sacrificed for the assessment of NCX1, NCX2 and NCX3 expression in the core, penumbra and subcortex of the right hemisphere.

In a subset of animals, arterial blood gas analysis was performed just prior to removal from the environmental chamber. In addition, MCAO was monitored by Laser Doppler flowmeter (MBF3, Moor Instruments, Axminster, UK).

Environmental chamber

All rats underwent adaptation for 1 week in the animal room. The environmental chamber comprised an air-tight box (650 × 350 × 450 mm) with a gas inlet and outlet port. Internal pressure was continuously monitored by a manometer. Oxygen (95%) or RA (by an aquarium pump) was delivered at a rate of less than 5 L/min through the inlet port. The oxygen concentration inside the container was continuously monitored with an oxygen sensor (Lutron-Do5510, Taiwan) and carbon dioxide cleared using soda lime (BDH Ltd., Poole, UK) at the bottom of the container. Oxygen concentration was maintained at 95% or 21% according to the experimental protocol.

Focal cerebral ischemia

The rats were weighed and anesthetized with chloral hydrate (Merck, Germany) (400 mg/1 kg; ip). MCAO was performed as described by Longa et al. (1989). Briefly, under a microscopic surgery, a 3–0 silicone-coated nylon (Nylon, home-made) suture was introduced through the external carotid artery stump. The occluder was advanced into the internal carotid artery 20–22 mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged in the anterior cerebral artery and blocked the blood flow to the MCA. Reperfusion was started by withdrawing the suture after 60 min of ischemia. Rectal temperature was monitored (Citizen-513w, CITIZEN, United Arab Emirates) and maintained at 37 °C by surface heating and cooling during surgery.

Neurobehavioral evaluation

After the suture was withdrawn, the rats were returned to their separate cages. 24 h later, the rats were assessed neurologically by an observer who was blinded to the animal groups. The neurobehavioral scoring was performed using the 6-point scale previously described by Longa et al. (1989): normal motor function = 0; flexion of contralateral forelimb on suspension vertically by tail or failure to extend forepaw = 1; circling to the contralateral side but normal posture at rest = 2; loss of righting reflex = 3; and no spontaneous motor activity = 4. Death was considered as score 5 only when a large infarct volume was present in the absence of subarachnoid hemorrhage. If the rats died as a result of subarachnoid hemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study.

Infarct volume assessment

After killing with chloral hydrate (800 mg/kg; ip), the animals were decapitated and the brains rapidly removed and cooled in 4 °C saline for 15 min. Eight 2-mm thick coronal sections were cut (Brain Matrix, Tehran, Iran) through the brain, beginning at the olfactory bulb. The slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride solution (Merck, Germany), and kept at 37 °C in a water bath for 15 min. The slices were then digitally photographed by a camera (Canon, DSC-W310) connected to a computer. Unstained areas were defined as infarct, and were measured using image analysis software (Image Tools, National Institutes of Health). The infarct volume was calculated by measuring the unstained and stained area in each hemisphere slice, multiplying by slice thickness (2 mm), and then summating all of the eight slices according to the method of Swanson et al. (1990)): corrected infarct volume = left hemisphere volume – (right hemisphere volume – infarct volume).

Brain sampling and protein extraction

At 48 h after pretreatment, intact and sham animals were killed by chloral hydrate (800 mg/kg) and decapitated for the measurement of protein expression. Core, penumbra and subcortex of the brain tissue were isolated as previously described (Lei