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In vivo normobaric hyperoxia preconditioning induces different degrees of antioxidant enzymes activities in rat brain tissue

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ABSTRACT

Recent studies suggest that intermittent and prolonged normobaric hyperoxia results in ischemic tolerance to reduce ischemia brain injury. In this research attempts were made to see the changes in antioxidant enzyme activities following prolonged and intermittent normobaric hyperoxia preconditioning. Rats were divided into four experimental groups, each of 21 animals. The first two were exposed to 95% inspired normobaric hyperoxia for 4 h/day for 6 consecutive days (intermittent normobaric hyperoxia) or for 24 h continuous (prolonged normobaric hyperoxia). The second two groups acted as controls, and were exposed to 21% oxygen in the same chamber. Each main group was subdivided to middle cerebral artery occlusion-operated, sham-operated (without middle cerebral artery occlusion), and intact (without any surgery) subgroups. After 24 h, middle cerebral artery occlusion-operated subgroups were subjected to 60 min of right middle cerebral artery occlusion. After 24 h reperfusion, neurologic deficit score, infarct volume were measured in middle cerebral artery occlusion-operated subgroups. Antioxidant enzyme activities were assessed in sham-operated and intact subgroups. Preconditioning with prolonged and intermittent normobaric hyperoxia decreased neurologic deficit score and infarct volume, and increased antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) significantly. Although further studies are needed to clarify the mechanisms of ischemic tolerance, the intermittent and prolonged normobaric hyperoxia seems to partly exert their effects via increase antioxidant enzymes activities.

1. 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. This phenomenon (ischemic preconditioning) has been demonstrated in a variety of organs including brain (Kitagawa et al., 1990, Bigdeli et al., in press). Ischemic preconditioning is clearly an attractive target for therapeutic development, and can be induced by means other than simple ischemia, such as exposure to diverse pharmacological agents, changes in inspired oxygen tension, and lipopolysaccharide-induced low-grade inflammation (Valen, 2003, Bigdeli et al., 2007).

Recent studies show that brain ischemic tolerance is mediated by the synthesis of proteins which promote neuronal survival, including heat shock protein 70 (Warner et al., 2004), Bcl-2 (Shimazaki et al., 1994), glutamate transporters transporters (Bigdeli et al., 2008 and Pradillo et al., 2006), superoxide dismutase (Toyoda et al., 1997, Bigdeli, 2009), antiapoptotic factors (Shimazaki et al., 1994), reactive oxygen species (Ravati et al., 2001), NF-κB and proinflammatory cytokines (Bigdeli and Khoshbaten, 2008).

One of the manifestations of central nervous system damage after cerebral ischemia is the formation of brain edema caused by the breakdown of the blood brain barrier that is improved by normobaric hyperoxia preconditioning (Bigdeli et al., 2007). Superoxide dismutase prevents vasogenic brain edema after several kinds of injuries (Kinouchi et al., 1991, Bigdeli, 2009), suggesting that O_2^- is an important factor for disruption. Superoxide dismutase is a metalloenzyme that catalyze the dismutation of superoxide anion (O^-_2) into O_2 and hydrogen peroxide (H_2O_2) in the cytosol, mitochondria and nucleus (Fridovich, 1986, Bigdeli, 2009). Subsequently, H_2O_2 is reduced to H_2O by glutathione peroxidase in the cytosol, or by catalase in the peroxisomes or in the cytosol. Another manifestation of central nervous system damage is the direct injury of neural cell including excitatory events that are induced by glutamate release after cerebral ischemia that is improved by normobaric hyperoxia preconditioning via upregulation of glutamate transporters (Bigdeli et al., 2008, in press). Glutamate elevates free calcium (Ca^{2+}), which activates Ca^{2+}-dependent enzymes and leads to free radical production (Orrenius et al., 1992).

In our laboratory, we have recently shown that pretreatment with intermittent and prolonged normobaric hyperoxia induces ischemic tolerance and upregulates glutamate transporters, serum TNF-α levels, and TNF-α converting enzyme in the rat brain (Bigdeli and

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Khoshbaten, 2008; Bigdeli et al., 2008) and confers different degrees of neuroprotection in the rat brain. Intermittent normobaric hyperoxia also reduces brain edema and Evans Blue extravasation significantly (Bigdeli et al., 2007).

This study was designed to explore the association of such brain ischemic tolerance with changes of antioxidant enzyme activities such as superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidaes.

2. Materials and methods

2.1. Animals and group assignment

All experimental animal procedures were conducted with the approval of the Ethics Committee of Shahid Beheshti University of Iran. Male Sprague–Dawley (250–380 g) rats were divided randomly into 4 groups of 21 animals. Two of these groups were placed in an environmental chamber and exposed to a hyperoxic atmosphere (95% oxygen: normobaric hyperoxic groups) either intermittently (for 4 h continuous of each day for each of six consecutive days, yielding a total hyperoxic exposure of 24 h) or for 24 h continuously. The two other groups were similarly placed in the environmental chamber and exposed to room–air equivalent (21% oxygen: normobaric normoxic groups, room air) for similar time periods: the full six days ('intermittent' room air) or for just 24 h ('prolonged' room air). After pretreatment, animals were then placed in ordinary room air (gap, Fig. 1). Therefore, There are 12 subgroups: first four subgroups (middle cerebral artery occlusion-operated subgroups) for assignment of infarct volume and neurologic deficit scores ($n=9$), second and third four subgroups (sham-operated subgroups, and intact subgroups, respectively) for measurement of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) activities (Fig. 1). In each sham-operated subgroup, all steps were similar to prolonged or intermittent groups, except of middle cerebral artery occlusion. In each intact subgroup, all steps were similar to prolonged and intermittent groups without any surgery procedure. Finally 48 h after pretreatment, sham-operated and intact subgroups animals were sacrificed for assessment of antioxidant enzyme activities in right hemisphere (ipsilateral) (Fig. 1).

In a subset of animals, arterial blood gas analysis was performed just prior to removal from the environmental chamber. In addition, middle cerebral artery occlusion was monitored by laser-Doppler flow meter (MBF3, Moor Instruments, Axminster, UK).

2.2. 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 room air (by an aquarium pump) were delivered at a rate of <5 L/min through the inlet port. The oxygen concentration inside the container was continuously monitored (Lutron-Do5510 oxygen sensor, Taiwan), and carbon dioxide cleared using soda lime (BDH Limited, Poole, England) at the bottom of the container.

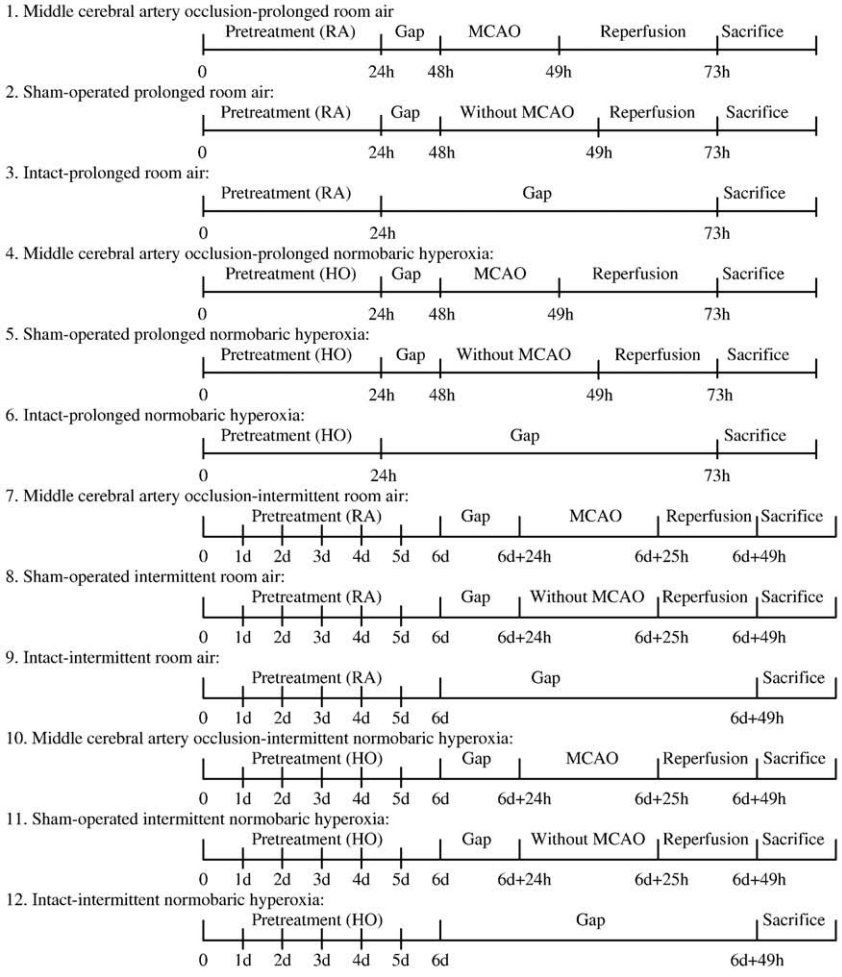

Fig. 1. prolonged (Pr) and intermittent (In) normobaric hyperoxia (HO) and normoxia (RA) in MCAO-operated (MCAO), sham-operated (S), and intact (I) subgroups are shown by a diagram, ordinary air room without any procedure (Gap), day (d), hour (h).

Oxygen concentration was maintained at 95% or 21% according to experimental protocol.

2.3. Focal cerebral ischemia

The rats were weighed and anesthetized with chloral hydrate (Merck, Germany) (400 mg/kg). Middle cerebral artery occlusion was performed as described by Longa et al., 1989. Briefly, under a microscopic surgery, a 3–0 silicone coated nylon suture was introduced through the external carotid artery stump. The occluder was advanced into the internal carotid artery 20 to 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 middle cerebral artery. Reperfusion was started by withdrawing the suture after 60 min of ischemia. Rectal temperature was monitored (Citizen-513w) and maintained at 37.0 °C by surface heating and cooling during surgery.

2.4. Neurobehavioral evaluation

After the suture was withdrawn, the rats of middle cerebral artery occlusion-operated subgroup were returned to their separate cages. 24 h later, the rats were assessed neurologically by an observer who was blind to the animal groups. The neurobehavioral scoring was performed using a six-point scale as was previously described by Bederson et al., 1986: normal motor function = 0; flexion of contralateral forelimb upon suspended vertically by tail or failure to extend forepaw = 1; circling to the contralateral side but have 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 due to subarachnoid hemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study.

2.5. Infarct volume assessment

After sacrifice with chloral hydrate (800 mg/kg), the animals of middle cerebral artery occlusion-operated subgroup 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, 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 photographed by the digital camera (Nokia 6630, Finland) connected to a computer. Unstained areas were defined as infarct, and were measured using image analysis software (Image Tools, NIH). The infarct volume was calculated by measuring the unstained and stained area in each hemisphere slice, and multiplied by slice thickness (2 mm), and then summating all of the eight slices according to the method of Swanson (Swanson et al., 1990): corrected infarct volume = left hemisphere volume − (right hemisphere volume − infarct volume).

2.6. Brain sampling and protein extraction

Samples (150 to 200 mg of right hemisphere tissue) were homogenized in 1 ml of buffer (0.32 mol/L sucrose, 1 mmol/L EDTA and 10 nmol/L Tris–HCl, pH 7.4) in a Teflon glass homogenizer. The homogenate was centrifuged at 13,600 × *g* for 30 min, and the supernatant was collected and used for the measurement of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activities (Xia et al., 1995). Protein was determined according to the method of Bradford (Bradford 1976) using Bovine serum albumin as standard.

2.7. Measurement of superoxide dismutase activity

The activity of total superoxide dismutase was measured by the method of Genet et al. (2002) with some modification. The assay mixture, in a final volume of 1 ml, contained: 50 mM sodium phosphate buffer, 0.1 mM EDTA, 0.48 mM pyrogallol and 20 µl enzymatic extract. The change in absorbance at 420 nm of the assay mixture was monitored for 1 min at 25 °C against a blank that contained all the ingredients except the homogenated tissue. One unit of enzyme is defined as the amount of enzyme that causes half maximal inhibition of pyrogallol autoxidation.

2.8. Measurement of catalase activity

The assay of catalase was performed by following the method of (Genet et al., 2002). The assay mixture, in a final volume of 1 ml, contained: 50 mM sodium phosphate buffer pH 7.0, 10 mM hydrogen peroxide and 20 µl enzymatic extract. The decrease in absorbance was then followed at 240 nm for 1 min at 25 °C against a blank containing all the ingredients without the homogenated tissue. One unit of catalase is defined as the amount of enzyme required to decompose 1 µmole of H_2O_2/min/mg protein.

2.9. Measurement of glutathione peroxidase activity

The activity of glutathione peroxidase was measured using a coupled enzyme assay system linked with glutathione reductase (Genet et al., 2002). The assay mixture, in a final volume of 1 ml, contained: 100 mM potassium phosphate buffer pH 7.0, 25 mM EDTA, 0.5 mM reduced glutathione, 2 mM sodium azide, glutathione reductase (1.5 IU), 0.1 mM NADPH and 50 µl enzymatic extract. The reaction was started by the addition of hydrogen peroxide and the decrease in absorbance was monitored for 1 min at 25 °C following the oxidation of NADPH at 340 nm. One unit of enzyme is defined as 1 µmole of NADPH oxidized/min/mg protein.

2.10. Measurement of glutathione reductase activity

Glutathione reductase activity was measured according to Romero et al. (2000) by following NADPH oxidation spectrophotometrically at 340 nm. The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.0, 2.5 mM GSSG and 125 mM NADPH at 25 °C. One unit of enzyme is defined as 1 µmol of NADPH oxidized/min/mg protein.

2.11. Activities staining of superoxide dismutase, catalase and glutathione peroxidase on PAGE gels

Polyacrylamide gel electrophoresis (PAGE) was performed at 4 °C, 120 V (for superoxide dismutase and glutathione peroxidase) and 140 V (for catalase) as described by Laemmli (1970). For superoxide dismutase and glutathione peroxidase the electrophoretic separation was performed on native 10% polyacrylamide gels and for catalase the enzyme solution was subjected to native PAGE with 7% polyacrylamide gel. Staining for superoxide dismutase was achieved using the procedure described by Beauchamp and Fridovich (1971). Electrophoretic patterns of catalase and glutathione peroxidase were determined as described by Dewir et al. (2006) and Lin et al. (2002), respectively. Briefly, Superoxide dismutase, catalase, and glutathione peroxidase were detected on polyacrylamide gels after native polyacrylamide gel electrophoresis. In glutathione peroxidase procedure, the gel was submerged in a 50 mM Tris–HCl buffer (pH 7.9) containing 13 mM glutathione and 0.004% hydrogen peroxide with gentle shaking for 10–20 min. The glutathione peroxidase activity was stained with a solution containing 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 1.6 mM phenazine

methosulfate for 10 min. In superoxide dismutase procedure, the gel equilibrated with 50 mMK-phosphate buffer (pH 7.8) containing $2.8\times10-5$ M riboflavin, 0.028 M *N,N,N′,N′*-tetramethyl ethylenediamine for 30 min. The gel was washed in distilled water for 1 min and submerged in a same solution containing 2.45 mM NBT for 10–20 min with gentle agitation in the presence of light, the enzymes appeared as colorless bands in a purple background. In catalase procedure, the gel was incubated in 0.01% H2O2 for 10–15 min and washed with distilled water twice and incubated for 15–20 min in 1% FeCl3 and 1% K3 [Fe(CN6)]. After staining gel was washed carefully with tap water.

2.12. Evans Blue staining

Focal cerebral ischemia confirmation was also evaluated by using Evans Blue (Sigma Chemicals, USA) dye extravasations, as described by Bigdeli et al. (2007). Briefly, the rats received 4 ml/kg of 2% Evans Blue solution in saline by tail vein injection 30 min after middle cerebral artery occlusion. 24 h after reperfusion, the thoracic cavity was opened under anesthesia. The rats were perfused with 250 ml saline transcardially to wash out intravascular Evans Blue until colorless perfusion fluid was obtained from the atrium. After decapitation, the brains were removed and the hemispheres sectioned coronally.

2.13. Laser Doppler velocitometry

Laser Doppler Flowmeter (MBF3D, Moor instrument, Axminster, UK) was used for the recording of regional cerebral blood flow (rCBF). The probe of Laser-Doppler flowmeter was positioned to the surface. Using a stereotaxic device and a low speed dental drill, a burr hole of 2 mm in diameter was made over the skull at 1 mm posterior and 5 mm lateral to the bregma on the right side. A needle shaped laser probe was placed on the dura away from visible cerebral vessels. Steady state baseline values were recorded before middle cerebral artery occlusion so that all regional cerebral blood flow data were expressed as percentages of the respective basal value (Chen and Cheung, 2002). Doppler flux was continuously measured from 30 min before middle cerebral artery occlusion until 30 min after reperfusion.

2.14. Statistical analysis

Superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activation, arterial blood gases, and infarct volume were compared using one-way ANOVA test. The neurologic deficit scores were analyzed using the Mann–Whitney *U* test. Data were expressed as mean ± SD. $P<0.05$ was considered significant.

3. Results

3.1. Experimental conditions parameters

Arterial blood gas analysis confirmed clinical hyperoxia and normoxia in the pretreated groups (Table 1). Cerebral blood flow was reduced to less than 24% of base line in each group.

Table 1
ABG tests at the end of pretreatment ($P<0.001$ = a).

Experimental groups	pH	PCO_2 (mmHg)	PO_2 (mmHg)	Respiratory Rate (Hz)
Intermittent normobaric normoxia	7.4 ± 0.0	41.6 ± 1.5	92.3 ± 2.5	1.61 ± 0.08
Intermittent normobaric hyperoxia	7.3 ± 0.0	39.0 ± 2.6	360 ± 14.9 a	1.3 ± 0.18
Prolonged normobaric normoxia	7.4 ± 0.0	40.3 ± 1.5	93.1 ± 1.7	1.59 ± 0.16
Prolonged normobaric hyperoxia	7.3 ± 0.0	39.3 ± 2.6	355 ± 10.4 a	1.29 ± 0.14

Table 2
The distribution of neurologic deficit score in each experimental group.

Experimental groups (group code)	NDS in each groups(N)						Total (N)	Median	Statistical results
	0	1	2	3	4	5			
Intermittent normobaric normoxia (1)	0	4	4	1	0	0	9	2	2 vs. 1 = sig.
Intermittent normobaric hyperoxia (2)	4	3	2	0	0	0	9	1	2 vs. 4 = nonsig.
Prolonged normobaric normoxia (3)	0	4	5	0	0	0	9	2	4 vs.3 = sig.
Prolonged normobaric hyperoxia (4)	4	3	2	0	0	0	9	1	–
Total	8	14	13	1	0	0	36	–	––

The neurologic deficit scores were analyzed using the Mann–Whitney *U* test.
NDS: neurologic deficit score; N: the number of cases in each groups; sig: significant; nonsig: nonsignificant.

3.2. Effects of normobaric hyperoxia-induced neuroprotection on neurologic deficit scores and infarct volume

Median neurologic deficit scores were reduced by hyperoxic exposure, being 1 (range: 0–2), 1 (range: 0–2) and 2 (range: 1–3) in the intermittent normobaric hyperoxia, prolonged normobaric hyperoxia and room air groups respectively, but these differences did not reach statistical significance (Table 2). Those with no deficit all showed Evans Blue extravasation, confirming the fact that focal cerebral ischemia had been induced. There was beneficial effect in all brain regions, but the neuroprotection exerted by normobaric hyperoxia was mainly seen in the penumbra (cortex) (Fig. 2).

3.3. Effects of normobaric hyperoxia-induced neuroprotection on superoxide dismutase (SOD) activation

Enzyme activity analysis showed that superoxide dismutase is expressed in the rat brains. The activation of superoxide dismutase was increased in intermittent and prolonged normobaric hyperoxia in sham-operated animals, when compared to the sham-operated room air groups (Figs. 3 and 7). Also, the activation of superoxide dismutase was increased in intermittent and prolonged normobaric hyperoxia in intact animals, when compared to the intact room air groups (Figs. 3 and 7). The activation of superoxide dismutase was not different between intact and sham-operated normobaric hyperoxia rats (Figs. 3 and 7). Therefore, it seems that exposure to normobaric hyperoxia caused an increase in the activation of superoxide dismutase (Figs. 3 and 7).

Superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activation were not different between intact and sham-operated rats in intact prolonged room air, intact intermittent room air, sham-operated prolonged room air, and sham-operated intermittent room air subgroups (Figs. 3–9).Therefore,

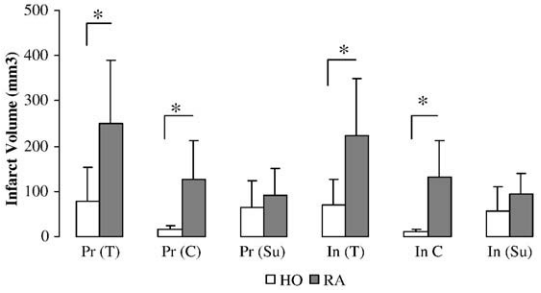

Fig. 2. The effect of normobaric hyperoxia (HO) and normobaric normoxia (RA) in prolonged (Pr) and intermittent (In) doses on infarct volume in total area (T), cortex area (C), and subcortex area (Su) (* = $P<0.05$).

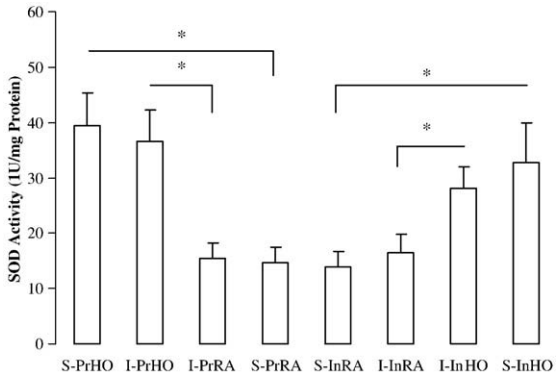

Fig. 3. Activities of superoxide dismutase (SOD) in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air; S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air; S-InRA: sham-operated intermittent room air (* = P<0.01).

experimental conditions and room air could not induce antioxidant enzyme activities in sham-operated and intact rats.

3.4. Effects of normobaric hyperoxia-induced neuroprotection on catalase (CAT) activation

Catalase is also expressed and overactivated in the rat brains from controls and experimental groups. The activation of catalase was increased in intermittent and prolonged normobaric hyperoxia in sham-operated animals, when compared to the sham-operated room air groups (Figs. 4 and 8). Also, the activation of catalase was increased in intermittent and prolonged normobaric hyperoxia in intact animals, when compared to the intact room air groups (Figs. 4 and 8). The activation of catalase in prolonged normobaric hyperoxia was more than that in intermittent normobaric hyperoxia in intact and sham-operated animals significantly (Figs. 4 and 8). Therefore, exposure to normobaric hyperoxia induced an increased in the activation of catalase (Figs. 4 and 8).

3.5. Effects of normobaric hyperoxia-induced neuroprotection on glutathione peroxidase (GPOX) activation

Glutathione peroxidase is also expressed and overactivated in the rat brains from controls and experimental groups. The activation of glutathione peroxidase was increased in intermittent and prolonged normobaric hyperoxia in sham-operated animals, when compared to the sham-operated room air groups (Figs. 5 and 9). Also, the activation of glutathione peroxidase was increased in intermittent and prolonged normobaric hyperoxia in intact animals, when compared to the intact room air groups (Figs. 5 and 9). The activation of glutathione peroxidase in prolonged normobaric hyperoxia was more than that in intermittent normobaric hyperoxia in intact and sham-operated animals significantly (Figs. 5 and 9). Therefore, exposure to normobaric hyperoxia induced an increased in the activation of glutathione peroxidase (Figs. 5 and 9).

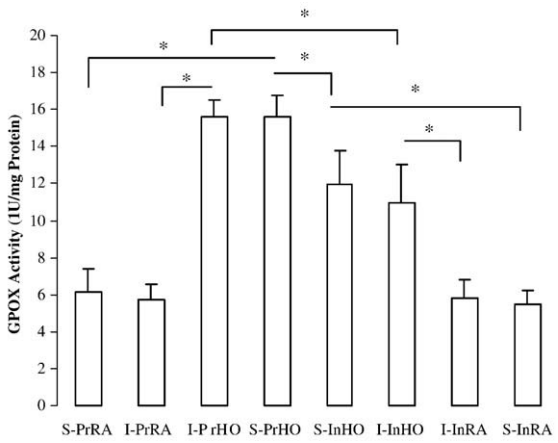

Fig. 5. Activities of glutathione peroxidase (GPOX) in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air; S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air; S-InRA: sham-operated intermittent room air (* = P<0.01).

3.6. Effects of normobaric hyperoxia-induced neuroprotection on glutathione reductase (GR) activation

Glutathione reductase is also expressed and overactivated in the rat brains from controls and experimental groups. The activation of glutathione reductase was increased in intermittent and prolonged normobaric hyperoxia in sham-operated animals, when compared to the sham-operated room air groups (Fig. 6). Also, the activation of glutathione reductase was increased in intermittent and prolonged normobaric hyperoxia in intact animals, when compared to the intact

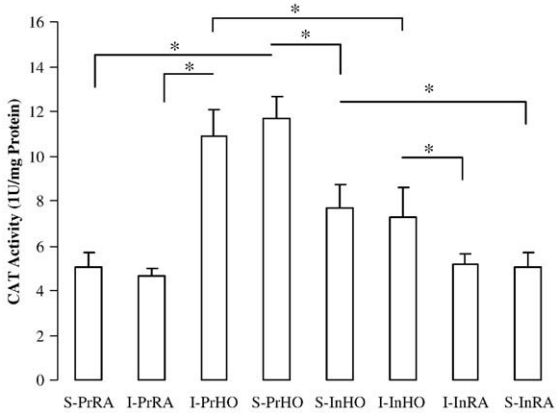

Fig. 4. Activities of catalase (CAT) in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air; S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air; S-InRA: sham-operated intermittent room air (* = P<0.01).

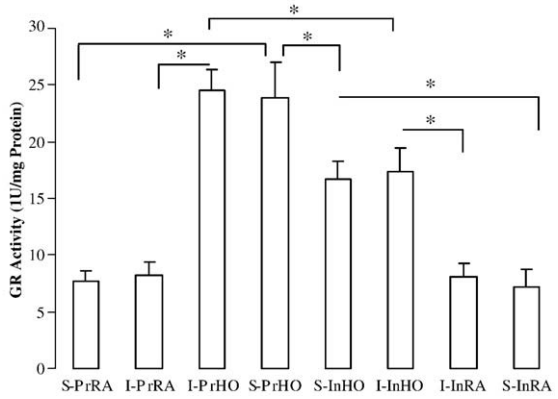

Fig. 6. Activities of glutathione reductase (GR) in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air; S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air; S-InRA: sham-operated intermittent room air (* = P<0.01).

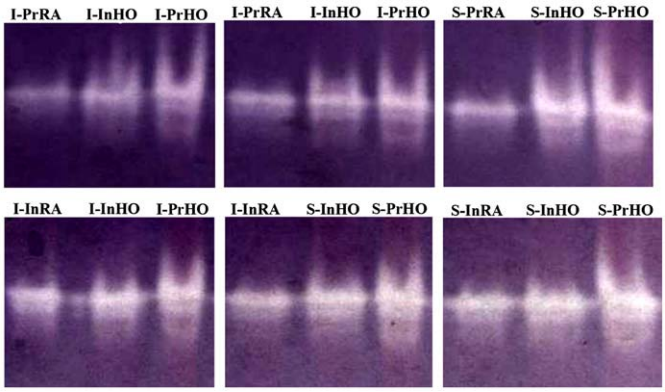

Fig. 7. Activities staining of superoxide dismutase (SOD) on PAGE gels in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air (loading control); S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air (loading control); S-InRA: sham-operated intermittent room air.

room air groups (Fig. 6). The activation of glutathione reductase in prolonged normobaric hyperoxia was more than that in intermittent normobaric hyperoxia in intact and sham-operated animals significantly (Fig. 6). Therefore, exposure to normobaric hyperoxia induced an increased in the activation of glutathione reductase (Fig. 6).

4. Discussion

The present study provides new insight into the cellular mechanism responsible for conferring increased brain ischemic tolerance to lethal ischemia injuries 48 h after in vivo prolonged and intermittent normobaric hyperoxia preconditioning. We have showed that pretreatment with normobaric hyperoxia in the rat produced significant protection against infarction 24 h later. This delayed protection was associated with a significant increase in brain antioxidant enzyme activities (Figs. 3–9), glutamate transporters expression (Bigdeli et al., 2008), serum TNF-α levels and TNF-α converting enzyme expression, nuclear factor-kappa B activation (Bigdeli and Khoshbaten, 2008). Taken together, these data strongly point to an important role for the induction of antioxidant enzymes in mediating the delayed infarct-limiting effects observed 48 h after in vivo normobaric hyperoxia preconditioning.

Although our data suggest that hyperoxia mediated neuroprotection is due to the reduction of post-ischemic infarct volume and brain edema, other mechanisms may be at work. Hyperoxia can produce angiogenesis and increase vessel density in the brain (Helms et al., 2005), and can inhibit intercellular adhesion molecule-1 (ICAM-1) expression and neutrophil accumulation (Ostrowski et al., 2005). Wada et al. (2000) described increase in expression of free radical oxygen as well as Bcl-2, an inhibitor of apoptosis, after repeated hyperbaric oxygen exposure in gerbils, which correlated with increased neural survival. On the other hand, the increase of free radical oxygen and superoxide dismutase were associated with decreased expression of hypoxia induce factor-1α, leading to improved blood brain barrier function via decreased vascular growth factor (Ostrowski et al., 2005). Therefore, normobaric hyperoxia can produce free radical oxygen and increase antioxidant enzyme activity in the brain. Free radical oxygen induced by normobaric hyperoxia explains the underlying possible mechanism by which normobaric hyperoxia induces antioxidant enzyme activities. Normally, antioxidant enzymes maintain high ratio of GSH/GSSH for antioxidant defense in the brain tissue. NF-κB is activated by various intracellular signals, including cytokines, TNF-α, neurotrophic factors, and neurotransmitters. Inhibition of NF-κB prior to hyperoxia abolishes protection (Leong and Karsan, 2000). NF-κB is a transcription factor for tumor necrosis factor-α which, in turn, induces activation of NF-κB in a positive feed back loop (Coward et al., 2002, Bigdeli and Khoshbaten, 2008).

4.1. Superoxide dismutase

Superoxide is key constituent in oxidative stress. It is derived from various sources at different stages of reperfusion. There are three major endogenous superoxide dismutases. Cu,Zn-superoxide dismutase is principally found in the cytosolic and lysosomal fractions, and mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001). Mn-superoxide dismutase is found in the mitochondrial matrix. Both Cu,Zn-superoxide dismutase and Mn-superoxide dismutase are abundant in the neural tissue and for this reason have received greatest scrutiny. Extracellular superoxide dismutase is also expressed in brain but in substantially lower concentration than Cu,Zn-superoxide dismutase and Mn-superoxide dismutase (Marklund 1984). Cu,Zn-superoxide dismutase overexpression reduces ischemic damage from ischemia/reperfusion (Yang et al., 1994). Cu,Zn-superoxide dismutase overexpression has been shown to inhibit post-ischemic mitogen activated protein kinase (Noshita et al., 2002), bad cell death signaling pathway (Saito et al., 2003) caspase activation (Sugawara et al., 2002), and fragmentation (Fujimura et al., 1999). These data indicate a potential proapoptotic role for superoxide in ischemia/reperfusion. This can be abated by superoxide dismutase overactivity and overexpression and potentially by pretreatment with superoxide dismutase overactivity mimic compounds such as normobaric hyperoxia and reactive oxygen species.

Therefore, ischemia/reperfusion presents numerous opportunities for formation of reactive oxygen/nitrogen species and resultant tissue injuries. Our results were shown that overactivating of superoxide dismutase induced by intermittent and prolonged normobaric hyperoxia partly reduces ischemia/reperfusion injuries via dismutation of superoxide derived from various sources at different stages of reperfusion (Figs. 3 and 7).

4.2. Catalase and glutathione peroxidase

Superoxide dismutase dismutates superoxide to hydrogen peroxide and oxygen. Hydrogen peroxide has modest oxidative potential and can freely cross cell membranes. Hydrogen peroxide can be converted to hydroxyl radical (Warner et al., 2004, Bigdeli, 2009). Therefore, elimination of hydrogen peroxide is critical to the efficacy of superoxide dismutase in reducing oxidative stress. Catalase and glutathione peroxidase serve this purpose. These enzymes are present in the brain although glutathione peroxidase activity is sevenfold greater than that

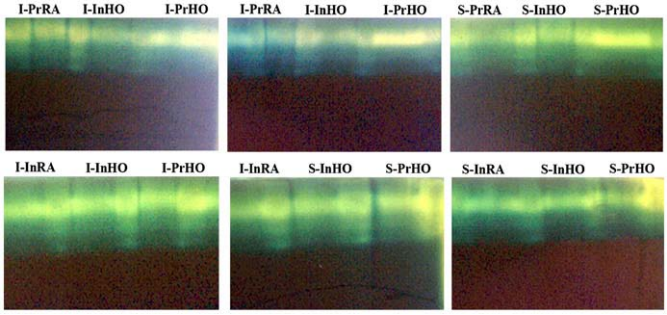

Fig. 8. Activities staining of catalase (CAT) on PAGE gels in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air (loading control); S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air (loading control); S-InRA: sham-operated intermittent room air.

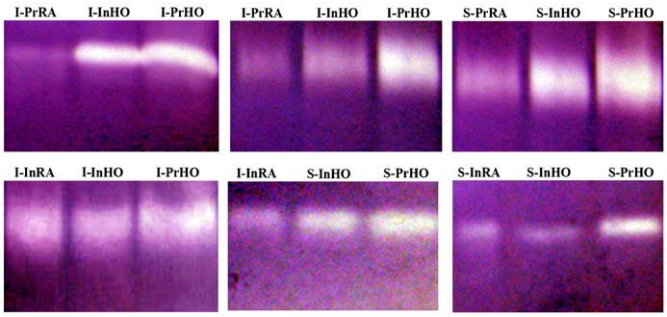

Fig. 9. Activities staining of glutathione peroxidase (GPOX) on PAGE gels in various experimental and control groups. S-PrHO: sham-operated prolonged normobaric hyperoxia; I-PrHO: intact prolonged normobaric hyperoxia; I-PrRA: intact prolonged room air (loading control); S-PrRA: sham-operated prolonged room air; S-InHO: sham-operated intermittent normobaric hyperoxia; I-InHO: intact intermittent normobaric hyperoxia; I-InRA: intact intermittent room air (loading control); S-InRA: sham-operated intermittent room air.

of catalase (Marklund et al., 1982). Further, while glutathione peroxidase is present in the cytosol, catalase is located mainly in peroxisomes. Both glutathione peroxidase-overexpressing and knock-out mice have been studied in the context of focal cerebral ischemia/reperfusion. Over-activating of glutathione peroxidase and catalase reduce necrotic and apoptotic cell death, astrocytic/microglial activation and inflammatory cell infiltration (Warner et al., 2004). The progeny of cross-breeding a glutathione peroxidase knock-out and a Cu,Zn-superoxide dismutase overexpressor caused a loss of protection that was otherwise afforded by overexpression of Cu,Zn-superoxide dismutase. However, the glutathione peroxidase knock-out alone was insufficient to worsen cerebral ischemia/reperfusion injuries (Warner et al., 2004), consistent with overlap in function with catalase. Our results were shown that overactivating of glutathione peroxidase and catalase induced by intermittent and prolonged normobaric hyperoxia partly reduce ischemia/reperfusion injuries (Figs. 4–9).

4.3. Glutathione reductase

Glutathione is a tripeptide (gamma-L-glutamyl-L-cysteinylglycine) that is the reductant for glutathione peroxidase. Oxidation of the cysteine sulfhydryl groups joins two glutathione (GSH) molecules with a disulfide bridge to form glutathione disulfide (GSSG). Glutathione reductase catalyzes recovery of glutathione (Warner et al., 2004). Normally, the brain maintains a high ratio of GSH/GSSG for antioxidant defense. Depletion of total glutathione and a decreased GSH/GSSG ratio are markers for oxidative stress in ischemia brain and as long as 72 h may be required to restore concentrations to normal values following an ischemia insult (Namba et al., 2001). Also, it has been shown that hyperbaric oxygen preconditioning decreased mortality rate, improved neurological recovery, lessened neuronal injury, reduced the level of MDA and increased the antioxidant activity of catalase and superoxide dismutase. These observations demonstrated that an upregulation of the antioxidant enzyme activity by hyperbaric oxygen preconditioning plays an important role in the generation of tolerance against brain ischemia–reperfusion injury (Li et al., 2008).

Prolonged durations of normobaric hyperoxia are associated with toxicity (Al-Motabagani 2005): exposure to 95% O_2 for 24 h has been suggested to result in severe pulmonary congestion with extravasations of red blood cell, edema, and alteration in the alveolar structure; recovery in room air for 2 weeks did not result in repair of distorted alveolar structure. Therefore, intermittent hyperoxia is less toxic to the lung that has prolonged hyperoxic exposure (Hendricks et al., 1977).

In conclusion, intermittent and prolonged normobaric hyperoxia results in increased reactive oxygen species production. In our study, it appears that normobaric hyperoxia induces ischemic tolerance via reduction of infarct volume and neurologic deficit scores. Reactive oxygen species and their accumulation presumably stimulate the activation of antioxidant enzymes including superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase. It may be proposed that antioxidant enzymes play an important role in the brain ischemic tolerance. In general, with consideration of evidence, the intermittent normobaric hyperoxia is more effective and safe than prolonged hyperoxia. Ultimately, it is hoped that novel cerebroprotective strategies may be developed for those at risk of stroke, or in whom cerebral perfusion is electively reduced perhaps at the time of surgery.

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