Eur J Appl Physiol (2010) 108:513–522
DOI 10.1007/s00421-009-1229-9

ORIGINAL ARTICLE

Hyperbaric oxygenation alleviates MCAO-induced brain injury and reduces hydroxyl radical formation and glutamate release

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Accepted: 22 September 2009 / Published online: 23 October 2009

Abstract The present study examined the effect of hyperbaric oxygen (HBO) on the formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), the products of salicylate trapping of hydroxyl free radicals, and glutamate release in the striatum during acute ischemia and reperfusion. Non-HBO rats ($n = 8$) were subjected to 1-h ischemia. Study rats ($n = 8$) were treated with HBO at 2.8 ATA for 1 h during ischemia. Artificial CSF solution containing 5 mM sodium salicylate was perfused at 1 μl/min. Samples were continuously collected at 15 min intervals and the levels of 2,3-DHBA, 2,5-DHBA, and glutamate were analyzed. The lesion volume was determined by TTC stain. Occlusion of the middle cerebral artery induced a significant increase in the levels of 2,3-DHBA and 2,5-DHBA. A peak of approximately two and fourfold of baseline levels was reached at 45 min and was maintained at elevated levels during reperfusion. The level of glutamate increased approximately two times at 30 min during ischemia, continued to increase, and reached approximately three times baseline level during reperfusion. HBO significantly alleviated brain injury associated with decreased levels of 2,3-DHBA, 2,5-DHBA and glutamate. This study suggests that the decreased glutamate release and the reduced formation of hydroxyl free radicals might contribute to the neuroprotective effect of HBO.

Keywords HBO · Ischemia reperfusion brain injury · Microdialysis · DHBA · Glutamate

Communicated by Dag Linnarsson.

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Introduction

Cerebral ischemia reperfusion injury refers to accelerated cellular damage that takes place in ischemic tissue once blood flow is reestablished. Evidence exists that the increased production of reactive oxygen species (ROS) plays an important role in ischemia reperfusion brain injury (Egashira et al. 1997; Olano et al. 1995). During the reperfusion phase, the hypoxanthine–xanthine oxidase system simultaneously generates superoxide. Superoxide then rapidly undergoes Fenton-type reactions in the presence of iron to generate hydroxyl radicals. Hydroxyl radicals are considered the most reactive and hazardous ROS (Halliwell 1992). The brain is particularly vulnerable to oxidative damage because it is rich in unsaturated fatty acids and iron, but relatively poor in antioxidant defenses with a low content of antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD) (Halliwell 1992). Excessive production of hydroxyl radicals can cause neuronal apoptotic nerve death, glutathione depletion, and release of excitatory aminoacids (Chen and Zeng 2000; Negishi et al. 2001; Skaper et al. 1999). It also has a detrimental effect on membrane integrity and function, which can further damage the brain (Aragno et al. 2000).

Excessive release of glutamate, a major excitatory neurotransmitter in the extracellular space, is known to be a contributing factor to the development of ischemic reperfusion brain injury (Choi 1988; Choi and Rothman 1990; Castillo et al. 1996). Attenuation of glutamate excitotoxicity by either blocking glutamate receptors or inhibiting glutamate release has been shown to alleviate ischemia reperfusion brain injury (Lees 2000). A linkage between glutamate excitotoxicity and enhanced ROS generation has been well recognized (Bondy and LeBel 1995; Kontos 2001; Yang et al. 1995). Hydroxyl radicals are difficult to measure directly because they are extremely reactive and have a short half-life. An indirect approach to demonstrate free radical production during cerebral ischemia has been developed (Choudray et al. 1995; Liu et al. 1997; McCabe et al. 1997). Salicylate, a hydroxyl radical trapping agent, reacts with hydroxyl radicals to form the stable hydroxylation products 2,3 dihydroxybenzoic acid (2,3-DHBA) and 2,5 dihydroxybenzoic acid (2,5-DHBA). These DHBAs can be used as an in vivo index of reactive hydroxyl radicals. They can be separated and quantified by the HPLC method with electrochemical detection.

The Undersea and Hyperbaric Medical Society has documented the beneficial effects of hyperbaric oxygen (HBO) therapy in 13 different diseases (Gesell 2008). The beneficial effect of HBO on ischemia–reperfusion brain injury has been evaluated in animal models (Badr et al. 2001; Yang et al. 2002) and in humans (Helms et al. 2005; Rockswold et al. 2001; Rusyniak et al. 2003; Zhang et al. 2003). The observed beneficial effects include improvement of brain metabolism (Golden et al. 2002), reduction of blood–brain barrier permeability and brain edema (Mink and Dutka 1995), decreasing intracranial pressure (Brown et al. 1988), attenuation of inflammatory response (Yin et al. 2002), and prevention of apoptotic cell death (Calvert et al. 2003; Yin et al. 2003). The search for better understanding of the mechanisms underlying the neuroprotective effect of HBO continues. In vivo microdialysis technique enables us to examine ongoing biochemical changes in response to HBO treatment. We previously observed that HBO ameliorated ischemia reperfusion brain injury associated with reduced dopamine release in the striatum (Yang et al. 2002). Excess dopamine release can react with oxygen to produce free radicals (Graham et al. 1978; Graham 1984), and react with hydroxyl radicals to form the dopaminergic neurotoxin 6-hydroxydopamine (Adams 1974; Slivka and Cohen 1985). The effect of HBO on hydroxyl radical formation in ischemic brain injury has not been investigated. In the present study, in vivo microdialysis technique was used to evaluate the effect of HBO on extracellular concentration of striatal 2,3-DHBA, 2,5-DHBA (the product of salicylate trapping of hydroxyl radicals) and glutamate in acute ischemia reperfusion brain injury in rats.

Materials and methods

Experimental animals

The proposed study was approved by the Institutional Committee for the Humane Use of Animals and was in accordance with the guidelines established by the National Institutes of Health. Pathogen-free male Sprague–Dawley rats weighing 300–350 g (Taconic Farm, Germantown, NY) were used. All rats were kept in wire mesh holding cages for a week to acclimate them to the study environmental conditions with a 12-h light/dark cycle. Standard rat chow (Diet #5008, Ralston Purina, St. Louis, MO) and tap water were provided ad libitum.

Intracerebral cannula guider placement

A schema of the experimental design is shown in Fig. 1. All rats were anesthetized by intramuscular injection of a rodent anesthetic mixture containing ketamine and xylazine (150:30 mg/ml) at a dose of 1.0 ml/kg body weight. The surgical region was shaved and the skin prepared with 10% solution of povidone–iodine (Betadine). An intracerebral cannula guide was implanted into the right striatum with a stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, CA). The stereotaxic coordinates from the bregma (+0.48 mm) were medial lateral, 3 mm from the middle line and dorsal–ventral, 4 mm ventral from the surface of the dura (Paxinos and Watson 1988). The cannula guider was fixed to the skull with acrylic dental cement. After the operation, the rats were allowed to recover for 7 days.

Brain ischemia–reperfusion injury model

On the experimental day, the rats were anesthetized again with ketamine and xylazine. Anesthesia was maintained by adding additional doses of the mixture as needed. The rats were placed in a supine position and a 1.5 cm longitudinal incision was made in the middle cervical skin. The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed and isolated. The distal portion of the ECA was ligated with 4-0 silk sutures. A 4-0 silk suture was tied loosely at the origin of the ECA. The CCA and ICA were temporarily clamped with Schwarz-microvascular clips. The MCA was occluded by inserting a 3-0 monofilament nylon suture (with tip rounded) via a small puncture, according to the method of Longa et al. (1989).

The skin was closed with 3-0 silk running sutures. After 1 h ischemia, reperfusion was achieved by pulling the 3-0 monofilament nylon suture to the origin of the ECA. The sham-operated rats had the same procedure except for the CCA and ICA clamps, and MCA occlusion (MCAO).

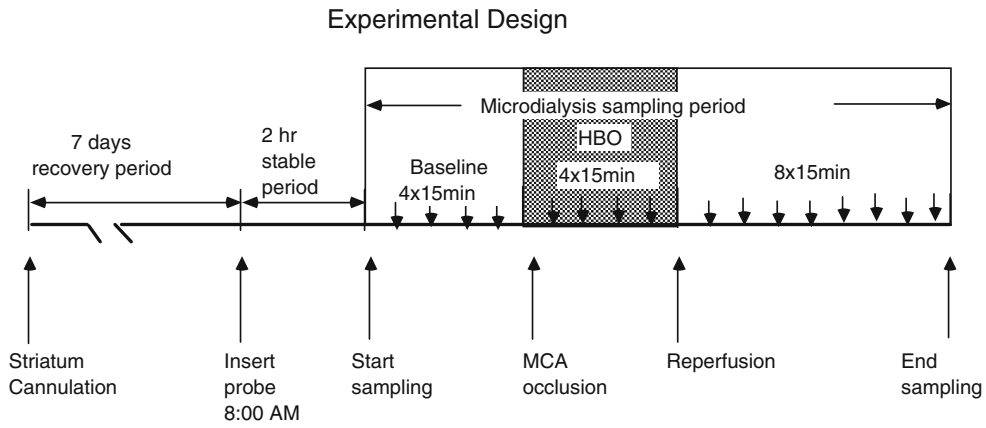

Fig. 1 Schema of the experimental design. For details, see "Materials and methods". *HBO* Administration of hyperbaric oxygenation, *MCA* middle cerebral artery

The left femoral artery was cannulated with polyethylene tubing (ID 0.58 mm, Becton-Dickinson Co., Parsippany, NJ) for monitoring systemic blood pressure. Due to the technical difficulty when rats were in the chamber under going HBO, blood pressure was measured only before and after HBO treatment.

Microdialysis procedure

Twenty-four rats were randomized into three groups: study rats (HBO, $n = 8$) underwent the MCAO procedure and were treated with HBO; control rats (Non-HBO, $n = 8$) underwent MCAO procedure without HBO; and sham-operated rats (Sham, $n = 6$) did not undergo MCAO and were treated with HBO. A microdialysis probe with a 2-mm membrane length and a 20,000-Da cut-off (CMA/12 CMA/Microdialysis, North Chelmsford, MA) was inserted through the cannula guide into the striatum. Artificial cerebrospinal fluid (CSF, CMA/Microdialysis, North Chelmsford, MA) was perfused at 1 μl/min by means of a CMA/100 microinjection pump (CMA Microdialysis, North Chelmsford, MA). Body temperature was monitored with a rectal probe and controlled at 37.5 ± 0.5°C with a heating pad and light. The microdialysis samples were continuously collected into CMA microvials (containing 10 μl of 0.4 mM perchloric acid for stabilization) on ice at 15 min intervals and measured immediately. At the end of 2 h equilibration period, salicylate (5 mM) was incorporated into the perfusion fluid. Four samples were collected as baseline values. HBO was administered as soon as MCAO was achieved as described previously (Yang et al. 2002). The Non-HBO group was kept in the chamber with the door open breathing room air.

Hyperbaric oxygenation administration

Immediately after MCA occlusion, the rats were placed in a cylindrical pressure chamber (Sechrist Model 1300B, Sechrist Industries, Inc. Anaheim, CA) and exposed to 100% oxygen at a pressure of 2.8 ATA for 1 h. The size of the HBO chamber was 0.037 m^3 (internal radius 33 cm and internal length 43.2 cm). When the designed pressure reached, the flow of oxygen was reduced to maintain constant pressure while allowing the flow out of the chamber. A tray of calcium carbonate crystals was used to reduce the accumulation of CO_2 in the chamber environment. Oxygen content was maintained at ≥98% and CO_2 at ≤0.03%.

Continuous microdialysis during HBO was achieved as described previously (Yang et al. 2002). Briefly, a Sechrist industries IV Pass Thru was cut to leave a wall connector with 5-cm-long tubing at each end. Two dialysis tubings were passed through the connector and sealed together with 734 Silicone (Dow Corning). These two tubings were connected at the inlet and outlet of the probe. The microdialysis flow was perfused via these two tubings. In this way, dialysate could be collected continuously outside of the chamber. This system was also tested to perform adequately under pressure of 2.8 ATA. Under this condition, the flow rate and the recovery rate of 2,3-DHBA, 2,5-DHBA and glutamate were not affected (as compared to the rate under normal environmental pressure). The sham-operated rats were treated as HBO rats.

Measurement of hydroxyl radical formation and glutamate release

Samples were immediately assayed after their collection, 2,3-DHBA and 2,5-DHBA were measured using reverse-phase liquid chromatograph with ESA Model 5014 high sensitivity analytical cell and ESA DHBA-250 column (15 × 3 mm ID). Mobile phase comprised of 50 mM sodium acetate, 50 mM citric acid, 25% methanol, 5% isopranol, pH was adjusted to 2.5 with phosphoric acid. Calibration curves were run daily with 2,3-DHBA and

2,5-DHBA standard. The concentrations of 2,3-DHBA and 2,5-DHBA were determined by comparing them with the peak areas of external standards run with each experiment. The CMA 600 analyzer (CMA/Microdialysis, North Chelmsford, MA) was used to analyze glutamate from the collected dialysate samples.

Determination of lesion volume

At the end of the experiment, anesthesia was again induced with a lethal dose of ketamine and xylazine and the rats were killed by intracardiac perfusion with 200 ml of normal saline. The brains were then carefully removed and cooled in ice-cold normal saline for 5 min, then dissected into coronal 2-mm sections using a Jacobowitz brain slicer (Zivic-Miller Laboratories, Inc., Allison Park, PA). The brain slices were incubated in isotonic phosphate-buffered saline (pH 7.4) containing 2% triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min and then stored in 10% neutral buffered formalin. The volume of the lesion area and hemispheric area of each section were traced using computer-assisted color image selection by Adobe Photoshop 6.0. The area was measured and analyzed using NIH image analysis software. The distribution of lesion area in the different brain slices was recorded. The lesion volume from each coronal section was mathematically reconstructed to give the total lesion volume as described by Lin et al. (1993).

Statistical analysis

The baseline of 2,3-DHBA, 2,5-DHBA and glutamate were determined by using a mean value of four consecutive samples prior to MCA occlusion. The changes in 2,3-DHBA, 2,5-DHBA and glutamate are expressed as percent variations from the mean baseline. All data are expressed as mean ± SE. ANOVA was used to analyze the time-course data within the groups, while Student's paired *t* test was used (Statview 5,0, Abacus Concepts Inc, CA), to compare the two groups. Differences with $P < 0.05$ were considered statistically significant.

Results

Effects of HBO on systemic blood pressure

The baseline mean arterial pressure (MAP) was similar among groups, being 87.3 ± 1.5, 88.3 ± 1.6 and 89.1 ± 1.4 mmHg in sham, control and study groups, respectively. There were no significant changes in MAP before and after HBO treatment among the groups.

Effects of HBO on lesion volume

All animals showed infarction in the right parietal cortex and caudate-putamen (MCA territory) on TTC stained slides at the end of the experiments. The lesion volume in the HBO group was 16.5 ± 5.1%, which was significantly lower than the 27.0 ± 7.2% in the non-HBO group ($P < 0.05$).

Effect of HBO on glutamate release in the striatum

According to our in vitro calibration test, a relative recovery rate was approximately 15% for glutamate at a rate of 1 μl/min. The baseline of glutamate level in the striatum varied from 1 to 18 μM.

Baseline levels of glutamate in the striatum of the two groups were not different. As shown in Fig. 2, MCA occlusion induced an immediate increase in glutamate release, which reached approximately 200% of baseline during 30 min of ischemia, continued to increase, and reached approximately threefold baseline level during the entire reperfusion period. HBO treatment significantly attenuated the increase in glutamate release during ischemia and reperfusion periods.

Effect of HBO on striatal 2,3-DHBA and 2,5-DHBA accumulations

Figure 3 shows the time-course of hydroxyl radicals formation, as monitored by the continuous administration of salicylate and collection of its hydroxylation products 2,3-DHBA and 2,5-DHBA via microdialysis probe. Baseline levels of 2,3-DHBA and 2,5-DHBA in the striatum of the two groups were not different. Baseline of 2,3-DHBA and 2,5-DHBA was approximately 15–20 pg/10 μl dialysate.

As shown in Fig. 3a, b, MCA occlusion induced an immediate increase of 2,3-DHBA and 2,5-DHBA levels, which reached approximately 300% of baseline during 15 and 45 min of ischemia, respectively, and remained elevated during the entire reperfusion period. HBO treatment almost completely inhibited the increase of 2,3-DHBA and 2,5-DHBA levels during ischemia. In the HBO-treated group, 2,3-DHBA gradually increased and reached approximately 200% of baseline at 60 min of reperfusion and remained at this level during the entire reperfusion period. However, this level was significantly lower than the non-HBO-treated group.

Discussion

The usefulness of HBO in the treatment of acute ischemic brain injury continues to be evaluated. Concomitant

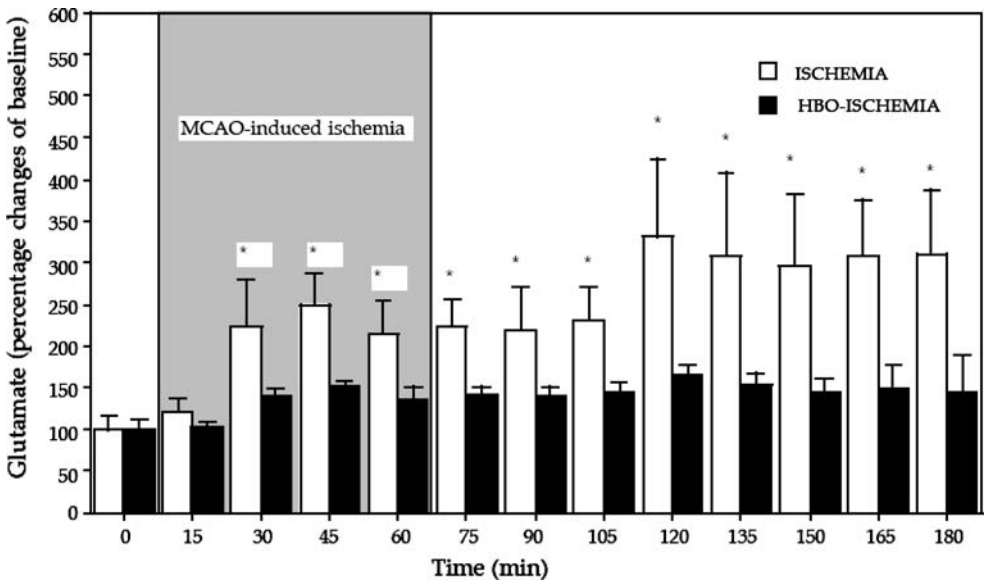

Fig. 2 The changes in striatal glutamate levels before (baseline), during (ischemia) and after (reperfusion) middle cerebral artery occlusion with or without HBO treatment. Occlusion of the middle cerebral artery induced an increase in glutamate level, which reached approximately 250% of baseline level during 30 min of ischemia and remained elevated during the entire ischemia reperfusion period. The increase of glutamate levels was significantly attenuated in HBO-treated rats during both the ischemia and reperfusion periods. *Open column* middle cerebral artery occlusion without HBO treatment, *dark column* middle cerebral artery occlusion with HBO treatment. $^{*}P < 0.05$ between groups. Results are expressed as mean ± SE

changes of focal free oxygen radical and glutamate in the striatum in response to HBO treatment during acute ischemia have not been described. In the present study, the effect of HBO on extracellular changes in hydroxyl radical formation reflected by 2,3-DHBA, 2,5-DHBA and glutamate release was continuously monitored in a rat model of MCAO-induced ischemia reperfusion brain injury. The main findings from this study are (1) MCAO-induced ischemia reperfusion brain injury was associated with significantly increased hydroxyl radicals formation (reflected by increased 2,3-DHBA and 2,5-DHBA levels) and glutamate release; (2) HBO per se had no significant effect on hydroxyl radicals formation (reflected by 2,3-DHBA and 2,5-DHBA levels; (3) HBO, when administered during ischemia, alleviated MCAO-induced ischemia reperfusion brain injury and attenuated hydroxyl radical formation and glutamate release.

In the present study, the lesion area was delineated by TTC stain. It has been questioned that TTC stain may overestimate the infarct area due to tissue edema (Lin et al. 1993). Severe brain edema evolves from 6 h to 7 days after the ischemic insult in MCAO model. In the present study, the brain was stained with TTC within 6 h after the ischemia insult. The term "lesion volume" was used in this study to include infarct area and possible edema area. The data from the present study are in agreement with our previous study (Yang et al. 2002), demonstrating the beneficial effect of HBO treatment in acute brain ischemia–reperfusion injury in young male adult rat.

It has been a concern that exposure to HBO at higher than atmospheric pressure may cause increased formation of oxygen radicals, which is in direct proportion to the increased oxygen tension. To minimize this side-effect, the therapeutic atmospheric pressures of HBO should not exceed 3 ATA (303 kPa) as recommended by the UMHS (Gesell 2008). One study examined the effect of HBO on formation of oxygen free radicals in the rat brain with in vivo microdialysis. The rats were exposed to 100% O_2 at a pressure of 3 ATA for 2 h. The formation of 2,3-DHBA in the hippocampal and striatal dialysates were not significantly different compared to that in the non-HBO group (Elayan et al. 2000). In another study, HBO was administered at 6 ATA for 40 min. There was no significant difference in formation of hydroxyl radicals in the striatum between HBO and non-HBO treatment (Amiridze et al. 1999). The present study is in agreement with the finding that HBO at 2.8 ATA for 60 min had no significant effect on hydroxyl radical formation (reflected by 2,3-DHBA and 2,5-DHBA levels) and glutamate release in the striatum On the other hand, Demchenko et al. reported that HBO at 5 ATA for 30 min increased hydroxyl radical production reflected by 2,3-DHBA by 56 ± 8% in the striatum (Demchenko et al. 2000). This discrepancy cannot be explained by the fact that different pressure of HBO was used in these studies because HBO is known to increase the production of ROS (Piantadosi and Zhang 1996), especially hydrogen peroxide (H_2O_2) production in the brain (Yusa et al. 1987). H_2O_2 can be reduced to the highly reactive hydroxyl

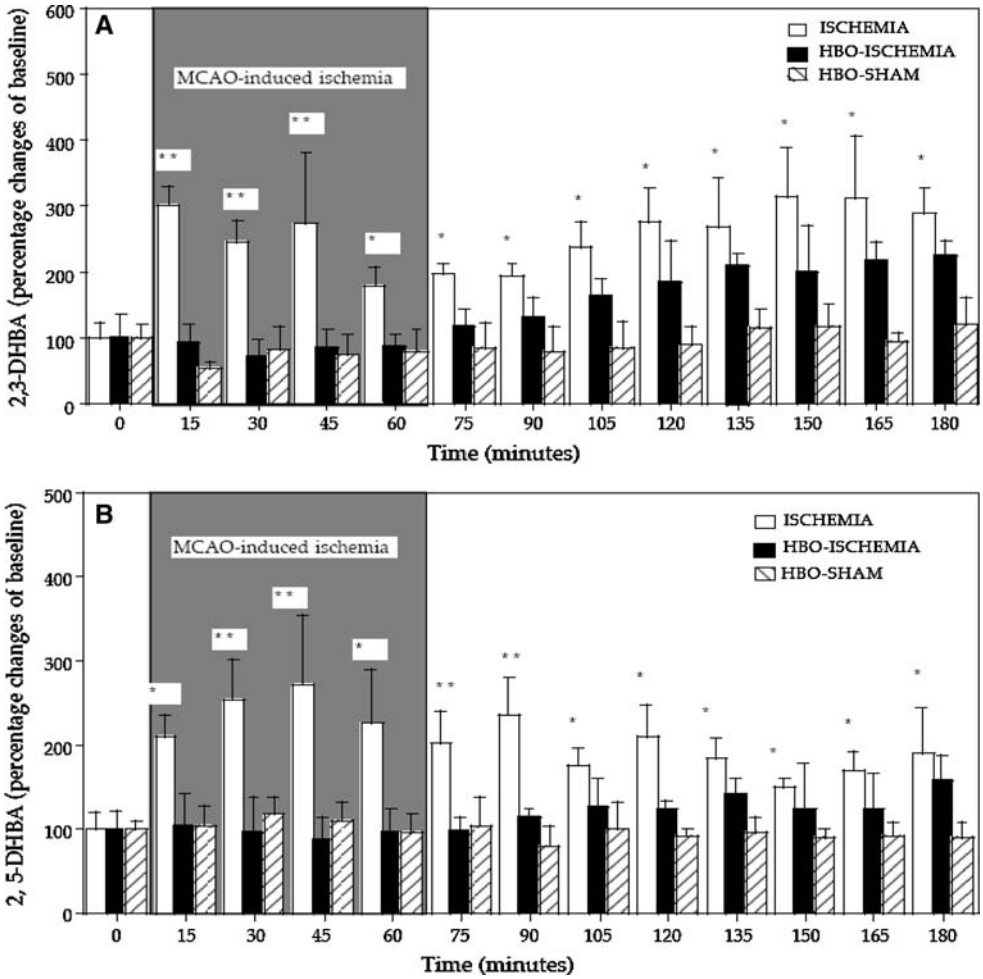

Fig. 3 The changes in striatal 2,3-DHBA (**a**) and 2,5-DHBA (**b**) levels before (baseline), during (ischemia) and after (reperfusion) middle cerebral artery occlusion with or without HBO treatment. Occlusion of middle cerebral artery induced an immediate increase in 2,3-DHBA and 2,5-DHBA levels, which reached approximately 300 and 200% of baseline during 15 min of ischemia and remained elevated during the entire ischemia reperfusion period. The increase of 2,3-DHBA and 2,5-DHBA levels was significantly attenuated in HBO-treated rats during both the ischemia and reperfusion periods. There were no significant changes in 2,3-DHBA or 2,5-DHBA levels in the sham group treated with HBO. *Open column* middle cerebral artery occlusion without HBO treatment, *dark column* middle cerebral artery occlusion with HBO treatment, *shaded column* without middle cerebral artery occlusion and with HBO treatment. $*P < 0.05$ and $**P < 0.01$ between groups (paired *t* test). Results are expressed as mean ± SE

radical in the presence of reduced transitional metals such as iron (Halliwell and Gutteridge 1990). It is possible that, in the present study, the increased production of H_2O_2 may not be reduced to hydroxyl radical because lack of enough released free metals, therefore affecting the detection of DHBAs. Another possibility is that one potential disadvantage of the salicylate trapping method is the low efficiency by which salicylate traps hydroxyl radicals. It is possible that we were unable to detect an increase in hydroxyl radical levels due to this disadvantage (Elayan et al. 2000).

The highly toxic hydroxyl radical has been implicated in ischemic neuronal injury through oxidization of cellular lipids, proteins, and nucleic acids (Halliwell 1992). Direct detection and quantization of the hydroxyl radical is extremely difficult because of its very short half-life. The salicylate-trapping method, combined with microdialysis, provides a practical technique to detect hydroxyl radicals in vivo (Obata 1997). Excessive formation of hydroxyl radical in global (Horiguchi et al. 2003; Yang et al. 1996) and focal ischemia (Wei and Quast 1998) reperfusion brain injury has been documented using this technique. In a study of focal ischemia reperfusion brain injury (Wei and Quast 1998), MCA was occluded for 120 min and reperfusion was resumed by the removal of the suture. Both 2,3-DHBA and 2,5-DHBA levels in the lateral striatum increased after onset of ischemia and continued to increase during reperfusion. Increased 2,3-DHBA and 2,5-DHBA peaks were noted throughout the 240-min observation period with the highest level of 2,3-DHBA approximately twofold of pre-ischemia at 75 min after MCAO and 30 min after reperfusion. In the present study, a similar profile of changes in 2,3-DHBA and 2,5-DHBA levels was observed. The occlusion of MCA induced significant increase in the levels of 2,5-DHBA and 2,3-DHBA and reached a peak at 45 min, being approximately twofold and fourfold of baseline levels, respectively, and maintained at elevated levels during reperfusion. This increased production of 2,3-DHBA and 2,5-DHBA exemplifies excessive formation of extracellular hydroxyl radicals in the striatum.

Horiguchi et al. evaluated the amount of hydroxyl radical produced in striatum by measurement of 2,3-DHBA and 2,5-DHBA in rats with forebrain ischemia reperfusion injury (Horiguchi et al. 2003). In animals whose post-ischemic

body temperature was maintained at 37°C, the levels of 2,3-DHBA and 2,5-DHBA significantly increased during ischemia and the reperfusion period. The peak levels of 2,3-DHBA and 2,5-DHBA were increased 2.9-fold and 2.7-fold above the corresponding baseline values, respectively. In animals whose temperature was lowered to 32°C within 10 min of reperfusion, the hydroxyl radical formation was almost completely inhibited during reperfusion. In the present study, HBO, when administered during ischemia, almost completely inhibited the formation of hydroxyl radicals generated by salicylate hydroxylation. In their and our studies, this inhibited hydroxyl radical formation was associated with significantly reduced ischemia reperfusion brain injury. Our present results suggest that the suppression of hydroxyl radical formation may be one of the key mechanisms underlying HBO-induced neuroprotection.

There are several possible explanations for the ability of HBO to influence the formation of hydroxyl radicals in the present study. Reduced glucose and oxygen supply result in decreased adenosine triphosphate (ATP) production and Na^+K^+ATPase activity during acute ischemia, which predispose the brain to the formation of ROS (Homi et al. 2002; Kim et al. 2002; Mrsic-Pelcic et al. 2004; Yufu et al. 1993). HBO increases the physically dissolved oxygen content of circulation, thus elevating the oxygen supply to damaged tissue, and resulting in an increase of oxygen in brain tissue (Sunami et al. 2000) and cerebrospinal fluid (Hollin et al. 1968). The beneficial effect of HBO in energy metabolism following hypoxic/ischemic events has also been demonstrated. In in vitro hypoxia-reoxygenation (Gunther et al. 2004) and ischemia (Gunther et al. 2002) studies, HBO has been reported to restore nucleotide status (ratio of ATP/ADP, guanosine triphosphate/guanosine diphosphate). In an acute global ischemia model, HBO significantly increases cerebral ATP level when administered at 2 ATA for 30 min (Shiokawa et al. 1986). HBO has also been shown to increase cerebral ATP levels and improve cognitive recovery after brain injury in the rat. In addition, HBO treatment improved cognitive recovery and reduced hippocampal neuronal cell loss after brain injury in rats (Zhou et al. 2007). A recent study suggests that oxygen treatment during the initial period of recovery from a hypoxia–ischemic insult is able to attenuate energy deficits in the brain, which ultimately leads to a reduction in brain injury (Calvert and Zhang 2007). Clinical observations also show that in severely brain-injured patients, HBO treatment decreased cerebrospinal fluid lactate levels (Rockswold et al. 2001) and lactate/glucose and lactate/pyruvate ratios (Tolias et al. 2004). Mitochondria are an important intracellular source of superoxide production under normal physiological and pathological conditions such as focal cerebral ischemia (Piantadosi and Zhang 1996). It is likely that HBO may alleviate MCAO-induced the mitochondria damage, therefore improves energy metabolism during ischemia and consequentially decreases ROS production from mitochondria.

Increased free radical formation, coupled with a reduced antioxidant defense, has been postulated to play a pivotal role in ischemic brain injury (McCord 1985). The neuroprotective mechanisms of HBO likely include enhanced antioxidant enzyme activity. The antioxidant enzyme activity of tissue affected by ischemia/reperfusion represents the primary endogenous defense against oxygen free radicals and involves the cooperative action of the three main intracellular antioxidant enzymes such as SOD, CAT and glutathione peroxidase (GPx) (Homi et al. 2002). Mrsic-Pelcic et al.'s study showed that global cerebral ischemia decreased the activities of SOD and Na^+K^+ATPase in hippocampal tissue. HBO was shown to preserve Na^+K^+ATPase activity and significantly enhance SOD activity (Mrsic-Pelcic et al. 2004). HBO may exert its neuroprotective effect by enhancing antioxidant defense to reduce oxygen free radicals formation.

It is well known that excessively released glutamate plays an important role in the development of tissue injury following cerebral ischemia (Choi 1988; Choi and Rothman 1990). A strong correlation has been suggested between glutamate excitotoxicity and damage due to free radicals in ischemia brain injury (Bondy and LeBel 1995; Kontos 2001). Yang and his colleagues investigated the in vivo interrelation between excitotoxicity and oxidative stress following cerebral ischemia in the cortex of anesthetized rats (Yang et al. 1995). In their study, formation of 2,3-DHBA and 2,5-DHBA, as well as glutamate content in the microdialysis perfusion solutions, were significantly increased following ligation of the bilateral common carotid arteries and the unilateral middle cerebral artery. Exogenous perfusion of authentic glutamate solutions through implanted microdialysis probes also resulted in increased hydroxyl radical formation. Their results suggest that ischemia-induced oxidative stress may also result from increased glutamate excitotoxicity. The effect of HBO on glutamate release during MCAO-induced ischemia reperfusion brain injury has been investigated by Badr et al. (2001). In their study, HBO was administered at 3 ATA for 2 h during reperfusion. The level of glucose, pyruvate, and glutamate in the striatum of awake and freely moving rats decreased to almost the level of the non-HBO group at 7, 10, and 24 h after reperfusion. In the present study, HBO was administered at 2.8 ATA for 60 min during ischemia and significantly reduced MCAO-induced glutamate release in the striatum. Energy failure and decreased Na^+K^+ATPase activity are partially responsible for excessive accumulation of extracellular glutamate during ischemia. HBO improves energy metabolism and enhances Na^+K^+ATPase activity, therefore reducing glutamate

accumulation during ischemia. Reduced glutamate accumulation may also contribute to the reduction of oxygen free radical formation. Another possible mechanism to explain the mitigating effect of HBO on hydroxyl radical generation is the suppression of dopamine accumulation. It has been hypothesized that excess dopamine release generates free radicals formation (Olano et al. 1995). We previously reported that HBO-alleviated MCAO-induced ischemia reperfusion brain injury was associated with significantly reduced dopamine accumulation in the striatum (Yang et al. 2002). The reduced dopamine release may also contribute to reduced hydroxyl radical formation following HBO treatment.

One limitation is that blood flow and oxygen supply to the brain were not measured in this study due to the technical difficulties of taking measurements in the HBO chamber. However, it has been reported that administration of HBO improved local microcirculation and increased the oxygen content in both the arterial and the venous blood in the ischemic cerebral area (Lin et al. 1998; Veltkamp et al. 2000; Li et al. 2008), which may contribute to the beneficial effect of HBO. The correlation between the production of 2,3-DHBA and 2,5-DHBA and the tissue oxygenation surrounding the microdialysis probe area during ischemia reperfusion process warrants further investigations.

A second limitation is that the mechanisms by which HBO influenced hydroxyl radical production in this study are not investigated. 2,3-DHBA is formed exclusively by hydroxyl radical radicals (non-enzymatic), whereas both hydroxyl radicals and cytochrome P450 (enzymatic) contribute to the formation of 2,5-DHBA. The biological activity of ROS is opposed by an array of antioxidant enzymes (such as SOD, CAT and GPx) and some important non-enzymatic antioxidants. The reduced production of 2,3-DHBA and 2,5-DHBA by HBO could be caused by increased antioxidant defenses, reduced basal production of oxidants, and reduction of radical leak during oxidative phosphorylation (Leeuwenburgh and Heinecke 2001). The effect of HBO on antioxidant defenses and oxidative phosphorylation should be evaluated in the further study.

A third limitation is that only the short-term beneficial effect of HBO therapy was investigated in the present study. Recent studies have showed that HBO when administered during reperfusion reduced infarct area and improved neurologic scores at 7 days after MCAO (Yin et al. 2003; Eschenfelder et al. 2008). As highlighted by DeBow et al. (2003), long-term follow-up is essential for any study of neuroprotection. Future studies should focus on the long-term neuroprotective effect of HBO in the treatment of ischemia reperfusion brain injury.

In conclusion, the present study shows that HBO, when administered during ischemia, offers significant neuroprotection against transient focal cerebral ischemia in the rat model. This mechanism appears to be correlated in part to reduced formation of hydroxyl radicals and extracellular accumulation of glutamate.

Acknowledgments This study was supported by the Department of Anesthesiology, Upstate Medical University, Syracuse, NY, USA. The authors wish to thank Dr. Danielle Masursky for her editorial assistance.

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