Published in final edited form as:

Neurobiol Dis. 2008 January ; 29(1): 1–13.

The hyperbaric oxygen preconditioning-induced brain protection is mediated by a reduction of early apoptosis after transient global cerebral ischemia

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Abstract

We hypothesized that the brain-protective effect of hyperbaric oxygen (HBO) preconditioning in a transient global cerebral ischemia rat model is mediated by the inhibition of early apoptosis.

One hundred ten male Sprague Dawley (SD) rats (300–350 g body weight) were allocated to the sham group and three other groups with 10 minutes of four-vessel occlusion, untreated or preconditioned with either 3 or 5 hyperbaric oxygenations. HBO preconditioning improved neurobehavioral scores and reduced mortality, decreased ischemic cell change, reduced the number of early apoptotic cells and hampered a conversion of early to late apoptotic alterations. HBO preconditioning reduced the immunoreactivity of phosphorylated p38 in vulnerable neurons and increased the expression of brain derived neurotrophic factor (BDNF) in early stage post-ischemia. However, preconditioning with 3 HBO treatments proved less beneficial than with 5 HBO treatments.

We conclude that HBO preconditioning may be neuroprotective by reducing early apoptosis and inhibition of the conversion of early to late apoptosis, possibly through an increase in brain BDNF level and the suppression of p38 activation.

Keywords

Hyperbaric oxygen preconditioning; Annexin V; Apoptosis; Global cerebral ischemia; BDNF; p38

Introduction

Hyperbaric oxygen preconditioning has been shown to have neuroprotective effects against focal and global cerebral ischemia (Xiong et al., 2000; Wada et al., 2001). It has been proposed as preconditioning treatment to prevent brain injury during major surgery (Wada et al.,

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2001). However the mechanism is not fully understood and more evidence is needed for HBO treatment to be accepted clinically (Prass et al., 2000).

The majority of studies examined HBO preconditioning effects on a delayed brain injury (Wada et al., 1996) that involves cell death in CA1 and layers 2 and 5 in the cerebral cortex occurring at least 12 hr after global ischemia (Lipton, 1999). However, the substantial benefit of HBO may occur in the early phase after ischemia, which, in turn, may be critical to the outcome of the protection against delayed brain injury. Additionally, the impact of HBO preconditioning on cortical damage has been investigated with less scrutiny than the effect on hippocampal cell death, despite established sensorimotor neurological deficits that occur acutely after global cerebral ischemia (Block 1999).

HBO preconditioning should have a powerful anti-apoptotic effect, as apoptosis is a dominant form of hippocampal cell death after global cerebral ischemia (Nitatori et al., 1995). Apoptosis has not yet been shown to occur in the early phase after global ischemic insult despite studies showing acute caspase activation, highly indicative of apoptotic pathway (Krajewska et al., 2004). Thus, hyperbaric oxygenation pre-conditioning may have an effect on early apoptosis but has yet to be examined for such an effect.

Neurotrophins are candidate genes underlying effects of HBO preconditioning. In the brain HBO can induce brain derived neurotrophic factor (BDNF) (Chavko et al., 2002) whereas HBO preconditioning has been shown to induce neurotrophin receptor p75 NTR (Hirata et al., 2007). Downstream effects of BDNF may involve a suppression of p38/MAPK activity by inhibiting p38/MAPK phosphorylation (Yamagishi et al., 2003). p38/MAPK qualifies as fast-response signal, as it is activated in vulnerable neurons within minutes after global cerebral ischemia (Sugino et al., 2000b). Apoptosis through p38/MAPK is induced along pathways involving the transcriptional factor AP-1, p53 phosphorylation and subsequent caspase activation (Chen et al., 2003). Inhibition of p38/MAPK has been proven beneficial for cell survival in conditions of focal and global cerebral ischemia (Sugino et al., 2000b; Barone et al., 2001). Therefore, it is conceivable that an increase in BDNF protein levels due to HBO may be part of an anti-apoptosis mechanism targeting a proapoptotic p-38-dependent pathway in neurons.

Past studies of HBO preconditioning used the 5 courses of HBO treatments (Wada et al., 1996). This may however be problematic for the practical reasons. Recently, three HBO treatments applied within 24 hr before anticipated brain insult established a clinically effective preconditioning regimen (Alex et al., 2005).

We hypothesized that HBO preconditioning reduces early apoptosis and apoptosis progression possibly through induction of BDNF, suppression of p38/MAPK phosphorylation and reduced caspase-3 activation in the rat model of transient global cerebral ischemia. We also evaluated the neuroprotective effects of 5 times HBO versus 3 times HBO, which seems more feasible in the clinical setting.

Material and Methods

Animal groups and a model of global cerebral ischemia

One hundred ten male SD rats were divided into four groups: a sham operation (n=23); global ischemia induced by four vessel occlusion (4VO, n=35), sacrificed at 2 hr 45 min and 6, 24, 72 hr and 7 days; and two global ischemic groups preconditioned with either 3 or 5 HBO treatments sacrificed as above (3HBO+4VO; n=29 or 5HBO+4VO; n=23). All surgical and euthanasia procedures were performed under deep anesthesia with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) i.p. injection. The animals were intubated and mechanically ventilated

during the surgical procedures. Atropine at a dose 0.05 mg/kg was given to reduce secretion in the respiratory tract. The four-vessel occlusion rat model (Pulsinelli, Brierley 1979) with modifications to the one stage anterior approach, recently established in our lab, was used (Yamaguchi et al., 2005). Briefly, the skin was incised on the neck and subcutaneous connective tissue and muscles were gently retracted. The trachea and esophagus were gently retracted to the right side. Cervical vertebral bodies were exposed and the bilateral vertebral arteries were occluded using electrosurgical coagulator between the second and third transverse processes. Next, both common carotid arteries were occluded with microvascular clips for a period of 10 minutes. The rectal temperature was maintained at 36.9–37.4°C by means of a heating lamp during surgery and continued to 2 hours after surgery. Femoral arteries were cannulated in subsets of rats for BP measurements and blood gas analyses. Blood Glucose levels were measured before and after ischemia or sham surgery. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University.

HBO preconditioning paradigms

Each course of hyperbaric therapy lasted 1 hr and involved pure oxygen at 2.5 atmospheres absolute (2.5 ATA). Rats were pressurized in a research hyperbaric chamber (1300B, Sechrist) with an oxygen flow of 22 L/min. Compression and decompression was maintained at a rate of 5 psi/min. Two regimens of preconditioning were used: 5 treatments with one treatment per day; the last dive 24 hr before ischemia (5HBO) or 3 treatments within 24 hr: at 24 hr, 12 hr and 4 hr before ischemic insult (3HBO).

Formalin perfusion and Nissl stain

For all histological studies, the rats were perfused intracardially with 60 mL of cold PBS, followed by 300 mL of cold buffered 10% formalin. Brains were postfixed for 72 hr in the formalin at 4°C, then cryoprotected in 30% sucrose/PBS until they sank. Ten micrometers thick frozen sections were cut in the cryostat as described previously (Ostrowski et al., 2005). For Nissl staining, the sections were dried, rehydrated and immersed in 0.5% cresyl violet for 2 min. After washing in water, the sections were dehydrated in graded alcohols, cleared in xylene and cover-slipped with Permount.

Annexin V histochemical staining

The detection of the early phase of apoptosis staining was performed according to the method developed by us previously, based on binding properties of annexin V to phosphatidylserine (Vermes et al., 1995) (Graupner et al., in preparation). Briefly, at 1 hr 45 min after the induction of global ischemia, the rats were reanesthetized and placed in a stereotaxic frame. A small burr hole was drilled in the skull and 10 µl of biotinylated annexin V in binding buffer (Beckman Coulter) was injected stereotaxically into the right hippocampus (coordinates: 3.7 mm posterior and 3.5 mm lateral to bregma and 3.5 mm below the dura (Shetty et al., 2005) at a rate of 2.5 µL/min over 4 min with a microinfusion pump (Harvard Apparatus). The infusion needle (Hamilton 26 S, 0.46 mm diameter) was kept *in situ* for an additional 30 min, then removed over 5 min. At 2 hr 45 min after ischemia, rats were sacrificed by formalin perfusion. Brains were postfixed in formalin for 72 hr, cryoprotected and sectioned in the cryostat (10 µm of thickness). To detect biotinylated annexin V bound to phosphatidylserine on the cell membrane of apoptotic cells, brain sections were incubated with Texas red-labeled streptavidin in the blocking serum (Texas Red-Streptavidin; Biomedica Corp.) at room temperature (RT) for 30 min, washed, cover-slipped and observed under a fluorescent microscope (Olympus BX51).

Cell counting

Four animals per group were used for the cell count study. Two slides were used from each brain: one anteriorly and another posteriorly to the injection level (approximately 3.2 mm and

4.2 mm posterior to bregma, respectively). Six visual fields of the cerebral cortex were photographed in each section (three on each side, magnification 200x), which resulted in 12 photographs from the anterior level and 12 from the posterior one for each brain. In total, we took 24 photographs from each brain and 96 per each group (Table 1). Sections were evaluated under an Olympus X51B fluorescent microscope. Cell counts were performed by the experimenters blinded to the study, with the aide of ImageJ software (NIH). Double fluorescence staining with annexin V and cell-specific markers, staining for two distinct apoptotic markers, and immunodetection of p38/MAPK was performed according to Graupner et al., (in preparation).

TUNEL method

Brain sections were pre-boiled in citric buffer, pH 6.0, for 15 min and labeled with an *In Situ* Cell Death Detection Kit (Roche). A mixture of FITC-labeled nucleotides and terminal deoxynucleotidyl transferase was applied onto brain sections for 60 min at 37° C in a dark humidified chamber as previously described (Sun et al., 2004; Matchett et al., 2007). Incubation with labeling solution without the enzyme served as negative labeling control.

BDNF immunofluorescence and ELISA

Sections from brains collected at 2 hr 45 min after ischemia were incubated with rabbit antiBDNF antibody diluted 1:100 for 1 hr at RT (Santa Cruz Biotech.), then probed with donkey anti-rabbit FITC-conjugated antibody from Jackson ImmunoResearch Laboratories (1 hr, RT), cover-slipped and observed under fluorescent microscope.

For BDNF ELISA, the rats were transcranially perfused with ice-cold PBS and brain structures including cerebral cortices, were separated, snap frozen, and kept at -80°C until analysis. BDNF ELISA procedure was performed using BDNF E_{max} ImmunoAssay System (Promega Corporation). Brain tissue was homogenated on ice in lysis buffer (137 mM NaCl, 20 mM Tris-HCl, 1% NP40, 10% glycerol, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium vanadate) and centrifuged. After measuring the protein concentration with a Dc kit (Bio-Rad), the supernatants (tissue extracts) were diluted in Dulbecco's PBS and processed according to the manufacturer's instructions. Briefly, Corning 96 well micro plates were coated with anti-BDNF monoclonal antibodies, and then blocked in a buffer. Two columns of each microplate were designated for the standard curve that used serial dilutions of BDNF standard provided, followed by serially diluted unknowns. Following incubation with polyclonal anti-BDNF antibody and anti-IgY HRP conjugate, enzyme substrate was added and incubated until a blue color formed. The reaction was stopped by the addition of 100 µl of 1N HCl (yellow color formed) and plates read within 30 min in Plate Reader (Bio Tek) at 450 nm. The BDNF concentrations were estimated from the standard curve and expressed as picograms per milligram protein (Gobbo, O'Mara 2004).

Neurobehavioral testing

To evaluate sensorimotor deficits, we used the Garcia score, modified for the evaluation of bilateral deficits (Garcia et al., 1995; Kusaka et al., 2004). Short term memory deficits, corresponding both with hippocampal damage and cortical injuries, were tested in the T-maze by the assessment of spontaneous alternation (Matchett et al., 2007). Briefly, rats were placed in the maze stem and allowed to explore for 1 min. Then 10 trials for spontaneous alternation of maze arms were done over 20 minutes. The results were expressed as percent of spontaneous alternation with respect to 50% reference (Gerlai 2001; Matchett et al., 2007).

Statistical Analysis

All quantitative data are expressed as mean $\pm$ SEM. The significance of differences between means was verified by ANOVA followed by Tukey test. For the analysis of cell count results and neurobehavioral scores, a non-parametric Kruskal-Wallis ANOVA was used, followed by Dunn's test. Mortality rates were analyzed using the chi square test.

Results

Mortality and neurological scores

The mean blood pressure and all blood gas parameters were equivalent in all experimental groups (data not shown). There was a significant increase in glucose level after 4VO both in untreated and preconditioned with HBO, as compared to preischemic levels and in comparison with those levels in the sham group. No significant differences in glucose levels were found between 4VO groups before and after ischemia regardless treated or not. The equivalent postischemic increase in glucose levels in preconditioned vs. non-preconditioned groups suggests that effects of HBO preconditioning were not confounded by hyperglycemia. A mortality of 17 % was recorded in the 4VO no treatment group. Most animals died within the first 24 hr after ischemic insult. As in the sham-operated group, no rat died in the 5HBO group ($p < 0.05$ vs. 4VO), whereas 2 animals (8.69%) died in the 3HBO group. The results of neurological, sensorimotor scoring are presented on Figure 1A as median scores. Sham animals had no neurological deficit throughout the observation period. After global ischemia the neurological score worsened significantly in the 4VO/no treatment group. The reduction of neurological function was smaller, although significant, in the 3HBO and 5HBO groups, with no significant differences between two preconditioned groups. However, unlike for the 5HBO group, the difference between the 3HBO group and the no treatment group reached statistical significance at only 24 hr. At 72 hr a certain degree of recovery was observed in all ischemic groups, although only in preconditioned groups was a full recovery present.

T-maze testing revealed that rats after ischemia had a significant decrease of spontaneous alternation by 64.71% as compared to the sham operated control (Fig. 1B). Sham operated animals and naïve controls (from unrelated study) presented equivalent numbers of spontaneous alternations. 5HBO rats and 3HBO rats showed insignificant decrease in the percentage of spontaneous alternations (by 11.75% and 17.75% vs. sham group, respectively) with no statistical differences between preconditioned groups.

Nissl stain

We found signs of degeneration in cortical neurons and pyramidal cells of CA1 as early as 24 hr after ischemia (Fig. 2A). Moderate shrinkage and darkening of cells were the most abundant changes in both regions. Similar changes were very few in the 5HBO group (Fig. 2B) and much reduced in the 3HBO group (Fig. 2C). At 72 hr after ischemia all CA1 neurons and a large population of cortical neurons were damaged in the 4VO/no treatment group (Fig. 2D), however only several damaged neurons were observed in the HBO preconditioned groups (Figs. 2E and 3F). Injured neurons with twisted axonal processes and cell loss were noted in the CA1 and cerebral cortex at day 7 after ischemia (Fig. 2G). In the 3HBO group, however, relatively numerous dark neurons were observed as compared to the 5HBO group (Fig. 2H) both in the hippocampus and in the cerebral cortex (Fig. 2I).

TUNEL and Caspase-3

TUNEL positive cells were observed in the cerebral cortex and in the CA1 of the hippocampus at 24 hr after global ischemia (Fig. 3A). Only a few TUNEL positive cells were present in the cortex in 5HBO and 3HBO preconditioned animals 24 hr after ischemia (Figs. 3B and 3C). At

72 hr, CA1 cells in 4VO untreated global ischemic rats was entirely TUNEL positive (Fig. 3G) and so was a large population of cortical neurons (Fig. 3D). Only a few apoptotic neurons were present in the CA1 and cortex in the 5HBO+4VO group (Figs. 3E and 3H). A decrease of TUNEL staining was also observed in the 3 times HBO preconditioned group although more apoptotic changes were associated with this regimen of preconditioning compared to 5HBO (Figs. 3F and 3I). Immunohistochemistry with monoclonal antibody that recognizes large fragments of activated caspase-3 showed a pattern of immunoreactivity that paralleled TUNEL positive changes. Strong signals for caspase 3 were observed in the no treatment group at 24 hr in the cortex (Fig. 3J) and at 72 hr in the cortex and hippocampus (Figs. 3M and 3P). Five treatments of HBO preconditioning abolished caspase-3 cleavage at 24 hr in the cortex (Fig. 3K) and reduced the number of caspase-3 positive neurons in both structures at 72 hr after ischemia (Figs. 3N and 3R). 3HBO preconditioning did not prevent the appearance of the foci of caspase-3 immunoreactive neurons in the hippocampus (Fig. 3S) and in the cortex (Figs. 3L and 3O).

Apoptotic cell count

Next we wanted to determine if HBO preconditioning protects against early apoptosis (Fig. 4). Histological panels show cells double stained for annexin V and NeuN as well as merged images of those two kinds of stains. Numerous apoptotic cells were present in the cerebral cortex of the no treatment group (Fig. 4B). Only several annexin V-positive cells were observed in the CA1 of the untreated 4VO group (data not shown). Panels 4C and 4D show largely reduced numbers of early apoptotic cells in preconditioned brains. Merging images revealed that a subpopulation of annexin V-expressing cells is not neuronal (Fig. 4J). The results of cell counting are presented in Figure 4M. Around 3% of neurons showed signs of early apoptosis in the sham group. In the ischemic group the overall number of apoptotic cells amounted 32.13% of all cells (7.12-fold increase compared to sham). More than half (16.54%) of those cells were neurons. In the 5HBO group, the number of apoptotic neurons was reduced markedly as compared to the 4VO untreated group even though it remained higher than in the sham group. In the 3HBO preconditioned rats the number of apoptotic cells was 9.98% of all cells and 7.81 % of neurons (significantly higher than the number of positive neurons in sham group); however it was reduced by 52.78% as compared to the number of apoptotic neurons in the 4VO no treatment group. Statistical analysis showed that in 5HBO and 3HBO groups the number of apoptotic cells was significantly different from the number in 4VO no treatment group. Additionally, in all groups, there was a significant increase in the number of apoptotic neurons as compared to the sham group.

Early apoptosis in astrocytes

The results of the cell counts for all early apoptosis suggest that better preservation of neurons in the preconditioned group is associated with improved survival of non-neuronal cells. Therefore, we performed a double fluorescence stain with annexin V and GFAP, astrocytic cell marker. Fig. 4J shows that abundant astrocytes were annexin V positive in non-preconditioned groups. Improved preservation of astrocytes was observed in the preconditioned brains (Figs. 5K and 5L).

Double apoptotic stain

Next we tested whether the protection against early apoptosis may contribute to a reduced extent of injury in later post-ischemic stages. In order to do so, we determined whether cells presenting the early apoptotic marker annexin V would progress to positive TUNEL change in the untreated vs. preconditioned groups. In the ischemic group there were few solely annexin V positive cells. On the other hand, especially in the preconditioned brains, solely annexin V positive cells were noted (Fig. 6L).

Phosphorylated p38

A double fluorescence staining was performed to detect neuronal immunoreactivity (IR) of phosphorylated p38 in the early phase post-ischemia. As shown on Figure 7I, only limited IR of neuronal p-p38 is present in the sham operated animals. In contrast, a strong signal was recorded in rat brains at 2 hr 45 min after ischemia (Fig. 7J). This signal was also observed in the CA1 hippocampal region (Fig. 7J inset). In the 5HBO preconditioned rats the IR of phospho-p38 was remarkably reduced, both in the cerebral cortex and in the CA1 (Fig. 7K). 3HBO preconditioning resulted in only limited suppression of p-p38; still, several foci of p-p38 reactive cells were present in the cerebral cortex and in the hippocampus (Fig. 7L).

BDNF

BDNF immunofluorescence at 2 hr 45 min after 4VO showed a stronger signal in the brains of 5HBO-preconditioned rats (Fig. 8A). BDNF ELISA showed a tendency towards BDNF depletion at 6 hr after global ischemia (Fig. 8B). However, in the HBO preconditioned group, the BDNF level was significantly higher than those in the not preconditioned rats. It was also higher than those in the sham group. These differences disappeared when 24 and 72 hr time point data were analyzed (Figs. 8C and 8D).

Discussion

There are several major observations of this study. The annexin V stain was positive for phosphatidylserine, providing evidence that apoptosis was occurring in cells in the cerebral cortex as early as 2 hr and 45 minutes after global cerebral ischemia. Cell counts for apoptotic cells revealed significantly less apoptotic cells in the preconditioned groups than in the non-treatment group. No significant difference between the percentages of apoptotic cells in the sham and 5HBO + 4VO groups indicates that HBO pre-conditioning is very effective in reducing early post-ischemic apoptosis. In contrast, around 10% of cortical cells were still apoptotic after 3HBO preconditioning. Although, versus 30% of apoptotic cells in the untreated group, it seems satisfactory, this regimen of preconditioning should be performed only when a longer pretreatment is undesirable. Although not quantified, brain hippocampi also showed higher abundance of early apoptotic cells in the untreated vs. preconditioned rats at 2 hr and 45 min after ischemia (data not shown). The overall abundance of early apoptotic cells in the hippocampi was however smaller than that in the cortex. Keeping in mind that the double apoptotic stain in the hippocampus was present at 16 hr, we assume that intense early apoptotic process in the hippocampus occurred later than 2 hr 45 min. Also TUNEL data seem to support the notion that HBO preconditioning protects against early apoptosis in the hippocampus, what is consistent with the improvement of hippocampus-dependent T-maze test after HBO preconditioning.

According to the manufacturer, the detection of early apoptosis in tissues, either cultured or isolated from humans or animals, requires 30 min incubation with biotinylated Annexin-V before washing and fixation (Beckman). The time between brain injection of annexin and fixation in our in vivo study was 1 hour, likely sufficient for annexin diffusion to remote areas of the brain. Moreover, positive stain with streptavidin conjugated Texas Red in the hemisphere contralateral to the injection strongly suggests effective diffusion of annexin and its binding with phosphatidylserine in this brain region.

The classical pattern of injury after global ischemia includes a delayed cell death in the hippocampus and layers II through VI of the cerebral cortex. This suggests that even relatively late intervention is capable of providing brain protection. We observed a very early apoptotic cell change (i.e. presenting phosphatidylserine for macrophage resolving) as early as 2 hr and 45 min after brain insult, which suggests that even quite early intervention (e.g. at 3–6 hr) may

be only partly effective as it cannot reduce the amount of the initial damage. We hypothesized that this amount may be critical for the further extent of injury and functional outcome. Therefore we demonstrated the conversion of early apoptotic change to later phase and examined the effect of HBO preconditioning. Indeed, annexin V positive cells did go on to the TUNEL detectible change. Since HBO reduced early apoptosis, thereby, assuming the evidenced conversion of early to late apoptotic change, it reduced the overall extent of delayed brain injury in this mechanism. Interestingly, it also reduced the conversion itself. The latter seems to be particularly promising as it has been postulated that the “inhibition of apoptosis might improve the energy state of the injured cell to such an extent that it may escape necrotic cell death” (Hossmann et al., 2001). The alternative explanation, that there is an occurrence of a delayed phase of apoptosis, is less likely taking into account largely reduced TUNEL signals in the corresponding regions of preconditioned brains at 24 and 72 hr post-ischemia.

Early apoptotic changes appeared earlier in the cerebral cortex than in the CA1. CA1 injury was still limited when annexin V positive cells displayed TUNEL detectible change (Graupner et al., in preparation). Such finding only partially corresponds with an established pattern of selective vulnerability that postulates greater resistance of cerebral cortex than CA1 to ischemia (Chen et al., 1998). Further studies are needed to determine if the early cortical injury may precipitate hippocampal damage. In these settings protection against early cortical damage would trigger a protection against a CA1 injury commonly observed in global ischemic models. Alternatively, the different timing of CA1 vs. cortical changes may occur due to a different quality of reperfusion that, in the cortex, is more efficient and produces immediate phosphatidylserine externalization. In experimental cardiological systems, such externalization has been evidenced as an immediate event after blood flow is restored (Reutelingsperger et al., 2002). In the hippocampus, reperfusion may be impaired, especially assuming permanent occlusion of vertebral arteries (Pulsinelli, Brierley 1979) and therefore injury occurs later, but comprises entire CA1. In addition, the injurious interaction between different brain regions might be vectored by astrocytic damage. Apoptosis in astrocytes has been evidenced after stroke and has received more attention recently (Prunell et al., 2005). Not surprisingly, HBO preconditioning protected also astrocytic cells, suggesting the preservation of their roles in supporting neurons and maintenance of blood brain barrier integrity amongst preconditioning effects (Trendelenburg, Dirnagl 2005).

Immuno-enzymatic and immunofluorescent evidence suggests that HBO pre-conditioning increases BDNF in the cerebral cortex and CA1 early after global ischemia. A previous study reported a decrease in BDNF protein after untreated transient forebrain ischemia (Kokaia et al., 1996). Our findings may indicate that HBO preconditioning decreases early apoptosis via the BDNF pathway. The upstream pathway for BDNF upregulation may include NF-kappaB, for which BDNF gene has a site in a promoter 3 region (Marini et al., 2004). Thus NF-kappaB, which has been shown upregulated by hyperoxia, might induce BDNF especially in the “permanent” 5HBO regimen (Tahepold et al., 2003). We did not investigate BDNF in the 3HBO group since our results suggested that BDNF could have only a limited positive impact in that group. Interestingly, immunofluorescence results suggested neuronal localization of the molecule, which is produced in the brain by glial cells (Leibrock et al., 1989). It is known, however, that BDNF can enter neurons by several mechanisms and neuronal BDNF immunoreactivity has been reported previously (Yanamoto et al., 2000). Although BDNF in our study increased before 3 hr and at 6 hr, Hirata et al. showed genomic change and protein synthesis for neurotrophin receptor that peaked at 12–24 hr post ischemia (Hirata et al., 2007).

We suggest that HBO preconditioning suppressed p-p38/MAPK levels possibly through BDNF overexpression, ascribing a predominantly pro-apoptotic function to p38/MAPK in ischemic neurons. Selective inhibition of p38 activity has been shown to protect neurons against

excitotoxic injury (Legos et al., 2002) and focal ischemic injury (Barone et al., 2001; Legos et al., 2001). Although one study showed that p38 is associated with acquired ischemic tolerance, p38 does not seem to be an effector of brain protection, as the same group found benefits of p38 inhibition post ischemia (Sugino et al., 2000a; Sugino et al., 2000b). However, another study found that pretreatment with p38 inhibitor may aggravate ischemic brain injury (Lennmyr et al., 2003). We observe that HBO preconditioning shifts the balance between pro-apoptotic and cell protective functions of p38/MAPK towards cell survival; we propose that HBO preconditioning allows for p38-mediated stimulation of protective signaling pathways while preventing pro-apoptotic, and perhaps excessive p38/MAPK activation in neurons after ischemia. In addition, our results showing suppressed levels of phosphorylated p38/MAPK at less than 3 hr may point to the dominant role of posttranslational modifications as a mediator of early effects of HBO preconditioning.

We have not analyzed likely downstream effectors in our ischemic model, but the involvement of p53 in global ischemic brain injury has lately been confirmed (Endo et al., 2006). Additionally, p38/MAPK mediates cytotoxicity in the endothelial cells (Lee, Lo 2003), and together with ERK1/2, can mediate secretion of MMP-9 in neuronal-astrocytic co-cultures (Wang et al., 2002).

In this present study the proteolytic activation of the caspase-3 was detected at 72 hr in the CA1 degenerating neurons and at 24 hr and 72 hr in the cortical neurons after transient global cerebral ischemia. One study could not find proteolytic activation of the caspase-3 precursor in the cerebral cortex after global ischemia (Chen et al., 1998). The discrepancy may be due to their longer duration of ischemia (15 min) that might trigger predominately necrotic or calpain-mediated cell death. However, consistent with previous studies caspase-3 activation is a process that underlies apoptotic cell death and lasts for days after global ischemia (Li et al., 2005).

According to some previous studies the second wave of caspase activation can occur 12 hr after stroke (Benchoua et al., 2001). We have found no evidence of a new wave of apoptosis that would be assumed if solely annexin V positive cells were observed at 16 hr after untreated ischemia (Graupner et al., in preparation). However, we cannot exclude that together with known late onset of brain inflammation, a new apoptotic wave may appear. Even so, our results seem to indicate that in the preconditioned group the effects of inflammation would be associated with an overall reduced apoptosis.

Our study shows weaker brain protection after short (3HBO) preconditioning and lesser activation of underlying candidate mechanisms. It indicates that longer (e.g. 5HBO) preconditioning should be considered for planned surgeries. However, even shorter HBO preconditioning regimes and exposure to oxygen offer safety advantages over hypoxic preconditioning (Freiberger et al., 2006). Recent studies by Xi and colleagues showed impaired performance after hypoxic preconditioning (Hua et al., 2005). Additionally hypoxic preconditioning procedures resulted in transiently reduced densities of dendritic spines on hippocampal CA1 neurons associated with open-field habituation impairments (Corbett et al., 2006). Our behavioral studies tested performance of animals associated with a function of cerebral cortex (sensory-motor tests) and hippocampus (T-maze) and found improvement in both instances, with no deterioration in neuro scores before ischemia. In this present study we did not calculate neurological scores at day 7 due to a small number of animals investigated at this time point. However in our another study with animals preconditioned and stroked in an identical fashion we found a significant improvement in neurological scores at 7 days as compared to untreated rats with 4VO. In relation to 72 hr time point, there was a further improvement in the preconditioned group which suggests a durable effect of HBO preconditioning (Ostrowski et al., in preparation). Based on our overall data, HBO

preconditioning appears as a very effective modality that may be used to achieve durable brain protection through a reduction of the early apoptosis after global cerebral ischemia.

Acknowledgements

This study is partially supported by grants from NIH NS52492 to J. Tang and NS53407, NS45694, and NS43338 to J. Zhang.

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* $P < 0.05$ vs. sham; # $P < 0.05$ vs. 5HBO; ¶ $P < 0.05$ vs. 3HBO

Figure 1.

(A) Neurological scoring showed functional deficit in rats after 4VO. Despite a tendency towards recovery, neurological impairment was still present in the untreated group at 72 hr after ischemia. HBO-preconditioned rats show a significant recovery that was noted at day 2 after the brain insult (sham: $n=14$; 4VO: $n=18$; 5HBO+4VO: $n=18$; 3HBO+4VO: $n=10$). (B) Testing rats in T-maze showed reduced percentages of spontaneous alternations in untreated rats at 72 hr after ischemia. HBO preconditioning resulted in a recovery of alternating behavior, irrespective of the HBO preconditioning regimen. * $P < 0.05$ vs. sham; # $P < 0.05$ vs. 4VO; ¶ $P < 0.05$ vs. 3HBO+4VO; $n=6$.

Figure 2.

Representative histological panels show ischemic cell change (arrowheads) in CA1 and in the cerebral cortex at 24 hr, 72 hr and 7 days after global cerebral ischemia (Figs. 2A, 2D and 2G). In the 5HBO preconditioned group, the majority of neurons presented undisturbed morphology (arrows; Figs. 2B, 2E and 2H). Global cerebral ischemia after 3HBO preconditioning was associated with the presence of foci of ischemic neurons both in the CA1 and cerebral cortex, increasingly till day 7 (Figs. 2C, 2F and 2I).

Figure 3.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) revealed multiple TUNEL positive cells in the cerebral cortex, showing enhanced immunoreactivity for large fragments of cleaved caspase-3 both at 24 and 72 hr after 4VO (Figs. 3A and 3J). At 72 hr but not at 24 hr, all CA1 cells were TUNEL positive in the no treatment group (Fig. 3G). Those cells also showed very strong expression of activated caspase-3 (Fig. 3P). Scarce TUNEL (Figs. 3B, 3E and 3H) and caspase-3 (Figs. 3K, 3N and 3R) positive cells were also seen in the 5HBO group in the cerebral cortex and in the CA1. More abundant TUNEL positive cells (Figs. 3C, 3F and 3I) and caspase-3 positive cells (Fig. 3L, 3O and 3S) were seen in the group preconditioned with 3 HBO treatments.

Figure 4.

The effect of HBO on early apoptosis in neurons (Figs. 4A-4L) and apoptotic cell counts at 2 hr 45 min after global cerebral ischemia (Fig. 4M). A significant increase in the number of annexin V positive cells, predominately neurons, occurred early after 4VO (Fig. 4J). Both 5HBO and 3HBO preconditioning reduced early apoptosis, although a number of apoptotic neurons in those groups was still significantly greater than in the sham-operated animals (Figs. 4K, 4L and 4M).

* P<0.05 vs. sham; #P<0.05 vs. 4VO; ¶ P<0.05 vs. 3HBO+4VO.

Figure 5.

The effect of HBO preconditioning on early apoptosis in astrocytes. Annexin V-positive astrocytes were largely absent in the sham-operated group (Fig. 5I). A great number of astrocytes showed a strong annexin V staining after ischemia (Fig. 5J). Partial preservation of astrocytes was achieved after preconditioning both in 3HBO and 5HBO regimen (Figs. 5K and 5L).

Figure 6.

HBO preconditioning targets progression of apoptosis. Double fluorescence imaging, including annexin V and TUNEL at 16 hr post-ischemia, suggests that cells exteriorizing phosphatidylserine (annexin V-positive) progress to DNA fragmentation in the cerebral cortex (Fig. 6J). Fig. 6K shows that the absence of annexin V-positive cells was associated with a lack of DNA fragmentation after 5HBO preconditioning. Foci of solely annexin V positive cells were noted predominately in the cerebral cortex of 3HBO preconditioned brains (Fig. 6L). Few cells, mostly in the 3HBO group, in CA1 showed double apoptotic staining (Fig. 6L inset).

Figure 7.

HBO preconditioning reduces the activation of p38 in neurons. Very minimal levels of immunoreactivity for phosphorylated p38 (p-p38) were detected in the cerebral cortex and CA1 of sham-operated animals (Fig. 7I). After 4VO there was a tremendous increase in the neuronal immunoreactivity of p-p38 (Fig 7J). Greater decreases in p-p38 immunoreactivity was observed in the 5HBO preconditioned group (Fig. 7K) than in rats preconditioned with 3 HBO treatments (Fig. 7L).

Figure 8.

BDNF immunofluorescence and ELISA. At 2 hr and 45 min. after 4VO, BDNF immunoreactivity was stronger in the preconditioned group (Fig. 8A). A significantly higher level of cortical BDNF was detected in the 5HBO preconditioned group compared to the no treatment group at 6 hr (Fig. 8B) but not at 24 hr (Fig. 8C) or 72 hr after ischemia (Fig. 8D).

* P<0.05 vs. 4VO.

Table 1

Parameters of Early Apoptotic Cell Counts

Experimental group			Slides per animal	Number of animals	Number of slides per group	Number of sections per group	Number of visual fields per section	Number of visual fields per group	Total number of counted cells per group
Sham			2	4	8	16	6	96	7856
4VO			2	4	8	16	6	96	7510
5HBO+4VO			2	4	8	16	6	96	7524
3HBO+4VO			2	4	8	16	6	96	7946