Effect of hyperbaric oxygen on apoptosis in neonatal hypoxia-ischemia rat model

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Calvert, John W., Changman Zhou, Anil Nanda, and John H. Zhang. Effect of hyperbaric oxygen on apoptosis in neonatal hypoxia-ischemia rat model. J Appl Physiol 95: 2072–2080, 2003; 10.1152/japplphysiol.00630.2003.—We have previously demonstrated that a transient exposure to hyperbaric oxygen (HBO) attenuated the neuronal injury after neonatal hypoxia-ischemia. This study was undertaken to determine whether HBO offers this neuroprotection by reducing apoptosis in injured brain tissue. Seven-day-old rat pups were subjected to unilateral carotid artery ligation followed by 2 h of hypoxia (8% oxygen). Apoptotic cell death was examined in the injured cortex and hippocampus tissue. Caspase-3 expression and activity increased at 18 and 24 h after the hypoxia-ischemia insult. At 18–48 h, poly(ADP-ribose) polymerase (PARP) cleavage occurred, which reduced the band at 116 kDa and enhanced the band at 85 kDa. There was a time-dependent increase in the number of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive cells. A single HBO treatment (100% oxygen, 3 ATA for 1 h) 1 h after hypoxia reduced the enhanced caspase-3 expression and activity, attenuated the PARP cleavage, and decreased the number of TUNEL-positive cells observed in the cortex and hippocampus. These results suggest that the neuroprotective effect of HBO is at least partially mediated by the reduction of apoptosis.

MATERIALS AND METHODS

Hypoxia-ischemia model and HBO treatment. The Animal and Ethics Review Committee at the Louisiana State University Health Sciences Center-Shreveport evaluated and approved the protocol used in this study. The model used in this study is based on the Rice-Vannucci model (38, 45), as previously described (12). Pups were housed with the dam under a 12:12-h light-dark cycle, with food and water available ad libitum throughout the study. Unsexed 7-day-old (day 0 = day of birth) Sprague-Dawley (Harlan) rats were anesthetized by inhalation with isoflurane (0.1%) in oxygen. The rats were kept at a temperature of 37°C as the right common carotid artery of each pup was exposed and ligated with 5-0 surgical sutures. The duration of the anesthesia did not exceed 20 min, and the pups were allowed to recover with their dams for 2 h. They were then placed in a jar perfused with a humidified gas mixture (8% oxygen-balance nitrogen) for 2 h. Both the jar and the gas mixture were kept at 37°C. The pups were returned to their dams after the hypoxic exposure. The pups that underwent HBO treatment were allowed to recover from the hypoxic exposure for 1 h before being placed in the HBO chamber (Sechrist Industries, Anaheim, CA). The HBO treatment of 100% oxygen was administered at a pressure of 3 atmospheres absolute (ATA) for 1 h, and the pups were then returned to their cages after the treatment. Only one HBO treatment was conducted for each pup.

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Experimental groups. The pups were divided into the following three groups: 1) control (no anesthesia, carotid ligation, hypoxia, or HBO exposure), 2) hypoxia-ischemia, and 3) hypoxia-ischemia + HBO. Each group was composed of pups from each litter to obtain parity within the groups. The brains were removed at various times after the hypoxia-ischemia insult: 12 h, 18 h, 24 h, 48 h, 2 wk, and 6 wk.

Paraffin embedding and TUNEL staining. Paraffin embedding was performed as described previously (31). In short, 10, 12, 18, and 24 h after the hypoxia-ischemia insult, pups (n = 3 for each group at each time point) were perfused with PBS under deep anesthesia, followed by 4% PAF in 0.1 M PBS. The brains were removed and placed in the same fixative solution for 1 wk before being embedded in paraffin. Paraffin-embedded brains were sectioned and processed for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. TUNEL staining was performed on paraffin-embedded sections by using the in situ cell death detection kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) as described previously (35, 39). Briefly, the procedure was carried out according to the manufacturer's instructions. The slides were dewaxed and rehydrated by heating the slides at 60°C. The slides were then washed in xylene and rehydrated through a graded series of ethanol and double-distilled water. Next, the slides were incubated for 30 min at 37°C in a 20 µg/ml proteinase K working solution. Then the slides were rinsed with PBS, and the area around the sample was dried. The slides were then incubated with 50 µl of the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase for 60 min in a dark, humidified atmosphere at 37°C. After the slides were rinsed three times with PBS, they were analyzed with a fluorescence microscope (515–565 nm). Two investigators who were blinded to the experimental protocol evaluated the TUNEL staining in sections from the cortical and hippocampal brain regions. These regions were selected because it is known that they are susceptible to hypoxia-ischemia injury (31). Regions in the contralateral hemisphere were also analyzed. Multiple fields (6 from each region) were analyzed, and data were represented as the number of TUNEL cells per high-power field previously described (13).

Caspase activity assay. Tissues from the cortex and hippocampus were taken from the lesioned hemispheres of P7 rat pups at 12, 18, 24, and 48 h after the hypoxia-ischemia insult (n = 4 for each group at each time point). The tissue was frozen in liquid nitrogen and stored at −80°C until use. Caspase-3 cellular activity was measured with caspase-3 cellular activity assay kit PLUS-AK-703 (BIOMOL Research Laboratories, Plymouth Meeting, PA) as described previously (30). The brain tissue was homogenized in ice-cold cell lysis buffer consisting of 50 mM HEPES, pH 7.4, 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM DTT, and 0.1 mM EDTA and centrifuged at 12,000 g for 10 min at 4°C. Protein content was measured by using the DC protein assay (Bio-Rad). Equal amounts of protein (20 µg) were loaded in each lane of polyacrylamide-SDS gels (7% for poly(ADP-ribose) polymerase (PARP), 12% for caspase-3). The gels were electrophoresed, followed by a transfer of the protein to a nitrocellulose membrane. The membrane was blocked with a blocking solution and then probed with rabbit polyclonal IgG PARP antibody (1:400) and rabbit polyclonal IgG caspase-3 antibody (1:400) (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then probed with anti-rabbit IgG-HPR antibody (1:2,500) (Santa Cruz Biotechnology). The membranes were then probed with the ImmunoStar horseradish peroxidase substrate kit (Bio-Rad), and densitometry analysis was performed with the ChemiDoc detection system (Bio-Rad) and Quantity One software (Bio-Rad). The membranes were also probed with rabbit polyclonal IgG α-tubulin antibody (1:400) (Santa Cruz Biotechnology) as an internal control.

Brain weight. Brain weight was determined as previously described (13). The pups (n = 7 for each group) were killed under deep anesthesia 2 or 6 wk after the hypoxia-ischemia insult. After removal of the brain, the cerebellum and brainstem were removed from the forebrain. The hemispheres were separated by a midline incision and then weighed on a high-precision balance (sensitivity ±0.001 g). Brain damage was expressed as the percent reduction of the ipsilateral (right) hemisphere compared with the contralateral (left) hemisphere.

Statistical analysis. The data are represented as means ± SE. Statistical differences were compared by using a one-way ANOVA and then, if a significant difference was found, a Student-Newman-Keuls method for multiple comparisons. A P value of <0.05 was considered to be statistically significant.

RESULTS

HBO treatment reduced changes in caspase-3 activity after hypoxia-ischemia insult. To confirm that HBO treatment offers its neuroprotective effects by reducing hypoxia-ischemia-induced apoptosis, we measured the amount of caspase-3 activity at 12, 18, 24, and 48 h after the hypoxia-ischemia insult (Fig. 1). The cleavage of Ac-DEVD-pNA, which reflects caspase-3 activity, was measured in both the cortex and hippocampus. Previously, it has been shown that caspase-3 activity increases 12 h after a hypoxia-ischemia insult, peaks between 24–36 h, and returns to baseline levels by 48 h (13). We found similar results in both the cortex and hippocampus (Fig. 1). After treatment with HBO, we found that the caspase-3 activity induced by hypoxia-ischemia was decreased in the cortex and hippocampus at 24 h after the hypoxia-ischemia insult (P < 0.05).
Furthermore, we investigated the expression of caspase-3 after a hypoxia-ischemia insult and subsequent HBO treatment by analyzing the active subunit of caspase-3 via Western blot analysis (Fig. 2). The p17 subunit of caspase-3 was present in both the cortex and hippocampus 18 and 24 h after the hypoxia-ischemia insult. Densitometric analysis of the immunoblots showed that the expression of caspase-3 increased in the cortex and hippocampus at 18 h ($P < 0.05$) and 24 h ($P < 0.05$) after the insult compared with the control levels (age-matched normal brain). Treatment with HBO decreased the hypoxia-ischemia-induced expression of caspase-3 in the cortex and hippocampus at both 18 h ($P < 0.05$) and 24 h ($P < 0.05$) after the insult.

**HBO treatment reduced changes in PARP cleavage after hypoxia-ischemia insult.** We next investigated how HBO affects cleavage of PARP, a caspase-3 substrate. Again, we used Western blotting and densitometric analysis to assess the expression of PARP after hypoxia-ischemia and subsequent HBO treatment (Fig. 3). We found that after a hypoxia-ischemia insult, PARP cleavage increased in both the cortex and hippocampus at 24 h ($P < 0.05$ for both regions) and 48 h.

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**Fig. 1.** Ac-DEVD-pNA cleavage activity was used to measure caspase-3 activity in the cortex (A) and hippocampus (B) at 12, 18, 24, and 48 h after a hypoxia-ischemia (HI) insult. Activity peaked ~24 h after the insult. Hyperbaric oxygen (HBO) treatment reduced the activity of caspase-3 in both the cortex and hippocampus. *$P < 0.05$ compared with control; †$P < 0.05$ compared with control and HI (ANOVA).

**Fig. 2.** A: representative immunoblot from 3 separate Western blot experiments showing the expression of the active subunit (p17 fragment) of caspase-3 in the cortex (a) and hippocampus (b). Lane 1 is control, lane 2 is HI, and lane 3 is HI + HBO. α-Tubulin was blotted to ensure that equal amounts of protein were loaded into each well. Expression of the active subunit (p17 fragment) of caspase-3 in the cortex (B) and hippocampus (C) 18 and 24 h after a HI insult was summarized. Data are represented as a percentage of the control levels, as measured by densitometry analysis. *$P < 0.05$ compared with control; †$P < 0.05$ compared with HI; †$P < 0.05$ compared with control and HI (ANOVA).
(P < 0.05 for both regions) over control levels (agematched normal brain). After HBO treatment, the hypoxia-ischemia-induced PARP cleavage in both the cortex and hippocampus was decreased at 24 h (P < 0.05) and at 48 h (P < 0.05).

**HBO treatment reduced DNA fragmentation after hypoxia-ischemia insult.** Cleavage of PARP leads to DNA fragmentation, and the fragmentation of nuclear DNA in cells has been identified extensively with TUNEL staining (7, 13, 25). We found that there was a time-dependent increase in the number of TUNEL-positive cells in the ipsilateral hemisphere (Fig. 4). Multiple fields (6 fields in each region) were analyzed, and the data were represented as the number of TUNEL-positive cells per high power field. In the cortex (Fig. 5A), there were ~4–5 cells per high power field by 12 h after the hypoxia-ischemia insult, ~20 cells per high power field by 18 h after the insult, and ~44 cells per high power field by 24 h after the insult. After HBO treatment, the number of TUNEL-positive cells observed in the cortex had decreased dramatically at both 18 and 24 h. Similar results were also observed in the hippocampus (Fig. 5B).

Under light microscopy, TUNEL-positive cells were markedly increased especially in hippocampus (Fig. 6E), and HBO reduced the number of TUNEL-positive cells (Fig. 6F). At higher magnification, the nuclei of cells were clearly stained in both hippocampus and cortex (Fig. 6, H and K). Again, HBO reduced the number of TUNEL-positive cells (Fig. 6, I and L). A few TUNEL-positive cells were identified in control slides (Fig. 6, G and J).

**HBO preserves brain weight.** Several pups in each litter were allowed to grow to 2 or 6 wk of age so that brain injury could be assessed on the basis of brain weight. Animals that were subjected to a hypoxia-ischemia insult showed brain atrophy and brain weight loss at 2 or 6 wk after hypoxia insult. The ipsilateral hemispheric weights at 2 wk after the insult were as follows: 0.529 ± 0.006 g for control pups, 0.253 ± 0.008 g for hypoxia pups, and 0.134 ± 0.006 g for hypoxia animals treated with HBO.
g (P < 0.05, ANOVA) for hypoxia-ischemia pups, and 0.396 ± 0.115 g (P < 0.05, ANOVA) for hypoxia-ischemia + HBO pups. The ipsilateral hemispheric weights at 6 wk after the insult were as follows: 0.608 ± 0.0065 g for control pups, 0.348 ± 0.412 g for hypoxia-ischemia pups, and 0.480 ± 0.264 g for hypoxia-ischemia + HBO pups. So a hypoxia-ischemia insult resulted in brain retardation up to 50% at 2 or 6 wk, and HBO treatment preserved brain growth up to 70–80%. Even though the brain weight in HBO-treated groups is significantly smaller (P < 0.05, ANOVA) than that of normal pups, it is significantly larger than those who suffered a hypoxia-ischemia insult and did not receive treatment (P < 0.05, ANOVA).

DISCUSSION

Because early brain injury occurs within 24 h after a hypoxia-ischemia insult (12), which might contribute to the delayed brain atrophy, we decided to study apoptotic changes from 12 to 48 h. We analyzed two different regions of the brain, the hippocampus and the cortex. These regions were selected because it is known that these regions are susceptible to hypoxia-ischemia injury (31), which is also consistent with our own observations (12). The results of the present study, using different detection methods including caspase-3 expression and activity, PARP cleavage, and TUNEL staining, demonstrate that HBO treatment affords neuroprotection partially by reducing apoptosis in the early stage of neonatal hypoxia-ischemia.

Apoptosis in hypoxia-ischemia. Patterns of cell loss after hypoxia-ischemia are likely to depend on the severity of the injury. Selective neuronal loss may develop after brief or acute injuries, whereas infarction or tissue necrosis, as well as glial loss, may result from more severe injuries. Different mechanisms of damage...
Fig. 6. TUNEL staining of tissue sections from the cerebral cortex and hippocampus of pups 24 h after a HI insult. Low magnification (×2) of age-matched control (A), HI section (B), HI + HBO section (C) showing the ipsilateral hemisphere (right) and contralateral hemisphere (left). D: age-matched control section showing the regions of the hippocampus (×10). E: TUNEL-positive cells (arrow) were observed in the hippocampus 24 h after a HI insult (×10). F: fewer TUNEL-positive cells (arrow) were observed in sections taken from pups treated with HBO (×40). G: age-matched control section showing the CA1 region of the hippocampus. H: TUNEL-positive cells (arrow) observed in the CA1 region of the hippocampus 24 h after a HI insult (×40). Inset represents a TUNEL-positive cell. I: fewer TUNEL-positive cells (arrow) were observed in sections taken from pups treated with HBO (×40). The same was observed in the cerebral cortex: age-matched control cortex (J), HI section (K), and HI + HBO section (×40) (L). Bars = 100 μm in A–C, 20 μm in D–F, and 5 μm in G–L.
are likely to be associated with these distinct patterns of cell loss (7–10, 16, 29, 34). Evidence has suggested that neuronal death, including ischemia-induced death, occurs via apoptosis as well as necrosis (29). Apoptosis has also been shown to play an important role in animal and human models of various diseases, including ischemic brain damage (7). Although several different models of neonatal hypoxia-ischemia have been developed in the past, the modified Levine preparation (38) in 7-day-old rats is the most commonly used model of cerebral hypoxia-ischemia in immature animals, and it has widely been used to assess the efficiency of putative neuroprotective agents (4). This model produces consistent cell death in the cerebral cortex, hippocampus, striatum, and thalamus, as well as white matter tracts (13, 37). Cell death after hypoxia-ischemia in this model is not the result of classic necrosis but may involve one or more components of the apoptotic pathway (21, 23). Now that it is established that apoptosis occurs in the neonatal brain after a hypoxia-ischemia insult, the question remains how to prevent its occurrence. Over the past several years, studies have been conducted with the idea of preventing or reducing apoptosis after a hypoxia-ischemia insult. Dexamethasone (13, 28), N-methyl-D-aspartate receptor open-channel blocker (43), group II metabotropic glutamate receptor agonist (11), N-tosyl-l-phenylalanyl-chloromethylketone (27), protein-disulfide isomerase (44), brain-derived neurotrophic factor (21), and hypothermia (1) have all been used with various degrees of success. These studies have provided an initial insight and understanding into the components that are involved in the apoptotic pathway. However, many questions remain unanswered.

Effect of HBO on hypoxia-ischemia-induced apoptosis. Present evidence seems to suggest that hypoxia in and of itself does not induce apoptosis, but rather it does so indirectly through energy depletion, altered ionic homeostasis, or oxygen-sensing molecules, which in turn activate the apoptotic pathway (7). Of the apoptotic activators, the cysteine proteases or caspases are of particular interest. Caspases are expressed as proenzymes containing three subunits that are activated after proteolytic processing and association of the large and small subunits (7, 9, 19). Once activated, caspases cleave proteins in a relatively substrate-specific manner, which provides for the morphological changes observed in cells during apoptosis (7, 9). Of the caspases, caspase-3 is the most widely studied in the neonatal hypoxia-ischemia model. Caspase-3 plays an effector role in neuronal cell death during normal brain development as well as after a hypoxia-ischemia insult. Caspase-3 and its downstream events were a main focus in the present study because caspase-3 is implicated in the apoptotic changes after neonatal hypoxia (19). Cheng et al. (13) showed that, in the ipsilateral hippocampus, caspase-3-like activity detected by Ac-DEVD-AMC increased 12 h after hypoxia-ischemia, peaked at ~24–36 h, and then returned to basal levels by 48 h. Han et al. (21) found that caspase-3 activation occurs predominantly in neurons and their processes in a specific and delayed time course after hypoxia-ischemia. Furthermore, Han et al. showed that caspase-dependent components of cell death do not peak until 12–24 h after the injury. Considering these studies, we looked at caspase-3 activity and the expression of caspase-3 around the peak times observed. We found that in both the cortex and hippocampus at 18 and 24 h after a hypoxia-ischemia insult, caspase-3 activity and expression were increased. HBO reduced the enhanced caspase-3 activity and expression.

Furthermore, we looked at PARP cleavage, a caspase-3 substrate. PARP has been shown to be synthesized after the activation of the apoptotic pathway by being degraded by caspases (42). The 116-kDa fragment of PARP cleaves to form fragments of 85 and 24 kDa. Cheng et al. (13) found that the 116-kDa fragment of PARP becomes cleaved after a hypoxia-ischemia insult. We detected the expression of the 116- and 85-kDa fragments of PARP after a hypoxia-ischemia insult and subsequent HBO treatment. At 24 and 48 h after a hypoxia-ischemia insult, there was a significant amount of PARP cleavage in both the cortex and hippocampus. However, after HBO treatment, PARP cleavage decreased in both regions at both time points.

Cheng et al. (13) found that cell injury is delayed 6–24 h after insult detected with both biochemical and anatomic methods designed to detect DNA damage. They also found that there was a time-dependent increase in the number of TUNEL-positive cells in the ipsilateral hemisphere, which peaked between 18 and 24 h after the insult. We also found similar results suggesting that the number of TUNEL-positive cells peaked ~18–24 h after the insult in both the cortex and hippocampus. Previously, our laboratory has confirmed apoptotic cells by transmission electron microcopy (12), a gold standard for apoptosis (41) in the dentate gyrus 24 h after a hypoxia-ischemia insult. After HBO treatment we observed far less apoptotic cells. Other studies (31, 34) have shown that apoptosis is present in the neonatal brain after hypoxia-ischemia and that it is delayed, which suggests that it could be an important target for treatment. We found that a single treatment of HBO (100% oxygen at 3 ATA) for 1 h reduced the number of TUNEL-positive cells present in the ipsilateral hemisphere of the cortex and hippocampus at 18 and 24 h after a hypoxia-ischemia insult.

In our laboratory’s previous study, we found that HBO not only attenuated the effects of hypoxia-ischemia on the neonatal rat brain by reducing the progression of neuronal injury, but it also preserved tissue function (12). Now, this study shows that a single treatment of HBO (100% oxygen, 3 ATA) for 1 h reduced the hypoxia-ischemia-induced increase of TUNEL-positive cells, the increase in the activity of caspase-3, the expression of caspase-3, and PARP cleavage. Taken together, the results of this present study suggest that HBO does in fact offer its neuroprotection by reducing hypoxia-ischemia-induced apoptosis. The rationale of using HBO as a treatment of hypoxia-ischemia comes from the evidence that HBO
increases tissue oxygen delivery, especially to areas of diminished flow, enhances neuronal viability, reduces brain edema, improves the integrity of the blood-brain barrier, and regulates postischemia metabolism (33). Whether HBO affords its neuroprotective effects by directly intervening in the apoptotic pathway somewhere upstream of caspase-3, by restoring the depletion of energy and the altered ionic homeostasis, or by changing the expression of oxygen-sensing molecules remains to be determined.

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DISCLOSURES
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