Oxygen treatment after experimental hypoxia-ischemia in neonatal rats alters the expression of HIF-1α and its downstream target genes

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Calvert, John W., Julian Cahill, Mitsuo Yamaguchi-Okada, and John H. Zhang. Oxygen treatment after experimental hypoxia-ischemia in neonatal rats alters the expression of HIF-1α and its downstream target genes. J Appl Physiol 101: 853–865, 2006. — Recently, mounting evidence has emerged to suggest that hyperbaric oxygenation (HBOT)-induced neuroprotection after experimental global ischemia and subarachnoid hemorrhage entails a decrease in the expression of hypoxia-inducible factor-1α (HIF-1α). Therefore, the purpose of this study was to test the hypothesis that oxygen-induced neuroprotection after neonatal hypoxia-ischemia involves alterations in the expression of HIF-1α. Seven-day-old rat pups were subjected to unilateral carotid artery ligation followed by 2 h of hypoxia (8% O2 at 37°C). Pups were then treated with HBOT (2.5 ATA) or normobaric oxygenation treatment (NBOT) for 2 h. The expression and phosphorylation status of HIF-1α was evaluated at intervals up to 24 h after the insult, as was the expression of glucose transporter (GLUT)-1, GLUT-3, lactate dehydrogenase (LDH), aldolase (Ald), and p53. The protein-protein interaction of HIF-1α and p53 was also examined. An elevated expression of HIF-1α, GLUT-1, GLUT-3, Ald, and LDH was observed after the insult. An increase in the dephosphorylated form of HIF-1α was followed by an increase in the association of HIF-1α with p53 and an increase in p53 levels. Both HBOT and NBOT reduced the elevated expression of HIF-1α and decreased its dephosphorylated form. Furthermore, both treatments promoted a transient increase in the expression of GLUT-1, GLUT-3, LDH, and Ald, while decreasing the HIF-1α-p53 interaction and decreasing the expression of p53. Therefore, the alteration of the HIF-1α phenotype by a single oxygen treatment may be one of the underlying mechanisms for the observed oxygen-induced neuroprotection seen when oxygen is administered after a neonatal hypoxic-ischemic insult.

brain; hyperbaric oxygenation; hypoxia-inducible factor-1α; normobaric oxygenation; neonatal hypoxia-ischemia

THE ABILITY TO ADAPT TO TIMES OF hypoxia in both the physiological and pathological setting is very important for the survival of cells. Hypoxia-inducible factor-1 (HIF-1) is a basic helix-loop-helix heterodimeric transcription factor, consisting of the HIF-1α and HIF-1β subunits, considered to be the most important factor involved in the cellular response to hypoxia. The expression of HIF-1α is tightly regulated by cellular oxygen concentration, because at normal oxygen concentrations the degradation domain of HIF-1α interacts with the product of the von Hippel-Lindau tumor suppressor gene, which functions as a multisubunit ubiquitin-protein ligase, promoting the degradation of HIF-1α by the proteasome. However, during times of hypoxia, HIF-1α is not degraded and plays a very general role by signaling the existence of hypoxia to the transcriptional machinery in the nucleus of all cells. Upon the dimerization of the two subunits, HIF-1 binds to the hypoxia-response element (HRE) in enhancers and promoters of many target genes such as erythropoietin, some glucose transporters (GLUTs), several glycolytic enzymes, vascular endothelial growth factor (VEGF), p53, BNIP3, and the inducible isoform of nitric oxide synthase (iNOS). So after a hypoxic episode HIF-1 can regulate the expression of both prosurvival and prodeath genes. Therefore, the purpose of this present study was to test the hypothesis that oxygen-induced neuroprotection after neonatal hypoxia-ischemia involves alterations in the expression of HIF-1α and its downstream target genes.

MATERIALS AND METHODS

The Animal and Ethics Review Committee at Loma Linda University approved and supported the protocol used in this study. Timed pregnant female Sprague-Dawley rats were obtained from a commercial breeder (Harlan Laboratories, Indianapolis, IN) and housed in individual cages. After birth, pups were housed with their dam under a 12:12-h light-dark cycle, with food and water provided ad libitum throughout the study. Treatment groups were assigned by 10.220.33.1 on August 15, 2017 http://jap.physiology.org/ Downloaded from

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artery of each pup was identified, exposed, and ligated with 5-0 surgical silk through a near-midline incision. The wound was then closed and the pups were allowed to recover from the anesthesia. Thereafter, 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 balanced nitrogen) for 2 h. Both the jar and the gas mixture were kept at 37°C to maintain a constant thermal environment. The pups were returned to their dams after the hypoxic exposure for intervals up to 72 h after the insult. The combination of unilateral common carotid artery ligation and 8% oxygen, as used in this model, results in a spectrum of brain damage primarily in the territory of the middle cerebral artery (MCA) of the ipsilateral cerebral hemisphere. Damage does not occur in the cerebral hemisphere contralateral to the carotid artery ligation or in either hemisphere when the animal is exposed to hypoxia or ligation alone (50).

**Oxygen treatment.** One hour after the end of the exposure to hypoxia a select number of pups were chosen randomly to be treated with 100% oxygen under hyperbaric or normobaric conditions as previously described (7, 8). The pups were placed in the chamber (Seachrist Industries, Anaheim, CA) with their dam to control body temperature changes. The Naive pups and HI pups were placed under a heating lamp to maintain a constant thermal environment. The temperature changes. The Naive pups and Hi pups were placed under a heating lamp to maintain a constant thermal environment. The separation of the pups from their dam does not cause any adverse effects, as preliminary studies in our lab have determined that the degree of injury to the HI pups when separated is the same as that for pups in litters that were not separated (data not shown). For HBOT, the chamber was allowed to stabilize at 1 ATA followed by an increase in the pressure at a rate of 0.5 ATA per minute until 2.5 ATA was reached. Once the desired pressure was achieved the rats were kept at this pressure for 2 h. The constant exchange of oxygen was used to reduce the accumulation of CO2 in the chamber environment. The chamber was then decompressed at 0.5 ATA per min at the end of the treatment. For NBOT, the pups were kept at a pressure of 1 ATA for 2 h.

**Subfractionation of cellular proteins for Western blot analysis.** Protein samples for Western blot analysis were prepared as described previously (7, 28). Pups were killed under deep anesthesia (intraperitoneal injection of ketamine, 80 mg/kg, and xylazine, 10 mg/kg) 0 h (immediately after the end of the hypoxia exposure), 4 h, and 24 h after the insult by decapitation. The brains were removed from the skull and separated into the following four regions: frontal-parietal cortex, basal ganglia, cortex, and hippocampus. The samples were flash frozen in liquid nitrogen and then stored at −80°C until needed. Samples (100–300 mg) of brain tissue were homogenized in 1 ml of ice-cold RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). The homogenate was then centrifuged at 1,330 g for 2 min to remove cellular debris. The supernatant was then transferred into a new tube and centrifuged at 16,000 g for 30 min at 4°C. The supernatant (postmitochondrial fraction) was collected. An aliquot of the postmitochondrial supernatant was then centrifuged at 100,000 g for 60 min at 4°C. The resulting pellet was resuspended in the same buffer supplemented with 1% Triton X-100. Protein concentration in the pellet (membrane) and supernatant (postmitochondrial fraction) was then measured by use of the DC protein assay (Bio-Rad Laboratories, Hercules, CA).

**Western blot.** Western blot analysis was performed as previously described (28). Equal amounts of protein (50 μg) were loaded in each lane of polyacrylamide-SDS gels. The gels were electrophoresed,

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Fig. 1. Western blot analysis of hypoxia-inducible factor-1α (HIF-1α) protein in the cortex (A), hippocampus (B), frontal-parietal cortex (C), and basal ganglia (D) from neonatal rats subjected to a hypoxic-ischemic insult. Representative immunoblots of HIF-1α protein from 0–24 h, as well as densitometry analysis, are shown for each brain region. The 0-h time point represents the time point immediately after the hypoxic exposure before any treatment was given. HIF-1α protein expression increased significantly after the hypoxic-ischemic (HI) insult in all brain regions examined. The HIF-1α protein levels in the samples taken from the animals administered either hyperbaric (HBOT) or normobaric oxygenation treatment (NBOT) were not elevated to those levels observed in the nontreatment samples. Values are means ± SE with 5 animals per group on 3 independent experiments normalized to α-tubulin and expressed as a percentage of the mean value of Naive controls. *P < 0.05 compared with Naive; †P < 0.05 compared with Naive and HI; ‡P < 0.05 compared with Naive, HI, and HI+NBOT (ANOVA with Tukey’s test).
followed by a transfer of the protein to a nitrocellulose membrane. The membrane was then blocked with a blocking solution and then probed with primary antibodies overnight at 4°C. The following antibodies were purchased from Santa Cruz Biotechnology: 1) goat polyclonal anti-HIF-1α (Y-15), 2) goat polyclonal anti-GLUT-1 (N-20), 3) goat polyclonal anti-GLUT-3 (M-20), and 4) goat polyclonal anti-p53 (FL-393). The following antibodies were purchased from Rockland (Gilbertsville, PA): 1) goat polyclonal anti-LDH and 2) goat polyclonal anti-Ald (where LDH is lactate dehydrogenase and Ald is aldolase). Immunoblots were then processed with secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham) to visualize signal, followed by exposure to X-ray film. Films were scanned and the optical density was determined with the Image J program (National Institutes of Health). As an internal control, α-tubulin (Santa Cruz Biotechnology) was blotted on the same membranes after stripping.

Coimmunoprecipitation. Whole cell lysates from brains were obtained as described (25). Whole cell lysates in buffer (20 mmol/l HEPES-KOH, 250 mmol/l sucrose, 10 mmol/l KCl, 1.5 mmol/l MgCl₂, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 0.1 mmol/l phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets; pH 7.9) were preincubated with 1 μg of control IgG and 20 μl of protein A-agarose (Santa Cruz Biotechnology) for 3 h at 4°C to remove nonspecific binding proteins. After centrifugation at 12,000 g for 20 s, the supernatants were incubated for 2 h at 4°C with 2 μg of anti-p53 antibody (Santa Cruz Biotechnology). Twenty microliters of protein A-agarose were then added, and each sample was incubated for 20 s, the supernatants were incubated for 2 h at 4°C with 2 μl of protein A-agarose (Santa Cruz Biotechnology) for 3 h at 4°C to remove nonspecific binding proteins. After centrifugation at 12,000 g for 3 min to dissociate the immune complexes, the samples were once again centrifuged at 12,000 g for 20 s and the supernatant was used for Western blot analysis as described above for the detection of HIF-1α.

Immunohistochemistry. At 4 and 24 h after the hypoxic-ischemic insult, pups were anesthetized [intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg)], the thorax was opened, and the left ventricle was perfused with 60 ml of 0.1 M PBS (pH 7.4) followed by 60 ml of 4% formalin in 0.1 M PBS (pH 7.4). After perfusion, the brains were removed and postfixed in the same fixative and stored at 4°C until use. Before sectioning, the brains were placed in 30% sucrose. Sections (2 mm) were made at the level of the hippocampus and basal ganglia and placed in tissue freezing medium (Triangle Biomedical Sciences). Ten-micrometer sections were cut using a cryostat (Leica LM 3050S).

Immunohistochemistry was performed on sections as previously described (52) using an ABC Staining System (Santa Cruz Biotechnology). Four series of sections were incubated in 3% hydrogen peroxide (H₂O₂) diluted in PBS (10 min) to prevent reaction with endogenous peroxidases. After 30 min in 3% normal serum in PBS, the sections were incubated with primary antibodies (1:200) overnight at 4°C. The following primary antibodies were purchased from Santa Cruz Biotechnology: 1) goat polyclonal anti-p53 (FL-393), 2) goat polyclonal anti-HIF-1α (Y-15), and 3) goat polyclonal anti-GLUT-1 (N-20). The goat polyclonal anti-Ald antibody was purchased from Rockland. After being rinsed with PBS, the sections were treated with a goat ABC kit (Santa Cruz Biotechnology). Twenty microliters of protein A-agarose were then added, and each sample was incubated overnight at 4°C. Pellets were then precipitated by centrifugation at 12,000 g for 20 s and washed three times with buffer. After boiling for 3 min to dissociate the immune complexes, the samples were once again centrifuged at 12,000 g for 20 s and the supernatant was used for Western blot analysis as described above for the detection of HIF-1α.

Fig. 2. Phosphorylation status of HIF-1α after hypoxia-ischemia. The phosphorylation status of HIF-1α was determined by measuring the optical density of each band that appeared on the immunoblot. The ratio of the phosphorylated to dephosphorylated band (Phosph/Dephosph) is given here for the cortex (A), hippocampus (B), frontoparietal cortex (B), and basal ganglia (D). Values represent means ± SE with 5 animals per group on 3 independent experiments. *P < 0.05 compared with Naive, †P < 0.05 compared with Naive and HI, ‡P < 0.05 compared with Naive, HI, and HI+NBOT (ANOVA with Tukey’s test).
avdin-peroxidase conjugate for 30 min. Peroxidase activity was revealed by dipping the sections for 5 min in a mixture containing diaminoben-
avidin-peroxidase conjugate for 30 min. Peroxidase activity was revealed
by the ABC kit (Santa Cruz Biotechnology) at room temper-
ature. Sections were then air-dried, dehydrated, and coveredslipped with
Permount. Images of the slides were captured using an Olympus BX51
microscope system (Olympus America). The application of a control
serum, instead of the primary antibody, on other sections of the same
samples provided a negative control for each staining.

Data analysis. All the data in this study were expressed as means ±
SE. Differences in data between the Naive and each group of hypoxia-
ischemia, with or without oxygen treatment, were compared by
one-way ANOVA and then, if a significant variance was found, the
Tukey’s test. A value less than 5% was considered significant.

RESULTS

HIF-α protein expression. To determine how oxygen treat-
ment would affect the level of HIF-1α protein after a hypoxic-
ischemic insult, Western blot analysis was performed on sam-
ple taken from the brains of the experimental groups (Fig. 1).
The four brain regions of the ipsilateral hemisphere examined
were the cortex, hippocampus, frontoparietal cortex, and basal
ganglia. HIF-1α expression was barely detectable in the Naive
control samples (Fig. 1A) probably owing to the degradation of
HIF-1α under normoxic conditions (30). A significant increase
(P < 0.05 compared with Naive) in the expression of HIF-1α
was observed in all brain regions sampled immediately after
the hypoxic exposure (0-h time point). This time point was
chosen to show that HIF-1α levels were increased before the
administration of either oxygen treatment. HIF-1α remained at
these elevated levels (P < 0.05 compared with Naive) in all of
these brain regions for up to 24 h after the insult. The admin-
istration of HBOT or NBOT 1 h after the insult significantly
(P < 0.05 compared with Naive and HI; ‡ P < 0.05 compared with Naive, HI, and HI+NBOT
(ANOVA with Tukey’s test).

Fig. 4. Western blot analysis of glucose transporter-1 (GLUT-1) protein in the cortex
(A), hippocampus (B), frontoparietal cortex (C), and basal ganglia (D) from neonatal rats
subjected to a hypoxic-ischemic insult. Representative immunoblots of GLUT-1 protein
at 4 and 24 h after the hypoxic-ischemic insult, as well as densitometry analysis, are
shown for each brain region. GLUT-1 protein expression increased significantly after
the hypoxic-ischemic insult in all brain regions examined. The GLUT-1 protein levels
in the samples taken from the animals admin-
istered either HBOT or NBOT demon-
strated a trend for a transient increase. Values
are means ± SE with 5 animals per
group on 3 independent experiments normal-
ized to α-tubulin and expressed as a percent-
age of the mean value of Naive controls.

*P < 0.05 compared with Naive; † P < 0.05
compared with Naive and HI; ‡ P < 0.05
compared with Naive, HI, and HI+NBOT
(ANOVA with Tukey’s test).

Fig. 3. HIF-1α immunostaining. Representative photomicrographs of HIF-1α immunohistochemistry in the cortex (A–F), basal ganglia (G–I), and hippocampus (M–R) 4 and 24 h after a hypoxic-ischemic insult. Strong immunoreactive cells were observed in each brain region of the HI pups (A, D, G, J, M, P). The expression of HIF-1α was reduced in all brain regions of the pups administered either HBOT (B, E, H, K, N, Q) or NBOT (C, F, I, L, O, R) compared with the HI pups. Insets in the top-left hand corner of A, D, G, J, M, and P represent photomicrographs taken from the cortex, basal ganglia, and hippocampus, respectively, from Naive control pups. Scale bars in the photomicrographs represent 50 μm (applicable to all panels).
expression of HIF-1α back to those levels observed in the Naive control brains ($P < 0.05$). Interestingly, HBOT was able to reduce the expression of HIF-1α to a lower level than NBOT in all the brain regions sampled at the 4-h time point ($P < 0.05$) and in the cortex, hippocampus, and basal ganglia at the 24-h time point ($P < 0.05$).

**HIF-1α phosphorylation status.** The phosphorylation status of HIF-1α determines whether its adaptive phenotype or pathological phenotype will predominate. Therefore, using the method described by Suzuki et al. (46), we determined which phosphorylation status of HIF-1α prevailed after a hypoxic-ischemic insult. The status is determined by calculating the ratio of the optical density for each band of HIF-1α that is detected by Western blot analysis. The slower migrating and the faster migrating bands are the phosphorylated and dephosphorylated forms, respectively. We observed a significant change ($P < 0.05$) in the phenotype of HIF-1α (Fig. 2) in all brain regions sampled from a more phosphorylated form toward a more dephosphorylated form, from 0 to 24 h after the insult. Interestingly, the phenotype became more phosphorylated in the basal ganglia from the 4- to 24-h time point. HBOT and NBOT significantly ($P < 0.05$) shifted the phenotype from a more dephosphorylated form seen immediately after the insult to a more phosphorylated phenotype at both 4 and 24 h after the insult. HBOT tended to increase ($P < 0.05$) the phosphorylation of HIF-1α to a greater extent than NBOT.

**HIF-1α immunostaining.** The cortex, basal ganglia, and hippocampus of the Naive control pups showed very weak HIF-1α immunostaining (insets in Fig. 3, A, D, G, J, M, and P). Extensive positive immunostaining of HIF-1α was observed in the cortex, basal ganglia, and hippocampus in samples taken from the HI pups 4 and 24 h after the insult. Marked attenuation of HIF-1α immunoreactivity was observed in the brain sections from those pups treated with either HBOT or NBOT.

**Protein expression of HIF-1α target genes.** To determine how oxygen treatment would affect the level of several downstream target genes of HIF-1α after a hypoxic-ischemic insult, Western blot analysis was performed on samples taken from the brains of the experimental groups. The expression of GLUT-1, GLUT-3, LDH, and Ald was measured. GLUT-1 and GLUT-3 protein expression was measured in the isolated membrane fractions. Expression of GLUT-1 was observed as a heavily glycosylated, high molecular mass (55 kDa) isoform (13) in all regions sampled in the Naive control brains (Fig. 4). After hypoxia-ischemia, a significant increase in the protein expression of GLUT-1 was observed in all brain regions at both 4 and 24 h after the insult. Interestingly, HBOT or NBOT did not significantly decrease the expression of GLUT-1 from

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Fig. 5. Western blot analysis of glucose transporter-3 (GLUT-3) protein in the cortex (A), hippocampus (B), frontoparietal cortex (C), and basal ganglia (D) from neonatal rats subjected to a hypoxic-ischemic insult. Representative immunoblots of GLUT-3 protein at 4 and 24 h after the hypoxic-ischemic insult, as well as densitometry analysis, are shown for each brain region. GLUT-3 protein expression increased significantly after the hypoxic-ischemic insult in all brain regions examined. The GLUT-3 protein levels in the samples taken from the animals administered either HBOT or NBOT demonstrated a trend for a transient increase. Values are means ± SE with 5 animals per group on 3 independent experiments normalized to α-tubulin and expressed as a percentage of the mean value of Naive controls. * $P < 0.05$ compared with Naive, † $P < 0.05$ compared with Naive and HI, ‡ $P < 0.05$ compared with Naive, HI, and HI+NBOT (ANOVA with Tukey’s test).
those levels observed in the HI samples at the 4-h time point. However, by 24 h after the insult the expression of GLUT-1 in all brain regions sampled from those pups administered HBOT or NBOT did not significantly decrease (P < 0.05) from the levels observed in the HI pups.

Similar results were noted for the protein expression of GLUT-3 (Fig. 5). GLUT-3 protein levels were also observed in all regions sampled in the Naive control brains. After hypoxia-ischemia, a significant increase in the protein expression of GLUT-3 was observed in all brain regions at both 4 and 24 h after the insult. Administration of HBOT or NBOT did not significantly decrease the expression of GLUT-3 from those levels observed in the HI samples at the 4-h time point in all regions sampled. By 24 h after the insult the expression of GLUT-3 in all brain regions sampled from those pups administered HBOT or NBOT had significantly decreased (P < 0.05) from the levels observed in the HI pups.

LDH (Fig. 6) and Ald (Fig. 7) protein levels were also observed in all regions sampled in the Naive control brains. After hypoxia-ischemia, a significant increase in the protein expression of LDH and Ald was observed in all brain regions at both 4 and 24 h after the insult. Administration of HBOT or NBOT did not significantly decrease the expression of either protein from those levels observed in the HI samples at the 4-h time point in all regions sampled. But, by 24 h after the insult the expression of LDH and Ald in all brain regions sampled from those pups administered HBOT or NBOT had significantly decreased (P < 0.05) from the levels observed in the HI pups.

GLUT-1 and Ald immunostaining. The cortex and basal ganglia of the Naive control pups showed detectable immunostaining for both GLUT-1 and Ald (insets in Fig. 8, A, D, G, and J). GLUT-1 immunostaining was observed throughout the cortex (Fig. 8A) and basal ganglia (Fig. 8D) in samples from the HI pups 24 h after the insult. It was characterized by a relatively strong staining of the wall of the blood vessels (arrows). Marked attenuation of GLUT-1 immunoreactivity was observed in the brain sections from those pups administered HBOT (Fig. 8, B and E) or NBOT (Fig. 8, C and F). Extensive positive immunostaining of Ald was also observed throughout the cortex (Fig. 8G) and basal ganglia (Fig. 8J) in samples from the HI pups 24 h after the insult. A marked attenuation of Ald immunoreactivity was also observed in the brain sections from those pups administered HBOT (Fig. 8, H and K) or NBOT (Fig. 8, I and L).
Protein expression of p53. To determine how oxygen treatment would affect the level of p53 after a hypoxic-ischemic insult, Western blot analysis was also performed on samples taken from the brains of the experimental groups. p53 expression was barely detectable in the Naive control samples (Fig. 9). A significant (P < 0.05) increase in p53 was observed at 4 and 24 h after the insult in all brain regions sampled from the HI pups, with the highest increase observed at the 24-h time point. The level of p53 in the brains of those pups administered HBOT or NBOT was significantly (P < 0.05) lower than those levels found in the HI pups. However, the administration of HBOT or NBOT did not decrease the expression of p53 to those levels found in the Naive control brains (P < 0.05). Coimmunoprecipitation (insets in Fig. 8) was performed to investigate the physical interaction between HIF-1α and p53 24 h after a hypoxic-ischemic insult by using whole-cell lysates from the cortex, hippocampus, frontoparietal cortex, and basal ganglia. The 24-h time point was chosen because the highest expression of p53 in all brain regions sampled was noted at this time point. Immunoprecipitation using an antibody against p53 brought down a significant (P < 0.05) amount of HIF-1α in all regions sampled from the brain of the HI pups. Significantly (P < 0.05) less HIF-1α was brought down in those regions sampled from the brain of the HBOT or NBOT pups. A significance (P < 0.05) between the HBOT and NBOT pups was noted for the hippocampus and basal ganglia. The cortex, basal ganglia, and hippocampus of the Naive control pups showed very weak p53 immunostaining (insets in Fig. 10, A, D, and G). In agreement with the Western blot analysis results, extensive positive immunostaining of p53 was observed in the cortex, basal ganglia, and hippocampus (Fig. 10, A, D, and G) in samples from the HI pups 24 h after the insult. We also noted a marked attenuation of p53 immunoreactivity in the brain sections from those pups administered either HBOT (Fig. 10, B, E, and H) or NBOT (Fig. 10, C, F, and I) 24 h after the insult.

**DISCUSSION**

The ability to sense oxygen and react to changes in the concentration of oxygen is a fundamental property of cellular physiology (38). In response to hypoxia, cells have the ability to regulate the expression of either adaptive or pathological genes depending on the magnitude and duration of the stimulus. The adaptive response, largely engineered by the transcription factor HIF-1α, is evident at the cellular level by the increased expression of glucose transporters and glycolytic enzymes, at the tissue level by enhanced angiogenesis, and at
Fig. 8. Immunohistochemistry of glucose transporter-1 (GLUT-1) and Ald 24 h after hypoxia-ischemia. Representative photomicrographs of GLUT-1 and Ald immunohistochemistry in the cortex (A–C and G–I) and basal ganglia (D–F and J–L) 24 h after a hypoxic-ischemic insult. Strong immunoreactivity, characterized by a relatively strong staining of the wall of blood vessels (arrows), for GLUT-1 were observed in both the cortex (A) and basal ganglia (D) of the HI pups. Expression of GLUT-1 was reduced in both brain regions of the pups administered either HBOT (B and E) or NBOT (C and F). Insets in the top left-hand corner of A and D represent photomicrographs taken from the cortex and basal ganglia from Naive control pups. Strong immunoreactive cells for Ald were also observed in the cortex (G) and basal ganglia (J) of the HI pups. Expression of Ald was reduced in both brain regions of the pups administered either HBOT (H and K) or NBOT (I and L) compared with the HI pups. Insets in the top left-hand corner of G and J represent photomicrographs taken from the cortex and basal ganglia from Naive control pups. Scale bars in the photomicrographs represent 50 μm (applicable to all panels).
the organism level by increased erythropoiesis (15). The role of HIF-1α as an adaptive response to hypoxia in the brain has been further supported by preconditioning modalities, because the upregulation of HIF-1α, GLUT-1, and some glycolytic enzymes after hypoxic preconditioning has been shown to protect the neonatal rat brain from a subsequent lethal hypoxic-ischemic insult (4, 20). However, there is mounting evidence to suggest that after ischemic insults in neuronal tissue HIF-1α mediates hypoxia-induced neuronal cell death either by enhancing the expression of Nip3, a proapoptotic member of the Bcl-2 family, or through a p53-mediated apoptotic cascade (15, 33). Recently, Helton et al. (16) confirmed that HIF-1α is prodeath in the brain after ischemia by demonstrating that mice with a late-stage brain deletion of HIF-1α were protected from hypoxic-induced cell death.

In contrast to the adult brain, HIF-1α after hypoxia or ischemia has not been studied extensively in the neonatal brain, except in the context of hypoxic preconditioning (4, 5, 20). In one of the few nonpreconditioning studies conducted, HIF-1α and its target gene VEGF were both shown to increase transiently after the occlusion of the MCA (30). In this present study, we found that HIF-1α protein levels were increased immediately after the exposure to hypoxia and that the elevated levels persisted up to 24 h after the insult. In agreement with Mu et al. (30), we also observed that for most of the brain regions examined the expression of HIF-1α increased during the initial reperfusion phase (from 0 to 4 h) and then declined as reperfusion persisted (from 4 to 24 h), possibly due to the reestablishment of flow and tissue normoxia. We then looked at several downstream targets of HIF-1α. VEGF is one of the most characterized target genes of HIF-1α, and an elevation in its expression is usually considered to be adaptive because it has been shown to induce vasculogenesis and angiogenesis in many organ systems (16). However, after an ischemic insult, an elevation in VEGF can be considered detrimental because it can enhance the permeability of the blood brain barrier (BBB) (31). After hypoxia-ischemia in neonatal rats, evaluation of the integrity of the BBB with IgG extravasation and MRI analysis revealed a disrupted BBB from 24 to 72 h after the insult (19, 27, 35). Therefore, in neonatal hypoxia-ischemia an increase in VEGF can enhance the disruption of the BBB, causing a worsening of vascular compliance further decreasing cerebral perfusion and oxygenation. This can cause secondary hypoxic-ischemic injury to the surrounding viable brain tissue, as well as edema formation and an increased inflammatory influx (23, 47). Because VEGF, in relation to HIF-1α, has already been shown to increase after neonatal hypoxia-ischemia (18, 30), we chose to examine the expression of several other gene targets of HIF-1α. We found that the expressions of GLUT-1, GLUT-3, LDH, and Ald were all elevated at both 4 and 24 h
after the insult in all brain regions examined. Therefore, as one might expect, after hypoxic-ischemic insults both adaptive and pathological target genes of HIF-1α can be upregulated.

It is generally considered that under mild conditions of hypoxia the stabilization of the HIF-1 complex leads to the transcriptional activation of mainly adaptive genes. However, under conditions of severe or sustained hypoxia HIF-1α can promote apoptotic cell death (14). So it is the severity and duration of hypoxia that actually determine the phenotype of HIF-1α (adaptive or pathological) (1). Furthermore, Suzuki et al. (46) have shown that it is the phosphorylation status of HIF-1α that determines which phenotype predominates, with the phosphorylated form being the adaptive phenotype and the dephosphorylated form being the pathological phenotype. Using the analysis of the immunoblots for HIF-1α as described by Suzuki et al., we found that the dephosphorylated form of HIF-1α tended to predominate after the hypoxic-ischemic insult in all brain regions examined. It has also been demonstrated that under hypoxic conditions HIF-1α stabilizes p53 (2) and that it is the dephosphorylated phenotype that plays a pivotal role in this stabilization and subsequent activation of the p53-dependent apoptotic pathway during hypoxia (46). The role of p53 in apoptosis is well established (10, 44), because an increase in the expression of p53 has been shown to precede cell death (15) in models of global ischemia (22, 24), spinal cord ischemia (49), and focal cerebral ischemia (11, 21, 36). Furthermore, a deficiency in p53 has been shown to lead to reduced infarction volumes after MCA occlusion (12). In this present study, we found that the expression of p53 was elevated up to 24 h after the insult in all brain regions examined. Additionally, we confirmed the interaction between HIF-1α and p53 by using coimmunoprecipitation experiments to demonstrate that the dephosphorylated form of HIF-1α associated with p53 after the hypoxic-ischemic insult.

Previously, we have shown that a single administration of HBOT attenuated brain damage caused by a hypoxic-ischemic insult on the neonatal rat brain by reducing the progression of apoptotic neuronal injury (6, 7). Theoretically, oxygen treat-

Fig. 10. Immunohistochemistry of p53 24 h after hypoxia-ischemia. Representative photomicrographs of p53 immunohistochemistry in the cortex (A–C), basal ganglia (D–F), and hippocampus (G–I) 24 h after a hypoxic-ischemic insult. Strong immunoreactive cells were observed in each brain region of the HI pups (A, D, G). The expression of p53 was reduced in all brain regions of the pups administered either HBOT (B, E, H) or NBOT (C, F, I) compared with the HI pups. Insets in the top left-hand corner of A, D, and G represent photomicrographs taken from the cortex, basal ganglia, and hippocampus, respectively, from Naive control pups. Scale bars in the photomicrographs represent 50 μm (applicable to all panels).
HBOT has also been shown to decrease elevated levels of HIF-1α and several of its downstream genes in experimental models of global ischemia (22) and SAH (31). In agreement with these studies, we also found that oxygen treatment at either hyperbaric or normobaric pressures was able to reduce the hypoxic-ischemic-induced elevation in the expression of HIF-1α at both time points examined after the insult. Interestingly, we found that either oxygen treatment only reduced the hypoxic-ischemic-induced elevated levels of GLUT-1, GLUT-3, LDH, and Ald at the 24-h time point and not initially after the treatment (4-h time point), suggesting that the adaptive genes are only transiently upregulated after oxygen treatment. Furthermore, we found that both HBOT and NBOT decreased the dephosphorylation of HIF-1α (pathological phenotype) after the insult. As a result of this, we found that both treatments decreased the association of HIF-1α with p53, thereby decreasing the cellular expression of p53.

HBOT-induced neuroprotection in models of global ischemia and SAH was shown to be associated with a decrease in the expression of HIF-1α. Ostrowski et al. (31) demonstrated that HBOT reduced early brain injury after SAH, through the inhibition of HIF-1α and its target genes VEGF and Nip3, which led to a decrease in apoptotic cell death and the preservation of the BBB function. Moreover, Li et al. (22) demonstrated that HBOT decreased the expression of HIF-1α and its target genes p53, caspase-9, and caspase-3, all apoptotic genes, which are associated with the harmful actions of HIF-1α. These studies clearly show that reducing the expression of both HIF-1α and p53 leads to a decrease in apoptotic cell death, a hypothesis that is further supported by others (14–16). Previously, we have shown that a single administration of HBOT decreased components of the apoptotic machinery [caspase-3 and poly(ADP-ribose)polymerase] and the number of terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling positive cells in the neonatal brain after hypoxia-ischemia (7).

So, taking into account the results of this present study and the results of our previous studies, we suggest that one potential mechanism for the observed oxygen-induced decrease in apoptotic cell death in the neonatal brain after hypoxia-ischemia is mediated through a decrease in the expression of HIF-1α. Specifically, we propose that oxygen treatment not only reduces the elevated expression of HIF-1α but also decreases the pathological phenotype of HIF-1α (dephosphorylated). Decreasing elevated levels of HIF-1α can lead to a reduction in the expression of Nip3 (31), whereas decreasing the pathological phenotype can lead to a decrease in the stabilization and expression of p53 (22). A decrease in all of these proteins can then lead to the observed decline in caspase-3 and caspase-9 (7, 22), which signals an attenuation of the apoptotic cell death cascade. This is further supported by the evidence that decreasing HIF-1α is associated with decreased neuronal cell death (15). Additionally, as previously demonstrated (31), oxygen treatment may also be able to preserve the function of the BBB after hypoxia-ischemia. However, the effect of oxygen treatment on BBB maintenance needs to be further clarified.

In regards to the expression and phosphorylation status of HIF-1α there appears to be a dose-response relationship between HBOT and NBOT. As noted above, HBOT was able to reduce the expression of HIF-1α to a lower level than NBOT in all the brain regions sampled at the 4-h time point ($P < 0.05$) and in the cortex, hippocampus, and basal ganglia at the 24-h time point ($P < 0.05$). Furthermore, HBOT tended to increase ($P < 0.05$) the phosphorylation of HIF-1α to a greater extent than NBOT. For the phosphorylation status significance was noted in the cortex, hippocampus, and frontoparietal cortex and a trend was noted in the basal ganglia. However, for the downstream target genes of HIF-1α a trend in difference between HBOT and NBOT was mostly noted. A significant difference between the two was observed for the expression of GLUT-1 in the cortex 24 h after the insult and for the expression of GLUT-3 in the hippocampus. NBOT has been used to as a treatment for cerebral ischemia and has achieved a similar level of brain protection as HBOT (42, 43). But, unlike HBOT, the administration of oxygen at normobaric pressure does not result in a large increase in the partial pressure of oxygen ($P_O_2$) of brain tissue. The $P_O_2$ does, however, increase in a linear fashion with normobaric oxygen, and this small increase may be able to offer the observed neuroprotection, because the critical oxygen tension for mitochondrial function is very low (43). So the differences between the HBOT and NBOT groups can most likely be attributed to a dose-response relationship.

In summary, a single administration of HBOT or NBOT dose-dependently reduced the hypoxic-ischemic-induced elevation of HIF-1α, as well as decreased the pathological phenotype of HIF-1α and decreased the association of HIF-1α with p53, thereby leading to a reduction in the cellular expression of p53. HBOT and NBOT also induced a transient increase in the expression of several adaptive target genes of HIF-1α. Therefore, the alteration of the HIF-1α phenotype by a single oxygen treatment may be one of the underlying mechanisms for the observed oxygen-induced neuroprotection seen when oxygen is administered after a neonatal hypoxic-ischemic insult.

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REFERENCES

HIF-1α TARGET GENE EXPRESSION AFTER OXYGEN TREATMENT


