Expression of hypoxia-inducible factor-1α in the brain of rats during chronic hypoxia

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Received 22 May 2000; accepted in final form 21 June 2000

EXPOSURE TO A LOW-OXYGEN ENVIRONMENT triggers several immediate and long-term adaptive mechanisms. At the systemic level, these include hyperventilation and polycythemia, which improve oxygen delivery to critical organs such as the brain (15, 20). However, these compensatory mechanisms are not sufficient to meet oxygen demand of the central nervous system, especially during prolonged exposure to hypoxia. The brain exhibits a remarkable capacity of structural and metabolic response to prolonged hypoxia. One of the most dramatic structural responses is the considerable remodeling of the cerebral microvascular network. Previous studies showed that 3 wk of exposure to hypobaric hypoxia caused a significant increase in microvessel density throughout the brain (2, 9, 15, 19). This structural plasticity is also accompanied by metabolic adaptation to low oxygen tension. For instance, increased glucose transport (7), increased cerebral metabolic rate for glucose (10), and cytochrome oxidase activity (4, 14) have been reported in rat brain after 3 wk of exposure to hypobaric hypoxia.

During prolonged exposure to hypoxia, HIF-1α should be expressed as long as the balance between oxygen supply and utilization in the tissue has not been reached. Our results showed that HIF-1α levels return to normoxic values after 3 wk of hypoxia, suggesting that the cerebral vascular remodeling and metabolic changes were able to compensate for brain tissue hypoxia.
MATERIALS AND METHODS

Exposure to chronic hypoxia. Male Wistar rats aged 2–3 mo were kept for up to 3 wk in hypobaric chambers maintained at a pressure of 380 Torr (0.5 atm, equivalent to 10% normobaric oxygen). In groups kept for >1 day, the chambers were opened 30 min each day for cage cleaning and food and water replenishment. The duration of hypoxia was 6 h, 12 h, or 1, 4, 7, 14, or 21 days. Each experimental group of rats had its own littermate control group, which was kept outside the hypobaric chambers but in the same location. A group of rats kept 21 days in the hypobaric chamber was further exposed to either 10% or 8% normobaric oxygen for 4 h immediately after being removed from the hypobaric chamber. Tail venous blood samples were obtained for hematocrit determinations before the rats were killed.

Western blot analysis. After hypoxic exposure, experimental and control rats were killed, and their brains were rapidly removed and frozen in liquid nitrogen. Cortical samples were dissected and homogenized in ice-cold buffer (20 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 M NaCl) supplemented with 0.2 mM dithiothreitol, 0.5 mM sodium vanadate, and protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride and 2 μg/ml each of leupeptin, pepstatin, and aprotilin). Subsequently, NaCl was added to a final concentration of 0.45 M, and the homogenate was centrifuged at 10,000 g for 30 min. Supernatants were collected and mixed with an equal volume of homogenization buffer containing 40% (vol/vol) glycerol before being stored at −80°C. Samples (total of 200 μg lysates) were subjected to electrophoresis in SDS-7% polyacrylamide gel and transferred to nitrocellulose membranes by standard procedures.

The membranes were blocked with 5% nonfat milk powder and incubated for 2 h at room temperature with the following antibodies: monoclonal anti-HIF-1α (1:500, Novus Biologicals, Littleton, CO), polyclonal anti-HIF-1β (1:1,000, Novus Biologicals), and polyclonal anti-VEGF (1:400, Santa Cruz Biotech, Palo Alto, CA). This was followed by incubation with secondary horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence detection (ECL, Amersham, Piscataway, NJ). Crude nuclear extracts of Hepa 1 cells (American Type Culture Collection, CRL-1830) exposed to 1% or 20% oxygen were used as positive controls (30 μg of protein) in Western blot analyses. Protein concentrations were determined by the Bradford protein assay (Bio-Rad).

RNA extraction and Northern blot analysis. Total RNA was extracted from brain cortex by using the RNAses Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. Equal samples (10 μg) of total RNA were electrophoresed in 1% agarose-formaldehyde gels, transferred to nylon membranes (Millipore, Bedford, MA), ultraviolet cross-linked, and hybridized with random prime-labeled probes. The probes were hybridized with Quickhyb solution (Stratagene, La Jolla, CA) and washed in 0.2× saline sodium citrate, 0.1% SDS at 55°C. VEGF and GLUT-1 probes were purchased from Research Genetics (GenBank accession numbers AA154722 and AA451073, respectively), and the oligonucleotide probe for 18S RNA was obtained from Life Technologies (Rockville, MD).

HIF-1α immunohistochemistry. Normoxic and hypoxic rats (4 and 21 days of hypoxia) were deeply anesthetized with Nembutal and perfused intracardially with ice-cold phosphate-buffered saline (pH 7.4) followed by 4% phosphate-buffered paraformaldehyde. Brains were removed and postfixed in 2% paraformaldehyde for 24 h and embedded in paraffin. Serial sections (6 μm) were cut on a microtome, mounted on gelatin-coated slides, air-dried, and stored at room temperature until they were processed for immunohistochemistry. To identify some of the cells expressing HIF-1α, double immunolabeling using two cellular markers, neuronal nucleus-specific antigen (NeuN) and glial fibrillary acidic protein (GFAP), was performed. Briefly, sections were deparaffinized, hydrated, and subjected to antigen retrieval at 90°C for 25 min using Target retrieval solution (Dako, Carpinteria, CA), according to the manufacturer's instructions. Mouse monoclonal antibody against HIF-1α (1:200, Novus Biologicals) was detected by use of a streptavidin-biotin-horseradish peroxidase system (catalyzed signal amplification system, Dako). The monoclonal anti-NGFAP (1:500, Sigma Chemical) and anti-NeuN (1:500, Chemicon, Temecula, CA) were detected by using Texas red and fluorescein-conjugated secondary antibodies, respectively (Vector, Burlingame, CA).

Statistical analysis. Data are reported as means ± SD. To calculate relative RNA abundance, optical densities of VEGF and GLUT-1 were normalized relative to 18S RNA signal. Normalized values were then averaged for the two replicated blots prepared from a single set of RNA samples. The densitometry values obtained for HIF-1α, HIF-1β, and VEGF immunoblots were used to calculate the percent increase relative to normoxic values of the same gel. The one-sample t-test was used to determine whether hypoxic-to-control ratios of protein densities were significantly greater than 1. Comparisons of the RNA and protein ratios at various time points were assessed by ANOVA with Tukey's correction (ONEWAY procedure, SPSS v8.0). In all cases, P < 0.05 was considered significant.

RESULTS

As expected, rats subjected to hypobaric hypoxia developed polycythemia. Hematocrit was elevated from 49 ± 4 to 56 ± 4 (not significant) after 4 days. It was significantly higher by 1 wk (62 ± 1) and continued to be higher than controls at 2 (69 ± 2) and 3 (73 ± 2) wk of hypoxic exposure (P < 0.05, n = 3 per time point).

HIF-1α and HIF-1β protein levels during chronic hypoxia. Immunoblot assays demonstrated that the HIF-1α antibody used in this study recognized an hypoxia-inducible protein of ~120 kDa in nuclear extracts from Hepa 1 cells as well as in brain cortical samples (Fig. 1A). Additional bands with higher molecular mass (125–130 kDa) were also observed in control and hypoxic samples. These bands were also induced by hypoxia and might correspond to posttranslational modifications of HIF-1α. Normoxic cortical samples showed a faint HIF-1α band that was strongly induced in hypoxic samples. A 9 to 10-fold increase was detected after 6 h, 12 h, 1 day, and 4 days of hypoxia (n = 3 per time point, P < 0.05). Thereafter, a fivefold induction of HIF-1α was found at 7 and 14 days of hypoxia (n = 3 per time point, P < 0.05). After 21 days, HIF-1α levels returned to the level of normoxic control (Fig. 1B). HIF-1α was detected with apparent molecular mass of 90 kDa. No statistically significant induction of HIF-1β was observed in brain cortex samples of hypoxic rats at any time point (Fig. 1).

HIF-1α induction in response to incremental hypoxia after adaptation. In the previous experiment, we showed that HIF-1α no longer accumulates in the brains of rats exposed for 21 days to hypobaric hypoxia.
The return of HIF-1α to baseline levels might be due to a successful restoration of oxygen tension in the tissue. To exclude the possibility that an impairment of the mechanism responsible for HIF-1α accumulation prevented its appearance, some rats were further exposed to either 10% or 8% normobaric oxygen (n = 3 each group) for 4 h immediately after being kept for 21 days in hypobaric hypoxia. As expected, 21 days of hypoxia caused HIF-1α levels to be similar to control values. Additional exposure for 4 h to 10% normobaric oxygen did not produce any changes in HIF-1α density compared with 21-day hypoxia-exposed cortical lysates (Fig. 2). These results indicate that a new balance between oxygen delivery and consumption was reached after 3 wk of exposure to hypoxia but that a further hypoxic stimulus was still capable of eliciting HIF-1α accumulation.

**HIF-1 target genes.** We also analyzed the activation of two HIF-1 downstream target genes: VEGF and GLUT-1. By Northern blot analyses, we were able to detect single transcripts for VEGF (4.5 kb) and GLUT-1 (4.8 kb). Both transcripts followed similar patterns of transient upregulation by chronic hypoxia (Fig. 3A), showing a three- to fourfold increase at 12 h, 1 day, and 4 days of exposure to hypoxia. After that, both transcripts started to decline. At 21 days of hypoxia, both transcripts were back to near normoxic levels. In addition, we also evaluated VEGF protein levels. A single band at 23 kDa was detected that probably

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**Fig. 1.** A: Western blot analysis of hypoxia-inducible factor (HIF)-1α, HIF-1β, and β-actin (loading control) in brain cortex of rats exposed to normoxic (C) and hypoxic (6 h to 21 days (d)) conditions. Crude nuclear extracts of Hepa 1 cells exposed to 20% (−) and 1% (+) oxygen were used as negative and positive controls, respectively, for the HIF-1α immunoblots. Indicated at right are the positions of molecular weight (MW) standards. B: ratios of HIF-1α (●) and HIF-1β (○) optical density (OD) relative to control values. Values are means ± SD from 3 experiments (n = 3 per time point; *P < 0.05 vs. control).

**Fig. 2.** HIF-1α response in brain cortex to incremental hypoxia after adaptation. Rats exposed to hypobaric hypoxia for 21 days (21d, n = 6) were subsequently exposed to 10% (n = 3) or 8% (n = 3) normobaric oxygen for 4 h (21d + 10%, 21d + 8%). Normoxia (C) and 4-day hypoxia (4d) samples are also shown. Nuclear extract of Hepa 1 cells exposed to 1% O2 was used as positive control (+).

**Fig. 3.** A: Northern blot analysis of vascular endothelial growth factor (VEGF) and glucose transporter-1 (GLUT-1) mRNA levels of brain cortical samples of rats exposed to normoxia (C) and hypobaric hypoxia (6 h to 21 days). Blots containing 10 μg of total RNA were hybridized with 32P-labeled cDNA probes specific for VEGF and GLUT-1. The same blot was hybridized with 18S RNA probe as a loading control. B: Immunoblot analysis of VEGF and β-actin protein levels in brain cortex of rats exposed to normoxic (C) and hypobaric hypoxia.
corresponds to the VEGF164 monomer. Levels of VEGF protein were also transiently induced, but its upregulation was delayed. A significant increase in VEGF protein was detected only after 24 h of hypoxia and it remained elevated for up to 14 days. As in the case of its mRNA, the VEGF protein level also decreased to control level at 21 days (Fig. 3B).

**Immunolocalization of HIF-1α in the brain.** We analyzed HIF-1α protein distribution in the rat forebrain. No significant levels of HIF-1α protein were detected in normoxic brains (Fig. 4A). In contrast, HIF-1α immunostaining was detected throughout the gray matter after 4 days of chronic hypoxia (Fig. 4, B, C, and D). White matter such as the corpus callosum showed no detectable labeling for HIF-1α after hypoxia (not shown). This immunostaining was primarily nuclear. Positive immunostaining was observed to be associated with small blood vessels and capillaries, suggesting that endothelial cells were also expressing HIF-1α (Fig. 4D). A strong nuclear staining was observed in the pial layer, ependymal cells lining the lateral and third ventricles, as well as in epithelial cells of the choroid plexus (Fig. 4, E and F). In agreement with the Western blot results, little if any positive staining for HIF-1α was observed after 21 days of hypoxia (not shown). HIF-1α-positive nuclei colocalize with NeuN (Fig. 5, A and B) and GFAP (Fig. 5, C and D), indicating that neurons and astrocytes express HIF-1α protein. Specificity of the immunostaining was confirmed by a number of controls. Staining was not evident when primary antibody was omitted, and two different secondary antibodies were tested, all yielding similar results.

**DISCUSSION**

The transcription factor HIF-1 has been identified as a critical component of the cellular and systemic response to hypoxia in mammals (22). It mediates oxygen-dependent expression of target genes encoding erythropoietin, glucose transporters, glycolytic enzymes, and VEGF, among others (3). The products of

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Fig. 4. Immunohistochemical localization of HIF-1α in normoxic and hypoxic rat brain. A: HIF-1α negative staining in normoxic brain sections. B: HIF-1α immunoreactivity in a hypoxic brain section showing intense nuclear staining (×100). C: higher magnification (×400) of a hypoxic brain section at the level of layer IV in the cortex. Positive staining is associated with round-shaped nuclei, presumably correspond to neuronal or glial nuclei. D: HIF-1α immunoreactivity associated with flat-shaped nuclei surrounding capillaries and small vessels (arrowheads), which might correspond to endothelial cell nuclei. Also shown are positive staining of pial (E) and positive HIF-1α immunostaining of the epithelial (F) cells of the choroid plexus and the ependymal cells lining the lateral ventricles of hypoxic brains. Scale bars, 50 μm, A and B are at ×100, C–F are at ×400.
these genes are involved in the regulation of hypoxic adaptive responses such as erythropoiesis, changes in energy metabolism, and angiogenesis.

HIF-1α is essential during embryonic development. HIF-1α−/− mouse embryos (homozygous for the null allele) die at midgestation, with major defects in cardiovascular development and massive cell death within the cephalic mesenchyme (12). Furthermore, the importance of HIF-1α in the physiological response to chronic hypoxia in organs other than the brain was demonstrated by using partially deficient adult mice (HIF-1α1/2). When exposed to 10% O₂ for 1–6 wk, HIF-1α1/2 mice demonstrated delayed development of polycythemia, pulmonary hypertension, and pulmonary vascular remodeling (28). Understanding the adaptive capacity of the brain to deal with oxygen deficiency is important because the molecular mechanisms responsible for this appear to be activated under many pathophysiological conditions such as tumors, ischemia-reperfusion injury, and stroke (5, 22). Angiogenesis and increased glycolysis represent tumor adaptations to a hypoxic microenvironment that are correlated with invasion, metastasis, and lethality. Glioblastomas and hemangioblastomas, which are the most malignant and highly vascularized tumors in the central nervous system, strongly express HIF-1α (29). In addition, induction of HIF-1α and transcriptional activation of its target genes occur in the ischemic penumbra area after permanent focal ischemia (1, 6).

We studied the expression of HIF-1α in the rat brain during adaptation to chronic hypoxia. Under normoxic conditions, we found low levels of HIF-1α protein by Western blot analysis, levels that were not detectable by the less sensitive immunohistochemistry. Our observations are in agreement with previous studies that reported basal expression of HIF-1α and transcriptional activity of HIF-1 in rodent brain (1, 26), human neuroblastoma cell lines (6), and purified murine cortical neurons (6).

The present study showed that HIF-1α protein accumulates significantly in the rat brain during hypobaric hypoxia. Immunostaining revealed that different brain cell types, including neurons, astrocytes, endothelial cells, and ependymal cells, express HIF-1α, suggesting a generalized tissue hypoxia (at least after 4 days of hypoxia) without cellular or regional heterogeneity. HIF-1α levels progressively declined during prolonged hypoxia but remained significantly elevated for at least 14 days and returned to near baseline by 21 days. Acute exposure to 8% but not 10% normobaric oxygen of animals adapted to hypobaric hypoxia for 3 wk resulted in renewed accumulation of HIF-1α. Consistent with the scenario that HIF-1α accumulation during hypoxia leads to transcriptional activation of HIF-1 target genes, we found an upregulation of GLUT-1 and VEGF transcripts that parallels the HIF-1α response. This observation is in agreement with previous studies showing reversible upregulation of VEGF and GLUT-1 expression in the brain of mice and rats exposed to hypobaric hypoxia (8, 13, 27).

It is important to note that oxygen tension is not homogeneously distributed in normal brain tissue but occurs as a log-normal distribution with values ranging from very low (<1–2 Torr) to >50 Torr, with a mean value of ~10–20 Torr (18, 24). The distribution is a direct consequence of the delivery of oxygen to the tissue by diffusion from the capillary network (17). Thus there are regions of normoxic brain tissue with...
low oxygen levels that could explain the small accumulation of HIF-1α in the control samples. Hypoxia would result in a left shift (i.e., toward lower oxygen tensions) of the distribution and more regions of tissue in which HIF-1α would accumulate. Reorganization of the capillary network after 3 wk of hypoxic exposure (2, 15) results in a right shift of the distribution through increased capillary densities and concomitant decreases in intercapillary and thereby in diffusional distances. HIF-1α does not accumulate in the hypoxic adapted tissue then, presumably because of the return to baseline tissue oxygen tensions. The effectiveness of angiogenesis together with polycythemia and metabolic changes to restore normal distribution of PtO2 during chronic hypoxia was predicted in a composite analytical model (16). In agreement with this prediction, our observation suggests that the signal triggering HIF-1α accumulation in the brain has disappeared despite a continuous systemic hypoxia. This suggests that the compensatory mechanism triggered in the brain during prolonged hypoxia was able to restore normal oxygen tension.

However, it is possible that transcription and translation of HIF-1α mRNA at 21 days was somehow reduced, leading to a lack of HIF-1α accumulation. This possibility was ruled out by the finding that, when an additional hypoxic stimulus was placed on the hypoxia-adapted tissue, HIF-1α did accumulate, indicating the continued ability of the tissue to respond to the presence of low tissue oxygen tension. Moreover, this showed that HIF-1α levels in the brain are regulated by signals reporting tissue, rather than systemic or ambient, oxygen deficiency.

This work was supported in part by National Institutes of Health grants NS-37111, NS-38632, and HL-56470.

REFERENCES


