Hyperbaric Oxygen Reduces Tissue Hypoxia and Hypoxia-Inducible Factor-1α Expression in Focal Cerebral Ischemia

Li Sun, MD; Hugo H. Marti, MD; Roland Veltkamp, MD

Background and Purpose—The usefulness of hyperbaric oxygen (HBO) and normobaric hyperoxia in acute ischemic stroke is being reexplored because both improve outcome in experimental cerebral ischemia. However, even the basic mechanisms underlying oxygen therapy are poorly understood. We investigated the effect of both oxygen therapies on tissue hypoxia and on the transcription factor hypoxia-inducible factor-1α.

Methods—Mice were subjected to filament-induced middle cerebral artery occlusion for 2 hours. Twenty-five minutes after filament introduction, mice breathed normobaric air, normobaric 100% O₂ (normobaric hyperoxia), or 100% O₂ at 3 ata (HBO) for 95 minutes. Hypoxic regions were mapped on tissue sections after preischemic infusion of the in vivo hypoxia marker EF-5. Hypoxia-inducible factor-1α protein was measured after 2-hour middle cerebral artery occlusion using immunofluorescence and immunoblotting. Vascular endothelial growth factor expression was analyzed using in situ mRNA hybridization.

Results—Severity of ischemia did not differ among groups. HBO (35.2 ± 10.4 mm²) significantly reduced the area of EF-5-stained hypoxic regions in focal cerebral ischemia compared with normobaric hyperoxia (46.4 ± 11.2 mm²) and air (49.1 ± 8 mm², P < 0.05, analysis of variance). Topographically, EF-5 fluorescence was decreased in medial striatum and in cortical ischemic border areas. Immunohistochemistry and immunoblotting revealed lower hypoxia-inducible factor-1α protein in the ischemic hemisphere of HBO-treated mice. Moreover, mRNA in situ hybridization showed lower expression of vascular endothelial growth factor in HBO and normobaric hyperoxia groups.

Conclusions—Measurement of extrinsic and intrinsic markers of hypoxia revealed that HBO improves penumbral oxygenation in focal ischemia. Modification of the transcription factor hypoxia-inducible factor-1α and its downstream targets may be involved in effects of HBO. (Stroke. 2008;39:1000–1006.)

Key Words: cerebral ischemia ▪ EF-5 ▪ HBO ▪ hypoxia-inducible factor-1α ▪ VEGF

Despite considerable advances in the understanding of the pathophysiology of cerebral ischemia over the past decades, the treatment options in acute ischemic stroke remain limited. Ischemia-induced hypoxia is a cardinal factor of primary and early secondary neuronal damage. Improving the oxygenation of hypoperfused tissue before reperfusion has been a simplistic but plausible therapeutic strategy for many years. The majority of clinical and experimental studies of “oxygen therapy” in stroke used hyperbaric oxygen (HBO). In most experimental studies, HBO had a protective effect on outcome. Three small clinical trials of HBO failed to show efficacy, but study designs have been criticized. Normobaric hyperoxia (NBO) has also a protective effect when started early during focal ischemia. NBO has the advantage of minimal logistic demands in the clinical setting. So far, however, clinical studies using NBO were either negative or too small to draw conclusions regarding efficacy.

Although potential targets of oxygen therapy, including prevention of apoptosis, inhibition of neuroinflammation, and blood–brain barrier damage, have been elucidated in recent years, the mechanisms of protection by HBO are largely unknown. Although under physiological condition, both NBO and HBO increase cerebral tissue oxygen tension, there is a lack of data supporting the fundamental hypothesis that HBO improves cerebral oxygenation during ischemia. Indeed, increasing the inspiratory oxygen concentration and partial pressure does not linearly increase the volume of O₂ dissolved in blood because blood oxygen content is largely determined by O₂ bound to hemoglobin. Also, hyperoxia induces cerebral vasoconstriction under otherwise physiological conditions and may therefore decrease cerebral blood flow in the ischemic brain.

The purpose of the present study was to determine the effect of NBO and HBO on cerebral oxygenation during focal cerebral ischemia. Hypoxic regions were delineated with the extrinsic hypoxia marker EF-5. Hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF)
expression were analyzed as intrinsic indicators of tissue hypoxia.

Materials and Methods
All experiments were performed on male C57BL/6 mice weighing 20 to 25 g (Charles River, Germany). Procedures were approved by the governmental animal care authorities. Mice underwent filament-induced middle cerebral artery occlusion (MCAO) with some modifications. Anesthesia was induced with 4% halothane in 70% N<sub>2</sub>O/30% O<sub>2</sub> and maintained with 1% halothane through face mask during surgery. A laser-Doppler flowmetry probe (Perimed) was positioned 2 mm posterior and 6 mm lateral from bregma. A silicone-coated 8–0 nylon filament was advanced into the internal carotid artery until resistance was felt and a decrease of relative cerebral blood flow was documented by laser-Doppler flowmetry. During surgery, rectal temperature was maintained at 37.0°C using a feedback heating pad. Separate groups of animals (n = 15) were operated on identically except for additional catheterization of the femoral artery for determination of physiological parameters.

Experimental Design
Mice underwent MCAO for 120 minutes. Animals were randomly assigned to one of 3 groups. Beginning 25 minutes after MCAO, awake mice were placed in a HBO chamber and breathed either normobaric air, normobaric 100% O<sub>2</sub> (NBO) or 100% O<sub>2</sub> at 3 ata for 120 minutes MCAO for determination of EF-5 distribution (n = 10/group). For HIF-1α immunoblotting (n = 5/group), HIF-1α immunofluorescence (n = 5/group), and for VEGF mRNA in situ hybridization (n = 4/group), animals were euthanized after 2-hour MCAO plus 2-hour reperfusion.

Normobaric Hypoxia
To obtain positive controls for determination of HIF-1α protein, additional mice (n = 8) were exposed to normobaric hypoxia at 6% O<sub>2</sub> for 6 hours. Hypoxia was induced by substituting nitrogen for oxygen using a Digamix SSA 18/3A pump (Woesthoff, Germany) after gradual adaptation over 1 hour. After hypoxic exposure, brains were removed for HIF immunostaining and immunoblotting, respectively.

EF-5 Immunofluorescence
To map hypoxic regions, 250 µL of the 2-nitromidazole compound EF-5 (10 mmol/L) was directly injected into the femoral vein 15 minutes before MCAO. EF-5 is reduced under hypoxic conditions, facilitating the formation of covalent linkages with cellular protein thiols, which can be detected with specific antibodies. Two hours after ischemia onset, mice (n = 10/group) were reanesthetized with 1.5% Avertin (2-2-2 tribromoethanol) and perfused with 0.9% NaCl. Brains were removed and frozen at −70°C. EF-5 accumulation was visualized using an ELK<sub>3</sub>–51 monoclonal Cy3 conjugated antibody (provided by S. Evans, University of Pennsylvania) as described. Sections were observed under a microscope (Leica) at 50-fold magnification. The entire sections were automatically scanned using a camera (Dage MTI). The area of hypoxic regions was measured on 3 sections at the levels of 1.52 mm, 0.26 mm, and −1.00 mm from bregma. EF-5 positivity was determined using a visual threshold and analyzed using MCID Elite 7.0 image analysis software (Imaging Research).

Hypoxia-Inducible Factor-1α Immunofluorescence
Anesthetized mice were perfused with phosphate-buffered saline containing zinc (concentration 3.9 mg/mL). Brains were postfixed in zinc fixation solution (0.1 mol/L TRIS, pH 7.4, 0.05% calcium acetate, 0.5% zinc acetate, 0.5% zinc chloride) overnight. After embedding in paraffin, 5-µm coronal sections were deparaffinized, hydrated, and incubated with 10% NGF. Sections were incubated with a rabbit polyclonal antibody against murine HIF-1α (NB100–449; Novus Biologicals) diluted 1:500 in NGS overnight at 4°C. After washing and incubation with a secondary goat-anti-rabbit antibody conjugated with fluorescent Cy3 (1:1000) for 1 hour, sections were coveredslipped and observed under a fluorescence microscope (544 to 590 nm; Olympus) at 200-fold magnification. HIF-1α fluorescence intensity was quantified using transfer into gray scale subtracting background using the MCID Elite 7.0 and ImageJ analysis program (Version 1.37V).

Hypoxia-Inducible Factor-1α Immunoblot
Brains were removed rapidly. Total protein was extracted using a kit (Active Motif). Hemispheres were homogenized on ice in 3 mL/g lysis buffer. Homogenates were incubated on ice for 30 minutes and centrifuged at 10 000 g for 10 minutes at 4°C. Supernatants were centrifuged again. Protein content was determined using a Bio-Rad protein assay. Protein samples containing 25 µg of protein were suspended in sample buffer (0.2 mol/L Tris-HCl, 4% sodium dodecyl sulphate, 20% glycerin, 0.001% Bromphenolblau, 200 mmol/L dithiotheriol), heated for 5 minutes at 95°C, and run on a 8% polyacrylamide gel at 250 V for 40 minutes. Proteins were transferred to a nitrocellulose membrane at 200 V for 4 hours by using a tank-blotting apparatus (Peqlab). Blots were blocked for 90 minutes, incubated overnight at 4°C with the HIF-1α primary antibody (NB100–449; Novus Biologicals), and diluted 1:500 in 5% nonfat dry milk. After rinsing in tris-buffered saline, blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000). The ECL Western blot detection reagents (Amersharm) were used for protein analysis. All blots were run in duplicate. Equal loading of lanes was verified with a β-actin antibody after stripping.

Protein signals were measured by densitometry. Bands were scanned with a scanner (Hewlett Packard). The density was measured using Adobe Photoshop 6.0 (Adobe). Background of the respective lane was subtracted and a quotient was calculated dividing ischemic by nonischemic hemisphere.

Vascular Endothelial Growth Factor mRNA In Situ Hybridization
After removal, brains were immediately frozen. VEGF in situ hybridization was performed as previously described. Single-stranded sense or antisense cRNA probes were generated by in vitro transcription using 35S-labeled UTP. Hybridization was performed on 10-µm coronal cryosections with 2.5×10<sup>6</sup> cpm/µL 35S-labeled mouse VEGF RNA probe overnight at 48°C. Sections were washed, dehydrated, and coated with Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY). After 14 days, sections were developed and counterstained with 0.02% toluidine blue. VEGF expression was detected using a dark field microscope (Leica) and digitized using MCID Elite 7.0. Gray scale intensity was measured in the dorsal cortex in both hemispheres using ImageJ. A quotient was calculated dividing ischemic by nonischemic side.

Statistical Analysis
All values are expressed as mean±SD. For comparison of data among groups, one-way analysis of variance was used followed by Tukey honestly significant difference post hoc analysis using SPSS analysis software. A probability value <0.05 was considered statistically significant.

Results
Animals of different groups underwent ischemia of the same severity as shown by laser-Doppler flowmetry measurements. Mean percentages of relative cerebral blood flow after MCAO compared with baseline were 17.0±2.7% in air, 15.0±3.2% in NBO, and 16.2±3.0% in HBO group. The Table shows physiological parameters. Arterial PaO<sub>2</sub> increased 3-fold in the NBO group and 4-fold in the HBO group.
arterial blood gases in the HBO group were measured immediately after opening the HBO chamber). All other physiological parameters remained within the normal range and were not significantly different among groups.

Hyperbaric Oxygen Reduces Penumbral EF-5 Immunofluorescence

As expected, EF-5 immunofluorescence depicted hypoxic regions in subcortical and cortical areas of the middle cerebral artery territory in air-treated mice (Figure 1, and supplemental Figure I, available online at http://stroke.ahajournals.org). EF-5 showed a typical intracellular staining (Figure 1D). Mean area of EF-5 immunofluorescence was 49.1±8 mm² in the air, 46.4±11.2 mm² in the NBO group, and 35.2±10.4 mm² in the HBO group. Thus, HBO significantly reduced the area of hypoxia in focal cerebral ischemia (P<0.05; Figure 2, and supplemental Figure II). In contrast, NBO did not affect EF-5 distribution. Topographic analysis of EF-5 fluorescence revealed that HBO prevented EF-5 deposition mainly in the medial striatum and in the cortical ischemic border areas in the majority of mice in the HBO group (Figure 2).

Hyperbaric Oxygen Reduces Hypoxia-Inducible Factor-1α in the Ischemic Hemisphere

To test whether reduction of tissue hypoxia resulted in changes of the intrinsic hypoxia marker HIF-1α, brain protein extracts were analyzed for HIF-1α expression. As a positive control, mice were exposed to 6% O₂ for 6 hours. As expected, in hemispheres harvested from hypoxic mice, a strong HIF-1α band was observed at 120 kDa, whereas protein extracts from nonischemic, normoxic control mice showed only a weak duplicate band (Figure 3, left, and supplemental Figure III). Duplication of this band is caused by different phosphorylation states of the HIF-1α protein.24 In focal ischemia experiments, HIF-1α levels in the nonischemic hemisphere were not different from nonischemic, normoxic mice. Also, no differences were noted between HIF-1α bands after MCAO in the nonischemic hemispheres among air-, NBO-, and HBO-treated mice (data not shown). Expression of HIF-1α in the ischemic hemisphere was lower than in the nonischemic hemisphere in all groups. When comparing the HIF-1α levels in the ischemic hemisphere, HBO resulted in a significant reduction of HIF-1α protein compared with air and NBO (Figure 3).

Hyperbaric Oxygen Decreases Hypoxia-Inducible Factor-1α Expression

To verify responsiveness of HIF-1α expression to tissue hypoxia, we compared HIF-1α immunofluorescence in nonischemic mice exposed to either air or hypoxia (6% O₂). As

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N=5 in each group.
*P<0.05 versus air.
†P<0.05 versus air and NBO.
‡Parameters were measured after decompression.
expected, HIF-1α immunoreactivity was substantially stronger in hypoxic (Figure 4B) compared with normoxic (Figure 4A) animals in the hippocampus, especially in CA1 and CA2. HIF-1α was also upregulated in the cortex of hypoxic (Figure 4D) compared with normoxic mice (Figure 4C).

After ischemia, constitutive expression of HIF-1α was observed in the nonischemic hemisphere, which was comparable to normoxic controls. HIF-1α immunostaining did not differ in the nonischemic hemisphere among air-, NBO-, and HBO-treated groups (data not shown). Compared with the nonischemic hemisphere, HIF-1α fluorescence was only detected in part of the cells in the ischemic hemisphere, presumably because nonfluorescent cells in the ischemic core were too severely damaged. In viable cells, however, HBO resulted in a pronounced decrease of HIF-1α immunoreactivity in the ischemic hemisphere (Figure 5A) compared with air (Figure 5C) and NBO (Figure 5B), consistent with the results of immunoblotting. The difference was pronounced in the dorsal cortex where HIF-1α fluorescence was 91±18 relative density units in air, 82±14 relative density units in NBO, and 55±18 relative density units in HBO (P<0.05; Figure 5D).

Hyperbaric Oxygen Attenuates Expression of Vascular Endothelial Growth Factor

Functional significance of altered HIF-1α expression was investigated by analysis of VEGF gene expression, because transcriptional regulation of VEGF is largely HIF-1α-dependent.25 VEGF mRNA expression was low and homogeneous throughout the nonischemic hemisphere. No difference of VEGF mRNA expression was detected in the nonischemic hemisphere among groups. In the ischemic hemisphere, VEGF expression was clearly upregulated in the dorsal cortex. In the ventral and lateral cortex, VEGF expression was only slightly increased. In the dorsal cortex of the ischemic hemisphere, a pronounced decrease of VEGF expression occurred in HBO- (Figure 6C) as compared with air-treated animals (Figure 6A). Ratio of VEGF mRNA density (ischemic/nonischemic side) was 2.0±0.2 in air, 1.5±0.1 in NBO, and 1.2±0.1 in HBO groups. Thus, both NBO and HBO induced a significant reduction of VEGF expression (P<0.05, analysis of variance). However, VEGF intensity was significantly lower in HBO- compared with NBO-treated mice.

Discussion

Hyperbaric, and more recently normobaric, hyperoxia have been advocated as potential treatments of ischemic stroke.4,26 Indeed, a plethora of experimental studies supports a beneficial effect of oxygen therapy in focal ischemia.10–14,27–31 Surprisingly, however, the most basic hypothesis regarding the protective mechanism of oxygen therapies—improved oxygenation of the ischemic brain—previously had not been
tested for HBO and only in one study for NBO.\textsuperscript{16} The major new finding of our study is that HBO significantly—and more effectively than NBO—reduces penumbral tissue hypoxia during the early phase of ischemia.

First, we used EF-5 as an extrinsic marker of tissue hypoxia. EF-5 increases with decreasing inspiratory pO\textsubscript{2} in vitro and in vivo.\textsuperscript{22,32} EF-5 deposition was augmented substantially when the inspiratory O\textsubscript{2} concentration was below 12%\textsuperscript{33} or at an intratumoral tissue pO\textsubscript{2} below 5 mm Hg, respectively.\textsuperscript{34} We have shown that binding of EF-5 increases in the ischemic penumbra after MCAO.\textsuperscript{23} Thus, EF-5 can be considered a reliable semiquantitative hypoxia marker in vitro and in vivo. EF-5 distribution was examined after 2-hour MCAO in the present study because we had previously found an improvement of in vivo MRI surrogate parameters in this setting.\textsuperscript{28} We and others have also shown that administration of HBO in the acute phase reduces infarct growth in transient ischemia in rats and mice.\textsuperscript{27–30} Remarkably, hypoxic area of EF-5 fluorescence was significantly smaller in HBO-treated mice compared with air and NBO. Topographic analysis of EF-5 fluorescence revealed that hypoxic regions were decreased in medial striatum and cortical ischemic border areas in HBO-treated mice, which represent the penumbra in this model. We could not compare the hypoxic area of EF-5 fluorescence with the infarct area in the same animal directly because mice were euthanized too early for histological infarct demarcation. However, comparison with infarct regions in a previous study with identical experimental protocol suggests that EF-5 fluorescent area exceeds ultimate infarct.

In contrast to HBO, NBO did not reduce the area of EF-5 fluorescence. Liu et al reported an increased tissue pO\textsubscript{2} in NBO-treated rats undergoing filament-induced MCAO.\textsuperscript{16} Presumably, methodological differences (Liu and coworkers used in vivo electron paramagnetic resonance oximetry) underlie these discrepant results regarding NBO.

To further corroborate these findings, we studied the protein level of HIF-1\textalpha in ischemic brain as an intrinsic marker of cellular hypoxia. Intracellular HIF-1\textalpha undergoes rapid proteasomal degradation under normoxic conditions.\textsuperscript{35} During hypoxia, HIF-1\textalpha is stabilized and translocated into the nucleus where it functions as a transcription factor for more than 100 genes, including VEGF.\textsuperscript{35} The specificity of the HIF-1\textalpha antibody was ascertained by detecting the appropriate band at 120 kDa and by demonstrating responsiveness to hypoxia on immunoblotting and immunofluorescence. Several groups have reported upregulation of HIF-1\textalpha protein concentration after global ischemia and in the ischemic hemisphere after focal ischemia in rats.\textsuperscript{24,33,36} In contrast, we found a 15% reduction of HIF-1\textalpha protein levels in the ischemic compared with the nonischemic hemisphere on immunoblots in mice breathing air. One explanation may be that total cerebral protein synthesis is suppressed during ischemia.\textsuperscript{37} However, this is unlikely to be the case for HIF-1\textalpha, because it contains an internal ribosomal entry site and is efficiently translated even under severe hypoxic conditions and even when general protein synthesis is inhibited.\textsuperscript{38,39} Instead, the reason for this reduction of HIF-1\textalpha expression below constitutive levels in the entire ischemic hemisphere became evident on immunofluorescence sections. Many cells with morphological features of neurons failed to express any HIF-1\textalpha in the ischemic area, presumably because they were too severely damaged.

Intriguingly, HBO resulted in a significant reduction of HIF-1\textalpha protein levels on immunoblots in the ischemic hemisphere compared with the NBO and air groups. This is consistent with reports showing reduced HIF-1\textalpha expression after HBO treatment in global ischemia\textsuperscript{46} and subarachnoid hemorrhage models.\textsuperscript{40} Comparing HIF-1\textalpha in the ischemic hemisphere among groups by immunofluorescence revealed that the difference in HIF-1\textalpha expression was predominantly localized in the dorsal cortex, which had also shown differ-
ences on EF-5 immunofluorescence. Theoretically, reduced expression of HIF-1α/H9251 in HBO-treated mice could also be interpreted as an indicator of more extensive cellular damage. However, we and other groups have previously shown that HBO-treated rodents have smaller infarcts and less apoptotic cells in the ischemic core and penumbra.5,29,36,40 Therefore, the calculated differences of HIF-1α/H9251 expression in the present study rather underestimates the actual attenuation of HIF-1α by HBO treatment in the ischemic hemisphere because more cells can be expected to have been viable and thereby capable of expressing HIF in the HBO group.

HIF-1α fluorescence was only slightly reduced in the NBO group compared with air, and the immunoblot revealed no significant difference between NBO- and air-treated mice. Thus, in accordance with the EF-5 immunohistochemistry data, NBO did not significantly reduce hypoxic–ischemic induction of HIF-1α protein compared with air.

In addition to comparing HIF-1α protein levels by immunohistochemistry and immunoblotting, we sought to determine the transcriptional activity of HIF-1α, which reflects hypoxia-induced stabilization and nuclear translocation of HIF-1α.35 Expression of VEGF, a target gene of HIF-1α, on in situ mRNA hybridized sections was substantially reduced in the cortical ischemic border areas in HBO-treated mice.

Interestingly, a significant but less effective reduction of VEGF mRNA was also noted in NBO-treated mice.

The primary goal of our study was to demonstrate the differential impact of oxygen therapies on ischemic tissue oxygenation. Conceivably, enhanced oxygenation supports cellular viability by improving tissue energy metabolism. It is tempting to speculate that the interaction of HBO with the HIF-1 signaling pathway is involved in its biological effects. Because of the large number of downstream effector proteins, the role of HIF-1 signaling in ischemia cannot be easily defined as either protective or deleterious. On the one hand, there is good evidence for endogenous neuroprotection by the HIF targets erythropoietin and VEGF.16,41 However, as a vascular permeability factor, VEGF is also involved in blood–brain barrier dysfunction and development of cerebral edema.42 Previously, we have shown that HBO reduces blood–brain barrier damage and edema after focal cerebral ischemia.29 Reducing VEGF expression by HBO might play a role in the protective effect of HBO on blood–brain barrier damage and edema. Moreover, HIF-1α may promote inflammatory processes and may be proapoptotic. Further studies are therefore needed to clarify the effect of HBO-induced reduction of HIF-1α.
In conclusion, our findings are of relevance for the treatment of ischemic stroke because they provide strong evidence for alleviation of penumbral tissue hypoxia by HBO.

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Disclosures
None.

References

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