Hyperbaric Oxygen Suppresses NADPH Oxidase in a Rat Subarachnoid Hemorrhage Model

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Background and Purpose—One of the major contributors to brain injury after subarachnoid hemorrhage (SAH) is oxidative stress, and 1 of the major enzymatic sources of superoxide anion production in the brain is NADPH oxidase. Therefore, we studied whether hyperbaric oxygen (HBO) suppresses neuronal NADPH oxidase in a rat model of SAH.

Methods—Eighty-three Sprague-Dawley male rats were assigned to sham, SAH, and SAH treated with HBO groups. SAH was induced by endovascular perforation. HBO (2.8 atmospheres absolutes for 2 hours) was started at 1 hour after perforation. Rats were euthanized at 6 or 24 hours, and brains were collected for histology, biochemistry, and molecular biology studies including NADPH oxidase activity, gp91phox mRNA expression, and lipid peroxidation assays. Mortality and neurological scores were evaluated.

Results—We observed an increased neuronal immunoreactivity of gp91phox at 24 hours after SAH. The upregulation of gp91phox mRNA was associated with increased oxidative stress. HBO decreased NADPH oxidase expression, activity, and the level of oxidative stress at 24 hours after SAH. HBO reduced neuronal injury and improved functional performance throughout the observation period.

Conclusion—HBO suppresses NADPH oxidase and oxidative stress in cerebral tissues at 24 hours after SAH. (Stroke. 2006;37:1314-1318.)

Key Words: hyperbaric oxygenation  ■  neuroprotection  ■  oxidative stress  ■  subarachnoid hemorrhage

We have described in one of our previous reports that hyperbaric oxygen (HBO) provided neuroprotection after subarachnoid hemorrhage (SAH) by inhibition of hypoxia-inducible factor–1α (HIF–1α) and its target genes, such as VEGF and BNIP3, which are involved for blood–brain barrier disruption and apoptotic cascades.1 Before moving into clinical trials, however, we need to address the possible brain oxidative stress related to HBO because extensive oxidative stress has been reported in SAH animal models,2 in SAH patients,3 and application of HBO may potentiate an existing oxidative stress after SAH. Three groups of contradicting reports of HBO in oxidative stress have been published; they state that HBO at pressures higher than 4 atmospheres absolutes (>4 ATA) increases oxidative stress,4 but at a lower atmosphere (≤3 ATA) does not enhance oxidative stress.5 If applied as a preconditioning strategy, HBO actually promotes antioxidant defenses by increasing the activity of superoxide dismutase.6 Therefore, we hypothesize that HBO, at a low atmosphere of 2.8 ATA, will reduce oxidative stress in brain tissues by suppression of NADPH oxidase, a major resource of oxidative stress, after SAH. NADPH oxidase is a membrane-bound enzyme, which, in nonphagocytic cells, may produce superoxide anions directed toward the cell interior.7 A phagocytic-like NADPH oxidase is present in neurons at the mRNA and protein level.8,9 It consists of the catalytic subunit flavocytochrome b558 formed by catalytic subunits gp91phox (NOX-2) and p22phox; it also consists of the following cytoplasmic subunits: adaptor protein p40phox, regulatory p47phox, and activator p67phox.10 In the present study, we examined the catalytic subunit gp91phox, NADPH oxidase activity, lipid peroxidation, and neuronal death in the presence and absence of HBO.

Materials and Methods

SAH Animal Model

Eighty-three male Sprague-Dawley rats weighing 300 to 350 g (Harlan; Indianapolis, Ind) were randomly assigned to the following groups: sham-operated, SAH without treatment, and SAH treated with HBO. Rats were intubated transorally and mechanically ventilated throughout the operation period. Rectal temperature was maintained at 37°C by a heating blanket. SAH was induced by left-sided endovascular perforation of the middle cerebral artery11 after being pretreated with 0.05 mg/kg atropine sulfate (SC) and then anesthetized by ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP). The left femoral artery was cannulated for blood pressure recordings and withdrawal of blood samples. Blood glucose, hematocrit, and blood gas were measured (1610 pH/Blood Gas Analyzer; Instrumentation Laboratories) before and after surgery. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) publication...
no. 85 to 23) and have been approved by the Animal Care and Use Committee at Loma Linda University.

Cerebral Blood Flow Measurements
We use the cerebral blood flow (CBF) measurement to evaluate the consistency of our SAH model. CBF in the contralateral cerebral cortex was measured by a laser Doppler flowmeter (Periflux System 5000; Perimed) as previously described.1 Recordings were started at 30 minutes before the induction of SAH and were continued for 1 hour after perforation. After CBF recordings, rats were either transferred to the HBO chamber or returned to their cages.

Neurological function was evaluated at 24 hours using the modified Garcia scoring system. The evaluation assessed motor function based on spontaneous activity, symmetry of limbs movements (scored 0 to 3) and climbing wall of wire cage (scored 1 to 3). A sensory score was derived from examination of responses to touch on vibrissae or sides of a trunk, each scored 1 to 3.12,13 Mortality was calculated by dividing the number of dead animals by the number of total animals used in each group at 6 and 24 hours after SAH.1

HBO Treatment
HBO (100% oxygen at 2.8 ATA for a 2-hour duration) was applied at 1 hour after SAH, using a small research hyperbaric chamber (Sechrist Industries) equipped with carbonate crystals to prevent CO2 accumulation.

Histology and Immunohistochemistry
The animals were anesthetized and then euthanized at 24 hours by transcardial perfusion with 200 mL of ice-cold 0.1 mol/L PBS, followed by 400 mL of 10% buffered formalin, to study the morphology of brain injury (Nissl, gp91phox immunohistochemistry). Brains were postfixed, cryopreserved and sectioned as described.1 For Nissl staining, sections were immersed in 0.1% cresyl violet for 2 minutes, dehydrated in the Flex tissue specimen system (Richard-Allan Scientific), cleared in xylenes, cover slipped with Permount, and observed under a light microscope (OLYMPUS BX51). For immunohistochemical analysis of gp91phox expression, sections were incubated overnight with goat polyclonal anti-gp91phox antibodies (Santa Cruz Biotechnology, Inc; Santa Cruz, Calif) diluted 1:100 at 4°C, followed by secondary antibody and DAB staining by using the ABC kit as described1 (Santa Cruz, Inc).

Lipid Peroxidation Assay
Under deep anesthesia, rat brains were perfused with 0.1 mol/L cold PBS at 6 and 24 hours after SAH. The level of malondialdehyde (MDA) in the left cerebral cortices was measured using a LPO-586 kit (Oxis Research) as previously described.14

NADPH Oxidase Activity
Left cerebral cortices collected at 6 and 24 hours after SAH were isolated and homogenized in Krebs-Ringer phosphate buffer at pH 7.4 (120 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L MgSO4, 2.2 mmol/L CaCl2, 0.1 mol/L phosphate buffer) with phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 500g for 5 minutes (4°C) and the pellet discarded. Supernatants were spun at 100 000 g in the Beckman L5-75B ultracentrifuge for 1 hour at 4°C. Cytosolic (supernatant) and membrane (pellet) fractions were separated.15 The pellet was resuspended in 150 μL of buffer and kept at −80°C until analysis.

For assaying NADPH oxidase enzymatic activity in membrane fraction, 10 μL of protein extract was added to each well of Luminometer (Microtutam LB96P; Berthold) microplate, which contained 100 μL of Krebs-Ringer phosphate buffer supplemented with 100 μmol/L lucigenin (Molecular Probes). The reaction was initiated by adding 10 μL of NADPH solution (Sigma) to the final concentration 100 μmol/L. Chemiluminescence counts were recorded every 90 seconds for 1 hour. Respective background counts were subtracted, and chemiluminescence was expressed in relative light units per micromgram protein.16

mRNA Expression of gp91phox
The mRNA expression of NADPH oxidase catalytic subunit gp91phox, in brain samples collected at 6 and 24 hours after SAH, was examined by RT-PCR as described.14 For the analysis of RT-PCR products, the densities of bands were determined by means of NIH graphic software, and results for gp91phox were expressed relative to the intensity of GAPDH bands. The RT-PCR experiments used the previously published primers.15

Statistical Analysis
Data are expressed as means±SEM. One-way ANOVA and Tukey tests were applied to verify statistical significance of differences between means. Differences in neurological scores were verified by the Means of Dunn Method. The Fisher exact test was used to analyze mortality data. A P<0.05 level was considered statistically significant.

Results
Physiological Variables
Blood gas parameters were not different among sham, SAH–no-treatment, and SAH+HBO groups (P>0.05, ANOVA; pO2: 151.3±9.5, 162.2±11.3, 152.2±8.0 mm Hg; pCO2: 35.8±2.1, 36.0±1.3, 35.3±1.3 mm Hg; pH: 7.34±0.01, 7.35±0.01, 7.35±0.01, respectively).

CBF
CBF decreased abruptly after SAH and reached its nadir in the first minute (19.46±2.73% and 19.23±2.48% of control in rats assigned to SAH–no-treatment and SAH+HBO groups, respectively; Figure 1). CBF then started to increase at a slow rate; from about 30 minutes after SAH, CBF was maintained within 60 to 70% of basal values until the end of measurements (60 minutes.). No statistical differences were obtained between SAH–no-treatment and SAH+HBO groups (P>0.05), indicating that the animals received similar brain insult after SAH.

Expression of mRNA for gp91phox
A significant rise of expression (1.7-fold) was observed at 24 hours after SAH (P<0.05 versus sham), and HBO suppressed the expression of gp91phox to pre-SAH level (P<0.05 versus SAH; P>0.05 versus sham; Figure 2). However, at 6 hours after SAH, the mRNA for gp91phox was slightly down-regulated at 0.8-fold regardless whether treated with HBO or not (P>0.05 versus sham; control: n=5; SAH and SAH+HBO: n=5 at 6 hours and n=6 at 24 hours).

Figure 1. CBF was recorded contralaterally to the ruptured artery in cerebral cortex acutely after SAH. CBF decreased below 20% of baseline level within the first minute after perforation. At 60 minutes after SAH the hypoperfusion was observed. Note no differences in CBF reduction between rats assigned to HBO and no-treatment groups (n=9 for each group).
NADPH Oxidase Activity
A peak of enzymatic activity (an increase by 117%) of NADPH oxidase in membrane fraction was noted at 24 hours after SAH ($P<0.05$ versus sham; Figure 3). HBO reduced activity in the cerebral cortex ($P<0.05$ versus SAH, $P<0.05$ versus sham). At 6 hours after SAH, the NADPH oxidase activity was insignificantly declined; however, it was within control limits after HBO (control: $n=5$; SAH and SAH+HBO: $n=5$ at 6 hours and $n=6$ at 24 hours).

**gp91** Immunohistochemistry
Traces of gp91 tissue expression were detected in control brain samples (Figure 4A). Strong enhancement of immunostaining of gp91 was observed in cortical neurons ipsilaterally to the ruptured artery at 24 hours after SAH (Figure 4A). Cerebral cortices from HBO–treated animals showed markedly decreased gp91 reactivity, which was confined to cell periphery (Figure 4B). Hippocampal neurons were not immunostained with gp91 antibodies in any of the experimental groups.

**Lipid Peroxidation**
Brain lipid peroxidation showed an increasing trend from 6 hours (252% of increase in MDA level) to an 8-fold increase at 24 hours after SAH ($P<0.05$ versus sham; Figure 5). HBO reduced MDA accumulation after SAH at 6-hour and 24-hour time intervals ($P<0.05$ versus sham, $P<0.05$ versus SAH).

**Mortality and Neurological Score**
A six-hour mortality amounted 7.41% (2 of 27 rats) in the SAH group and 3.85% (1 of 26 rats) in SAH+HBO rats (Figure 6). A remarkable difference in mortality was present at 24 hours after SAH (31.82% in SAH versus 11.76% in SAH+HBO).

Neurological scores decreased at 24 hours after no-treatment SAH and SAH treated with HBO (control: $n=13$; SAH and

**Nissl Staining**
Nissl staining showed neuronal injury predominately in the left cerebral cortex and in the left hippocampus at 24 hours after SAH (Figure 4C and 4E; $n=3$). In contrast, HBO–treated animals had well-preserved neurons in both structures. Single dark neurons were observed in brain samples after SAH treated with HBO (Figure 4D and 4F).

**Figure 2.** Top panels show representative bands of PCR products related to mRNA gp91 expression (upper bands) and to a housekeeping gene GAPDH (lower bands) in the control, SAH and HBO groups in given time intervals. Graph below presents quantitation of bands by densitometry. Expression of gp91 mRNA increased 1.7-fold at 24 hours after SAH but was at a control level in SAH treated with HBO group. Sham operated rats served as a control /c/. In Figures 2, 3 and 5: n=5 except for 24 hours for which n=6 in both SAH groups. *P<0.05 versus sham; #P<0.05 versus SAH.

**Figure 3.** NADPH oxidase activity shows a significant increase at 24 hours after SAH. HBO suppressed enzymatic activity at 24 hours but had no effect at 6 hours after SAH.

**Figure 4.** A weak expression of gp91 was observed in the control cerebral cortex. Features of cell injury (C and E) were accompanied by a strong gp91 immunostaining in the cerebral cortex at 24 hours (A) after SAH. Photographs of brain sections in HBO group show a reduction of cortical gp91 immunoreactivity (B) in parallel to a good preservation of neurons (D and F). Ctl indicates control; scale bars: A through D=200 μm; E and F=300 μm; all insets=30 μm; n=3 in each group.

**Figure 5.** MDA accumulation in left cerebral cortex is present throughout the observation period, with a tremendous increase at 24 hours (8-fold). HBO inhibited lipid peroxidation at 6 and 24 hours after SAH.
monoxide hypoxia. So far, there are no data showing that ATA reduced formation of brain free radicals after carbon monoxide oxidation in the brain. On the contrary, normobaric ATA induces seizures, increases lipid peroxidation and enhanced in the injured brain by HBO. Oxygen at 100% and 5 ATA induced increased lipid peroxidation at 6 hours after SAH, which was accompanied by an elevated gp91phox mRNA expression; (2) lipid peroxidation enhanced at 6 hours and further increased at 24 hours after SAH; (3) HBO reduced NADPH oxidase enzymatic activity and gp91phox mRNA at 24 hours after SAH and decreased lipid peroxidation at 6 and 24 hours; (4) HBO improved neurological function at 24 hours after SAH.

We have made the following observations: (1) Neuronal NADPH oxidase enzymatic activity increased at 24 hours after SAH, which was accompanied by an elevated gp91phox mRNA expression; (2) lipid peroxidation enhanced at 6 hours and further increased at 24 hours after SAH; (3) HBO reduced NADPH oxidase enzymatic activity and gp91phox mRNA at 24 hours after SAH and decreased lipid peroxidation at 6 and 24 hours; (4) HBO improved neurological function at 24 hours after SAH.

One major concern is that oxidative stress may be enhanced in the injured brain by HBO. Oxygen at 100% and 5 ATA induces seizures, increases lipid peroxidation and protein oxidation in the brain. On the contrary, normobaric therapy with 100% oxygen can be neuroprotective and does not increase oxidative stress after stroke. Very limited neuroprotection was detected after hyperbaric treatment (3 ATA) with air (21% oxygen). However, treatment with 100% oxygen under pressure between 2 and 3 ATA has been proven strongly neuroprotective and did not affect lipid peroxidation after stroke. Moreover, 100% HBO at 1.5 ATA reduced formation of brain free radicals after carbon monoxide hypoxia. So far, there are no data showing that HBO at low ATA reduces brain oxidative stress after stroke. The results from the present study addressed this issue. HBO did not enhance oxidative stress in the injured brain but rather suppressed oxidative stress by decreasing NADPH oxidase enzymatic activity. HBO treatment also reduced cell injury in the cortex and hippocampus (Figure 4). This neuroprotective effect of HBO has been translated into neurological functional improvement and a trend towards reduction of mortality after SAH.

The mechanisms for HBO induced suppression of NADPH oxidase, especially the enzymatic subunit of gp91phox, are not clear. A previous study has shown that gp91phox is part of the cytochrome b556 that belongs to the cytochrome b5 family, which has been found down-regulated at mRNA level in lung tissue after hyperoxia. Suppression of NADPH oxidase activity by HBO may derive from limited gp91phox mRNA availability because HBO reduced gp91phox mRNA at 24 hours after SAH (Figure 2). In addition, it may be secondary to HBO-induced down-regulation of other known activators of NADPH oxidase, including protein kinase C, cytokines, endothelin-1, and angiotensin II. Thirdly, HBO reduced NADPH oxidase is an oxygen-sensing agent, and it is likely that it may be down-regulated by an excess of oxygen supplied by HBO. Indeed, our previous study demonstrated that HBO reduced HIF-1α that is known to be stabilized by NADPH oxidase on hypoxia. Therefore, suppression of NADPH oxidase by HBO may mediate redox-dependent HIF-1α degradation, subsequent HIF-1α target genes involved in cell death and blood–brain barrier disruption after SAH.

One of the difficulties of studying HBO in stroke is that the neuroprotective effect of HBO is not specific but rather complex, even though theoretically HBO should influence oxygen sensitive genes such as HIF-1α and NADPH oxidase (present study). This pan-neuroprotective effect of HBO is also an advantage because brain injury is not caused by 1 factor; by interacting with multiple signaling pathways, HBO may provide more pronounced neuroprotection. As expected, NADPH oxidase is an important resource for the production of oxidative stress; however, other mediators of lipid peroxidation are involved in the cerebral oxidative stress because we detected increased lipid peroxidation at 6 hours after SAH despite a substantially unchanged expression of catalytic gp91phox subunit and activity of NADPH oxidase at that time period. Therefore, the inhibitory effect of HBO on lipid peroxidation at 6 hours after SAH is not mediated by the effect of HBO on NADPH oxidase but possibly by restoration of high-energy phosphates or rapid activation of antioxidant defenses. In addition to this, there might be a reduction of gp91phox mRNA, NADPH oxidase enzymatic activity, and oxidative stress, partially attributable to diminished microglial proliferation or leukocyte infiltration after HBO treatment. Our study showed the important role of NADPH oxidase in early brain injury at 24 hours after SAH, and that HBO may provide a crucial neuroprotection at this acute stage.

We performed CBF measurements in all animals and confirmed reproducibility of this SAH model. A similar degree of CBF impairment immediately after SAH, in rats assigned to either no treatment or HBO, revealed a robust effect of 1 HBO treatment on mortality and neurological function (Figure 6). This observation is consistent with a recent report that 1 (2.5 ATA, 90 minutes) early treatment

**Figure 6.** The effect of HBO on mortality (A) and neurologic score (B) after SAH: No animal died after sham operation. Less than 10% mortality was recorded within 6 hours and gross of deaths occurred between 6 and 24 hours. Twenty-four-hour mortality showed a remarkable difference between no treatment and HBO treated groups. Neurologic score decreased significantly after SAH; however, a smaller reduction was observed in HBO group; n=13 in control and n=15 in both SAH groups.

SAH+HBO: n=15). HBO improved neurological performance; however, a full recovery was not observed.

**Discussion**

We performed CBF measurements in all animals and confirmed reproducibility of this SAH model. A similar degree of CBF impairment immediately after SAH, in rats assigned to either no treatment or HBO, revealed a robust effect of 1 HBO treatment on mortality and neurological function (Figure 6). This observation is consistent with a recent report that 1 (2.5 ATA, 90 minutes) early treatment
is capable of reducing infarct size after permanent focal ischemia.26

For future studies we plan to determine the effects of HBO at later time-points after SAH and on vascular NADPH oxidase, because a major cause of brain injury after SAH is arterial vasospasm. Prior report stated that in vascular NADPH oxidase, the translocation of cytosolic subunit p47\textsuperscript{phox} to the membrane appears to be important and no change was found in gp91\textsuperscript{phox} expression at 72 hours after the initial hemorrhagic event.28 On the contrary, others found an increase in gp91\textsuperscript{phox} mRNA and protein expression in cerebral vessels at 12 and 24 hours after SAH,15 which seems to correlate with our findings in cerebral tissues. However, the endovascular model of SAH is not suitable to study vasospasm. We will study effects of HBO on vasospasm using 2-hemorrhage model.

We measured oxidative stress in the left cerebral cortices, ipsilaterally to the perforated artery. However, we need to point out that SAH produces global ischemia and global oxidative stress. The suppressive effect of HBO on oxidative stress is also global, affecting both hemispheres. Because long-term application of 100% oxygen may affect normal brain tissues, we used a short-term HBO for 2 hours, which is similar to HBO applications to different disorders, including wound healing, that no oxidative stress in brain tissues was indicated. Previously, the up-regulation of NADPH oxidase had been linked to brain injury in cerebral ischemia and intracerebral hemorrhage.9,14 Further studies should verify whether the suppression of NADPH oxidase is the universal mechanism of HBO-induced brain protection.

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