Hyperbaric Oxygen Reduces Blood–Brain Barrier Damage and Edema After Transient Focal Cerebral Ischemia

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Background and Purpose—Hyperbaric oxygen (HBO) has been shown to protect the brain parenchyma against transient focal cerebral ischemia, but its effects on the ischemic microcirculation are largely unknown. We examined the potential of HBO to reduce postischemic blood–brain barrier (BBB) damage and edema.

Methods—Wistar rats and C57/BL6 mice underwent occlusion of the middle cerebral artery (MCAO) for 2 hours. Forty minutes after filament introduction, animals breathed either 100% O_2 at 3.0 atmospheres absolute (ata; HBO group) or at 1.0 ata (control) for 1 hour in an HBO chamber. In rats, MRI was performed 15 minutes after MCAO and after 15 minutes and 3, 6, 24, and 72 hours of reperfusion. In mice, BBB permeability for sodium fluorescein was measured after 24-hour reperfusion.

Results—Increased BBB permeability on postcontrast T1-weighted (T1w) images had a biphasic pattern. HBO reduced volumes and intensity of enhancement. Mean abnormal enhancing volumes were 71 ± 10 mm^3 (control) versus 47 ± 10 mm^3 (HBO) at 15 minutes; 111 ± 21 mm^3 versus 69 ± 17 mm^3 3 hours; 147 ± 44 mm^3 versus 83 ± 21 mm^3 6 hours; 150 ± 37 mm^3 versus 89 ± 14 mm^3 24 hours; and 322 ± 52 mm^3 versus 215 ± 21 mm^3 72 hours (all $P<0.05$). Interhemispheric quotients of mean gray values on T1w were at 1.73 ± 0.11 versus 1.57 ± 0.07 15 minutes; 1.74 ± 0.07 versus 1.60 ± 0.06 at 3 hours; 1.77 ± 0.07 versus 1.62 ± 0.06 at 6 hours; 1.79 ± 0.10 versus 1.60 ± 0.05 at 24 hours; and 1.81 ± 0.10 versus 1.62 ± 0.07 at 72 hours (all $P<0.05$). HBO-treated mice had significantly lower postischemic BBB permeability than mice treated with either normobaric hyperoxia or room air. Vasogenic edema assessed on T2w images and histologic sections was significantly lower in HBO-treated rats.

Conclusions—Intraischemic HBO therapy reduces early and delayed postischemic BBB damage and edema after focal ischemia in rats and mice. **(*Stroke*. 2005;36:1679-1683.)**

Key Words: cerebrovascular disorders ■ hypoxia ■ magnetic resonance imaging ■ microcirculation

The permeability barrier characteristics of the cerebral microvasculature are formed by tight interendothelial cell connections, the intact subtending basal lamina, and astrocyte end feet.[1] In vitro and in vivo studies have shown that hypoxia and ischemia damage both the primary endothelial barrier and the secondary barrier which consists of the basal lamina and the integrin-mediated interactions of cells with the extracellular matrix.[2–7] The temporal course of blood–brain barrier (BBB) damage in transient focal ischemia follows a biphasic pattern.[8,9] Whereas alterations of endothelial tight junctions and integrin expression as well as the activation of matrix metalloproteinases occur within the first hours after transient ischemia, dissolution of basal lamina components is more delayed.[1–7] Alterations of the microvascular barrier function contribute to many of the secondary pathophysiological cascades of ischemia including influx of inflammatory cells and mediators, edema formation and hemorrhage.[1,10,11] Consequently, protection of the cerebral microcirculation in general and of the BBB in particular has become an important target of experimental therapeutic stroke interventions.

Since its introduction into both experimental and clinical focal cerebral ischemia in the 1960s, considerable uncertainty continues concerning the pathophysiological targets and therapeutic mechanisms of hyperbaric oxygen (HBO). The majority of experimental studies focused on putative protection of the brain parenchyma.[12–16] However, HBO has also been associated with protection of the ischemic BBB and thus reduction of edema formation.[16] Essentially, however, this concept is based on a single study in *global* cerebral ischemia which examined only one time point after 4 hours of reperfusion.[17] In contrast, the effect of HBO on the microvascular permeability barrier in the early and delayed phases after *focal* cerebral ischemia is currently unknown.

The purpose of the present study was to examine the effect of intraischemic HBO on the BBB and edema using repetitive

Received January 12, 2005; revisions received April 16, 2005; accepted April 26, 2005.

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Stroke **is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000173408.94728.79**

in vivo MRI (MRI) after transient middle cerebral artery occlusion (MCAO) in rats. The effect of HBO on postischemic BBB permeability was confirmed by measurement of a fluorescent tracer in mice.

Materials and Methods

Surgical Preparation

All experiments were performed on male Wistar rats (n=54) weighing 280 to 320 g and C57Bl/6 mice (n=20) weighing 20 to 25 g, respectively (Charles River, Germany). The procedures were approved by the governmental animal care authorities. Focal cerebral ischemia was induced using the reversible filament occlusion model as introduced by Zea-Longa et al[18] with some modifications. Anesthesia was induced with 4% halothane in O_2, and then continued via a facial mask using 0.5 to 1.0% halothane in 70% N_2O/balance O_2. During surgery, rectal temperature was held at 37.0±0.5°C. In rats, femoral artery and vein were cannulated for continuous blood pressure recording, to provide samples for blood gas measurements, and to inject the MR contrast agent.

Following filament introduction and closure of the neck, rats were placed into a MRI scanner for perfusion weighted MRI (see below). After MRI, anesthesia was discontinued and rats breathed 100% O_2 followed by oxygen enriched air. Forty minutes later, awake animals were transferred into a rodent HBO chamber

Experimental Design

Rats were randomly assigned to one of two groups. The control group breathed 100% O_2 at ambient pressure (ie, 1 atmosphere absolute; ata). Animals in the HBO group received 100% O_2 at 3.0 ata. In mice, a third additional group was treated with room air. In the HBO group, compression was started 40 min after filament introduction and was performed over 5 min. Decompression was begun 105 minutes after filament introduction at the same rate. Thus, the overall "diving time" at 3.0 ata in the chamber was 60 min.

After decompression, animals were reanesthetized and the filament was removed 120 min after introduction. In rats, a second MRI was performed 15 min after reperfusion. Animals were allowed to awaken in an O_2 enriched environment and subsequently transferred to their cages. At 3, 6, 24, and 72 hour after filament removal, rats were transiently reanesthetized for MRI scanning.

Magnetic Resonance Imaging

The animals were examined in a 2.35-T scanner (Biospec 24/40, BRUKER Medizintechnik, Ettlingen, Germany) using a previously described configuration.[19] The MR-protocol consisted of a diffusion-weighted spin-echo echo-planar imaging (SE-EPI) sequence (b-values=200, 300, 400, 500, 600, 700 s/mm^2) and a multi-spin-echo sequence (12 echoes with echo times of 8, 16, 24, 96 ms). For perfusion weighted imaging (PWI), we used a gradient-echo echo-planar imaging (GE-EPI) sequence (repetition time=1 s, echo-time=15 ms, 20 repetitions with a time resolution of 1 s/image data set) for monitoring the bolus passage of 1 mmol/kg of a paramagnetic contrast agent (Omniscan, Nycomed Amersham, Oslo, Norway). The T1-Spin Echo sequence was performed with a repetition time (TR) of 400ms, echo time (TE) of 15 ms, a flip angle of 90°, a matrix of 128x128, field of view (FOV) 4 cm x 4 cm number of slices was 6 and slice thickness=2 mm, number of averages was 8. The parameters for the T2*-fast low angle shot (FLASH) sequence were: TR=300ms, TE=20ms, flip angle=20°, matrix 128x96, FOV=4 cm x 4 cm, number of slices=6, and NA=4.). T1 weighted imaging was performed 10 min after injection of the contrast agent.

Data Analysis

During MR-imaging, a 6-slice data set was obtained covering the MCA territory. The first axial slice was selected containing the olfactory bulb. The following slices were placed at 2 mm intervals posterior to the first slice.

After optimal adjustment of contrast, DWI, T2w and T1w data were analyzed by visually identifying and encircling areas of abnormal signal intensity for each MR section using a side to side comparison on the screen. Volume of abnormal hyperintense signal on DWI, T2w and enhancement on postcontrast T1w images was calculated by multiplying the total area with section thickness.

As a surrogate parameter for intensity of postcontrast enhancement on T1w images, we calculated a interhemispheric quotient (ischemic/nonischemic) of mean gray values (MGV). For each slice, the enhancing area on T1w and the corresponding area in the contralateral hemisphere were separately encircled as the regions of interest. This method of analysis was also performed for more operator-independent analysis of MRI raw data.

For analysis of PWI during ischemia and 15 min after reperfusion, the relative cerebral blood volume and the relative mean transit time were calculated in two predefined regions of interest in the parietal cortex and the striatum in both hemispheres from the signal-time-curve determined from the PWI data set as previously described.[20]

Determination of Infarct Size

Seven rats in each group were euthanized following the MRI scan 24 hour after ischemia. Postmortem, 20 μm coronal cryosections were cut at 400 μm intervals, stained with the high-contrast silver infarct method as described[21] and analyzed using the public domain Scion Image program (release beta 4.0.2). Focal edema in the ischemic hemisphere was determined according to Swanson et al.[22]

Determination of Vascular Permeability in Mice

To quantify vascular permeability of brain vessels, 200 μL of sodium-fluorescein (Sigma) at a concentration of 6 mg/mL in PBS was injected through the tail vein 24 hour after reperfusion as previously described.[23] Sodium-fluorescein (MW 376.3) is a fluorescent tracer that does not cross the intact BBB. Thirty minutes later, mice were reanesthetized and then perfused with PBS (20 mL). Both hemispheres of the brain were removed and frozen in liquid nitrogen. To assess fluorescence, brain hemispheres were homogenized in 0.5 mol/L borate buffer (pH 10) and centrifuged (3000 rpm) for 15 min at 4°C. The supernatant was added to 1.2 mL of ethanol to precipitate proteins. Probes were again centrifuged (13000 rpm) for 20 min at 4°C and the fluorescence of the supernatant was measured at 485 nm at an excitation wavelength of 330 nm using the Safire fluoroscope (Tecan). Results are presented as a quotient of relative fluorescence units (rfu) per mg of brain tissue.

Statistical Analysis

All values are expressed as mean±standard deviation (SD). For comparison of physiological values, MRI and fluorescence data, ANOVA was used followed by Bonferroni post hoc analysis using SPSS analysis software. Histological edema was compared using t-test. A probability value <0.05 was regarded as statistically significant.

Results

Physiological parameters before MCAO and after 30 min of reperfusion were not significantly different in both groups (data not shown). Perfusion deficit during ischemia was not significantly different in cortex and striatum in the control and the HBO treatment group. For example, ischemic striatal rCBV was 0.63±0.14 in the control group versus 0.58±0.18 in the HBO group. Similarly, reperfusion after filament retraction did not differ significantly between groups. Thus, animals in both groups were exposed to ischemia of similar severity.

Abnormal hyperintense signal on postcontrast T1w images was detected already 15 min after reperfusion. Initially, volume of enhancement doubled until 6 hour, but almost no additional increase of BBB permeability was observed be-

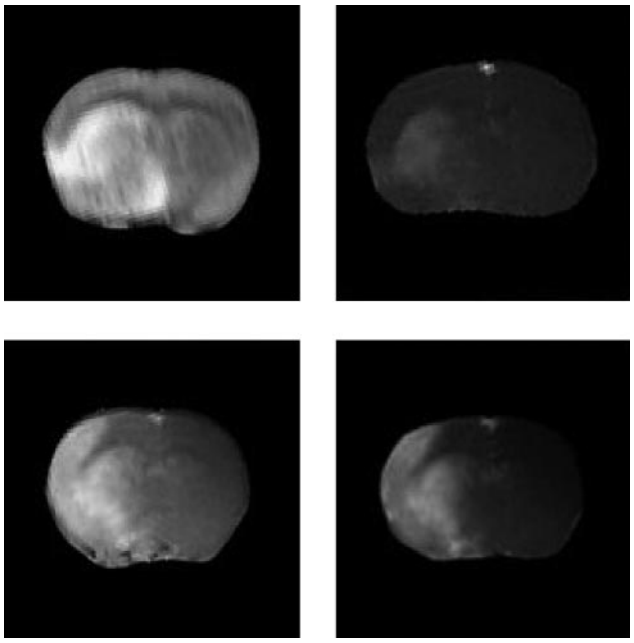

Figure 1. Axial MRI sections from rats after MCAO. Upper panel, left, After 3 hours of reperfusion, diffusion-weighted image reveals decreased diffusion mainly in the striatum. The corresponding postcontrast T1w image (right) reveals faint enhancement in part of the area with abnormal diffusion signal. Lower panel, T2w image (left) 72 hours after MCAO demonstrates extensive subcortical and cortical hyperintensity encompassing ischemic infarct and edema. BBB damage on the corresponding postcontrast T1w image (right) now also includes part of the cortex.

tween 6 hour and 24 hour. Thereafter, there was again a twofold increase until 72 hour of reperfusion (Figures 1 and 2) suggesting a biphasic pattern of BBB damage (Figures 1 and 2). HBO reduced volume of early and delayed BBB damage as measured on postcontrast T1w images (Figure 2). Postischemic BBB damage on postcontrast T1w images was separately analyzed in subcortex and cortex at 2 time points. Volumes of enhancement on T1w images in subcortex were 130 ± 43 mm^3 in control versus 73 ± 23 mm^3 (HBO; $P<0.05$) at 6 hour and 166 ± 56 mm^3 (control) versus 107 ± 13 mm^3 (HBO; $P<0.05$) at 72 hour, respectively. Volumes of enhancement on T1w in the cortex were at 6 hour: 12 ± 9 mm^3

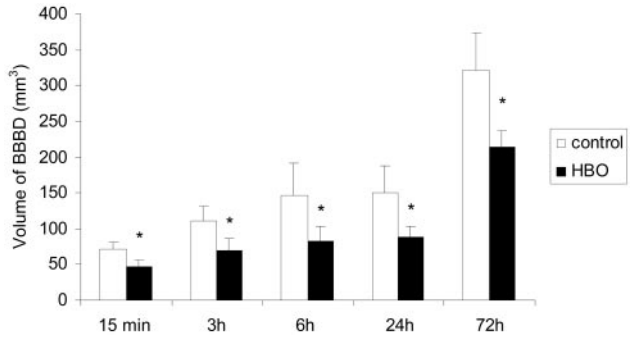

Figure 2. Volume of enhancement on postcontrast T1w MR images at various time points after reperfusion in control (100% O_2) and HBO-treated groups. HBO reduces BBB permeability in the early (first 24 hours) and late (24 to 72 hours) phases after MCAO. *$P<0.05$; ANOVA.

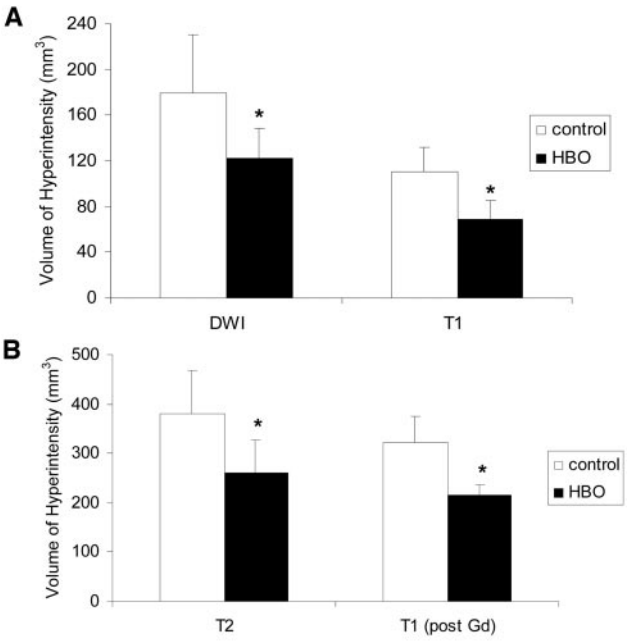

Figure 3. Comparison of hyperintense volumes on MR diffusion-weighted images at 3 hours (A) and T2w images at 72 hours after MCAO (B), with hyperintense volumes of postcontrast T1w enhancement at these time points. *$P<0.05$, ANOVA.

(control) versus 6 ± 5 mm^3 (HBO; n.s.) and at 72 hour 155 ± 48 mm^3 versus 113 ± 13 mm^3 (HBO; $P<0.05$). Thus, BBB damage at 6 hour was almost entirely located in the striatum.

The quotients of MGV were significantly lower in the HBO treated animals at all time points. Quotients of MGV were at 15 minutes of reperfusion: 1.73 ± 0.11 (control) versus 1.57 ± 0.07 (HBO); at 3 hour: 1.74 ± 0.07 versus 1.60 ± 0.06; at 6 hour: 1.77 ± 0.07 versus 1.62 ± 0.06; at 24 hour: 1.79 ± 0.10 versus 1.60 ± 0.05; at 72 hour: 1.81 ± 0.10 versus 1.62 ± 0.07 (all $P<0.05$).

At all time points, the area of postcontrast enhancement on T1w images was located within the hyperintense areas on DWI and T2w images, respectively (Figures 1, 3a, and 3b). Volume of hyperintensity on DWI at 6 hour corresponds closely to parenchymal damage on silver stained histological sections at 24 hour in this paradigm (data not shown). Because HBO also significantly reduced MRI lesion volume on DWI and T2w images, ratios of BBB-damage and parenchymal damage were calculated for two time points. At 6 hour we divided volume of postcontrast enhancement on T1w images by volume of hyperintensity on DWI. At 72 hour, volume of enhancement on T1w volume was divided by volume of hyperintensity on T2w images. At 6 hour of reperfusion, the ratio was 0.70 ± 0.16 in the control group versus 0.59 ± 0.15 in the HBO group ($P>0.05$). At 72 hour, the ratio was 0.85 ± 0.23 (control) versus 0.87 ± 0.17 (HBO).

To confirm our MR findings, postischemic BBB permeability was measured fluoroscopically after injection of the tracer Na-Fluorescein. After 24 hour of reperfusion, BBB permeability measured as the interhemispheric quotient (ischemic/nonischemic) of relative fluorescence units was sig-

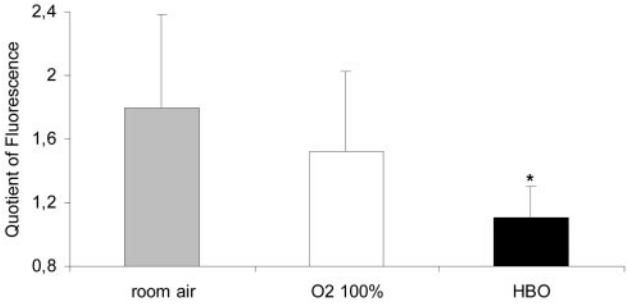

Figure 4. Postischemic BBB permeability 24 hours after reperfusion in mice. Fluorescence was measured postmortem as relative fluorescence units (rfu) in each hemisphere 30 minutes after injection of sodium fluorescein. Interhemispheric quotients of rfu were calculated for each animal. *$P<0.05$, ANOVA.

nificantly larger in mice receiving either room air or normobaric hyperoxia than in HBO treated mice (Figure 4).

The effect of HBO on ischemic focal brain edema was estimated by two ways. Hyperintense areas on T2w images at 24 hour and at 72 hour represent both parenchymal damage and brain edema. Volume of hyperintensity in the ischemic hemisphere was significantly larger in control than in HBO treated animals on T2w images 72 hour after MCAO (Figure 3b). Furthermore, edema formation was calculated for a subgroup of animals which were euthanized already 24 hour after ischemia (n=7 per group). Acccording to the formula for measurement of histological infarct size adjusted for edema introduced by Swanson et al,[22] volume attributable to focal edema was 72 ± 29 mm^3 in the control and 41 ± 14 mm^3 in the HBO group ($P<0.05$; Figure 5).

In contrast to a previous report,[24] no hypointense areas on gradient echo susceptibility weighted images (T2*) corresponding to punctate parenchymal hemorrhage could be detected in any animal in either group after 24 hour and 72 hour of reperfusion in our study.

Discussion

The effect of HBO on the postischemic BBB so far has only been studied by Mink and Dutka who examined brain vascular permeability at a single time point as early as 4 hours after *global* cerebral ischemia.[17] The major new finding of the present study is that intraischemic HBO reduces early and delayed BBB damage and edema after transient *focal* cerebral ischemia in rats. This effect was confirmed with a second BBB permeability assay in mice.

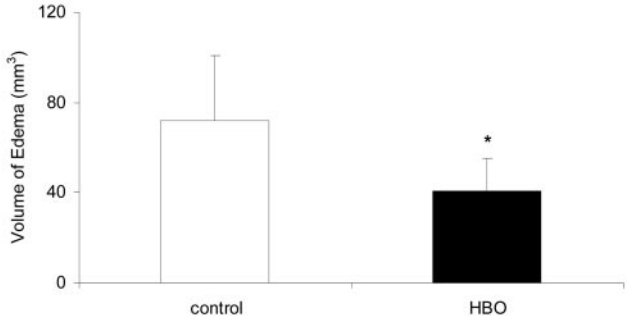

Figure 5. Estimation of focal cerebral edema on histologic sections for a subset of rats (n=14) humanely killed after 24-hour reperfusion according to Swanson et al[22].*$P<0.05$, *t* test.

The present study used enhancement on repetitive postcontrast T1w MRI as a surrogate parameter for postischemic BBB damage. Similar to previous experimental MRI studies,[24,25] we found weak contrast enhancement beginning in the early reperfusion period which increased until 6 hour of reperfusion, then did not progress until 24 hour, but doubled thereafter until 72 hour of reperfusion. This biphasic pattern is consistent with studies using other methods and compounds for measurement of BBB permeability such as Evans-Blue stain and other tracers.[8,9] Intriguingly, HBO reduced BBB damage during both phases. The methods used in our present study did not allow identification of the mechanisms underlying BBB protection by HBO but its effect on both phases suggests multiple pathways of action interfering with BBB breakdown. Loss of the permeability barrier function in the early phase of hypoxia-ischemia involves dysfunctioning of endothelial tight junctions which is associated with relocation and upregulation of the tight junction proteins occludin and ZO-1.[2,3] Also, integrins that mediate the interaction between endothelial cells, astrocytes and the extracellular matrix rapidly decrease after ischemia.[4–6] In contrast, reduction of basal lamina components such as collagen 4 and laminin has been documented at later time points.[4–6] How HBO may interact with this molecular machinery is unknown.

Consistent with studies using MRI or correlation of tissue histology with endogenous albumin extravasation,[25–27] the area of postcontrast enhancement on T1w images in our experiments was located within the DWI hyperintensity at 6 hour and almost as extensive as T2w hyperintensity at 72 hour. Indeed, after volumes of increased BBB permeability were adjusted for parenchymal lesion size, no significant differences were found between groups. This is consistent with findings by Singhal et al who compared normobaric hyperoxia with room air.[28] The protective mechanisms of oxygen therapies may ameliorate ischemic damage in various cell types in parallel. Alternatively, because the neuronal-glial and the microvascular compartment are pathophysiologically interwoven in the neurovascular unit,[29] HBO may trigger a sequence of protective events originating in one and secondarily affecting the other compartment.

A major concern with HBO therapy is increased production and detrimental effect of reactive oxygen species which play an important role in ischemic microcirculatory damage. Although we did not directly measure oxygen radical effects such as lipid peroxidation, the present study does not support a net negative effect of HBO-induced reactive oxygen species generation on the postischemic BBB. Similar findings were reported by Singhal and coworkers using normobaric hyperoxia in cerebral ischemia.[28]

Protection of the BBB by HBO during the first three days after ischemia may be of relevance for clinical stroke. Damage of the BBB has been associated with important complications of ischemia such as brain edema and secondary hemorrhage.[1,10,11] Intriguingly, focal brain edema in the ischemic hemisphere was reduced by HBO suggesting a protective potential especially in large MCA infarction.

In summary, early administration of HBO therapy can reduce rapid and delayed BBB damage and brain edema after experimental transient focal cerebral ischemia. The precise molecular and cellular targets of this HBO effect and its potential implications for reduction of secondary microvascular complications of ischemic stroke require further investigation.

Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (VE 196/2-1). The authors thank Petra Freudenmacher for excellent technical assistance.

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