Oxygen therapy improves energy metabolism in focal cerebral ischemia

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ABSTRACT

Oxygen therapy (OT) with hyperbaric oxygen (HBO) or normobaric hyperoxia (NBO) improves the oxygenation of penumbral tissue in experimental ischemic stroke. However, whether this results in the improvement of energy metabolism is unclear. We investigated the effect of both OTs on tissue acidosis and on ATP production. Beginning 25 min after filament middle cerebral artery occlusion (MCAO), mice breathed either air, 100% O\textsubscript{2} (NBO), or 100% O\textsubscript{2} at 3 ata (HBO) for 60 min. Regional tissue pH was measured using the umbelliferone fluorescence. Regional ATP concentration was depicted by substrate-specific bioluminescence. Severity of ischemia did not differ among groups in laser-Doppler flowmetry. Both NBO (70.1±14.0 mm\textsuperscript{3}) and, more effectively, HBO (57.2±11.9 mm\textsuperscript{3}) significantly reduced volume of tissue acidosis compared to air (89.4±4.0 mm\textsuperscript{3}, p<0.05). Topographically, acidosis was less pronounced in the medial striatum and in the cortical ischemic border areas. This resulted in significantly smaller volumes of ATP depletion (77.8±7.7 mm\textsuperscript{3} in air, 61.4±15.2 mm\textsuperscript{3} in NBO and 51.2±14.4 mm\textsuperscript{3} in HBO; p<0.05). In conclusion, OT significantly improves energy metabolism in the border zones of focal cerebral ischemia which are the areas protected by OT in this model.

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ATP depletion
Tissue acidosis
Hyperbaric oxygen
Normobaric hyperoxia

1. Introduction

Despite considerable advances in the understanding of the pathophysiology of cerebral ischemia over the past decades (Dirnagl et al., 1999; Lo et al., 2005; Mies et al., 1991), treatment of acute ischemic stroke remains limited. Ischemia-induced hypoxia and the consequent bioenergetic failure are cardinal factors of primary and early secondary neuronal damage (Dirnagl et al., 1999; Hara et al., 2000; Hata et al., 1998; Siesjo, 1992). Under ischemic conditions, the decreased oxygen delivery limits oxidative phosphorylation in the mitochondria, the major pathway of cellular ATP generation. The resulting shortage of ATP generation leads to a disturbance of a stable membrane potential which results in peri-infarct depolarizations (PIDs) (Mies et al., 1993; Shimizu et al., 2000; Takano et al., 2007) and further energy failure induces terminal ischemic cell membrane depolarization. Other energy-dependent processes, such as the reuptake of excitatory amino acids, are also impaired. These pathophysiological cascades escalate to a vicious circle of excitatory neurotoxicity, calcium overload and further metabolic challenges of the comprised tissue leading to secondary ischemic brain damage (Iijima et al., 1992; Kristian, 2004; Mies et al., 1991; Siesjo, 1992).

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Improving the oxygenation of hypoperfused tissue has been a simplistic but plausible therapeutic strategy for many years. In many experimental studies, oxygen therapy (OT) with hyperbaric oxygen (HBO) or more recently normobaric hyperoxia (NBO) provided consistent neuroprotection if OT was started early after ischemia-onset (Poli and Veltkamp, 2009; Singhal, 2007; Zhang et al., 2003), but the effectiveness of OT is greater in reperfusion models (Veltkamp et al., 2005, 2006). 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 underlying mechanisms of protection by HBO remain largely unknown (Zhang et al., 2005). In the past, the most basic concept of OT, namely improved availability of oxygen and consecutive improvement of cerebral energy metabolism, has barely been investigated. In a previous study, we showed that HBO reduced penumbral tissue hypoxia during the early phase of ischemia and improved oxygenation of the ischemic brain (Sun et al., 2008). Similarly, Liu et al. (2006) reported an increased cerebral tissue pO2 in the penumbra in NBO treated animals undergoing MCAO. However, whether improved oxygenation improves energy metabolism during ischemia so far is unknown.

The purpose of the present study, therefore, was to determine the effect of NBO and HBO on cerebral energy metabolism in transient focal cerebral ischemia.

2. Results

Animals of different groups underwent ischemia of the same severity as shown by LDF measurements. Relative CBF dropped to 16.0±4.7% of baseline in the air, 14.0±3.2% in the NBO, and 17.2±3.0% in the HBO group (n=9/group, p>0.5, ANOVA). Table 1 shows physiological parameters. Arterial paO2 increased threefold in the NBO and fourfold in the HBO group (arterial blood gasses in the HBO group were measured immediately after opening of the HBO chamber). All other physiological parameters remained within the normal range and were not significantly different among groups.

2.1. Oxygen therapies reduce ATP depletion in the ischemic hemisphere

Regions of ATP depletion were sharply demarcated in subcortical and cortical areas of the MCA territory in air treated mice (Fig. 1). Mean volume of ATP depletion was 77.8±7.7 mm³ in the air, 61.4±15.2 mm³ in the NBO, and 51.2±14.4 mm³ in the HBO group. Thus, HBO and NBO significantly reduced the volume of ATP depletion in focal cerebral ischemia (p<0.05; ANOVA). No significant difference was observed between both oxygen therapies (Fig. 2). Furthermore, topographic analysis of ATP depletion revealed that ATP production remained relatively intact in the medial striatum and the cortical ischemic border areas of oxygen treated mice (Fig. 3). No difference of ATP depletion was observed between HBO treated animals with and without decompression (data not shown).

2.2. NBO and HBO reduce penumbra tissue acidosis

Umbelliferone fluorescence revealed a large volume of severe acidosis which clearly demarcated the ischemic hemisphere (Fig. 4). Consistent with a previous report (Back et al., 2000; Hossmann, 1994), severely acidotic areas extended further into the medial striatum and the dorsolateral cortex compared to areas of ATP depletion (Fig. 2). Mean tissue pH inside the acidic area was 6.19±0.05, 6.22±0.05 and 6.21±0.07, respectively (p>0.1). Mean volume of tissue acidosis was 89.4±4.0 mm³ in the air, 70.1±14.0 mm³ in the NBO, and 57.2±11.9 mm³ in the HBO group (p<0.05, ANOVA). Thus, both NBO and HBO induced a significant reduction of tissue acidosis. HBO decreased tissue acidosis more effectively compared to NBO (p=0.047). Topographic analysis revealed that oxygen therapy prevented enlargement of tissue acidosis mainly in the medial striatum and in the dorsal cortex of the ischemic hemisphere in the majority of mice in therapy groups (Fig. 5).

3. Discussion

This study shows for the first time, that: 1) NBO and HBO significantly decrease the ischemic area in which ATP is depleted. 2) Both oxygen therapies significantly reduce the area of tissue acidosis during the early phase of ischemia.

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### Table 1 – Physiological parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Air</th>
<th>NBO</th>
<th>HBO ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>paO2 (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min before MCAO</td>
<td>127.5±12.3</td>
<td>124.3±15.6</td>
<td>117.5±13.6</td>
</tr>
<tr>
<td>120 min after MCAO</td>
<td>116.1±15.8</td>
<td>419.5±26.4*</td>
<td>667.2±29.3**</td>
</tr>
<tr>
<td>paCO2 (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min before MCAO</td>
<td>46.6±3.9</td>
<td>47.2±3.6</td>
<td>47.8±4.4</td>
</tr>
<tr>
<td>120 min after MCAO</td>
<td>47.3±4.1</td>
<td>48.0±3.3</td>
<td>46.4±3.9</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min before MCAO</td>
<td>7.39±0.04</td>
<td>7.37±0.05</td>
<td>7.39±0.03</td>
</tr>
<tr>
<td>120 min after MCAO</td>
<td>7.38±0.03</td>
<td>7.38±0.04</td>
<td>7.39±0.05</td>
</tr>
</tbody>
</table>

N=5 in each group.
* P<0.05 versus air.
** P<0.05 versus air and NBO.
*** Parameters were measured after decompression.
Normal tissue ATP content is an important indicator of intact cellular energy metabolism. After onset of focal cerebral ischemia, ATP content rapidly decreases during the first 5 min and falls to approximately 15–30% of the ATP concentration measured in the non-ischemic hemisphere during the first 2 h after ischemia-onset (Folbergrova et al., 1992; Paschen et al., 2000). Regions in which the ipsilateral ATP concentration fell below 30% of the contralateral hemisphere correlated well with the reduction of regional cerebral blood flow below 20 mL/100 g/min which have been considered as ischemic core (Back et al., 2000; Paschen et al., 2000). In the present study, the volume of ATP depletion was significantly smaller in both HBO and NBO treated mice compared to air. We and others have previously shown that oxygen therapy reduces tissue hypoxia in the acute phase of ischemia (Liu et al., 2006; Sun et al., 2008). Hypoxic regions were decreased in medial striatum and cortical ischemic border areas which represent the tissue at risk in this model (Sun et al., 2008). Intriguingly, the topographic distribution of hypoxia in our previous experiments and the area of ATP depletion in the ischemic hemisphere in the present study overlapped. Although we did not control effects on regional cerebral blood flow, these findings suggest that improving oxygenation in the ischemic border zones maintains oxidative energy metabolism in these areas.

Fig. 2 – Effect of OT on total volumes of ATP depletion and tissue acidosis among groups. A: Volume of ATP depletion was significantly reduced in both OT groups. There was no difference between HBO and NBO. B: NBO and, more effectively, HBO reduced volume of tissue acidosis (*significant difference compared to air, **significant difference compared to air and NBO, p<0.05, ANOVA).

Fig. 3 – Topographic distribution depicted as percentage range of animals in each group with ATP depletion in respective area. Dark red: >80%; bright red: 40% to 80%; yellow: <40% of animals within respective group. Both OTs reduce ATP depletion in medial striatum and in cortical ischemic border areas.

Fig. 4 – Representative sections of tissue pH on umbelliferone fluorescence in same animals of air, NBO and HBO group that corresponded to those in Fig. 1. Area of tissue acidosis with pH values below 6.4 was sharply demarcated in ischemic hemisphere and was more extended compared to area of ATP depletion.
Ischemia-induced tissue acidosis is associated with secondary neuronal damage (Back et al., 2000; Siesjo, 1992). In cerebral ischemia, acidosis mainly results from an increased production of lactic acid by anaerobic glycolysis. Accordingly, the cerebral tissue pH can be considered as a parameter reflecting the state of brain energy metabolism. In the present study, both oxygen therapies reduced the volume of acidotic brain tissue in the ischemic hemisphere substantially. Interestingly, previous studies showed a close correlation between tissue acidosis with pH values below 6.4 and a cerebral blood flow threshold of \(32 - 35 \text{ L/100 g/min}\) (Hossmann, 1994). Although the relationship between cerebral blood flow and the different surrogate markers of energy metabolism is also time-dependent, the mismatch area between the regions depleted of ATP and those being severely acidotic, respectively, has been viewed as "metabolic penumbra" (Hossmann, 1994) as well as the mismatch between cerebral protein synthesis inhibition and ATP depletion (Mies et al., 1991). In the present study, topographic analysis revealed a reduction of tissue acidosis in the OT groups. Taken together, our findings suggest that OT in the early phase of ischemia attenuates the metabolic consequences of ischemic blood flow thresholds resulting in a reduced size of the ischemic core and a topographic shift of the penumbra. This is consistent with previous studies showing rapid neuroprotective effects in the early phase of focal cerebral ischemia using diffusion-weighted MR imaging in the very early phase of ischemia (Henninger et al., 2006; Veltkamp et al., 2005) and with extension of the time window for thrombolysis, respectively (Kim et al., 2005; Sun et al., 2010).

Beyond being the central mechanism of early cell death, hypoxia-induced energy failure is involved in important secondary pathophysiological cascades of cerebral ischemia including peri-infarct depolarizations, accumulation of glutamate, and calcium flux in acute ischemia (Dirnagl et al., 1999; Iijima et al., 1992; Kristian, 2004; Mies et al., 1991; Siesjo, 1992). Indeed, there is convincing evidence that OT is affecting some of these key processes substantially. Hypoxia was associated with prolonged cortical spreading depression while increasing the \(O_2\)-availability shortened the duration of cortical spreading depression (Takano et al., 2007). Another study reported that NBO largely prevented peri-infarct depolarizations in focal cerebral ischemia in mice (Shin et al., 2007). Moreover, using microdialysis in a rat MCAO model, HBO decreased glucose, pyruvate and glutamate after transient ischemia (Badr et al., 2001). In stroke and traumatic brain injury patients, respectively, HBO reduced the lactate concentration in cerebrospinal fluid (Hollbach et al., 1972; Rockswold et al., 2001).

In conclusion, our findings provide strong evidence for a beneficial effect of OT on energy metabolism in the border zones of focal ischemia. Because it has also protective effects on the ischemic microcirculation, OT could be viewed as a promising complementary treatment strategy combined with thrombolytic treatment in the clinical setting.

4. Experimental procedures

All experiments were performed on male C57BL/6 mice, weighing 20 to 25 g (Charles River, Germany). The procedures were approved by the governmental animal care authorities (Regierungspräsidium Karlsruhe). Mice underwent filament-induced middle cerebral artery occlusion (MCAO) as previously described (Longa et al., 1989) with some modifications (Sun et al., 2008). Anesthesia was induced with 4% halothane in 70% \(N_2O/30\% O_2\) and maintained with 1% halothane via facial mask during surgery. A laser-Doppler flowmetry probe (LDF, Perimed, USA) was positioned 1.5 mm posterior and 3 mm lateral from bregma. A silicone-coated 8-0 nylon filament was advanced into the internal carotid artery toward the middle cerebral artery (MCA) until a resistance was felt and a decrease of relative CBF (rCBF) was documented by LDF. During surgery, rectal temperature was maintained at 37.0±0.5 °C using a feedback heating pad.

Separate groups of animals (n=15) were operated identically except for additional catheterization of the femoral artery for determination of physiological parameters.

4.1. Experimental design

Animals were randomly assigned to one of three groups (n=9/group). Beginning 25 min after MCA occlusion, awake mice were placed in an experimental HBO chamber which breathed either normobaric air, normobaric 100% \(O_2\) (NBO) or 100% \(O_2\) at 3 atmospheres absolute (ata, HBO) for 60 min. At the end of the
90 min ischemia period, mice were taken out from the chamber, anesthetized and then euthanized by immersion in liquid nitrogen to prevent catabolism of ATP. Postmortem, brains were removed in a cold box at −20 °C and kept at −80 °C in a freezer until further processing. Brains were cut at −20 °C in a cryostat into 20 μm thick coronal sections at six defined levels that encompassed the entire ischemic lesion and covered the bregma coordinates from 1.78 mm to −3.02 mm.

To rule out that ATP is already partially catabolized during decompression period in the HBO group, a separate group of animals (n = 10) underwent hyperbaric oxygen therapy at 3 ata in a human hyperbaric chamber (Haux, Germany). Immersion of anesthetized mice in liquid nitrogen was performed in the chamber without decompression at the end of HBO therapy.

4.2. Tissue ATP imaging

Substrate-specific bioluminescence was used to determine the regional concentrations of ATP on brain sections as described (Mies et al., 1999). Briefly, after heating to 50 °C for 10 min, 10 mL of arsenate buffer solution (1 mol/L HEPES, 1 mol/L sodium) was mixed with glycerol (100 mg), gelatine (200 mg) and polystyrene (100 mg). Then, 220 mL of firefly lantern (Sigma Aldrich) and 20 μL of MgCl₂ (1 mol/L) were added. After centrifugation at 20,000 g for 2 min, the supernatant containing the necessary enzymes, coenzymes and cofactors for substrate-specific biochemical reaction was transferred into a template and frozen into a block. The frozen block was sliced into sections of 60 μm thickness at −20 °C. Each brain section was covered with a frozen enzyme solution to evoke the bioluminescent reaction upon thawing. Substrate-dependent bioluminescence was recorded with a 50 mm lens. Bioluminescence images were recorded using an image processor (Field-Illumination-System, Photonics, Germany). ATP content was evaluated by comparing the optical density of the ischemic tissue with that of the opposite hemisphere.

4.3. Tissue pH imaging with umbelliferone fluorescence

Tissue pH was visualized using the previously described umbelliferone fluorescence technique (Csiba et al., 1983). Briefly, electrophoresis filter paper was immersed in an umbelliferon solution (250 mg umbelliferone, Carl Roth, Germany, diluted in 100 mL ethanol) for 60 min. After drying at room temperature, filter paper was cut and mounted on a square cover glass. Immediately after cryosecting, brain sections were placed inside the cryostat on umbelliferone-impregnated filter paper. After transferring to the illuminator via the liquid nitrogen bath, sections and different pH standards (0.1 mol/L NaHPO₄, 0.5% albumin and 0.01% umbelliferone, pH 6.3, 6.6, 6.8, 7.0, 7.2 and 7.4) were illuminated with 340- and 370-nm ultraviolet light, and two fluorescence photographs were taken by using a 450 nm barrier filter. For qualitative evaluation of tissue pH changes, the brightness of the pH-dependent 370-nm fluorescence image was compared with that of the contralateral hemisphere. For quantitative analysis, the optical densities of the fluorescence images obtained at 370 and 340 nm were subtracted by using the above described processor (Field-Illumination-System, Photonics, Germany), and the resulting image was calibrated by plotting optical density against the pH of the standards.

4.4. Data analysis

ATP depletion and tissue acidosis were defined as the decline to less than 30% of the mean value of the contralateral side and a pH below 6.4, respectively (Back et al., 2000). The areas of tissue acidosis and ATP depletion were measured on six 20 μm thick sections at the levels of 1.78 mm to −3.02 mm from bregma. The volume was calculated by integration of the lesion areas at six equidistant levels with a distance of 0.8 mm among sections.

4.5. Statistical analysis

All values are expressed as mean ± standard deviation (SD). For comparison of data among groups, one-way ANOVA was used followed by post hoc Fisher’s protected least significant difference test using SPSS analysis software. A p value <0.05 was considered as statistically significant.

Disclosure/conflict of interest

The authors report no conflicts of interest.

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REFERENCES


