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WHEN THE BRAIN GOES DIVING: GLIAL OXIDATIVE METABOLISM MAY CONFER HYPOXIA TOLERANCE TO THE SEAL BRAIN

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Abstract—Deep diving mammals have developed strategies to cope with limited oxygen availability when submerged. These adaptations are associated with an increased neuronal hypoxia tolerance. Brain neurons of the hooded seal *Cystophora cristata* remain much longer active in hypoxic conditions than those of mice. To understand the cellular basis of neuronal hypoxia tolerance, we studied neuroglobin and cytochrome c in *C. cristata* brain. Neuroglobin, a respiratory protein typically found in vertebrate neurons, displays three unique amino acid substitutions in hooded seal. However, these substitutions unlikely contribute to a modulation of O_2 affinity. Moreover, there is no significant difference in total neuroglobin protein levels in mouse, rat and seal brains. However, in terrestrial mammals neuroglobin resided exclusively in neurons, whereas in seals neuroglobin is mainly located in astrocytes. This unusual localization of neuroglobin is accompanied by a shift in the distribution of cytochrome c. In seals, this marker for oxidative metabolism is mainly localized in astrocytes, whereas in terrestrial mammals it is essentially found in neurons. Our results indicate that in seals aerobic ATP production depends significantly on astrocytes, while neurons rely less on aerobic energy metabolism. This adaptation may imbue seal neurons with an increased tolerance to hypoxia and potentially also to reactive oxygen species, and may explain in part the ability of deep diving mammals to sustain neuronal activity during prolonged dives.

Key words: astrocytes, cytochrome c, deep diving, lactate shuttle, neuroglobin, seal.

Energy metabolism of mammalian brains is fueled by carbohydrates and ketone bodies. For many years, it was thought that glucose constitutes the sole energy substrate for neurons. Glucose was assumed to be directly provided to neurons via the extracellular space by the cerebral circulation. Recently, it has been proposed that—in addition to glucose—neurons largely rely on lactate to sustain their activity (for review, see Pellerin, 2005, 2008). According to this "lactate shuttle" hypothesis, glycolysis occurs predominantly in glial cells (astrocytes), which produce substantial amounts of lactate (Peng et al., 1998). Lactate is taken up by neurons, which appear to have a preference to oxidize imported lactate instead of producing lactate/pyruvate by their own glycolysis (Peng et al., 1998; Itoh et al., 2003). Thus most oxygen is consumed by the respiratory chain in the mitochondria of brain neurons. As mammals rely on oxidative metabolism to meet their energy demands, an acute lack of oxygen (hypoxia) usually leads to severe consequences for the animal, especially in hypoxia-sensitive tissues. Most terrestrial mammals show massive brain dysfunctions already after seconds of oxygen shortage, resulting from decreasing ATP levels, which affects ion homeostasis and therefore the excitation of neurons (Katsura et al., 1994; Lutz and Nilsson, 2004).

Interestingly, diving mammals have developed various physiological adaptations to cope with severe hypoxia (for review, see: Blix and Folkow, 1983; Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007). High concentrations of hemoglobin (Hb) in blood and myoglobin (Mb) in skeletal and heart muscle increase the capacity to store oxygen (Scholander, 1940; Lenfant et al., 1970; Snyder, 1983; Polasek and Davis, 2001). Selective vasoconstriction assures the blood circulation in O_2-sensitive organs such as the brain and reduces the O_2-consumption in other organs (e.g. kidney) to a minimum. Some species may further reduce their metabolic rate by regional hypothermia (Butler and Jones, 1997). Moreover, seals may also actively cool their brains to reduce oxygen demand (Odden et al., 1999). On the cellular level, almost nothing is known concerning how diving mammals maintain neuronal activity during severe hypoxia (Ramirez et al., 2007).

The hooded seal (*Cystophora cristata*) is known to dive as deep as 1000 m for up to 1 h (Folkow and Blix, 1999). Recently, Folkow et al. (2008) showed that cortical neurons from the hooded seal are able to cope with severe oxygen deprivation and remain much longer active under hypoxic stress than mouse neurons. Folkow et al. (2008) speculated that neuroglobin (Ngb) (Burmester et al., 2000) contributes to the remarkable hypoxia tolerance of the seal's brain. Ngb, a globin related to Mb and Hb, is expressed in neurons of the CNS and peripheral nervous system (Burmester et al., 2000). Ngb binds oxygen reversibly via an iron-ion with an oxygen affinity that is roughly similar to

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Abbreviations: CNS, central nervous system; Cyt c, cytochrome c; GFAP, glial fibrillary acidic protein; Hb, hemoglobin; Mb, myoglobin; Ngb, neuroglobin; nHb, nerve hemoglobin; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline.

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that of Mb (Burmester et al., 2000; Dewilde et al., 2001). Several studies have shown that Ngb has neuroprotective properties (Sun et al., 2001, 2003; Khan et al., 2006). On the cellular level, Ngb might have a similar role as Mb in muscle cells, thus acting as respiratory protein that supplies O_2 to the metabolically highly active neurons. Alternatively, Ngb may scavenge reactive oxygen or nitrogen species (ROS/RNS) (e.g. Herold et al., 2004; Wang et al., 2008). Various other or additional functions of Ngb have been proposed (for review, see: Hankeln et al., 2005; Burmester and Hankeln, 2009), but regardless of its actual physiological role, there is convincing evidence that Ngb is tightly linked to the oxidative metabolism (Schmidt et al., 2003; Bentmann et al., 2005).

Here we have investigated the presence and distribution of Ngb in the hooded seal (*C. cristata*). Ngb levels did not differ in brains of seals, rats and mice, but we found an unprecedented localization of Ngb and cytochrome c (Cyt c) in astrocytes of the seal neocortex. We hypothesize that in seals, the cerebral energy metabolism fundamentally differs from that of terrestrial mammals in that astrocytes assume at least partly oxidative metabolism, while neurons largely function anaerobically.

EXPERIMENTAL PROCEDURES

Animals

Adult rats (Sprague–Dawley, $n=2$) and mice (Balb/C; C57BL/6, $n=6$) were maintained under constant conditions (12-h light/dark cycle, room temperature 21±1 °C; food and water *ad libitum*). Rats and mice were killed with an overdose of isoflurane at the middle of the light period. Adult female hooded seals (*C. cristata*, $n=5$) were collected off the coast of East Greenland under permits of the national authorities of Denmark and Norway. The seals were decapitated under full anesthesia (i.m. injection of zolazepam/tiletamine, 1.5–3.0 mg per kg of body mass), as approved by the National Animal Research Authority of Norway. The procedures concerning rats, mice and seals reported in this study were conducted to minimize the number of animals used and their suffering. They complied with German, Norwegian and European laws for the protection of animals.

Cloning and sequencing of hooded seal Ngb-cDNA

Seal brain tissue was cut into small pieces and stored frozen. Total RNA was extracted using the RNeasy Mini-Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Oligonucleotide primers were designed according to the conserved segments of the aligned mammalian Ngb-cDNA sequences (Burmester et al., 2004): 5′-GAGCTGATCCGGCAGAGCTGGCG-3′ and 5′-GCCATGAGTCGAGGCTGGGA-3′. Ngb-cDNA fragments were amplified by reverse transcription–PCR experiments employing the Qiagen OneStep-kit according to the manufacturer's instructions. Missing 5′- and 3′-ends of the cDNA were obtained using the GeneRacer™ Kit (Invitrogen, Karlsruhe, Germany). The PCR products were cloned into the pCR4-TOPO TA vector (Invitrogen, Karlsruhe, Germany). Sequences were obtained from both strands using a commercial sequencing service (GENterprise, Mainz, Germany). The seal cDNA sequence was deposited at the GenBankTM/EMBL database under the accession number FM999827.

A homology model of *C. cristata* Ngb was built applying the online facility SwissModel (GlaxoWellcome Experimental Research, Geneva, Switzerland) at the following address: http://www.expasy.ch/spdbv/ using the known human Ngb crystal structures (Pesce et al., 2003) as template. The structure was visualized by the aid of POLYVIEW-3D (Porollo and Meller, 2007).

Immunohistochemistry

For immunohistochemistry, rat and mouse brain tissues were fixed by perfusion with 4% paraformaldehyde (Reuss et al., 2002). Fresh seal brain tissue (neocortex, cerebellum) was cut into appropriate pieces ($15\times15\times15$ mm^3) and fixed overnight in 4% paraformaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and stored in cold PBS until use. Brain samples from mouse, rat and seal were immersed overnight in 30% sucrose/PBS at 4 °C and cryo-sectioned at 40 μm thickness. Brain sections were incubated free-floating with the first antibody diluted in PBS/0.1%–0.4% Triton X-100/1% bovine serum albumin overnight at room temperature. A commercial polyclonal rabbit antibody against Ngb (1:500, Sigma Aldrich, Deisenhofen, Germany) was used. In addition, we employed a monoclonal mouse antibody against glial fibrillary acidic protein (GFAP; 1:200, Abcam, Cambridge, UK) and a polyclonal anti–Cyt c antibody produced in sheep (1:8, Sigma Aldrich). The sections were washed three times 10 min in PBS and incubated for 90 min at room temperature in the dark with the corresponding secondary antibodies, which had been diluted in PBS: donkey anti-rabbit $F(ab)_2$-fragment coupled to Cy3 (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), donkey anti-mouse $F(ab)_2$-fragment coupled to Cy2 (1:200 in PBS; Jackson ImmunoResearch) or donkey anti-sheep $F(ab)_2$-fragment coupled to Cy2 (1:200 in PBS; Jackson ImmunoResearch). Sections were washed three times 10 min in PBS, mounted on glass slides and embedded in Mowiol (Calbiochem, Darmstadt, Germany). The Hoechst dye 33258 (0.3 μg/ml) was added to the Mowiol to stain the nuclei. Sections were analyzed using an Olympus BX51 research microscope equipped with a digital camera. Images were combined using Adobe Photoshop 7.0.

Western blotting

For Western blot analyses, frozen mouse and seal brains were microdissected to obtain samples enriched in tissues of cerebrum, cerebellum, and medulla spinalis. If adequate, we separated grey (cortical) from underlying white (medullary) matter. Proteins were extracted by homogenizing the tissues in PBS with 0.1% SDS. Protein concentrations were estimated according to Bradford (1976). Tissue extracts (about 80 μg protein per lane) were diluted with sample buffer (65 mM Tris–HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol) and denatured at 95 °C for 5 min. Recombinant mouse Ngb was used as positive control. After gel electrophoresis on a 15% SDS–polyacrylamide gel, the proteins were transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked by incubation for 1 h with pure soy milk. The membranes were incubated with a custom antibody raised against a conserved Ngb peptide (amino acid positions 55–70: H_2N-CLSSPEFLDHIRKVML-CONH2; Eurogentec, Seraing, Belgium) diluted 1:100 in soy milk at 4 °C overnight. The membranes were then washed four times 10 min in TBS (10 mM Tris–HCl, 150 mM NaCl, pH 7.5) and incubated with a goat anti-rabbit antibody coupled with alkaline phosphatase (Dianova, Hamburg, Germany; 1:10,000 in TBS) for 45 min at room temperature. The membranes were washed in TBS as above and detection was carried out with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

RESULTS

Sequencing of hooded seal Ngb cDNA

Degenerated primers were deduced from a multiple sequence alignment of known mammalian Ngbs (Burmester et al., 2004). A 323 bp fragment of *C. cristata* Ngb cDNA