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Hypoxia induces a complex response of globin expression in zebrafish (*Danio rerio*)

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Summary

Unlike most mammals, many fish species live and survive in environments with low or changing levels of oxygen. Respiratory proteins like hemoglobin or myoglobin bind or store oxygen, thus enhancing its availability to the respiratory chain in the mitochondria. Here we investigate by means of quantitative real-time PCR the changes of hemoglobin, myoglobin, neuroglobin, cytoglobin and globin X mRNA in zebrafish (*Danio rerio*) exposed to mild (P_{O_2}=~8.6 kPa) or severe (P_{O_2}=~4.1 kPa) hypoxia. Neuroglobin and myoglobin protein levels were investigated by western blotting. Whereas mild hypoxia caused only minor changes of mRNA levels, strong hypoxia enhanced mRNA levels of the control genes (lactate dehydrogenase A and phosphoglycerate kinase 1). Surprisingly, levels of hemoglobin α and β mRNA were significantly reduced under severe hypoxia. Myoglobin mRNA and protein in heart mildly increased, in line with its proposed oxygen supply function. Likewise, neuroglobin mRNA and protein significantly increased in brain (up to 5.7-fold at the protein level), but not in eye. This observation, firstly, suggests physiological differences of zebrafish eye and brain under hypoxia, and secondly, indicates an important role of neuroglobin in oxidative metabolism, probably oxygen supply within neurons. There was little change in the expression of the two cytoglobin genes. Globin X mRNA significantly decreased under hypoxia, pointing to a functional linkage to oxygen-dependent metabolism.

Supplementary material available online at
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Key words: cytoglobin, hemoglobin, myoglobin, neuroglobin, oxygen, zebrafish, *Danio rerio*.

Introduction

The aerobic lifestyle of most animals requires a constant supply of sufficient oxygen, and low oxygen levels (hypoxia) constitute a major environmental threat. Fish live in water, which has a low oxygen capacitance (1/30th compared to air) and poor oxygen diffusibility. Aqueous environments are also prone to temporal and seasonal changes of oxygen partial pressures. Therefore, many fish species have evolved various behavioral, anatomical, physiological, biochemical and molecular adaptations that enable them to cope with periods of hypoxia (for reviews, see Nikinmaa, 2002; Nilsson and Renshaw, 2004; Cossins and Crawford, 2005; Nikinmaa and Rees, 2005). Low oxygen levels cause a decrease of activity, enhanced ventilation and reduction of the metabolic rate (Jensen et al., 1993; Dalla Via et al., 1994; van den Thillart and van Waarde, 1985; Smith et al., 1996; Ton et al., 2003; van der Meer et al., 2005). Prolonged hypoxia, which extends more than a few days, may also induce anatomical changes such as expansion of the gill surface (Sollid et al., 2005; van der Meer et al., 2005).

It is not surprising that hypoxia causes major changes of gene expression in fish (Gracey et al., 2001; Ton et al., 2003; Nikinmaa and Rees, 2005; van der Meer et al., 2005), which allow the animals to save oxygen and to better cope with periods of oxygen shortage. For example, genes encoding enzymes for glycolysis and fermentation were found to be more strongly expressed after long term hypoxia in adult goby fish, *Gillichthys mirabilis* (Gracey et al., 2001), adult zebrafish, *Danio rerio* (van der Meer et al., 2005), and after 24 h of anoxia (0% O_2) in zebrafish embryos (Ton et al., 2003). By contrast, among others, genes required for oxygen-dependent energy production (e.g. the TCA cycle or the mitochondrial respiratory chain) and protein translation were repressed. Hypoxia also causes developmental arrest, as reflected by the repression of cell cycle-related genes (Padilla and Roth, 2001; Ton et al., 2003).

Respiratory proteins enhance the availability of oxygen to the electron transport chain in the mitochondria. All respiratory proteins of vertebrates belong to the superfamily of globins, which harbor a porphyrin-ring with a Fe^{2+} ion. Five globin

classes are known to be present in fish (Fig. 1). Hemoglobin (Hb) consists of two α and two β chains, is located in the erythrocytes and greatly increases the oxygen capacitance of the blood. It enables the efficient transport of oxygen from the respiratory surfaces (lungs, gills, skin) to the inner organs. The monomeric myoglobin (Mb) is mainly situated in striated muscle and heart. It is supposed to store oxygen and to facilitate intracellular oxygen diffusion (Wittenberg and Wittenberg, 2003). Neuroglobin (Ngb) is located in the central and peripheral nervous system (Burmester et al., 2000), the retina (Schmidt et al., 2003) and some endocrine tissues (Reuss et al., 2002). Ngb may have a Mb-like role in enhancing the availability of oxygen to the metabolically active neurons, although other functions such as the detoxification of reactive oxygen or nitrogen species (ROS, RNS) or oxygen signaling have been proposed (for reviews, see Burmester and Hankeln, 2004; Hankeln et al., 2005). Cytoglobin (Cygb) is expressed in fibroblast-related cells and some neurons (Burmester et al., 2002; Schmidt et al., 2004). Its function may be related to ROS detoxification or oxygen supply to particular enzymatic reactions (Schmidt et al., 2004; Hankeln et al., 2005). Globin X (GbX) is restricted to fish and amphibia (Roesner et al., 2005). Its function is currently unknown.

It may be expected that altered oxygen levels have a significant impact on the expression of globins. For example, Hb levels were shown to have increased in mammals under high altitude conditions (e.g. Samaja et al., 2003), whereas data on Mb expression under hypoxia are variable (Hoppeler and Vogt, 2001; Levine and Stray-Gundersen, 2001). Results on hypoxia regulation of Ngb in mammalian systems are also not consistent. Whereas Ngb was found to be more highly expressed in hypoxic cell and tissue culture systems (Sun et al., 2001; Fordel et al., 2004), no changes in Ngb mRNA levels were found in whole animal experiments (Mammen et al., 2002). Cygb mRNA levels increase in hypoxic liver and heart of rat and mice (Schmidt et al., 2004), whereas the observed changes of Cygb mRNA levels in the brain were not significant (Fordel et al., 2004) (A. Avivi, F. Gerlach, S. Reuss, T. Burmester, E. Novo and T. Hankeln, unpublished data).

Most mammals are not adapted to environments with low or changing oxygen partial pressures. Therefore, at least some of the data on globin expression, particularly those on Ngb and Cygb, have been obtained from animals that will never be subject to any hypoxic conditions during their adult live. Here, we investigate the response of globin mRNA and protein (for Mb and Ngb) levels to hypoxia in the zebrafish *Danio rerio*. The zebrafish may actually experience low oxygen levels in its warm and tropical environment and it has an astonishing ability to withstand hypoxia (Padilla and Roth, 2001; Rees et al., 2001; Pelster, 2002; Ton et al., 2003; van der Meer et al., 2005). Moreover, *D. rerio* has become a prime model for investigation of physiological adaptations at the molecular level.

Materials and methods

Animal maintenance

Danio rerio Hamilton (Cypriniformes) were obtained from a local pet shop and kept in activated carbon filtered water at a density of approximately 1 fish per 2 l, with a 14 h:10 h light:dark cycle and a temperature of 25°C. Fish were fed TetraMin flakes (Tetra, Melle, Germany) once per day. Water was filtered with a thermofilter (Ekip 350, Hydor, Bassano del Grappa, Italy). Water quality (total and carbonate hardness, pH and nitrite and nitrate content) was checked periodically and partial water changes were carried out when necessary. Temperature acclimation was performed by cooling 1°C per day. Zebrafish were acclimated to 22°C for at least 10 days before hypoxia treatment. Animal handling and experiments were conducted according to a protocol that had been approved by the county-government office (Bezirksregierung Rheinhessen-Pfalz, AZ 1.5 177-07/021-30).

Hypoxic treatment

Groups of four fish were randomly assigned to hypoxia treatment or control groups. Hypoxia treatment was performed in a 10 l glass aquarium, covered with loosely fitting acrylic covers. Gas mixtures (2% O_2 in N_2 or 5% O_2 in N_2, Air liquide,

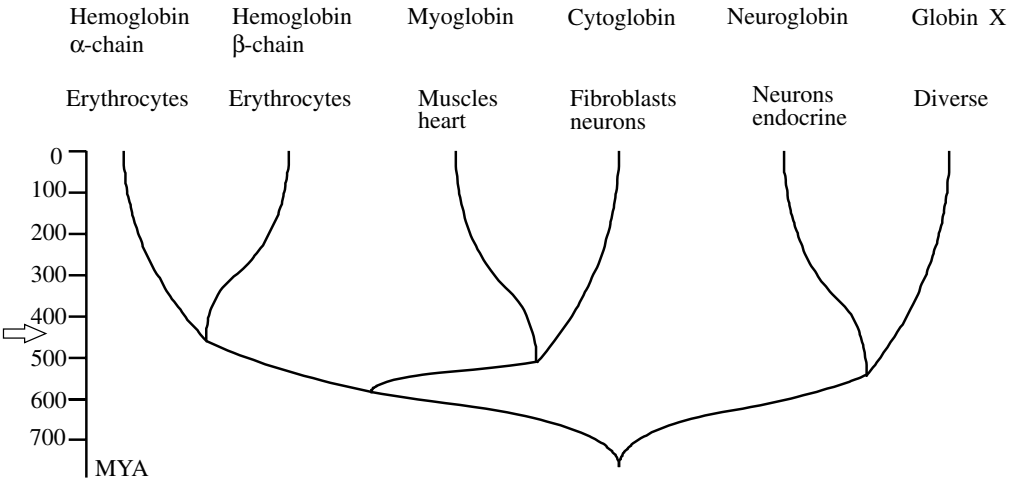

Fig. 1. Fish respiratory proteins. The scheme provides an overview on the expression and evolution of fish globins. MYA, million years ago. The arrow indicates the approximate time of divergence of teleost fish and tetrapods.

Dusseldorf, Germany) were bubbled through water. A thermopump (Ekip 200, Hydor, Bassano Del Grappa, Italy) was used to circulate the water and to keep the temperature constant at 22°C. Oxygen partial pressure and temperature were measured every 5 min by an oxygen sensor (Oxi 340i, WTW, Weilheim, Germany). Fish were not fed for 24 h before the start of, or during, the experiment. Experiments started at an oxygen partial pressure of 18.4 kPa (138 Torr). The water was aerated to achieve 4.1 kPa (~31 Torr, bubbled with 2% O_2) or ~8.6 kPa (65 Torr, bubbled with 5% O_2), respectively, after 1 h. Oxygen partial pressure remained constant (±0.6–0.8 kPa) throughout the experiment. Control zebrafish were kept under the same conditions, but the water was gassed with room air (P_{O_2}=~18.4 kPa). After 24 h or 48 h, fish were directly frozen in liquid nitrogen. For the experiments employing total eyes, brain or heart, the specimens were cooled on ice and killed by decapitation. For each fish, organs were removed within less than 2 min, shock-frozen in liquid N_2 and kept at –80°C until use.

RNA extraction

RNA samples from total animals or single organs were extracted using the RNeasy Mini Kit by Qiagen (Hilden, Germany). Fish were weighed and homogenized in the required volume of RLT buffer (Qiagen) with a rotor stator. To avoid contamination with genomic DNA, a DNase digestion was performed on the column (Qiagen). Quality and amount of RNA were checked photometrically and with RNA gel electrophoresis.

cDNA cloning and sequencing

The complete cDNA sequences of the genes used in this study [acidic ribosomal protein [*ARP* (also known as *rplp0*), EMBL/GenBank acc. no. BC049058], cyclophilin (AY391451), Hbα [*hba* (*hbaa1*), NM_131257), Hbβ [*hbb*(*ba1*), NM_131020], Mb (AY337025), Ngb (NM_131853), Cygb1 (AJ320232), Cygb2 (AJ635229) and GbX (NM_001012261)] were obtained by amplification from total RNA with the OneStep RT-PCR kit (Qiagen) using specific primers (see Table S1 in supplementary material). After cloning into standard vectors (pCR4-TOPO, Invitrogen, Karlsruhe, Germany, or pGEM Teasy, Promega, Mannheim, Germany) the sequences were determined by a commercial service (Genterprise, Mainz, Germany). The cDNA plasmids were used for generating standard curves for use in real-time PCR (see below). The cDNAs of the genes used as positive controls [lactate dehydrogenase A (*ldha*, NM_131246) and phosphoglycerate kinase 1 (*pgk1*, NM_213387)] were not cloned.

Quantitative real-time PCR

RNA extractions and cDNA synthesis were carried out from single specimens, except for the control group for which the RNA samples were combined before cDNA synthesis. In the experiments with brains or total eyes, both the hypoxia and control group animals were treated individually. Quantitative real-time RT–PCR was performed with a two-step protocol. First, total RNA was converted into cDNA employing Superscript II RNase H^- reverse transcriptase (Invitrogen) and an oligo(dT_{16}) primer according to manufacturer's instruction. The cDNA samples were diluted with the same volume of DNase-free water. Real-time RT–PCR experiments were carried out on an ABI Prism 7000 SDS (Applied Biosystems, Darmstadt, Germany) using the ABsolute™ QPCR SYBR® Green ROX Mix (Abgene, Hamburg, Germany). Levels of mRNA of ARP, cyclophilin, LDHA, PGK1, Hba, Hbb, Mb, Ngb, Cygb1, Cygb2 and GbX were evaluated. *ARP* and cyclophilin were employed as putatively non-regulated reference genes (Simpson et al., 2000). To avoid amplification of genomic DNA, all primer pairs included one intron-spanning oligonucleotide. The oligonucleotide primers were obtained from Sigma-Genosys (Hamburg, Germany) (see Supplemental Table S1 in supplementary material). Reactions were run in triplicate with one or two repetitions, using 1 μl of diluted cDNA as template in a reaction volume of 30 μl. Primer concentrations were 0.13 μmol l^{-1} for each oligonucleotide. The Taq-polymerase was activated for 15 min at 95°C, followed by 40 cycles of a standard PCR protocol (94°C 15 s, 60°C 30 s, 72°C 30 s). Efficiency of reaction was measured by the slope of a standard curve. For standard curves, only duplicates were run, using fivefold cDNA dilutions (positive controls *ldha* and *pgk1*) or tenfold dilutions of plasmids (all other genes). Specificity of the amplification reaction was analyzed using dissociation curves with a temperature range from 60°C to 95°C. First evaluation of results was performed in the ABI Prism 7000 SDS program; for normalization and calibration, data were exported to qBase (http://www.medgen.ugent.be/qbase/). Final data analyses were carried out with the Microsoft Excel XP spreadsheet program. The significance of the data was evaluated using Student's *t*-test.

Protein extraction and western blotting

After the hypoxia treatment, zebrafish used for protein analyses were cooled down on ice and immediately dissected. Total eyes, brains and hearts were removed and stored at –20°C. Tissues were homogenized in 1× phosphate-buffered saline (PBS; 140 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 8.1 mmol l^{-1} Na_2HPO_4, 1.5 mmol l^{-1} KH_2PO_4) with ultrasonication and precipitated for 10 min at 13,000 ***g*** at 4°C. Protein concentrations of the supernatant were determined with the Bradford method (Bradford, 1976).

Protein extracts (100 μg per lane) were heat-denatured in sample buffer (31.25 mmol l^{-1} Tris–HCl, pH 6.8, 1% SDS, 2.5% β-mercaptoethanol, 5% glycerol) for 5 min at 95°C and loaded onto a 15% SDS polyacrylamide gel. Antibody detection was carried out on protein samples transferred to nitrocellulose membrane for 2 h at 0.8 mA cm^{-2}. Non-specific binding sites were blocked by incubating for 45 min with 2% bovine serum albumin (BSA) in TBS (10 mmol l^{-1} Tris, pH 7.4, 140 mmol l^{-1} NaCl). Membranes were then incubated for 2 h with anti-Ngb antibodies (Fuchs et al., 2004) or anti-

zebrafish-Mb serum (raised against recombinantly expressed *D. rerio* Mb; unpublished), both diluted 1:500 in 2% BSA/TBS, and washed four times for 5 min with TBS. Membranes were incubated with the goat anti-rabbit antibody coupled with alkaline phosphatase (Dianova, Hamburg, Germany) for 1 h, diluted 1:10 000 in 2% BSA/TBS, and washed as described above. Detection was carried out with nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate salt as substrates. The filters were scanned at 1,200 d.p.i. and the images were imported into the Scion Image program (version Beta 4.02) and an analysis of grey values was performed to obtain an estimate of the protein levels. The mean grey values of the background of empty gel lanes were subtracted from the measurements of the Ngb or Mb protein levels. Data were imported into Microsoft Excel XP and Student's *t*-test was used to assess significance.

Results

We first followed the survival rate of adult *D. rerio* after exposure to hypoxia. We found that P_{O_2}=~2.4 kPa (~18 Torr) was lethal for more than 80% of the animals after 24 h (N=8). At ~4.1 kPa O_2 partial pressure and at 22°C, the survival rate was >80% after 48 h hypoxia exposure (N=43). Therefore, the latter regime was applied as maximum (severe) hypoxic conditions.

Changes of gene expression in the hypoxic zebrafish

Evaluation of real-time RT–PCR results showed that ARP expression does not change under hypoxia (P_{O_2}=~4.1 kPa or ~8.6 kPa), whereas we observed a mild but significant upregulation of cyclophilin (Figs 2 and 3). Therefore, all expression levels were subsequently normalized according to ARP. Microarray data had demonstrated that expression levels of LDH-A and PGK1 mRNA were highly enhanced in zebrafish under hypoxia (Ton et al., 2003). These mRNAs were used in our experiments as positive controls to verify hypoxia exposure and response. In fact, at P_{O_2}=~4.1 kPa for 48 h, we saw significant upregulation of the expression of both LDHA and PGK1 mRNA by a factor of 2.5 and 3.8, respectively (Figs 2 and 3). PGK-1 levels increase even under mild hypoxia (48 h, P_{O_2}=~8.6 kPa; 1.9-fold compared with the normoxic control) or after a shorter time-period (24 h, P_{O_2}=~4.1 kPa) (2.1-fold compared with the normoxia). The large standard deviations reflect differences of mRNA levels among individuals. However, the results were significant according to a Student's *t*-test, as indicated in Figs 2 and 3.

Globin-mRNA levels in zebrafish under different hypoxia regimes

We found only minor changes in globin mRNA levels in animals exposed to P_{O_2}=~8.6 kPa for 48 h (Fig. 2). There was an approximate 50% decrease in Hbα and Hbβ mRNA levels, which was, however, not significant. Under severe hypoxia (P_{O_2}=~4.1 kPa), the changes in globin mRNA were much more pronounced (Fig. 3). Hb mRNAs decreased after 24 h by 20%, to 30% compared to the normoxia control, and after 48 h at P_{O_2}=~4.1 kPa they were reduced by between 65% and 75% (P<0.005 and P<0.001). Mb mRNA levels increased about 2.5-fold under short- and long-term severe hypoxia, although this change was not significant because of the large standard deviation. Ngb mRNA increased after 24 h strong hypoxia by about fourfold, but the standard deviation results in a borderline significance (P=0.065). After 48 h at 4.1 kPa P_{O_2}, the Ngb mRNA levels were nearly back to normoxia levels, with only about a 1.3-fold increase. Zebrafish possess two paralogous Cygb genes (Fuchs et al., 2005). For Cygb1 mRNA, we see a mild upregulation under hypoxia, which was, however, not significant. Cygb2 mRNA levels remained almost unaltered. GbX mRNA expression was unchanged at

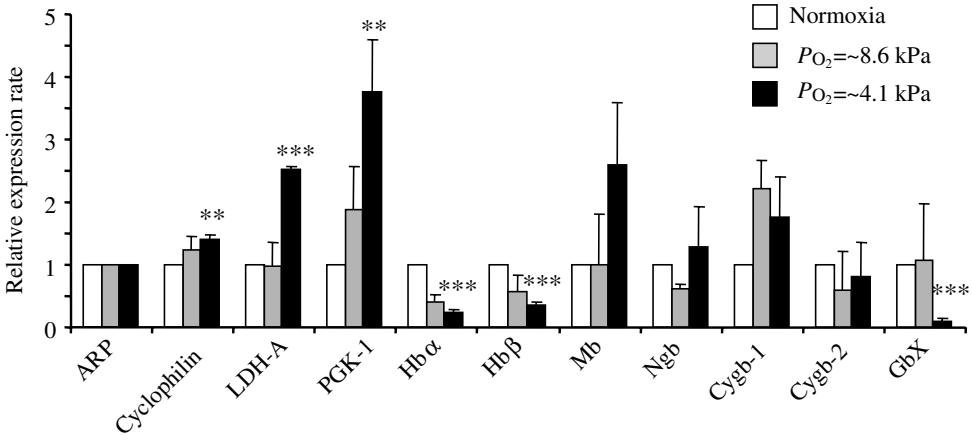

Fig. 2. Expression of *D. rerio* globins at different oxygen levels. mRNA quantities were determined by quantitative real-time PCR. The white columns represent mRNA levels from zebrafish kept for 48 h at normoxia (P_{O_2}=~18.4 kPa), grey columns are mRNA levels from zebrafish kept for 48 h at P_{O_2}=~8.6 kPa, black columns are mRNA levels from zebrafish kept for 48 h at P_{O_2}=~4.1 kPa. Values are means ± s.d. The significance of the data was estimated with a Student's *t*-test, with N=3 or 4 individuals for each data point. ***P<0.001; **P<0.01. ARP, acidic ribosomal protein; Cygb, cytoglobin; GbX, globin X; Hb, hemoglobin; LDHA, lactate dehydrogenase A; Mb, myoglobin; Ngb, neuroglobin; PGK1, phosphoglycerate kinase 1.

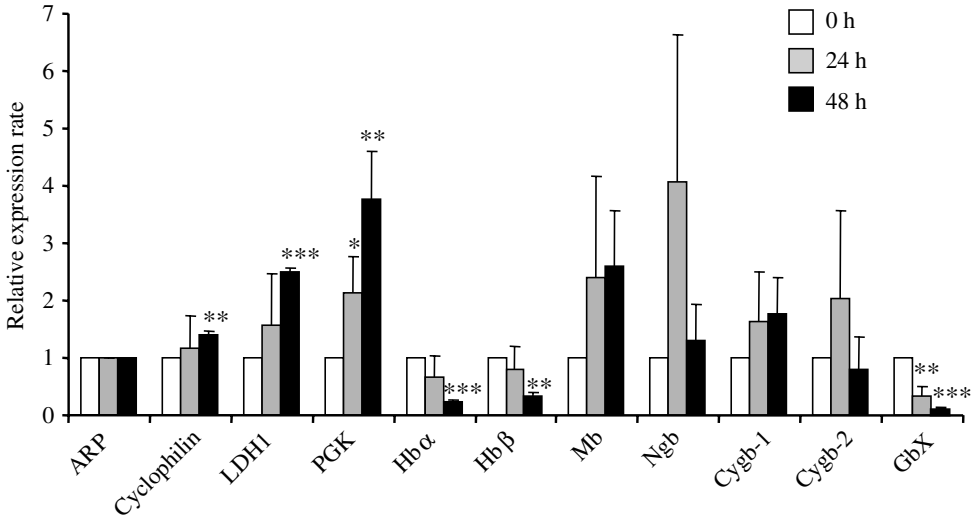

Fig. 3. Rate of expression of *D. rerio* globin expression under hypoxia. Zebrafish were kept at P_{O_2}=~4.1 kPa for 24 h (grey columns) and 48 h (black columns). The white columns represent the normoxia control. mRNA quantities were determined by quantitative real-time PCR. Values are means ± s.d. ***P<0.001; **P<0.01; *P<0.05 (t-test). For abbreviations, see Fig. 2.

P_{O_2}=~8.6 kPa, but significantly decreased at P_{O_2}=~4.1 kPa to a level of about 10% of the normoxia control after 48 h (P<0.001).

Expression of Ngb mRNA in brain and eye

We further analyzed the expression of Ngb mRNA separately in brain and eye of zebrafish that had been kept for 24 h at P_{O_2}=~4.1 kPa. Total brains and eyes were immediately removed from zebrafish and shock-frozen in liquid N_2. Expression of selected genes was analyzed in individuals by quantitative real-time PCR. Again, we found a strong variation in gene expression levels between individuals of the same group (Fig. 4). The positive-control genes *ldha* and *pgk1* were found to be significantly upregulated in both tissues (data not shown). Ngb mRNA levels were essentially the same in the normoxic and hypoxic eyes (Fig. 4). However, in brain we saw about threefold higher mRNA level in tissues from hypoxic animals compared to the control (P<0.01).

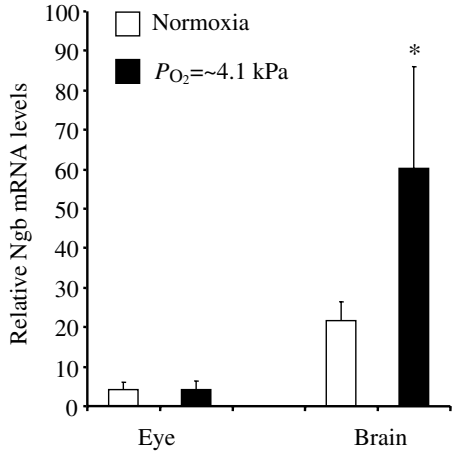

Fig. 4. Expression of neuroglobin (Ngb) mRNA in zebrafish eye and brain. Zebrafish were kept for 24 h at normoxia (white columns) or P_{O_2}=~4.1 kPa (black columns). Ngb mRNA levels from eye and brain were quantified separately by real-time PCR (N=4). The units of the y-axis are arbitrary. *P<0.05 (t-test).

Quantitative western blotting

Proteins were extracted from brains, total eyes and hearts of individual zebrafish that had been kept for 48 h at severe hypoxia (P_{O_2}=~4.1 kPa). To analyze changes in protein levels, we performed quantitative western blots, applying a constant amount of total protein extracts (100 μg per lane; Fig. 5). We observed a mildly (20%) but significantly (P<0.05) higher level of myoglobin in the hearts from hypoxic zebrafish compared to control animals that had been kept for the same time under normoxia (P_{O_2}=~18.4 kPa). Ngb protein levels were somewhat higher in the protein extracts from the hypoxic eye than in those of the normoxia control (1.6-fold), but this increase was not significant (P=0.11). In the brain, however, we consistently observed 5.7-fold more Ngb protein in the hypoxic than in the normoxic animals. This increase was highly significant, as estimated by a Student's t-test (P<0.001).

Discussion

Zebrafish at low oxygen concentrations

Fish have many different adaptations that allow them to cope with the oscillations in P_{O_2} that may occur in the aquatic environment (Nikinmaa, 2002; Nilsson and Renshaw, 2004; Cossins and Crawford, 2005). A previous study reported that adult zebrafish survive a P_{O_2} of 2 kPa at 22°C for 48 h (Rees et al., 2001), whereas in our hands P_{O_2}=~2.4 kPa at 22°C caused >80% mortality after less than 24 h. This discrepancy

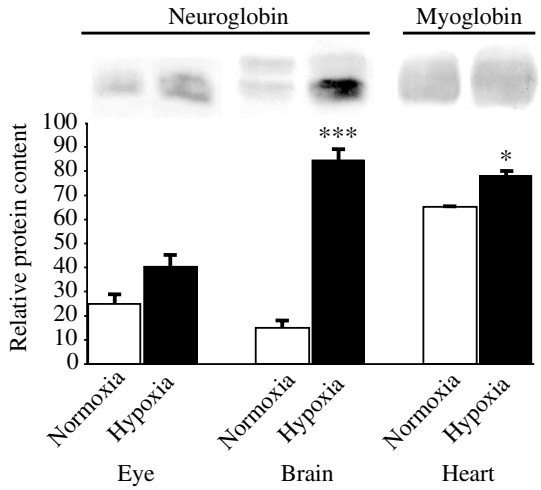

Fig. 5. Expression of neuroglobin (Ngb) in zebrafish eye and brain, and myoglobin (Mb) in heart. Zebrafish were kept for 48 h at normoxia (white columns) or P_{O_2}=~4.1 kPa (black columns). Ngb protein levels from eye and brain, and Mb levels from heart, were quantified separately (100 μg protein per lane; *N*=3). The units of the *y*-axis are arbitrary. ***P<0.001; *P<0.05 (*t*-test).

may be explained by differences in zebrafish strains. We have chosen an oxygen partial pressure of ~4.1 kPa (4.15 kPa) as the severe hypoxic condition for our experiments. The strong upregulation of the typical hypoxia-responsive genes *ldha* and *pgk1*, which is in line with a recent microarray study (Ton et al., 2003), demonstrates that under these conditions zebrafish were actually experiencing serious hypoxic stress.

We observed a large standard deviation in all our quantitative real-time PCR experiments employing zebrafish. A similarly large variability has been noted for various fish species in a number of microarray studies (Oleksiak et al., 2002; Cossins and Crawford, 2005). These variations in mRNA levels certainly reflect genetic, physiological or behavioral differences between individuals. Nevertheless, most changes in globin mRNA levels were significant, and, in the case of Mb and Ngb, agreed with parallel western blot experiments. Moreover, we observed very similar values for the expression levels of Hbα and Hbβ, as expected for the products of such tightly co-regulated genes (Hardison, 1998). It should also be noted that variability in Mb and Ngb protein content was much smaller than the variation in mRNA levels, suggesting additional regulation at the translational level.

Understanding the role of respiratory proteins in hypoxia response

Maintaining a constant flow of oxygen from the water to the respiratory chain of the mitochondria is a major challenge for fish living under hypoxia. Respiratory proteins already enhance oxygen availability under normoxia, and changes in their concentration or physiological properties are expected under hypoxia. It is well established that Hb transports oxygen in the circulatory system and that Mb enhances oxygen supply to striated muscle cells, which is, in the case of fish, mainly the heart. Much less is known about the recently discovered novel members of the globin family, Ngb, Cygb and GbX (Burmester and Hankeln, 2004; Hankeln et al., 2005), which are present in fish (Awenius et al., 2001; Burmester et al., 2002; Fuchs et al., 2004; Fuchs et al., 2005; Roesner et al., 2005). Their expression response in an animal system that is evolutionarily adapted to at least temporarily low oxygen partial pressures provides hints to their general physiological functions in vertebrates and their particular roles in hypoxic response.

Hemoglobin mRNA is downregulated under hypoxia

A surprising finding of our study is that Hbα and β mRNA levels were significantly lower after 2 days of hypoxia compared to the normoxia controls. The response of Hb to hypoxia exposure in fish is not clear (Nikinmaa and Rees, 2005). Whereas some studies reported an increase of Hb under hypoxia (e.g. Timmerman and Chapman, 2004), others did not see any change in Hb concentration (e.g. Person-Le Ruyet et al., 1998), or a differential responses depending on exposure times (e.g. Affonso et al., 2002). Thus hypoxia response of Hb in fish may be species specific. Down-regulation of Hb mRNA in zebrafish has previously been reported in a microarray study employing embryos (Ton et al., 2003). The authors explained this observation by stating that zebrafish embryos, 24 h post-fertilization, did not require blood flow, but oxygen uptake was achieved by diffusion. However, there is no doubt that adult zebrafish, which display the same decrease of Hb mRNA (Figs 2 and 3), rely on blood circulation. Therefore, an alternative hypothesis to explain Hb regulation in hypoxic zebrafish is required.

First, we have to emphasize that we only monitored Hb mRNA. Protein levels may actually behave quite differently. Another explanation could be the induction of distinct Hb chains. Database searches revealed six copies of the Hbα gene and five of the Hbβ gene. According to the annotation, two α and three β hemoglobins are embryonic hemoglobins, leaving multiple copies of each gene that are potentially expressed in the adult zebrafish. The oligonucleotide primers we used for quantitative PCR were designed to amplify all adult Hbα and β chains. Even a differential behavior of adult Hb genes would not alter the observed overall down-regulation of Hb. We cannot exclude that hypoxia, for example, induces the expression of embryonic Hbs. In fact, it has been observed by gel electrophoresis that hypoxia changes the pattern of Hb chains in trout (Marinsky et al., 1990). However, based on the observation of Ton et al. (Ton et al., 2003) of a down-regulation of embryonic Hbs in embryos, we consider this explanation less likely.

Another possible explanation is that under hypoxia the amount of Hb exceeds the available oxygen. Therefore, a rise in Hb concentration and thus oxygen carrying capacity would have only minor effect on oxygen delivery, but would be very energy costly. Numerous studies have demonstrated that hypoxia induces in fish a change in oxygen binding properties of Hb (Weber and Jensen, 1988; Nikinmaa, 2001; Jensen, 2004). Under hypoxia, the concentrations of the modulators

adenosine triphosphate (ATP) and guanosine triphosphate (GTP) fall in red blood cells, leading to an increased O_2 affinity of Hb. This is a more efficient mechanism that improves oxygen uptake by the blood. The decrease of Hb mRNA could be explained by an energy-saving mechanism that ceases Hb synthesis. However, we should note that we only investigated short-term changes, up to 2 days hypoxia. It is possible that other regimes will reveal a distinct pattern of Hb expression. Clearly, additional studies are required to fully understand the peculiar behavior of Hb mRNA under hypoxia.

Hypoxia induces myoglobin expression in zebrafish heart

The function of Mb is to facilitate diffusion of oxygen from the capillaries to mitochondria and to store oxygen, but it may also detoxify NO (Wittenberg and Wittenberg, 2003). In mammals, the pattern of Mb regulation under hypoxia is not conclusive. It is held that low-oxygen conditions, as they occur e.g. during high altitude training, increase Mb levels (Hoppeler and Vogt, 2001), but not all agree (e.g. Levine and Stray-Gundersen, 2001). In zebrafish, we observe that severe hypoxia induces Mb mRNA and protein (Figs 2, 3 and 5). This observation is in line with a major oxygen supply role of Mb for the heart. Because Mb is a monomeric protein, it does not display an Hb-like cooperativity that would enhance its oxygen affinity. The only possible mechanism to increase oxygen flow into the cardiac muscle is therefore to raise Mb concentrations.

Hypoxia regulation of neuroglobin and implication for its function

In this study, we have investigated for the first time the changes of Ngb expression in fish. Although this respiratory protein has been intensively studied in recent years, its exact role in vertebrate neurons is far from understood (Burmester and Hankeln, 2004; Hankeln et al., 2005). The phylogenetic relationship of Ngb to invertebrate nerve Hbs (Burmester et al., 2000), positive correlation with the intensity of cellular metabolism (Schmidt et al., 2003), co-localization with mitochondria (Bentmann et al., 2005) and ability to promote neuronal survival under hypoxia or ischemia (Sun et al., 2001) point to an Mb-like O_2 supply function of Ngb. However, other functions for Ngb, such as the detoxification of RNS and NO or signaling have been proposed (for reviews, see Burmester and Hankeln, 2004; Hankeln et al., 2005).

The published data disagree on the regulation of expression of Ngb under low oxygen conditions in mammalian systems. Sun et al. reported a 2.5-fold upregulation of Ngb mRNA and protein under nominal anoxia in a primary neuronal cell culture from mouse (Sun et al., 2001). An upregulation of Ngb mRNA was also obtained in a mouse neuronal cell line (Fordel et al., 2004), although the changes were not significant. By contrast, mice kept under sustained hypoxia (10% O_2) for up to 2 weeks showed no change in Ngb mRNA in the brain (Mammen et al., 2002). However, in rat, we observed a down-regulation of Ngb mRNA by about 40–60% after 5 h at 6% O_2, or after up to 44 h at 10% O_2 (A. Avivi, F. Gerlach, S. Reuss, T. Burmester, E. Nevo and T. Hankeln, unpublished data).

We show here that Ngb behaves differentially in the zebrafish brain and eye. Whereas in brain, Ngb mRNA levels showed a short-term upregulation after 24 h at P_{O_2}=~4.1 kPa (Fig. 4), no effect was observed in the whole eye. This pattern perfectly matches the western blot data (Fig. 5), which show a 5.7-fold increase of Ngb protein after 48 h severe hypoxia in brain but not in eye. In the eye, the protein levels only slightly increased. The differences might be explained by distinct behavior of these organs under hypoxia. The crucian carp *Carassius carassius*, which also belongs to the Cypriniformes, becomes blind under anoxia (Johansson et al., 1997), whereas the brain still functions (Nilsson, 2001). By inference, we assume that hypoxia causes a major decrease of metabolic activity of the zebrafish eye, whereas the brain is more or less active. We have previously demonstrated that the expression levels of Ngb in mammals are closely linked to metabolic activity and oxygen consumption (Schmidt et al., 2003; Bentmann et al., 2005). Therefore, high levels of Ngb are obviously required to preserve the oxidative metabolism in the zebrafish brain.

Our data show for the first time that Ngb is actually a hypoxia-inducible gene *in vivo* under naturally occurring conditions. This observation perfectly agrees with the hypothesis of Ngb being an O_2 supply protein of the neurons, similar to the function of Mb in the muscle cells (Burmester et al., 2000; Sun et al., 2001; Schmidt et al., 2003; Bentmann et al., 2005). In fact, Ngb behaves in the brain in a largely similar way to Mb in the heart. Nevertheless, it should also be considered that several studies reported that under low oxygen conditions (hypoxia or ischemia) the concentrations of ROS and NO may increase (Jezek and Hlavata, 2005; Wenger, 2006). Therefore, it is still conceivable that Ngb breaks down these deleterious compounds (Herold et al., 2004; Brunori et al., 2005). Regardless of its actual function at the molecular level, Ngb is presumably neuroprotective for both fish and mammals.

The other globins: cytoglobin and globin X

Fish possess two paralogous Cygb genes, which duplicated early in teleost evolution (Fuchs et al., 2005). In mammals, which have only a single gene copy (Burmester et al., 2002), Cygb expression increases under hypoxia in a variety of tissues (Schmidt et al., 2004; Fordel et al., 2004). We did not observe significant changes of Cygb1 or Cygb2 mRNA levels (Figs 2 and 3), indicating that Cygb is not involved in acute hypoxia response of zebrafish. GbX has only recently been discovered in fish and amphibians (Roesner et al., 2005). It is expressed at a low level in a variety of tissues, but its function is uncertain. The severe down-regulation of GbX mRNA in hypoxic zebrafish points to a close relationship of the gene to oxygen-dependent metabolism, but it is unlikely to be involved in oxygen supply.

Conclusions

We have presented the first comprehensive study on the regulation of globins under hypoxia in a single organism.

Because zebrafish may actually experience hypoxic periods in its environment, it is probable that the changes in mRNA and protein levels we observed here reflect physiological responses. This is in contrast to many mammalian studies, which have investigated species that are unlikely to experience any hypoxic situation during adult life or have used cell culture systems. The down-regulation of Hb mRNA in zebrafish under hypoxia certainly requires additional studies. The increased expression of Mb and Ngb under hypoxia is consistent with a proposed function of these proteins in oxygen supply to the heart and brain, respectively. This finding is remarkable in the case of Ngb, the function of which is still a topic of hot debate. GbX gene expression is markedly reduced under oxygenated conditions, providing a first hint to its physiological role.

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