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Nitric oxide metabolites in goldfish under normoxic and hypoxic conditions

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SUMMARY

Nitric oxide (NO), produced by nitric oxide synthases (NOS enzymes), regulates multiple physiological functions in animals. NO exerts its effects by binding to iron (Fe) of heme groups (exemplified by the activation of soluble guanylyl cyclase) and by *S*-nitrosylation of proteins – and it is metabolized to nitrite and nitrate. Nitrite is used as a marker for NOS activity but it is also a NO donor that can be activated by various cellular proteins under hypoxic conditions. Here, we report the first systematic study of NO metabolites (nitrite, nitrate, *S*-nitroso, *N*-nitroso and Fe-nitrosyl compounds) in multiple tissues of a non-mammalian vertebrate (goldfish) under normoxic and hypoxic conditions. NO metabolites were measured in blood (plasma and red cells) and heart, brain, gill, liver, kidney and skeletal muscle, using highly sensitive reductive chemiluminescence. The severity of the chosen hypoxia levels was assessed from metabolic and respiratory variables. In normoxic goldfish, the concentrations of NO metabolites in plasma and tissues were comparable with values reported in mammals, indicative of similar NOS activity. Exposure to hypoxia [at P_{O_2} (partial pressure of O_2) values close to and below the critical P_{O_2}] for two days caused large decreases in plasma nitrite and nitrate, which suggests reduced NOS activity and increased nitrite/nitrate utilization or loss. Tissue NO metabolites were largely maintained at their tissue-specific values under hypoxia, pointing at nitrite transfer from extracellular to intracellular compartments and cellular NO generation from nitrite. The data highlights the preference of goldfish to defend intracellular NO homeostasis during hypoxia.

Key words: hypoxia, iron-nitrosyl, nitric oxide, nitrate, nitrite, *S*-nitroso compounds.

INTRODUCTION

Nitric oxide (NO) is a signaling molecule with important roles in cardiovascular homeostasis, neurotransmission, immune defense and other physiological functions (Moncada and Higgs, 2006). NO is produced from L-arginine by nitric oxide synthases (NOS enzymes) in a process that requires O_2 and NADPH (Alderton et al., 2001). NO typically exerts its physiological functions by binding to iron (Fe) of heme (Denninger and Marletta, 1999) or by *S*-nitros(yl)ation of proteins (Foster et al., 2009) but it also participates in a number of other biochemical reactions (Hill et al., 2010). As an illustration, NO produced in the vascular endothelium diffuses to the underlying vascular smooth muscle cells where it binds to the heme of soluble guanylyl cyclase and induces muscle relaxation (vasodilation); however, NO also diffuses into the blood, where it reacts with O_2 to form nitrite, binds to ferrous heme of hemoglobin (Hb) in red blood cells (RBCs) to form iron-nitrosylhemoglobin or it reacts with oxygenated Hb to form nitrate (Kim-Shapiro et al., 2006; Bryan and Grisham, 2007). Thus, NO is metabolized to metabolites such as nitrite, nitrate, iron-nitrosyl (FeNO), *S*-nitroso (SNO) and *N*-nitroso (NNO) compounds. The physiological half-life of NO ranges from 2 ms to 2 s, depending on the availability of reactants of NO (Hill et al., 2010), and NO homeostasis is therefore typically assessed from measurements of the relatively more stable NO metabolites (Bryan and Grisham, 2007). In particular, the level of nitrite in plasma has been widely used as a reliable marker of constitutive NOS activity in mammals (Kleinbongard et al., 2003).

Over the past decade it has become clear that nitrite is more than just an end product of NO metabolism. Nitrite functions as a reservoir of NO activity that can be activated by a number of cellular proteins under hypoxic and/or acidic conditions (Gladwin et al., 2005; Lundberg et al., 2008). The proteins that convert nitrite into NO include deoxygenated Hb (Cosby et al., 2003), myoglobin (Mb) (Shiva et al., 2007), neuroglobin (Petersen et al., 2008), xanthine oxidoreductase (Millar et al., 1998) and carbonic anhydrase (Aamand et al., 2009). The mechanisms have variable potency and functions at different P_{O_2} (partial pressure of O_2) values but collectively they allow the production of NO from nitrite in hypoxic situations, where NOS-catalyzed NO formation may be compromised by a shortage of the substrate O_2. In mammalian models, nitrite-derived NO has been shown to contribute to hypoxic vasodilation, modulation of cellular respiration and cytoprotection during hypoxic/anoxic insults (Lundberg et al., 2008).

Teleost fish express NOS enzymes, and albeit conflicting results are available in the literature, it appears that the functional roles of NO in teleost fish and mammals are comparable (Tota et al., 2005; Agnisola, 2005; Pelster, 2007; Olson and Donald, 2009). Similarly, nitrite may function as a NO donor under hypoxic conditions in fish, and the importance could exceed that in mammals, given that many fish species naturally experience fluctuating or chronic low ambient P_{O_2} values (Jensen, 2009a). However, compared with mammals, relatively little is known about NO homeostasis in fish.

Basal levels of nitrite and other NO metabolites are well documented in mammalian blood and to some extent also in tissues. In fish, basal plasma nitrite concentrations have only been sparsely reported, using methods that have the required sensitivity to assess physiological concentrations in the sub-micromolar range, and information on levels of nitrite and other NO metabolites in tissues are, to our knowledge, absent.

One primary purpose of the present study was – for the first time in a fish species – to obtain systemic insight into NO homeostasis by determining the basal levels of all major NO metabolites in plasma and multiple intracellular compartments. A

further main goal was to evaluate the influence of hypoxia on NO metabolites. Our hypothesis was that NO production *via* constitutive NOS enzymes will decrease in hypoxia and that NO generation from nitrite will compensate for this, which should be reflected in the levels of NO metabolites. It has been suggested that nitrite reduction to NO may be particularly relevant in hypoxia-tolerant animals, and that nitrite-derived NO could be important in the acclimation of fish to hypoxia (Jensen, 2009a). We therefore decided to use the highly hypoxia-tolerant goldfish as the experimental animal and to investigate the effects of different P_{O_2} levels in an acclimated (two days) steady-state situation. The severity of the employed hypoxia levels was assessed from measurements of metabolic and respiratory variables.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated.

Animals and experimental protocol

The use of animals followed the guidelines of the Danish Law on Animal Experiments and was approved by the Danish Ministry of Justice (permission no. 2008/561–1470).

Goldfish (*Carassius auratus* L.) weighing 62.5±6.2 g (mean ± s.d., *N*=18) were obtained from a local supplier and held for 7 days in a 200 l aquarium with aerated tap water at 22°C and a 12 h:12 h light:dark cycle. A minimum of 80 l of water was exchanged twice daily, and the fish were fed once a day with commercial fish food (TetraMin®, Melle, Germany). Five days prior to the beginning of experiments, fish were randomly assigned to three aquaria with six fish in each. Each aquarium contained 70 l of aerated experimental water made by diluting tap water with demineralized water in a ratio of 1:4. The chloride concentration in experimental water was 280 μmol l^{-1}, and temperature was controlled at 22°C. During acclimation to experimental water, 40 l of the 70 l of water in each aquarium was exchanged twice daily.

The experimental design involved the exposure of goldfish to three different levels of environmental P_{O_2} for 2 days. One group of goldfish was maintained in normoxic water [P_{O_2}=147 mmHg (1 mmHg=133 Pa)]; the second group was exposed to a P_{O_2} close to the critical oxygen tension (~30 mmHg at 22°C) (Fry and Hart, 1948); and the third group was exposed to a P_{O_2} below the critical P_{O_2}. The desired P_{O_2} values were obtained by bubbling air (normoxia) or humidified mixtures of air and N_2 (hypoxia) through the water. Gas mixtures were delivered by Wösthoff (Bochum, Germany) gas mixing pumps, and water P_{O_2} was measured with Radiometer (Copenhagen, Denmark) E5046 electrodes connected to a PHM 73 monitor with data aquisition on a computer (AD Instruments PowerLab and Chart software, Bella Vista, NSW, Australia). Water P_{O_2} decreased to the desired hypoxia levels within 6 h and then remained stable up to 48 h at values of 29±1 and 19±1 mmHg, respectively. The water surfaces in the aquaria were covered with expanded polystyrene to limit gas exchange and to prevent the goldfish from gulping air. Water was not exchanged during the 48 h experimental period. The measured water nitrite concentration was 0.073±0.042 μmol l^{-1} at the beginning of the experiments and increased to 0.43±0.28 μmol l^{-1} by the end. The increase in water nitrite can be seen as a consequence of bacterial nitrification (in the water) of ammonia excreted by the fish (Jensen, 2003). However, even though water [NO_2^-] increased slightly, its absolute value remained low and considerably below water [Cl^-] (280 μmol l^{-1}), whereby problems with nitrite uptake across the gills (due to competition between Cl^- and NO_2^- for the branchial Cl^- uptake mechanism) were considered insignificant (Jensen, 2003).

Sampling of blood and tissues

Goldfish were individually netted and anesthetized in MS-222 (ethyl 3-aminobenzoate methanesulfonate). A blood sample was taken from the caudal vessels with a heparinized syringe, where after the fish was euthanized by cutting the spinal cord. The blood was immediately processed, while, at the same time, a number of tissues were quickly dissected out.

Blood was transferred to pre-weighed Eppendorf tubes, and subsamples were taken for measurements of hematocrit (Hct), Hb and nucleoside triphosphates (NTPs). The remaining blood was centrifuged (2 min, 16,000 ***g***, 5°C), and the plasma was transferred to a new Eppendorf tube. A subsample of plasma was taken for lactate measurement, and the remaining plasma was frozen in liquid N_2 for later measurements of NO metabolites. The tube containing the RBCs was weighed to determine RBC mass and then instantly frozen in liquid N_2.

Samples of the following tissues were dissected out for measurements of NO metabolites: gills (left side second gill arch), white skeletal muscle, the heart ventricle, the liver, the kidney, and the brain. The samples were washed in a phosphate-buffered saline [50 mmol l^{-1} phosphate buffer pH 7.8; 85 mmol l^{-1} NaCl; 2.4 mmol l^{-1} KCl; 10 mmol l^{-1} *N*-ethylmaleimide (NEM); 0.1 mmol l^{-1} diethylenetriaminepentaacetic acid (DTPA)], and then dried on a paper towel, weighed and frozen in liquid N_2. Additionally, the right side first gill arch was dissected out and placed in Bouin's solution (BHD Prolabo, Haasrode, Belgium) for later analysis of gill morphology, and the right side second gill arch was placed in SEI-buffer solution (300 mmol l^{-1} sucrose; 20 mmol l^{-1} Na_2EDTA; 50 mmol l^{-1} imidazole buffer pH 7.3) for later measurement of gill Na^+/K^+-ATPase activity.

Homogenization of tissues and preparation of RBCs

Tissue samples were thawed in four times their mass of a 50 mmol l^{-1} phosphate buffer (pH 7.3) that contained NEM (10 mmol l^{-1}) and DTPA (0.1 mmol l^{-1}) and immediately homogenized with a tissue grinder (Struers, Heidolph, Denmark). The homogenate was centrifuged (6 min, 16,000 ***g***, 2°C) and the supernatant was frozen in liquid nitrogen for later measurements of NO metabolites. The presence of NEM and DTPA prevents post-sampling reactions of SNO compounds. NEM binds free thiols, and DTPA stabilizes existing SNO by chelating copper and other trace metals that can lead to degradation of SNO compounds (Yang et al., 2003). For measurements on RBCs, it was necessary to stabilize SNO more extensively and to avoid possible reactions of nitrite with Hb during the handling of samples. For that purpose the frozen RBCs were thawed in nine times their mass of a nitrite/SNO-preservation solution (5 mmol l^{-1} $K_3[Fe(CN)_6]$; 10 mmol l^{-1} NEM; 0.1 mmol l^{-1} DTPA; 1% NP-40), whereupon the hemolysate was vortexed and centrifuged. The potassium ferricyanide ($K_3[Fe(CN)_6]$) oxidizes ferrous heme to ferric heme, which prevents reactions of nitrite with ferrous heme and stabilizes SNO-Hb (Yang et al., 2003; Dejam et al., 2005). The oxidation of heme groups accelerates NO release from Fe-nitrosyl-Hb, whereby this compound is not measured in RBCs treated with the preservation solution (Yang et al., 2003).

Analytical techniques

NO metabolites were measured with a highly sensitive chemiluminescent method, using a Sievers (Boulder, CO, USA) Nitric Oxide Analyzer (NOA, model 280i), as outlined earlier

(Samouilov and Zweier, 1998; Feelisch et al., 2002; Yang et al., 2003; Bryan and Grisham, 2007; MacArthur et al., 2007). The approach was to inject a sample into the NOA purge vessel, which contained a reducing agent that converted NO metabolites to NO. The NO was transported with a carrier gas (N_2) to the NOA reaction cell, where NO reacted with ozone to form O_2 and electronically excited nitrogen dioxide (NO_2*), which (on decay to its ground state) emitted light in the near-infrared region. The emitted light was detected by a photomultiplier, and the amplified signal was sent to a computer. An injected sample resulted in a peak, and the area under the peak was determined by integration. By comparing the area with areas produced by known nitrite standards, the amount of NO produced from the injected sample could be calculated.

We used the tri-iodide (I_3^-, made by adding NaI and I_2 to acetic acid) assay at 25°C to release NO from nitrite, SNO, FeNO and NNO compounds (Yang et al., 2003). To differentiate between the different compounds, each sample was divided into three aliquots. One aliquot was used for direct injection to assess the sum of nitrite, SNO, FeNO and NNO. The second aliquot was first incubated for 3 min with acidified sulfanilamide, which eliminated nitrite from the sample. The last aliquot was first incubated for 2 min with $HgCl_2$ (which converts SNO to nitrite) and then for 3 min with acidified sulfanilamide, giving a peak that represented the mercury-resistant compounds FeNO and NNO (Feelisch et al., 2002; Yang et al., 2003). Thus, by comparing the peak areas it was possible to assess [nitrite], [SNO] and [FeNO + NNO]. Nitrate was determined with vanadium(III) chloride in 1 mol l^{-1} HCl as a reducing agent and the purge vessel was heated to 95°C (Yang et al., 2003). This treatment converted all concerned NO metabolites to NO, and nitrate was accordingly obtained from the peak by subtraction of the sum of nitrite, SNO, FeNO and NNO determined in the I_3^- assay.

Gill Na^+/K^+-ATPase activity was determined by a semi-micro method (McCormick, 1993), using a microplate reader (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA, USA). The enzyme activity was normalized to the protein content of the sample and expressed as μmol ADP mg^{-1} protein h^{-1}. Protein concentration was measured by a standard method (Lowry et al., 1951) modified for plate reader.

Gills fixed in Bouin's solution for 24 h were stored in 70% ethanol until a piece was dehydrated by transfer to gradually higher ethanol concentrations, cleared in Tissue-Clear (Sakura, Alphen aan den Rijn, The Netherlands) and embedded in paraffin. Sections of 5 μm were cut, rehydrated and stained with Mayer's hematoxylin and eosin and analyzed using a light microscope (Leica Microsystems, Wetzlar, Germany).

Hct was measured by centrifugation of blood (3 min at 13,700 ***g***) in glass capillaries, and the concentration of Hb was measured with the cyanmethemoglobin method, using an extinction coefficient of 11 l $mmol^{-1}$ cm^{-1} at 540 nm. Red cell NTPs (includes both ATP and GTP) were measured spectrophotometrically *via* enzyme coupled reactions (Sigma Bulletin No. 366-UV), using neutralized supernatants from blood that had been deproteinized in 12% trichloroacetic acid. The [NTP]/[Hb_4] ratio was obtained from the corresponding [Hb_4] (concentration of tetrameric Hb). Plasma lactate was determined by the lactate dehydrogenase enzymatic method after deproteinization of plasma with 0.6 mol l^{-1} perchloric acid.

Statistical analysis and data processing

Results are presented as means ± s.e.m., and statistical differences between means were evaluated by one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. Differences between means were considered significant at $P<0.05$. The data generally fulfilled the assumptions of normality (judged graphically) and homogeneity of variances (Levene's test) but a few parameters were log-transformed to fulfill these assumptions. Statistical analyses were performed with SAS (SAS Institute Inc., Cary, NC, USA) version 9.1.

Due to the low concentration of some NO metabolites and the limited sample volume that is obtainable from small-sized goldfish, some samples triggered relatively small NO peaks in the NOA. We therefore transferred raw data from the NOA program to Origin 7.0 (OriginLab Corporation, Northampton, MA, USA), which allows accurate assessment of the area under small peaks (Yang et al., 2003).

RESULTS

Metabolic indicators and gill characteristics

The impact of hypoxia was evaluated through examination of various metabolic indicators at the three ambient oxygen levels. Plasma lactate did not increase significantly above the normoxic level in goldfish exposed to hypoxic water with a P_{O_2} of 29 mmHg whereas plasma lactate was significantly increased in goldfish exposed to a P_{O_2} of 19 mmHg (Fig. 1). This agreed with the experimental idea that one hypoxia level should be close to the critical P_{O_2} of goldfish (~30 mmHg at 22°C) (Fry and Hart, 1948) whereas the most severe hypoxia level should be below the critical P_{O_2}, whereby anaerobic metabolism was stimulated.

The blood [Hb_4] was not significantly altered with decreasing P_{O_2} (Fig. 2A), which was also the case with Hct (data not shown). The RBC [NTP]/[Hb_4] ratio, which reflects the erythrocytic NTP (ATP + GTP) content, decreased significantly during exposure to hypoxia (Fig. 2B). The NTP level was graded to ambient P_{O_2}, and the largest decrease occurred between 29 mmHg and 19 mmHg (Fig. 2B).

The branchial Na^+/K^+-ATPase activity was similar in goldfish exposed to P_{O_2} values of 147 mmHg and 29 mmHg but decreased significantly when P_{O_2} was lowered from 29 mmHg to 19 mmHg (Fig. 3). The statistics were, however, not entirely conclusive (Fig. 3).

Gill morphology differed between the normoxic group and the two hypoxic groups. Normoxic goldfish had their lamellae embedded in an interlamellar cell mass that reduced the respiratory

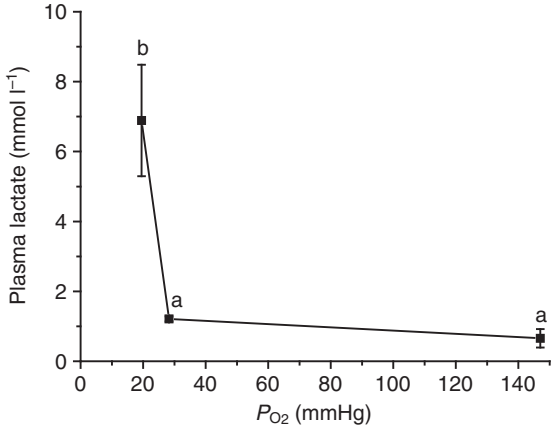

Fig. 1. Plasma lactate concentration as a function of water oxygen tension (P_{O_2}) in goldfish acclimated to normoxic water (P_{O_2}=147 mmHg) or exposed to hypoxia (P_{O_2}=29 mmHg or 19 mmHg) for two days. Values are means ± s.e.m. (N=6 at each P_{O_2}). Different letters at points signify a significant difference ($P<0.05$).

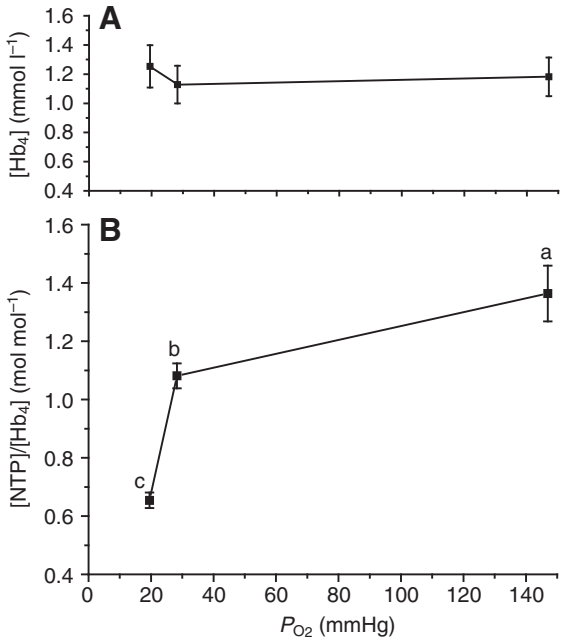

Fig. 2. Concentration of hemoglobin tetramers ([Hb_4]) in whole blood (A) and red blood cell [NTP]/[Hb_4]-ratio (B) in goldfish exposed to different water P_{O_2} (partial pressure of O_2) values. Other details as in Fig. 1.

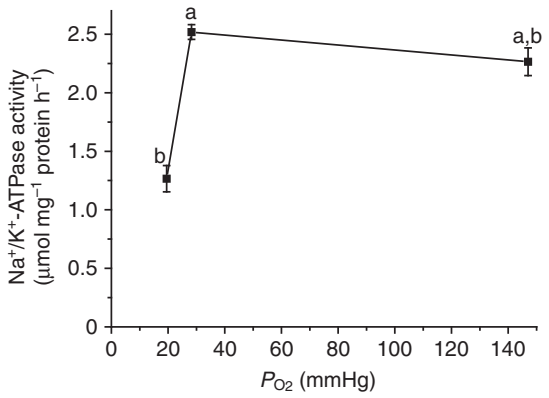

Fig. 3. Gill Na^+/K^+-ATPase activity in goldfish exposed to three different water P_{O_2} (partial pressure of O_2) values. The normoxic control *versus* the lowest P_{O_2} group were not significantly different (P=0.072). Other details as in Fig. 1.

surface area (Fig. 4A) compared with the two hypoxic groups, where the interlamellar cell mass was gone and the lamellae appeared longer (Fig. 4B).

NO metabolites in plasma and RBCs

In normoxic goldfish, the basal concentration of nitrite in plasma was 0.75 μmol l^{-1} (Fig. 5A). Hypoxia induced a pronounced and significant decrease in plasma nitrite, and the magnitude of the decrease clearly depended on the degree of hypoxia (Fig. 5A). At the lowest ambient P_{O_2}, plasma nitrite was reduced by 84% compared with the normoxic group. The plasma concentrations of SNO, FeNO and NNO compounds were low, and only their sum was plotted. The sum amounted to 25 nmol l^{-1} in normoxic fish and did not change significantly with hypoxia (Fig. 5B). Nitrate was present at 45 μmol l^{-1} in plasma of normoxic goldfish, and – like nitrite – the concentration was strongly and significantly decreased by hypoxia (Fig. 5C). At the most severe hypoxia level, plasma nitrate was reduced by 91% compared with the normoxic level.

Interestingly, the decline in extracellular nitrite in hypoxic goldfish was not accompanied by reduced intracellular levels of nitrite in the RBCs (Fig. 6A). The RBC nitrite concentration remained relatively constant and actually showed a tendency to increase at low P_{O_2}, although this was not significant (Fig. 6A). The distribution ratio of nitrite ($[NO_2^-]_{RBC}/[NO_2^-]_{plasma}$) was 0.41±0.06 in normoxic goldfish and increased to 1.0±0.2 at P_{O_2} 29 mmHg and further to 5.1±0.9 at the lowest P_{O_2}. While this is not the true water-based ratio, because plasma concentrations refer to l^{-1} plasma and RBC values refer to l^{-1} RBCs (i.e. rather than cell water), the ratio is useful for analysis (see Discussion).

The concentration of SNO compounds inside the RBCs showed a significant increase in the two hypoxic groups when compared with the normoxic group (Fig. 6B) whereas the RBC concentration of nitrate decreased significantly in hypoxic goldfish (Fig. 6C).

NO metabolites in tissues

Concentrations of NO metabolites were determined in the different organs on a wet mass basis and expressed as absolute concentrations in μmol l^{-1} (assuming a tissue density of 1 kg l^{-1}). For this reason, it is important to evaluate whether water shifts from extracellular to intracellular compartments influenced the results (e.g. acute hypoxia may convert glycogen macromolecules into multiple lactic acid molecules and osmotically draw water into the cells). We therefore plotted nitrite concentrations in liver, kidney and white skeletal muscle both as absolute concentrations and as values normalized to the protein content of the tissue (Fig. 7A–C). This resulted in equivalent curves (Fig. 7A–C), showing that altered cell water content was not an influential factor, probably because any water shift that may have occurred during acute hypoxia had been compensated by cellular volume regulatory mechanisms during the two days of acclimation. Hence, the absolute concentrations (μmol l^{-1}) are judged to reflect the intracellular tissue NO metabolite content.

In liver, kidney and muscle, the values for [nitrite], [SNO] and [FeNO + NNO] were maintained relatively constant at the three P_{O_2} levels (Fig. 7). Nitrate (~35, ~25 and ~30 μmol l^{-1} in liver, kidney and muscle, respectively) also did not change significantly with P_{O_2} (not illustrated). One exception from this overall picture was muscle nitrite, which showed a significant decrease at the lowest P_{O_2} (Fig. 7C).

The levels of NO metabolites also generally stayed constant with decreasing P_{O_2} in brain, heart ventricle and gill tissues (Fig. 8). The notable exception was that the concentration of SNO compounds decreased significantly in the heart ventricle during hypoxia (Fig. 8B).

The levels of the individual NO metabolites differed between tissues. With regard to nitrite, the concentrations were significantly higher in brain, heart and gill tissues (Fig. 8) than in liver, kidney and white skeletal muscle tissues (Fig. 7) but within each of these two organ groupings the nitrite levels were similar. It is also worth noting that while SNO and FeNO + NNO compounds were present in most tissues, the values were very low in white skeletal muscle (Fig. 7F) and undetectable in the gills (Fig. 8C).

The general picture emerging from the tissue results was that the cellular concentrations of NO metabolites were sustained during hypoxia despite large significant decreases in extracellular nitrite and nitrate.

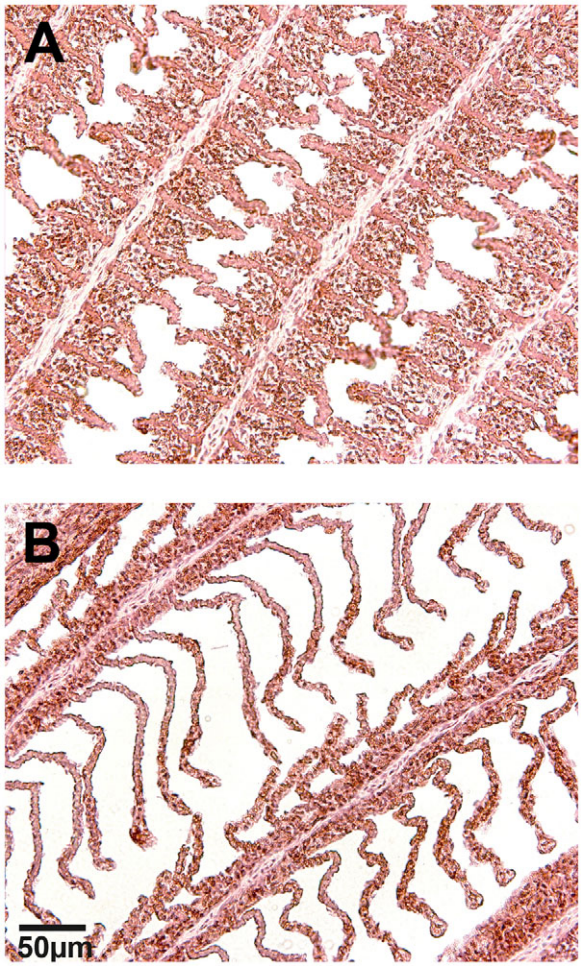

Fig. 4. Light micrographs of gills from goldfish exposed to normoxic water (A) and hypoxic water (B; representative for both hypoxia levels). Temperature was 22°C, and the exposure time to hypoxia was two days. Magnification was ×200. Three fish were examined at each P_{O_2} (partial pressure of O_2) level.

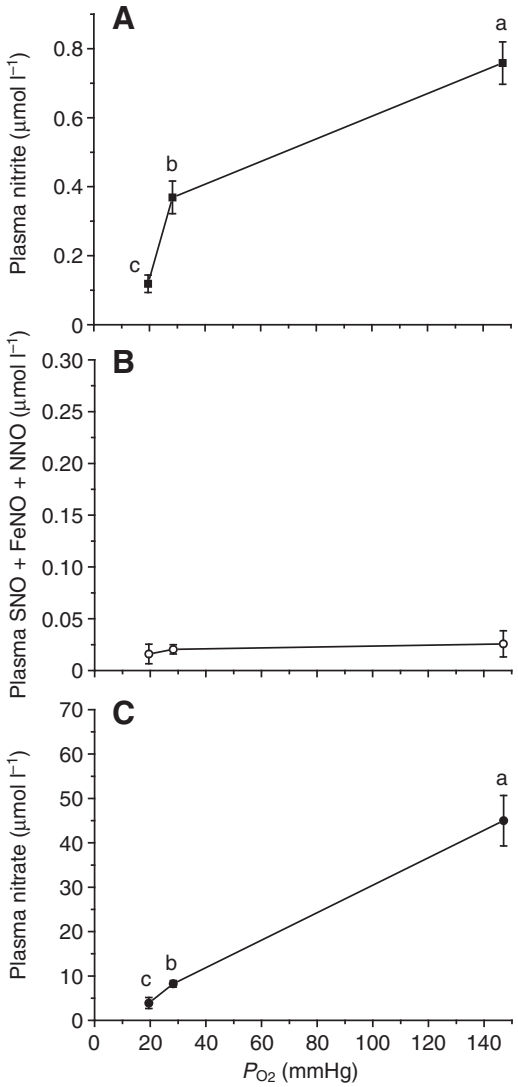

Fig. 5. Concentrations of nitric oxide (NO) metabolites in plasma from goldfish, showing nitrite (A), the sum of *S*-nitroso (SNO), iron-nitrosyl (FeNO) and *N*-nitroso (NNO) compounds (B) and nitrate (C) at three levels of environmental P_{O_2} (partial pressure of O_2). Other details as in Fig. 1.

DISCUSSION

This is the first systematic study that assesses the levels of all main NO metabolites in blood (plasma and RBCs) and multiple tissues of a fish under normoxic and hypoxic conditions. In order to evaluate the influence of hypoxia it is important that the hypoxia levels and their impact on the physiology of the animals are well defined. We therefore measured various metabolic and respiratory parameters to ascertain the severity of hypoxia.

Respiratory parameters

Exposure of fish to hypoxia causes a global decrease in P_{O_2} that challenges aerobic metabolism and induces a variety of compensatory mechanisms that aim to secure a sufficient O_2 transport from the environment to the tissue cells in spite of the decreased O_2 availability. The acute decrease in arterial O_2 content can be compensated for by increasing blood [Hb] (e.g. *via* release of RBCs from the spleen) or by increasing blood O_2 affinity but different fish species exploit these possibilities to a variable degree (Weber and Jensen, 1988). The unchanged blood [Hb] (Fig. 2A) shows that goldfish (like other cyprinids) do not increase blood [Hb] in response to hypoxia. Instead, they rely on increasing Hb O_2 affinity (and thus arterial O_2 saturation), partially by decreasing the erythrocyte NTP content (Jensen, 2004). The decrease in NTP was graded to P_{O_2} and was particularly prominent at the lowest P_{O_2} (Fig. 2B). This amplified drop in NTP coincides with P_{O_2} decreasing below the critical P_{O_2}, where respiratory compensations are not sufficient to maintain a fully aerobic metabolism and anaerobic metabolism is stimulated, as reflected by the increased plasma lactate values (Fig. 1).

A drop in P_{O_2} below the critical value also seemed to depress gill Na^+/K^+-ATPase activity in goldfish (Fig. 3). A similar hypoxia-induced decrease in branchial Na^+/K^+-ATPase activity was recently reported in Amazonian oscar and European flounder (Richards et al., 2007; Lundgreen et al., 2008) and may be reminiscent of a general metabolic suppression strategy in severe hypoxia (Bickler and Buck, 2007). The possible role of NO in decreasing Na^+/K^+-ATPase activity (Tipsmark and Madsen, 2003; Ebbesson et al., 2005) cannot be deduced from the present results.

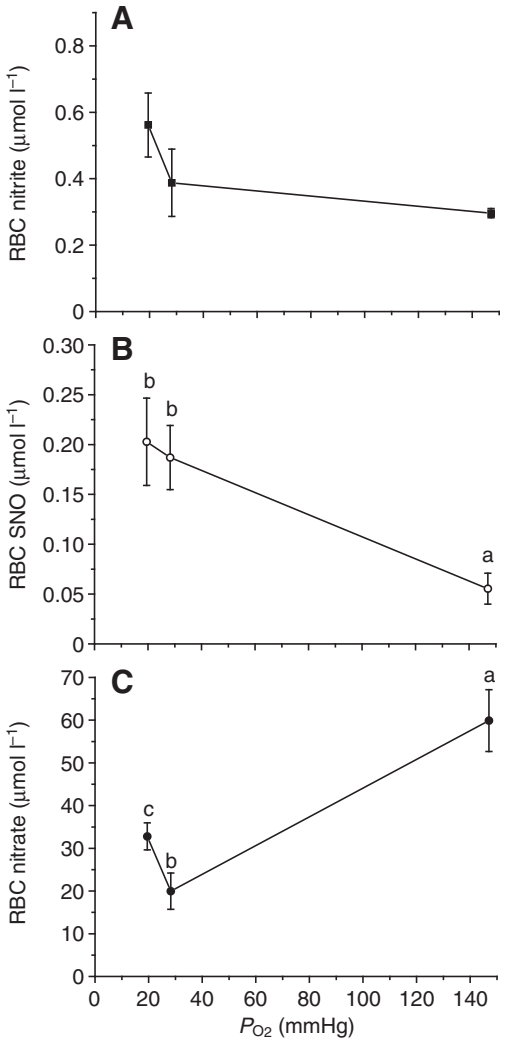

Fig. 6. Red blood cell (RBC) concentrations of nitrite (A), *S*-nitroso (SNO) compounds (B) and nitrate (C) in goldfish at three levels of environmental P_{O_2} (partial pressure of O_2). Other details as in Fig. 1.

The gills of normoxic goldfish had their lamellae embedded in an interlamellar cell mass that disappeared under hypoxia (Fig. 4). This is a well-known response in goldfish and its close relative, crucian carp (Nilsson, 2007). The interlamellar cell mass reduces gill surface area and limits gill ion loss in situations where O_2 is plentiful whereas its disappearance increases gill surface area and favors branchial O_2 uptake in situations where O_2 is limiting (Nilsson, 2007). The interlamellar cell mass is absent not only in hypoxia but also at high temperatures, where O_2 demand is increased (Sollid et al., 2005). Our finding of an interlamellar cell mass in normoxic goldfish at 22°C differs from an earlier report of protruding lamellae in normoxic goldfish at 15 and 20°C (Sollid et al., 2005). The difference between the two studies may, however, be explained by our goldfish being larger [62.5 g *versus* 8–16 g in Sollid et al. (Sollid et al., 2005)] and thus having a lower mass-specific O_2 demand.

From the measured respiratory parameters it is evident that the chosen experimental O_2 conditions cover normoxia, a level of strong hypoxia, where respiratory compensations allows aerobic metabolism, and a level of severe hypoxia, where the organism partially relies on anaerobic metabolism.

Basal normoxic values of NO metabolites

The steady-state concentration of nitrite (or any other NO metabolite) in a given body compartment represents a balance between its rate of formation (or uptake) and its rate of consumption (or excretion). Due to the long-term exposure to the different P_{O_2} levels, the measured concentrations of the NO metabolites are assumed to reflect true steady-state values.

In mammalian species, natural plasma nitrite concentrations range from ~0.1 to 0.8 μmol l^{-1}, and the majority of this nitrite is derived from constitutive NOS activity (Kleinbongard et al., 2003). Thus, albeit dietary intake can cause some variation in basal levels (Lundberg et al., 2008), plasma nitrite is generally a reliable marker of constitutive NOS activity. Basal plasma nitrite levels have only been measured in a limited number of fish species, using methods that are sufficiently sensitive to assess values in the sub-micromolar range. Plasma nitrite concentrations in the two marine teleost fish European flounder (0.27 μmol l^{-1}) and eelpout (0.23 μmol l^{-1}) fall within the mammalian range (Jensen, 2009a) whereas the value in the elasmobranch spiny dogfish (4.5 μmol l^{-1}) is higher (Swensson et al., 2005). When measuring basal plasma nitrite levels in fish – particularly freshwater fish – care must be taken to avoid nitrite contamination of the ambient water, because nitrite can be taken up actively across the gills, which will add to the endogenous nitrite that is produced *via* oxidation of NOS-derived NO (Jensen, 2003; Jensen, 2009a). In our experiments, ambient nitrite was kept sufficiently low (see Materials and methods) to allow the assignment of plasma nitrite primarily to an endogenous production. The determined value of 0.75 μmol l^{-1} in normoxic goldfish (Fig. 5A) therefore supports the idea that the basal plasma nitrite levels resulting from constitutive NOS activity may be similar in teleosts and mammals (Jensen, 2009a).

The values for plasma nitrate (45 μmol l^{-1}, Fig. 5C) and [SNO + FeNO + NNO] (0.025 μmol l^{-1}) in goldfish are also comparable with the values reported in mammals (Kelm, 1999; Lundberg et al., 2008; Rassaf et al., 2002).

The intracellular RBC nitrite concentration has been reported as 0.1–0.7 μmol l^{-1} in rats (Bryan et al., 2004; Bryan et al., 2005) and 0.3 μmol l^{-1} in humans (Dejam et al., 2005), which compares with the basal normoxic level (0.3 μmol l^{-1}) found in goldfish (Fig. 6A). However, the literature on the ratio between nitrite concentrations in RBCs and plasma is conflicting. Some investigations report higher RBC nitrite levels than plasma nitrite levels (Bryan et al., 2004; Dejam et al., 2005) whereas others report that RBC [NO_2^-] is lower than extracellular [NO_2^-] (Bryan et al., 2005; Feelisch et al., 2008; Jensen and Rohde, 2010) as observed in normoxic goldfish. Nitrite appears to be rapidly transported across the RBC membrane *via* both nitrous acid (HNO_2) diffusion and anion exchanger 1-mediated facilitated NO_2^- diffusion, which predicts that the equilibrium distribution ratio between intracellular (RBC) and extracellular (ex) nitrite is related to the proton distribution according to: $[NO_2^-]_{RBC}/[NO_2^-]_{ex}=[H^+]_{ex}/[H^+]_{RBC}=10^{(pH_{RBC}-pH_{ex})}$ (Jensen and Rohde, 2010). The lower pH inside RBCs than outside therefore predicts a ratio lower than 1, implying $[NO_2^-]_{RBC}<[NO_2^-]_{ex}$. The ratio, however, refers to free nitrite, and if intracellular nitrite to some extent is bound to proteins, higher intracellular than extracellular values are possible. Anion binding to Hb inside RBCs is favored by deoxygenation (Jensen and Rohde, 2010), which may explain why $[NO_2^-]_{RBC}/[NO_2^-]_{ex}$ increases from values below 1 in normoxic goldfish to values above 1 in hypoxic goldfish (see below).

Reported values for SNO compounds in mammalian RBCs have been at variance due to differences in employed experimental procedures and methodologies but recent literature argues that

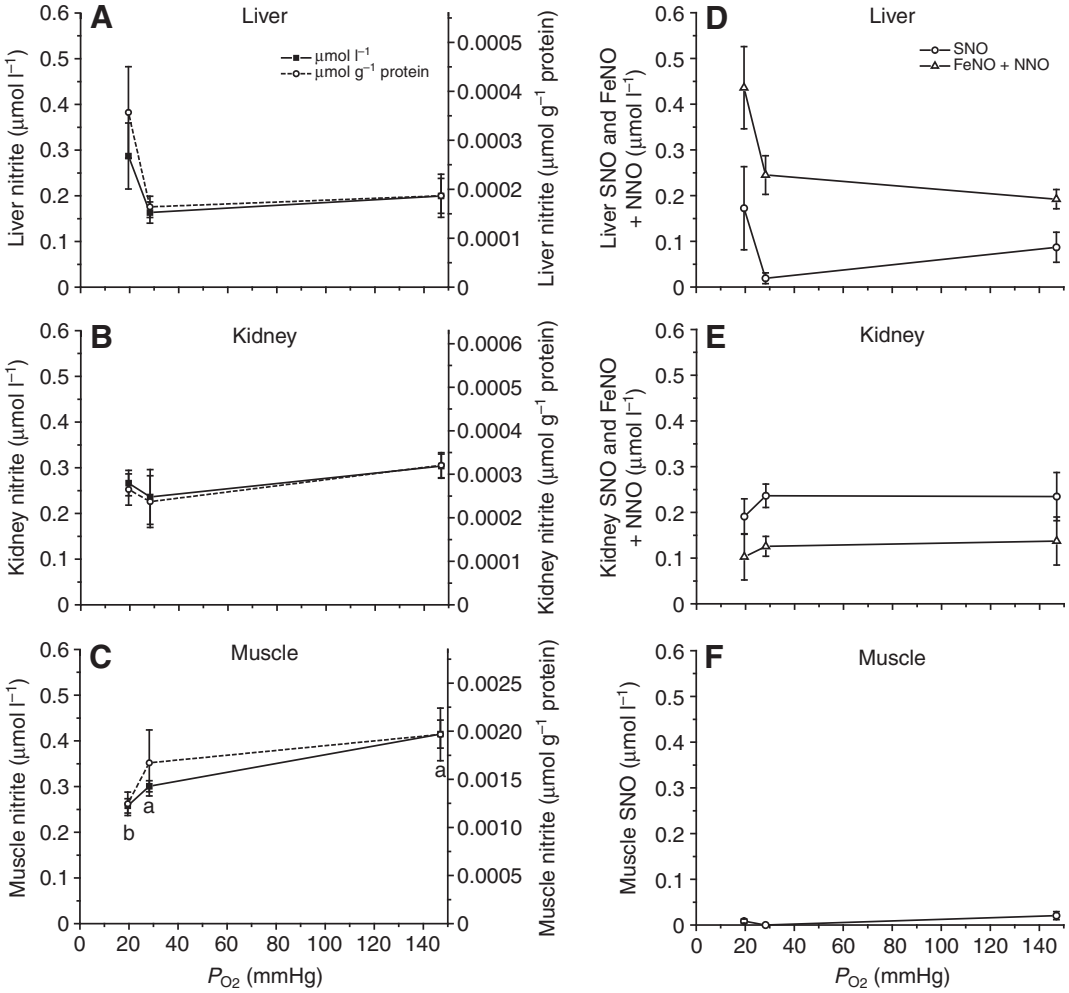

Fig. 7. Concentration of nitrite in goldfish liver (A), kidney (B) and white skeletal muscle (C) tissues. Values were determined on a wet weight basis and shown as absolute concentrations (filled symbols, left *y*-axis) in μmol l^{-1} (assuming a tissue density of 1 kg l^{-1}) and normalized to protein content (open symbols, right *y*-axis). The corresponding concentrations of *S*-nitroso (SNO) compounds and *N*-nitroso (NNO) + iron-nitrosyl (FeNO) compounds in liver, kidney and muscle are shown in panels D, E and F.

the level of SNO compounds measured with reductive chemiluminescense is ~0.05 μmol l^{-1} (Yang et al., 2003; MacArthur et al., 2007), which corresponds with the SNO concentrations in normoxic goldfish RBCs (Fig. 6B).

Levels of NO metabolites in tissues of mammals have been considerably less studied than levels in blood but it is clear that values vary from tissue to tissue (Bryan et al., 2004; Bryan et al., 2005) as also observed here in goldfish (Figs 7 and 8). We find higher nitrite concentrations in brain, heart and gill (Fig. 8) than in liver, kidney and white skeletal muscle (Fig. 7), and both the absolute tissue nitrite concentrations and the tissue differences compare well with findings in rats (Bryan et al., 2005). If HNO_2 diffusion is a main mechanism for nitrite transport across tissue cell membranes, then pH will influence cellular nitrite concentrations (Samouilov et al., 2007; Jensen and Rohde, 2010). A higher intracellular pH in goldfish brain and heart tissues than in muscle, as reported in channel catfish (Cameron and Kormanik, 1982), would consequently contribute to a higher nitrite concentration in the former tissues. It is also possible that tissues with high nitrite concentrations are richer in constitutive NOS, leading to a larger NO and NO_2^- production that translates into higher steady-state nitrite concentrations. Indeed, such a correlation seems present in rat tissues (Bryan et al., 2004). In fish, significant NOS expression has been reported in brain, heart and gills (Brüning et al., 1995; Holmqvist et al., 2000; Tota et al., 2005; Ebbesson et al., 2005; Hyndman et al., 2006), the tissues with the highest nitrite levels in goldfish. The values of [SNO] and [FeNO + NNO] in tissues range from practically zero (below detection level) in the gills (Fig. 8C) to values that are comparable with nitrite concentrations in kidney and liver (Fig. 7), which points to significant differences in the extent and importance of nitros(yl)ation reactions among tissues. The physiological significance of this must await further study.

The overall picture from the normoxic results is that basal levels of all NO metabolites in both plasma, RBCs and various tissues of goldfish compares well with the corresponding values in mammalian species, which leads to the conclusion that both the production of NO and its biochemical fates are rather similar in fish and mammals.

Influence of hypoxia on NO metabolites

A salient result of the present study is the dramatic decreases in plasma nitrite and plasma nitrate following two days of hypoxia exposure (Fig. 5). A similar decrease in plasma nitrite was reported in European flounder exposed to hypoxia for two days (Jensen, 2009a). Likewise, in humans, both plasma nitrite and plasma nitrate

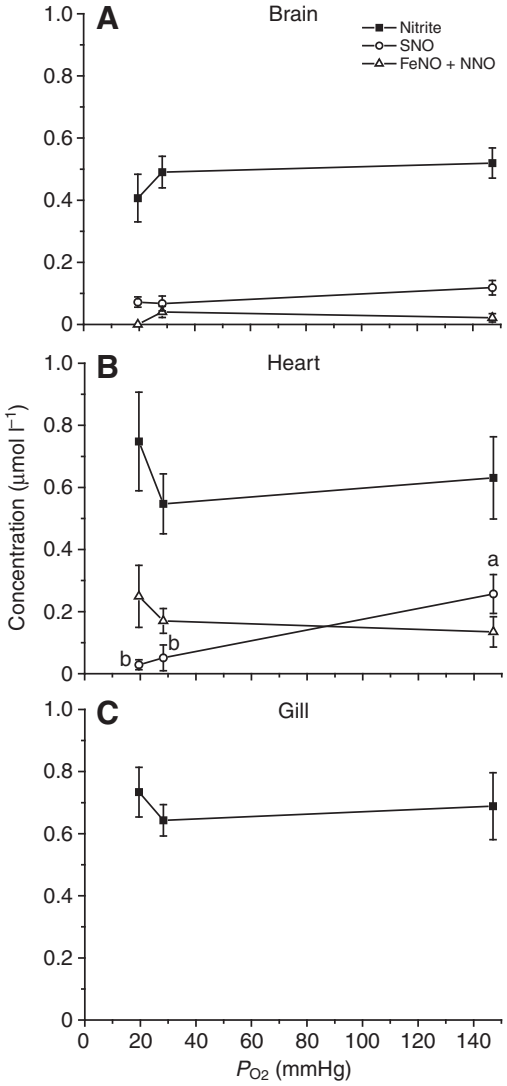

Fig. 8. Concentrations of nitrite, *S*-nitroso (SNO) compounds and iron-nitrosyl (FeNO) + *N*-nitroso (NNO) compounds in goldfish brain (A), heart ventricle (B) and gill (C) tissues at different ambient P_{O_2} (partial pressure of O_2) levels. Values were determined on a wet mass basis and shown in μmol l^{-1}, assuming a tissue density of 1 kg l^{-1}. Levels of [SNO] and [FeNO + NNO] were below the detection limit (~6 nmol l^{-1} with the available sample volumes) in the gills.

decrease by close to 40% following exposure to hypoxia (12.9% O_2) for 9 h (Bailey et al., 2009). These results seem to support the idea that NOS activity is compromised by the lack of O_2. A reduced NOS-catalyzed NO production during hypoxia would decrease both nitrite production from the reaction of NO with O_2 and nitrate production from the reaction of NO with oxygenated Hb (i.e. less oxyHb available under hypoxia). The decrease in plasma nitrite furthermore is compatible with the idea that nitrite is consumed by being utilized as a cellular NO donor during hypoxia whereas the decrease in plasma nitrate hints at the possibility that vertebrates can reduce nitrate to nitrite and then further to NO under hypoxia (Jansson et al., 2008). In addition to these explanations, it should also be considered whether an enhanced urinary excretion or efflux *via* the gills might contribute to lower extracellular nitrite and nitrate in hypoxia. A recent study on goldfish, however, showed that hypoxia actually inhibits passive branchial ion efflux in spite of an increased respiratory surface area of the gills (Mitrovic et al., 2009), and in another hypoxia-tolerant fish (Amazonian oscar), both urinary and branchial ion excretion rates were decreased rather than increased by hypoxia (Wood et al., 2009).

Contrasting with the picture that extracellular levels of nitrite and nitrate are reduced in goldfish, European flounder and humans during relatively long-term hypoxia are reports that acute hypoxia (duration ≤60 min) increases plasma nitrite in dogfish (Swenson et al., 2005) and the sum of plasma nitrite and nitrate in rainbow trout (McNeill and Perry, 2006). While these differences may relate to differences in the degree and duration of hypoxia they also draw attention to species variation. NOS activity may be decreased by the lack of O_2 to a variable extent among species, and a reduced NO formation by individual NOS enzymes can be compensated for by an increased expression of NOS, as seems to be the case in rainbow trout (McNeill and Perry, 2006). In addition, the cellular utilization of nitrite during hypoxia may vary between species (Jensen, 2009a).

In the present study, nitrite was measured both in the extracellular space and in multiple tissues, and the integrated results propose a reduced whole body nitrite content during hypoxia, supporting a decreased nitrite production from NOS-derived NO and an increased nitrite consumption. The data furthermore points at a transfer of nitrite from extracellular to intracellular compartments, because extracellular [NO_2^-] decreases while intracellular [NO_2^-] is relatively stable. Indeed, a cellular consumption of nitrite will establish a diffusion gradient from the extracellular to the intracellular space. The RBCs provide a special case. As nitrite is consumed in RBCs, new nitrite rapidly diffuses in, re-establishing the equilibrium distribution across the membrane (Jensen and Rohde, 2010). This distribution is guided by pH conditions (see above). Intracellular pH_{RBC} increases during hypoxia due to deoxygenation of the Hb (large Haldane effect in teleost fish) and due to the reduction in erythrocytic NTP (Jensen, 2004). This will increase the distribution ratio $[NO_2^-]_{RBC}/[NO_2^-]_{ex}$ but cannot explain values that exceed 1, as is actually observed at the lowest P_{O_2}. However, as mentioned above, such high ratios would be possible by an increased binding of nitrite to Hb (or other proteins) upon deoxygenation, because it is the free nitrite that attains an equilibrium distribution.

It is striking that the tissue concentrations of nitrite and other NO metabolites remain relatively stable in hypoxic goldfish. During acute hypoxia one would expect that nitrite utilization should decrease the intracellular nitrite concentration and increase the concentrations of FeNO and SNO compounds, as observed following a few minutes of global hypoxia in rat (Bryan et al., 2004; Feelisch et al., 2008). The general absence of such changes following two days of hypoxia suggest the involvement of regulatory mechanisms that defend intracellular NO homeostasis. While acute hypoxia may have caused divergence in NO metabolites, a longer exposure time (hypoxia acclimation) seems to establish a new steady state with similar tissue NO metabolite levels as in normoxia. Nitrite influx from the extracellular space together with an increased tissue expression of NOS may help normalize cellular nitrite levels. We hypothesize that this may be supplemented by increased nitrite binding to proteins (perhaps *via* expression of specific nitrite-binding proteins), which keeps intracellular nitrite stable in the face of declining extracellular values.

It is increasingly realized that *S*-nitrosylation of specific protein targets play important roles in the normal physiology of mammals and that hypo- or hyper-*S*-nitrosylation can be associated with malfunctions (Foster et al., 2009). The ability to maintain SNO

compounds relatively stable at low P_{O_2} may therefore be a significant aspect of hypoxia tolerance in goldfish.

While tissue NO metabolites were generally constant, there were a few significant changes with hypoxia that deserves notion. In the RBCs, [SNO] increased during hypoxia (Fig. 6B). The SNO concentrations encompass both low molecular weight thiols (e.g. glutathione) and proteins but a main candidate for *S*-nitrosation inside RBCs is Hb. In mammals, it has been proposed that Hb nitrosated at Cys93 of the β chain represents a blood-borne NO activity that can be released upon deoxygenation in hypoxic tissues and contribute to hypoxic vasodilation (Jia et al., 1996). Fish Hbs do not contain this particular cysteine but may be nitrosated at other cysteines (Jensen, 2009b). Goldfish Hb contains four Cys residues in the β chain, of which three are internal whereas one is placed in a surface crevice of the Hb molecule (Reischl et al., 2007) and therefore is potentially available for *S*-nitrosation. The rise in RBC [SNO] with hypoxia is, however, opposite to the decrease with deoxygenation predicted from the SNO-Hb hypothesis in mammals (Jia et al., 1996), obliterating a function of *S*-nitrosated Hb in hypoxic vasodilation in goldfish. The increase in erythrocyte SNO in goldfish during hypoxia probably is a consequence of increased deoxyHb-mediated nitrite reduction to NO in the RBCs, which increases the formation of N_2O_3 (Basu et al., 2007), a potent nitrosating agent. The nitrite reductase activity of deoxyHb is particularly prominent in hypoxia-tolerant fish with high O_2 affinity (Jensen, 2009a), and the nitrite-derived NO may contribute to the regulation of vascular tone in hypoxic conditions (Cosby et al., 2003; Jensen, 2009b).

In the heart, the concentration of SNO compounds decreased with hypoxia (Fig. 8B). This might reflect a reduced NO production from NOS combined with an insufficient compensation *via* nitrite reduction to NO. Deoxygenated Mb is probably the main nitrite reductase in the ventricle (Shiva et al., 2007). In goldfish, the expression of Mb in the heart does not change with hypoxia (Roesner et al., 2008) and the Mb O_2 affinity may be so high that Mb does not deoxygenate sufficiently at the prevailing tissue P_{O_2} to generate enough NO (and N_2O_3) to maintain *S*-nitrosylation of proteins. This may be of significance, because *S*-nitrosylation of proteins has been reported to modulate the inotropic response of fish hearts (Garofalo et al., 2009).

Concluding remarks

Our results suggest that nitrite and other NO metabolites are homeostatically regulated in tissues, and that goldfish have a profound capacity for defending intracellular NO homeostasis during severe hypoxia. This appears to involve the transfer of nitrite to intracellular compartments and usage of nitrite as a NO donor. Furthermore, even though NOS-mediated NO formation is compromised by a shortage of the substrate O_2, the expression of tissue NOS may go up as a compensation that helps to keep cellular NO levels stable. Future experiments will need to address these possibilities in more detail.

LIST OF ABBREVIATIONS

DTPA	diethylenetriaminepentaacetic acid
FeNO	iron-nitrosyl
Hb	hemoglobin
Hct	hematocrit
Mb	myoglobin
NEM	*N*-ethylmaleimide
NNO	*N*-nitroso
NO	nitric oxide
NOS	nitric oxide synthases
NTP	nucleoside triphosphates
P_{O_2}	partial pressure of O_2
RBCs	red blood cells
SNO	*S*-nitroso

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