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RESEARCH ARTICLE

The roles of tissue nitrate reductase activity and myoglobin in securing nitric oxide availability in deeply hypoxic crucian carp

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ABSTRACT

In mammals, treatment with low doses of nitrite has a cytoprotective effect in ischemia/reperfusion events, as a result of nitric oxide formation and *S*-nitrosation of proteins. Interestingly, anoxia-tolerant lower vertebrates possess an intrinsic ability to increase intracellular nitrite concentration during anoxia in tissues with high myoglobin and mitochondria content, such as the heart. Here, we tested the hypothesis that red and white skeletal muscles develop different nitrite levels in crucian carp exposed to deep hypoxia and assessed whether this correlates with myoglobin concentration. We also tested whether liver, muscle and heart tissue possess nitrate reductase activity that supplies nitrite to the tissues during severe hypoxia. Crucian carp exposed to deep hypoxia ($1<P_{O_2}<3$ mmHg) for 1 day increased nitrite in red musculature to more than double the value in normoxic fish, while nitrite was unchanged in white musculature. There was a highly significant positive correlation between tissue concentrations of nitrite and nitros(yl)ated compounds. Myoglobin levels were 7 times higher in red than in white musculature, but there was no clear correlation between nitrite and myoglobin levels. Finally, we found a low but significant nitrate reductase activity in liver and white muscle, but not in cardiomyocytes. Nitrate reduction was inhibited by allopurinol, showing that it was partly catalyzed by xanthine oxidoreductase.

KEY WORDS: Ethanol, Hypoxia, Nitrate reduction, Nitric oxide, Nitrite, Red muscle

INTRODUCTION

Nitric oxide (NO) is a vital signaling molecule that exerts its physiological effects by reversible binding/reacting with hemes, thiols or amines, forming iron-nitrosyl (FeNO), *S*-nitroso (SNO) and *N*-nitroso (NNO) compounds (Hill et al., 2010). Furthermore, NO is short-lived and excess NO is rapidly oxidized to nitrite and nitrate. Under normoxic conditions, NO is produced from the reaction of L-arginine with molecular oxygen, catalyzed by nitric oxide synthase (NOS) enzymes, and this reaction is vulnerable to hypoxia because of the requirement for O_2 (Moncada, 1993; Bryan, 2006; Lundberg et al., 2008). However, it has been established that both nitrite and nitrate can be reduced back to NO, which provides an alternative pathway for NO generation under hypoxia (Gladwin et al., 2005; Lundberg et al., 2009; van Faassen et al., 2009). Accordingly, NO is generated by different mechanisms depending on oxygen levels and also tissue type, pH and redox status (Gladwin et al., 2005; Feelisch et al., 2008; Hill et al., 2010). The preservation of NO availability by nitrite reduction is indeed important in hypoxia, where it contributes to hypoxic vasodilation and cytoprotection (Cosby et al., 2003; Shiva and Gladwin, 2009). Several studies have documented that administration of nitrite can reduce cell death and infarct size after ischemia/reperfusion in heart and liver tissues of mammals (Webb et al., 2004; Duranski et al., 2005; Hendgen-Cotta et al., 2008; Shiva and Gladwin, 2009). This relates to the binding of nitrite-derived NO to complex IV of the mitochondrial respiratory chain, which reduces respiration rate and extends the O_2 gradient, as well as *S*-nitrosation of complex I, which limits generation of reactive oxygen species (ROS) at the onset of reoxygenation (Shiva et al., 2007; Murillo et al., 2011; Chouchani et al., 2013). Interestingly, anoxia-tolerant vertebrates (crucian carp and freshwater slider turtles) seem to naturally exploit these mechanisms by elevating nitrite and nitros(yl)lated compounds in, for example, the heart during deep hypoxia and anoxia (Sandvik et al., 2012; Jensen et al., 2014; Hansen et al., 2016).

We have previously shown that goldfish and crucian carp maintain tissue nitrite concentration during hypoxia and increase it in the heart during deep hypoxia and anoxia (Hansen and Jensen, 2010; Sandvik et al., 2012; Hansen et al., 2016). This occurs in the face of a decrease in extracellular nitrite concentration. We suggested that nitrite is transferred from the extracellular to the intracellular space and that this could be facilitated by intracellular binding of nitrite to proteins, which would keep the cytosolic concentration of free nitrite low and permit inward diffusion (Hansen and Jensen, 2010). Because anoxia increases tissue nitrite in the heart of crucian carp and red-eared slider turtles, as well as in red pectoral muscle of turtles, but not white muscle of crucian carp, we hypothesized that myoglobin (Mb) or mitochondria, both present at high levels in heart and red musculature, play a role (Jensen et al., 2014). Specifically we suggested that the increased binding of negatively charged nitrite during anoxia could be explained by a progressively more positively charged Mb due to H^+ buffering caused by anoxia-induced acidosis (Jensen et al., 2014). One aim of the present study was to measure nitrite levels in red and white muscle of normoxic and deeply hypoxic crucian carp to validate a difference in nitrite levels between the two muscle types within the same species. Further, we measured Mb concentrations in red and white muscle to test for a possible correlation between tissue nitrite and Mb levels.

As mentioned above, extracellular nitrite is shifted into tissues of hypoxic and anoxic fish. But the extracellular pool of nitrite is sparse, and nitrite needs to be supplemented from other sources to maintain or increase tissue nitrite concentration during long-term

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hypoxia. We recently showed that crucian carp can utilize ambient nitrite by taking it up across the gills and transporting it via the blood to tissues, where it enters cells, notably in hypoxia (Hansen et al., 2016). Another potential source of tissue nitrite is tissue nitrate. Mammalian tissues have been shown to possess nitrate reductase activity (mediated by xanthine oxidoreductase and possibly other proteins) that can reduce nitrate to nitrite, and this is most prominent during hypoxic/anoxic conditions (Jansson et al., 2008; Huang et al., 2010; Piknova et al., 2015). The existence of tissue nitrate reductase activity in fish remains to be established, but it could be a valuable additional source of nitrite (and thus NO) in crucian carp, when they experience long-term hypoxia and anoxia in ice-covered ponds during winter (Vornanen et al., 2009). For this reason, a major aim of the present study was to investigate nitrate reductase activity in selected tissues from normoxic and deeply hypoxic crucian carp. The liver was examined because of its relatively high nitrate reductase activity in mammals (Jansson et al., 2008; Piknova et al., 2015). The heart was tested for nitrate reductase activity because of the distinctive increase in nitrite concentration in this tissue during anoxia and deep hypoxia (Sandvik et al., 2012; Hansen et al., 2016). Finally, white skeletal muscle was examined because it constitutes some 50% of the fish mass, and because it has a unique role in anoxic crucian carp in converting lactate to ethanol (for subsequent excretion across the gills), thereby limiting acidosis during anaerobic metabolism (Shoubridge and Hochachka, 1980; Johnston and Bernard, 1983; Vornanen et al., 2009). To ascertain ethanol production in deep hypoxia, we measured ethanol concentration in muscle and plasma.

MATERIALS AND METHODS

Animals, treatment and sampling

Crucian carp, *Carassius carassius* (Linnaeus 1758), of mixed sex, weighing 45.4±1.85 g (mean±s.e.m., N=42) were caught in a local pond (Langsted, Funen, Denmark) in July and transferred to two 200 l holding tanks, where pond water was gradually changed to experimental water (Odense tap water mixed with demineralized water in a 1:4 ratio). The fish stayed in the tanks for 17 days and were fed with commercial trout pellets (Inicio, Biomar, Denmark), while being acclimated to 15°C and a 12 h:12 h light:dark cycle in normoxic (P_{O_2}>140 mmHg) water. Normoxia was obtained by bubbling air, and water was exchanged daily.

The fish were subsequently moved to four normoxic experimental aquaria (100 l, with 10–11 fish in each) for 5 days without feeding; 60 l of water was exchanged twice daily. Two aquaria were maintained normoxic (P_{O_2}>140 mmHg) for one additional day, while the two other aquaria were bubbled with N_2 for 1 day to expose the fish to deep hypoxia (1<P_{O_2}<3 mmHg). The water surface was covered with expanded polystyrene, and deep hypoxia was reached within 4 h. Water P_{O_2} was measured using an optical Hach Lange optode (HQ 40d, Loveland, CO, USA). Water nitrite concentration stayed below 0.5 µmol l^{-1} and water Cl^- concentration was 260 µmol l^{-1}.

Fish were caught individually and anesthetized in 2‰ MS222 (ethyl-3-aminobenzoate methanesulfonate) dissolved in experimental water. The fish were weighed and blood was taken from the caudal vessels, after which tissues were dissected out in the following order: heart, liver and muscle. The muscle was divided into red muscle (primarily slow oxidative fibers) and white muscle (primarily fast glycolytic fibers). The red muscle (musculus lateralis superficialis trunci) is situated in a thin layer underneath the skin along the lateral line of the fish (Hamoir, 1953). Blood was centrifuged (2 min, 8000 ***g***, 15°C) and plasma was separated. Tissues were rinsed in 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)], dried on tissue paper and weighed. All samples were snap frozen in liquid nitrogen and stored at −80°C until further analysis.

The procedures and experiments were conducted in accordance with Danish laws on animal experimentation. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

Nitrate reduction and determination of NO metabolites

The method for determination of tissue nitrate reductase activity was mostly conducted as previously described (Jansson et al., 2008). In short, subsamples of tissues weighing between 19 and 200 mg (depending on the tissue) were thawed and homogenized in 3 times their weight of phosphate buffer (100 mmol l^{-1}, pH 7.0) and 1 vol % protease inhibitor (P2714, Sigma-Aldrich). Heart and liver tissues were homogenized using zirconium oxide beads (ZROB05, 5 mm, Next Advance, NY, USA) in a Bullet Blender Blue (Next Advance) and muscle tissue was homogenized with a tissue grinder (Struers, Heidoloph, Denmark). Homogenates were centrifuged (10 min, 500 ***g***, 4°C), and supernatants were decanted and divided into subsamples that were snap frozen, or used for determination of protein concentration, using the BioRad (München, Germany) protein assay based on Bradfords assay, with bovine serum albumin as standard (Bradford, 1976). Final concentrations of protein (7 mg ml^{-1}), cofactors (1 mmol l^{-1} NADPH, 500 µmol l^{-1} NADH, 500 µmol l^{-1} NAD^+, 500 µmol l^{-1} GSH) and nitrate (300 µmol l^{-1}) were achieved by mixing appropriate amounts of buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris-HCl, pH 7.0), homogenate, cofactor mix and nitrate in the given order. The volume of buffer was adjusted to fit the final volume in incubations with or without nitrate. Immediately after mixing, one aliquot was used to determine NO metabolites at time zero and the other subsample was subsequently bubbled with pure nitrogen for 2 min (heart and liver) or 7 min (muscle), sealed with Parafilm® and incubated for 5 h at 25°C before a further determination of NO metabolites. To ascertain a persistent N_2 atmosphere, we also performed experiments with liver homogenates in shaking Eschweiler (Kiel, Germany) tonometers receiving a continuous flow of humidified N_2. The role of xanthine oxidoreductase (XOR) in nitrate reduction was examined by adding the XOR inhibitor allopurinol at 2 mmol l^{-1}. Nitrite and nitros(yl)ated compounds were determined by chemiluminescence (NO analyzers: Model CLD 77 AM, Eco Physics, Duernten, Switzerland; and Model 280i, Sievers, Boulder, CO, USA), as previously described (Yang et al., 2003; Hansen and Jensen, 2010). Nitrate was assessed with a vanadium chloride assay, and nitrite and nitros(yl)ation compounds (SNO+FeNO+NNO) were determined in a triiodide assay. To distinguish between nitrite and [SNO]+[FeNO]+[NNO], the samples were treated with sulfanilamide (Hansen and Jensen, 2010). NO-metabolites were calculated as absolute concentrations in µmol l^{-1} assuming a tissue density of 1 kg l^{-1}. Protein determinations in liver, muscle and heart did not differ between normoxic and deeply hypoxic fish, indicating that hypoxia did not cause a significant water shift and hence did not influence NO metabolite concentrations.

Muscle Mb concentration

Mb concentration was determined spectrophotometrically using the method developed by Reynafarje (1963) with small modifications (Helbo and Fago, 2012). White muscle (~200 mg) and red muscle

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(~100 mg) were homogenized in 4.25 and 9.25 times their mass of hypotonic buffer (40 mmol l^{-1} $KHPO_4$, pH 6.60), respectively. We used a knife homogenizer (ultra-turrax T25, IKA-labortechnic, Staufen, Germany) on ice, 3 times 30 s with a 30 s break in between, to avoid heating up the homogenates. After centrifugation (50 min, 15,000 ***g***, 4°C), homogenates containing Mb were equilibrated with CO for 3 min and ~0.001 g dithionite was added to reduce any ferric heme (Helbo and Fago, 2012). Absorbance spectra were collected from 700 to 400 nm, and Mb concentration (mg g^{-1} wet mass) was determined from the difference in absorbance at 538 and 568 nm [which is zero for carboxyhemoglobin (HbCO) but not for carboxymyoglobin (MbCO)], using reported extinction coefficients for the corresponding dilution (Reynafarje, 1963).

Ethanol

Ethanol was determined in plasma and white muscle homogenates using a commercial ethanol assay kit (MAK076, Sigma-Aldrich) and a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Statistics and data processing

Graphing and statistical analyses were performed in Prism 5.0 (GraphPad Software, San Diego, CA, USA) and Origin 8.5 (OriginLab Corporation, Northampton, MA, USA). Results are presented as means±s.e.m. and statistical differences between means were evaluated using unpaired *t*-test, one-way analysis of variance (ANOVA) with Bonferroni *post hoc* test or two-way ANOVA, as appropriate. Furthermore, linear regression and one-sample *t*-test (to compare means with zero) were used. We applied a significance level of $P<0.05$ and data that did not fulfil the assumption of equal variances (Bartlett's test) were log-transformed prior to analysis.

RESULTS

Crucian carp exposed for 1 day to deep hypoxia showed slightly lower plasma nitrite concentration (Fig. 1A) and plasma [SNO+FeNO+NNO] (Fig. 1B) compared with normoxic fish. Plasma nitrate concentration (Fig. 1C) did not differ between normoxic and deeply hypoxic groups.

In red skeletal muscle, nitrite concentration increased to more than double the normoxic value after exposure for 1 day to deep hypoxia, whereas it stayed constant in white skeletal muscle at a value that was similar to that in normoxic red muscle (Fig. 2A). The rise in red muscle nitrite concentration during deep hypoxia was paralleled by a significant increase in the concentration of nitros(yl)ated compounds (SNO+FeNO+NNO; Fig. 2B). In white muscle, [SNO+FeNO+NNO] did not change (Fig. 2B). A plot of all individual muscle [SNO+FeNO+NNO] values against corresponding nitrite concentrations revealed a highly significant (R^2=0.811) linear increase in [SNO+FeNO+NNO] with increasing nitrite concentration (Fig. 3). Tissue nitrate concentration was slightly higher in white than in red muscle but only decreased non-significantly during deep hypoxia (Fig. 2C).

The level of Mb was significantly higher in red muscle than in white muscle but was unaffected by the level of ambient oxygen (Fig. 4). Hence, red muscle Mb was 3.68±0.369 mg g^{-1} (corresponding to 0.22 mmol l^{-1}) in normoxic fish and 2.98±0.362 mg g^{-1} in deeply hypoxic fish, which is 7–8 times higher than in white muscle, where Mb was 0.499±0.104 mg g^{-1} in normoxic fish and 0.361±0.075 mg g^{-1} in deeply hypoxic fish. When connected values of muscle nitrite and muscle Mb concentration from all individual fish are plotted against each other, it is evident that there is no linear correlation between muscle nitrite and Mb concentration, either when combining all groups or when analyzing them independently (Fig. 5). The main difference is that nitrite levels in deeply hypoxic red muscle are raised above the values in the other groups (Fig. 5).

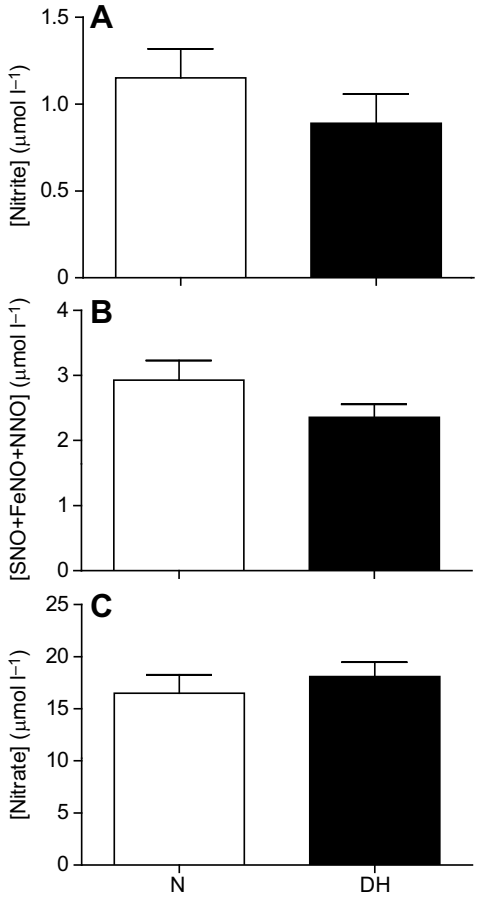

Fig. 1. Plasma nitric oxide (NO) metabolites in normoxic and deeply hypoxic crucian carp. Concentrations of nitrite (A), *S*-nitroso (SNO)+iron-nitrosyl (FeNO)+*N*-nitroso (NNO) compounds (B) and nitrate (C) in plasma of crucian carp after 1 day exposure to normoxia (N; P_{O_2}>140 mmHg) or deep hypoxia (DH; 1<P_{O_2}<3 mmHg). Values are means±s.e.m. (*N*=15 for each group).

In white skeletal muscle homogenates from both normoxic and deeply hypoxic fish, there was no significant nitrite production during 5 h hypoxic incubation in the absence of exogenous nitrate (Fig. 6A,E and C,G). However, when 300 µmol l^{-1} nitrate was added, the muscle tissue from deeply hypoxic fish showed a significant nitrite production (Fig. 6D,H). In muscle from normoxic fish, the change in nitrite concentration was not significant (P=0.07) (Fig. 6B,F). In the liver, we obtained similar results from N_2 incubations in Eppendorf tubes and in rotating tonometers with continuous flow of N_2 (Fig. 7). The nitrite production from nitrate in liver was significant after 5 h incubation in the presence of added nitrate (Fig. 7), and the nitrate reductase activity was higher than that in muscle. For comparison, we also tested nitrite production in a single mouse liver and found a 3 times higher production than in crucian carp (data not shown). The nitrite production in liver homogenates from crucian carp was significantly inhibited by allopurinol (Fig. 8). We did not observe nitrate reduction in the heart, and the nitrite concentration actually decreased by about 2 µmol l^{-1} during 5 h of incubation (Fig. 9).

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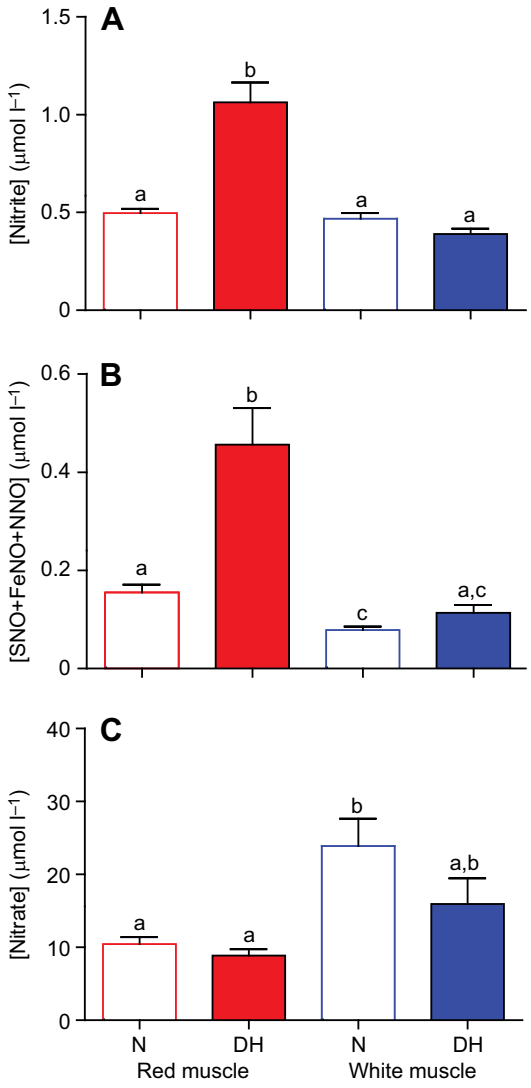

Fig. 2. Red and white muscle NO metabolites in normoxic and deeply hypoxic crucian carp. Concentrations of nitrite (A), SNO+FeNO+NNO (B) and nitrate (C) in red muscle and white muscle of crucian carp exposed to normoxic (N; P_{O_2}>140 mmHg) and deeply hypoxic (DH; 1<P_{O_2}<3 mmHg) water for 1 day. Values are means±s.e.m. (N=15 for each group). Differences between groups were tested using a one-way ANOVA, followed by a Bonferroni *post hoc* test, and different letters signify statistical differences between groups.

Ethanol was not detected in white muscle and plasma from normoxic fish, whereas ethanol increased to 10.5±1.1 mmol l^{-1} (mean±s.e.m., N=8) in muscle and 3.9±0.3 mmol l^{-1} (mean±s.e.m., N=8) in plasma of crucian carp exposed to deep hypoxia for 1 day.

DISCUSSION

A major finding of the current study is that tissue nitrate reduction can take place in crucian carp during deep hypoxia, indicating that nitrate reductase activity, albeit low, may participate in supplying nitrite for cytoprotection in the deeply hypoxic crucian carp. Furthermore, we found that deep hypoxia induces increased concentrations of nitrite and nitros(yl)ation compounds in red muscle but not white muscle. The Mb concentration was considerably higher in red muscle than in white muscle and it was unaffected by hypoxia. As discussed below, we suggest that tissue nitrate reductase activity supplements other nitrite supply

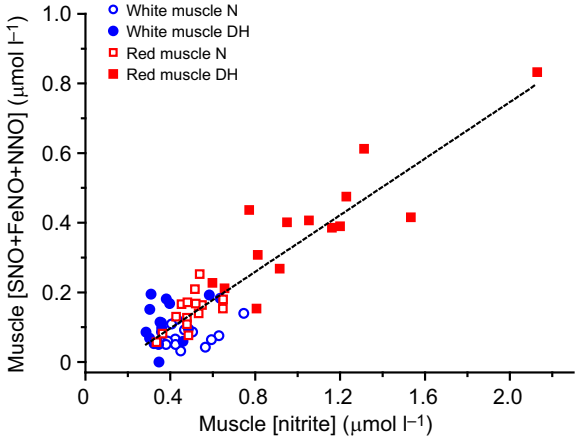

Fig. 3. Relationship between SNO+FeNO+NNO and nitrite concentration in muscle tissues from normoxic and deeply hypoxic crucian carp. Connected values of SNO+FeNO+NNO and nitrite concentration in red muscle (red symbols) and white muscle (blue symbols) of individual crucian carp exposed to normoxic (N; P_{O_2}>140 mmHg) and deeply hypoxic (DH; 1<P_{O_2}<3 mmHg) water. The dashed line represents the overall linear regression (y=0.406x−0.066, R^2=0.811, N=60).

routes in deeply hypoxic crucian carp, and that the Mb concentration is not directly responsible for increased tissue nitrite accumulation.

Mb and NO metabolites in red and white muscle

The present study is the first to examine NO metabolite and Mb levels in both red and white muscle in a hypoxia-tolerant lower vertebrate. We found considerably higher Mb concentrations in red muscle than in white muscle, and the level of nitrite increased significantly with exposure to deep hypoxia in red muscle, but not in white muscle. The latter observation corroborates the idea that tissues rich in Mb and mitochondria, such as red muscles and the heart, develop increased nitrite levels during severe O_2 deprivation (Jensen et al., 2014). Thus, hypoxia or anoxia has previously been found to increase nitrite concentration in pectoral muscle (having a high proportion of red muscle fibers) of slider turtles and in cardiomyocytes from slider turtles and crucian carp, whereas white

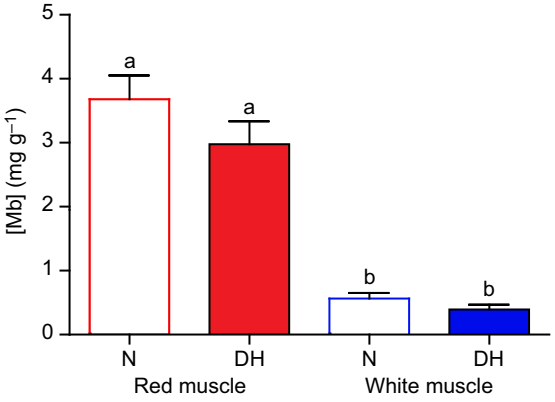

Fig. 4. Myoglobin (Mb) concentration in red and white muscle from normoxic and deeply hypoxic crucian carp. Mb concentration is given in mg per g wet mass for crucian carp exposed to normoxia (N; P_{O_2}>140 mmHg) and deep hypoxia (DH; 1<P_{O_2}<3 mmHg) for 1 day. Values are means±s.e.m. (N=15 for each group). Differences between groups were tested using a one-way ANOVA, followed by a Bonferroni *post hoc* test, and different letters signify statistical differences between groups.

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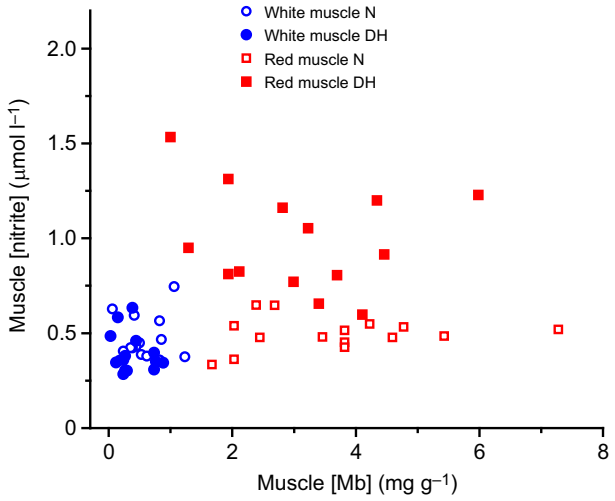

Fig. 5. Red and white muscle nitrite levels as a function of Mb concentration. Interrelated values of nitrite and Mb concentration in red muscle (red symbols) and white muscle (blue symbols) of individual crucian carp exposed to normoxia (N; P_{O_2}>140 mmHg) and deep hypoxia (DH; 1<P_{O_2}<3 mmHg). *N*=60 in total.

muscle nitrite concentration in crucian carp and goldfish was maintained or tended to drop (Hansen and Jensen, 2010; Sandvik et al., 2012; Jensen et al., 2014).

The hypoxia-induced increase in red muscle nitrite concentration was mirrored by an increase in nitros(yl)ation products (Fig. 2) and a strong linear correlation between muscle nitrite concentration and [SNO+FeNO+NNO] was found to apply to both normoxic and deeply hypoxic red and white myocytes (Fig. 3). This substantiates the nitrosative power of nitrite, where nitrite (via nitrosating species like N_2O_3) generates SNO compounds, and supports that NO originating from nitrite reduction nitrosylates heme groups to form FeNO compounds. The overall response of increased nitrite and nitros(yl)ation compounds in red muscle during deep hypoxia resembles the increased nitrite/NO activity found in cardiomyocytes from deeply hypoxic and anoxic crucian carp and slider turtles (Sandvik et al., 2012; Jensen et al., 2014; Hansen et al., 2016).

As outlined in the Introduction, we recently hypothesized that Mb may be involved in maintaining/increasing intracellular nitrite levels in hypoxia-tolerant species (Jensen et al., 2014), and the present study therefore tested for a correlation between muscle nitrite and Mb levels. The levels of Mb in red muscle and white muscle of normoxic crucian carp (Fig. 5) are similar to those reported in other fish species (Reynafarje, 1963; Giovane et al., 1980; Jaspers et al., 2014). Additionally, 1 day of deep hypoxia did not change the Mb protein level, which is in accordance with other studies on Mb expression during hypoxia, where 2–5 days of hypoxia in killifish, goldfish and carp did not affect Mb concentration in heart or red muscle (Cossins et al., 2009; Borowiec et al., 2015) or *Mb* mRNA levels (Roesner et al., 2008; Okogwu et al., 2014). If Mb concentration had a direct influence on muscle nitrite levels, one would expect a positive correlation between muscle nitrite and muscle Mb concentration. Such a correlation was clearly not present (Fig. 5). The data reflect an increased red muscle nitrite concentration that is independent of muscle Mb concentration (Fig. 5).

Other possible mechanisms for elevating intracellular nitrite levels should also be considered. As high mitochondrial content is also a shared property of red muscle and cardiomyocytes, mitochondria could be involved in elevating intracellular nitrite concentration during deep hypoxia. Sequestering of nitrite inside

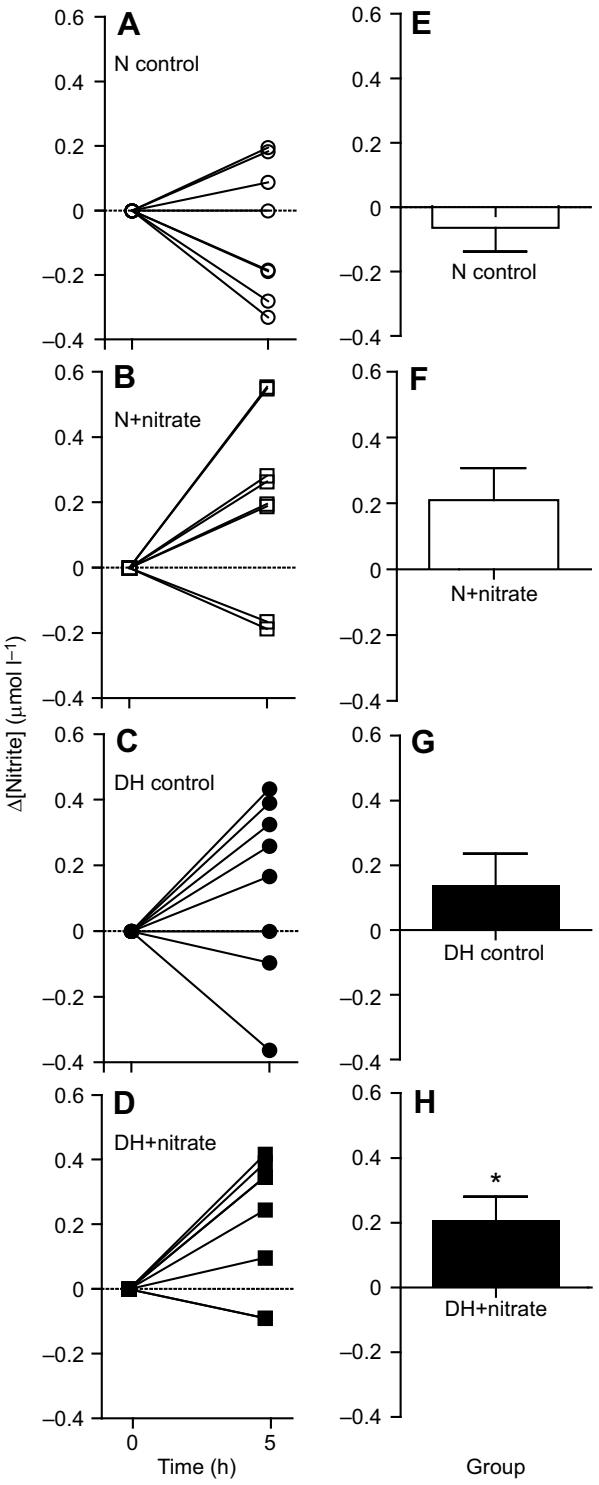

Fig. 6. Nitrate reductase activity in white muscle homogenates. Muscle homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol l^{-1} exogenous nitrate added to the homogenates. (E–H) Group-specific changes as means±s.e.m. A one-sample *t*-test was performed to compare group means with zero; asterisks signify a statistical difference (*P*<0.05; *N*=8 for each group).

mitochondria would be much in line with the fact that the cytoprotective effects of nitrite are largely directed at the mitochondria (Halestrap, 2004; Walters et al., 2012; de Lima Portella et al., 2015). In the mitochondria, nitrite *S*-nitrosates

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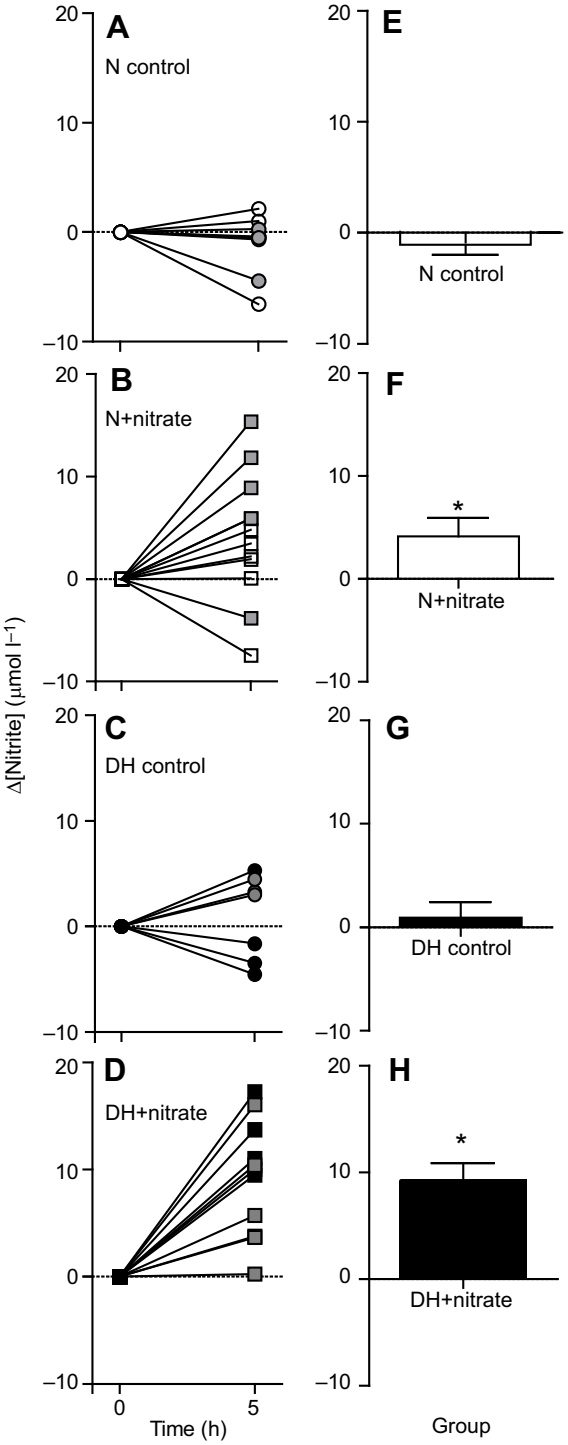

Fig. 7. Nitrate reductase activity in liver homogenates. Liver homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol l^{-1} exogenous nitrate added to the homogenates. Black and white symbols are from incubations in Eppendorf tubes and gray symbols signify incubation in tonometers (see Materials and methods). (E–H) Group-specific changes as means±s.e.m. A one-sample *t*-test was performed to compare group means with zero; asterisks signify a statistical difference (P<0.05; N=7–11 for each group).

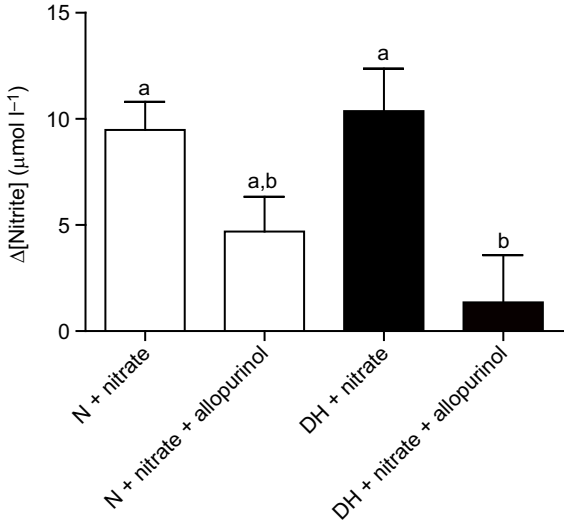

Fig. 8. Effect of allopurinol on nitrate reductase activity in liver homogenates. Liver homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. All groups (means±s.e.m.) were incubated with 300 μmol l^{-1} nitrate for 5 h with or without 2 mmol l^{-1} allopurinol (N=5 in each group). Differences between groups were tested using a one-way ANOVA, followed by a Bonferroni *post hoc* test, and different letters signify statistical differences between groups.

complex I, attenuating ROS generation during early reperfusion (Dezfulian et al., 2009; Chouchani et al., 2013), and nitrosylates complex IV, which inhibits oxygen consumption rates (Hendgen-Cotta et al., 2008). However, a role of nitrite binding/storage by mitochondria is not known and future studies are needed to clarify the possible role of mitochondria in elevating cellular nitrite levels during hypoxia and anoxia.

Tissue nitrate reductase activity

The major sources of nitrate in the body are oxidation of NOS-generated NO (i.e. reaction with oxygenated heme proteins) and uptake from the diet, producing internal nitrate concentrations in the micromolar range well above nitrite concentrations (Lundberg et al., 2008). This larger pool of nitrate is a potential source of nitrite, provided nitrate can be reduced to nitrite in the tissues. Nitrate reduction was previously thought to be attributed solely to bacterial nitrate reductase enzymes and, thus, nitrate reduction in mammals was believed to be restricted to commensal bacteria in the oral cavity (Lundberg and Govoni, 2004). Recent studies have changed this belief and it is now known that mammals possess inherent tissue nitrate reductase activity that complements the more efficient nitrite reduction by oral bacteria (Li et al., 2003; Jansson et al., 2008; Huang et al., 2010; Piknova et al., 2015). The present study expands on this knowledge, by showing that among ectotherms, the crucian carp is also able to reduce nitrate to nitrite. The nitrate reduction activity was higher in liver (Fig. 7) than in muscle (Fig. 6) and it was absent in the heart (Fig. 9). In the heart, the nitrite concentration actually decreased, pointing at a dominating nitrite reduction under the assay conditions. We did not test for nitrate reductase activity in red muscle because the limited amount of red muscle tissue was totally consumed by the measurements of NO metabolites and Mb. However, given the low nitrate reduction activity in white muscle and its absence in the heart (resembling red muscle metabolically), the nitrate reduction activity may also be low in red muscle, which is supported by the absence of a decrease in red muscle nitrate concentration in deeply hypoxic fish (Fig. 2C). The higher nitrate reduction in the liver will supply nitrite to the anoxic liver, but it may additionally function to export nitrite to other tissues, including the heart, via the circulation, along with the function of the liver to

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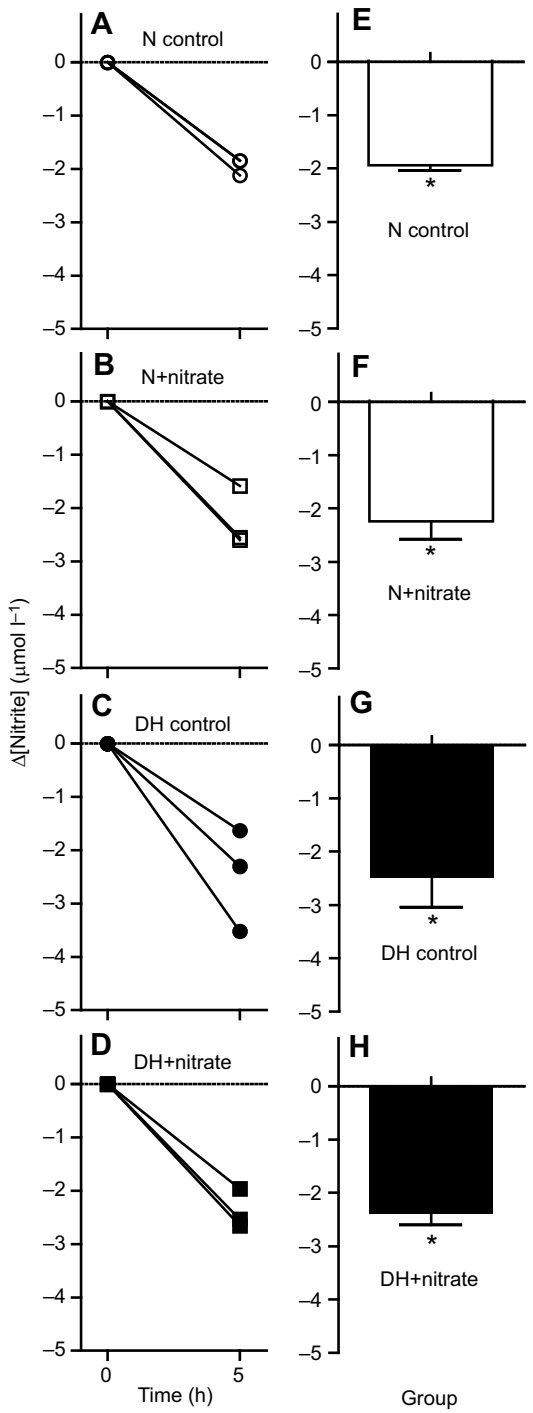

Fig. 9. Test for nitrate reductase activity in heart tissue. Ventricle homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol l^{-1} exogenous nitrate added to the homogenates. (E–H) Group-specific changes as means±s.e.m. A one-sample *t*-test was performed to compare group means with zero; asterisks signify statistical difference (P<0.05). Because of the small ventricle size, ventricles from two fish were pooled in each individual homogenate (N=3 for each group).

deliver glucose to sustain glycolytic metabolic activity during deep hypoxia.

The difference in nitrate reductase activity between tissues corresponds to findings in mice and rats, where liver nitrate reductase activity is high compared with that in heart and muscle (Jansson et al., 2008; Piknova et al., 2015). Interestingly, in the current study, the nitrate reductase activity appeared higher in tissues from crucian carp exposed to deep hypoxia for 1 day than in tissues obtained from normoxic fish (Figs 6D,H and 7D,H), which suggests that the enzyme(s) responsible for nitrate reduction in crucian carp may be upregulated with transition to deep hypoxia. One main enzyme responsible for nitrate reduction is XOR, as reflected by a significant (but incomplete) inhibition of nitrate reductase activity using the XOR inhibitor allopurinol (Fig. 8). This is similar to mammals (Jansson et al., 2008; Piknova et al., 2015), where a role for XOR is further substantiated by the finding of high nitrate reductase activity in the liver and gastrointestinal tract (Jansson et al., 2008), which have the highest XOR levels (Harrison, 2004). In mammals, XOR expression is upregulated by hypoxia (Kelley et al., 2006), and also in germ-free mice, where nitrate reduction by commensal bacteria is absent (Huang et al., 2010), and XOR is known to also be expressed in teleost fish (Basha and Rani, 2003; Garofalo et al., 2015). Thus, XOR is a likely candidate for nitrate reductase activity in crucian carp, and given that the expression pattern is similar to that in mammals, this would explain the difference between nitrate reductase activity in crucian carp liver and muscle. An upregulation of XOR (and/or another nitrate reductase enzyme) in crucian carp would explain the increase in tissue nitrate reductase activity in tissues from deeply hypoxic fish compared with normoxic fish. It is also possible that other nitrate reductase enzymes, expressed solely during hypoxia, contribute to nitrate reduction in crucian carp. This awaits future study.

As a test of the nitrate reduction assay, we analyzed a mouse liver homogenate concurrently with crucian carp liver (data not shown), and found mouse liver nitrate reductase activity to be around 3 times higher than crucian carp liver nitrate reductase activity. Thus, there indeed appears to be a lower nitrate reduction activity in crucian carp tissue compared with mammalian tissue. An explanation could be that crucian carp utilize other sources of nitrite mobilization. Alternative nitrite sources in crucian carp, compared with mammals, are nitrite uptake from the ambient water (Hansen et al., 2016) and higher basal plasma nitrite levels (Fago and Jensen, 2015). Indeed, crucian carp take up ambient nitrite across the gills and direct it to tissues such as the heart during deep hypoxia (Hansen et al., 2016). By having access to an ambient pool of nitrite, crucian carp may be less dependent on internal nitrate reduction as a nitrite source. The ability to keep up internal nitrite levels is important for securing NO availability (e.g. nitrite reduction to NO) during deep hypoxia and anoxia, where NOS enzymes cannot produce NO because of the absence of O_2. A number of cellular proteins can reduce nitrite to NO, including deoxygenated Mb (Lundberg et al., 2008; Fago and Jensen, 2015). In the deeply hypoxic fish in the present study, the tissue oxygen tension will be practically zero and Mb will be deoxygenated and serve as an effective nitrite reductase.

Nitrate reductase activity in crucian carp muscle was low and insignificant in normoxic fish but significant in fish exposed to deep hypoxia. In rats, muscle nitrate reductase activity tended to increase after 24 h incubation with nitrate, and even though only low levels of nitrite were produced, it was suggested that muscle nitrate reductase activity could play a role in whole-body nitrite generation because of the large mass of skeletal muscle (Piknova et al., 2015). Likewise, the low levels of nitrite produced from nitrate in crucian carp muscle may sum to make a difference, because white muscle constitutes around 50% of the fish mass.

Concluding remarks

In summary, the main finding of the present study is the documentation of a low nitrate reductase activity in crucian carp tissues that supplements other nitrite supply routes and probably contributes to cytoprotection in deep hypoxia. Additionally, we found that red musculature – like cardiac musculature but in contrast to white musculature – increases nitrite levels and thus NO availability during deep hypoxia. This is not directly explained by high Mb levels and future studies will determine whether this increase in intracellular nitrite may relate to the high mitochondria content that characterizes cardiomyocytes and red muscle fibers.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.N.H., F.B.J. and J.O.L. conceived and designed the experiments; M.N.H., F.B.J., M.F., A.F. and N.M.G.C. performed the experiments; M.N.H., F.B.J., M.F., A.F. and N.M.G.C. analyzed the data; M.N.H. and F.B.J. wrote the paper; M.N.H., F.B.J., J.O.L., M.F., A.F. and N.M.G.C. revised the manuscript.

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