Nitric oxide availability in deeply hypoxic crucian carp: acute and chronic changes and utilization of ambient nitrite reservoirs

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Hansen MN, Gerber L, Jensen FB. Nitric oxide availability in deeply hypoxic crucian carp: acute and chronic changes and utilization of ambient nitrite reservoirs. Am J Physiol Regul Integr Comp Physiol 310: R532–R540, 2016. First published January 13, 2016; doi:10.1152/ajpregu.00515.2015.—Recent research suggest that anoxia-tolerant fish transfer extracellular nitrite into the tissues, where it is used for nitric oxide (NO) generation, iron-nitrosylation, and S-nitrosation of proteins, as part of the cytoprotective response toward prolonged hypoxia and subsequent reoxygenation. We hypothesized that crucian carp take up ambient nitrite and use it as a source of cellular NO availability during hypoxia. Fish were exposed for 1 day to normoxia (PO2 > 140 mmHg) and deep hypoxia (1 < PO2 < 3 mmHg) at both low (< 0.2 μM) and moderately elevated (10 μM) ambient nitrite to decipher NO metabolites in plasma and several tissues. We also compared NO metabolite changes during acute (10 min) and chronic (1 day) exposures to three different O2 levels. Plasma nitrite decreased with decreasing O2, while the cellular concentrations of nitrite and nitrosoylated compounds either increased or remained constant, depending on O2 level and tissue type. Nitrite was notably increased in the heart during deep hypoxia, and the increase was amplified by elevated ambient nitrite. Raised nitrite also increased gill nitrite and decreased mRNA expression of an inducible nitric oxide synthase-2 gene variant. The data support that defending intracellular levels of NO metabolites is part of the strategy toward lack of oxygen and reoxygenation. Hence, hypoxic goldfish maintain intracellular NO metabolites at the expense of decreasing plasma levels, and anoxic crucian carp and anoxic freshwater slider turtles even increase nitrite, SNO, and FeNO+NNOH in the heart and selected other tissues (24, 37). This points at an adaptive trait, protecting the tissues against damage during anoxia and subsequent reoxygenation (11), and complementing other adaptive traits of hypoxia/anoxia-tolerant animals (2, 31). Whether the remarkable increase in cardiac NO metabolites is dependent on complete O2 absence is unknown, and one objective of the present study was, therefore, to investigate whether deep hypoxia (1 < PO2 < 3 mmHg) can elicit a similar response.

Extracellular nitrite appears to be shifted into tissues of hypoxic and anoxic fish, where it is used for NO and SNO formation (16, 37). The extracellular reservoir is, however, not sufficient to maintain or increase cellular nitrite during long-term O2 lack, unless it is supplemented from other sources. As freshwater fish can take up nitrite actively across the gills, and ambient nitrite often is moderately elevated during environmental hypoxia, it has been hypothesized that ambient nitrite could be taken up and used internally for NO generation during extended hypoxia/anoxia (22). One major aim of the present study was to test this idea by exposing crucian carp to normoxia and deep hypoxia at low (< 0.2 μM) and moderately elevated (10 μM) ambient nitrite concentrations.

The steady-state concentration of nitrite and other NO metabolites in a given body compartment reflects a balance between production and uptake rates and consumption and excretion rates. Previous studies on NO metabolites in hypoxic/anoxic fish have addressed chronic (i.e., days) exposures, where NO metabolites are assumed at steady state. However, it is also of interest to gain insight into acute changes. Another purpose of the present study was, therefore, to expose crucian carp to both acute (10 min) and chronic (1 day) hypoxia to allow an evaluation of possible differences between the two types of hypoxia challenge.

Nitrite is naturally produced endogenously as an oxidative metabolite of NO produced by NOS enzymes in normoxia and moderate hypoxia, where sufficient O2 for the NOS reaction is available. Nitrite can, therefore, be considered a natural product of the NOS reaction. We recently reported a strong inhib-
hibition of gill Nos2 mRNA expression in brown trout exposed to nitrite, suggesting negative-feedback regulation of Nos2 gene expression by nitrite (23). A final aim of the present study was to test whether a similar inhibition of Nos2 expression occurs in crucian carp by only moderately elevated nitrite concentrations.

MATERIALS AND METHODS

Animals

Two batches of crucian carp (Carassius carassius) of mixed sex, weighing 38.92 ± 8.22 g (means ± SD; n = 49) and 36.61 ± 7.12 g (means ± SD, n = 27), were obtained from a local pond (Langsted, Funen, Denmark) in two subsequent years (series 1 and series 2 below). Fish were transferred to a holding tank, where pond water was gradually changed to experimental water (Odense tap water mixed with demineralized water in a 1:4 ratio) over a period of 24 h. The experimental water [Cl–] was 0.26 mM, and [HCO3–] was 0.96 mM. The fish were held in the holding tank for 6 days and fed with commercial trout pellets (Inicio, Biomar, Denmark), while being acclimated to 15°C and a 12:12-h light-dark cycle in normoxic (PO2 > 140 mMgHg) water. The water was exchanged daily.

Experimental Protocol

Following acclimation to experimental water, the fish were distributed among a series of 100-liter experimental aquaria (eight or nine fish in each) and acclimated for further 5 days to normoxia without feeding. Water was exchanged twice daily to avoid accumulation of nitrogen wastes in the water. Hereafter, the fish were exposed to either continued normoxia, hypoxia, or deep hypoxia for variable periods, depending on experimental series. Normoxia (PO2 > 140 mMgHg) was obtained by bubbling of air. Hypoxia [at PO2 = 18 mMgHg, which is below the critical PO2 of about 38 mMgHg in crucian carp at 15°C (42)] was achieved with a Loligo OXY-REG oxygen analyzer and regulator system (Loligo SystemsAps, Tjele, Denmark), using a set point of 0.26 mM, and [HCO3–] was 0.96 mM. The fish were held in the holding tank for 6 days and fed with commercial trout pellets (Inicio, Biomar, Denmark), while being acclimated to 15°C and a 12:12-h light-dark cycle in normoxic (PO2 > 140 mMgHg) water. The water was exchanged daily.

Series 1: acute and chronic exposures to different O2 levels. Chronic exposures for 1 day to normoxia, hypoxia, and deep hypoxia were performed in the experimental 100-liter aquaria. Changes in PO2 from normoxic to hypoxic levels were completed in 4 h. No water was exchanged during the 1-day exposures, and water nitrite concentrations were measured as 0.06 μM (normoxia), 0.11 μM (hypoxia), and 0.17 μM (deep hypoxia) at the end of experiments. Acute exposure for 10 min to the different O2 levels was performed by transferring individual fish from a normoxic aquarium to small 5-liter aquaria, with the desired oxygen level 10 min prior to sampling. Water nitrite concentrations were below 0.1 μM at all O2 levels.

Series 2: role of ambient nitrite in normoxic and deeply hypoxic fish. Crucian carp were exposed for 1 day to normoxia (PO2 > 140 mMgHg) and deep hypoxia (1 < PO2 < 3 mMgHg) both in the absence and presence of moderately elevated ambient [nitrite]. Nitrite was added as NaNO2 to a concentration of 10 μM. No water was exchanged in the four experimental aquaria during the final 1-day exposures. At the end of the experiments, the measured water nitrite concentrations were below 0.2 μM in the absence of nitrite addition, while they were 9.8 μM and 10.6 μM in normoxic and deep hypoxic nitrite-added aquaria, respectively.

Blood and Tissue Sampling and Preparation

Fish were individually anesthetized in 0.3% MS-222 (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, Steinheim, Germany) that was dissolved in experimental water to maintain exposure PO2 during anesthesia. A blood sample was taken from the caudal vessels with a heparinized syringe, after which the fish was euthanized by cutting the spinal cord. The protocol and procedures were in accordance with Danish laws of animal experimentation and a permit granted by the Danish Animal Experiments Inspectorate. Tissues (in the following order: left and right side second gill arches, heart ventricle, liver, kidney, brain, and muscle) were dissected out and washed (except right side gill arch, which was frozen directly for later Nos mRNA evaluation) in PBS [50 mMol/l phosphate buffer pH 7.8; 85 mMol/l NaCl; 2.4 mMol/l KCl; 10 mMol/l N-ethylmaleimide (NEM); 0.1 mMol/l diethylthreomipentacetonic acid (DTPA)]. The tissues were quickly dried on filter paper, weighed, and frozen in liquid nitrogen. Blood was centrifuged (2 min, 13,600 g, 5°C), allowing separation of plasma and red blood cells (RBC) to be frozen separately.

For analysis of NO metabolites, frozen tissues were thawed and homogenized in four times their mass of a 50 mM phosphate buffer (pH 7.3) that contained NEM (10 mMol/l) and DTPA (0.1 mMol/l) to stabilize S-nitrosoglutathione (46). The homogenates were centrifuged (6 min, 16,000 g, 0°C), and the supernatants were frozen in liquid nitrogen and stored at −80°C until measurements. RBC samples were thawed and vortexed in nine times their volume of a nitrite/SNO preservation solution containing 5 mMol/l K2[Fe(CN)6], 10 mMol/l NEM, 0.1 mMol/l DTPA, and 1% NP-40 (16, 46).

Analytical Techniques

The NO metabolites nitrite, nitrate, and SNO+FeNO+NONO were assessed by reductive chemiluminescence, using a Sievers (Boulder, CO) nitric oxide analyzer (model 280i) and previously described procedures to distinguish between the individual compounds (16, 46). Blood hemoglobin and hematocrit, red cell nucleotide triphosphates (NTP), and plasma lactate were determined as earlier outlined (16).

Procedures for evaluating the mRNA expression of different Nos gene isoforms in gill tissue were conducted, as described recently (23). In short, TRIzol reagent (Bioline) was used to isolate total RNA, and first-strand cDNA was synthesized from DNase-treated RNA (QI Q1 RNase-free DNase, Promega, Madison, WI) using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster, CA). SYBR Green detection (LightCycler 480 SYBR green I Master from Roche, Indianapolis, IN) was used to follow cDNA amplification during qPCR (Mx3000P instrument; Stratagene, La Jolla, CA), and the relative mRNA levels were calculated using Efiα as the housekeeping gene. We used our previously designed crucian carp Nos1, Nos2A, Nos2Bi, and Nos2Bit primers (37) and Salmo salar Ef1α primers (27). Because of the lack of Efiα nucleotide sequences from crucian carp, we compared the Efiα nucleotide sequence from the closely related goldfish (Carassius auratus; GenBank: AB056104) with the Efiα-encoding DNA sequence from Salmo salar (GenBank: AF321836) to confirm the specificity of the Efiα primers. The alignment revealed a 98% similarity, suggesting that Efiα gene is highly conserved and that the Salmo salar Efi1α primers can be used.

Statistics

Results are presented as means ± SE. A two-way ANOVA and a Bonferroni post hoc test was used to evaluate the significance (P < 0.05) of treatments. Data that did not fulfill the assumptions of equal variances (Bartlett’s test, P < 0.05) were log10 transformed prior to analysis. Statistical analyses were done with GraphPad Prism (GraphPad Software, La Jolla, CA, version 5.02).

RESULTS

Acute Versus Chronic Exposure to Normoxia, Hypoxia, and Deep Hypoxia

Plasma [nitrite] decreased gradually with lowered ambient PO2 in fish exposed to normoxia (PO2 > 140 mMgHg), hypoxia...
(PO₂ ~18 mmHg), and deep hypoxia (1 < PO₂ < 3 mmHg). This was the case during both acute (10 min) and chronic (1 day) exposures, but plasma [nitrite] was higher overall in the acute challenge (Fig. 1A). The plasma concentrations of nitros(y)lated compounds (SNO+FeNO+NNO) and nitrate did not change appreciably with PO₂, but were also generally higher in acutely exposed fish (Fig. 1, B and C).

In the heart ventricle, the cellular levels of nitrite and other NO metabolites were unaffected by O₂ level during acute exposures (Fig. 2). Chronic exposure to deep hypoxia induced significant increases in ventricle [nitrite] and [SNO+FeNO+NNO] (Fig. 2, A and B), whereas heart NO metabolite levels in other tissues (liver, brain, muscle, gill, and kidney) were typically unaffected by O₂ level in acute and chronic exposures, but there was a tendency of higher SNO+FeNO+NNO levels in acutely exposed fish (data not shown).

Fig. 1. Plasma NO metabolites in crucian carp after acute and chronic exposures to different oxygen levels. Concentration of nitrite (A), SNO+FeNO+NNO (B), and nitrate (C) after 10 min (10m) or 1 day (1d) exposures to normoxia (N, PO₂ >140 mmHg), hypoxia (H, PO₂ ~ 18mmHg) and deep hypoxia (DH, 1 < PO₂ < 3 mmHg). Values are expressed as means ± SE, and n = 8 or 9 for each group. A two-way ANOVA was used to evaluate significant (P < 0.05) effects of exposure time (€) and oxygen level (†). Interaction between the two factors is indicated by an X. Different letters (lowercase or capital) indicate a significant difference (Bonferroni post hoc test).

Fig. 2. NO metabolites in the heart ventricle of crucian carp after acute and chronic exposures to different oxygen levels. Concentration of nitrite (A), SNO+FeNO+NNO (B), and nitrate (C) in the heart after 10 min (10m) or 1 day (1d) normoxia (N), hypoxia (H), or deep hypoxia (DH). Values are expressed as means ± SE, and n = 8 or 9 at each column. A two-way ANOVA was used to evaluate significant (P < 0.05) effects of exposure time (€) and oxygen level (†). Interaction between the two factors is indicated by an X. Different letters indicate a significant difference (Bonferroni post hoc test).
Influence of Ambient [Nitrite] During Chronic Exposure to Normoxia and Deep Hypoxia

To evaluate the role of ambient nitrite as a source of cellular NO availability, we exposed a new series of crucian carp to normoxia and deep hypoxia for 1 day under basal (< 0.2 μM) and moderately elevated (10 μM) ambient nitrite concentrations. Plasma [nitrite] was slightly lower in these fish than in the previous series, but plasma nitrite decreased in response to hypoxia as previously, and the decrease was more prominent at elevated ambient [nitrite] (Fig. 3A). Plasma nitrite increased with nitrite exposure, notably in normoxic fish (Fig. 3A), but plasma levels were well below ambient levels. Other plasma NO metabolites only changed slightly (Fig. 3, B and C).

Heart [nitrite] increased significantly with both deep hypoxia and with elevated ambient nitrite (Fig. 4A). Thus, the combined deep hypoxia and nitrite treatment increased heart [nitrite] five- or six-fold compared with normoxic controls and two or three times compared with deep hypoxic controls, reaching a concentration close to 6 μM (Fig. 4A). The rises in heart [nitrite] were associated with elevated cardiac [SNO+FeNO+NNO] (Fig. 4B). Changes in [nitrate] were insignificant (Fig. 4C). In RBCs and liver tissue, the cellular concentrations of nitrite and nitrosylated compounds likewise tended to increase with both deep hypoxia and elevated ambient [nitrite] (Fig. 5, A–D). The levels of nitrite reached about 4 μM in RBCs (Fig. 5A), which is well below values that could significantly elevate methemoglobin levels, because of the effective methemoglobin reductase activity inside RBCs, and because the erythrocyte heme concentration (~18 mM) by far exceeds the nitrite concentration. Changes in NO-metabolites in brain and kidney were modest (Fig. 5, E–H). Gill tissue [nitrite] increased with nitrite exposure, whereas gill [SNO+FeNO+NNO] increased with both deep hypoxia and elevated ambient nitrite (Fig. 5, I–J).

In the gill, the mRNA expression of Nos2Bi decreased significantly with elevated ambient nitrite treatment (Fig. 6C), while the mRNA levels of other Nos variants (Nos1 and Nos2A) were unaffected by treatment (Fig. 6, A and B) or below detection limit (Nos2Bi).

Metabolic Indicators

Plasma lactate increased significantly in deep hypoxia and were unaffected by the moderate elevation of ambient [nitrite] (Fig. 7A). The RBC [NTP][Hb₄] ratio, which reflects the erythrocytic NTP (ATP + GTP) content, decreased significantly from 1.6 in normoxia to 0.8 in deep hypoxia both at basal and at moderately elevated ambient [nitrite] (Fig. 7B).

DISCUSSION

Role of Ambient Nitrite and the Degree of Hypoxia

The main finding of the present study is that crucian carp take up ambient nitrite and use it as a source of cellular NO availability during hypoxia in a tissue-specific manner, with the heart being a central target. Thus, a moderate elevation of ambient [nitrite] increases gill [nitrite] (Fig. 5) and plasma [nitrite] (Fig. 3), in support of nitrite uptake across the gills. The residence in plasma is temporary, and nitrite is further transported into tissues, particularly during hypoxia. This is reflected by the maintenance or increase of intracellular [nitrite] (Figs. 4 and 5) in the face of decreasing extracellular [nitrite] in hypoxia (Fig. 3A), as previously observed in hypoxic goldfish (16) and anoxic crucian carp (37). Nitrite seems selectively redistributed to the heart but also to RBCs and the liver, as shown by increased cellular [nitrite] in these tissues during deep hypoxia (Figs. 4 and 5). This selective increase in cellular [nitrite] occurs at basal ambient [nitrite] and becomes amplified by moderately elevated ambient [nitrite]. Thus, ambient nitrite appears utilized as a source of cellular nitrite in
hypoxia at PO2 values of 29 and 19 mmHg (16), while cardiac tained relatively constant in goldfish exposed for 2 days to underpinning the selective nitrite uptake into cardiomyocytes. concentrations in deep hypoxia compared with normoxia (Fig. 4), \[\text{[nitrite]}\]. We found a three-fold increase in heart nitrite con-
both situations but more so in deep hypoxia at elevated ambient [nitrite]. We found a three-fold increase in heart nitrite concentrations in deep hypoxia compared with normoxia (Fig. 4), underpinning the selective nitrite uptake into cardiomyocytes.

Previous studies have shown cardiac [nitrite] to be main-
tained relatively constant in goldfish exposed for 2 days to hypoxia at PO2 values of 29 and 19 mmHg (16), while cardiac [nitrite] increased during 1–5 days of full anoxia in crucian carp (37). The present study both corroborates and expands on these studies. Thus, cardiac [nitrite] was maintained constant in crucian carp exposed for 1 day to hypoxic water at PO2 18 mmHg (Fig. 2), while it increased in deep hypoxia (1 < PO2 < 3 mmHg) both in experimental series 1 (Fig. 2) and series 2 (Fig. 4). This highlights that deep hypoxia (rather than full anoxia) is sufficient to stimulate an elevation of cardiac [nitrite] and suggests the existence of a threshold PO2 somewhere below 18 mmHg, where this increase is triggered.

The transport of nitrite from low extracellular concentration to high intracellular concentrations during deep hypoxia could point at an active transport mechanism. Alternatively, nitrite may bind to intracellular proteins during hypoxia, which would lower the cytosolic concentration of free nitrite, and, hence, allow a passive uptake of nitrite down a concentration gradient of free nitrite despite the fact that total cellular [nitrite] is increased. We recently suggested that negatively charged ni-
trite binds to positive charges on myoglobin (Mb) to increase total cellular [nitrite] (24). This proposal was based on the fact that anoxic freshwater turtles elevate tissue [nitrite] in both the heart and red skeletal muscle (24), which are characterized by high Mb and mitochondria contents, whereas anoxic fish only elevate [nitrite] in the heart but not in white musculature (37), which has low Mb and mitochondria contents. An increase in nitrite binding to Mb during anoxia would be aided by cellular acidification that increases the positive charge on Mb by H\(^+\) buffering (24). One may also envisage an increased Mb mRNA expression during hypoxia (36) that increased Mb protein levels and, thus, nitrite binding, allowing nitrite influx down a concentration gradient in free nitrite. There is, however, not a uniform relationship between Mb expression and hypoxia in heart tissue of fish (17). The exact mechanism(s) involved in cardiac nitrite uptake during deep hypoxia and anoxia must await future studies.

While it is clear that an increase in cardiac nitrite to some extent is supplied from ambient reservoirs, other possibilities should also be considered. One possibility would be increased NOS activity and autoxidation of the produced NO to nitrite. However, this pathway is implausible, because both the NOS reaction and autoxidation require molecular oxygen, which is very scarce in deep hypoxia and fully absent in anoxia, and cardiac [nitrite], nevertheless, increases in both situations [Fig. 4 (37)]. It is also conceivable that cellular nitrate could be reduced to nitrite under low O2 conditions (21). This is not supported by a decrease in cellular [nitrate] in deep hypoxia compared with normoxia (Fig. 4D), but as the expected concentration change is low, it would be difficult to detect, given the relatively high background concentrations of nitrate.

We are currently exploring this possibility in a separate study, using alternative methods.

Inside the heart and other tissues, nitrite enters NO- and SNO-generating pathways in hypoxia, as reflected by elevated levels of nitros(yl)ated compounds (Figs. 4 and 5). In the heart, Mb is the primary nitrite reductase (18, 30, 34, 39). Formation of NO from nitrite leads to increased iron-nitrosylation of heme proteins, while formation of nitrosating agents, such as N\(_2\)O\(_3\), leads to S-nitrosation of exposed protein thiols. These post-translational modifications are central to the cytoprotection offered by NO and nitrite during severe hypoxia followed by reoxygenation.

A number of mammalian studies have documented reduced cell death and infarct size following myocardial ischemia and reperfusion, when [nitrite] is moderately elevated before ischemia (8, 18, 40, 45) and before reoxygenation (15). A similar protection applies to other tissues, including the liver (8). Much of the protection provided by nitrite is
HYPOXIC CRUCIAN CARP DIRECT AMBIENT NITRITE TO THE HEART

Fig. 5. Cellular NO metabolites in crucian carp under different oxygen and nitrite regimes. [Nitrite] (top) and [SNO] (RBC) or [SNO+FeNO+NNO] (bottom) in RBC, liver, brain, gill, and kidney. Values are expressed as means ± SE; n = 6–8 in each group. † and *Significant statistical difference (P < 0.05) of oxygen and nitrite level, respectively (two-way ANOVA). Different letters signify significant difference between groups (Bonferroni post hoc test).

directed at the mitochondria, where inhibition of complex IV by iron-nitrosylation may prolong O2 availability in severe hypoxia (39), while S-nitrosation of complex I strongly reduces mitochondrial ROS production and oxidative damage during reoxygenation (7, 18, 30, 33). In mammals, the protection relates to therapeutic administration of nitrite in a diseased state, whereas the mechanisms appear inherently exploited in anoxic-tolerant crucian carp (37) and freshwater turtles (24) that naturally experience deep hypoxia/anoxia and reoxygenation in their life cycle. The boosting of cardiac nitrite levels in deep hypoxia by elevation of ambient [nitrite] (Fig. 4) is also ecologically relevant, as hypoxic/anoxic aquatic habitats often have elevated nitrite levels generated by bacterial processes (3, 9). In addition to the cytoprotective effect of moderately elevated cardiac [nitrite], nitrite-derived NO can assist cardiac function by increasing myocardial efficiency (32) and mechanical performance (20).

In RBC and the remaining tissues investigated, we found a tissue- and product-specific increase in NO metabolites during exposure to severe hypoxia combined with elevated ambient nitrite (Figs. 4 and 5). This is in accordance with previous studies on rats (4, 12), goldfish (16), crucian carp (37), and freshwater turtles (24), in which concentration and nitrite metabolism vary from tissue to tissue.

Acute Versus Chronic Exposure

Crucian carp tolerate long-term severe hypoxia/anoxia, and the O2 depletion typically develops gradually (44). Therefore, we have previously (and here) focused on NO metabolite changes during longer (i.e., days) exposures, reflecting an acclimated steady state. However, it is also of interest to investigate changes during acute hypoxia challenge for comparison, and therefore, we exposed crucian carp to 10 min of hypoxia at two PO2 levels below the critical PO2. We hypothesized an acute response similar to rats (4, 12), where 2–10 min of global hypoxia (by cervical dislocation) would cause plasma and tissue [nitrite] to decrease and nitrosylation products to increase. We did observe a progressive decrease in plasma [nitrite] with lowered PO2 (Fig. 2A), whereas tissue nitrosylation products did not change acutely but only chronically (Fig. 2), suggesting that more than 10 min is required to establish the change. Similarly, although the characteristic increase in cardiac [nitrite] was present after 1 day of deep hypoxia, it was not established after 10 min, showing that a longer duration of deep hypoxia is required (Fig. 2A). This may be partly explained by a lower temperature (15°C in crucian carp compared with 37°C in mammals) that decreases the rates of involved transport processes and reactions (e.g., nitrite reduction). Also, internal O2 stores from the normoxic prehistory will postpone establishment of global deep hypoxia inside the fish.

One surprising observation was that NO metabolites in both plasma (Fig. 1) and tissues (Fig. 2) were generally higher in the 10-min exposure groups compared with the respective 1-day exposure groups. We speculate that this is an effect of stress and increased swimming activity associated with the relocation of fish to new experimental aquaria in the 10-min groups. Contrary to fish exposed for 1 day, fish subjected to 10 min of varying PO2 swam vigorously immediately prior to sampling. In mammals, it is known that exercise increases the expression and phosphorylation status of NOS3, resulting in elevated NO metabolite levels (6), and 10 min of high-intensity ergometer exercise has been reported to increase circulating plasma [nitrite] in humans (35). Furthermore, a single injection of adrenaline increases the levels of nitrite and nitrosothiols in plasma and heart of mice (5). The present results point at a similar response to exercise and stress in crucian carp, involving either NOS1 or NOS2 isoforms, because teleost fish do not possess the Nos3 gene (1). The influence of exercise and stress hormones on NO homeostasis in fish deserves further future study.

The gradual decrease in plasma [nitrite] with PO2 (Fig. 1) is partly explained by reduced NOS activity and NO autoxidation as O2 becomes more and more limiting. The higher plasma [nitrite] in the acute vs. chronic exposure also during deep hypoxia may, therefore, seem unexpected due to the extreme O2 lack. However, while the acute drop in water PO2 will be registered immediately via gill chemoreceptors (10), triggering a stress response, the internal O2 reserves (from the normoxic prehistory) will last for some time before deep hypoxia is fully transferred to the entire fish. This will allow an increased...
NOS-derived NO production and NO autoxidation to nitrite for a period and explain the higher [nitrite] in acutely exposed fish also in deep hypoxia.

Branchial mRNA Expression of NOS

Moderate elevation of ambient nitrite significantly inhibited mRNA expression of Nos2Bi in the gills of crucian carp (Fig. 6C). This response resembles the strong inhibition of branchial Nos2 expression that we recently reported in brown trout exposed to higher ambient [nitrite] (23). Hence, our data support the suggestion of negative feedback regulation of Nos2 gene expression by nitrite (23), which may be due to S-nitrosation of the nuclear transcription factor NF-κB (26). In crucian carp, the inhibition was significant for Nos2Bi but not for Nos2A (Fig. 6). As [nitrite] was only moderately elevated, this suggests that the inhibition is concentration-dependent and varies among Nos2 variants. Nitrite did not influence Nos1 expression (Fig. 6A), which is similar to brown trout (23).

Whereas hypoxia increases gill Nos2 mRNA levels in brown trout (23), we did not observe an increase in crucian carp (Fig. 6), as earlier reported (41). The present degree of hypoxia was much more severe than used in brown trout, and an increased Nos2 expression translating into increased protein would not benefit NO production due to lack of the NOS substrate O2. In fact, we have previously observed a decrease in Nos2A mRNA levels in gills of anoxic crucian carp (37).

Metabolic Indicators

We measured plasma [lactate] and RBC [NTP]/[Hb4] as metabolic indicators (Fig. 7) to underpin the strong O2 limitation in deep hypoxia. At first sight, the relatively large increase in plasma [lactate] in deep hypoxia may appear unexpected, given that crucian carp is well known for its ability to produce ethanol as an anaerobic end product to avoid acidosis from lactic acid accumulation (2, 31). Ethanol is, however, only produced in skeletal muscle, while other organs produce lactate that is subsequently transported in the blood to the muscles for transformation into ethanol (44). A rise in plasma lactate is, therefore, predictable. The plasma [lactate] observed after 1 day of deep hypoxia (~14 mM) compares with the value...
reported after 2.5 h anoxia (12 mM) at the same temperature (13) and might reflect a steady-state level attained. The decrease in RBC [NTP]/[Hb 4] with hypoxia (Fig. 7) is a general response in fish that serves to increase blood O 2 affinity, which improves O 2 extraction and arterial O 2 saturation under environmental O 2 shortage (25).

Perspectives and Significance

Our results support the idea that crucian carp use ambient nitrite as a source of internal NO availability during hypoxia. The external supply of nitrite appears essential during long-term severe hypoxia, when NOS-catalyzed NO production is suspended and internal nitrite reservoirs have limited lifetime. Nitrite is particularly directed to the heart, where it predictively offers cytoprotection during deep hypoxia and reoxygenation through NO generation, iron-nitrosylation, and S-nitrosation of proteins. This is analogous to the cardioprotective effect of nitrite administration during ischemia/reperfusion in mammals, with the notable difference that in crucian carp the nitrite treatment is a natural part of its physiology during deep hypoxia. In many freshwater fish, the branchial entry of nitrite is via the active Cl\(^{-}\) uptake mechanism, with the potential danger that excessive elevations in ambient [NO\(_2\)\(^{-}\)] could cause internal [NO\(_2\)\(^{-}\)] to rise from therapeutic to toxic levels (22, 23). However, the elevation of ambient [NO\(_2\)\(^{-}\)] in hypoxic habitats are typically moderate and seldom exceed a few micromolar. Furthermore, it is possible that anoxia-tolerant fish have evolved regulatory mechanisms that adjust nitrite uptake. Future studies should address to what extent branchial nitrite uptake is controlled and what the mechanisms and signaling pathways are.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.N.H. and F.B.J. conceived and design of research; M.N.H., L.G., and F.B.J. performed experiments; M.N.H., L.G., and F.B.J. analyzed data; M.N.H. and F.B.J. interpreted results of experiments; M.N.H. prepared figures; M.N.H. and F.B.J. drafted manuscript; M.N.H., L.G., and F.B.J. edited and revised manuscript; M.N.H., L.G., and F.B.J. approved final version of manuscript.

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