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Review

The role of nitrite in nitric oxide homeostasis: A comparative perspective

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ABSTRACT

Nitrite is endogenously produced as an oxidative metabolite of nitric oxide, but it also functions as a NO donor that can be activated by a number of cellular proteins under hypoxic conditions. This article discusses the physiological role of nitrite and nitrite-derived NO in blood flow regulation and cytoprotection from a comparative viewpoint, with focus on mammals and fish. Constitutive nitric oxide synthase activity results in similar plasma nitrite levels in mammals and fish, but nitrite can also be taken up across the gills in freshwater fish, which has implications for nitrite/NO levels and nitrite utilization in hypoxia. The nitrite reductase activity of deoxyhemoglobin is a major mechanism of NO generation from nitrite and may be involved in hypoxic vasodilation. Nitrite is readily transported across the erythrocyte membrane, and the transport is enhanced at low O_2 saturation in some species. Also, nitrite preferentially reacts with deoxyhemoglobin rather than oxyhemoglobin at intermediate O_2 saturations. The hemoglobin nitrite reductase activity depends on heme O_2 affinity and redox potential and shows species differences within mammals and fish. The NO forming capacity is elevated in hypoxia-tolerant species. Nitrite-induced vasodilation is well documented, and many studies support a role of erythrocyte/hemoglobin-derived NO. Vasodilation can, however, also originate from nitrite reduction within the vessel wall, and at present there is no consensus regarding the relative importance of competing mechanisms. Nitrite reduction to NO provides cytoprotection in tissues during ischemia–reperfusion events by inhibiting mitochondrial respiration and limiting reactive oxygen species. It is argued that the study of hypoxia-tolerant lower vertebrates and diving mammals may help evaluate mechanisms and a full understanding of the physiological role of nitrite.

1. Introduction

Nitric oxide is a gaseous free radical that functions as an important signal molecule in cardiovascular homeostasis [1]. It is produced in the endothelium from the reaction of L-arginine with oxygen catalysed by endothelial nitric oxide synthase (eNOS):

$$\text{L}-\text{Arginine} + O_2 \xrightarrow{\text{eNOS}} \text{NO} + \text{L}-\text{citrulline}. \quad (1)$$

From the endothelium NO can diffuse to the underlying vascular smooth muscle, activate guanylyl cyclase and cause smooth muscle relaxation, which vasodilates the vessel and increases local blood flow. NO formation and its physiological roles have primarily been studied in mammalian models, but nitric oxide synthases and NO signaling are known to be present also in the cardiovascular system of lower vertebrates, including fish [2,3].

NO produced in the endothelium diffuses not only into vascular smooth muscle but also into the blood, where it reacts with plasma O_2 to form nitrite [4]:

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (2)$$

NO also reacts with oxygenated hemoglobin (Hb) to form ferric methemoglobin (metHb) and nitrate, and it binds with strong affinity to deoxygenated ferrous heme groups to form nitrosylhemoglobin (HbNO):

$$Hb(Fe^{2+})O_2 + NO \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (3)$$

$$Hb(Fe^{2+}) + NO \rightarrow Hb(Fe^{2+})NO \quad (4)$$

These two NO-scavenging reactions with Hb limit the amount of free NO [5] and are important for paracrine NO signaling by restricting the effects of NO to the local area of its production [1]. The formation of nitrite and nitrate by reactions 2 and 3 imply that these compounds are naturally present in animals at low concentrations. Nitrite originating from NO oxidation was considered relatively inert, until it became clear that nitrite can be converted back to NO by a variety of mechanisms under hypoxic and/or acidic conditions. NO is regenerated from nitrite by acidic disproportionation [6] and by enzymatic reduction via xanthine oxidoreductase [7], mitochondrial enzymes [8,9], or deoxygenated hemoglobin [10,11], myoglobin [12] and neuroglobin [13]. Recently, eNOS was also found capable of reducing nitrite to NO under anoxia [14]. The mechanisms that reduce nitrite to NO are all favored by low oxygen tension and/or low pH, and the concept has emerged that endogenous nitrite constitutes a reservoir

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of NO activity that is activated and plays an important role under hypoxic conditions [15]. Nitrite-derived NO has been suggested to be involved in hypoxic vasodilation [10,11,16] and it has been shown to protect cells and tissues against ischemia–reperfusion injury [17–19]. The fact that NO generation from nitrite is activated by multiple mechanisms at low Po_2 points to a general role of nitrite-derived NO during hypoxia, where the NOS-catalysed NO production is compromised due to the lack of the substrate oxygen.

Environmental hypoxia is seldom encountered by humans and other terrestrial mammals, apart from at high altitude or in the underground burrows of burrowing species; but internal hypoxia is an integral component of various human diseases, and the therapeutic role of nitrite in such states has received much interest [20]. Environmental hypoxia is, on the other hand, a common phenomenon in many aquatic habitats, where oxygen-depletion is a natural consequence of heavy microbial degradation of organic matter. Many fish may thus naturally encounter hypoxia in their habitat, and some species have become evolutionary adapted to live in habitats with severe periodic or chronic hypoxia. Such hypoxia-tolerant fish may be good animal models for disclosing mechanisms of hypoxia protection mediated by nitrite [21]. Mechanisms of nitrite reduction to NO are evolutionary ancient [22,23], and much may be learned about their physiological role by studying early vertebrate groups. The present article gives an overview of the role of nitrite in NO homeostasis from a comparative point of view, with focus on mammals and fish. It is the hope that the treatise will stimulate interest in the comparative physiology of nitrite.

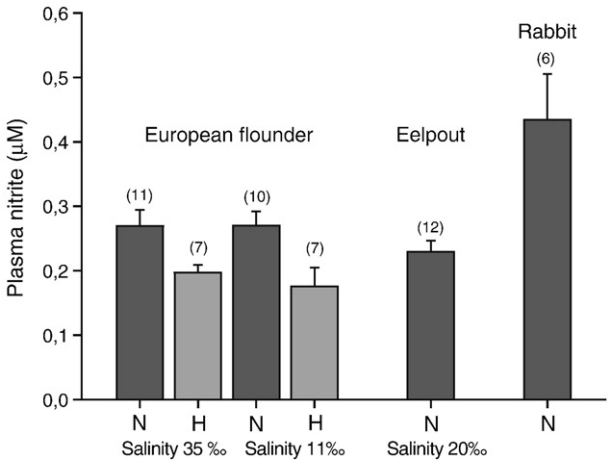

Fig. 1. Plasma nitrite concentrations in two marine teleost fish species (European flounder and eelpout) and one mammal (rabbit). European flounder (*Platichthys flesus*) were exposed to normoxia (N, inspired $Po_2 \geq 150$ mm Hg) or 2 days of hypoxia (H, inspired $Po_2 = 54$ mm Hg) at 10 °C in full strength seawater (salinity 35‰) or seawater with 11‰ salinity. The plasma was available from a recent study in our laboratory [29]. Female eelpout (*Zoarces viviparus*) were kept at 10 °C in running normoxic seawater with salinity 20‰ at our marine research station in Kerteminde, Denmark. The plasma was obtained by centrifugation of freshly drawn blood. Rabbit (*Oryctolagus cuniculus*) plasma was obtained from the Biomedical Laboratory, University of Southern Denmark. Nitrite was measured by reductive chemiluminescence, using a Sievers (Boulder, CO, USA) NO analyzer (NOA 280i). Data are means + SEM, with number of animals indicated in brackets (F.B. Jensen, unpublished data).

2. Natural nitrite levels

Apart from being endogenously produced as an oxidative metabolite of NO (cf. above), nitrite may also enter the body across the intestine due to its presence in the diet. Also, in mammals, nitrate from food and drinking water is reduced to nitrite by bacteria in the oral cavity [20]. Natural levels of plasma nitrite in mammals are typically 0.1–0.8 μM, and the levels seem to reflect the constitutive NOS activity [24]. Basal levels of plasma nitrite have not been systematically investigated in fish. Reported values for freshwater fish are normally in the low micromolar range (<10 μM) during control conditions [25,26], which would predict that plasma nitrite levels are higher in freshwater fish than in mammals. This probably reflects the fact that freshwater fish have an additional nitrite supply route compared to mammals, namely the direct uptake of nitrite from the ambient water across the gills [27]. Freshwater fish are hyperosmotic to their environment and need an active uptake of Na^+ and Cl^- across the gills to compensate for passive ion losses across the gills and in the urine [28]. Nitrite is naturally present at low concentration (typically<1 μM) in aquatic habitats, because it is an intermediate component in the ecosystem nitrogen cycle. Nitrite has, however, an affinity for the active Cl^- uptake mechanism, whereby small amounts of nitrite in the ambient water are likely to raise internal nitrite levels [27]. This complication is not encountered with seawater fish, because they are hypoosmotic to the environment and actively excrete NaCl across the gills. Furthermore, the high chloride concentration in full strength seawater (550 mM) will out-compete transport of nitrite via shared transport routes [27].

Measurements of nitrite in seawater fish can therefore be predicted to provide insight into the basal nitrite levels that result from the endogenous production due to NOS activity. This idea was recently tested by measuring plasma nitrite in two marine teleost fishes and one mammal, using reductive chemiluminescence, which has the required sensitivity to accurately assess plasma nitrite in the nanomolar range (Fig. 1). Under normoxic conditions, the mean values for European flounder (0.27 μM) and eelpout (0.23 μM) were inside the 0.1–0.8 μM range reported for mammals, and the mean value for rabbit (0.43 μM) agreed with the earlier reported value [24] for this species (Fig. 1). The data accordingly suggests that the basal plasma nitrite levels from constitutive NOS activity may be similar in fish and mammals. The measurements on European flounder gave some further interesting information. Plasma nitrite was the same in normoxic fish acclimated to full strength seawater (salinity 35‰) and brackish water with salinity of 11‰, but at both salinities there was a significant decrease in plasma nitrite following exposure to hypoxia for two days (Fig. 1). This is the first observation of changes in the internal nitrite stores with environmental hypoxia, and it supports the idea that nitrite is consumed during hypoxia through its conversion to NO, and that this may play a role in the acclimation of fish to hypoxia.

3. Nitrite accumulation in freshwater fish

Generation of NO from nitrite is favored by low pH and low Po_2, but it is also favored by increased nitrite concentration. This situation can become relevant in freshwater fish due to the uptake of nitrite via the active Cl^- uptake mechanism in the gills. So, whenever nitrite is present in the ambient water, part of the Cl^- uptake will be shifted to nitrite uptake. Nitrite can therefore be accumulated across the gills to concentrations that are well above the ambient concentration [30,31]. In nitrite-contaminated water, plasma nitrite concentrations in the millimolar range can eventually develop. Nitrite is toxic at such high concentrations and several critical physiological disturbances are induced that affect ion regulatory, respiratory and endocrine processes [27]. This illustrates that there is a trade-off between potential positive effects of nitrite at low concentrations and harmful effects at high concentrations.

The toxic actions of nitrite are beyond the scope of the present treatise, except for the possibility that nitrite accumulation in plasma can be hypothesized to cause a massive NO production from nitrite. This idea was recently tested in zebrafish. The fish were exposed to different ambient nitrite levels for variable time periods, and changes in blood HbNO levels were used as a biomarker of internal NO production [32]. Indeed, very high levels of HbNO were found in nitrite-exposed zebrafish, which testifies to a substantial production of NO from nitrite. The large NO formation in nitrite-exposed fish

predicts that disturbance of NO homeostasis is part of the toxic action of nitrite at high concentrations [32]. High nitrite-derived NO levels could perturb NO-based signaling and a large number of NO-influenced physiological processes, and it may cause nitrosative stress in tissues, resulting in high levels of S-nitrosylated proteins and damage to cellular structures. Furthermore, the high blood levels of HbNO will contribute to disturbances in blood O_2 transport, as they add to even higher levels of metHb in lowering the amount of functional (O_2 carrying) Hb in nitrite-exposed fish [32].

The fact that nitrite can be accumulated across the gills leads to the relevant question whether nitrite can function as a reliable reservoir of NO activity in freshwater fish. Ambient nitrite levels are low in most freshwater ecosystems, and rises in water nitrite are typically temporary events produced by transient imbalances in bacterial nitrification and/or denitrification processes in the milieu [27]. The natural internal nitrite levels in freshwater fish may therefore be slightly elevated compared to seawater fish and mammals, but they are normally not disproportionately high. As the endogenous nitrite pool primarily is activated by hypoxia, slightly elevated nitrite levels could actually prove positive. Furthermore, moderate elevation of ambient nitrite levels often coexist with environmental hypoxia [33], which would give freshwater fish an accessible pool of nitrite to be used internally during prolonged periods of hypoxia. Indeed, some freshwater fish species naturally experience weeks or months of severe environmental hypoxia, and my benefit from having access to environmental nitrite that can be used as an internal NO source after uptake across the gills. Future studies are required to fully evaluate these potential interactions between ambient nitrite levels and internal nitrite/NO homeostasis in chronic hypoxia.

4. The potential role of erythrocytes and hemoglobin in nitrite-induced vasodilation

Among the various mechanism that are capable of reducing nitrite to NO, the reaction of nitrite with deoxygenated Hb to form metHb and NO has attracted particular interest:

$$Hb(Fe^{2+}) + NO_2^- + H^+ \rightarrow Hb(Fe^{3+}) + NO + OH^- \quad (5)$$

This nitrite reductase activity of deoxyHb has been suggested to participate in hypoxic vasodilation [10,11]. The idea is that the RBCs will experience an increased degree of deoxygenation when passing the microcirculation of hypoxic tissues, and that this is coupled to an increased production of a vasodilator (NO) that upon release from the RBCs can increase blood flow according to need. In this way the RBCs functions not only as carriers of O_2 both also as sensors of local O_2 conditions and appropriate mediators of local blood flow. There is both in vivo and in vitro experimental evidence that supports the involvement of Hb/RBCs in nitrite-induced vasodilation at near-physiological concentrations [10,34–36], but the mechanism also has controversial aspects that remain to be solved. The mechanism in principle involves four steps: (1) transport of nitrite across the RBC membrane, (2) reaction of nitrite with Hb, (3) escape of NO from the RBCs and (4) induction of vasodilation.

4.1. Erythrocyte nitrite transport

The transport of nitrite across the RBC membrane exhibits substantial differences between species.

Early studies on carp RBCs showed that the transport is strongly oxygenation-dependent in this species. At physiological pH, nitrite extensively enters deoxygenated red blood cells, whereas it hardly permeates oxygenated cells [37,38]. When pH is lowered, nitrite starts to enter oxygenated red blood cells, but at much lower rates than in deoxygenated cells [38]. A similar oxygenation dependency was found in tench and whitefish erythrocytes [27]. Thus, in these fish species, nitrite preferentially permeates erythrocytes with low oxygen saturation (So_2), which appears to supply nitrite for deoxyHb-mediated NO generation in an appropriate manner. A similar difference between fully oxygenated and deoxygenated erythrocytes is not seen in mammalian (pig and sheep) erythrocytes [39,40]. In pig RBC suspensions, added nitrite quickly permeates and equilibrates across the membrane, and then continues to enter the cells at a rate that depends on the intracellular removal of nitrite (via its reactions with Hb), which creates a continued diffusion gradient from the extracellular to the intracellular space [39]. Interestingly, whereas the influx of nitrite is similar into oxygenated and deoxygenated sheep RBCs, the influx is increased at 50% HbO_2 saturation [40]. This seems to support the finding that the reaction rate between nitrite and mammalian Hb is maximal around 50% HbO_2 saturation [41]. We have observed a similar elevated nitrite influx at intermediate So_2 in rabbit RBCs, whereas there is a gradual increase in influx with decreasing So_2 in carp RBCs (S. Rohde and F.B. Jensen, to be published). Two major factors that govern the transport of nitrite across the RBC membrane can therefore be identified: namely the permeability of the membrane to nitrite and the rates of the intracellular reactions of nitrite with Hb. In mammalian erythrocytes the permeability seems to be relatively unaffected by HbO_2 saturation, but because the intracellular reaction rates increase at intermediate So_2, the influx of nitrite also increases at these saturations. In fish erythrocytes the reaction rates also vary with O_2 saturation, but additionally the membrane permeability increases with decreasing So_2 in some species. This selective change in membrane permeability then becomes instrumental in the oxygenation dependency of nitrite entry. Indeed, when nitrite enters carp erythrocytes at low So_2, a subsequent full oxygenation is able to switch off further nitrite entry [42].

The transport mechanism seems to involve both NO_2^- diffusion (possibly employing the anion exchanger AE1) and HNO_2 diffusion, but application of DIDS (an AE1 inhibitor) or pH changes have not been able to provide conclusive answers regarding the relative importance of transport routes (cf. [39,43] for recent discussion).

4.2. NO formation by hemoglobin nitrite reductase activity

Inside the RBCs, nitrite reacts with Hb, but the reaction products depend on the oxygenation status of the cells. In oxygenated cells the reaction will be with oxyHb, which leads to the oxidation of nitrite to nitrate and oxidation of oxyHb to metHb with three of the heme-bound O_2 becoming reduced [44]:

$$4Hb(Fe^{2+})O_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb(Fe^{3+}) + 4NO_3^- + O_2 + 2H_2O \quad (6)$$

The mechanism is, however, more complex than suggested by the stoichiometry in Eq. (6). The reaction kinetics show an initial slow 'lag' phase followed by an autocatalytic increase in reaction rate, and the reaction proceeds via a series of steps that produce reactive intermediates such as H_2O_2, NO_2 and ferrylhemoglobin, with the free radical NO_2 functioning as the autocatalytic propagatory species [45].

Nitrite reacts with deoxyHb to form metHb and NO, as shown in Eq. (5), and the NO subsequently binds to an adjacent ferrous heme to form HbNO (Eq. (4)). Therefore, the reaction with the fully deoxygenated Hb will lead to the formation of metHb and HbNO in equal amounts [21,41,46]. MetHb that is formed by both the deoxyHb and the oxyHb reactions is incapable of O_2 transport, but inside RBCs it will quickly be reduced back to functional Hb by metHb reductase systems under normal physiological circumstances, where the nitrite concentration is low.

The potential involvement of nitrite reduction in hypoxic vasodilation has stimulated much recent scrutiny of the reaction between nitrite and deoxyHb under anaerobic conditions. One prominent finding is that when nitrite is applied in excess to deoxyHb, the deoxyHb concentration decreases in a sigmoid fashion with time

rather than showing an exponential decrease, as would be expected from a simple second-order reaction [41]. This phenomenon has been explained by allosteric autocatalysis [16,41]. The Hb molecule will initially be in the T structure, but during the reaction there will be an allosteric transition to the R structure due to the formation of metHb and HbNO. Deoxygenated hemes have a lower redox potential (better ability to reduce nitrite) in the R structure than in the T structure, and the reaction rate will therefore speed up during the reaction [16,41]. Under physiological conditions, this behavior should result in the fastest Hb-mediated nitrite reduction around 50% O_2 saturation, as indeed reported with human Hb and RBCs [34,41]. The sigmoid decrease in [deoxyHb] with time during the reaction of nitrite with fully deoxygenated Hb applies to other mammalian Hbs (Fig. 2) and to carp Hb [21]. It appears, however, that the sigmoid nature is more prominent in mammalian (rabbit and harbor porpoise) than in fish (carp and rainbow trout) Hbs when studied under the same experimental conditions (Fig. 2).

In the arterial–venous circulation, the Hb molecule cycles between full and intermediate oxygen saturations, and it will not become fully deoxygenated. It is therefore important to understand how the reaction between nitrite and Hb proceeds at intermediate oxygen saturations, where nitrite can react with both oxyHb and deoxyHb. Interestingly, when excess nitrite reacts with Hb at intermediate So_2 values, the deoxyHb concentration decreases faster than the oxyHb concentration (Fig. 3), showing that the reaction of nitrite with deoxyHb is favored over that with oxyHb [21,46]. The preferential reaction of nitrite with deoxyHb leads to the production of significant amounts of NO and HbNO also at intermediate So_2. It is in fact HbNO formed in the deoxyHb reaction that inhibits the oxyHb reaction at intermediate So_2 [21,46].

Given that nitrite reduction may be important during hypoxia, it is relevant to evaluate how the reaction is influenced by oxygen affinity, and whether the nitrite reductase capability of Hb shows adaptive traits that correlate with hypoxia tolerance. Hypoxia-tolerant fish, such as carp, have evolved Hb with very high O_2 affinity [47], which can be hypothesized to give the Hb a high R-state character and low redox potential that should promote deoxyHb-mediated nitrite reduction to NO. Indeed, carp Hb with high O_2 affinity reacts faster with nitrite than rabbit Hb with lower O_2 affinity, and higher amounts of NO and HbNO are produced with carp Hb than with rabbit Hb at any given intermediate So_2 level [21]. Thus, a high O_2 affinity seems to be associated with a high nitrite reductase capability. In order to elaborate further on this idea, I have recently tested rainbow trout Hb under the same experimental conditions. Rainbow trout Hb has a much lower O_2 affinity than both carp Hb and rabbit Hb, and accordingly would be hypothesized to have a low nitrite reductase

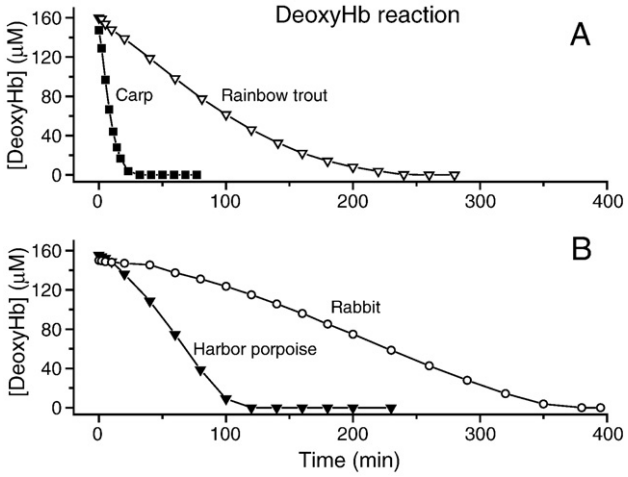

Fig. 2. Time-dependent decline in [deoxyHb] during the reaction of nitrite with fully deoxygenated Hb from two teleost fish (A, common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss*) and two mammals (B, Harbor porpoise *Phocoena phocoena* and rabbit *Oryctolagus cuniculus*). In parallel with the decline in [deoxyHb] there was a 1:1 increase in metHb and HbNO, each reaching half the initial deoxyHb concentration by the end of the reaction for each species (not shown). All experiments were conducted under the same conditions. Hemoglobin concentration was ~155 μM on heme basis, and the nitrite/heme concentration ratio was 2.7. Temperature was 25 °C. Measurements were made in 0.05 M Tris buffer with 0.1 M KCl at a pH of 7.3. Carp and rabbit data: [21]; rainbow trout data: (F.B. Jensen, unpublished); Harbor porpoise: (M.N. Hansen and F.B. Jensen, unpublished).

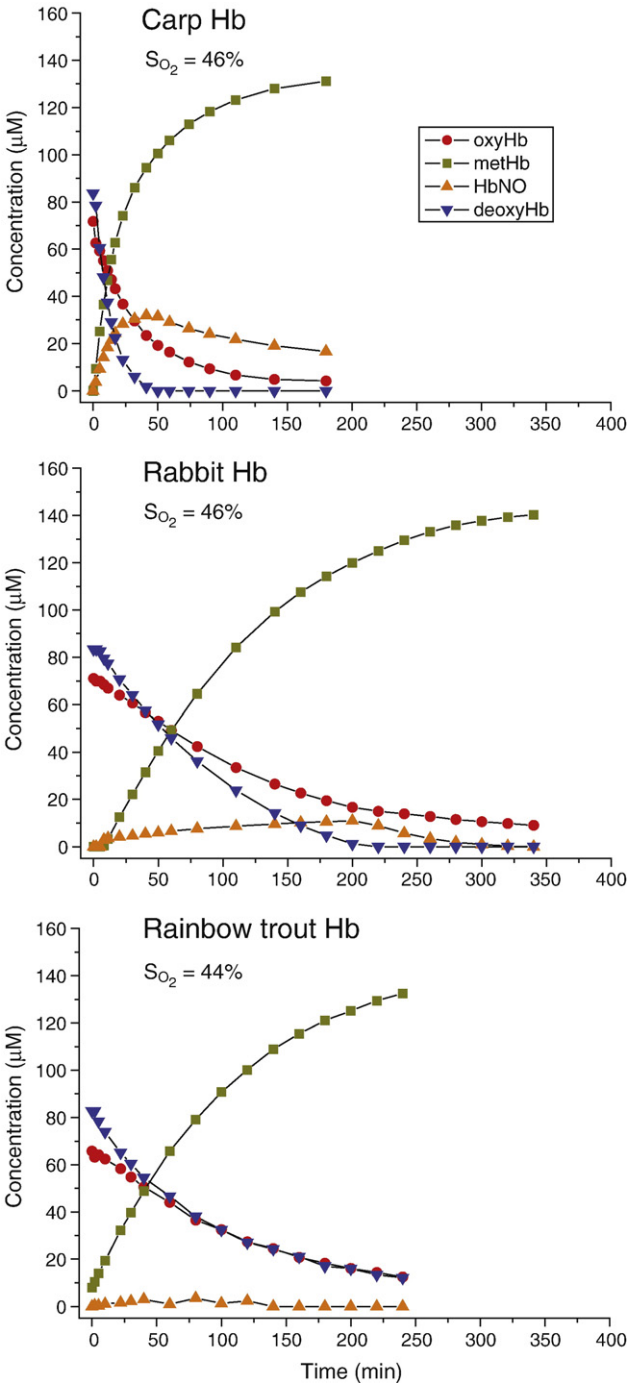

Fig. 3. The reaction of nitrite with carp, rabbit and rainbow trout Hb at an intermediate oxygen saturation (So_2) of 44–46%. Time-dependent changes in oxygenated hemoglobin, methemoglobin, nitrosylhemoglobin and deoxygenated hemoglobin are shown. All experiments were conducted under the same conditions. Hemoglobin concentration was ~155 μM on heme basis, and the nitrite/heme concentration ratio was 2.7. Temperature was 25 °C. Measurements were made in 0.05 M Tris buffer with 0.1 M KCl at a pH of 7.3. Carp and rabbit data: [21]; rainbow trout data: (F.B. Jensen, unpublished).

capability. The reaction of nitrite with rainbow trout deoxyHb is certainly slower than that with carp Hb (Fig. 2), and the production of HbNO at an intermediate So_2 of 44% is definitely lower than in carp and rabbit Hb (Fig. 3). An illustrative overview of these species differences can be obtained by plotting the maximal HbNO concentration during the reaction as a function of the initial O_2 saturation (Fig. 4). The production of HbNO decreases in a curvilinear fashion with increasing So_2 in all three species, but at any given intermediate So_2 the $[HbNO]_{max}$ level is highest in carp Hb (high O_2 affinity), lower in rabbit Hb (reduced O_2 affinity) and lowest in trout Hb (low O_2 affinity). The NO/HbNO production stays significant in carp even up to 80% So_2, whereas a significant HbNO production is only observed at very low So_2 in rainbow trout (Fig. 4). This clear difference would suggest that the deoxyHb-mediated NO formation could be particularly relevant in hypoxia tolerant species such as carp, whereas it may have less relevance in hypoxia intolerant species such as rainbow trout.

Apart from showing species differences, which may have been selected for during evolution, the nitrite reductase activity of deoxyHb will also vary with cellular factors. Addition of ATP to carp Hb lowers O_2 affinity by T state stabilization, and this induces a decrease in the deoxyHb-mediated nitrite reduction rate and NO production [21] (Fig. 4). This ATP effect underscores the dynamic influence of the T ↔ R allosteric equilibrium and Hb O_2 affinity on nitrite reduction, but it is also of physiological interest, because nucleoside triphosphates (ATP and GTP) are used as allosteric modifies of Hb O_2 affinity in fish RBCs. During acclimation to hypoxia the erythrocyte concentration of nucleoside triphosphates is lowered in order to increase O_2 affinity [47]. This improves the arterial O_2 saturation at low Po_2, but at the same time the decreased concentration of nucleoside triphosphates can be predicted to increase the nitrite reductase capability of Hb. Both responses would appear adaptive in supplying the required O_2 to the tissues, assuming that the produced NO is vasoactive.

Species differences in Hb-mediated nitrite reduction can in principle be quantified from the bimolecular rate constant for the reaction. However, due to the sigmoid kinetics of the deoxyHb reaction with nitrite, which results from the different reactivity of T-state and R-state hemes, the apparent bimolecular rate constant increases during the reaction [16]. The initial second-order rate constant, determined at the beginning of the reaction, however, provides a useful measure for species comparisons. For the reactions between fully deoxygenated Hb and nitrite at 25 °C shown in Fig. 2 the initial second-order rate constants calculates as 2.5 $M^{-1}s^{-1}$ in carp, 0.15 $M^{-1}s^{-1}$ in rainbow trout, 0.2 $M^{-1}s^{-1}$ in Harbor porpoise and 0.06 $M^{-1}s^{-1}$ in rabbit. These values corroborate the high nitrite reductase capability of carp Hb but also suggest that differences may be present among mammalian species. As a diving mammal, Harbor porpoise employ extensive peripheral vasoconstriction and experience tissue hypoxia during long dives, which is followed by reperfusion/oxygenation upon surfacing for breathing. One may speculate that a somewhat elevated nitrite reductase capability could play a role in this situation, in line with the suggested positive effects of nitrite-derived NO in clinical ischemia–reperfusion events [17–19].

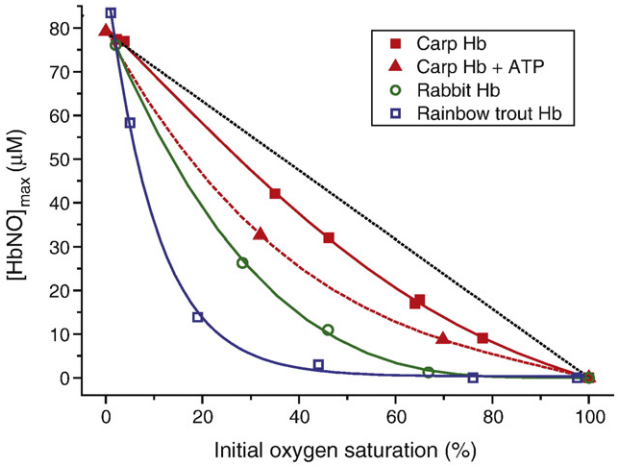

Fig. 4. The maximal HbNO concentration during the reaction of nitrite with hemoglobin is dependent on initial oxygen saturation and species-specific differences in heme oxygen affinity and redox potential. Data are shown for carp Hb ((■, high O_2 affinity: P_{50} = 1.2 mm Hg), rabbit Hb (○, lower O_2 affinity: P_{50} = 5.1 mm Hg) and rainbow trout Hb (□, low O_2 affinity: P_{50} = 32 mm Hg); and for carp Hb in the presence of ATP (▲), where oxygen affinity is lowered (P_{50} = 6 mm Hg) by T state stabilization of the Hb. The upper dotted line represents the possible maximum HbNO value if all NO formed during the reaction of nitrite with Hb at intermediate oxygen saturations binds to vacant deoxy hemes and no NO reacts with oxyHb or escapes the system (cf. [21]). Hemoglobin concentration was ~155 μM on heme basis, and the nitrite/heme concentration ratio was 2.7. Temperature was 25 °C. Measurements were made in 0.05 M Tris buffer with 0.1 M KCl at a pH of 7.3. Carp and rabbit data: [21]; rainbow trout data: (F.B. Jensen, unpublished).

4.3. Escape of NO

Even though several studies have documented that the Hb molecule and RBCs possess mechanisms that guide nitrite towards the reaction with deoxyHb to produce NO under hypoxic conditions, it remains uncertain to what extent this NO will be able to escape the RBCs and induce vasodilation.

NO binds to deoxygenated heme groups with very high affinity and the rate of dissociation of NO from HbNO is low [48]. Thus, deoxyHb exerts a NO scavenging rather than releasing role. An additional sink for RBC-produced NO is the reaction with oxyHb to form metHb and nitrite (Eq. (3)). Mathematical modeling shows that nitric oxide is unlikely to be exported from RBCs directly as NO in amounts that are sufficient to activate soluble guanylyl cyclase in vascular smooth muscle cells [49]. In spite of this dilemma, there is accumulating evidence that sufficient NO activity can escape Hb scavenging and produce vasodilation [10,34–36]. The export of NO activity from RBCs could be eased via a localized reaction between deoxyHb and nitrite directly at the membrane [50]. DeoxyHb is known to bind to the N-terminal cytoplasmic domain of the erythrocyte anion exchanger AE1 [51], where it would be ideally placed to reduce incoming nitrite (assuming that nitrite enters via AE1; cf. [43]). AE1-bound Hb was recently found to react faster with nitrite than Hb in solution, supporting the possibility of a preferential NO generation and release directly at the membrane [52]. It has also been suggested that NO activity could escape the RBCs in the form of N_2O_3 [49,53]. A nitrite-metHb intermediate with NO_2 radical properties is formed during the nitrite-Hb reaction, which can react with NO to produce N_2O_3 [54]. This N_2O_3 can diffuse out and re-form NO outside the RBCs by N_2O_3 homolysis to NO and NO_2 [49,53,54]. N_2O_3 also readily forms S-nitrosothiols, which provides an alternative avenue for export of NO activity [53–55]. In any case, the escape of only a small amount of the NO that is produced inside RBCs may suffice to produce vasodilation.

4.4. Nitrite-induced vasodilation

Nitrite was previously considered a week vasodilator that only induced vasodilation at high concentrations. Some studies have supported this view by failing to document vasodilation upon nitrite infusion in humans at relative low concentration [56]. However, a number of other recent studies have shown that in vivo nitrite infusion in humans and other primates causes vasodilation at near-physiological concentrations in a manner that seems consistent with a contribution from RBC-derived NO [10,57,58]. Furthermore, hypoxia (breathing of 12% O_2) augments the nitrite-mediated vasodilation in humans in vivo [58]. Nitrite infusion experiments with rats also agree with a role for RBC nitrite reduction in regulation of vascular tone [35]. Additional support for a coupling between nitrite-induced vasodilation and Hb/RBC deoxygenation has been obtained with in vitro

bioassays using aortic rings [10,34,36]. Contrasting data have, however, also been reported, suggesting that nitrite vasorelaxation of aortic rings is mainly intrinsic to the vessel wall, perhaps via formation of vasoactive S-nitrosothiols from nitrite within the smooth muscles, rather than being dependent on Hb [59]. Nitrite alone (i.e. in the absence of Hb or RBCs) has a high potency for relaxation of aortic rings at low Po_2 [36,59], and in addition to the possible involvement of S-nitrosothiols [59] one may envisage a possible involvement of smooth muscle deoxymyoglobin [36] or other proteins such as xanthine oxidoreductase or eNOS [60]. There is an increased realization that nitrite reduction to NO in the vascular wall contributes to nitrite-induced vasodilation, and that there accordingly are several routes of NO formation from nitrite within the circulation [43,59–62]. At present there is no clear consensus regarding the importance of competing mechanisms, and more studies are needed to weigh the relative importance of the vessel wall versus that of Hb/RBCs in nitrite-induced vasodilation.

There is only limited information on nitrite-induced vasodilation in fish. Rainbow trout respond to nitrite exposure with an acute sharp increase in heart rate, and this happens in the absence of an adrenergic stress response and before significant rises in metHb or extracellular $[K^+]$ have developed [63]. A likely explanation of the tachycardia is the appearance of nitrite in plasma, which causes a systemic vasodilation (via the formation of NO) that becomes countered by increased cardiac pumping to reestablish blood pressure [63]. The potential role of RBCs in reducing nitrite to vasoactive NO has been tested in the coronary circulation of the isolated rainbow trout heart. Perfusion of the coronaries with hypoxic saline resulted in NO production and vasodilation, and both these responses could be inhibited by the NOS inhibitor L-NA, showing that NO was vasoactive and probably of endothelial origin [64]. Subsequent perfusion with RBCs and nitrite resulted in Hb deoxygenation, consumption of nitrite and a rise in metHb during passage of the coronary tree, which was paralleled by an NO production that was not inhibited by L-NA. These changes would seem to support the production of NO from nitrite in the RBCs, but it cannot be excluded that the heart itself generated NO from nitrite [64]. The NO formation associated with nitrite was found have no effect on coronary flow. Apparently, the nitrite-derived NO was produced in the capillaries after passage of the resistance vessels, and the signal was not conducted upstream to arterioles [64]. It is possible that the effect of nitrite varies between different microvasculatory beds, and therefore other microcirculations need to be examined in fish. Also, rainbow trout may not be the best model choice among fish, given the reduced capacity for Hb-mediated nitrite reduction that was recently discovered in this species (cf. above). It may be more rewarding to study carp or other hypoxia-tolerant species in future work.

5. Tissue nitrite and its role in cytoprotection

Nitrite is ubiquitous present in mammalian tissues at concentrations that are either comparable (brain and heart), slightly lower (liver, kidney and lung) or higher (aorta; ~10 μM) than in plasma [23]. Nitrite plays an important physiological role in tissues as a NO donor but also as a signaling molecule on its own that regulates vital cell functions, including gene expression [23]. In mammals, particular focus has been on the cytoprotection that nitrite exerts after ischemia and blood-flow reperfusion events in various tissues. The protective effect on cellular necrosis and apoptosis following long periods of restricted blood flow seems related to the reduction of nitrite to NO, which in ischemic tissue may be mediated both by acidic disproportionation (i.e. low intracellular pH) and enzymatic reduction via e.g. xanthine oxidoreductase or myoglobin [17–19,65]. A recent study sheds light on the mechanism and the role of myoglobin (Mb) in myocardial tissue. By using Mb wild-type and knockout mice, Mb-mediated reduction of nitrite to NO was found to protect against ischemia–reperfusion injury by inhibiting mitochondrial respiration (conserving the limited O_2) and limiting the generation of reactive oxygen species (formed during re-oxygenation), which reduces oxidative protein damage [19]. The data accordingly provides Mb with an important nitrite reductase function in cytoprotection that adds to its functions in O_2 storage and delivery [66]. From a comparative viewpoint it is tempting to speculate that the high Mb levels found in the skeletal muscles of diving mammals and birds could play a similar role. The high Mb levels serve as an O_2 reservoir that keeps the muscles aerobic during most dives, even though the muscles are restricted from blood flow by vasoconstriction; but the muscles turn anaerobic during long dives [67]. In this setting, the nitrite reductase activity of muscle Mb could lower respiration at low muscle Po_2 and limit the surge in reactive oxygen species when the muscles re-oxygenate upon surfacing. Consequently, in diving mammals, both the reactions of nitrite with Mb and Hb (cf. above) seem worth future study.

The cytoprotection that nitrite mediates through its reduction to NO may be supplemented by additional mechanisms. Thus, nitrite has been shown to up-regulate the expression of heat shock protein 70, a molecular chaperone that repairs damaged proteins and protects against cellular stress [23].

Information on NO production from nitrite in tissues of lower vertebrates is not yet available. We have work in progress that aims at assessing nitrite levels and nitrite reduction in tissues of fish and the importance of this in hypoxia. Metabolic suppression and free radical defenses are part of the overall strategy that allows some fishes, amphibians and reptiles to tolerate severe hypoxia and fluctuations in oxygen availability [68]. Given that nitrite reduction to NO can inhibit mitochondrial respiration [12,19] and limit damage from reactive oxygen species [19], it seems possible that tissue nitrite reduction supplements other mechanisms involved in providing hypoxia tolerance.

6. Concluding remarks

Most studies on nitrite-dependent hypoxic signaling and the function of nitrite in NO homeostasis have been conducted with mammalian models, but available data supports that nitrite-derived NO is important also in fish. There is accumulating evidence for the involvement of nitrite in hypoxic vasodilation and in cytoprotection during hypoxia and oscillations in O_2, but there are still many questions to answer before we fully understand the mechanisms and their physiological importance. According to the August Krogh principle [69], much may be learned by studying hypoxia-tolerant lower vertebrates. More data on these will help evaluate mechanisms and document whether nitrite reduction to NO is part of an evolutionary ancient strategy for coping with hypoxia.

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