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Nitric oxide formation from nitrite in zebrafish

Frank B. Jensen
Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark
e-mail: fbj@biology.sdu.dk

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Summary

Nitrite is a potential nitric oxide (NO) donor and may have important biological functions at low concentrations. The present study tests the hypothesis that nitrite accumulation across the gills in fish will cause a massive NO production from nitrite. Zebrafish were exposed to three different nitrite levels for variable time periods, and changes in blood nitrosylhemoglobin (HbNO), methemoglobin (metHb), oxygenated hemoglobin (oxyHb) and deoxygenated hemoglobin (deoxyHb) were evaluated by spectral deconvolution. Blood HbNO (a biomarker of internal NO production) was low in controls, increased to a stable level around 3.7% of total Hb in fish exposed to 0.6 mmol l^{-1} nitrite, and to 12.1% (at day 2) in fish exposed to 2 mmol l^{-1} nitrite. The very high HbNO levels testify to an extensive conversion of nitrite to NO. With deoxyHb-mediated reduction of nitrite being a major NO-producing mechanism, the data reveal the significance of this mechanism, when hemoglobin cycles between full and intermediate oxygen saturations in the arterial–venous circulation. Fish exposed to 0.6 mmol l^{-1} nitrite for up to 5 days could be divided into responding (with elevated metHb) and non-responding individuals. Exposure to 2 mmol l^{-1} nitrite caused a time-dependent increase in metHb to 59% of total Hb within 2 days. Taking HbNO into account, the functional (potential O_2 carrying) Hb was reduced to 29% at this stage. Total blood [Hb] was also significantly decreased. In spite of the reduced blood O_2 capacitance, and the possibility that excess NO may inhibit mitochondrial respiration, whole animal routine oxygen consumption was not depressed.

Key words: hemoglobin, methemoglobin, nitric oxide, nitrite, nitrosylhemoglobin, oxygen consumption, spectral deconvolution.

Introduction

The study of biological actions of nitrite has received increasing interest in recent years. Nitrite is recognized as a toxic compound that can induce a number of physiological disturbances, when its concentration in the organism is high (Jensen, 2003). Nitrite is, however, also a natural constituent in the body, and recent research has suggested that nitrite has important biological functions at its natural low endogenous concentrations (Gladwin et al., 2005). Thus, nitrite may participate in local blood flow regulation (hypoxic vasodilation), protect against ischemic insults, and it may act as a signaling molecule and regulate gene expression (Bryan et al., 2005; Gladwin et al., 2005). Nitrite physiology therefore seems to be a balance between toxic disruption of functions at high concentrations and beneficial effects at low concentrations (Jensen, 2007).

Nitrite is produced inside the body as an oxidative metabolite of the physiological messenger molecule nitric oxide (NO). Nitric oxide produced in the vascular endothelium exerts its biological function by diffusing to the underlying vascular smooth muscle, causing their relaxation and thus local vasodilation. NO diffusing into the blood can react with plasma oxygen and form nitrite. Other sources of nitrite include intake *via* the diet and (in mammals) reduction of nitrate (present in diet or drinking water) to nitrite by bacteria in the oral cavity (Lundberg and Weitzberg, 2005). An important additional route in fish is the direct uptake of nitrite from the ambient water to the blood across the gills. Nitrite has an affinity for the active branchial chloride uptake mechanism in freshwater fish, whereby even minor elevations of nitrite in the environment can lead to massive accumulation of nitrite in the fish (Margiocco et al., 1983; Jensen, 2003).

Nitrite originating from NO oxidation was considered relatively inert, until it became clear that nitrite may function as a storage pool of NO activity (Cosby et al., 2003). Thus, NO can be regenerated from nitrite by non-enzymatic acidic reduction (Zweier et al., 1999) and by enzymatic reduction *via* xanthine oxidoreductase (Millar et al., 1998) or deoxygenated hemoglobin (Cosby et al., 2003). These reactions are favored by low pH and low oxygen tension (P_{O_2}), pointing to their possible role in vasodilation during hypoxia and exhausting exercise (Gladwin et al., 2005; Gladwin et al., 2006; Fago and Jensen, 2007). The formation of NO from nitrite and its physiological role in blood flow regulation has mainly been examined and documented in mammalian models (Cosby et al., 2003; Crawford et al., 2006), but some information is also available for fish (Aggergaard and Jensen, 2001; Jensen and Agnisola, 2005).

NO formation from nitrite is favored not only by low pH and P_{O_2} but also by high [NO_2^-]. This is seldom considered, because nitrite typically is present in the sub-micromolar or low micromolar range in plasma or tissues (Kleinbongard et al.,

2003). However, in nitrite-exposed fish, plasma [NO_2^-] can increase to the millimolar range, and it seems likely that this could lead to excess NO production (Jensen, 2003). This implies that disturbance of NO homeostasis may be a major impact of nitrite exposure in fish. The main aim of the present study was to test the hypothesis that a massive production of NO occurs in nitrite-exposed fish.

NO reacts with deoxygenated heme groups of hemoglobin (Hb) to form iron-nitrosyl Hb (HbNO). The reaction is rapid and has a strong binding constant, whereas the rate of dissociation is low (Antonini and Brunori, 1971). Thus, the level of HbNO can be used as a 'meter' for the internal NO level (Gladwin et al., 2005). The present study therefore used the blood HbNO concentration as a biomarker for the internal NO production and NO load. By performing spectral deconvolution on spectra of hemolysates obtained from zebrafish exposed to three nitrite levels for variable time periods, it was possible to evaluate dose- and time-dependent changes in HbNO, methemoglobin (metHb), functional Hb and total Hb during nitrite exposure.

It is well documented that blood gas transport is disturbed by increased metHb levels and decreased total [Hb] during nitrite exposure (Jensen, 2003), but little is known about how this affects O_2 consumption in fish. Metabolism may also be influenced by inhibition of mitochondrial respiration *via* NO formed from nitrite (Crawford et al., 2006; Shiva et al., 2007). Consequently, a further aim of the present study was to evaluate whether overall metabolic rate, as reflected by routine oxygen consumption, is influenced by nitrite exposure. Nitrite exposure can induce K^+ efflux from intracellular to extracellular compartments, which has been hypothesized to cause an overall potassium deficit (Knudsen and Jensen, 1997). A final aim was, therefore, to analyze whole body ion content during nitrite exposure. Zebrafish were chosen as experimental animals, because their high activity level should make changes in metabolic rate from limitations in O_2 transport easy to detect. Furthermore, zebrafish are used as model animals in many biological disciplines, and their rearing and maintenance can be associated with occasional nitrite exposure, which (as in nature) results from nitrite built-up during imbalance in bacterial nitrification or denitrification processes (Jensen, 2003). Thus, the information on nitrite effects and tolerance in zebrafish appeared a relevant side reward of using this species to study NO formation from nitrite in fish.

Materials and methods

Experimental fish

Zebrafish *Danio rerio* Hamilton, body mass 0.44±0.16 g (mean ± s.d., N=111), were obtained from a local aquarium store and acclimated for 10 days to 26°C and a 12 h:12 h light:dark cycle in aquaria containing 60 l aerated freshwater and 25–30 fish. The experimental freshwater was city tapwater diluted 1:9 with deionized water in order to reduce the high hardness of ordinary tapwater. Water composition in experimental freshwater was (in mmol l^{-1}): Cl^- 0.14, Na^+ 0.15, K^+ 0.017, Ca^{2+} 0.3, HCO_3^- 0.4. The fish were fed twice daily with commercial fish food (TetraMin®, Malle, Germany) and the water was exchanged daily. Feeding was withheld for 2 days before experimental sampling.

Experimental groups and sampling of blood and whole animals

The experimental design involved three exposure groups. Zebrafish were exposed to either (i) control water (no nitrite addition, measured [NO_2^-] ≤0.005 mmol l^{-1}), (ii) 0.6 mmol l^{-1} nitrite or (iii) 2.0 mmol l^{-1} nitrite. The required amount of nitrite was added as dissolved $NaNO_2$, and the water nitrite concentration was confirmed by spectrophotometric measurements (Gries reaction). The fish were sampled after 0, 1 and 2 days of exposure at each of these three nitrite conditions. Fish exposed to 0.6 mmol l^{-1} nitrite were additionally sampled after 5 days of exposure.

Upon sampling, individual fish were netted and anesthetized in MS-222 (ethyl 3-aminobenzoate methanesulfonate; Sigma, Steinheim, Germany). Following full anesthesia, the tail was cut posterior to the anal fin and blood was sampled into a heparinized capillary tube held toward the exposed caudal vessels. This resulted in the collection of 2–8 μl blood. Blood was immediately processed for spectral analysis (cf. below), and, at the same time, the whole fish (including the tail) was transferred to a mortar for instant freezing in liquid nitrogen and pulverization with a pestle. The powdered fish was transferred to a pre-weighed vial for determination of tissue wet mass. To each vial, 2 ml of 8% perchloric acid was added and samples were allowed to digest for 48 h with intermittent mixing for the extraction of ions. The homogenates were centrifuged and the supernatant was used for measurements of whole body ion concentrations. Potassium was measured by atomic absorption spectroscopy (AAnalyst 100, Perkin-Elmer, Waltham, MA, USA), sodium was measured using a flame photometer (Instrumentation Laboratory 243) and chloride was measured with a Radiometer (Copenhagen, Denmark) CMT 10 chloride titrator. Ion concentrations were expressed as mmol kg^{-1} wet mass.

Spectral scans and spectral deconvolution

Directly after blood sampling, an accurate amount of blood was pipetted into 1 ml 20 mmol l^{-1} phosphate buffer (pH 7.3). Cell debris was removed from the hemolysate by centrifugation (1 min at 12 750 ***g***), and the supernatant was transferred to a 1 cm, 1 ml cuvette. A spectral scan was made from 480 to 700 nm in 0.2 nm steps, using a Cecil CE2041spectro-photometer (Cambridge, UK). Scans were completed in less than 3 min from blood sampling to exclude any potential post-sampling changes in concentration of methemoglobin or HbNO.

The hemoglobin (Hb) solution from each individual zebrafish was assumed to be a mixture of oxygenated Hb (oxyHb), methemoglobin (metHb), iron-nitrosyl Hb (HbNO) and deoxygenated Hb (deoxyHb). Using the Lambert-Beer law, the measured absorbance (A) at any wavelength (λ) will be the sum of the contribution of each individual Hb species, according to:

$$A_\lambda = [(C_{\text{oxyHb}} \times \epsilon_{\text{oxyHb},\lambda}) + (C_{\text{metHb}} \times \epsilon_{\text{metHb},\lambda}) + (C_{\text{HbNO}} \times \epsilon_{\text{HbNO},\lambda}) + (C_{\text{deoxyHb}} \times \epsilon_{\text{deoxyHb},\lambda})] \times l, \quad (1)$$

where C is the millimolar concentration of the Hb species (on heme/monomer basis), ϵ signifies the millimolar extinction coefficient of the given Hb species at wavelength λ, and l is thickness of the absorbing layer (=1 cm). The use of this

equation to evaluate the concentration of each Hb derivative required information on ϵ values for each Hb type at all concerned wavelengths.

A reference spectrum for oxyHb was obtained by hemolysing 8 μl freshly drawn zebrafish blood in 1.0 ml air-equilibrated (P_{O_2}=155 mmHg; 1 mmHg=133.3 Pa) 20 mmol l^{-1} phosphate buffer (pH 7.3). Cell debris was removed by centrifugation (1 min at 12 750 ***g***), and 900 μl supernatant was transferred to a 1 cm, 1 ml cuvette. A spectral scan was made from 480 to 700 nm in 0.2 nm steps. The resulting oxyHb spectrum had the two characteristic peaks situated at 540.8 nm and 576.6 nm. The Hb concentration (mmol heme l^{-1}) in the cuvette was calculated from the absorbance at these two peaks and the reported millimolar extinction coefficients of 13.8 and 14.6 l $mmol^{-1}$ cm^{-1} (Antonini and Brunori, 1971). A reference spectrum for metHb was obtained by adding 0.00014 g $K_3[Fe(CN)_6]$ to the oxyHb solution and mixing with a spatula. The reaction was completed within 30 min. DeoxyHb was obtained by adding a few crystals of sodium dithionite ($Na_2S_2O_4$) to an oxyHb solution and mixing with a spatula. Nitrosyl hemoglobin (HbNO) was obtained by adding 1.0 ml of pure NO gas to deoxyHb in a closed, gas tight 1 ml cuvette. The millimolar extinction coefficients for each Hb type at all the concerned wavelengths were computed from the reference spectra as $A_\lambda/(C\times l)$, where C is total [Hb] and l is 1 cm. The obtained reference spectra (ϵ *versus* λ) for the four zebrafish Hb derivatives are shown in Fig. 1A.

The concentrations of oxyHb, metHb, HbNO and deoxyHb in the hemolysates obtained from experimental zebrafish were determined by spectral deconvolution. Each individual zebrafish spectrum, consisting of A values from 480 nm to 700 nm in 0.2 nm steps (1101 data points) was fitted to Eqn 1, using nonlinear least squares curve fitting based on the Levenberg–Marquardt algorithm (Origin 7, OriginLab Corporation). In this procedure, A was assigned the dependent variable, and ϵ_{oxyHb}, ϵ_{metHb}, ϵ_{HbNO} and $\epsilon_{deoxyHb}$ the independent variables. The output of the fitting procedure was the values of C_{oxyHb}, C_{metHb}, C_{HbNO} and $C_{deoxyHb}$ that minimizes the sum of squares of differences between the observed spectrum and the calculated fit. The spectra were fitted with the constraints that the concentration of each Hb derivative should be larger or equal to zero. At first, all parameters to be fitted (i.e. C_{oxyHb}, C_{metHb}, C_{HbNO} and $C_{deoxyHb}$) were allowed to vary, but if one of the parameters converged towards zero after one to three iterations (which was often the case for $C_{deoxyHb}$, as expected from the high P_{O_2} in the hemolysate), then this variable was set to zero, and the iteration procedure was continued.

The determined values of C_{oxyHb}, C_{metHb}, C_{HbNO} and $C_{deoxyHb}$ were used to calculate total [Hb] in the original blood sample (the sum of the four Hb species, taking into account the dilution factor). The percentages of oxyHb, metHb, HbNO and deoxyHb in the sample were calculated from the fraction each Hb species constituted of the total Hb. The calculated values for metHb and HbNO reflected their original *in vivo* values in the blood, as these derivatives are stable within the time frame here considered (3 min). The sum of oxyHb and deoxyHb gave the functional (potential O_2 carrying) Hb of the sample.

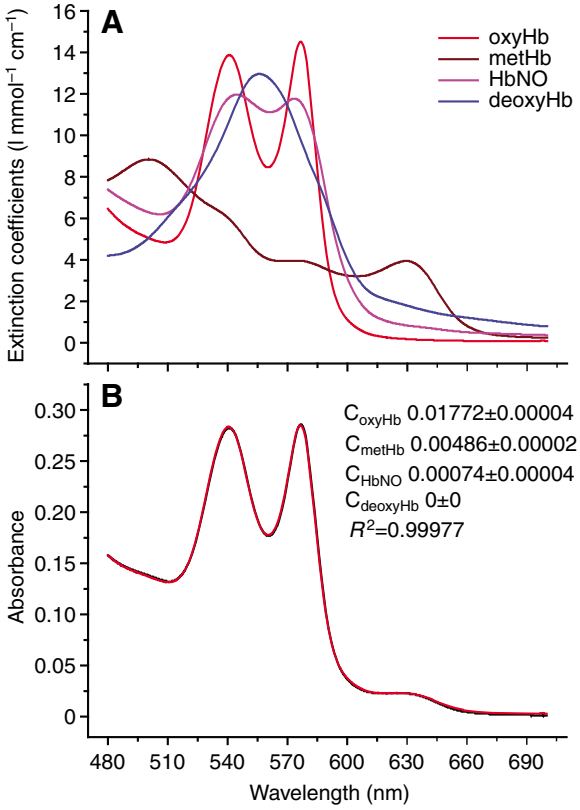

Fig. 1. (A) Reference spectra depicting the millimolar extinction coefficients of zebrafish oxygenated hemoglobin (oxyHb), methemoglobin (metHb), nitrosylhemoglobin (HbNO) and deoxygenated hemoglobin (deoxyHb) as function of wavelength at pH 7.3. (B) Representative result from the nonlinear least-squares curve-fitting procedure. The black curve shows the experimental spectrum of the diluted hemolysate from a nitrite-exposed zebrafish, and the red curve gives the fitted curve. The estimated millimolar concentrations of oxyHb, metHb, HbNO and deoxyHb in the cuvette, and the error on the parameter values, are shown in the insert.

Oxygen consumption

Fish were anaesthetized in MS-222, weighed and placed in closed chambers each holding three or four fish. The water volume of each chamber (around 260 ml) was determined by weighing the water required to fill the chamber while containing the three or four fish. The chambers were submerged in a 26°C thermostatted bath and covered to avoid visual disturbance but yet allowing light to enter during the light phase of the 24 h cycle. Water was pumped from an experimental aquarium through the chambers, using peristaltic pumps (type 110, Ole Dich Instrumentmakers, Denmark) with a flow rate of 25 ml min^{-1}. The ingoing water tube exited at the bottom of the chambers (to create mixing) and the outlet from the chamber was in the top. The fish were allowed to acclimate to chamber condition for at least 24 h before the start of measurements. Measurements of oxygen consumption were started 24 h before nitrite exposure (–24 h). A 1-ml water sample was drawn through a needle inserted into the outlet from each chamber and oxygen tension (P_{O_2}) was measured with Radiometer (Copenhagen, Denmark) E5046 electrodes in D616

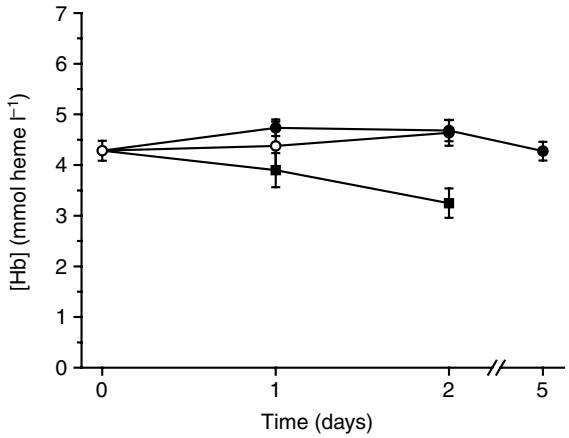

Fig. 2. Total hemoglobin concentration in blood from zebrafish exposed to control water (○), 0.6 mmol l^{-1} nitrite (●) and 2 mmol l^{-1} nitrite (■) for variable time periods. Values are means ± s.e.m. ($5 \leqslant N \leqslant 13$ at individual points).

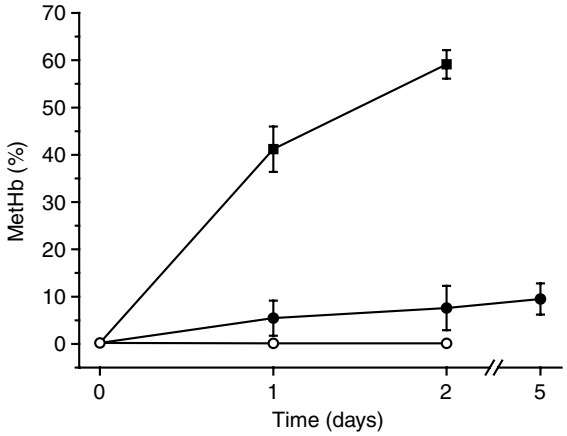

Fig. 3. Blood methemoglobin (metHb) content in zebrafish exposed to control water (○), 0.6 mmol l^{-1} nitrite (●) and 2 mmol l^{-1} nitrite (■) for variable time periods. Values are means ± s.e.m. ($5 \leqslant N \leqslant 13$ at individual points).

thermostatted (26°C) cells, with signals displayed on PHM 73 monitors and REC 80 recorders. The flow through the chambers was then stopped for exactly 30 min. 5 s following re-commencement of flow a new 1-ml sample was drawn for P_{O_2} measurement. Fish movements and the re-establishment of flow ensured that samples were of truly mixed chamber water. The rate of oxygen consumption ($\dot{M}_{O_2}$) was calculated as $(V_w \times \alpha o_2 \times \Delta P_{O_2})/(t \times M_b)$, where V_w is chamber water volume, αo_2 is O_2 solubility at 26°C [1.64 μmol l^{-1} $mmHg^{-1}$ (Boutilier et al., 1984)], t is elapsed time and M_b is fish body mass. Measurements of oxygen consumption were done at –24, –18 and –1 h in control water. Nitrite was then added to the experimental aquarium and further measurements were done at 3, 23 and 47 h of nitrite exposure. Two different series examined exposure to 0.6 mmol l^{-1} nitrite (N=6 chambers) and 2 mmol l^{-1} nitrite (N=4 chambers).

Statistics

Results are presented as means ± s.e.m. unless otherwise stated. Statistical analysis of blood and whole body ion variables in the three exposure groups at days 0, 1 and 2 was done with two-way analysis of variance (ANOVA) followed by a Bonferoni-adjusted Fisher's LSD test. Differences between means were considered significant at $P<0.05$. To include values measured at day 5 in the 0.6 mmol l^{-1} nitrite exposure group, an additional one-way ANOVA followed by a Bonferroni-adjusted Fishers LSD test was performed on this group. Values measured at day zero (i.e. fish in control water) did not differ between the three treatment groups and have been pooled to one overall mean in the figures for clarity. Oxygen consumption data were analyzed with two-way ANOVA. Statistical analyses were performed with SAS version 9.1.

Results

Spectral deconvolution

The employed nonlinear least-squares curve-fitting procedure resulted in very good fits to the spectral data, as reflected by high R^2 values of the fits and small errors on the fitted parameters (cf. Fig. 1B for an example). The average R^2 for the fits was 0.99958±0.00004 (mean ± s.e.m., N=66), which supports that parameters were estimated with high accuracy and that the analysis took into account all relevant Hb species. Thus, spectral deconvolution was a powerful and reliable method for estimating the concentration of the individual Hb derivates, as experienced in other studies of Hb mixtures of similar or alternative composition (Völkel and Berenbrink, 2000; Huang et al., 2005).

Blood parameters

Total [Hb] of whole blood was around 4.4 mmol heme l^{-1} (1.1 mmol tetramers l^{-1}) and did not change significantly in controls or zebrafish exposed to 0.6 mmol l^{-1} nitrite (Fig. 2). In zebrafish exposed to 2 mmol l^{-1} nitrite for 2 days, however, total [Hb] was reduced significantly below that in the other two experimental groups (Fig. 2).

Methemoglobin was low (0.2% of total Hb) and unchanged with time in control zebrafish (Fig. 3). Exposure to 0.6 mmol l^{-1} nitrite induced a small increase in the mean metHb level, but the variability was large, and the increase failed to be significant. In zebrafish exposed to 2 mmol l^{-1} nitrite the blood metHb level increased significantly with time and values at day 1 (41% of total Hb) and day 2 (59% of total Hb) were also significantly higher than in the other groups (Fig. 3).

The cause of the large variability in metHb values of nitrite-exposed fish was analyzed using a box chart of the pooled metHb data for each of the three groups. In zebrafish exposed to 0.6 mmol l^{-1} nitrite, the mean values at days 1, 2 and 5 were similar (Fig. 3), but it was clear from the box chart that the fish could be divided into responding and non-responding animals (Fig. 4). Thus, more than half the fish exposed to 0.6 mmol l^{-1} nitrite had metHb levels that did not differ from the low level observed in controls, whereas the remaining fish in this group had metHb levels elevated to a variable degree (Fig. 4). Thus, the data were not normal distributed and the median differed substantially from the mean (Fig. 4). In zebrafish exposed to 2 mmol l^{-1} nitrite all individuals showed elevated metHb levels, but to a variable degree (Fig. 4). This was partly due to the

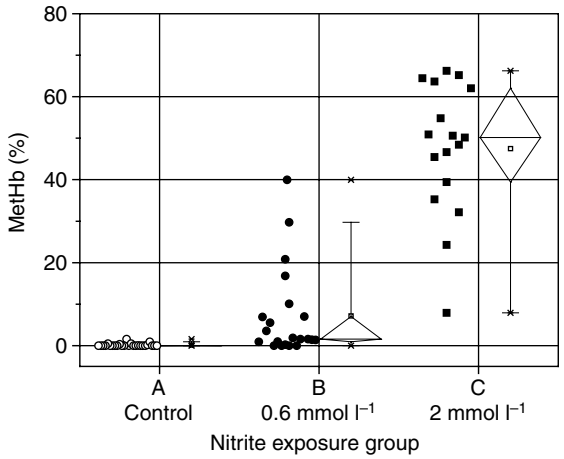

Fig. 4. Box chart of the pooled methemoglobin (metHb) values from (A) control zebrafish, (B) zebrafish exposed to 0.6 mmol l^{-1} nitrite for 1, 2 and 5 days, and (C) zebrafish exposed to 2 mmol l^{-1} nitrite for 1 and 2 days. The boxes are determined by the 25th and 75th percentiles, whiskers show the 5th and 95th percentiles, and crosses the 1st and 99th percentiles. The median is given by the horizontal line in the diamond boxes, and the mean for each pooled group is given by open squares. The filled symbols to the left of the boxes show the individual binned data points in each of the three groups.

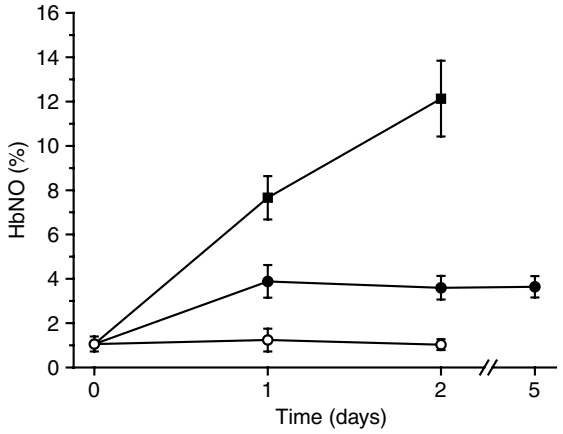

Fig. 5. Nitrosylhemoglobin (HbNO) content in blood from zebrafish exposed to control water (○), 0.6 mmol l^{-1} nitrite (●) and 2 mmol l^{-1} nitrite (■) for variable time periods. Values are means ± s.e.m. ($5 \leqslant N \leqslant 13$ at individual points).

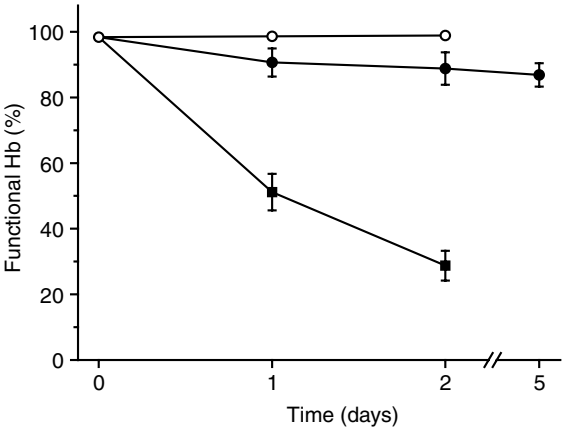

Fig. 6. Functional hemoglobin content in blood from zebrafish exposed to control water (○), 0.6 mmol l^{-1} nitrite (●) and 2 mmol l^{-1} nitrite (■) for variable time periods. Values are means ± s.e.m. ($5 \leqslant N \leqslant 13$ at individual points).

significant increase in metHb from day 1 to day 2, but individual variation in response magnitude was still present.

The pattern of changes in nitrosylhemoglobin showed some resemblance with that for metHb. Thus, HbNO was low and constant in controls, rose to a somewhat higher but constant level in fish exposed to 0.6 mmol l^{-1} nitrite, and increased to very high levels in fish exposed to 2 mmol l^{-1} nitrite (Fig. 5). This increase in HbNO was significant both with time and when compared to the other groups at each individual day. The HbNO level reached a surprising high value of 12% of the total Hb in zebrafish exposed to 2 mmol l^{-1} nitrite for 2 days (Fig. 5).

The significant increases in both metHb (Fig. 3) and HbNO (Fig. 5), which are non-functional Hb derivatives as regards O_2 transport, means that there was a major and significant decrease in functional Hb, particularly in the zebrafish exposed to the highest nitrite concentration. In this group functional Hb was lowered to 29% of total Hb at day 2 (Fig. 6).

Oxygen consumption and whole body ions

Routine oxygen consumption of zebrafish varied around 15 μmol h^{-1} g^{-1} both in control water and during nitrite exposure (Fig. 7). The $\dot{M}_{O_2}$ values showed no significant differences with either time or between fish exposed to 0.6 mmol l^{-1} and 2 mmol l^{-1} nitrite.

The whole body concentrations of potassium, sodium and chloride were relatively stable with $[K^+]>[Na^+]>[Cl^-]$ (Fig. 8). The concentration of the individual ions did not vary significantly with time or between the exposure groups.

Discussion

Nitric oxide formation during nitrite exposure

A major finding of the present study was the documentation of very high levels of HbNO in the blood of nitrite-exposed zebrafish (Fig. 5). This result confirms the primary hypothesis of the study and provides the first evidence for a massive production of NO from nitrite in nitrite-exposed fish.

In the blood, a main mechanism for HbNO formation will be the reaction of nitrite with deoxygenated ferrous heme groups to form NO and ferric heme (metHb), with NO subsequently being captured by unoxidized deoxygenated heme groups to form nitrosylhemoglobin (Cosby et al., 2003):

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

$$Hb(Fe^{2+}) + NO \rightarrow Hb(Fe^{2+})NO . \quad (3)$$

This mechanism does not require the Hb to be fully deoxygenated and will proceed at intermediate oxygenation degrees (as found in venous blood) with both mammalian (Grubina et al., 2007) and fish (F.B.J., unpublished data) Hbs. In addition to this mechanism, some of the HbNO detected in the blood may result from reduction of nitrite to NO *via* other heme proteins, xanthine oxidoreductase or non-enzymatic

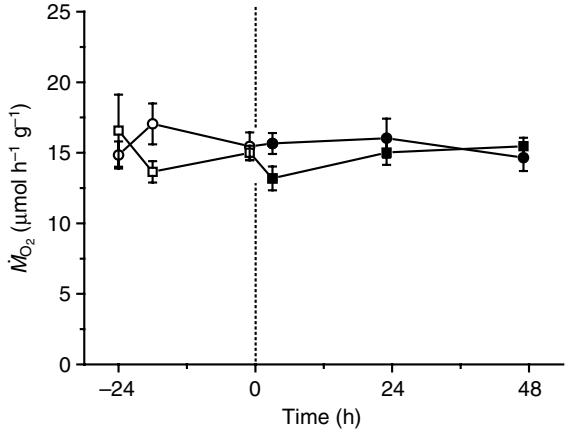

Fig. 7. Routine oxygen consumption in zebrafish before (open symbols) and after (filled symbols) exposure to nitrite at 0.6 mmol l^{-1} (circles) or 2 mmol l^{-1} nitrite (squares). The dotted line indicates the onset of nitrite exposure. See text for further details.

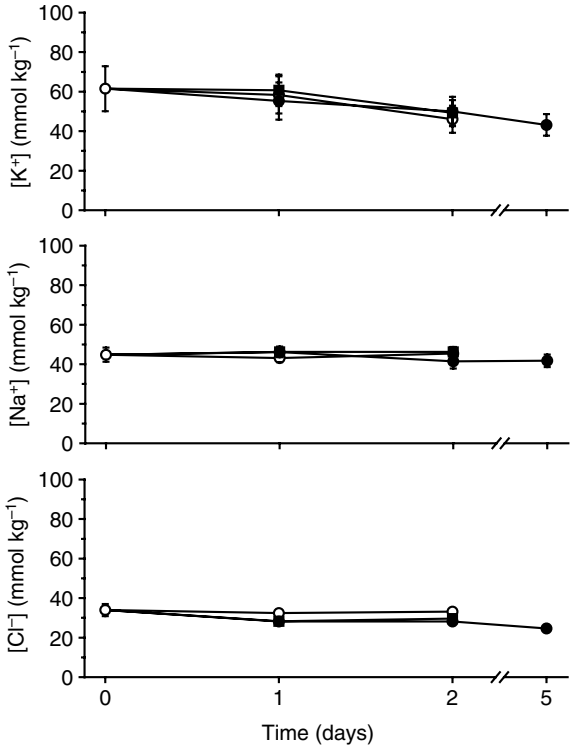

Fig. 8. Whole body concentrations (mmol kg^{-1} wet mass) of potassium, sodium and chloride in zebrafish exposed to control water (○), 0.6 mmol l^{-1} nitrite (●) and 2 mmol l^{-1} nitrite (■) for variable time periods.

acidic disproportionation (Zweier et al., 1999; Gladwin et al., 2005). The latter will, however, require rather acidic conditions (Modin et al., 2001), which are not developed in nitrite-exposed cyprinids (Jensen et al., 1987).

Whereas the reaction of nitrite with fully deoxygenated Hb leads to a 1:1 formation of HbNO and metHb (Eqn 2 and Eqn 3), the *in vivo* situation is more complex. The blood O_2 saturation cycles between practically full saturation in arterial blood and intermediate saturations in venous blood. At full O_2 saturation, nitrite reacts with oxyHb to form metHb and nitrate (Kosaka and Tyuma, 1987), whereas nitrite reacts with both oxyHb and deoxyHb at intermediate O_2 saturations (Grubina et al., 2007) (F.B.J., unpublished). The production of HbNO is therefore lower than that of metHb, even though HbNO formation stays significant even at relatively high O_2 saturations (F.B.J., unpublished). The oxidation of Hb is countered *in vivo* by the presence of metHb reductase systems inside the red blood cells, which can keep pace with the metHb formation when nitrite concentrations are low. During nitrite exposure in fish, however, the nitrite concentration continuously increases, and metHb levels are forced upwards, albeit a quasi balance exist between Hb oxidation and Hb reduction at each nitrite concentration (Jensen, 2003). On this background, the finding of higher metHb levels (Fig. 3) than HbNO levels (Fig. 5) in nitrite-exposed zebrafish is expected. The astonishing result is the very high HbNO levels that actually develop in the circulating blood (Fig. 5).

In mammals, the basal concentration of HbNO is in the sub-micromolar range but has been reported to increase to values ranging from 1 to 40 μmol l^{-1} after administration of small doses of nitrite *via* infusion, inhalation or food (Cosby et al., 2003; Hunter et al., 2004; Tsuchiya et al., 2005). The HbNO level of 12% reached in nitrite-exposed zebrafish (Fig. 5) corresponds to some 400 μmol l^{-1} at the prevailing total [Hb] (Fig. 2). This high level is the result of the timely extended exposure to nitrite at much higher concentrations than in mammals. Thus, whereas nitrite administration in mammals typically leads to a transient increase in plasma nitrite in the micromolar range, nitrite exposure in fish leads to a continuous increase in plasma nitrite to values that can reach several millimolar (Jensen, 2003).

The HbNO level in control zebrafish was about 1% of total Hb (Fig. 5). This may, however, not be the real basal level. Some of the control fish had HbNO levels indistinguishable from zero, whereas HbNO was detectable in others, producing the average of 1%. Control animals were kept in aquaria without biological filters, and the measured water nitrite concentration was 1–5 μmol l^{-1} in spite of the daily changes of water. Because zebrafish could be divided into responding and non-responding animals (cf. results and below), it seems plausible that responding fish took up some nitrite from the low concentration in the control water, which would slightly elevate internal nitrite and HbNO without affecting control metHb (due to red blood cell metHb reductase activity). That NO formation may precede significant elevations of metHb is supported by *in vivo* observations. In rainbow trout, heart rate increases early during nitrite exposure, before metHb elevation or other physiological disturbances are significant (Aggergaard and Jensen, 2001). It was suggested that the appearance of small amounts of nitrite in the blood causes NO formation that triggers vasodilation and a decrease in blood pressure, which quickly becomes countered by increased cardiac pumping to re-establish blood pressure (Aggergaard and Jensen, 2001).

A major fraction of the NO formed during nitrite exposure is either captured by ferrous deoxyHb, forming HbNO, or reacts with oxyHb to produce metHb and nitrate. These NO

scavenging reactions of Hb limit the amount of free NO (Kim-Shapiro et al., 2006). Furthermore, some NO reacts with thiol groups to form nitrosothiols (RSNO). NO can subsequently be released from HbNO and RSNO compounds to some extent (Grubina et al., 2007; Kim-Shapiro et al., 2006). Indeed, the deoxyHb-mediated formation of NO is believed to participate in local blood flow regulation through release of some of the NO from the red cells (Cosby et al., 2003). The very high HbNO level in nitrite-exposed zebrafish testifies to a substantial production of NO from nitrite, creating a NO load that probably disturbs normal NO homeostasis. Thus, the multitude of functions that are influenced by NO, ranging from blood flow regulation and hemostasis to neurotransmission, may all potentially be affected by excess NO production. At present, however, little is known about these consequences. The rapid rise in heart rate in rainbow trout lends some support to an interference of nitrite-derived NO with cardiovascular function (Aggergaard and Jensen, 2001). NO inhibits platelet aggregation in mammals, and with the similarities of hemostatic pathways and thrombocyte function in mammals and fish (Jagadeeswaran et al., 1999), one may hypothesize that excess NO formation during nitrite exposure could increase bleeding from damaged blood vessels. Blood [Hb] decreases during nitrite exposure (Fig. 2), which has been proposed to reflect an increased removal of damaged red blood cells from the circulation (Jensen, 2003). It may be speculated that internal bleeding could also contribute. Evaluation of this and other potential disturbances of NO homeostasis must, however, await further study.

Blood gas transport and oxygen consumption

The formation of high metHb levels (Fig. 3), with a consequent reduction in arterial oxygen content, are well-known effects in nitrite-exposed fish (cf. Jensen, 2003). A lowering of total Hb, as observed in zebrafish exposed to 2 mmol l^{-1} nitrite (Fig. 2), has also been reported in other fish, and will contribute to limitations in blood O_2 transport (Jensen, 2003). An important new realization from the present study is that nitrosylhemoglobin also contributes significantly to reduce blood O_2 capacitance. Owing to the tight binding and low rate of dissociation of NO from HbNO, this Hb derivative has a long half-life and does not participate in O_2 transport. The high levels of HbNO (Fig. 5) therefore add to the high metHb levels (Fig. 3) in lowering the amount of functional Hb (Fig. 6) in nitrite-exposed fish. Earlier studies have only considered metHb formation and have therefore underestimated the amount of non-functional Hb.

On basis of the declines in functional Hb and total Hb in nitrite-exposed zebrafish, one may envisage that whole animal O_2 consumption could be reduced. The routine oxygen uptake was, however, not influenced by nitrite exposure (Fig. 7). Absolute values varied around 15 μmol h^{-1} g^{-1}, which compares well with earlier reported $\dot{M}_{O_2}$ values in zebrafish (Lucas and Priede, 1992). Thus, it appears that neither the reduction of arterial O_2 content nor the possibility that the internal NO load could inhibit mitochondrial respiration (Crawford et al., 2006; Shiva et al., 2007) were of sufficient magnitudes to lower routine O_2 consumption. It is predictable, however, that exercise capability and hypoxia tolerance will be compromised by the lowered amount of functional Hb.

Nitrite tolerance in zebrafish

Zebrafish appeared relatively robust towards nitrite exposure. The mean metHb level did not exceed 10% of total Hb during exposure to 0.6 mmol l^{-1} nitrite (Fig. 3). Many other fish species would develop much higher metHb levels when exposed to 0.6 mmol l^{-1} nitrite in water of the present ionic composition. When ambient nitrite was elevated to 2 mmol l^{-1}, the metHb content in zebrafish blood rose to 59% in 2 days (Fig. 3). For comparison, metHb rose to 83% in carp after 2 days exposure to a lower nitrite concentration (1 mmol l^{-1}), even though water [Cl^-] was higher (which limits nitrite uptake) (Jensen et al., 1987), and in rainbow trout metHb also increased to higher values than in zebrafish at lower ambient nitrite and higher ambient [Cl^-] (Aggergaard and Jensen, 2001). Carp is normally considered rather resistant towards nitrite, whereas rainbow trout is fairly sensitive. The above comparison therefore reveals a comparatively large tolerance towards nitrite in zebrafish. This is supported by toxicological data that show high LC_{50} values in zebrafish (Voslárova et al., 2006). The underlying mechanism could be a relatively low Cl^- uptake rate across the gills in zebrafish, because it is known that the branchial Cl^- uptake rate is lower in resistant than in sensitive fish species (Williams and Eddy, 1986; Tomasso and Grosell, 2005). Alternatively, the affinity of nitrite for the active branchial Cl^- uptake mechanism could be reduced in zebrafish.

Interestingly, zebrafish could be divided into responding and non-responding individuals when exposed to 0.6 mmol l^{-1} nitrite (Fig. 4). In rainbow trout, individuals can similarly be divided into two groups, with some individuals showing faster nitrite accumulation and more pronounced physiological disturbances than others (Stormer et al., 1996; Aggergaard and Jensen, 2001). The intraspecific difference in trout correlated with higher branchial nitrite uptake rates in the more sensitive individuals (Jensen, 2003). A similar explanation may be applicable to the individual difference observed in zebrafish.

Nitrite exposure reduces the K^+ content of skeletal muscle in rainbow trout and carp (Stormer et al., 1996; Knudsen and Jensen, 1997). The release of K^+ to the extracellular space elevates extracellular [K^+] (Jensen et al., 1987; Stormer et al., 1996; Knudsen and Jensen, 1997) but less than expected from the amount of K^+ released from skeletal muscles, which suggests a further transport of K^+ to the environment and the development of a potassium deficit (Knudsen and Jensen, 1997). The whole body ion content was analyzed in zebrafish to test this possibility, but a significant reduction in K^+ content was not detected (Fig. 8). It may be that the high tolerance of zebrafish also correlates with a reduced K^+ release compared to rainbow trout and carp.

Concluding remarks

By using the blood level of HbNO as a biomarker of NO production, the present study documents an extensive *in vivo* formation of NO in nitrite-exposed zebrafish. The main mechanism is the reduction of nitrite to NO by deoxygenated heme groups inside red blood cells, with the subsequent binding of NO to adjacent deoxygenated ferrous heme groups, forming HbNO. This suggests that deoxyHb-mediated nitrite reduction is significant in the *in vivo* arterial–venous circulation, where

Hb cycles between full and intermediate O_2 saturations. This mechanism has been proposed to be involved in blood flow regulation at natural low nitrite concentrations (Cosby et al., 2003), but the excess NO formation in nitrite-exposed zebrafish predicts that disturbance of NO homeostasis is part of the toxic action of nitrite at high concentrations.

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