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RESEARCH ARTICLE

Circulating nitric oxide metabolites and cardiovascular changes in the turtle *Trachemys scripta* during normoxia, anoxia and reoxygenation

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SUMMARY

Turtles of the genus *Trachemys* show a remarkable ability to survive prolonged anoxia. This is achieved by a strong metabolic depression, redistribution of blood flow and high levels of antioxidant defence. To understand whether nitric oxide (NO), a major regulator of vasodilatation and oxygen consumption, may be involved in the adaptive response of *Trachemys* to anoxia, we measured NO metabolites (nitrite, *S*-nitroso, Fe-nitrosyl and *N*-nitroso compounds) in the plasma and red blood cells of venous and arterial blood of *Trachemys scripta* turtles during normoxia and after anoxia (3 h) and reoxygenation (30 min) at 21°C, while monitoring blood oxygen content and circulatory parameters. Anoxia caused complete blood oxygen depletion, decrease in heart rate and arterial pressure, and increase in venous pressure, which may enhance heart filling and improve cardiac contractility. Nitrite was present at high, micromolar levels in normoxic blood, as in some other anoxia-tolerant species, without significant arterial–venous differences. Normoxic levels of erythrocyte *S*-nitroso compounds were within the range found for other vertebrates, despite very high measured thiol content. Fe-nitrosyl and *N*-nitroso compounds were present at high micromolar levels under normoxia and increased further after anoxia and reoxygenation, suggesting NO generation from nitrite catalysed by deoxygenated haemoglobin, which in turtle had a higher nitrite reductase activity than in hypoxia-intolerant species. Taken together, these data indicate constitutively high circulating levels of NO metabolites and significant increases in blood NO after anoxia and reoxygenation that may contribute to the complex physiological response in the extreme anoxia tolerance of *Trachemys* turtles.

Key words: nitrite, *S*-nitrosothiol, iron-nitrosyl, haemoglobin, nitrite reductase, thiol, adaptation, oxygen.

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INTRODUCTION

The free radical nitric oxide (NO) is a universal signalling molecule in living organisms (Schmidt and Walter, 1994; Hill et al., 2010). In vertebrates, NO controls a vast array of metabolic and systemic responses, including vasodilatation and cellular metabolic activities (Moncada et al., 1991), particularly within the heart (Tota et al., 2005; Gödecke, 2006; Flögel et al., 2010). These responses involve two major specific targets of NO, namely soluble guanylate cyclase in the vasculature, and cytochrome *c* oxidase (complex IV) in the mitochondria (Hill et al., 2010). During normal tissue oxygenation, NO is synthesized from L-arginine and molecular O_2 by a family of enzymes, the nitric oxide synthases (NOSs) (Alderton et al., 2001). However, when O_2 (a substrate for NOS) becomes limiting, nitrite and *S*-nitrosothiols (RSNO), which are relatively stable *in vivo* products of NO, function as recyclable sources of NO (Bryan et al., 2005; Hess et al., 2005) and contribute to hypoxic vasodilatation (Crawford et al., 2006; Dalsgaard et al., 2007). The conversion of nitrite to NO may occur non-enzymatically (Zweier et al., 1995) or may be catalysed by a number of enzymes (reviewed by Lundberg et al., 2008; van Faassen et al., 2009), including deoxygenated haemoglobin (Hb) (Cosby et al., 2003). The NO generated from nitrite under hypoxia has the dual effects of causing local vasodilatation to rescue O_2 supply and of reducing mitochondrial O_2 consumption by temporary inhibition of cytochrome *c* oxidase (Moncada and Erusalimsky, 2002).

We speculate that NO and its products may be of particular importance in animals that have evolved to survive prolonged lack of O_2 and subsequent reoxygenation. In contrast to mammals, where particularly the heart and brain are extremely sensitive to even brief periods of anoxia, freshwater turtles, including *Trachemys scripta*, survive weeks of anoxia during winter when submerged under ice-covered ponds or several hours at room temperature (Ultsch and Jackson, 1982; Herbert and Jackson, 1985). This is achieved by a core of adaptations: (1) strong metabolic depression (whereby a reduced anaerobic energy supply is at balance with a reduced energy demand), (2) reorganization of blood flow to the most vital organs, (3) utilization of the shell calcium carbonate to buffer metabolic acidosis and high lactate levels, and (4) constitutively high levels of antioxidants, including thiols, that act as a redox buffer against reactive oxygen species generated at reoxygenation (Jackson, 2000; Hermes-Lima and Zenteno-Savín, 2002; Nilsson and Lutz, 2004; Bickler and Buck, 2007; Overgaard et al., 2007; Jackson and Ultsch, 2010). However, the molecular mechanisms underlying these extreme adaptive responses to low O_2 remain to be fully understood.

To better understand whether NO-related metabolites may contribute to the extreme anoxia-tolerance of *Trachemys scripta*, we measured the concentration of the major NO metabolites in the

venous and arterial blood of turtles during normoxia, anoxia and reoxygenation, while monitoring O_2 content, pH, heart rate and arterial and venous blood pressures. We also determined the content of red blood cell thiols and the enzymatic activity of deoxy-Hb as a nitrite reductase. In analogy with seminal studies showing the contribution of blood *S*-nitrosothiols (Stamler et al., 1997) and nitrite (Cosby et al., 2003) in the oxygenation-linked regulation of blood flow, we focused on blood as a source of NO bioactivity during the extreme O_2 lack experienced by anoxic turtles.

MATERIALS AND METHODS

Red-eared sliders *Trachemys scripta* (Gray) with a body mass of 351±32 g (mean ± s.d.) were obtained from Lemberger (Oshkosh, WI, USA) and sent by air-freight to Aarhus University (Denmark), where they were held for several months before experimentation. Animals were kept at 21°C in large aquaria with free access to dry platforms under infrared lamps for thermoregulation. Turtles were fed fish and mussels. Food was withheld for at least three days before experimentation. Procedures were in accordance with the laws of animal care and experimentation in Denmark and were approved by the Animal Experimentation Board, Danish Ministry of Justice.

Surgical procedures

Animals were anaesthetized by an injection of 0.15 mg kg^{-1} propofol injected into the supravertebral sinus. If the surgery lasted more than 40 min, the turtles were intubated with soft rubber tubing for artificial ventilation with 1–4% isoflurane in air using an HI 665 Harvard Apparatus Respirator (Cambridge, MA, USA). An approximately 4×4 cm portion of the plastron was excised using a bone saw, allowing for occlusive cannulation (PE-50 containing saline, 100 i.u. ml^{-1} heparin) of the jugular vein and the carotid artery. The venous catheter was guided into the sinus venous of the heart allowing for sampling of mixed venous blood and measurements of central venous pressure. The excised plastron was placed in its original position using surgical tape and fast-drying epoxy resin.

After surgery, turtles were ventilated with room air until they resumed spontaneous ventilation and were allowed to recover at 21°C with free access to air for at least 24 h in individual boxes (40×30×30 cm), kept in climate chambers to minimize disturbance. The turtles were kept in the same boxes during and following experimentation.

Experimental protocol

Catheters were guided out of the boxes and climate chambers and connected to pressure transducers (Baxter Edward, model PX600, Irvine, CA, USA) to monitor arterial and central venous pressures, as well as heart rate. Pressure transducers were calibrated daily against a static water column. The signals were amplified using an in-house-built pre-amplifier and recorded with a Biopac MP100 data acquisition system (Biopac Systems, Goleta, CA, USA) at 100 Hz. Turtles were then exposed to normoxia (1 h), anoxia (3 h) and reoxygenation (30 min) at 21°C. Turtles were made anoxic by bubbling the water with N_2, while pulmonary ventilation was prevented by a metal grid 2–3 cm below the water surface. At the end of the anoxic period, water was removed and the animals regained access to air (reoxygenation).

Processing of blood samples

At the end of each period, a blood sample (~0.5–0.8 ml) was withdrawn using EDTA-containing 1-ml syringes from arterial and venous catheters to measure O_2 content, pH and NO metabolites. pH was measured with a Radiometer (Copenhagen, Denmark) BMS Mk2 capillary pH electrode connected to a Radiometer PHM 73 meter, while the O_2 content of blood samples was measured by the Tucker method (Tucker, 1967). Separate normoxic samples were used to measure haematocrit and concentration of thiol groups in red blood cells.

Sulfhydryl measurements

Total thiol concentration in red blood cells was obtained by the stoichiometric reaction of 4,4′-dithiodipyride (4-PDS) with thiols, which generates 4-thipyridone (4-TP) (Grassetti and Murray, 1967; Bonaventura et al., 1998). Washed red blood cells were lysed on ice for 30 min with a 10-fold excess volume of 10 mmol l^{-1} phosphate buffer, pH 7.6, and centrifuged at high speed to remove membrane debris prior to 4-PDS addition. A 4-molar excess of 4-PDS over haem was used and the reaction was allowed to proceed for 1 h at room temperature. The reaction product 4-TP was quantified spectrophotometrically at 324 nm using the extinction coefficient 19.8 mmol l^{-1} cm^{-1} (Grassetti and Murray, 1967).

NO metabolite measurements

Arterial and venous aliquots of blood samples for determination of NO metabolites were immediately centrifuged at 2000 ***g*** for 15 min at 5°C to separate plasma and red blood cells. Plasma was transferred into a separate tube and frozen in liquid N_2 before measurements of NO metabolites. Red blood cells (200 µl) were incubated with 1 ml of SNO-stabilizing solution (Gladwin et al., 2002): 10 mmol l^{-1} KCN, 10 mmol l^{-1} $K_3[Fe(CN)_6]$, 20 mmol l^{-1} *N*-ethylmaleimide (NEM), 1 mmol l^{-1} EDTA and 0.05% Triton X-100. Fresh solutions were made every day and nitrite contamination was checked spectrophotometrically with the Griess reagent (Alexis Biochemicals, Enzo Life Sciences, AH Diagnostics, Aarhus, Denmark) (Bryan and Grisham, 2007) or by chemiluminescence, as described below. Immediately after mixing with the stabilizing solution, the samples were flash-frozen in liquid N_2 and thawed to favour haemolysis, and then incubated for 1 h in the dark at room temperature. Samples were then centrifuged at high speed for 10 min to remove membrane debris, and the supernatant stored in liquid N_2 for later analysis of NO metabolites by chemiluminescence.

Nitrite concentration in plasma was measured spectrophotometrically at 540 nm (extinction coefficient 50 mmol l^{-1} cm^{-1}) using the Griess reagent. To eliminate background and improve signal-to-noise ratio, plasma (240 µl) was previously deproteinized by adding 12 µl of $ZnSO_4$ (1.86 µmol l^{-1}), and centrifuged at high speed after 5 min of incubation at room temperature. Concentrations of nitrite, *S*-nitrosothiols (RSNO), FeNO (haem-bound NO or iron-nitrosyl) and RNNO (*N*-nitrosamines) in red blood cells were measured by reductive chemiluminescence using a Nitric Oxide Analyzer (NOA) (Sievers, Model 280, Boulder, CO, USA) and the tri-iodide method, following previously described protocols (Yang et al., 2003; Hansen and Jensen, 2010). In brief, each sample was divided into three aliquots: the first was injected directly in the NOA to obtain [nitrite+RSNO+FeNO+RNNO]. The second aliquot was first incubated with acidified sulfanilamide (3 min) to eliminate nitrite, in order to obtain [RSNO+FeNO+RNNO]. The third was first incubated with $HgCl_2$ (2 min) and acidified sulfanilamide (3 min) to eliminate RSNO and nitrite, in order to obtain [FeNO+RNNO]. To improve accuracy, peaks were integrated using the software program Origin 7.0 (OriginLab Corporation, Northampton, MA, USA) (MacArthur et al., 2007). Differences between the values obtained in the three aliquots correspond to the individual levels of NO metabolites.

Calibrations were done daily using freshly made standard solutions of nitrite.

To assess the stability of haem-bound NO (FeNO) in Hb, turtle haemolysate was prepared from frozen washed red blood cells by hypotonic lysis and centrifugation. Addition of purified NO gas to prepare FeNO (nitrosyl) Hb and quantification by spectral deconvolution using reference spectra for pure oxy-, nitrosyl- and met (ferric)-Hb were as previously described (Fago et al., 2003).

Nitrite reductase activity of haemoglobin

Purified Hb solutions (in 0.05 mol l^{-1} Tris buffer, pH 7.3 and 0.1 mol l^{-1} KCl) were prepared as previously described (Jensen, 2008). The nitrite reductase activity of the fully deoxygenated Hb (at 150 μmol l^{-1} haem concentration) was recorded spectrophotometrically (in duplicate) at 25°C by adding nitrite to a 2.7-fold molar excess over haem and assessing the concentrations of deoxy-, oxy-, nitrosyl- and met-Hb in the course of the reaction, using spectral deconvolution (Jensen, 2008). The experimental conditions matched those used previously on carp, rabbit and rainbow trout haemoglobins (Jensen, 2008; Jensen, 2009), allowing a direct comparison of the nitrite reductase activity of turtle Hb with these species.

Statistics

All data are presented as means ± s.e.m. Statistical differences between mean values of NO metabolites were determined by one-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test. Statistical differences in heart rate and blood pressure were determined by the *t*-test. The level of significance was $P<0.05$.

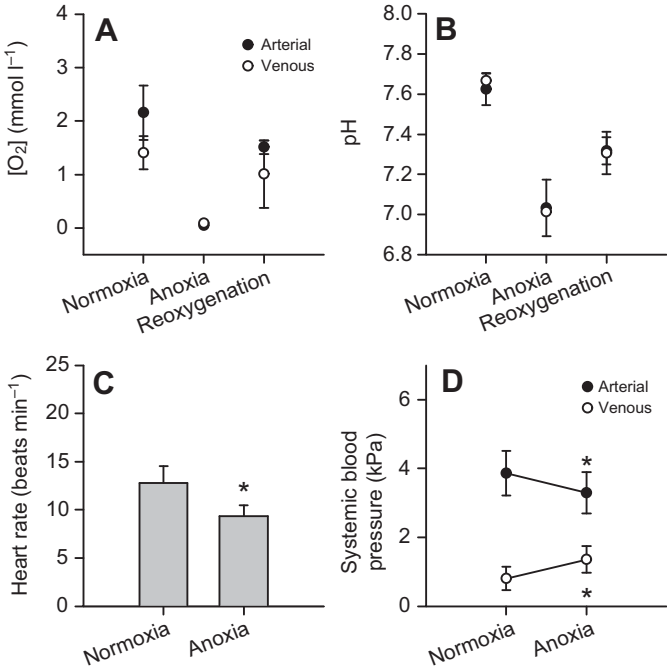

Fig. 1. Arterial and venous blood O_2 content (A), pH (B) during normoxia and after anoxia and reoxygenation, heart rate (C) and arterial and venous systemic blood pressure (D) during normoxia and anoxia in *Trachemys scripta*. Data are means ± s.e.m. with N=2–4 (A,B) and N=6–8 (C,D). Statistically significant differences (*t*-test) are indicated (*$P<0.05$).

RESULTS

In normoxic turtles, haematocrit was 18.5±1.9% and total red blood cell thiols (including those present on Hb and glutathione) measured by reaction with 4-PDS were 24±1 mmol l^{-1}. Changes in blood O_2 content, pH, heart rate and systemic blood pressure (arterial and venous) for turtles before and after anoxia are reported in Fig. 1. The marked reduction of arterial and venous blood O_2 contents towards zero (<0.08 mmol l^{-1}) at the end of the 3 h anoxic period indicates that the animals had become truly anoxic (Fig. 1A), in agreement with a previous study using the same experimental protocol and temperature (21°C) (Stecyk et al., 2004). Furthermore, blood pH decreased from 7.67±0.03 under normoxia to 7.01±0.08 after 3 h of anoxia (Fig. 1B), a value that is somewhat lower than found in previous studies (Herbert and Jackson, 1985) and indicating the onset of anaerobic metabolism and accumulation of lactic acid. A partial recovery of blood O_2 content and pH was observed after the 30 min reoxygenation period (Fig. 1A,B). Anoxia also caused a reduction in heart rate (by 27%; Fig. 1C) and in systemic arterial blood pressure (by 16%), whereas venous blood pressure increased considerably (by 76%, from 0.80 to 1.36 kPa; Fig. 1D). The reported normoxic heart rate values corresponded to periods with the lowest and most stable heart rates, presumably associated with spontaneous breath-holding episodes.

Fig. 2 shows individual and mean arterial–venous concentrations of plasma nitrite and of red blood cell nitrite, RSNO and FeNO (including RNNO compounds) under normoxia, anoxia and subsequent reoxygenation measured by reductive chemiluminescence and the Griess reaction (plasma nitrite). Plasma RSNO and FeNO+RNNO compounds were below detection levels (approximately 0.001 μmol l^{-1} with the used sample volume). Plasma nitrite measured by chemiluminescence and the Griess method gave similar results. As shown in Fig. 2, there was no significant arterial–venous difference for any of the measured NO metabolites, regardless of the oxygenation condition. Overall, NO metabolites were present at high concentrations (micromolar range) in turtle blood (Figs 2,3). In normoxic arterial blood, plasma nitrite concentration was 2.91±1.02 μmol l^{-1} and red blood cell nitrite, RSNO and FeNO+RNNO were 1.12±0.32, 0.22±0.08 and 1.96±0.70 μmol l^{-1}, respectively (Fig. 3). In arterial blood, exposure to anoxia and subsequent reoxygenation had no significant effect on nitrite and RSNO (Fig. 3A–C), but caused FeNO+RNNO levels to increase significantly to ~21 μmol l^{-1} in anoxia and ~30 μmol l^{-1} after reoxygenation (Fig. 3D). The chemiluminescence measurements of red blood cell NO metabolites involved treatment with a SNO-stabilizing solution that preserves labile SNO groups but tends to oxidize the haem with consequent decrease of FeNO (Yang et al., 2003). To test the resistance of the FeNO derivative of turtle Hb to the SNO-stabilizing solution, we incubated a FeNO sample of turtle Hb (84% FeNO) with the stabilizing solution under identical conditions (1 h at room temperature in the dark). After the incubation, the FeNO saturation of the Hb was 65%, indicating a loss of only ~20% of the iron-bound NO (data not shown). Consequently, the red blood cell FeNO+RNNO fraction measured by chemiluminescence contained appreciable levels of FeNO. In venous blood, NO metabolites did not change significantly after anoxia and reoxygenation (not shown).

After 3 h of anoxia, the circulating Hb was fully deoxygenated (Fig. 1A) and contained higher levels of FeNO (Fig. 3D), indicating that NO was generated in the anoxic blood. We therefore measured the nitrite reductase activity of deoxygenated turtle Hb. Addition of nitrite to deoxy-Hb in solution resulted in a progressive decrease in the concentration of deoxy-Hb to zero and a concomitant 1:1 increase in the concentrations of met-Hb and nitrosyl-Hb to half the initial concentration of deoxy-Hb (Fig. 4). This is exactly the

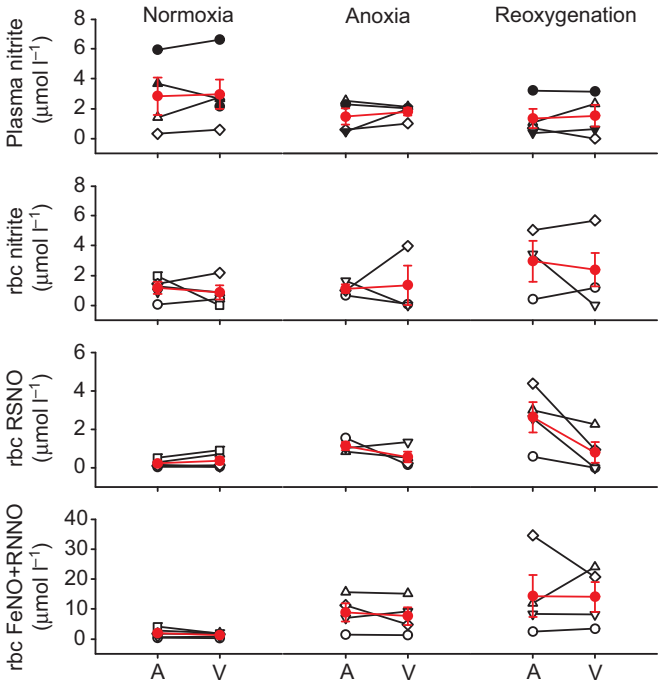

Fig. 2. Individual arterial–venous (A–V) differences in plasma nitrite and red blood cell (rbc) nitrite, RSNO and FeNO+RNNO concentrations during normoxia and after anoxia and reoxygenation. Identical symbols refer to the same animal. Mean (±s.e.m.) values are indicated in red.

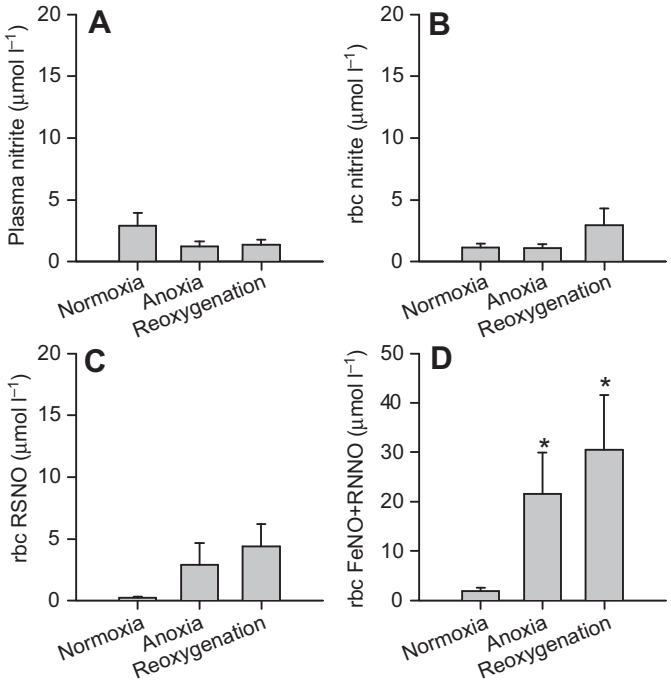

Fig. 3. Plasma (A) and red blood cell nitrite (B), RSNO (C) and FeNO+RNNO (D) concentrations in arterial blood during normoxia and after anoxia and reoxygenation. Data are means ± s.e.m. with *N*=6–8 (A), *N*=3–5 (B), *N*=4–5 (C) and *N*=5–6 (D). It was not always possible to obtain sufficient arterial (and venous) samples from the same animal under all conditions examined and therefore the number of individual measurements varies. Statistically significant differences from normoxia are indicated (**P*<0.05). Other paired comparisons were not statistically different (one-way ANOVA).

expected outcome when nitrite reacts with a vacant ferrous (Fe^{2+}) haem group in deoxy-Hb to form met (Fe^{3+})-Hb and NO, with subsequent rapid binding of the formed NO to deoxy-ferrous haem present:

$$Hb(Fe^{2+}) + NO_2^- + H^+ \rightarrow Hb(Fe^{3+}) + NO + OH^- , \quad (1)$$

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

The initial second-order rate constant was calculated as $0.9\,l\,mol^{-1}\,s^{-1}$.

DISCUSSION

The extreme anoxia tolerance of turtles from the genus *Trachemys* is remarkable among air-breathing animals. The results of this study show that prolonged (3 h) anoxia and reoxygenation in turtles at room temperature is linked to large changes in arterial blood NO that may be due to deoxy-Hb functioning as a nitrite reductase and source of NO during anoxia. We propose that this circulating NO may play a role in the extreme anoxia tolerance of turtles, including the redistribution of blood flow and tissue metabolic depression.

Basal NO metabolites and thiols in the blood

Under normoxic conditions, basal levels of plasma nitrite are a good indicator of the constitutive endothelial NOS activity (Kleinbongard et al., 2003). Plasma nitrite is in the range 0.1–0.2 μmol l^{-1} in humans (MacArthur et al., 2007) and 0.2–0.3 μmol l^{-1} in the marine teleosts European flounder (*Platichthys flesus*) and eelpout (*Zoarces viviparus*) (Jensen, 2009), while goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*), which are anoxia tolerant, have higher plasma nitrite levels (0.75 and 1.75 μmol l^{-1}, respectively) (Hansen and Jensen, 2010; Sandvik et al., 2012). As summarized in Table 1, in the normoxic turtle we found higher levels of plasma nitrite (2.9 μmol l^{-1}), which suggest a high constitutive NOS activity. Even higher values of plasma nitrite have been found in the spiny dogfish (*Squalus acanthias*) (4.5 μmol l^{-1}) (Swenson et al., 2005) and in Tibetan human natives living at high altitudes (4.8-11 μmol l^{-1}) (Erzurum et al., 2007). In turtle, normoxic red blood cells contained high levels of nitrite (1.1 μmol l^{-1}) as in crucian carp (1.4 μmol l^{-1}) (Sandvik et al., 2012) (Table 1). Normoxic red blood cell values of RSNO (0.2 μmol l^{-1}) were in between those of humans and goldfish (~0.05 μmol l^{-1}) (Hansen and Jensen, 2010; MacArthur et al., 2007) and those of crucian carp (~1 μmol l^{-1}) (Sandvik et al., 2012) and Tibetan human populations (2.4 μmol l^{-1}) (Erzurum et al., 2007). Notably, while being present at very low (often undetectable) concentrations in other species, FeNO+RNNO complexes in red blood cells were present in appreciable amounts (1.9 μmol l^{-1}) in *Trachemys* turtles under normoxic conditions (Table 1), indicating that the fate of NO is different in different species. From these comparisons, a trend emerges where anoxia- or hypoxia-tolerant species appear to have constitutively high levels of most, if not all, circulating NO metabolites, from which NO can be regenerated under hypoxia and anoxia. As previously suggested (Hermes-Lima and Zenteno-Savín, 2002; Bickler and Buck, 2007), anoxia tolerance in turtles also involves very high levels of antioxidants, including thiols that are involved in the protection against oxidative stress at reoxygenation. Consistently, we found much higher levels of red blood cell thiols (24 mmol l^{-1}) than in other vertebrate species (1–3 mmol l^{-1}) (Nikinmaa, 1990). Interestingly, the high content of thiol groups in *Trachemys* red blood cells is equivalent to that (26 mmol l^{-1}) reported for another hypoxia-

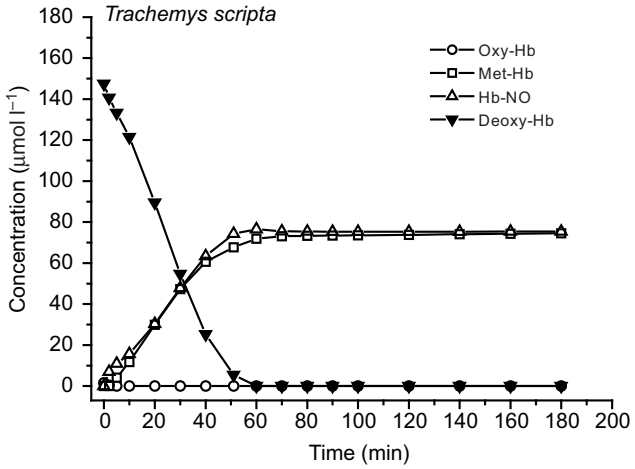

Fig. 4. Time-dependent changes in the concentrations of oxy-Hb, met-Hb, Hb-NO and deoxy-Hb during the reaction of fully deoxygenated turtle Hb with nitrite. The haem concentration was 150 μmol l^{-1} and the nitrite/haem concentration ratio was 2.7. Measurements were made in 0.05 mol l^{-1} Tris buffer with 0.1 mol l^{-1} KCl at pH 7.3 and 25°C.

tolerant turtle species (Reischl, 1986). Although the adaptive role of such high content of thiols in turtles remains to be investigated in detail, the present data show that RSNO products are not exceedingly high, suggesting a tight control mechanism for their formation (Foster et al., 2009).

The absence of any arterial–venous difference of NO metabolites in the turtle (Fig. 2) differs from humans and rats, where nitrite and RSNO are typically higher on the arterial side of the circulation and reflect the differences in the O_2-linked generation of products of NO metabolism in the arterial circulation and consumption in the venous circulation (Stamler et al., 1997; Gladwin et al., 2000; McMahon et al., 2002; Cosby et al., 2003). The lower metabolic rate of turtles compared with mammals, reflected by the small arterial–venous gradient in the O_2 content of turtles even in normoxic animals (Fig. 1A), may explain the absence of significant arterial–venous gradients in NO metabolites.

O_2-linked changes in NO metabolites: haemoglobin as a nitrite reductase

When O_2 availability decreases, the activity of endothelial NOS decreases and nitrite is progressively converted to NO by several enzymes, including deoxy-Hb, generating FeNO as a side product. Notably, in the absence of oxygen in the blood, as during anoxia, the fate of any produced NO is not nitrite (a major oxidative end-product of NO during normoxia), but to bind to the vacant haem sites of circulating Hb to generate FeNO. Accordingly, after anoxia and reoxygenation, the concentration of FeNO+RNNO in the turtle blood increases significantly, indicating that NO is generated and that deoxygenated Hb, which is by far the most abundant protein present (millimolar levels) in the O_2-deprived blood of anoxic turtles (Fig. 1), might be responsible for the reduction of nitrite to NO. Previous studies on humans and rats have also ascribed the formation of FeNO in red blood cells to the nitrite reductase activity of deoxy-Hb during acute hypoxia (Cosby et al., 2003; Feelisch et al., 2008). In other studies on goldfish and crucian carp, plasma nitrite decreases while erythrocyte nitrite is unchanged or increased during days of exposure to hypoxia or anoxia (Hansen and Jensen, 2010; Sandvik et al., 2012). In contrast to these studies, however, nitrite did not change significantly in plasma or red blood cells after the turtles had been exposed to 3 h of anoxia (Fig. 3). In the turtle, it is possible that nitrite for erythrocyte NO generation might have originated from some of the tissues that were perfused by blood during anoxia. It is known that nitrite permeates cellular membranes and is transported into red cells and tissues upon an elevation of extracellular levels (Bryan et al., 2005; Feelisch et al., 2008; Jensen and Rohde, 2010) or exposure to reduced O_2 levels (Hansen and Jensen, 2010; Sandvik et al., 2012). Measuring nitrite in tissues other than blood in future studies will help to establish the role of nitrite as a source of NO during anoxia in turtles.

When nitrite reacted with turtle deoxy-Hb (Fig. 4), the decline in deoxy-Hb concentration with time was slower than in carp Hb but faster than in rainbow trout Hb examined under the same conditions (Jensen, 2009). The calculated initial second-order rate constant was 0.91 mol^{-1} s^{-1} for turtle Hb, which is in between the values of 2.51 mol^{-1} s^{-1} for carp Hb and 0.151 mol^{-1} s^{-1} for rainbow trout Hb (Jensen, 2009). For comparison, human deoxy-Hb has an initial second-order rate constant of 0.23–0.41 mol^{-1} s^{-1} for the reaction with nitrite (Huang et al., 2005). Taken together, these results indicate higher rate constants (and thus higher nitrite reductase capabilities) in haemoglobins from species that are tolerant to severe hypoxia, such as the carp and the turtle.

Interestingly, the levels of FeNO+RNNO metabolites measured after 30 min of reoxygenation increased further compared with normoxic values (Fig. 3D). This increase may be caused by a reactivation of NOS enzymes, providing a new burst of NO, and by the fact that the FeNO derivative of the Hb is highly stable due to the low NO dissociation rate (Sharma and Ranney, 1978), whereby FeNO disappears slowly from the blood. Possibly, the high NO tone at reoxygenation would help to maintain the mitochondria in a partially inhibited state and prevent formation of excess reactive oxygen species (Moncada and Erusalimsky, 2002) when the animals are allowed to breathe again after the anoxic period.

Cardiovascular changes and role of circulating NO metabolites in anoxia tolerance

The heart rate of the turtles in the present study, both during normoxia and anoxia, was lower than previous studies on the same species of turtles at similar temperatures (e.g. Hicks and Wang, 1998;

Table 1. Mean concentrations (μmol l^{-1}) under normoxia of plasma and red blood cell (rbc) NO metabolites in selected species measured by reductive chemiluminescence

Species	Plasma nitrite	rbc nitrite	rbc RSNO	rbc FeNo+NNO	Reference
Turtle	2.9	1.1	0.2	1.9	Present study (Fig. 3)
Crucian carp	1.75	1.4	~1	n.d.	Sandvik et al., 2012
Goldfish	0.75	0.3	0.055	n.d.	Hansen and Jensen, 2010
Humans	0.1–0.2[a]	0.3[a]	0.05[a]	<0.05[b]	[a]MacArthur et al., 2007; [b]Cosby et al., 2003
Humans (Tibetans)	4.8–11.0	0.7	2.4	n.d.	Erzurum et al., 2007

n.d., not determined/not detected.

Stecyk et al., 2004; Overgaard et al., 2007). Large variation in heart rates amongst studies can be expected due to the pronounced tachycardia during pulmonary ventilation and the bradycardia associated with apnoea (e.g. Wang and Hicks, 1996), and all turtles in the present study exhibited pronounced changes in heart rate, although ventilation was not measured directly. To better compare with anoxia, where the turtles did not ventilate their lungs, we report the low and stable heart rates in normoxic turtles, presumably associated with periods of apnoea. Also, the lower heart rate in the present study is probably due to long breath-holds and high vagal tone, which indicates that the turtles were undisturbed and unstressed. Consistent with previous studies on turtles, anoxia caused significant reductions in heart rate and systemic arterial pressure (Hicks and Wang, 1998; Stecyk et al., 2004; Overgaard et al., 2007). Although blood flow was not measured in the present study, earlier studies at a similar temperature have demonstrated a peripheral vasoconstriction mediated by increased sympathetic tone on the systemic vessels (Stecyk et al., 2004), and that H_2S generation may play some role, particularly at low temperatures (Stecyk et al., 2010). Our study is the first to report on venous pressures in anoxic turtles. The significant rise in venous pressure during anoxia (Fig. 1D) may partially result from relocation of blood from the arterial to the venous side as arterial blood pressure declined. This rise in filling pressure of the heart may serve to enhance filling and hence recruit cardiac contractile force by the Frank–Starling response and hence alleviate the reduction in cardiac contractility caused by oxygen lack, acidosis and hyperkalaemia (Overgaard et al., 2005). Increased filling pressure could thus be an important component in the maintenance of stroke volume during anoxia (Hicks and Wang, 1998).

In *Trachemys* turtles, anoxia causes profound redistribution of blood flow to the most vital organs, including brain, heart and skeletal muscle, while the perfusion of lungs, gut and kidneys is drastically reduced (Stecyk et al., 2004). The shell, being essential for the buffering lactic acid in anoxia (Jackson and Ultsch, 2010), receives a large proportion of cardiac output in anoxic turtles (Stecyk et al., 2004). Conversely, it is possible that the high levels of circulating NO found here induce vasodilatation in specific tissues and hence sustain their perfusion during anoxia. The vasodilating activity of the NO-donor sodium nitroprusside in the systemic but not in the pulmonary circulation (which is virtually not perfused during anoxia) of anaesthetized *Trachemys* turtles appears to support this hypothesis (Crossley et al., 2000). In another study, topically added sodium nitroprusside and L-NAME, a NOS inhibitor, had no effect on the cerebral blood flow in anoxic turtles (Hylland et al., 1996), which can be explained on the basis of the present data, suggesting that the high endogenous NO levels present in the blood may already elicit maximal vasodilatation. If the vasoactive action of NO is local, then its major target, the enzyme soluble guanlyate cyclase, would be expected to have a different tissue-specific expression pattern in the turtle vasculature. This aspect remains to be investigated.

Another major target of NO is the electron transport chain of the mitochondria (Moncada and Erusalimsky, 2002), where it reduces the rate of O_2 consumption and the mitochondrial generation of reactive oxygen species. Both of these inhibitory mechanisms may contribute to anoxia tolerance because they prolong O_2 availability (i.e. during the transition to anaerobic energy metabolism) and limit oxidative damage at reoxygenation. In a previous study we found that generation of NO from NOS (by adding L-arginine) significantly decreased O_2 consumption in the *Trachemys* myocardium, thereby increasing myocardial efficiency (i.e. ratio of developed force to O_2 consumption), particularly at low oxygenation (Misfeldt et al., 2009). Such effects of NO would be of importance not only in maintaining cardiac function during anoxia but also in favouring metabolic depression in other tissues as well. Thus during anoxia, circulating NO may contribute to maintain mitochondria in an inhibited state in the tissues that are perfused, particularly brain, heart and skeletal muscle.

The adaptive strategy that enables turtles to survive anoxia involves high levels of circulating bicarbonate to buffer changes in blood pH (Jackson, 2004) and high expression of constitutive antioxidant defences, including red blood cell thiols (as found here) and antioxidant redox enzymes to reduce oxidative stress at reoxygenation (Hermes-Lima and Zenteno-Savín, 2002; Bickler and Buck, 2007). In addition, as reported in this study, high levels of circulating NO at anoxia and reoxygenation may be of further help to orchestrate blood flow distribution, depress metabolic activity and limit oxidative damage after anoxia.

LIST OF ABBREVIATIONS

4-PDS	4,4′-dithiodipyride
4-TP	4-thipyridone
FeNO	iron nitrosyl
Hb	haemoglobin
Met	ferric haemoglobin
NO	nitric oxide
NOS	nitric oxide synthase
RNNO	*N*-nitrosamine
RSNO	*S*-nitrosothiol

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