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Exploring pathways of NO and H$_2$S signaling in metabolic depression: the case of anoxic turtles

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Running head: NO and H$_2$S signaling in the anoxic turtle

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Abstract

In contrast to most vertebrates, freshwater turtles of the genera *Trachemys* and *Chrysemys* survive oxygen deprivation for long periods of time. This remarkable tolerance makes them ideal August Krogh’s model animals to study adaptations to survive oxygen deprivation. The gasotransmitters nitric oxide (NO) and hydrogen sulfide (H$_2$S) and their metabolic derivatives are central in regulating the physiological responses to oxygen deprivation. Here, we explore the role of these signaling molecules in the anoxia tolerance of the freshwater turtle, including metabolic suppression and protection against oxidative damage with oxygen deprivation. We describe the interaction of NO and H$_2$S with protein thiols and specifically how this regulates the function of central metabolic enzymes. These interactions contribute both to metabolic suppression and to prevent oxidative damage with oxygen deprivation. Furthermore, NO and H$_2$S interact with ferrous and ferric heme iron, respectively, which affects the activity of central heme proteins. In turtles, these interactions contribute to regulate oxygen consumption in the mitochondria, as well as vascular tone and blood flow during oxygen deprivation. The versatile biological effects of NO and H$_2$S underscores the importance of these volatile signaling molecules in the remarkable tolerance of freshwater turtles to oxygen deprivation.
**Introduction**

Among the very few vertebrates able to tolerate long periods completely deprived of O$_2$, freshwater turtles of the genera *Trachemys* and *Chrysemys* are animals of choice when searching for the molecular mechanisms of hypoxia and anoxia tolerance. Following the August Krogh principle that ‘For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied’ (Krogh, 1929), the cornerstone of comparative physiology, we present fundamental mechanisms underlying tolerance to O$_2$ deprivation in turtles exposed to anoxia. The focus of this short review will be on the crucial role of two ubiquitous gasotransmitters, nitric oxide (NO) and hydrogen sulfide (H$_2$S) and their metabolites, in controlling metabolic suppression and protection against oxidative damage under O$_2$ deprivation, a condition that can be conveniently studied in turtles. Understanding these mechanisms will provide insight into the prevention of ischemia and reperfusion injury in humans.

The exceptional ability of turtles to tolerate hypoxia and anoxia (Jackson et al., 1984) derives from a ‘core triad of adaptations’ (Bickler & Buck, 2007). These include: 1) strong anaerobic metabolic depression, balancing low ATP consumption with low ATP production, thereby prolonging liver glycogen stores; 2) increased ability to buffer lactic acid, the end product of anaerobic energy metabolism, by the bone tissue of the shell; and 3) preventing tissue oxidative damage from reactive oxygen species (ROS) at reoxygenation (Fig. 1). As explained in detail below, two key factors may prevent ROS generation at reoxygenation: the high thiol (-SH) content of turtle red blood cells (RBCs) acting as a redox buffering system and the low succinate accumulation combined with an almost maintained ATP/ADP ratio in the heart during anoxia (Stecyk et al., 2009) (Fig. 1). These responses - in particular metabolic suppression and ROS avoidance - are in part mediated by the concerted action of NO and H$_2$S signaling on specific protein targets and heme groups. The unique ability of turtles to tolerate anoxia provides useful insights into reactivity, metabolism and transport of these signaling molecules.

**NO and H$_2$S: origin and protein targets involved in metabolic depression**

NO and H$_2$S are ubiquitous signaling molecules (forming a triad of ‘gasotransmitters’ together with carbon monoxide) involved in numerous physiological responses, including regulation of vascular tone, neuronal function and respiration (Kolluru et al., 2017). Both molecules are generated by enzymes from amino acid substrates (Fig. 2). In normoxia, NO and H$_2$S are constantly produced for signaling purposes, oxidized to less reactive products, including nitrite and nitrate (NO) and thiosulfate (H$_2$S). In hypoxic conditions, NO and H$_2$S oxidation is inhibited and availability for signaling (possibly via persulfides; see Olson, this issue) increases. Although low O$_2$ also inhibits NO synthesis, as this requires O$_2$ as co-substrate, NO can be efficiently ‘recycled’ from its end product nitrite by the nitrite reductase activity of deoxygenated hemoglobin (Hb) in the RBCs and myoglobin (Mb) in cardiomyocytes. Similarly, H$_2$S can be regenerated by reduction of protein-bound polysulfides and persulfides (Fig. 2). Thus, both gasotransmitters are present in tissues at low concentrations as ‘free’ (nM NO, µM H$_2$S) – especially in hypoxia - and at higher levels as ‘bound’ storage pools.

NO and H$_2$S have two major cellular targets: protein thiols and heme groups. Protein Cys residues are selectively modified in the presence of NO and H$_2$S to form labile protein-bound S-nitrosyl (SNO) and persulfides (SSH) or polysulfides (S$_n$SH), respectively (Fig. 2). An important target enzyme is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), containing a reactive Cys in its active site.
Accumulation of its substrate glyceraldehyde 3-phosphate in the heart of anoxic *Trachemys* turtles suggests that this glycolytic enzyme is inhibited (Bundgaard et al., 2019a), possibly by S-nitrosation at the active site Cys. Conversely, persulfidation (SSH) of GAPDH increases the Cys reactivity (Mustafa et al., 2009). Other factors like high NADH/NAD<sup>+</sup> ratio, lactate and proton concentrations during anoxia can also allosterically inhibit the enzyme (Bundgaard et al., 2019a, 2020b). Another target of inhibitory S-nitrosation is the mitochondrial complex I (Fig. 2, 3). This modification would limit electron access to the electron transport chain when O<sub>2</sub> is not available, and is key in preventing the burst of ROS at reoxygenation (Chouchani et al., 2013). In the heart of *Trachemys* turtles, complex I is S-nitrosated to the same extent in normoxia and anoxia (Bundgaard et al., 2018), suggesting a constitutive rather than anoxia-induced inhibition. Interestingly, ATP synthase complex V is inhibited in heart mitochondria from anoxic *Trachemys* (Galli et al., 2013) and is also partly S-nitrosated (Bundgaard et al., 2018), but regardless of the oxygenation state. The functional consequences of S-nitrosation of complex V are not known. It has not been examined if these complexes are also targets of persulfidation (SSH).

The second type of target of NO and H<sub>2</sub>S is the heme group. While free NO binds ferrous heme almost irreversibly, free H<sub>2</sub>S binds reversibly ferric heme (Fig. 2). In mitochondria, NO and H<sub>2</sub>S can inhibit O<sub>2</sub> consumption by binding to complex IV (Fig. 3). Although their mechanism of action and heme affinity differs (Cooper and Brown, 2008), both are capable of reversibly depressing O<sub>2</sub> consumption rates during hypoxia, when their inhibitory effect is more pronounced, and redistribute available oxygen for respiration among mitochondria. Furthermore, NO increases myocardial efficiency in turtles (Misfeldt et al., 2009) (Fig. 3). Evidence based on high levels of heme-bound NO in anoxic turtle heart indicates sufficiently high levels of free NO to inhibit complex IV in anoxia (Jacobsen et al., 2012; Jensen et al., 2014).

Altogether, these labile but potent protein modifications may act to depress glycolysis and mitochondrial oxidative phosphorylation, thereby reducing ATP production and rate of O<sub>2</sub> consumption.

Preventing ROS damage: keeping succinate low and ADP high

A hallmark of anaerobic metabolism is the accumulation of mitochondrial succinate, which in mammals causes ROS overproduction by complex I via reverse electron transfer from complex II to complex I (Fig. 3), a major cause of the ischemia reperfusion injury (Chouchani et al., 2014). This process is favored by a high proton-motive force across the inner mitochondrial membrane (as occurring when electrons are fueled to the electron transport chain but oxygen is not consumed) and by the lack of sufficient ADP as a substrate for complex V to allow dissipation of the proton gradient. In the anoxic turtle heart, mitochondrial integrity is maintained (Bundgaard et al., 2019b) and there is only a modest succinate build up. In addition, the adenylate pool is not depleted and, ADP is maintained (Bundgaard et al., 2019a), which at reoxygenation would drive electron flow in the forward direction by dissipating the proton gradient through complex V rather than complex I (Bundgaard et al., 2019a). We have shown that isolated *Trachemys* turtle heart mitochondria are in fact capable of generating ROS *in vitro* at rates comparable to those of mouse heart mitochondria (Bundgaard et al., 2019a), indicating that low succinate and high ADP as mitochondrial substrates are crucial for preventing ROS in the turtle heart (Fig. 3). As expected, *in vitro* inhibition of turtle complex I by S-nitrosation almost completely abolishes ROS after anoxia and reoxygenation of mitochondria (Bundgaard et al., 2018), although no changes in endogenous S-nitrosation levels of complex I could be detected (Fig. 3). Interestingly, in heart mitochondria from *Trachemys* turtle,
bearded dragon and python, a large fraction of complex I is consistently assembled with complex III to form a ‘supercomplex’ (Bundgaard et al., 2018, 2020a), which might further contribute to limit reverse electron transfer and ROS production. It has been repeatedly proposed that *Trachemys* turtles possess constitutively high levels of antioxidant enzymes (Willmore and Storey, 1997), but a rigorous comparison with other species has not been made.

**The blood as a redox buffer and as a storage pool of bound sulfide**

Contributing to the overall high resistance of turtles to oxidative damage, *Trachemys* turtle RBCs contain high levels of reactive thiols, partly as Cys residues of the Hb (Fig. 4). In particular one of these thiols is readily oxidized to form intermolecular disulfide bonds and extended polymers of Hb molecules, without affecting oxygen affinity and cooperativity (Petersen et al., 2018). Thus, turtle Hb may function as a circulating redox buffer, and since turtle RBCs contain mitochondria and are regularly exposed to large variations in oxygen levels (Bickler and Buck, 2007; Jackson et al., 1984), this would effectively protect erythrocytes against endogenous and exogenous ROS (Fig. 4).

A consequence of the high thiol content of turtle RBC is the high capacity of carrying high levels of sulfide (as persulfide and polysulfide) bound to the Hb molecule, as found in normoxic and anoxic *Trachemys* turtles (Jensen et al., 2019) (Fig. 4). In addition, the minor fraction of Hb containing a ferric heme (~1%) can also act as a reversible carrier of H2S in the blood (Jensen and Fago, 2020, 2018), consistent with high measured levels of free H2S (~20 µM) in turtle RBCs (Jensen et al., 2019). Although these values appear exceedingly high compared to those found in other vertebrates, it is important to keep in mind that these levels correspond to max 0.5% of total Hb thiol persulfidation and to max 10% of ferric heme bound to H2S, which are not unrealistic considering estimates of 10-25% persulfidation in other less abundant proteins (Mustafa et al., 2009). Thus, the blood may act as a carrier and storage of H2S for other tissues, and since binding of H2S to ferric Hb is reversible, binding and release would be controlled by existing H2S concentration gradients in the circulation. Since levels of ferric Hb are not affected by oxygen, this feature would help mediate H2S vasoactive effects in the circulation independently of local oxygen tensions. More specifically, H2S acts a potent vasoconstrictor in the turtle circulation (Fig. 2), apparently eliciting maximal effect during anoxia (Stecyk et al., 2010) and produced constitutively, independently on anoxia (Melleby et al., 2020). In contrast to Hb, the *in vitro* redox reaction of ferric Mb with H2S produces ferrous Mb and a thiyl radical (HS·), which would readily persulfidate target proteins (Mishanina et al., 2015) (Fig. 4). The seemingly important *in vivo* consequences of this reaction for heart function remain to be investigated.

**The conversion of nitrite to NO in anoxic turtles**

When *Trachemys* turtles are exposed to anoxia, the heart accumulates high levels of nitrite, SNO and heme-bound NO, deriving either from an increased intracellular NO synthesis during the transition from normoxia to anoxia, or from plasma nitrite and subsequent conversion in the cardiomyocytes (Jensen et al., 2014). This pattern of NO metabolites is consistent with intracellular nitrite forming at low pH either SNO (via N2O3, a strong S-nitrosating agent) or free NO to target protein and enzymes involved in metabolic suppression and ROS protection (Fig. 4). The conversion from nitrite to NO in anoxia is favored by increased desaturation of Mb, which in the turtle has an oxygen affinity similar to that of other reptiles (Helbo et al., 2015). Since NO has a very strong affinity for ferrous heme, part of the NO produced by the nitrite reductase activity of deoxy Mb will rebind to the ferrous heme of deoxy Mb present in excess and is therefore detected experimentally as heme-
bound NO. This reaction has also importance in the blood of anoxic turtles and in hypoxia in general. According to the current view (Jensen and Rohde, 2010), plasma nitrite enters the RBCs through the anion exchanger (and via HNO₂ diffusion) and react with deoxy Hb in anoxic RBCs (Fig. 4). The NO generated in the process may either bind to deoxy Hb or escape the RBC and diffuse into the vasculature, where it activates soluble guanylate cyclase and initiates vasodilation (Fig. 2). This process contributes to the observed blood-dependent component of hypoxic vasodilation, whereas the vasodilatory effect of nitrite alone occurs via nitrite to NO conversion catalyzed by the low amount of Mb expressed in the endothelium. Depending on differences in the localization of soluble guanylate cyclase, the target of NO-dependent vasodilation, in the vascular tree, this process would help redistribute blood volume in the circulation, counteracting H₂S-mediated and adrenergic vasoconstrictions.

**Conclusion**

NO and H₂S are important key signaling molecules involved in the tolerance to oxygen deprivation with overlapping and distinct targets and mode of actions, but also showing significant cross-talk (Cortese-Krott et al., 2015; Kolluru et al., 2017). Although much remains to be understood, we show here that anoxia tolerant turtles are excellent August Krogh models to unravel causes of anoxia survival at the molecular level and identify potential targets for human intervention against ischemia and reperfusion.

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**Abbreviations**

- 3MST: 3-mercaptopyruvate sulfurtransferase
- CBS: Cystathionine \(\beta\)-synthase
- CSE: Cystathionine \(\gamma\)-lyase
- H\(_2\)S: Hydrogen sulfide
- Hb: Hemoglobin
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- Mb: Myoglobin
- NO: Nitric oxide
- NOS: Nitric oxide synthase
- RBCs: Red blood cells
- ROS: Reactive oxygen species
- SNO: S-nitrosyl
- SSH: Persulfides
- S\(_{1}\)SH: Polysulfides
- SQR: Sulfide quinone reductase
References


NO and H2S signaling in the anoxic turtle


NO and H\textsubscript{2}S signaling in the anoxic turtle
Figure legends

Figure 1. Adaptations to anoxia in the freshwater turtle.
Anoxia-tolerant freshwater turtle species can survive prolonged periods of O₂ deprivation by relying on a strong anaerobic metabolic depression. The anaerobic end product lactic acid is tolerated via buffering by calcium carbonate from the shell. Metabolic suppression in the heart is associated with a high NADH/NAD⁺ ratio and low succinate accumulation in cardiomyocyte mitochondria, and during reoxygenation, the low succinate together with a maintained ATP/ADP pool may prevent the formation of ROS and thus protect the heart against oxidative damage. Moreover, thiol-rich RBCs act as redox buffer in the blood.

Figure 2. Origin and protein targets of the signaling molecules NO and H₂S.
The signaling molecules NO and H₂S are continuously produced by several enzymatic reactions in various tissues. Nitric oxide synthases (NOS) catalyze the conversion of L-Arginine to NO in the presence of O₂, and during low O₂, NO can be recycled from nitrite by nitrite reductase activity of deoxygenated Hb in the RBCs and myoglobin (Mb) in cardiomyocytes. H₂S is synthesized independent of O₂ from several thiol-containing substrates via cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST), and can further be regenerated from the bound sulfane sulfur pool consisting of thiosulfate, persulfide and polysulfides. NO and H₂S share two major cellular targets: protein thiols and heme groups. Reactive protein thiols can be modified to protein-bound S-nitrosyl (SNO) and persulfides (SSH) or polysulfides (SₓSH), respectively, which can alter the activity of key enzymes involved in e.g. glycolysis, mitochondrial respiration and regulation of vascular tone. These physiological processes can also be regulated by NO and H₂S heme binding. While free NO binds ferrous heme almost irreversibly, free H₂S binds reversibly to ferric heme.

Figure 3. The effects of H₂S and NO on the mitochondrial electron transport system in turtles.
H₂S can act as an electron donor via sulfide quinone reductase (SQR), contributing to mitochondrial electron transfer and energy production. Both H₂S and NO can act as inhibitors of complex IV (cytochrome c oxidase), inhibiting oxygen consumption. During hypoxia, inhibition of complex IV by NO increases myocardial efficiency, as NO inhibits oxygen consumption without affecting myocardial force development in the turtle heart (Misfeldt et al., 2009). During prolonged anoxia, however, overall mitochondrial oxygen consumption rate and production capacity for reactive oxygen species (ROS) is inhibited by another mechanism (Bundgaard et al., 2018). While NO can inhibit production of ROS by S-nitrosation of complex I, this does not seem to be the mechanism behind lower ROS production of heart mitochondria isolated from anoxic turtles (Bundgaard et al., 2018).

Figure 4. Physiological targets and effects of NO and H₂S and their metabolites in the freshwater turtle.
Changes with anoxia are indicated in green arrows. During anoxia, levels of NO metabolites increase in red blood cells (RBCs), plasma, heart, brain and liver (Jacobsen et al., 2012; Jensen et al., 2014), suggesting an increase in NO signaling (upper left panel). In contrast, the high levels of H₂S metabolites do not change with anoxia (Jensen et al., 2019), suggesting that H₂S signalling is unaltered during anoxia. Instead, high levels (24 mM) of reactive thiols in turtle RBCs (Jacobsen et
al., 2012) may act as redox buffer and contribute to antioxidant defense. Turtle Hb contain six thiols, and exposure to the reactive oxygen species H$_2$O$_2$ leads to oxidation of Cys5α of the major Hb isoform, which leads to protein polymerization (lower left panel) (Petersen et al., 2018). Both NO and H$_2$S may also directly affect the heart. Increase in deoxy Mb and in nitrite in the anoxic turtle heart may increase NO levels and NO signaling (upper right panel). The same reactions will take place in the blood catalyzed by deoxyHb. Ferric Mb may increase H$_2$S-dependent protein modifications in the heart via formation of reactive thyl radical HS$^\cdot$ (lower right panel), while ferric Hb may function as reversible H$_2$S carrier in the blood, in analogy with in vitro studies on other Mb and Hb systems (Jensen and Fago, 2018).
NO and H₂S signaling in the anoxic turtle

Adaptations to anoxia
1. Strong metabolic suppression
2. Increased lactic acid buffering
3. Preventing oxidative damage

Figure 1

Figure 2
NO and H₂S signaling in the anoxic turtle

Figure 3

Figure 4
Graphical abstract: