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SHORT COMMUNICATION

Tissue-dependent variation of hydrogen sulfide homeostasis in anoxic freshwater turtles

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ABSTRACT

Hydrogen sulfide (H_2S) controls numerous physiological responses. To understand its proposed role in metabolic suppression, we measured free H_2S and bound sulfane sulfur (BSS) in tissues of the freshwater turtle *Trachemys scripta elegans*, a species undergoing strong metabolic suppression when cold and anoxic. In warm normoxic turtles, free H_2S was higher in red blood cells (RBCs) and kidney (~9–10 µmol l^{-1}) than in brain, liver and lung (~1–2 µmol l^{-1}). These values overall aligned with the tissue H_2S-generating enzymatic activity. BSS levels were similar in all tissues (~0.5 µmol l^{-1}) but ~100-fold higher in RBCs, which have a high thiol content, suggesting that RBCs function as a circulating H_2S reservoir. Cold acclimation caused significant changes in free and bound H_2S in liver, brain and RBCs, but anoxia had no further effect, except in the brain. These results show tissue-dependent sulfide signaling with a potential role in brain metabolic suppression during anoxia in turtles.

KEY WORDS: Anoxia, Gasotransmitter, Red blood cell, Metabolic suppression, *Trachemys scripta*

INTRODUCTION

Hydrogen sulfide (H_2S) has emerged as an important signaling molecule belonging to the family of gasotransmitters together with nitric oxide (NO) and carbon monoxide (Kolluru et al., 2017; Olson and Straub, 2016; Wang, 2002). First discovered as a modulator in the brain (Abe and Kimura, 1996), H_2S was later found to control other key physiological functions, such as vascular tone and cytoprotection (Elrod et al., 2007; Yang et al., 2008; Zhao et al., 2012). H_2S conveys its biological activity by binding reversibly to ferric heme proteins (Jensen and Fago, 2018; Pietri et al., 2011) and by *S*-sulfhydration of thiol groups (Mustafa et al., 2009; Paul and Snyder, 2012), but also via other sulfur-containing compounds, such as persulfides (R-S-SH), polysulfides (R-S_n-R, n>2) and thiosulfate ($S_2O_3^{2-}$) (Kimura, 2017; Olson et al., 2013; Shen et al., 2017). Endogenous H_2S is produced in the cytosol enzymatically by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), using (homo)-cysteine and cystathionine as main substrates. In the mitochondria, 3-mercaptopyruvate sulfurtransferase is the primary enzyme producing H_2S from mercaptopyruvate assisted by cysteine aminotransferase, though this enzyme machinery is also present in the cytosol (Nagahara et al., 1998). In addition to enzymatically produced H_2S, free H_2S can also be regenerated by reduction of bound stores of persulfides, polysulfides and thiosulfate, collectively representing the bound sulfane sulfur (BSS) fraction (Koike and Ogasawara, 2016; Shen et al., 2012, 2015). During low O_2 conditions, H_2S can be regenerated from thiosulfate (Olson et al., 2013), as part of a potential oxygen-sensing mechanism (Olson, 2013), as well as from persulfides and polysulfides (Ishigami et al., 2009; Kimura, 2014a).

The discovery that exposure to exogenous H_2S gas triggers reversible suspended animation in mice (Blackstone et al., 2005) has fostered the idea that endogenous H_2S may control the biological metabolic suppression of hibernating animals. To fit with this proposed *in vivo* role, H_2S is an *in vitro* reversible inhibitor of cytochrome *c* oxidase (Collman et al., 2009; Cooper and Brown, 2008; Pietri et al., 2011), thus being able to modulate mitochondrial oxygen consumption. In a previous analysis of blood samples from free-ranging summer active and winter-hibernating brown bears (Revsbech et al., 2014), we detected changes in free H_2S and BSS during hibernation, consistent with the generation of free H_2S from circulating storage pools that may contribute to the suppression of mitochondrial respiration in tissues. Moreover, the substrate cysteine shifted towards the synthesis of RBC glutathione (GSH), which increased dramatically (Revsbech et al., 2014).

In hibernating bears, O_2 consumption rate falls to ~25% of basal levels (Tøien et al., 2011); in comparison, some turtle species are capable of overwintering in complete anoxia, sustaining even more pronounced metabolic suppression, down to 5–10% of basal levels (Bickler and Buck, 2007; Ultsch, 2006). Among these turtle species, the red-eared slider, *Trachemys scripta elegans* (Wied-Neuwied 1839), is among the most extreme vertebrates as it can survive for weeks in anoxia at low temperatures, relying only on glycolysis for energy production (Bundgaard et al., 2019; Ultsch, 1989, 2006; Warren et al., 2006). Thus, we hypothesized that *T. scripta elegans* endogenous levels of free H_2S and BSS in various tissues should change more markedly upon cold acclimation and anoxia than found in the brown bears (Revsbech et al., 2014). Furthermore, NO metabolites are known to increase dramatically in the blood (Jacobsen et al., 2012) and tissues (Jensen et al., 2014) of anoxia-acclimated *T. scripta elegans* and may contribute further to the strong metabolic suppression of this species (Fago and Jensen, 2015), although not necessarily to cytoprotection against oxidative stress at reoxygenation, at least in the heart (Bundgaard et al., 2018).

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

Animals

Adult red-eared sliders of both sex (*Trachemys scripta elegans*) kept in aquaria at the animal care facility at Zoophysiology, Aarhus

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Received 25 March 2019; Accepted 14 May 2019

Journal of Experimental Biology (2019) 222, jeb203976. doi:10.1242/jeb.203976

List of abbreviations

BSS	bound sulfane sulfur
CBS	cystathionine β-synthase
CSE	cystathionine γ-lyase
GSH	glutathione
H_2S	hydrogen sulfide
Hb	hemoglobin
MBB	monobromobimane
NMADR	*N*-methyl-D-aspartate receptor
NO	nitric oxide
RBC	red blood cell
R-S-SH	persulfides
R-S_n-R, n>2	polysulfides
SDB	sulfide dibimane

University, were used for experiments. While fasted, the turtles were progressively acclimated to low temperature (5°C) followed by randomized exposure to either normoxic (N=5, 0.66±0.13 kg) (termed 'cold normoxia') or anoxic conditions (N=4, 0.64±0.07 kg) (termed 'cold anoxia') for 9 days, according to a previously described protocol (Bundgaard et al., 2018; Bundgaard et al., 2019; Jensen et al., 2014). A control group (N=5, 0.61±0.04 kg) of non-fasting turtles taken directly from the aquaria at 25°C was also included in the study (termed 'warm normoxia'). All procedures were performed in accordance with regulations of animal care and experimentation in Denmark under permit 2015-15-0201-00544.

Tissue sampling

Turtles were killed as previously described (Bundgaard et al., 2018). In brief, an overdose of pentobarbital (50 mg kg^{-1}) was given via the supravertebral venous sinus. When reflexes were lost, the head was cut off and an opening was made in the plastron. After removing the heart (used for another study), a blood sample was quickly collected in heparin-filled syringes from the thoracic cavity and centrifuged (2000 ***g***, 3 min) to separate RBCs from plasma; 250 µl RBCs were diluted in 800 µl 100 mmol l^{-1} Tris-HCl buffer (pH 9.5, 0.1 mmol l^{-1} DTPA) and snap-frozen in liquid N_2. Tissue samples (~150 mg), including liver, brain, lung and kidney) were harvested and conserved in 800 µl of 100 mmol l^{-1} Tris-HCl buffer (pH 9.5, 0.1 mmol l^{-1} DTPA) followed by snap-freezing in liquid N_2. All samples were weighed to calculate the exact dilution factor. The whole procedure was completed within 20 min. Samples were shipped on dry ice to Louisiana State University Health Sciences Center, Shreveport, LA, USA, where H_2S metabolite analysis took place. Additional tissue samples were collected and stored at −80°C for enzyme activity measurements at Aarhus University.

H_2S metabolites

Bioavailable biochemical forms of H_2S including free H_2S and BSS (including persulfides, polysulfides and thiosulfate) were measured by a fluorescent monobromobime (MBB) assay coupled with RP-HPLC as previously described (Shen et al., 2011, 2012, 2015). This method has been extensively refined to determine specific incubation pH conditions and reaction times (<30 min) of MBB to derivatize biological sulfide and its various forms with minimal interference or artifact (Shen et al., 2011). The MBB assay reported here was performed under exacting specifications to ensure minimal chemical interference and maintain the chemical identity and abundance of various sulfide species, as previously confirmed in a separate publication using LC/MS non-radioisotopic standardization (Shen et al., 2014). In this method, the tissue homogenates were prepared in alkaline 100 mmol l^{-1} Tris-HCl buffer (to convert all free H_2S gas to the HS^- anionic form). HS^- was then derivatized by incubation with excess MBB (10 mmol l^{-1} in CH_3CN) for 30 min; 1 mmol l^{-1} TCEP [tris(2-carboxyethyl)phosphine hydrochloride] was used to liberate total stored sulfide (Shen et al., 2012, 2015). The whole procedure was done at room temperature at 1% O_2 in a hypoxic chamber in the dark. All measurements were obtained in technical triplicate. MBB was separated from the product, sulfide dibimane (SDB), by a RP-HPLC equipped with a fluorescence detector (λ_{ex}: 390 nm and λ_{em}: 475 nm). Retention time was 16.5 and 17.6 min, respectively. The amount of SDB in each sample was quantified from a standard curve. H_2S is a weak acid in solution (pK_{a1} ~6.8, 37°C) (Li and Lancaster, 2013), and under physiological conditions, ~80% of total sulfide is present as hydrosulfide anion. When using the MBB method, the equilibrium $H_2S \rightleftharpoons HS^- + H^+$ is right-shifted as a result of alkaline conditions, and thus the method measures both H_2S and HS^-. For simplicity, the two species are here collectively referred to as H_2S.

Enzyme activity

The activity of H_2S-producing enzymes was measured in warm normoxic turtles. Tissue homogenates were prepared in 100 mmol l^{-1} potassium phosphate buffer+1 mmol l^{-1} EDTA, pH 7.4, mixed with 90 mmol l^{-1} pyridoxal 5′-phosphate and incubated in a 1 ml glass chamber with continuous stirring (500 rpm) at room temperature. A final concentration of 250 mmol l^{-1} cysteine was added to initiate the reaction. Production of H_2S over time was measured with an amperometric H_2S-specific microsensor (Unisense A/S, Aarhus, Denmark), calibrated with a freshly made Na_2S stock solution of known concentration (r^2=0.99). Activity was expressed as nanomoles of H_2S produced per milligram total protein per hour. Protein content was measured by a colorimetric assay using bovine serum albumin as a standard. Protein concentration in RBCs was measured as Hb concentration determined from the oxyHb extinction coefficients at 541 nm of 13.8 l $mmol^{-1}$ cm^{-1}, 577 nm of 14.6 l $mmol^{-1}$ cm^{-1} and 415 nm of 125 l $mmol^{-1}$ cm^{-1}.

Lactate

Plasma lactate concentrations were measured in a subsample of normoxic and anoxic turtles by a colorimetric L-lactate assay kit (ab65331, Abcam, Denmark).

Statistics

Statistical analyses were done in Prism (GraphPad Software Inc., La Jolla, CA, USA). Shapiro–Wilk normality test was applied to determine whether data were normally distributed. Significant differences between warm normoxic turtles, cold normoxic turtles and cold anoxic turtles were assessed by one-way ANOVA with Dunnett's multiple comparisons test (normally distributed) and the few non-normally distributed datasets were assessed by non-parametric Kruskal–Wallis test with Dunn's multiple comparisons test (Table S1). The significance level was P<0.05. All data are reported as means±s.e.m.

RESULTS AND DISCUSSION

To examine for changes in H_2S metabolism during the extreme metabolic suppression of cold-acclimated and anoxic turtles, we kept turtles at 25°C under normoxia and at 5°C under either normoxia or anoxia for 9 days, as described previously (Bundgaard et al., 2019). We then measured *in vivo* pools of free H_2S and BSS in kidney, brain, liver, lung and RBC samples using the MBB method

(Shen et al., 2012, 2015). An increase in plasma lactate from 0.21 ±0.04 mmol l^{-1} (cold normoxic turtles) to 19.4±9.7 mmol l^{-1} (cold anoxic turtles) confirmed that glycolysis was upregulated and that turtles were anoxic. Physiological basal levels (expressed in µmol l^{-1}) of free H_2S and BSS in kidney, brain, liver and lung homogenates of freshwater turtle are shown in Fig. 1. An overview of H_2S levels normalized to total protein content is given in Fig. S1. To our knowledge, this is the first time that H_2S metabolites have been measured in a reptile.

Free H_2S in kidney, brain, liver and lung

Our analysis showed that the levels of free H_2S in tissues of warm normoxic turtles were highest in the kidneys (9.3±0.4 µmol l^{-1}) and lowest in the brain, liver and lung (0.9–2.5 µmol l^{-1}) (Fig. 1A). Upon cold acclimation of turtles to 5°C, these values did not change, except in the liver, where free H_2S decreased significantly (Fig. 1A). No increase was observed in free H_2S after acclimation of turtles to anoxia for 9 days in any of these tissues (Fig. 1A). This finding suggests that free H_2S is not involved in overall metabolic suppression, although we cannot exclude that the lack of O_2 would enhance the inhibitory effect of H_2S on cytochrome *c* oxidase activity. O_2 and H_2S levels are inversely linked (Olson and Straub, 2016), as a result of the oxidative degradation of H_2S (Hildebrandt and Grieshaber, 2008), and H_2S lifetime increases when O_2 is limiting.

Although not in our experimental setup, the natural environment of overwintering anoxic turtles is presumably sulfide rich, which could add to the *in vivo* H_2S levels. A recent study on amphibian fish inhabiting sulfide-rich mangroves found no change in aquatic H_2S sensitivity between wild-caught and laboratory-reared fish in terms of behavior (Cochrane et al., 2019); however, further studies are needed on the potential adaptations to environmental H_2S in vertebrates.

That free H_2S is high in the kidneys (Fig. 1A) is not unique to turtles, as mammals also exhibit abundant H_2S levels (Shen et al., 2013). The enzyme D-amino acid oxidase constitutes an additional enzymatic pathway for H_2S production and is highly expressed in mammalian kidneys (Shibuya et al., 2013). In mammals, endogenous H_2S is proposed to be important for normal kidney function (Lobb et al., 2015) and H_2S has been shown to increase glomerular filtration rate (Xia et al., 2009) and salt excretion by inhibition of Na^+/K^+-ATPase (Ge et al., 2014), and to suppress renin release (Cao and Bian, 2016). Given that H_2S is an ancient vasoregulatory molecule across multiple vertebrate clades (Dombkowski et al., 2004), our data indicate that H_2S could also have a similar regulatory role in the renal function of turtles. Protein content in the kidney did not change during acclimation, but free H_2S normalized to total protein decreased significantly in cold anoxic turtles compared with warm normoxic ones (Fig. S1). Compared with other tissues such as brain and liver, the kidneys of anoxic turtles exhibit the highest reduction in blood flow (Stecyk et al., 2004), suggesting H_2S-mediated regulation of vascular tone in this organ.

BSS in kidney, brain, liver and lung

BSS levels were overall similar in tissues of all three groups of turtles, except for the brain, where BSS decreased significantly when cold-acclimated turtles were exposed to anoxia (Fig. 1B) without increasing free H_2S (Fig. 1A). In mammals, H_2S and H_2S-derived polysulfides in the brain are implicated in neuroprotection and have been shown to regulate the activity of essential receptors such as *N*-methyl-D-aspartate receptor (NMADR) and transient receptor potential ankyrin (TRPA) by persulfhydration (Abe and Kimura, 1996; Kimura, 2014b; Kimura et al., 2013; Li et al., 2017). In the turtle brain, NMDAR is strongly inhibited during anoxia by phosphorylation (Bickler and Buck, 2007) and cysteine *S*-nitrosation (Takahashi et al., 2007) to limit neuronal activity and protect the turtle brain from damage and cell death (Bickler and Buck, 2007). One can speculate that an increased persulfhydration may enhance the activity of NMDAR (Abe and Kimura, 1996) and other brain receptors at low temperatures in normoxia (Fig. 1B) and promote subsequent inhibition by increased *S*-nitrosation in hypoxia and anoxia (Fago and Jensen, 2015; Jensen et al., 2014). There is emerging evidence of complex H_2S/NO cross-talk of protein cysteine redox modifications that remains to be investigated in detail (Cortese-Krott et al., 2015, 2017; Hosoki et al., 1997; Miyamoto et al., 2017). Besides possible interactions including production of polysulfides (Miyamoto et al., 2017), the two gasotransmitters have been suggested to have several overlapping functions (Kolluru et al., 2013).

H_2S and BSS in RBCs

The level of free H_2S in RBCs was 10.5±0.8 µmol l^{-1} in warm normoxic turtles (Fig. 2A), which is not far from the value reported for human RBCs measured using a MBB-based method (3.8± 1.0 µmol l^{-1}) (Tan et al., 2017). RBC free H_2S increased 2-fold in cold-acclimated turtles in normoxia but did not change further in anoxia (Fig. 2A), as for the other tissues (Fig. 1A). BSS concentrations in RBCs were remarkably high in warm normoxic turtles (68±3 µmol l^{-1}) compared with those of other tissues and increased even more in cold-acclimated normoxic and anoxic turtles (121±8 and 91±9 µmol l^{-1}, respectively) (Fig. 2B). These high values of BSS in RBCs appear to be consistent with the high content (~24 mmol l^{-1}) of total reactive thiols in *T. scripta elegans* RBCs, including those of hemoglobin (Hb) and GSH (Damsgaard et al., 2013; Jacobsen et al., 2012; Petersen et al., 2018). Thiols can potentially be partly sulfhydrated (i.e. R-S-SH) and contribute to the

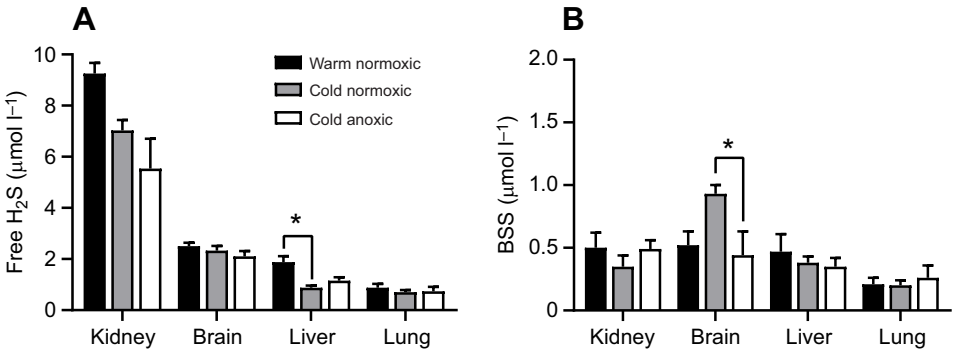

Fig. 1. Free H_2S and bound sulfane sulfur (BSS) in tissues of *Trachemys scripta elegans*. Tissue concentration of (A) free H_2S and (B) BSS in warm normoxic turtles (*N*=5 brain, liver, lung; *N*=3 kidney) and cold normoxic turtles (*N*=5 brain, liver, lung; *N*=4 kidney) and cold anoxic turtles (*N*=4 for all tissues). Data are means±s.e.m. Significant differences (ANOVA/Kruskal–Wallis tests) are indicated (*P<0.05).

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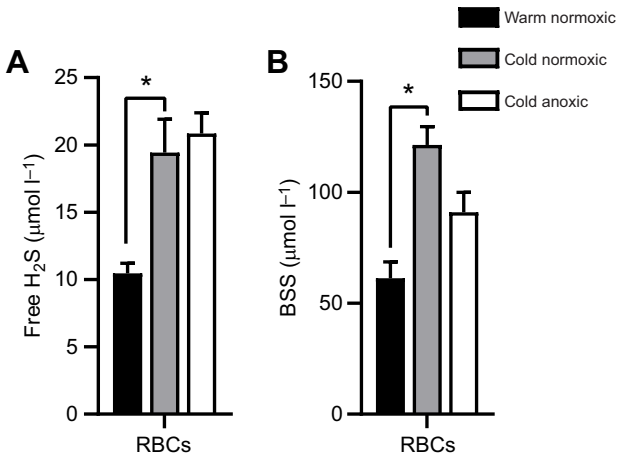

Fig. 2. Free H_2S and BSS in *T. scripta elegans* red blood cells (RBCs). Concentration in RBCs of (A) free H_2S and (B) BSS in warm normoxic turtles (*N*=4), cold normoxic turtles (*N*=5) and cold anoxic turtles (*N*=4). Data are means±s.e.m. Significant differences (ANOVA/Kruskal–Wallis tests) are indicated (*P<0.05).

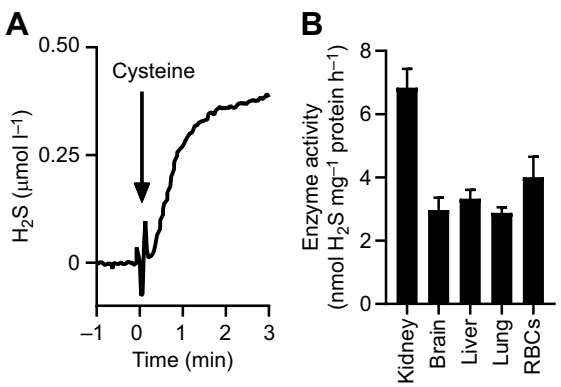

Fig. 3. H_2S enzyme activity in tissues of *T. scripta elegans*. (A) Representative trace from a H_2S microsensor. (B) Enzymatic production of H_2S in tissues and RBCs of warm normoxic turtles. *N*=5 brain, liver, lung; *N*=4 RBCs; *N*=3 kidney. Data are means±s.e.m.

BSS pool, and in *T. scripta elegans* Hb, we have previously identified bound persulfide and polysulfides by mass spectrometry (Petersen et al., 2018). Considering that protein sulfhydration is a pervasive modification (Mishanina et al., 2015) estimated to be up to 10–25% for certain proteins (Mustafa et al., 2009), the values of ∼70–120 µmol l^{-1} detected here indicate a maximum of 0.25–0.5% of total protein thiol sulfhydration in turtle RBCs, which is not unrealistic. Sulfur extraction from GSH is expected to be only 0.01% under the conditions used in the MBB methods (Montoya et al., 2015), and would therefore not contribute much. In addition to protein sulfhydration, some of the BSS pool may originate from H_2S coordinated to the ferric heme in metHb (Jensen and Fago, 2018). MetHb in freshwater turtles is ∼1.0% of total Hb (Maginniss et al., 1983), which corresponds to ∼20 µmol l^{-1} ferric heme capacity available for H_2S binding and release (Jensen and Fago, 2018). Therefore, these data suggest that *T. scripta* RBCs may act as a circulating storage pool of bound sulfide, that can either take up or regenerate free H_2S by varying cellular redox conditions, pH and body temperature (Fig. 2).

Enzymatic H_2S production

We added cysteine to tissue homogenates to detect the generation of H_2S by the combined enzymatic activity of CBS and CSE (Vicente et al., 2016) by using a H_2S-specific microsensor (Fig. 3A). The enzymatic activity of H_2S production was highest in the kidneys, which aligns with the high concentration of free H_2S in this tissue (Fig. 3B). RBCs, brain, liver and kidney all showed comparable enzymatic activity (Fig. 3B). This finding implies that the high levels of free H_2S and BSS detected in RBCs (Fig. 2) are not due to a particularly high capacity for enzymatic production, and further supports a role for RBCs as a circulating H_2S reservoir.

H_2S versus NO

H_2S and NO are involved in numerous physiological functions. NO upregulation may contribute to metabolic suppression in *T. scripta* (Fago and Jensen, 2015; Jensen et al., 2014), but apparently not in hibernating brown bears (Revsbech et al., 2014). In contrast, free H_2S and BSS decrease in the blood of hibernating bears (Revsbech et al., 2014), whereas in turtle blood, free H_2S and BSS increase during cold acclimation, but show no further change during anoxia (Fig. 2). This suggests that NO may be the major gasotransmitter regulating physiological responses in turtles during anoxia. But, H_2S could play a role in cytoprotection, as suggested by the enlarged BSS storage pool of cold-acclimated turtles (Fig. 2B).

Concluding remarks

In conclusion, we observed tissue-specific changes in endogenous levels of free H_2S and BSS upon cold acclimation and 9 days of anoxia in *T. scripta elegans* liver and brain and especially RBCs. In warm normoxic turtles, the levels of free H_2S were highest in RBCs and kidney and lowest in brain, liver and lung, which matched the observed enzymatic production rates of H_2S. BSS levels were 100-fold higher in RBCs than in other tissues, indicating that turtle RBCs may function as a circulating reservoir for bioactive H_2S bound to Hb's ferric heme and thiols as persulfide and polysulfides. Besides changes in brain BSS, free H_2S and BSS levels increased during cold acclimation only in the RBCs, where they remained high during anoxia. Interestingly, levels of NO metabolites have been found to increase dramatically in the blood (Jacobsen et al., 2012) of anoxia-acclimated *T. scripta*. In contrast, in plasma of hibernating bears, free H_2S and BSS decrease, while nitrite did not change significantly (Revsbech et al., 2014). These differences suggest distinct effects on aerobic or anaerobic metabolic suppression mediated by NO and H_2S, respectively, that are yet to be fully unraveled.

Acknowledgements

We thank Amanda Bundgaard for assisting with turtle acclimation, Inge Grønvall Revsbech for support during tissue sampling and Elin E. Pedersen for technical assistance with lactate measurements.

Competing interests

The authors declare no competing or financial interests. C.G.K. has intellectual property regarding sulfide measurement technologies and a commercial interest in Innolyzer Labs, LLC, Shreveport, LA 71103, USA.

Author contributions

Conceptualization: B.J., A.F.; Methodology: B.J., S.P., C.G.K.; Formal analysis: B.J., S.P.; Investigation: B.J., S.P.; Resources: C.G.K., A.F.; Writing - original draft: B.J.; Writing - review & editing: B.J., C.G.K., A.F.; Supervision: C.G.K., A.F.; Funding acquisition: B.J., C.G.K., A.F.

Funding

This work was supported by Det Frie Forskningsråd | Natur og Univers (grant 4181-00094 to A.F.), by Aarhus Universitets Forskningsfond (NOVA grant AUFF-E-2016-9-37 to A.F.) and by a Company of Biologists travel grant to B.J. Results in this report were also supported by an Institutional Development Award (IDeA) from the National

Institute of General Medical Sciences of the U.S. National Institutes of Health under grant number P20GM121307. Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.203976.supplemental

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