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Mitochondrial responses to anoxia exposure in red eared sliders (*Trachemys scripta*)

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A B S T R A C T

When deprived oxygen, mitochondria from most vertebrates transform from the main site of ATP production to the dominant site of cellular ATP use due to the reverse functioning of the F1FO-ATPase (complex V). The anoxia-tolerant freshwater turtle *Trachemys scripta* however, has previously been shown to inhibit complex V activity in heart and brain in response to anoxia exposure, but the regulatory mechanism is unknown. To gain insight into the putative regulatory mechanisms underlying the anoxia-induced inhibition of complex V in *T. scripta*, we examined the effects of two weeks anoxia exposure at 4 °C on the mitochondrial proteome and candidate mechanisms that have been shown to regulate complex V in other organisms. In *T. scripta*, we confirmed that anoxia exposure resulted in a > 80% inhibition of complex V in heart, brain and liver. Incubation of mitochondria with the nitric oxide donor, s-nitrosoglutathione, did not affect complex V activity despite showing the expected inhibition in mice. Proteomics analysis showed anoxia-induced decreases in three peripheral stalk subunits of complex V, possibly pointing to a unique site of regulation. Proteomics analysis also revealed differential expression of numerous enzymes involved with the electron transport system, the tricarboxylic acid cycle, as well as lipid and amino acid metabolism in response to anoxia exposure.

1. Introduction

For the vast majority of vertebrates, oxygen is a pre-requisite for life because of its vital role in mitochondrial oxidative phosphorylation and energy metabolism. As a result, survival in environments devoid of oxygen (anoxia) is in the order of minutes and even short anoxia exposure can cause irreversible damage to organs systems especially the heart and brain. The mitochondrion has long been recognized as the *sine qua non* for hypoxia/anoxia related cell dysfunction. In most hypoxia/anoxia-intolerant organisms, oxygen deprivation at the mitochondria results in an inner-mitochondrial membrane depolarization due to the inability of the ETS to oxidize substrates (Griffiths, 2012). As a result of the membrane depolarization, the mitochondrial F_1F_O-ATP synthase (complex V of the ETS) runs in reverse hydrolyzing ATP, consequently turning the mitochondria into a major site of ATP consumption in the cell (Rouslin et al., 1990; Boutilier and St-Pierre, 2002). This, along with the hypoxia/anoxia-linked reduction in ATP production, ultimately leads to a cellular energy deficit, failure of essential ATP dependent processes, and ultimately cell death (Sanderson et al., 2013; Penna et al., 2013).

While this anoxia-sensitivity applies to most vertebrates, there are groups of facultative anaerobes that can survive anoxia for extended periods. The North American freshwater turtles, *Trachemys scripta* and *Chrysemys picta*, are among the champions of vertebrate facultative anaerobes because of their ability to survive for up to 4 months in cold, anoxic, ice-covered ponds (Jackson, 2002). The biochemical strategies that *T. scripta* and *C. picta* employ to survive anoxia have been well studied and involve a strong metabolic rate depression (Hochachka et al., 1996; Hochachka and Lutz, 2001), storage and use of large glycogen reserves for oxygen-independent ATP production (Storey, 2004; Bickler and Buck, 2007), buffering of acidosis and lactate accumulation by the turtle shell (Jackson et al., 2000), and a remodeling of the mitochondria to reduce aerobic capacity and wasteful ATP consumption (Galli et al., 2013; Pamenter et al., 2016).

Anoxia-induced inhibition of complex V has been observed in mitochondria from both heart (Galli et al., 2013) and brain (Pamenter et al., 2016) of *T. scripta* following two weeks exposure to anoxia at 4 °C. A similar anoxia-induced inhibition of complex V has also been

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; Complex I, NADH dehydrogenase; Complex II, succinate dehydrogenase; Complex III, coenzyme q - cytochrome *c* reductase; Complex IV, cytochrome *c* oxidase; Complex V, F1Fo-ATPase; CS, citrate synthase; ETS, electron transport system; GSNO, s-nitrosoglutathione; IF_1, inhibitory factor-1; LC-MSMS, liquid chromatography mass spectrometry mass spectrometry; NO, nitric oxide; SP3, Single-Pot Solid-Phase-enhanced Sample Preparation; STAGE, STop And Go Extraction; TCA, tricarboxylic acid

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reported in skeletal muscle of another facultative anaerobe, *Rana temporaria* (Boutilier and St-Pierre, 2000), indicating that it might be a common strategy for energy conservation among facultative anaerobes. Despite this potentially conserved response, the mechanisms responsible for this inhibition remain unknown. In mammals, complex V activity has been shown to be regulated through direct protein s-nitrosylation. Indeed, incubation of mitochondrial fractions from mouse heart with s-nitrosoglutathione (GSNO), a nitric oxide (NO) donor, decreased complex V activity in a dose dependent manner (Sun et al., 2007). NO signaling has previously been implicated in the adaptive response of turtles to anoxia because of the anoxia-induced increases in circulating nitrates and s-nitrosylated compounds during anoxia exposure (Jacobsen et al., 2012; Fago and Jensen, 2015).

Beyond the inhibition of complex V, anoxia exposure in *T. scripta* (20 h anoxia at 7 °C) resulted in an up regulation of the mitochondrial genes *MT-NAD5* [subunit 5 of NADH dehydrogenase (complex I)] and *MT-COX1* [subunit 1 of cytochrome C oxidase (complex IV)] in heart, liver, and kidney (Cai and Storey, 1996) and increased expression of *MT-NAD4* (subunit 5 of complex I) and *MT-CYTB* (cytochrome *b* of complex III) in liver (Willmore et al., 2001). These studies suggest that anoxia exposure may also affect the function of several ETS complexes and possibly other aspects of mitochondrial function. It is not known however, whether changes in gene expression of mitochondrial proteins during anoxia actually affects protein content. Whole cell proteomics (2D-gel electrophoresis) from brain homogenates of 24 h anoxia exposed *C. picta* (18 °C) revealed a general decrease in glycolytic enzymes and apoptotic proteins but did not detect mitochondrial proteins (Smith et al., 2015). Thus, a more focused analysis of the mitochondrial proteome in anoxia turtles is needed to better understand how anoxia affects mitochondrial function in turtles.

The objectives of the present study were two fold. First, we adopted a candidate-systems approach to determine the mechanism(s) responsible for the anoxia-induced down-regulation of complex V in *T. scripta* (Galli et al., 2013). Specifically, we characterized enzyme kinetics in isolated mitochondria, the pH sensitivity of complex V activity, and investigated the role of nitrosylation in regulating complex V. Second, we adopted a discovery-based proteomics approach in heart mitochondria isolated from normoxic and anoxic turtles to identify changes in the mitochondrial proteome that may be involved in the inhibition of aerobic metabolism and complex V.

2. Materials and methods

Thirty adult turtles, *Trachemys scripta*, (250 to 500 g) were obtained from Niles Biological (Sacramento, CA, USA) and transported to The University of British Columbia (Vancouver BC Canada). Upon arrival, turtles were placed in 100 L holding tanks at 18 °C with basking platforms equipped with UVA/UVB heat lamp (12 h:12 h, light:dark) with continuous access to food. Turtles were allowed to recover from transport for 8 weeks before experimentation. To begin the experiment, turtles were paired and transferred to plastic aquaria (30 cm × 15 cm) held within a temperature-controlled chamber. Water was added to each aquarium to a depth of ~2.5 cm (water not exceeding the plastron). Over the subsequent 2 weeks, temperature was gradually reduced from 18 °C to 4 °C and light:dark cycles were shifted to 8 h:16 h. Turtles were held under these final conditions for an additional 4 weeks after which the turtles were randomly assigned to one of two groups: anoxia (n = 15) and normoxia (n = 15). To induce anoxia, individual turtles were enclosed in weighted mesh cages and submerged in water-filled glass aquaria. The aquaria were then fitted with lids sealed using vacuum grease and bubbled with N_2 gas. The excess N_2 gas was allowed to escape the aquaria through a small one-way valve. The dissolved oxygen was measured daily using a handheld dissolved oxygen probe (Oakton DO 110 Series, IL, USA) and never exceeded 1% air saturation. The anoxia-exposed turtles were held in complete darkness to avoid light-induced increases in activity (Madsen et al., 2013). The remaining (normoxic) turtles were held at 4 °C in 8:16 light:dark for an additional 2 weeks. After the exposures, individual turtles were removed from their aquaria and euthanized *via* decapitation. The brain and liver were quickly dissected and frozen in liquid N_2. A portion of the heart ventricle was also frozen in liquid N_2 and the remainder from some animals were used for mitochondrial isolation. Not all turtle samples were used in all analyses (n values for each analysis is given in figure captions). All experimental procedures were approved by The University of British Columbia animal care committee under Animal Use Protocol A13-0254.

2.1. Tissue preparation

2.1.1. Whole tissue

In order to characterize the effects of anoxia on complex V activity and identify potential regulators, we chose to work with brain, liver, and heart tissue homogenates as well as isolated heart mitochondria (procedures for mitochondrial isolation are below). Tissue homogenates were prepared and assayed as previously described (Galli et al., 2013). For enzymatic assays, tissues where ground into a fine powder under liquid N_2 using a mortar and pestle. A100 mg aliquot of ground tissue was transferred to a 1.5 ml microcentrifuge tube filled with 500 µl of ice-cold hypotonic medium (25 mM K_2HPO_4, 5 mM $MgCl_2$, pH 7.2) and samples were sonicated for three 10-s bursts on ice (Kontes sonicator, Vineland, NJ, USA). The resulting homogenate was centrifuged at 600*g* for 10 min at 4 °C and the supernatant transferred to a new micro centrifuge tube and the centrifugation repeated and the final supernatant distributed among several microcentrifuge tubes and frozen at −80 °C. Protein concentration in the final supernatant was determined by the Bradford protein assay (Bradford, 1976). These samples were used for analysis of tissue homogenate complex V activity and effects of s-nitrosylation.

2.1.2. Isolated heart mitochondria

Mitochondria were isolated from heart tissue as previously described by (Almeida-Val et al., 1994). Heart ventricle muscle was dissected from connective tissue and rinsed with isolation buffer (250 mM sucrose, 0.5 mM NaEDTA, 10 mM Tris, 0.5% fatty acid free BSA, pH 7.4, 4 °C) to remove blood. Tissue was minced on ice using scissors and fresh isolation buffer. Minced tissue was then digested using 10 ml of Trypsin (Type IX, Sigma-Aldrich) for 8 min, then resuspended in Trypsin Inhibitor (Type I-S, Sigma-Aldrich) for 2 min. The digested tissue samples was then transferred to a glass mortar and homogenized in isolation buffer using a loose fitting Teflon pestle for 30 s on ice. Homogenate was transferred to a polycarbonate centrifuge tube and centrifuged at 600*g* for 10 min at 4 °C. Supernatant was filtered through glass wool and centrifuged again at 9000*g* for 10 min at 4 °C. Supernatant was discarded and the resulting pellet was gently washed with isolation buffer, resuspended in buffer and centrifuged again at 9000*g* for 10 min at 4 °C. The final pellet was resuspended in 400 µl of isolation buffer. Protein levels were determined by Bradford protein assay (Bradford, 1976) then the mitochondrial suspension was aliquoted into several micro centrifuge tubes and frozen at −80 °C. These samples were used for analysis of Michaelis-Menten kinetics of complex V activity, pH sensitivity, and proteomics analysis.

2.2. Enzymatic assays

2.2.1. Complex V activity

Complex V activity was determined spectrophotometrically using a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) in assay buffer (25 mM K_2HPO_4, 5 mM $MgCl_2$, 100 mM KCl, 2.5 mg/ml BSA; pH 7.4). Activity was measured as oxidation of NADH (340 nm) for 20 min (5 mM ATP, 2 mM PEP, 2 mM NADH, 3 U μl^{-1} lactate dehydrogenase 3 U μl^{-1} and pyruvate kinase) in the absence or presence of 0.5 mg ml^{-1} oligomycin. The effects of different ATP concentrations (0.05, 0.1, 0.5, 1.0, 2.5, 5 mM ATP, pH 7.4) on the reaction rate of

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