Mitochondrial responses to prolonged anoxia in brain of red-eared slider turtles

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Mitochondria are central to aerobic energy production and play a key role in neuronal signalling. During anoxia, however, the mitochondria of most vertebrates initiate deleterious cell death cascades. Nonetheless, a handful of vertebrate species, including some freshwater turtles, are remarkably tolerant of low oxygen environments and survive months of anoxia without apparent damage to brain tissue. This tolerance suggests that mitochondria in the brains of such species are adapted to withstand prolonged anoxia, but little is known about potential neuroprotective responses. In this study, we address such mechanisms by comparing mitochondrial function between brain tissues isolated from cold-acclimated red-eared slider turtles (Trachemys scripta elegans) exposed to two weeks of either normoxia or anoxia. We found that brain mitochondria from anoxia-acclimated turtles exhibited a unique phenotype of remodelling relative to normoxic controls, including: (i) decreased citrate synthase and F1F0-ATPase activity but maintained protein content, (ii) markedly reduced aerobic capacity, and (iii) mild uncoupling of the mitochondrial proton gradient. These data suggest that turtle brain mitochondria respond to low oxygen stress with a unique suite of changes tailored towards neuroprotection.

1. Introduction

Mitochondria are the lynchpin of aerobic metabolism. In normoxia, mitochondria consume more than 90% of the oxygen acquired by an organism to facilitate the pumping of protons (H+) across the inner mitochondrial membrane [1]. This work generates the proton-motive force that energizes the phosphorylation of ADP to ATP via the F1F0-ATPase [2]. Through this action, mitochondria generate the majority of a cell’s energy in an oxygen-dependent manner and are thus well suited to serve as biological oxygen sensors [2]. Fittingly, mitochondria also coordinate downstream cellular responses to hypoxia. For example, mitochondria (i) are the primary source of cellular reactive oxygen species (ROS) generation, which can trigger hypoxia-inducible factor-dependent gene transcription, and also directly modulate membrane protein activity; (ii) are a major sink for cellular Ca2+, a potent second messenger that mediates neuronal excitability and signalling; and (iii) affect the cellular energy balance and thereby the activity of AMP-activated protein kinase, a master switch of cellular energetics [2]. Through such mechanisms, mitochondria function as a signalling hub that coordinates the cells’ defence strategy against low oxygen stress [2].

In anoxia, however, mitochondria become a liability. Deprived of oxygen to serve as the terminal electron acceptor in the electron transport chain (ETC), the mitochondrial F1F0-ATPase reverses, hydrolysing ATP into ADP in order to maintain the proton-motive force, and thereby robbing the cell of valuable fuel reserves [3]. In addition, mitochondrial dysfunction is a central contributor to hypoxic/anoxic cell death; either by triggering programmed cell death pathways or by generating deleterious bursts of ROS upon reoxygenation [2]. Nonetheless, hypoxic and anoxic environments are common, particularly in...
The dearth of information regarding mitochondrial adaptations responses to light cues consistent with a spring thaw [8]. The functionality in prolonged anoxia to facilitate behavioural indeed, anoxia-tolerant turtle neural networks retain some cell function cannot be entirely shut down in anoxia, and avoid deleterious increases in cytosolic Ca²⁺ also of the mitochondrial H⁺ unique in that maintenance of neuronal energy charge and phosphorylation [6]. Brain cells are also physiologically anoxia as it produces a large majority of its ATP via oxidative particular interest because brain is exquisitely sensitive to brains of anoxia-tolerant species. Such mechanisms are of little is known regarding mitochondrial adaptations in the mitochondrial adaptations to anoxia in muscular tissue, very aquatic habitats, and these niches are populated by species with physiological adaptations allowing them to tolerate a lack of oxygen. Not surprisingly, studies of these species have revealed important adaptations at the mitochondrial level that limit the deleterious effects of hypoxia. For example, F₁F⁰-ATPase activity is reduced by approximately 95% in skeletal muscle of the anoxia-tolerant frog Rana temporaria [4] and by approximately 85% in the heart of red-eared slider turtles (Trachemys scripta elegans) [5]. This adaptation is thought to prolong cellular viability by limiting ATP consumed by reversed activity of the F₁F⁰-ATPase in anoxia.

Despite recent advances in our understanding of mitochondrial adaptations to anoxia in muscular tissue, very little is known regarding mitochondrial adaptations in the brains of anoxia-tolerant species. Such mechanisms are of particular interest because brain is exquisitely sensitive to anoxia as it produces a large majority of its ATP via oxidative phosphorylation [6]. Brain cells are also physiologically unique in that maintenance of neuronal energy charge and also of the mitochondrial H⁺ gradient are obligatory to avoid deleterious increases in cytosolic Ca²⁺, which can trigger excitotoxic cell death [7]. As a result of these demands, brain cell function cannot be entirely shut down in anoxia, and indeed, anoxia-tolerant turtle neural networks retain some functionality in prolonged anoxia to facilitate behavioural responses to light cues consistent with a spring thaw [8]. The dearth of information regarding mitochondrial adaptations in the brain of anoxia-tolerant species represents a major gap in our understanding of naturally evolved cellular anoxia-tolerance. Therefore, we exposed cold-acclimated red-eared slider turtles, which are among the most anoxia-tolerant vertebrates identified [9], to two weeks of chronic anoxia and examined the impact of this treatment on brain mitochondrial function.

2. Abridged methodology

Twenty-two adult female red-eared slider turtles were cold-acclimated to 5°C for four to five weeks and then randomly divided into two groups: normoxic and anoxic (n = 11 each). Turtles were held at these conditions for two weeks before experimentation.

A complete description of experimental approaches can be found in the electronic supplemental material section. Briefly, following treatment turtles were decapitated and whole brains were extracted, homogenized and then permeabilized with 4 mM saponin for 45 min on ice [10]. F₁F⁰-ATPase and citrate synthase (CS) enzyme activity and protein content were assessed in whole brain using spectrophotometric assays or Western blot approaches, respectively [5]. Permeabilized brain cell mitochondrial respiration and membrane potential (Vₘ) were measured with an Oroboros Oxygraph 2k high-resolution respirometry system (Oroboros

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**Figure 1.** ETC flux and complex V (F₁F⁰-ATPase) activity are decreased following two weeks of anoxia. (a,b) Summary of CS activity (a) and protein expression (b) in brains from turtles exposed to two weeks of normoxia (black bars) or anoxia (white bars). (c) Sample blots of CS protein expression. (d,e) Summaries of complex V activity (d) and protein expression (e). (f) Sample blots of complex V protein expression. (g) Mitochondrial respiratory flux rates. (h) Total ETC capacity. (i) Individual complex respiratory rates. Data are means ± s.e.m. Numbers in parentheses indicate n. Asterisks indicate significant differences between normoxia- and anoxia-acclimated turtles (p < 0.05).

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**Diagram:**

- **(a)**: Enzyme activity (nmol min⁻¹ mg⁻¹) for citrate synthase.
- **(b)**: Protein expression (normalized to GAPDH) for citrate synthase.
- **(c)**: Sample blots of citrate synthase (52 kDa) and GAPDH (36 kDa).
- **(d)**: Enzyme activity (nmol min⁻¹ mg⁻¹) for complex V.
- **(e)**: Protein expression (normalized to GAPDH) for complex V.
- **(g)**: Mitochondrial respiration rate (% ETC) for normoxia and anoxia.
- **(h)**: Citrate synthase activity (nmol min⁻¹ mg⁻¹) for different states (II, III, IV).
- **(i)**: Mitochondrial respiration rate (µmol O₂ min⁻¹ mg⁻¹) for different complex states (I, II, III, IV).
### 3. Results and discussion

(a) **Citrate synthase and F$_1$F$_0$-ATPase enzyme activity, but not protein content, are decreased in anoxic brain**

We measured CS activity from brain tissue as a marker for oxidative capacity. In anoxic mitochondria, CS activity decreased by approximately 20% relative to normoxic animals (figure 1a; $t_{10} = 2.71$, $p = 0.02$). This suggests an overall reduction in the aerobic capacity of brain in response to two weeks of anoxia. This change is tissue-specific to turtle brain because CS activity does not change in turtle heart following acclimatization to anoxia [5]. The anoxic decrease in CS activity is likely not indicative of a change in mitochondrial volume density, however, because total CS protein expression was not different between treatments (figure 1b; $t_{10} = 0.78$, $p = 0.45$). The mechanism by which CS activity is decreased in anoxia may involve post-translational modifications but further experiments are required to evaluate this possibility. In addition, F$_1$F$_0$-ATPase activity decreased by approximately 80% in anoxic samples (figure 1c; $t_{10} = 0.04$, $p = 0.97$), which is similar to the 85% decrease previously reported in anoxic turtle heart mitochondria [5]. F$_1$F$_0$-ATPase protein expression was unchanged by anoxia (figure 1d; $t_{10} = 7.79$, $p < 0.0001$), indicating that post-translational modification of the F$_1$F$_0$-ATPase or cellular inhibitory factors may regulate F$_1$F$_0$-ATPase activity in anoxic brain [4].

(b) **Electron transport chain flux and $\Psi_m$ are reduced by chronic anoxia**

Next, we confirmed that anoxic brain mitochondria have decreased oxidative capacity by examining ETC respiratory flux and $\Psi_m$ using a SUIT protocol. A two-way repeated measures ANOVA revealed a significant treatment effect between normoxia and anoxia on ETC respiratory flux (treatment: $F_{1,14} = 52.11$, $p < 0.0001$; interaction: $F_{1,14} = 0.86$, $p = 0.64$). Further analysis with Bonferroni post-tests revealed specific changes in state II (substrate-fuelled), III (ADP-fuelled) and IV$_{oligo}$ (succinate-fuelled in the presence of oligomycin A) mitochondrial respiration rates, which were 24%, 55% and 26% lower, respectively, in anoxia-acclimated brain (figure 1g). Furthermore, analysis of the maximum total respiration capacity of the ETC in fully uncoupled mitochondria (by FCCP (carbonyl cyanide-4-phenylhydrazone addition) revealed a 31.5% reduction in ETC capacity in anoxic brain (figure 1h). Measurements of the individual components of the ETC revealed that complex I activity decreased by 59% in anoxic brain but the activities of complexes II–IV were not different when these were normalized to their respective total ETC capacities (figure 1i). Therefore, the anoxic decreases in ETC capacity and O$_2$ consumption are likely the result of reverse inhibition due to downregulation of the F$_1$F$_0$-ATPase in anoxia, with an additional minor contribution from decreased CS activity that likely inhibits complex I respiration. In addition, a two-way repeated measures ANOVA revealed a significant treatment effect between normoxia and anoxia on $\Psi_m$ (treatment: $F_{1,14} = 10.88$, $p < 0.0001$; interaction: $F_{1,14} = 0.92$, $p = 0.38$), matching the treatment effect observed for ETC flux; however, this trend did not reach significance with any individual treatment (electronic supplementary material, figure S1).

Overall, the respiratory flux pattern observed in figure 1 is divergent from anoxic turtle heart mitochondria [5]: anoxia-mediated changes in turtle brain result in a more robust downregulation of the ETC as a whole, whereas in turtle heart, reductions in the respiration rates of individual ETC complexes are observed. Conversely, the decrease in F$_1$F$_0$-ATPase activity is similar between turtle brain and heart, but reduced relative to skeletal muscle from hypoxia-
acclimated mitochondria from anoxic turtles, the decrease in F_{O}F_{O}ATPase activity is significantly greater than the associated decreases observed in ETC flux, indicating that even when F_{O}F_{O}ATPase activity is markedly reduced during prolonged anoxia, there is still sufficient capacity in complex V to match ETC function.

(c) The mitochondrial H\(^{+}\) gradient is less tightly coupled in anoxia and \(\psi_{m}\) is depolarized

Next, we compared kinetics of the mitochondrial H\(^{+}\) gradient between treatments. In general, a two-way repeated measures ANOVA revealed that mitochondria from anoxia-acclimated turtles had higher rates of O\(_{2}\) consumption and a more depolarized \(\psi_{m}\) than mitochondria from normoxia-acclimated turtles (figure 2a; treatment: \(F_{1,11} = 9.22, p < 0.0001\); interaction: \(F_{1,11} = 2.39, p = 0.18\)). This indicates either that anoxic brain mitochondria are leakier to H\(^{+}\) or that other ion pumps are functioning at the expense of the H\(^{+}\) gradient. For example, in vitro, turtle brain mitochondria become depolarized when exposed to acute anoxia due to the activation of mitochondrial ATP-sensitive K\(^{-}\) (mKATP) channels [12]. mKATP channel activation increases mitochondrial membrane permeability to K\(^{-}\), and the resulting K\(^{+}\) flux through these channels must be opposed by the activity of H\(^{+}\)-fuelled antiporters. This futile K\(^{+}\) cycle thereby mildly uncouples the H\(^{+}\) gradient from ATP production but initiates glutamatergic channel arrest [13], a key neuroprotective mechanism against anoxia in turtle brain. Channel arrest persists for at least the first three weeks of anoxic exposure in turtles [14]; mild uncoupling of the mitochondrial H\(^{+}\) gradient due to mKATP channel activation would explain the observed shift.

(d) Anoxia- and normoxia-acclimated brain mitochondria are equally tolerant of acute anoxic stress

Finally, we tested the sensitivity of mitochondria to an acute anoxic challenge by comparing respiration rates in substrate-fuelled state III-respiring mitochondria before and after 20 min of anoxia with a repeated measures two-way ANOVA. We found no difference between respiration rates within or between groups (figure 2b; \(F_{1,11} = 5.64, p = 0.84\)), indicating that both sets of mitochondria were undamaged by the anoxia/reperfusion stress. In support of this conclusion, previous examinations of mitochondrial ROS generation in anoxic turtle brain demonstrated decreased ROS levels during acute anoxia in vitro and deleterious bursts of ROS are not observed during reperfusion [15]. These data suggest that a unique suite of cytoprotective mechanisms are involved in preventing deleterious reoxygenation injury in turtle brain.

(e) Summary

We demonstrate that turtle brain mitochondria respond to two weeks of anoxia-acclimatization by decreasing: (i) CS and F_{O}F_{O}ATPase activity, (ii) ETC respiratory flux, and (iii) H\(^{+}\) gradient leak. However, brain mitochondria from normoxic and anoxic turtles are equally tolerant of an acute anoxic challenge, suggesting that these mitochondria possess endogenous defence mechanisms that are chronically activated and do not require mitochondrial remodelling during prolonged anoxic exposure. These results highlight that mitochondria from different species can respond very differently to similar environmental challenges. By reducing the respiratory capacity of mitochondria during prolonged anoxia, turtle brains are likely able to realize energy savings, while still retaining sufficient scope to resume normal function rapidly upon the detection of light cues indicating a spring thaw [8]. Our results demonstrate that turtle brain mitochondria exhibit a hybrid adaptive response to anoxia that is likely tailored to the specific limitations and demands of brain function during anoxia.

References


