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REVIEW

Mitochondria from anoxia-tolerant animals reveal common strategies to survive without oxygen

Gina L. J. Galli · Jeffrey G. Richards

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Abstract The mitochondrion plays a critical role in the development of Oxygen (O_2)-related diseases. While research has predominantly focused on hypoxia-sensitive mammals as surrogates for humans, the use of animals which have naturally evolved anoxia tolerance has been largely ignored. Remarkably, some animals can live in the complete absence of O_2 for days, months and even years, but surprisingly little is currently known about mitochondrial function in these species. In contrast to mammals, mitochondrial function in anoxia-tolerant animals is relatively insensitive to in vitro anoxia and reoxygenation, suggesting that anoxia tolerance transcends to the level of the mitochondria. Furthermore, long-term anoxia is associated with marked changes in the intrinsic properties of the mitochondria from these species, which may afford protection against anoxia-related damage. In the present review, we highlight some of the strategies anoxia-tolerant animals possess to preserve mitochondrial function in the absence of O_2. Specifically, we review mitochondrial Ca^{2+} regulation, proton leak, redox signaling and mitochondrial permeability transition, in phylogenetically diverse groups of anoxia-tolerant animals. From the strategies they employ, these species emerge as model organisms to illuminate novel interventions to mitigate O_2-related mitochondrial dysfunction in humans.

Keywords Mitochondria · Anoxia · Ectothermic · Electron transport chain · Mitochondrial permeability transition pore · Proton leak

Introduction

Oxygen (O_2) is used as the terminal electron acceptor in the mitochondrial electron transport chain and it is, therefore, essential for the generation of metabolic energy (ATP) via oxidative phosphorylation. When O_2 becomes limited, the organism must rely on anaerobic respiration to fulfill their energy demands which yields <1/10th of the ATP produced by oxidative pathways. Due to the high energy demands of the brain and heart, ATP is rapidly exhausted in the absence of O_2, and energy-dependent processes associated with ion transport soon fail, leading to membrane depolarization and loss of intracellular ion homeostasis. This process initiates a deadly cascade of events within the mitochondria, beginning with Ca^{2+} overload and reactive O_2 species (ROS) generation and culminating in cellular apoptosis and necrosis (Griffiths 2012). For many animals, however, the availability of O_2 is a common environmental challenge. A reduction in O_2, termed hypoxia, can occur in a wide range of aquatic (intertidal, estuarine, swamps and floodplains) and terrestrial (high altitude and sealed burrows) habitats (Diaz and Breitburg 2009). Whether an animal becomes hypoxic in these environments depends on the species, as hypoxia is defined as the partial pressure of O_2 at which physiological function and metabolic rate cannot be maintained (Richards 2011). However, under extreme circumstances, many of these environments become completely devoid of O_2 (anoxic). When faced with anoxia,

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G. L. J. Galli (✉)
Faculty of Medicine and Human Sciences, University of Manchester, 2.4 Core Technology Facility, 46 Grafton St, Manchester M139NT, UK
e-mail: gina.galli@manchester.ac.uk

J. G. Richards
Department of Zoology, The University of British Columbia, 6270 University Blvd, Vancouver, BC V6T 1Z4, Canada

animal survival is dependent upon the continued functioning of anaerobic metabolic processes which yields <1/10th of the ATP produced by oxidative pathways. Nevertheless, some animals have successfully exploited anoxic environments and can survive without O_2 for hours, days, months and even years (Bickler and Buck 2007; Clegg 1997; Duerr and Podrabsky 2010; Jackson 2002; Nilsson and Renshaw 2004; Tattersall and Ultsch 2008).

The apparent disparity in anoxia tolerance between animals raises an interesting and important question: How do mitochondria from anoxia-tolerant animals avoid the pathological consequences of O_2 deprivation? As anoxia is associated with numerous pathologies in humans, including ischemia, angina and stroke, the answer to this question has far reaching, clinically relevant implications. In fact, the mitochondrion is now regarded as the lynchpin in the progression of anoxia-related pathologies in humans (Walters et al. 2012). As such, research efforts have been focused on developing the potential therapeutic applications of mitochondrial targeting (Walters et al. 2012). While this research has predominantly focused on anoxia-sensitive mammals as surrogates for humans, the use of animals which have naturally evolved anoxia tolerance has been largely ignored. Not surprisingly, anoxia-tolerant animals have evolved a diverse collection of unique physiological and biochemical adaptations that permit sustained life for prolonged periods without O_2 (Bickler and Buck 2007; Jackson 2000; Nilsson and Lutz 2004). It is these remarkable, anoxia-tolerant animals that we now turn to for understanding strategies of anoxia tolerance at the level of the mitochondrion.

The purpose of this review is to highlight strategies that animals employ to preserve mitochondrial function in the absence of O_2. While previous work and reviews on anoxia-tolerant animals have concentrated on the biochemical adaptations involved with glycolysis (Lutz 1992; Lutz and Nilsson 1997; Perez-Pinzon et al. 1997; Savina et al. 2009; Storey and Storey 1990), the effects of anoxia on oxidative phosphorylation and the electron transport chain have been largely ignored, presumably because these processes are O_2-dependent. However, cellular damage associated with anoxia/reoxygenation in mammals is mostly confined to the mitochondrial machinery involved with oxidative phosphorylation (see Fig. 1), and recent evidence suggests that anoxia-tolerant animals differ from mammals in this respect. Therefore, our review will concentrate exclusively on anoxia survival strategies that are specific to the apparatus associated with oxidative phosphorylation.

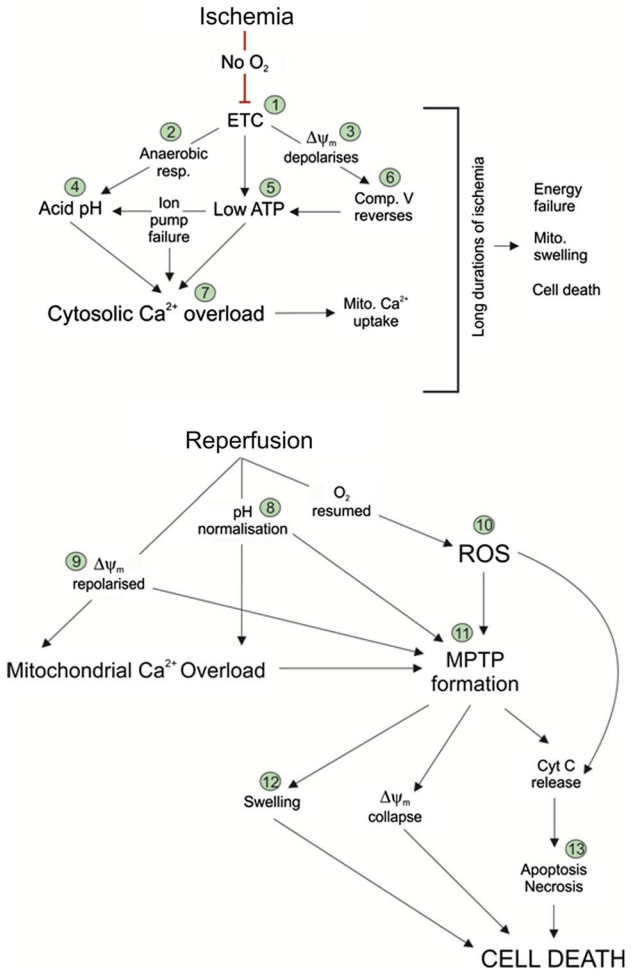

Fig. 1 The effects of ischemia and reperfusion on mitochondrial function in mammalian cardiomyocytes. During ischemia, a lack of O_2 inhibits the electron transport chain (ETC), which reduces ATP production and depolarises mitochondrial membrane potential ($\Delta\Psi_m$). The F_1F_o-ATPase (Complex V) runs in reverse and hydrolyses ATP in an attempt to maintain $\Delta\Psi_m$, further reducing ATP reserves. The reduction in ATP causes ion pump failure and the switch to anaerobic respiration leads to intracellular acidosis. Both of these factors contribute to cytosolic Ca^{2+} overload and mitochondrial Ca^{2+} uptake. If ischemia persists, energy reserves are exhausted, mitochondrial swelling occurs and cell death ensues. During reperfusion of a previously ischemic region, pH normalization and repolarization of $\Delta\Psi_m$ cause mitochondrial Ca^{2+} overload, and the reintroduction of O_2 causes the generation of reactive O_2 species (ROS). These factors contribute to the formation of the mitochondrial permeability transition pore (MPTP) which causes mitochondrial swelling, $\Delta\Psi_m$ collapse, cytochrome *c* (Cyt *c*) release and the activation of apoptopic and necrotic factors, ultimately leading to cell death. See text for full details. Information collated from (Brenner and Moulin 2012; Griffiths 2012; Penna et al. 2013; Walters et al. 2012). *Green circles* with *numbers* represent aspects of the cascade which have been studied in anoxia-tolerant animals; references are given in Table 1 (color figure online)

Mitochondrial dysfunction in anoxic-intolerant animals

Before we turn our attention to anoxia-tolerant animals, it is first instructive to review the sequence of events leading to mitochondrial dysfunction in an O_2-deprived mammalian mitochondria. The following section will broadly summarize the effects of O_2 deprivation on mammalian

mitochondrial function and the reader is referred to the following reviews for a more in-depth analysis (Griffiths 2012; Sanderson et al. 2013; Walters et al. 2012). Due to the clinical implications, most of the research concerning O_2 deprivation in mammalian mitochondria has concentrated on the effects of ischemia (cessation of blood flow) and reperfusion. Therefore, the description below is mainly derived from experiments on ischemic mitochondria. It should be noted that although anoxia is a consequence of ischemia, the two conditions are not identical, as ischemia is also associated with alterations in plasma ions, metabolites, and proteins (for example glucose, Ca^{2+}, K^+ and Mg^{2+}). A schematic diagram representing the effects of ischemia and reperfusion on mammalian cardiomyocyte function is given in Fig. 1 and summarized in the sections below.

Under aerobic conditions, the mitochondrion is the primary site of ATP production. Mitochondria produce ATP in two major steps (for reviews see (Gledhill and Walker 2006; Papa et al. 2012). First, electrons from substrate oxidation are donated to the electron transport chain and as they pass along the chain to the final electron acceptor (O_2), protons are pumped or consumed by metalloproteins (Complexes I, III and IV) from the matrix to the intermembrane space. The loss of protons from the mitochondrial matrix results in a transmembrane proton electrochemical gradient. Subsequently, protons flow back down their electrochemical gradient through a fifth protein complex (the ATP synthase, or F_1F_o-ATPase), which harvests the proton gradient to generate ATP from ADP and Pi (Papa et al. 2012). Without O_2, the final electron acceptor is not available and the electron transport chain will come to a halt (Griffiths 2012). As a consequence, protons will accumulate in the mitochondrial matrix and mitochondrial membrane potential ($\Delta\Psi_m$) depolarizes. The F_1F_o-ATPase, which normally produces ATP, switches into a reverse mode during ischemia and consumes ATP in an attempt to re-establish $\Delta\Psi_m$. Up to 50–80 % of the ATP consumed during ischemia, dependent on the species (Rouslin et al. 1990), is from the reversal of the F_1F_o-ATPase, transforming the mitochondrion from an ATP producer to the dominant ATP consumer. As ischemia progresses, ATP-dependent ion transporters (e.g., Na^+/K^+ ATPase, Ca^{2+} ATPase) fail and intracellular acidification occurs. As extracellular free Ca^{2+} concentrations are approximately 10,000 times that of the cytosol, Ca^{2+} entry is favored culminating in cytosolic Ca^{2+} overload. Once the maximum rate of Ca^{2+} uptake into the mitochondrial matrix exceeds the rate of efflux (Bernardi 1999), Ca^{2+} enters the matrix through the mitochondrial Ca^{2+} uniporter (Penna et al. 2013). Since the mitochondrial Ca^{2+} uniporter is electrophoretic and inhibited by a depolarized membrane, Ca^{2+} entry is limited during ischemia. Therefore, the fall in ATP and the increase in Ca^{2+} do not necessarily result in irreversible injury, at least not during moderate durations of ischemia (Murphy and Steenbergen 2008). However, if ischemia persists, high [Ca^{2+}] can activate degrading enzymes leading to the destruction of the membrane, and ATP depletion will lead to the failure of all energy requiring processes causing mitochondrial swelling and cell death (Penna et al. 2013).

Although essential for survival, reperfusion of a previously ischemic region paradoxically causes additional, substantial damage (Penna et al. 2013; Sanderson et al. 2013). The reintroduction of O_2 causes a burst of ROS, and the restoration of $\Delta\Psi_m$ drives entry of Ca^{2+} that accumulated during ischemia through the uniporter. These factors, coupled with the restoration of pH, initiate activation of the mitochondrial membrane permeability transition pore (MPTP) (Brenner and Moulin 2012). When open, the MPTP permits a sudden increase in the permeability of the inner membrane to solutes with a molecular mass <1,500 Da (Hunter et al. 1976). Proteins can now move freely across the inner membrane exerting a colloidal osmotic pressure that causes the mitochondria to swell (Halestrap et al. 2004), and the membrane becomes freely permeable to protons, causing the total collapse of $\Delta\Psi_m$. Further damage occurs as a result of ROS facilitating the detachment of cytochrome *c* from cardiolipin (Ott et al. 2007). Cytochrome *c* is then released into the cytosol due to the breakdown of the outer mitochondrial membrane as a consequence of MPTP opening. The release of cytochrome *c* into the cytosol activates mitochondrial apoptotic factors (e.g., apoptosis-inducing factor (AIF) and caspases), and eventually leads to apoptosis, necrosis and cell death (Murphy and Steenbergen 2011).

It should be noted that the precise sequence of events leading to ischemia-related cell death is dependent, among other things, on the duration of ischemia, the tissue type, and the species under investigation. In particular, opening of the MPTP is dependent on many interrelated factors (oxidative stress, voltage, pH, peptides and other small molecules) and will ultimately depend on the delicate balance between MPTP activators and inactivators acting in concert. For example, in the mammalian heart, it is generally accepted that factors leading to MPTP formation during ischemia (high matrix Ca^{2+} and depolarized $\Delta\Psi_m$) are offset by inhibitory factors (intracellular acidosis), so that MPTP opening is more likely to occur during reperfusion. However, this may not be the case in the brain (Gouriou et al. 2011). Thus, the duration of ischemia and the timing of events will be crucial in determining whether MPTP formation occurs. Nevertheless, despite the obvious clinical implications, this sequence of events has not been characterized in an anoxia-tolerant animal. Although information is available for certain aspects of this cascade from various different animals (see green circles in Fig. 1; Table 1), a

Table 1 Selected references for research conducted on aspects of mitochondrial function in anoxia-tolerant animals

	Aspect	Organism	References
1	Electron transport chain activity (anoxia)	Painted turtle, red eared slider, annual killifish, Eastern oyster, common frog	Birkedal and Gesser (2004), Duerr and Podrabsky (2010), Galli et al. (2013), Kurochkin et al. (2009), St-Pierre and Boutilier (2001)
2	Anaerobic metabolism (anoxia)	Selected reviews on various vertebrates and invertebrate strategies	De Zwaan and Putzer (1985), Hochachka et al. (1996), Lutz and Nilsson (1997), Perez-Pinzon et al. (1997)
3	Mitochondrial membrane potential (anoxia)	Red eared slider turtle, painted turtle, common frog	Galli et al. (2013), Hawrysh and Buck (2013), St-Pierre et al. (2000b)
4	Cytosolic pH regulation (anoxia)	Red eared slider turtle, common carp, brine shrimp, goldfish	Bickler (1992), Kwast et al. (1995), Stecyk et al. (2009), van den Thillart et al. (1989)
5	ATP levels (anoxia)	Annual killifish, brine shrimp, red eared slider turtle	Podrabsky et al. (2012), Stecyk et al. (2009)
6	Complex V reversal (anoxia)	Red eared slider turtle, painted turtle, common frog, annual killifish	Duerr and Podrabsky (2010), Galli et al. (2013), Hawrysh and Buck (2013), St-Pierre et al. (2000b)
7	Cytosolic Ca^{2+} (anoxia)	Red eared slider turtle, painted turtle	Bickler (1992), Bickler and Buck (1998), Bickler et al. (2000), Hawrysh and Buck (2013)
8	Cytosolic pH regulation (reoxygenation)	Painted turtle, goldfish, common carp, crucian carp	Van Den Thillart and Van Den Waarde (1991), Wasser et al. (1990)
9	Mitochondrial membrane potential (reoxygnation)	Red eared slider turtle	Galli et al. (2013)
10	ROS generation (anoxia and reoxygenation)	Red eared slider, painted turtle, epaulette shark, goldfish	Hickey et al. (2012), Krivoruchko and Storey (2010), Lushchak et al. (2001), Milton et al. (2007), Pamenter et al. (2007)
11	MPTP	Brine shrimp, ghost shrimp, green goby, Eastern oyster, rainbow trout, zebrafish, painted turtle	Adiele et al. (2012), Azzolin et al. (2010), Hand and Menze (2008), Hawrysh and Buck (2013), Holman and Hand (2009), Menze et al. (2005), Sokolova et al. (2004), Toninello et al. (2000)
12	Mitochondrial swelling (sensitivity to activators)	Brine shrimp, ghost shrimp, brine shrimp, green goby	Holman and Hand (2009), Menze et al. (2005), Toninello et al. (2000)
13	Apoptopic factors (anoxia and reoxygenation)	Red eared slider turtle, painted turtle, crucian carp	Kesaraju et al. (2009), Krivoruchko and Storey (2010), Nayak et al. (2011), Pamenter et al. (2012), Smith et al. (2009)

Numbers in first columns correspond to aspects highlighted in Fig. 1

complete sequence has yet to be determined. Several interesting and potentially important questions remain to be fully addressed; does mitochondrial Ca^{2+} overload occur in anoxia-tolerant mitochondria? Is $\Delta\Psi_m$ maintained? How are ROS regulated? How does the cell survive reoxygenation? Is the MPTP present and if so, is it activated? New information has recently come to light regarding these questions, which is summarized in the following sections.

Mitochondrial function in anoxia-tolerant animals

Anoxic environments and the animals that inhabit them

In freshwater systems, anoxia commonly occurs in small ponds and lakes during the winter where ice and snow cover block photosynthesis and O_2 is exhausted by organisms trapped under the ice. Remarkably, certain freshwater species, such as the water-breathing Northern European crucian carp (*Carassius carassius*) and air-breathing American turtles of the genera *Trachemys* and *Chrysemys*, can survive this anoxic environment at cold temperatures for periods lasting up to 5 months (Jackson 2000; Nilsson and Renshaw 2004). O_2 depletion is also common in salt lakes and ponds as a result of the interaction between salinity and dissolved O_2 concentration. In the Great Salt Lake of Utah, the indigenous brine shrimp, *Artemia franciscana*, lays eggs in 52 ppt salinities and embryos of this species can survive in anoxia for a staggering 4 years and still, upon return to favorable conditions, continue development and give rise to viable nauppli (Clegg 1997). Ephemeral ponds in tropical environments are also prone to anoxia, with O_2 concentrations changing radically during the transition from the rainy to the dry season. During the dry season in the Maracaibo Basin in Venezuela, the annual killifish, *Austrofundulus limnaeus*, deposits their embryos into the muddy pond substrate where O_2 is consumed by microbial activity (Myers 1952; Nico and Thomerson 1989). The embryos of *A. limnaeus* can survive in this anoxic state for periods lasting several months (Podrabsky and Hand 1999; Simpson 1979). Within marine environments, animals occupying intertidal and estuarine habitats regularly experience dramatic fluctuations in O_2 on a daily and seasonal basis. Tide pools can become anoxic during emersion at night, while during the day O_2 tensions can become hyperoxic. The eastern oyster, *Cassostrea virginicia*, is frequently subjected to bouts of anoxia and can survive for several days to weeks without O_2 during neap tides in intertidal zones (Kurochkin et al. 2009). Lastly, daily anoxic bouts are also experienced in animals that burrow in estuarine tidal flats and tidal streams. The ghost shrimp, *Lepidophthalmus louisianensis*, constructs burrows several metres deep which become anoxic once the tide recedes, and when peak low tides or storm surges occur, *L. louisianensis* can survive for 2–3 days without O_2 (Holman and Hand 2009).

Even when body temperature is taken into account, the animals described above are roughly a 1,000 times more anoxia-tolerant than mammals (Nilsson and Lutz 2004), which rarely survive longer than a few minutes of anoxia before severe organ damage and death ensues. Common to all of the anoxia-tolerant animals is a coordinated reduction in whole animal metabolism (hypometabolism) during O_2 deprivation. By reducing energy consumption, the organism can match ATP demand to the reduced ATP supply associated with anaerobic respiration (Hochachka 1986). Hypometabolism is a fundamental and highly successful strategy which is implemented across the animal kingdom and appears to be reflected at all levels of biological organization. In conjunction with hypometabolism, the physiology and plasticity of an animal's mitochondria are likely to be paramount in determining anoxic survival times. Indeed, it appears that anoxia-tolerant animals possess robust mitochondria which are inherently tolerant of O_2 deprivation.

In vitro mitochondrial tolerance to anoxia and reoxygenation

Traditionally, in vitro mitochondrial anoxia tolerance has been assessed by measuring respiratory function before and after an anoxia/reoxygenation trial. The classic experiment incubates mitochondria in air tight chambers fitted with O_2 electrodes and maximal respiration rates are measured in different mitochondrial states [reviewed in (Brand and Nicholls 2011)]. Mitochondria are first supplied with substrate (e.g., succinate, malate, pyruvate, glutamate), termed State II conditions, and then given saturating levels of ADP to allow the electron transport chain to accelerate and the F_1F_oATPase to produce ATP. Under these conditions, the mitochondria are exhibiting maximum ADP-stimulated respiration, designated as State III. Once all of the ADP has been consumed, respiration rate decreases to a steady-state, known as State IV, whereby O_2 consumption compensates mainly for proton leak. The State III respiration divided by State IV respiration is defined as the respiratory control ratio (RCR), which provides a measure of how coupled the mitochondria are, and their capacity for substrate oxidation. Lastly, the ratio of ADP phosphorylated to atoms of O_2 consumed (P/O ratio) can be determined, which estimates the efficiency of ATP synthesis coupled to cell respiration. Once this protocol has been completed, the mitochondria can be left to consume all of the remaining O_2, thereby entering into anoxia, and when O_2 is reintroduced (reoxygenation) the protocol can be repeated to assess the effect of anoxia/reoxygenation on respiratory function.

When mammalian mitochondria are subjected to 1–30 min of in vitro anoxia/reoxygenation, maximal rates

Table 2 Effect of anoxia/ischemia and reoxygenation/reperfusion on mitochondrial respiratory function

Animal	Tissue	Time in anoxia/ischemia (min)	State III (% change)	State IV (% change)	RCR (% change)	P/O ratio (% change)	References
Rat	Heart	An: 1	−64	52	−56	−24	Ascensao et al. (2006)
Rat	Heart	An: 20	−52	83	–	–	Ozcan et al. (2001)
Rat	Liver	An: 10	−20 to 25	30 to 80	–	−22 to 32	Du et al. (1998)
Rat	Brain	Is: 30	−59	22	−66	–	Sciamanna et al. (1992)
Rat	Brain	An: 5	−24	36	−50	–	Zini et al. (2002)
Pig	Lung	Is: 45	−43	−2	−36	−17	Willet et al. (2000)
Epaulette shark	Heart	An: 10	−14	–	–	–	Hickey et al. (2012)
Slider turtle	Heart	An: 20	−3	30	−34	−14	Galli et al. (2013)
Shovelnose ray	Heart	An: 10	7	–	–	–	Hickey et al. (2012)

Values are given as percentage change before and after the stated exposure to anoxia (An) or ischemia (Is) followed by reoxygenation. Endothermic and ectothermic species are shaded in blue and green, respectively. Species: Rat, *Rattus norvegicus*; pig, *Sus domestica*; shark, *Hemiscyllum ocellatum*; slider turtle, *T. scripta*; shovelnose ray, *Aptychotrema Rostrata*. Abreviations: State III, maximal ADP-stimulated respiration; State IV, respiration following complete ADP phosphorylation; RCR, respiratory control ratio (State III/State IV); P/O ratio, the ratio of ADP phosphorylated to atoms of O_2 consumed. Data are taken from the papers referenced in the final column

of O_2 consumption under state III conditions are reduced by ~20–64 % and State IV respiration is increased by up to 80 % (Table 2; Fig. 2a). Furthermore, the RCR and P/O ratio declines by ~36–66 and 17–32 %, respectively (Table 2). This loss of respiratory function, termed "mitochondrial dysfunction", can eventually collapse $\Delta\Psi_m$ and lead to cell death (Fiskum et al. 1999; Sanderson et al. 2013). In contrast, isolated mitochondria from the heart of *Trachemys scripta* can endure 20 min of anoxia followed by reoxygenation without any impact on subsequent ADP-stimulated O_2 consumption and only minor effects on the P/O ratio [Table 2; Fig. 2b (Galli et al. 2013)]. Although anoxia/reoxygenation leads to a 30 % increase in State IV respiration in *T. scripta* (corresponding to a 34 % decline in RCR), this value is in the lower range of those reported for anoxia-sensitive animals [Table 2; Fig. 2b (Galli et al. 2013)]. It should be noted that turtle mitochondria were investigated at 13 °C [as opposed to room temperature (Ascensao et al. 2006; Du et al. 1998) or 30 °C (Ozcan et al. 2001)], which may afford some protection against anoxic damage, but the same experiment repeated at 37 °C yielded similar results (Galli et al. 2013). Another hypoxia-tolerant animal, the epaulette shark (*Hemiscyllum ocellatum*), only suffers a 14 % decline in State III respiration after anoxia/reoxygenation of ventricular permeabilized fibers (Hickey et al. 2012). Thus, data from the turtle and epaulette shark point towards inherent specializations at the mitochondrial level that are common to hypoxia/anoxia-tolerant animals. However, State III respiration rate actually increased after anoxia/reoxygenation in the shovelnose ray [*Aptychotrema rostrata*, Fig. 2d (Hickey et al. 2012)], which is a species known to be hypoxia-sensitive. Thus, mitochondrial in vitro tolerance to anoxia/reoxygenation may be a property that is common among ectotherms and not exclusive to hypoxia or anoxia-tolerant animals (Table 2).

As anoxia progresses into the long-term (weeks to months), marked changes in the intrinsic properties of the mitochondria could potentially lengthen the amount of time an animal could survive without O_2. Indeed, in contrast to brief periods of anoxia/reoxygenation, chronic anoxia is associated with a non-pathological downregulation of mitochondrial respiratory capacity in anoxia-tolerant animals. Following 2 weeks of anoxia at 5 °C, *T. scripta* exhibits a profound reduction in maximal State II, III and IV respiration rates in cardiac permeabilized fibers and isolated mitochondria (Fig. 3a, b) (Galli et al. 2013). Similarly, 6 days of anoxia in the eastern oyster led to a reduction in State III respiration but no change in State IV respiration of gill mitochondria (Kurochkin et al. 2009). The common frog, *Rana temporaria*, which is capable of surviving anoxia for several days at cold temperatures (Pinder et al. 1992), inhibits State III and State IV respiration rates by ~60 % when subjected to severe hypoxia (St-Pierre et al. 2000a). Lastly, diapausing embryos of the annual killifish, *A. limnaeus*, can endure months of anoxia at 25 °C (Duerr and Podrabsky 2010) by reducing mitochondrial oxidative capacity by up to 84 % (Duerr and Podrabsky 2010). In aggregate, the data collected from ectothermic animals support the view that chronic anoxic survival is associated with a depression of mitochondrial respiratory capacity. Thus, whole-animal hypometabolism is reflected at the level of the mitochondria.

The mechanisms behind the reduction in respiratory capacity in these animals vary. In anoxic *T. scripta,* there is no change in the enzyme activities of Complex I, II, IV or citrate synthase, and the downregulation of respiratory

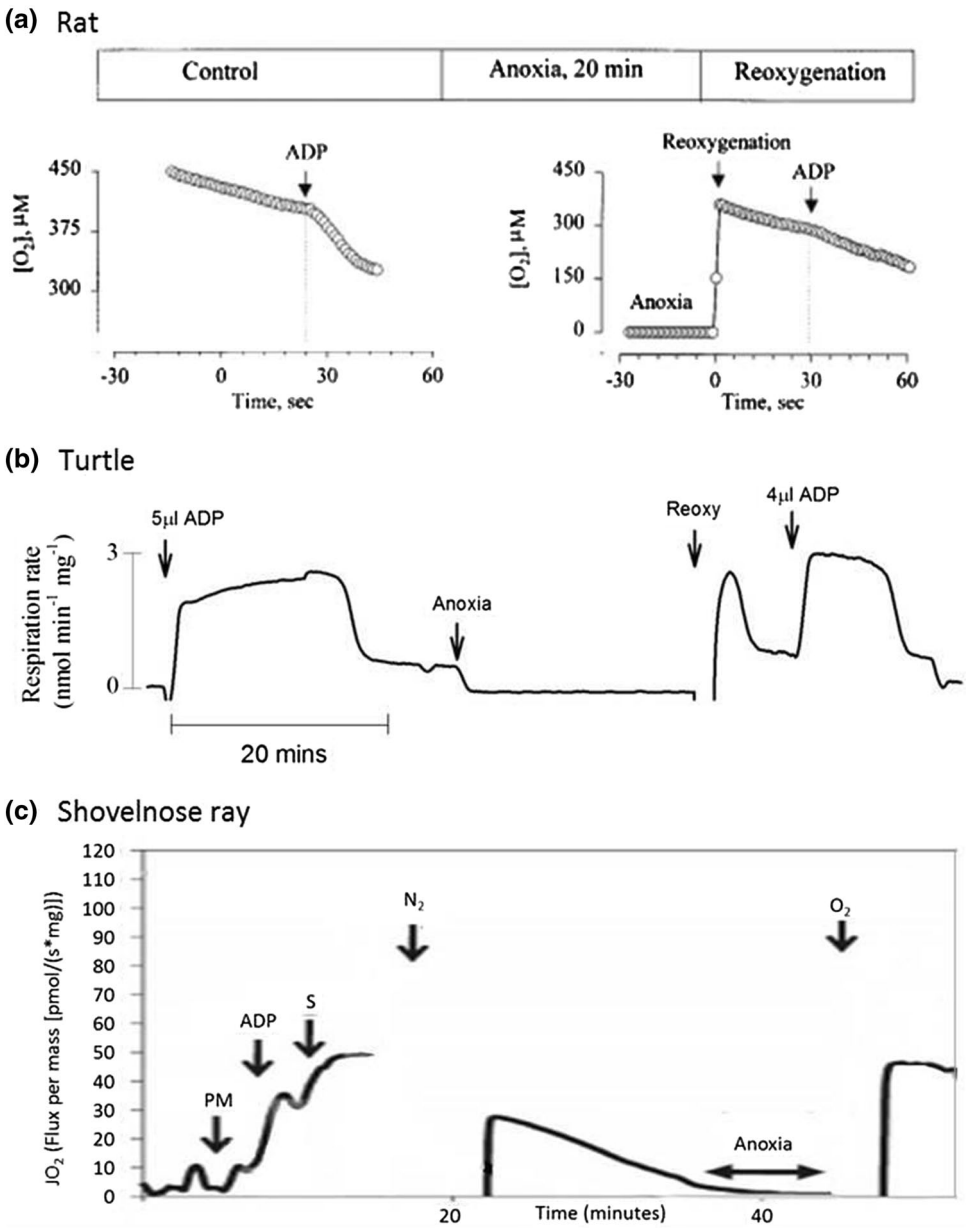

Fig. 2 The effects of in vitro acute anoxia and reoxygenation on mitochondrial respiration rate in a range of vertebrates. Mitochondria or permeabilized fibers were incubated in a microrespirometer with substrates for oxidative phosphorylation. ADP-stimulated respiration was measured before and after anoxia and reoxygenation. **a** Rat (*Rattus norvegicus*) ventricular mitochondria: The concentration of O_2 ($[O_2]$ in µM) was monitored in control conditions (*left panel*) or after 20 min of anoxia and reoxygenation (*right panel*). Addition of ADP in mitochondria respiring on malate and pyruvate induced a rapid decline in $[O_2]$ under control conditions, but had a minor effect after anoxia/reoxygenation. Trace adapted from (Ozcan et al. 2001). **b** Turtle (*Trachemys scripta*) ventricular mitochondria: ADP-stimulated respiration rate with malate and pyruvate was similar before and after 20 min of anoxia and reperfusion. Trace adapted from (Galli et al. 2013). **c** Shovelnose ray (*Aptychotrema rostrata*) permeabilized ventricular fibers: ADP-stimulated respiration was measured in the presence of malate and pyruvate (PM) and succinate (S). O_2 was removed from the chamber via N_2 injection, and anoxia was maintained for 10 min before reoxygenation. Trace adapted from (Hickey et al. 2012). NB: experiment in panel **a** was performed in a traditional airtight multichannel chamber, while the experiments for panel **b** and **c** were performed in a more modern high-resolution oxygraph (Oroboros microrespirometer)

capacity can be entirely attributed to a profound reduction in the activity of Complex V (the F_1F_o-ATPase pump) [Fig. 3c (Galli et al. 2013)]. In severely hypoxic *R. temporaria,* enzyme activities of cytochrome *c* oxidase (Complex IV of the electron transport chain) and succinate dehydrogenase (Complex II of the electron transport chain) are downregulated (St-Pierre and Boutilier 2001), and citrate synthase (a proxy for mitochondrial content) was

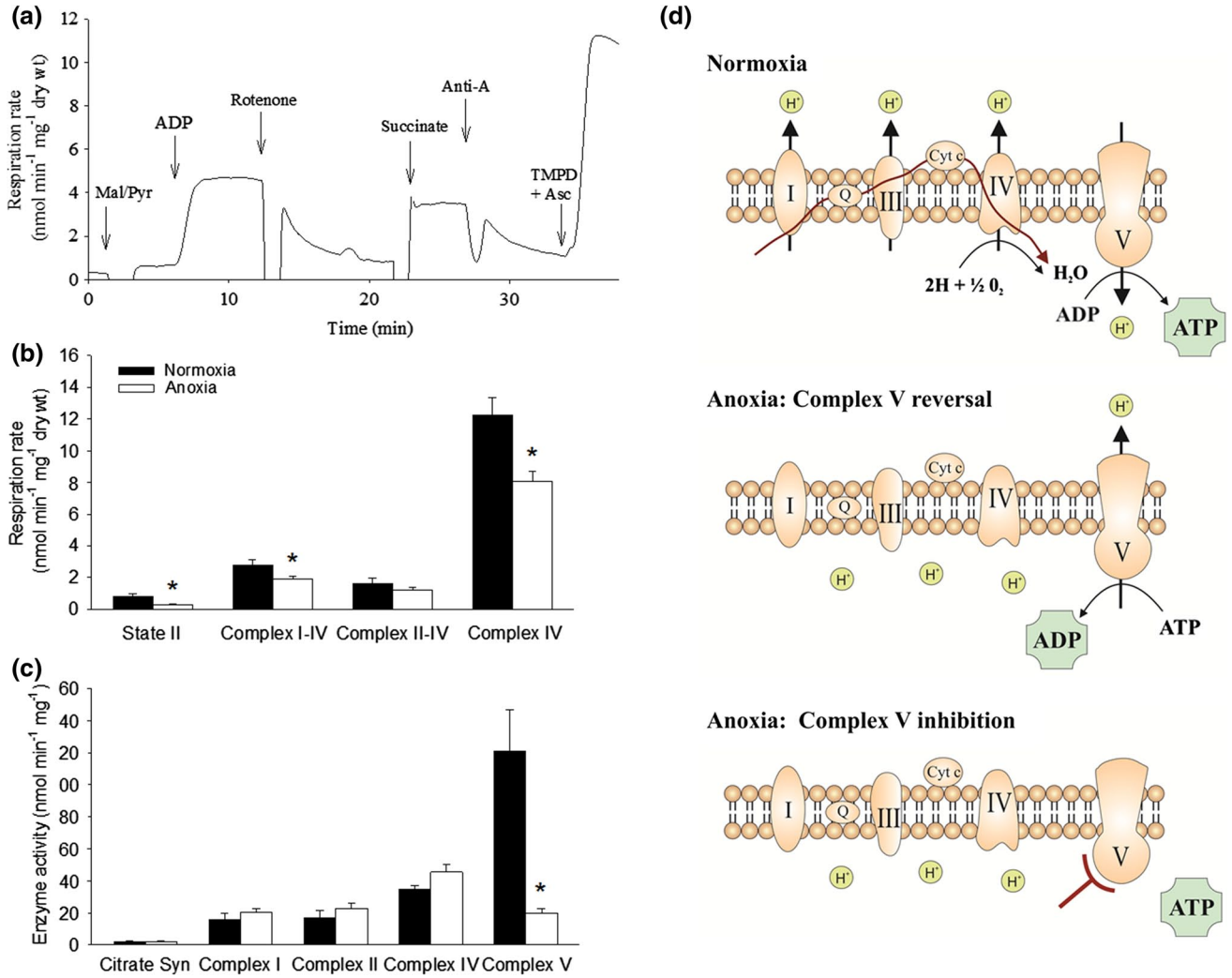

Fig. 3 The effect of chronic anoxia (2 weeks) on ventricular mitochondrial function in the freshwater turtle, *Trachemys scripta.* Following 2 weeks of anoxic acclimation at 5 °C, maximal respiration rates in permeabilized fibers from the turtle heart were dramatically reduced (panel **a** and **b**) by a downregulation of the F_1F_o-ATPase, or Complex V (panel **c**). This strategy avoids ATP depletion by inhibiting the reverse mode action of Complex V which consumes ATP (panel **d**). **a** Original trace from a normoxic exposed turtle: Ventricular permeabilized fibers were incubated in an Oroboros microrespirometer and maximal respiration rates were measured through different Complexes of the electron transport chain. Malate and pyruvate (Mal/Pyr) were added to the chamber (*State II*) and respiration rate through Complex I–IV was measured by the addition of ADP. Rotenone was then injected to inhibit Complex I, and succinate was added to measure respiration rate through Complex II–IV. Antimycin A was then added to block Complex II and flux through Complex IV was assessed by adding tetramethyl-*p*-phenylene-diamine (TMPD) and ascorbate. **b** Averages for anoxic exposed (2 weeks, *black bars*, $n = 6$) and normoxic exposed (*white bars*, $n = 6$) turtle ventricular permeabilized fibers subjected to the protocol given in Panel **a**. Data are mean ± SEM respiration rates under *State II*, nonphosphorylation conditions, as well as maximum respiration rates through Complex I–IV, Complex II–IV and Complex IV. Asterisks indicate a significant difference between normoxia- and anoxia-exposed animals ($P < 0.05$). **c** The effect of chronic anoxia on the activity of citrate synthase and Complexes I, II, IV and V in normoxia (*black bars*, $n = 6$) and anoxia exposed (*white bars*, $n = 6$) turtle ventricular tissue. Data are mean ± SEM. *Asterisks* indicate a significant difference between normoxia- and anoxia-exposed animals ($P < 0.05$). **d** Schematic diagram representing the effect of inhibiting the F_1F_o-ATPase during chronic anoxia. *Upper panel* when O_2 is present, electrons from substrate oxidation are donated to the electron transport chain and as they pass along the chain to the final electron acceptor (O_2), protons are pumped by Complexes I, III and IV from the matrix to the intermembrane space. Subsequently, protons flow back down their electrochemical gradient through a Complex V (the F_1F_o-ATPase) which harvests the proton gradient to generate ATP from ADP. Middle panel: Without O_2, the final electron acceptor is not available and the electron transport chain will come to a halt. As a consequence, protons will accumulate in the mitochondrial matrix and mitochondrial membrane potential ($\Delta\Psi_m$) depolarises. The F_1F_o-ATPase, which normally produces ATP, switches into a reverse mode during ischemia and consumes ATP in an attempt to re-establish $\Delta\Psi_m$. *Lower panel* by inhibiting Complex V of the electron transport chain, animals can limit ATP consumption during anoxia by inhibiting reverse mode action of the F_1F_o-ATPase which consumes valuable ATP. Schematic diagram drawn by G. Galli

significantly lower (St-Pierre and Boutilier 2001). Lastly, diapausing embryos of *A. limnaeus* downregulate respiratory capacity with a reversible reduction in the activity of Complexes I, II, IV and V (Duerr and Podrabsky 2010). Whatever the mechanism, it is clear that downregulating respiratory capacity during anoxia is an energy sparing mechanism which contributes to the hypometabolic condition characteristic of anoxia-tolerant animals. However, in addition to ATP conservation, inhibition of the electron transport chain may protect the cell from other anoxia-related injuries. In the mammalian heart, blockade of individual Complexes (mainly Complex I) of the electron transport chain can attenuate ROS generation which limits oxidative damage and lowers the probability of MPTP opening (Szczepanek et al. 2012). This is because Complex I and III of the electron transport chain are major sources of ROS generation during ischemia (Chen et al. 2008). Furthermore, inhibition of Complex I preserves the integrity of the inner and outer mitochondrial membranes following reperfusion and inhibits cytochrome *c* release (Lesnefsky et al. 2004). While these possibilities have not been directly tested in an anoxia-tolerant animal, it is possible that downregulation of respiratory capacity also serves to limit ROS generation and cytochrome *c* release. From these in vitro studies, it becomes clear that anoxia tolerance transcends to the level of the mitochondria. The strategies that underlie this tolerance are described below.

Regulation of the F_1F_O-ATPase (Complex V)

Although reducing respiratory capacity will conserve ATP supplies during anoxia, anoxia-tolerant animals are still at risk of energy depletion due to the reverse activity of the F_1F_o-ATPase. To defend ion homeostasis, mammalian mitochondria maintain $\Delta\Psi_m$ at the expense of ATP via reversal of the F_1F_o-ATPase (Fig. 3d, middle panel). This is a costly process at a time when energy producing pathways are limited (St-Pierre et al. 2000b), and will eventually lead to an energy crisis during long periods of anoxia. Early reports from anoxic frog (*R. temporaria*) skeletal muscle mitochondria (St-Pierre et al. 2000b) demonstrated that anoxia-tolerant animals have solved this problem via inhibition of the F_1F_o-ATPase during anoxia. After 30 min of anoxia, the decline in ATP concentration catalyzed by the frog mitochondrial F_1F_o-ATPase was only 15 % of the expected loss (St-Pierre et al. 2000b). However, when this experiment was repeated in the presence of a mitochondrial uncoupler, the decrease in ATP concentration catalyzed by the frog F_1F_o-ATPase was considerable. Since proton conductance of frog mitochondria was not altered in anoxia, the authors concluded that the F_1F_o-ATPase had been inhibited during anoxia to limit ATP consumption. Since these early investigations, an inhibition of Complex V during anoxia has been reported in chronically anoxic *T. scripta* [Fig. 3 (Galli et al. 2013)] and diapausing *A. limnaeus* embryos (Duerr and Podrabsky 2010), suggesting that this strategy is commonly utilized in anoxia-tolerant animals. By downregulating Complex V of the electron transport chain, animals can further limit ATP consumption during anoxia by inhibiting reverse mode action of the F_1F_o-ATPase which consumes valuable ATP (Fig. 3d, lower panel).

Interestingly, regulation of the F_1F_o-ATPase has been implicated in the cardioprotective effects of ischemic preconditioning in mammalian models of anoxia tolerance (for reviews see (Faccenda and Campanella 2012; Kane and Van Eyk 2009; Lippe et al. 2009). Activation of endogenous protective mechanisms by brief periods of ischemia before (preconditioning) or after (postconditioning) a more severe ischemic bout increases myocardial tolerance to the ischemic injury (Gross and Auchampach 2007; Murry et al. 1986). ATP levels in preconditioned hearts fall more slowly and $\Delta\Psi_m$ is depolarized to a greater extent during ischemia compared with controls (Murry et al. 1990; Ylitalo et al. 2000), leading to less ultrastructural injury. While the mechanism for the downregulation of Complex V in preconditioning models is controversial, it has been proposed that Complex V is either directly inhibited through the binding of the inhibitory factor (IF) (Vander Heide et al. 1996) or through S-nitrosylation (Sun et al. 2007). However, similar mechanisms have not been investigated in anoxia-tolerant animals and should be addressed in future studies.

Proton leak

Proton leak is the basal or induced movement of protons across the inner mitochondrial membrane that results in partial dissipation of the $\Delta\Psi_m$ and uncoupling of oxidative phosphorylation (Divakaruni and Brand 2011). Under normoxic conditions in mammals, proton leak accounts for 20–25 % of basal metabolic rate. There are numerous pathways available to protons to transverse the inner mitochondrial membrane, besides the ATP-generating F_1F_o-ATPase. These include specific proteins that facilitate uncoupled respiration, termed uncoupling proteins, as well as proton movement through non-specific proteins such as the adenine nucleotide translocase. Direct proton movement through the membrane is also possible, which will be influenced by the phospholipid composition.

In theory, decreases in mitochondrial proton leak during periods of O_2 deprivation should help to stabilize $\Delta\Psi_m$ and assist in preventing the F_1F_o-ATPase from running in reverse in its effort to maintain the transmembrane potential and cellular energy status. Indeed, decreases in proton leak via inner mitochondrial membrane remodeling have been shown to occur in animals in response to environmental

challenges (Casey et al. 2002; Kelly et al. 2008; Nadtochiy et al. 2006; Quarrie et al. 2011; Stuart et al. 1998), facilitating the maintenance of $\Delta\Psi_m$. However, in ischemic reperfusion scenarios in mammals, there is accumulating evidence to suggest that mild increases in proton leak, although energetically wasteful, exert a protective effect on the cell by maintaining the flow of electrons through the electron transport chain, thus limiting the production of ROS generation both during the ischemic event and during reperfusion (Cunha et al. 2011). Increases in proton leak have also been documented in other mammalian models of ischemia tolerance (Kelly et al. 2008; Nadtochiy et al. 2006), and in mitochondria isolated from diapausing killifish embryos (Duerr and Podrabsky 2010), but not all studies agree with this conclusion. For example, neonatal cardiomyocytes from rats are known to reduce proton leak following exposure to moderate levels of hypoxia (Casey et al. 2002). Clearly, however, during long-term anoxia, as with freshwater turtles, ROS generation will not be possible and anoxia survival in this case will be dependent on the maintenance of cellular energy balance. Therefore, the most reasonable prediction is that proton leak will be decreased during long-term anoxia exposure.

In the anoxia-tolerant turtle (Galli et al. 2013), the state IV O_2 consumption rate required to maintain a set $\Delta\Psi_m$ (Fig. 4) was not different between normoxic and anoxic (2 weeks) exposed turtles. These data clearly suggest that turtle proton leak is not modified in response to anoxia acclimation, despite the fact that $\Delta\Psi_m$ appears to be preserved during long-term anoxia exposure (Galli et al. 2013). The mechanisms behind this preservation of $\Delta\Psi_m$ without changes in proton leak are unknown, but it has been proposed that the mitochondria from ectothermic animals may, in general, be less prone to proton leak than homeothermic endotherms (Brookes et al. 1998; St-Pierre et al. 2000a). In comparisons across fish, reptiles, birds, and mammals, ectothermic animals with their lower standard metabolic rates had lower proton leak, which was related to their inner mitochondrial membrane being composed of more monounsaturated phospholipid composition (Brookes et al. 1998). In comparison, endotherms with high standard metabolic rates have high measured proton leak rates, and higher levels of polyunsaturated phospholipids. As a result, the inherent "tightness" of the ectothermic mitochondrial membrane may serve to limit proton leak during anoxia, even in the highly depolarized mitochondria, and alleviate the need for membrane remodeling as seen in endotherms. Furthermore, mitochondria from the liver of ectotherms also appear to maintain a lower $\Delta\Psi_m$ relative to endotherms, due to the lower activity of enzymes involved in substrate oxidation (Brookes et al. 1998). Thus, part of the basis of anoxia tolerance in the few truly anoxia-tolerant animals may be related to aspects which are inherent to ectotherms.

Ca^{2+} homeostasis

Maintenance of ionic homeostasis, particularly Ca^{2+}, is a major challenge during anoxia, as ion pumping is energetically expensive and must be maintained despite large-scale reductions in ATP supply. This challenge proves too great for anoxia-sensitive animals, and the limited ATP supply coupled with intracellular acidosis activates numerous Ca^{2+} transporters leading to a progressive rise in cytosolic Ca^{2+} and eventually Ca^{2+} overload [reviewed in (Garcia-Dorado et al. 2012)]. Recent estimates in the mammalian ischemic heart suggest that intracellular Ca^{2+} may increase as much as fourfold, with diastolic and systolic levels reaching 1.2 and 1.8 μM, respectively (Stamm et al. 2003; Venkataraman et al. 2012). Despite the fact that mitochondria have an impressive capacity to accumulate Ca^{2+} during cellular stress, this level of cytosolic Ca^{2+} overload will eventually lead to a pathological rise in mitochondrial matrix Ca^{2+} (Garcia-Dorado et al. 2012; Sanderson et al. 2013). Indeed, elevations in mitochondrial Ca^{2+} are observed during ischemia in the mammalian brain and myocardium, and this is potentiated once reperfusion occurs and the membrane repolarizes (Garcia-Dorado et al. 2012; Sanderson et al. 2013). The increase in mitochondrial Ca^{2+} contributes to formation of the MPTP and subsequent activation of apoptopic and necrotic pathways. Thus, regulating mitochondrial Ca^{2+} during anoxia/reperfusion is likely to be a crucial aspect of anoxia survival.

To our knowledge, direct measurements of mitochondrial Ca^{2+} in an anoxia-tolerant animal have not been made, but in comparison to mammals, cytosolic Ca^{2+} is reasonably well defended in the anoxic turtle brain (Bickler 1992; Bickler and Buck 1998). In rat cortical brain slices, intracellular Ca^{2+} increases from 300 to 1,000 nM within 5 min of anoxia, while 2–5 h of anoxia actually led to a decrease in intracellular Ca^{2+} in turtle brain slices [*T. scripta*, (Bickler 1992)] or a moderate increase of 35 % (Bickler et al. 2000). Furthermore, turtle neurons subjected to nearly 6 weeks of anoxia exhibited only a slight increase in intracellular Ca^{2+} which recovers to normoxic levels towards the end of the anoxic bout (Bickler and Buck 1998). The mechanism by which Ca^{2+} is maintained in the anoxic turtle brain is through suppression of ion channels associated with Ca^{2+} transport, a phenomenon termed "channel arrest" (Hochachka 1986). This process involves a coordinated reduction in the activity or expression of ion transporters to match ATP demand to the reduced ATP supply associated with anoxia. Numerous ion channels are downregulated in the anoxic turtle [for reviews see (Buck et al. 2012; Milton and Prentice 2007)], but perhaps the most important is suppression of glutamate receptors (NMDA and AMPA) in the brain.

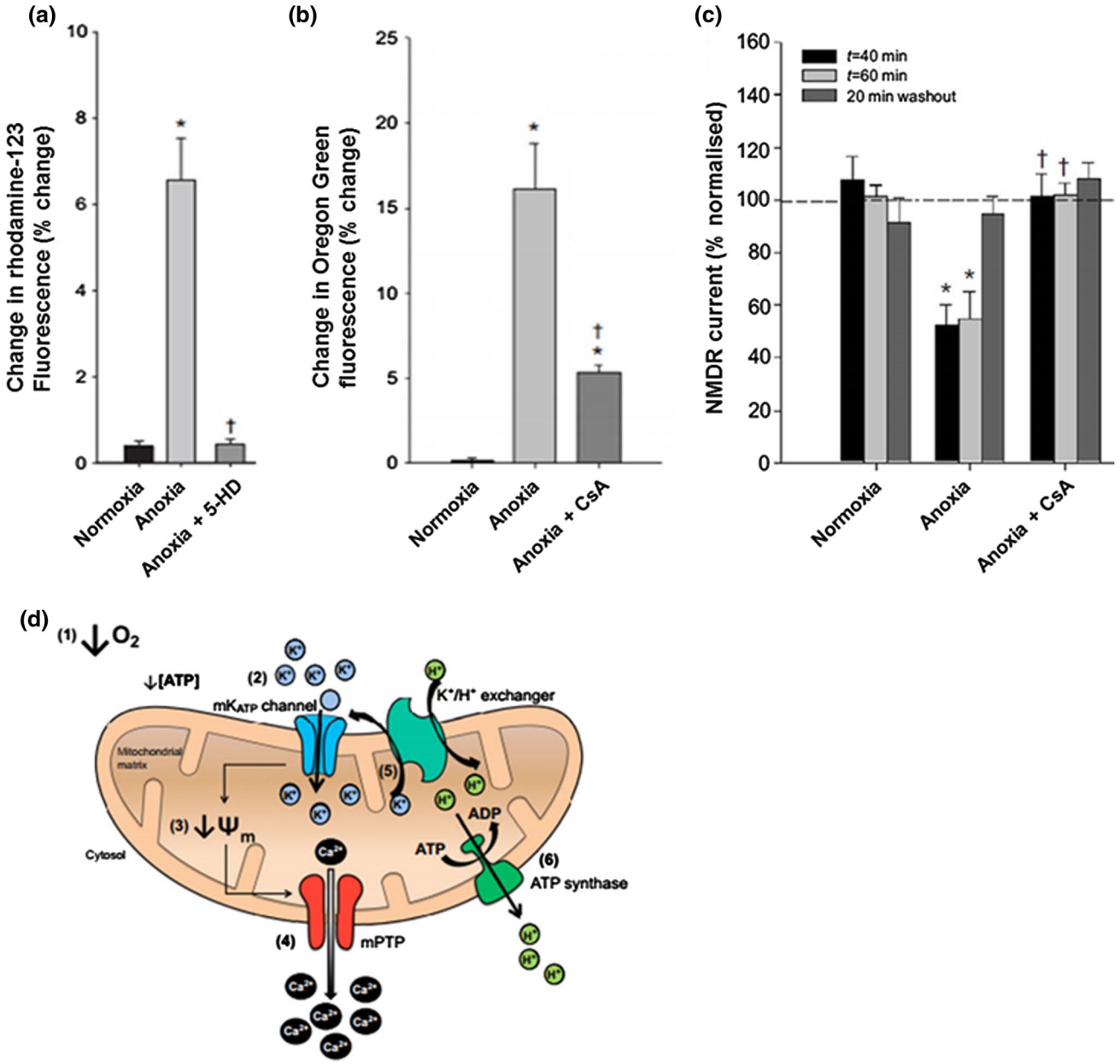

Fig. 4 The effect of anoxia on NMDR receptor activity, intracellular Ca^{2+} and mitochondrial membrane potential in cortical slices from the painted turtle, Chrysemys picta bellii. **a** Rhodamine-123 fluorescence (as a measure of mitochondrial membrane potential) increases in response to anoxia and can be abolished by the mitochondrial ATP-sensitive K^+ channel blocker 5-hydroxydecanoic acid (5-HD). Symbols indicate values different from normoxic controls (*asterisks*) and anoxic controls (*dagger*). **b** Percentage normalized *Oregon Green fluorescence* (as a measure of intracellular Ca^{2+}) increases in response to anoxia and this can be inhibited by the mitochondrial permeability transition pore (MPTP) inhibitor, cyclosporin A (CsA). Symbols indicate values different from normoxic controls (*asterisks*) or anoxic controls (*dagger*). **c** NMDAR currents are reduced during anoxia and this reduction can be inhibited by CsA. NMDAR currents were recorded after 30 min ($t = 40$ min, *black bars*) and 50 min ($t = 60$ min, *light gray bars*) of treatment, followed by 20 min of washout (*dark gray bars*). The dashed line represents normoxic controls taken at $t = 10$ min ($N = 47$). *Symbols* indicate data significantly different from normoxic controls taken at $t = 10$ min (*asterisks*) and anoxic controls taken at corresponding time points (*dagger*). **d** Simplified schematic illustration to represent the factors leading to mitochondrial Ca^{2+} release in the anoxic turtle brain. Anoxia leads to a decline in ATP via oxidative phosphorylation (*1*) which results in a reduction in local (ATP) which stimulates mK^+ATP channel opening and increases K^+ in the mitochondrial matrix (*2*). This depolarizes mitochondrial membrane potential [Ψ_m, (*3*)], which transiently opens the MPTP and causes Ca^{2+} release (*4*). While mK^+ATP channels remain open, the K^+ influx is balanced by K^+/H^+ exchange (*5*). The imbalance in proton concentration is maintained by reversal of the F_1F_0-ATPase (ATP synthase) (*6*), which prevents collapse of the proton gradient and maintains Ψ_m at a newly depolarized set point. Data and schematic diagram were compiled from (Hawrysh and Buck 2013) (color figure online)

Under normoxic conditions, NMDA and AMPA receptors transmit a presynaptic excitatory input to a second messenger via Ca^{2+} entry, but during anoxia/ischemia, membrane depolarization leads to the release of excessive glutamate causing excitotoxicity which activates NMDA/AMPA receptors to allow Ca^{2+} influx (Cross et al. 2010). In anoxia-sensitive animals, this process results in Ca^{2+} overload and excitotoxic cell death. In the turtle brain, however, whole-cell patch-clamp experiments have shown NMDA and AMPA currents are reversibly reduced by ~45–65 % during 30–40 min of anoxia (Buck et al. 2012; Hawrysh and Buck 2013; Pamenter et al. 2008; Shin and Buck 2003), thereby limiting Ca^{2+} entry. Inhibition of the NMDA receptor during anoxia is proposed to occur via Ca^{2+} signaling between the mitochondria and the cytosol in response to a depolarization of $\Delta\Psi_m$ (Hawrysh and Buck 2013). When turtle cortical slices are exposed to anoxia, oxidative phosphorylation ceases and the decline in ATP activates mitochondrial ATP-sensitive K^+ channels (mK_{ATP}). When open, mK_{ATP} channels allow entry of K^+ into the mitochondrial matrix causing a depolarization of $\Delta\Psi_m$ (Garlid et al. 2009). To avoid the complete collapse of $\Delta\Psi_m$, which can initiate apoptopic processes, the turtle F_1F_o-ATPase runs in reverse to pump protons back into the intramembrane space and regulates $\Delta\Psi_m$ to a mildly depolarized level (Hawrysh and Buck 2013). The mild depolarization of $\Delta\Psi_m$ triggers the transient opening of the MPTP in a low conductance confirmation which only allows the release of molecules of ~0.3 kDa or less, such as Ca^{2+} (Hunter and Haworth 1979). In the low conductance confirmation, opening of the MPTP is a protective measure, which is not associated with mitochondrial swelling and the release of apoptotic factors, as in the high conductance state. In the turtle brain, the Ca^{2+} released from the MPTP during anoxia subsequently binds to calmodulin which dephosphorylates NMDR and AMPA receptors and reduces their activity (Buck et al. 2012). Thus, inhibition of NMDR receptors in the anoxic turtle brain is a Ca^{2+}-dependent process which begins with depolarization of $\Delta\Psi_m$ and ends with the release of Ca^{2+} from the MPTP. Evidence to support this sequence of events in the turtle brain is given in Fig. 4, which shows that the anoxia-induced depolarization of $\Delta\Psi_m$ can be completely abolished with 5-Hydroxydecanoic Acid (5-HD, an inhibitor of mK_{ATP} channels, Fig. 4a) and the increase in Ca^{2+} and reduction in NMDR activity can be abolished by CsA (Fig. 4b, c). A schematic representation of the sequence of events leading to mitochondrial Ca^{2+} release during anoxia (outlined above) is given in Fig. 4d.

While a modest increase in cytosolic Ca^{2+} may be characteristic of the anoxic turtle brain, other tissues may not defend Ca^{2+} to the same extent. Contrary to the brain, channel arrest does not occur in the turtle heart after 2 weeks of anoxia (Stecyk et al. 2007, 2008), suggesting that Ca^{2+} transport mechanisms remain active. In support of this contention, unpublished data from our laboratory suggests that systolic Ca^{2+} levels in isolated turtle cardiomyocytes are increased ~3 fold during 20 min of acute anoxia with average levels rising to ~750nM. Nevertheless, a threefold increase in intracellular Ca^{2+} may not constitute Ca^{2+} overload or elevate mitochondrial Ca^{2+}, and may in fact constitute an important signaling mechanism, similar to the turtle brain. Clearly, direct measurements of mitochondrial Ca^{2+} in the anoxic turtle heart are necessary, with parallel experiments on anoxia-sensitive species, before any meaningful conclusions can be drawn.

Redox signaling

Discussion of redox signaling during anoxia warrants an entire review (for excellent reviews see Krivoruchko and Storey 2010; Leveelahti et al. 2014; Manzanero et al. 2013; Pagliaro et al. 2011; Raedschelders et al. 2012; Storey 1996) and will only be discussed briefly here. In healthy mitochondria, a small but significant quantity of O_2 (0.1–5 %, depending on the tissue) during oxidative phosphorylation is transformed into ROS superoxide (O_2^-) at complexes I and III of the electron transport chain (Arnaiz et al. 1999; Boveris and Cadenas 2000; Penna et al. 2013). O_2^- gets converted to H_2O_2 by the enzyme superoxide dismutase (SOD), and freely diffuses out of the mitochondria, acting as an intracellular messenger (Penna et al. 2013; Rice 2011). However, under pathological circumstances, excessive ROS can damage the cell through a variety of mechanisms, including activation of proteolytic enzymes, lipid peroxidation, protein oxidation and nitration, DNA damage and activation of the MPTP, ultimately leading to cell death (Raedschelders et al. 2012). Thus, ROS are a double edged sword that must be carefully regulated to a concentration that allows protective signaling cascades to operate but does not initiate cell death pathways. To achieve this, the cell can directly regulate ROS generation at specific sites of the electron transport chain and eliminate ROS with neutralizing enzymes or scavenging molecules (anti-oxidant defences) (Manzanero et al. 2013). During normoxia, the cell achieves the delicate balance of ROS formation and elimination, but during anoxia/reoxygenation, elevations in scavenging molecules cannot compensate and antioxidant defences are overwhelmed. Although myocardial ischemia is associated with a restriction of O_2 to the affected area, the partial pressure of residual O_2 rarely falls below 4 Torr (Becker et al. 1999), so that ROS can be generated during ischemia and damage the electron transport chain [mainly at Complexes I and IV (Racay et al. 2009)]. However, the greatest and most dangerous burst of ROS generation occurs at reperfusion. When O_2 is replenished and the electron transport chain resumes function, the

O_2^- that was generated from the damaged electron transport chain is free to be converted into large quantities of lethal H_2O_2.

Due to the central role that ROS play in the development of ischemic injury, their regulation became an obvious target to reveal adaptations in anoxia-tolerant animals. Obviously, long-term anoxia is associated with the complete absence of O_2, which makes ROS formation impossible. Interestingly, scavenging of residual O_2 in the mammalian heart during ischemia improves heart function subsequent to reperfusion, suggesting that truly anoxic conditions are somewhat protective (Vanden Hoek et al. 1997). However, ROS generation during the transition to anoxia and subsequent reoxygenation is expected to be a major challenge for anoxia-tolerant animals. One of the ways to evaluate whether oxidative stress has occurred during anoxia is to measure lipid peroxidation, as elevations in the end products of lipid peroxidation suggest that existing antioxidants have been overwhelmed. However, this was not the case in various different turtle tissues during 20 h of anoxia followed by reoxygenation, suggesting that no free radical damage had occurred (Willmore and Storey 1997). Indeed, the same investigation demonstrated that *T. scripta* possessed constitutively high levels of antioxidant enzymes, such as catalase, SOD, glutathione-S-transferase, and glutathione peroxidase, (Rice et al. 1995; Willmore and Storey 1997) which are comparable to mammalian activities despite much lower overall metabolic rates (Storey 1996). Furthermore, while mammals respond to anoxia by increasing antioxidant enzymes (e.g., SOD, catalase), the turtle actually decreases them during anoxia (Storey 1996). Thus, existing antioxidant defences in the turtle may be sufficient to protect tissues from ROS damage.

More recent studies have attempted to directly measure the production of ROS during anoxia and reoxygenation with the use of fluorometry. These studies have shown ROS production in cortical brain sheets from *C. picta* declines during the transition from normoxia to anoxia, and although a burst of H_2O_2 occurs during reperfusion after 10 min of anoxia, values do not exceed those recorded in normoxia (Pamenter et al. 2007). This reduction in ROS production in *C. picta* brain, which is in stark contrast to the mammalian paradigm, is proposed to play a key role in cell signaling by initiating cytoprotective mechanisms and acting as an O_2 sensor during the transition to anoxia (Pamenter et al. 2007). Importantly, low rates of ROS production at rest, and following anoxia/reperfusion, have also been observed in the epaulette shark (Hickey et al. 2012), despite the fact that lipid peroxidation is known to increase after 24 h of reoxygenation in this species with no change in the activity of antioxidant enzymes (Renshaw et al. 2012). Assuming this pattern of ROS production also occurs in the heart of *T. scripta*, these results suggest that ample antioxidant reserves, and possibly the regulation of ROS, are a critical component to avoiding mitochondrial dysfunction following acute anoxia/reoxygenation.

Similar to ROS, the delicate balance of reactive nitrogen species (RNS) production and degradation plays an important role in cell signaling and the development of O_2-related diseases in mammals. RNS are produced by the reaction of NO with O_2^- to form many different reactive species, including peroxynitrite ($ONOO^-$) and nitrogen dioxide radicals (NO_2) which have cytotoxic effects and reduce the availability of NO (Pagliaro et al. 2011). This is important, as NO has numerous beneficial and protective effects within mitochondria, especially during ischemia and reperfusion (Davidson and Duchen 2006). In particular, NO interacts with the MPTP to limit postischemic myocardial damage and lower ROS-dependent damage. Anoxia is known to suppress NO production as the absence of O_2 and intracellular acidosis inhibits nitric oxide synthase (NOS) (Raedschelders et al. 2012). However, NO generation actually increases during long periods of ischemia, and cannot be abolished by NOS inhibition (Zweier et al. 1995a), suggesting NO production via NOS-independent pathways. It was later found that the source of NO was the reduction of nitrite, a reaction that naturally occurs when the mitochondria are subjected to low pH and hypoxia (Zweier et al. 1995b). Interestingly, anoxia is associated with a dramatic increase in nitrite levels in the heart of the crucian carp and the pectoral muscle, red blood cells and heart of the red eared slider turtle (Jensen et al. 2014; Sandvik et al. 2012). It is now known that nitrite and its bioactive form, NO, modulate mitochondrial function and confer cardioprotection during ischemia and reperfusion (Murillo et al. 2011). S-nitrosylation of Complex I by NO during ischemia inhibits ROS generation, and this effect is reversed slowly when O_2 is reintroduced which allows the "gradual wakeup" of the mitochondrial machinery, thereby avoiding oxidative damage during reperfusion (Burwell et al. 2009). Furthermore, S-nitrosylation is implicated as the mechanism underlying the protective inhibition of the F_1F_o-ATPase during ischemic preconditioning (Sun et al. 2007). These findings suggest that nitrite is an important aspect of cytoprotection.

The recalcitrant mitochondrial permeability transition pore

Recent data collected from anoxia-tolerant invertebrates suggest that anoxia-tolerant animals may possess a recalcitrant MPTP that is insensitive to Ca^{2+}. In rat liver mitochondria, the addition of 0.1 mM external Ca^{2+} causes Ca^{2+} efflux from the matrix with associated mitochondrial swelling and release of cytochrome *c* (Menze et al. 2005). However, the addition of 0.1 mM Ca^{2+} in mitochondria from *A. franciscana* did not release

internal stores of Ca^{2+} (no MPTP opening), and did not elicit swelling or a release of cytochrome *c* (Menze et al. 2005). In fact, *A. franciscana* mitochondria continued to actively load Ca^{2+} even when challenged with 1 mM external Ca^{2+} (Menze et al. 2005). Importantly, positive controls revealed swelling could be artificially manifested in *A. franciscana* mitochondria, suggesting that the MPTP existed but was not opened by concentrations of Ca^{2+} known to induce pore opening in mammals (Menze et al. 2005). Thus, the lack of a typical mammalian MPTP may be an adaptive trait to tolerate long periods of anoxia. In support of this hypothesis, a similarly recalcitrant MPTP was found in the ghost shrimp, *L. louisianensis*, which can survive anoxia at room temperature for several days (Holman and Hand 2009) and a regulated MPTP may also be absent from the anoxia-tolerant oyster, *Crassostrea virginica* (Sokolova et al. 2004). Nevertheless, the recalcitrant version of the MPTP seen in *A. Franciscana*, *C. virginica* and *L. Louisianensis* may be a feature of many invertebrates and not necessarily a trait exclusively associated with anoxia tolerance (Hand and Menze 2008). For example, the insensitive MPTP in the embryonic form of *A. franciscana* is also present in their nauppli counterparts (Menze et al. 2005), which are not anoxia-tolerant. Further investigations are needed to determine whether this trait is a general trait of invertebrates or if there is an association with anoxia tolerance and survival.

Very little is known about the functionality of the MPTP in non-mammalian vertebrates, especially with regard to truly anoxia-tolerant animals. Within teleosts, a functional MPTP has been demonstrated in the giant green goby [*Zosterisessor ophiocephalus* (Toninello et al. 2000)], the rainbow trout [*Oncorhynchus mykiss* (Adiele et al. 2012) and the zebrafish (*Danio rerio* (Azzolin et al. 2010)]. When incubated in the presence of Ca^{2+} and phosphate, liver mitochondria from *Z. ophiocephalus* undergo a complete collapse of $\Delta\Psi_m$ accompanied by swelling of the matrix and a release of ions such as Mg^{2+}, Ca^{2+} and K^+ (Toninello et al. 2000). This effect can be completely abolished with cyclosporin A, a known inhibitor of the MPTP, suggesting that induction of the giant goby MPTP has characteristics similar to those observed in mammalian liver mitochondria. Importantly, however, liver mitochondria from the giant goby needed a higher Ca^{2+} concentration (above 100 μmol L^{-1}) for the induction of the MPTP compared to rat liver mitochondria (within 20–50 μmol L^{-1}) suggesting a lower binding affinity for Ca^{2+} (Toninello et al. 2000). Although this species is not known to be anoxia-tolerant, the giant goby burrows into the sediments of marine environments and can withstand large seasonal fluctuations in oxygenation levels, heavy metals and other contaminants. Thus, the Ca^{2+} insensitivity of the goby MPTP may be associated with hypoxia tolerance and/or pollutant tolerance. Ca^{2+} exposure (as low as 5 μM) of rainbow trout hepatocytes leads to mitochondrial swelling which can be abolished by cyclosporin A, suggesting a role for the MPTP in the rainbow trout (Adiele et al. 2012). However, the swelling of *O. mykiss* mitochondria was modest relative to that observed with mammalian mitochondria, suggesting that the mechanisms/kinetics of MPTP induction in this species might be different from the mammalian paradigm (Adiele et al. 2012). In zebrafish mitochondria, the addition of low concentrations of Ca^{2+} (20 μmol L^{-1}) causes a profound mitochondrial depolarization which is prevented by the addition of cyclosporin A (Azzolin et al. 2010). The Ca^{2+}-dependent change in conductance in zebrafish mitochondria also responds in the appropriate manner to the key modulators of the mammalian MPTP pore (such as voltage, pH, ligands of the adenine nucleotide translocator) (Azzolin et al. 2010).

Taken together, these studies suggest that a functional MPTP is evident in a wide range of ectothermic animals, suggesting that the MPTP may have been conserved throughout evolution. At least, some of these ectothermic animals have a desensitized MPTP which requires very high concentrations of Ca^{2+} to activate pore opening. Interestingly, mammalian neonates, which are significantly more tolerant of anoxia compared to their adult counterparts, demonstrate significant ontogenetic differences in the role of the MPTP (Milerova et al. 2010; Ostadal et al. 2009). Cardiac mitochondria isolated from neonatal rats exhibit a Ca^{2+}-dependent and Cyclosporin A-sensitive MPTP which is less sensitive to Ca^{2+} compared with adults (Milerova et al. 2010). Furthermore, mitochondria from different regions of the mammalian brain have different sensitivities to Ca^{2+}-induced swelling, which correlates with differential sensitivities to ischemia/reperfusion (Friberg et al. 1999). This suggests that there is selective vulnerability in the brain to ischemia/reperfusion and which may represent the susceptibility of mitochondria to Ca^{2+}-induced MPTP opening. Collectively, the findings from mammals and anoxia-tolerant species suggest that a desensitized MPTP may constitute a physiological adaptation which limits pore opening during periods of high intracellular Ca^{2+}, such as ischemia/anoxia.

Summary

While very little is known about mitochondrial function in anoxia-tolerant animals, significant advances in our understanding have recently been made. Mitochondria isolated from anoxia-tolerant animals are relatively insensitive to in vitro acute anoxia and reoxygenation, and marked changes in the intrinsic properties of the mitochondria occur in response to long-term anoxia. A downregulation of

respiratory capacity is a common strategy during chronic anoxia which conserves energy and contributes to the hypometabolic condition. By simultaneously downregulating Complex V of the electron transport chain, animals can further limit ATP consumption during anoxia by inhibiting reverse mode action of the F_1F_o-ATPase which consumes ATP. Although an increase in proton leak may be protective in some models of ischemia and anoxia tolerance, it appears that the inherent "tightness" of the ectothermic mitochondrial membrane may limit proton leak during anoxia and alleviate the need for membrane remodeling. While cytosolic Ca^{2+} overload plays a major role in ischemia/reperfusion injury in mammals, intracellular Ca^{2+} is only marginally elevated in the anoxic turtle brain due to a reduction in NMDR receptor activity brought about by Ca^{2+} signaling between the mitochondria and the cytosol. ROS may also be limited in anoxia-tolerant animals by strong anti-oxidant defences, the activation of mK_{ATP} channels and via protective signaling molecules, such as NO. Lastly, anoxia-tolerant animals may possess a desensitized MPTP which may constitute a physiological adaptation that limits pore opening during periods of high intracellular Ca^{2+}, such as hypoxia/anoxia. In conclusion, it is evident that many of the mitochondrial strategies involved in natural anoxia tolerance are effective in reducing tissue damage in mammalian O_2-related diseases. Anoxia-tolerant animals have, therefore, emerged as unique models which could aid in the understanding and treatment of pathological conditions involved in oxidative stress.

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