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REVIEW

Mitochondria: a multimodal hub of hypoxia tolerance[1]

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Abstract: Decreased oxygen availability impairs cellular energy production and, without a coordinated and matched decrease in energy consumption, cellular and whole organism death rapidly ensues. Of particular interest are mechanisms that protect brain from low oxygen injury, as this organ is not only the most sensitive to hypoxia, but must also remain active and functional during low oxygen stress. As a result of natural selective pressures, some species have evolved molecular and physiological mechanisms to tolerate prolonged hypoxia with no apparent detriment. Among these mechanisms are a handful of responses that are essential for hypoxia tolerance, including (*i*) sensors that detect changes in oxygen availability and initiate protective responses; (*ii*) mechanisms of energy conservation; (*iii*) maintenance of basic brain function; and (*iv*) avoidance of catastrophic cell death cascades. As the study of hypoxia-tolerant brain progresses, it is becoming increasingly apparent that mitochondria play a central role in regulating all of these critical mechanisms. Furthermore, modulation of mitochondrial function to mimic endogenous neuroprotective mechanisms found in hypoxia-tolerant species confers protection against otherwise lethal hypoxic stresses in hypoxia-intolerant organs and organisms. Therefore, lessons gleaned from the investigation of endogenous mechanisms of hypoxia tolerance in hypoxia-tolerant organisms may provide insight into clinical pathologies related to low oxygen stress.

Key words: AMP kinase, apoptosis, channel arrest, facultative anaerobe, GABA, glutamate, glycolysis, hypoxia inducible factor, ischemia, metabolic depression, preconditioning, proton gradient, reactive oxygen species, spike arrest, synaptic transmission, uncoupling.

Résumé : Une disponibilité réduite d'oxygène nuit à la production d'énergie par les cellules et, en l'absence d'une réduction coordonnée et proportionnelle de la consommation d'énergie, entraîne rapidement la mort de l'organisme. Les mécanismes qui protègent le cerveau des lésions hypoxiques sont particulièrement intéressants parce que, en plus d'être l'organe le plus sensible à l'hypoxie, le cerveau doit demeurer actif et fonctionnel en situation de stress hypoxique. En réponse à des pressions de sélection naturelle, certaines espèces ont développé des mécanismes moléculaires et physiologiques de tolérance à l'hypoxie soutenue sans effet délétère. Parmi ces mécanismes figurent différentes réactions clés de la tolérance à l'hypoxie, dont (*i*) des capteurs qui détectent les variations de la disponibilité d'oxygène et déclenchent des réactions de protection, (*ii*) des mécanismes de conservation d'énergie, (*iii*) le maintien d'une fonction cérébrale de base et (*iv*) l'évitement des cascades de mort cellulaire catastrophiques. Au fil de l'étude des cerveaux tolérants à l'hypoxie, il est de plus en plus évident que les mitochondries jouent un rôle central dans la régulation de tous ces mécanismes critiques. En outre, la modulation de la fonction mitochondriale pour imiter des mécanismes neuroprotecteurs endogènes observés dans des espèces tolérantes à l'hypoxie confère une protection contre des stress hypoxiques par ailleurs mortels dans les organes et organismes intolérants à l'hypoxie. Aussi, les leçons tirées de l'étude des mécanismes endogènes de tolérance à l'hypoxie dans des organismes tolérants à l'hypoxie peuvent jeter un nouvel éclairage sur les pathologies cliniques associées au stress hypoxique. [Traduit par la Rédaction]

Mots-clés : protéine kinase activée par l'AMP, apoptose, arrêt des canaux, anaérobie facultatif, GABA, glutamate, glycolyse, facteur induit par l'hypoxie, ischémie, dépression métabolique, préconditionnement, gradient de protons, forme réactive de l'oxygène, arrêt des pointes, transmission synaptique, découplage.

Introduction

Oxygen-limited environments are common on earth and many organisms experience periods of intermittent or prolonged hypoxia or anoxia in their daily and (or) annual life cycles (Bickler and Buck 2007). Such environments present a daunting challenge to oxygen-dependent organisms. This challenge is primarily manifested at the cellular level where mitochondria require the potential energy of oxygen to produce adenosine triphosphate (ATP) through the electron transport chain (ETC) via the biochemical process of oxidative phosphorylation. In normoxia, abundant oxygen is readily available for the synthesis of sufficient ATP to meet cellular energy requirements; however, during periods of hypoxia or anoxia, oxygen availability becomes limited and oxidative phosphorylation is halted. As a result, metabolic throughput along the ETC is greatly decreased (or abolished in the case of anoxia) and the cell must rely on anaerobic energy production to make up this energy deficit. Organisms that are tolerant to long-term low oxygen stress typically undergo a coordinated reduction in energy demand to match the hypoxic decrease in energy production (Buck and Pamenter 2006). Recently, considerable advances have been made in our understanding of the molecular and biochemical mechanisms that enable severe reductions in metabolic rate during anoxia, but the signaling mechanisms that detect changes in oxygen availability and subsequently initiate and coordinate this metabolic depression are only beginning to be

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discovered. In particular, evidence supporting a central role for mitochondria in the initiation and coordination of biochemical and metabolic responses to low oxygen stress has begun to accumulate. Indeed, due to the intimate relationship between oxygen and mitochondrial function, mitochondria are well situated to detect changes in oxygen availability. Furthermore, the electrochemical apparatus that underlies ATP production (i.e., the ETC) also imbues mitochondria with pivotal roles in the maintenance of cellular ionic balance, free-radical production, cell division, and cellular fate determination. Through these interactions, mitochondria are also able to communicate changes in oxygen availability to the rest of the cell and coordinate cytoprotective cellular responses to low oxygen stress. Furthermore, mitochondria can also mediate entry into cell death pathways via changes in the regulation of ionic balance, free radicals, and other signaling intermediates.

Studies of species that are highly tolerant to hypoxia and anoxia provide prime examples of this central role for mitochondria in signaling oxygen variability and the subsequent coordination of cellular responses to hypoxia. In these organisms, mitochondria regulate a variety of neuroprotective mechanisms that reduce cellular energy expenditure and enable prolonged tolerance of low oxygen stress (Buck and Pamenter 2006). These pathways mediate diverse responses including changes in transcription, organelle, and synaptic function, as well as intercellular communication. Conversely, in hypoxia-intolerant species, such mitochondria-regulated cytoprotective pathways are not activated. Instead, during periods of low oxygen stress, mitochondrial ion gradients in these species are disrupted, leading to ionic and free-radical imbalances that damage mitochondria and contribute to the induction of cell death (Crompton 2000; Goyal et al. 2007). Importantly, however, mitochondria are evolutionarily ancient structures and their architecture is highly conserved across species; thus, the signaling apparatus that induces and regulates cytoprotective mechanisms in hypoxia-tolerant species also exists, albeit in a generally inactivated state, in hypoxia-intolerant species. Therefore, it is not surprising that interventions targeted to the mitochondria that activate or mimic the endogenous responses of hypoxia-tolerant species have been shown to provide protection across a range of low oxygen and ischemic stresses in hypoxia-intolerant species as varied as the nematode worm *Caenorhabditis elegans* (Maupas, 1900) and the common laboratory rat (*Rattus norvegicus* (Berkenhout, 1769)) (see sections "Ca^{2+} buffering and regulation of channel arrest" and "Ischemic preconditioning in hypoxia-intolerant species" below). Considerable progress has been made recently in understanding the role that mitochondria play both in endogenous neuroprotective mechanisms in facultative anaerobes and also in inducible neuroprotective and cardioprotective mechanisms in the brain and heart of hypoxia-intolerant species. These studies have revealed a remarkable degree of overlap between endogenous and inducible mechanisms of hypoxia tolerance.

The aim of this review is to examine discoveries regarding the role of mitochondria as a cellular hub that coordinates both cytoprotective and deleterious cellular responses to low oxygen stress. Differences in the activation of these pathways between hypoxia-tolerant and hypoxia-intolerant species will be highlighted. Further, similarities between endogenous neuroprotective strategies employed in facultative anaerobes and inducible mechanisms of cytoprotection in hypoxia-intolerant species will also be examined. While studies of the function of isolated mitochondria have been performed in a wide variety of hypoxia-tolerant species, examinations of mitochondrial function and mitochondria-mediated cellular pathways in intact organs or whole animals have been conducted in only a few species. In particular, models of hypoxia tolerance in hypoxia-adapted fruit flies *Drosophila melanogaster* Meigen, 1830 and also in the brain of the most hypoxia-tolerant vertebrates identified—the Western Painted Turtle (*Chrysemys picta bellii* (Gray, 1831)), the Red-eared Slider (*Trachemys scripta elegans* (Wied-Neuwied, 1839)), and the crucian carp (*Carassius carassius* (L., 1758))—have yielded the most complete profile of the role of mitochondria in regulating cellular responses to low oxygen stress in hypoxia-tolerant species. Therefore, this review will focus primarily on research in these organisms to inform discussion of mitochondrial mechanisms of hypoxia tolerance and to contrast such mechanisms to those elucidated in hypoxia-intolerant species, which have received considerably greater research attention.

It is important to note that the definition of hypoxia is a relative concept that varies considerably between species. Hypoxia is generally defined as a level of oxygen that is not sufficient to meet the energetic needs of an organism and that is detrimental without the induction of some form of metabolic depression. Typically, any oxygen level below room air (~21% oxygen) is termed "hypoxic" in the literature, largely due to a prevailing clinical or mammalian bias. However, in the natural environment of many hypoxia-tolerant organisms, oxygen levels considerably lower than 21% are common. Indeed, in anoxia-tolerant turtle models, significant metabolic suppression and many tissue-level adaptations to decreased oxygen do not occur until the animal is completely anoxic (Buck and Pamenter 2006). Therefore, in this organism, levels of oxygen that are commonly referred to as hypoxic relative to the human experience are in fact normoxic or even hyperoxic relative to the turtle's basal milieu, and complete anoxia may be the only true form of hypoxia (i.e., an oxygen level that is insufficient and requires metabolic adaptation) to this organism. For the purpose of this review, "hypoxia" is used to describe low oxygen environments, including anoxia, as appropriate to the species being discussed.

Related to hypoxia is ischemia, which is typically caused by blood vessel occlusion. Ischemia results from obstructed blood flow to a region of cells and tissues and is a common cause of pathological cell death due to stroke or heart attack. Local oxygen delivery is limited by ischemia, and in some cases is completely ablated, resulting in tissue hypoxia or anoxia; however, obstructed blood flow also limits the delivery of energetic substrates and the removal of acidic end products from the ischemic tissue region. Due to the important hypoxic component, studies of the effects of low oxygen stress and ischemia are often linked in the mammalian literature. Conversely, comparative research in hypoxia-tolerant organisms has largely been focused on mechanisms of hypoxia tolerance, as this is a naturally occurring stress in the lives of many organisms; however, a handful of studies have examined ischemic stress in facultative anaerobes. In general, the results from these studies support the concept that endogenous cytoprotective mechanisms of hypoxia tolerance also imbue tolerance against more extreme ischemic insults in the heart and brain of a variety of species (e.g., Dave et al. 2006; Kesaraju and Milton 2009; Pamenter et al. 2012*a*). Furthermore, as will be discussed below, remarkable similarities exist between inducible cellular mechanisms that are cytoprotective against both hypoxia and ischemia in hypoxia-intolerant species, and endogenous mechanisms of hypoxia tolerance in facultative anaerobes. Therefore, for the purpose of this review, these stresses will be considered similar, although an effort is made to indicate which stress is being examined in each species under discussion.

A blueprint for a hypoxia-tolerant brain

It is generally accepted that the key to surviving prolonged periods of low oxygen stress is achieving a sustainable balance between energy production and consumption (Hochachka 1986; Buck and Pamenter 2006). Glycolysis is the primary energy-producing pathway in both aerobic and anaerobic organisms, producing two molecules of ATP from the conversion of glucose into pyruvate (three molecules of ATP from glycogen). During aerobic conditions, these end products are further catabolized via oxidative phosphorylation to produce 30–32 ATP molecules per glucose molecule, including glycolytic production (Hochachka and Dunn 1983). During periods of low oxygen stress, however, oxidative phosphorylation ceases, resulting

in the production of only two to three molecules of ATP from the catabolism of each glycogen molecule. During hypoxic conditions where energy production is severely diminished, organisms can either upregulate glycolytic throughput or decrease energy expenditure to achieve energy balance at the cellular level. An increase in glycolytic rate for the production of additional ATP is known as the Pasteur effect (Ainscow and Brand 1999). The Pasteur effect is not a sustainable long-term protective mechanism because maintaining ATP production rates at normoxic levels with an incremental increase in glycolysis is generally not possible for more than a few minutes. Furthermore, increased glycolytic rates lead to secondary survival bottlenecks such as (*i*) the ability of the organism to handle accumulation of the acidic end products of glycolysis (i.e., lactate), (*ii*) the limited available store of glycogen, and (*iii*) the ability of the organism to maintain delivery of glucose to energy-requiring tissues. Together, these factors limit the practical utility of this strategy to organisms that experience relatively short-term hypoxic bouts (Hochachka and Dunn 1983). Conversely, during periods of long-term hypoxia, most tolerant organisms rely primarily on a coordinated reduction of energy demands to match decreased energy supply, a phenomenon known as metabolic depression (Hochachka et al. 1996; Buck and Pamenter 2006). Through reliance upon metabolic depression, some vertebrates, including freshwater turtles and some fish and amphibian species, display a remarkable tolerance to low oxygen stress (Bickler and Buck 2007). This tolerance is achieved via a plethora of protective mechanisms at the cellular level (e.g., ion channel arrest or electrical depression) (Hochachka 1986; Pérez-Pinzón et al. 1992*a*; Bickler and Buck 2007). Metabolic depression is complimented by numerous systemic-level adaptations (e.g., increased glycogen stores, enhanced capacity to buffer acidic metabolic end products, maintained heart rate and cerebral blood flow) that are also individually critical to long-term survival in low oxygen environments (Jackson 2002; Bickler and Buck 2007); however, perhaps the most important mechanisms are cell-level adaptations that enable large-scale reductions in energy requirements of the brain—the most oxygen-sensitive tissue.

Based on oxygen consumption, the brain is the body's single greatest consumer of ATP in humans. Although it comprises only ~2% of total body mass, neuronal metabolism utilizes ~20% of the human body's total energy at rest (Schmidt-Nielson 1984; Rolfe and Brown 1997). These percentages are generally lower in non-primates, in which the brain typically accounts for 0.1%–1.5% of body mass but still accounts for a disproportionate amount of total body energy consumption, typically ranging from 1.5% to 8.5% (van Ginneken et al. 1996; Rolfe and Brown 1997; Soengas and Aldegunde 2002). The brain is highly aerobic, producing 95% of its ATP via oxidative metabolism in mammals (Erecińska and Silver 1989) and a similarly large majority of ATP via oxidative phosphorylation in lower poilkiothermic vertebrates, including reptiles, fish, and amphibians (Suarez 1988). The brain does not store significant quantities of glycogen or oxygen (Erecińska and Silver 1989). Therefore, the brain is both the largest consumer of ATP and the first tissue to suffer cell damage as a result of oxygen deprivation.

In the brain, ATP is primarily consumed for the functioning of numerous transmembrane pumps, which maintain electrochemical gradients across cell membranes, a requirement for neuronal signaling and survival. It has been estimated that 50%–60% of the energy budget of the brain during normoxia is spent on ion pumping across various cell and organelle membranes (Attwell and Laughlin 2001). Furthermore, where metabolism in most other tissues in the body can essentially be halted to conserve energy during prolonged hypoxia (e.g., skeletal muscle), maintenance of brain function at some reduced level is critical because it is the regulator of autonomic life-support systems in the body, and also because energy charge must be maintained in brain as neurons and glia undergo cell death if their electrical charge is not maintained. Therefore, maintenance of some degree of energy balance and function is obligatory in the brain during hypoxia, and a strategy to drastically reduce energy demand is particularly important in this organ.

Based on these requirements, it is possible to assemble a list of essential criteria to form a blueprint of a hypoxia-tolerant brain. These basic requirements are (*i*) a mechanism to sense changes in oxygen availability and communicate these changes within the cell and tissue region, (*ii*) mechanisms to downregulate energy consumption of the afflicted cells in a manner that is coordinated with the limited energy production capabilities during hypoxia, but that still (*iii*) enables maintenance of basic neuronal function. Furthermore, hypoxia-tolerant brains should have mechanisms to (*iv*) avoid catastrophic cell death cascades that are characteristic of hypoxia-intolerant brains. As study of the hypoxia-tolerant brain progresses, it is becoming increasingly apparent that mitochondria play central roles in regulating all of these critical mechanisms. A fifth major component of a hypoxia-tolerant brain is a mechanism to tolerate or beneficially regulate pH. Anaerobic metabolism leads to the marked accumulation of acidic end products and hypoxia-tolerant species have evolved diverse mechanisms to handle this. For example, turtles utilize extensive buffering systems based on bone and shell sequestration of ions (Jackson et al. 2000), while carp are able to convert acidic end products to ethanol and excrete these via their gills (Shoubridge and Hochachka 1980). Although critical to hypoxia tolerance, these mechanisms are typically not regulated by mitochondria and are therefore not discussed in the present review.

Mitochondria as cellular oxygen meters

Mitochondria consume more than 90% of the oxygen taken up by cells for oxidative phosphorylation (Rolfe and Brown 1997). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet}$), are chemically reactive molecules that are formed as a by-product of the cellular reduction of oxygen (Turrens 2003). Physiologically, ROS are primarily produced during mitochondrial respiration and it is estimated that 2%–3% of oxygen consumed by mitochondria are incompletely reduced, yielding ROS (Turrens 2003). Due to the high sensitivity of this process to oxygen levels, ROS are excellent candidates to regulate cellular responses to changes in systemic or tissue-level oxygen availability. Indeed, ROS are important second messengers that regulate a myriad of intra- and extra-cellular signaling pathways (Kakkar and Singh 2007). For example, ROS are able to function as direct signaling molecules by modifying target proteins via the oxidation of reactive -thiol groups. This process results in the formation of disulfide bonds, which reversibly alter protein structure and thus function (Forman 2009). Examples of such target proteins include phosphatases that modify signal transduction pathways (e.g., mitogen-activated protein kinases (MAPKs), proteins that regulate embryogenesis, nuclear transcription factors, RNA-binding proteins that effect DNA methylation, and also proteins involved in processes related to histone acetylation, deacetylation, or methylation (i.e., epigenetic modifications), among many others (Kovtun et al. 2000; Winterbourn and Hampton 2008; Shlomai 2010; Sundar et al. 2010; Ufer et al. 2010).

In addition to such roles as potent biomessengers, ROS also have deleterious effects on cells or organelles during periods of environmental or metabolic stress, as they communicate cellular distress or induce damage directly. For example, in mammalian stroke pathology, ROS generation from neurons peaks during the reperfusion phase. This spike in ROS production leads to local DNA damage and also deleterious activation of cell death pathways in surrounding tissue (the penumbra) (Flamm et al. 1978; Barzilai 2007; Kakkar and Singh 2007). Furthermore, ROS generated following ischemic stroke contribute to the breakdown of the blood–brain barrier, inducing edema and facilitating penumbral spread (Fraser 2011). Therefore, ROS generation also plays an important role in the initiation of cell death pathways and the

propagation of cell death via myriad cellular and intercellular interactions.

It has been hypothesized that hypoxic inhibition of mitochondrial oxidative phosphorylation alters ROS production, signaling cellular oxygen tension changes (Rounds and McMurtry 1981). This theory has been challenged by studies showing that oxygen-dependent responses are initiated at oxygen tensions above those required to inhibit mitochondrial respiration (Buescher et al. 1991). Indeed, in the anoxic turtle brain, ROS production is significantly decreased from controls after 10 min (Milton et al. 2007; Pamenter et al. 2007), whereas cytoprotective mechanisms against anoxic stress such as ion channel arrest and spike arrest may be initiated within a shorter time scale (Bickler et al. 2000; Pamenter et al. 2011). However, this discrepancy may be due to detection limitations of the tools for measuring ROS that are currently available. It is possible that small or regional changes in ROS, which are beyond the detection capacity of commonly used fluorescence-based assays, induce protective changes very rapidly soon after oxygen availability decreases. Future studies that take advantage of emerging technologies with the resolution to detect ROS changes in micro domains within cells will help to resolve this question.

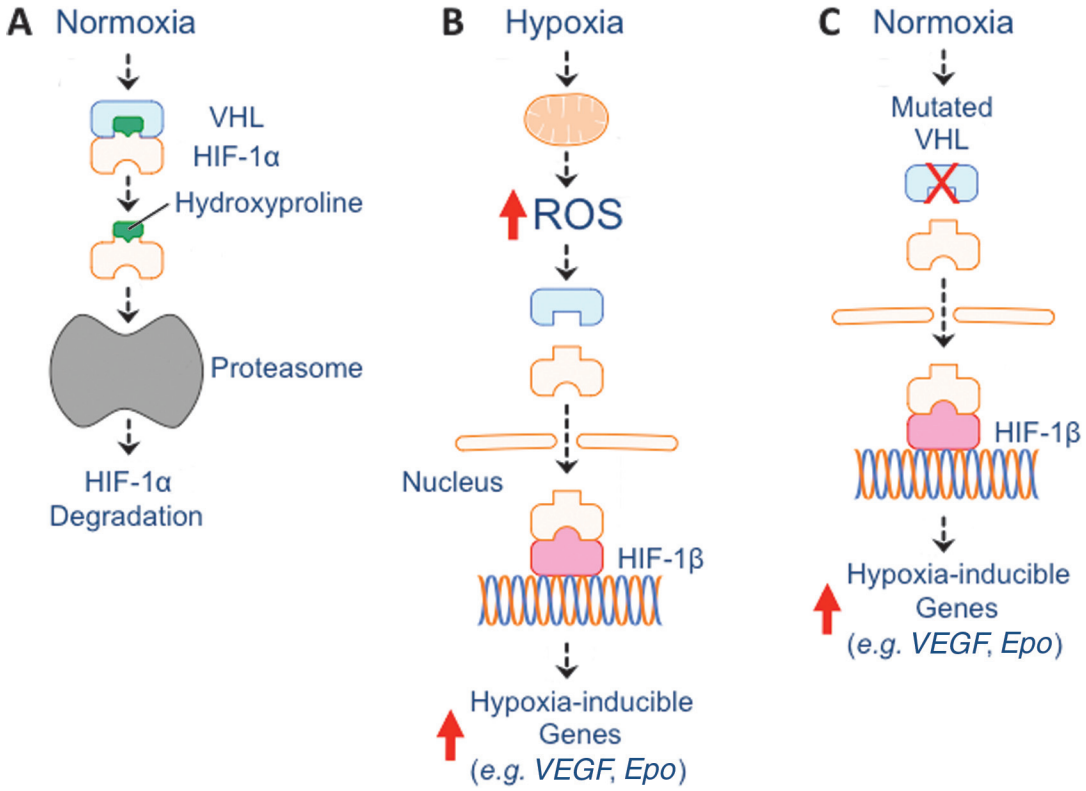
In addition to direct effects of ROS on protein or gene function, another key set of cellular responses induced by changes in oxygen that are regulated by mitochondria is the array of cellular changes mediated by hypoxia-inducible factor-1 (HIF-1) activity. HIF-1 was initially identified by its binding to the hypoxia response element (HRE) of the erythropoietin (*Epo*) gene after hypoxic exposure (Wang et al. 1995). HIF-1 is a heterodimeric transcription factor composed of an α and a β subunit (HIF-1α, HIF-1β). HIF-1α is ubiquitously expressed and its stability is oxygen-sensitive, such that during normoxia HIF-1α is continuously synthesized and degraded, whereas during hypoxia the degradation of HIF-1α is retarded (Weidemann and Johnson 2008). The interface between oxygen and HIF is provided by oxygen-dependent prolyl hydroxylation of the α subunit (Weidemann and Johnson 2008; Prabhakar and Semenza 2012). This enzymatic reaction in turn regulates the activity of Von Hippel – Lindau (VHL) tumor suppressor protein, which ubiquitinalates HIF-1α, targeting it for proteolysis (Fig. 1A). In hypoxia, hydroxylation of HIF-1α and thus recognition by VHL is prevented and HIF-1 is translocated to the nucleus where it binds to HREs, leading to the transcription of a wide variety of hypoxia-related genes involved in angiogenesis, cell proliferation, erythropoiesis, glucose transport, glycolytic metabolism, and cell survival, among others (Fig. 1B) (Semenza et al. 2006). For example, HIF-1 is central to the switch between oxidative and anaerobic metabolism and it increases the expression of the key glycolytic enzymes lactate dehydrogenase A and pyruvate dehydrogenase kinase 1, which increase the synthesis of ATP in anaerobic conditions (Semenza 2011). HIF-1 also promotes vascular endothelial growth factor (*VEGF*) function, which is a key mediator of angiogenesis and promotes endothelial cell migration to hypoxic areas. This is particularly important in the formation of the vasculature in embryonic development (Semenza 2011) and may play a role in increased vascularization of oxygen-exchange surfaces in facultative anaerobes (Sollid et al. 2006). The precise mechanism of HIF-1α stabilization during low oxygen stress remains poorly understood but is thought to involve mitochondria-derived ROS since free-radical scavengers abolish the hypoxic HIF response (Chandel et al. 1998). A leading model to explain HIF-1 stabilization by mitochondria posits that during hypoxia, increases in mitochondria-generated ROS prevent HIF-1α protein degradation and thereby communicate a change in oxygen availability to the HIF machinery. This subsequently activates the myriad of cellular responses to hypoxia mediated by HIFs (Chandel et al. 1998; Klimova and Chandel 2008).

Despite the clear importance of HIFs in regulating responses to hypoxia, surprisingly little research has examined the role of HIFs in initiating protective mechanisms during periods of low oxygen stress in facultative anaerobes and other hypoxia-tolerant species. Although discovered in 1995, HIF-1α was not cloned in a vertebrate facultative anaerobe until 2006 when Nilsson's group identified it in crucian carp gill (Sollid et al. 2006). These researchers found that hypoxia increased HIF-1 expression in carp gill and they also reported a high basal expression of HIF-1α in gill during normoxia, which is uncharacteristic of hypoxia-intolerant species. However, these authors were unable to definitively link hypoxic increases in HIF-1α to gill remodeling in the hypoxic carp. Conversely, another study from the same group found that hypoxia increased HIF-1α expression in numerous carp tissues at cold temperatures, suggesting a functional link between temperature acclimatization and hypoxic stress in hypoxia-adapted species (Rissanen et al. 2006). These studies may indicate that HIFs in facultative anaerobes play a role in regulating cellular responses during normoxia but that environmental factors other than hypoxia are more important in the regulation of HIF induction during hypoxia in these species. Thus, mechanisms other than HIF-1α may be more critical in activating adaptive mechanisms of hypoxia tolerance in facultative anaerobes. Unfortunately, neither of these studies examined HIF changes in the brain, which is the most anoxia-sensitive organ in the body. Further studies examining the role of HIF-1α in the brain of crucian carp and other facultative anaerobes will likely yield interesting results that may help to illuminate the point of deviation between aerobic and anaerobic energetic pathways in such species.

A clear indicator of the role of HIFs in mediating protective responses to hypoxia may be more apparent in studies of less hypoxia-tolerant species, such as mole-rats, which exhibit an intermediate degree of hypoxia tolerance relative to anoxia-tolerant facultative anaerobes. Blind subterranean mole rats of the genus *Spalax* Guldenstaedt, 1779 and the naked mole-rat *Heterocephalus glaber* Rüppell, 1842 are generally considered to be among the most hypoxia-tolerant mammals described to date. These animals often tolerate burrow oxygen concentrations as low as 5%–7% during the rainy season and survive in the laboratory at oxygen concentrations as low as 3% for hours to days without any observable change in their behavior (Sherman et al. 1991; Shams et al. 2004). Relative to rats, *Spalax* typically have far greater *VEGF* expression, enhanced mitochondrial and capillary density, and also higher erythrocyte counts and *Epo* expression relative to hypoxia-intolerant rats (Arieli et al. 1986; Avivi et al. 1999; Shams et al. 2004). These adaptations are largely due to enhanced hypoxia-mediated recruitment of HIF-1 in *Spalax* relative to rats (Shams et al. 2004). Similar to crucian carp, basal *HIF-1α* expression is substantially higher in the normoxic mole-rat; in response to hypoxic exposures, *HIF-1α* expression increases significantly during the first 4–12 h of exposure and these increases are markedly greater than increases observed in rat tissue. Importantly, the degree to which *HIF-1α* expression increases correlates with the degree of environmental hypoxia typically experienced between different subspecies of *Spalax*, such that in species that typically experience less severe hypoxia in their natural environment, smaller increases in the expression of *HIF-1α* are observed. Similarly, graded responses were found for the expression of *Epo*, which is a HIF-dependent gene. In *Spalax*, *Epo* expression increases up to 250-fold during hypoxia, a change that is 5-fold greater than that observed in rat, and this increase is sustained through ~40 h of hypoxic exposure (Shams et al. 2004). Also similar to HIF-1, the degree of hypoxia-induced changes in *Epo* correlates directly with the relative degree of hypoxia tolerance between subspecies of *Spalax*. The relatively high expression of HIF and *Epo* in *Spalax* compared with rats also persists throughout developmental stages such that *Spalax* exhibits relatively high basal expression of *Epo* and HIF-1 mRNA at the embryonic, newborn, and adult stages of development, and hypoxic exposure induces larger increases in both genes in multiple tissues at all developmental stages relative to rats (Shams et al. 2005).

The recent sequencing of the naked mole-rat, which is considered to be the most hypoxia-tolerant of the mole-rat species,

Fig. 1. HIF-mediated oxygen sensing is regulated by mitochondrial reactive oxygen species (ROS). (A) In the presence of oxygen, hypoxia-inducible factor-1α (HIF-1α) binds to Von Hippel – Lindau (VHL) tumor suppressor protein, which ubiquitinates HIF-1α. Ubiquitinated HIF-1α is degraded by the proteasome. (B) In most hypoxia-intolerant species, hypoxia increases mitochondrial ROS generation, which stabilizes HIF-1α, allowing it to heterodimerize with hypoxia-inducible factor-1β (HIF-1β). This HIF dimer translocates to the nucleus and binds to hypoxia response elements to upregulate the transcription of hypoxia-inducible genes, such as vascular endothelial growth factor (*VEGF*) and erythropoietin (*Epo*). (C) In the hypoxia-tolerant naked mole-rat (*Heterocephalus glaber*), HIF-1α expression is high even during normoxia. In this organism VHL is mutated, which would prevent binding of HIF-1α to VHL and degradation of HIF-1α.



provides some insight into the mechanism behind the high basal level of HIF expression reported in this species. Sequence analysis of the naked mole-rat *HIF-1α* revealed a mutation in the VHL protein-binding domain that is unique among animals studied to date (Kim et al. 2011). As described above, VHL mediates ubiquitin-dependent degradation of HIF-1α during normoxia. The reported mutation occurs at a functional site of VHL that would impair HIF-1α degradation and likely underlies the high expression of HIF-1α in normoxic mole-rat (Fig. 1C), which may lead to a hypoxia-like profile of gene expression in the naked mole-rat during normoxic conditions. It remains to be determined if similar mutations occur in HIFs of other hypoxia-tolerant species (carp would be of considerable interest given the high basal expression of HIF reported previously in this organism), but as the genomes of more such species are being sequenced (e.g., Shaffer et al. 2013), it will become possible to answer this question.

While research investigating mitochondrial ROS signaling and in particular HIF-1α-mediated signaling in hypoxia-intolerant species is reasonably advanced, efforts to examine these mechanisms in facultative anaerobes and other hypoxia-tolerant species is still in relatively early stages. Initial results suggest that these mechanisms play some role in mediating the hypoxia tolerance of adapted species, but this role may be dependant on other environmental factors, such as temperature gradients, and may also be overshadowed by other signaling mechanisms. Elevated basal expression of HIFs during normoxia relative to intolerant animals appears to be a common feature of hypoxia-tolerant species, but the sample size for comparison is too small to draw meaningful conclusions, while the mechanisms mediating this high basal expression are only beginning to be understood. One approach that holds considerable promise in determining the role of HIFs in hypoxia adaptation is the study of lines of fruit flies that have been selected through multiple generations for hypoxia tolerance (Zhou et al. 2007). Unfortunately, the study of HIF expression changes between these hypoxia-selected flies and naïve flies has yet to be undertaken. In the race to delineate cellular oxygen sensors, the role of mitochondria remains to be determined, and in particular, examination of the interplay between mitochondrial ROS signaling and HIF stabilization in hypoxia-tolerant species remains unexplored. Nonetheless, the architecture of mitochondria provides this organelle with a myriad of signaling capabilities beyond HIF-mediated signaling that allows coordination of cellular responses to oxygen. Examples of this will be discussed in the following sections.

Mitochondrial architecture: the proton-motive force and the mild uncoupling hypothesis

The architecture of mitochondria is complex and imbues this organelle with a diverse array of signaling capabilities. Morphologically, two membranes functionally divide the mitochondria. In turn, these two membranes define two sub-mitochondrial compartments: the intermembrane space located between the inner and the outer membranes, and the mitochondrial matrix located within the inner membrane. The outer membrane contains a number of proteins that make it permeable to molecules up to 10 kDa in size. Conversely, the inner membrane is composed of a higher percentage of specialized proteins and is the primary permeability barrier between the cytosol and the mitochondrial ma-

trix. There is a large voltage gradient across the inner membrane, with the matrix being approximately –180 mV with regard to the outside of the cell (O'Rourke 2000, 2007). This voltage gradient is due primarily to the proton (H^+) gradient, which results from the reduction of oxygen by the ETC. The impermeability of the inner mitochondrial membrane to H^+ allows the energy of H^+ traveling back along their concentration gradient via specialized channels and pumps to be harnessed to power the movement of other ions across the membrane against their own respective concentration gradients. For example, the mitochondrial membrane potential (Ψ_m) is generated primarily by the pumping of K^+ ions out of the matrix via a H^+/K^+ antiporter, which is powered by this H^+ gradient. The combination of the ion concentration and electrical gradients results in a proton-motive force, which energizes the phosphorylation of adenosine diphosphate (ADP) to ATP via the ATP synthetase (O'Rourke 2000, 2007). Thus, the H^+ gradient is tightly coupled to ATP production and any perturbation that dissipates the gradient for physiological purposes other than ATP production is termed an uncoupling mechanism.

In addition to powering ATP production, the proton-motive force is directly harnessed by ion transporters to power the movement of ions across the mitochondrial membrane (via the movement of H^+ ions along their gradient). In particular, mitochondrial Ca^{2+} sequestration is highly dependent on the proton-motive force (Mitchell and Moyle 1967). A balance between mitochondrial Ca^{2+} uptake and Ca^{2+} efflux mechanisms determines the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$), and these mechanisms are dependent on the mitochondrial H^+ gradient. Unique to the mitochondria, Ca^{2+} entry into this organelle is mediated by ion movement along its ion gradient via gated channels, while Ca^{2+} exchangers mediate efflux. This is contrary to the movement of most cations across organelle membranes and is due to the very negative membrane potential across the mitochondrial membrane. Ca^{2+} uptake into the mitochondria occurs electrophoretically via the activity of the mitochondrial Ca^{2+} uniporter (MCU), which has been recently identified after more than 30 years of extensive research efforts (Baughman et al. 2011; De Stefani et al. 2011). Patch-clamp experiments on the inner mitochondrial membrane of mitoplasts (i.e., mitochondria that have been stripped of their outer membrane) demonstrate that the uniporter has a very high affinity for Ca^{2+} (2 nmol/L; Kirichok et al. 2004), suggesting that this channel functions at a resting rate under normal physiological conditions and increases the rate of Ca^{2+} uptake into the mitochondria under pathological conditions when the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) is elevated. Calcium influx leads to a net inward movement of two positive charges and thus a temporary dissipation of Ψ_m, which is counterbalanced by an opposing flux of H^+ ions. This leads to alkalinization of the mitochondrial matrix that eventually inhibits further Ca^{2+} uptake.

Conversely, mitochondrial Ca^{2+} efflux occurs via the activity of a Na^+/Ca^{2+} exchanger and its associated Na^+/H^+ antiporter in non-excitable cells, whereas Ca^{2+} efflux is mediated in excitable tissues primarily via the activity of a Na^+/Ca^{2+} exchanger (Palty et al. 2010). The activity of the Na^+/Ca^{2+} exchanger is electrogenic with a stoichiometry of three Na^+ ions transported into the mitochondria for each Ca^{2+} ion transported out (Pizzo et al. 2012). Calcium equilibrium therefore results in a futile Ca^{2+} cycle that dissipates the mitochondrial H^+ gradient (Drago et al. 2011). Calcium release from mitochondria has also been demonstrated to occur through a low-conductance conformation of the mitochondria permeability transition pore (MPTP), potentially as a pressure-release mechanism during periods of stress to essentially remove accumulated Ca^{2+} from mitochondria and prevent or delay the formation of the high-conductance MPTP conformation and ensuing cell death (Huser and Blatter 1999). Mitochondrial Ca^{2+} cycling is a well-reviewed field and further information can be found elsewhere (Gunter et al. 1998; O'Rourke 2007; Pizzo et al. 2012).

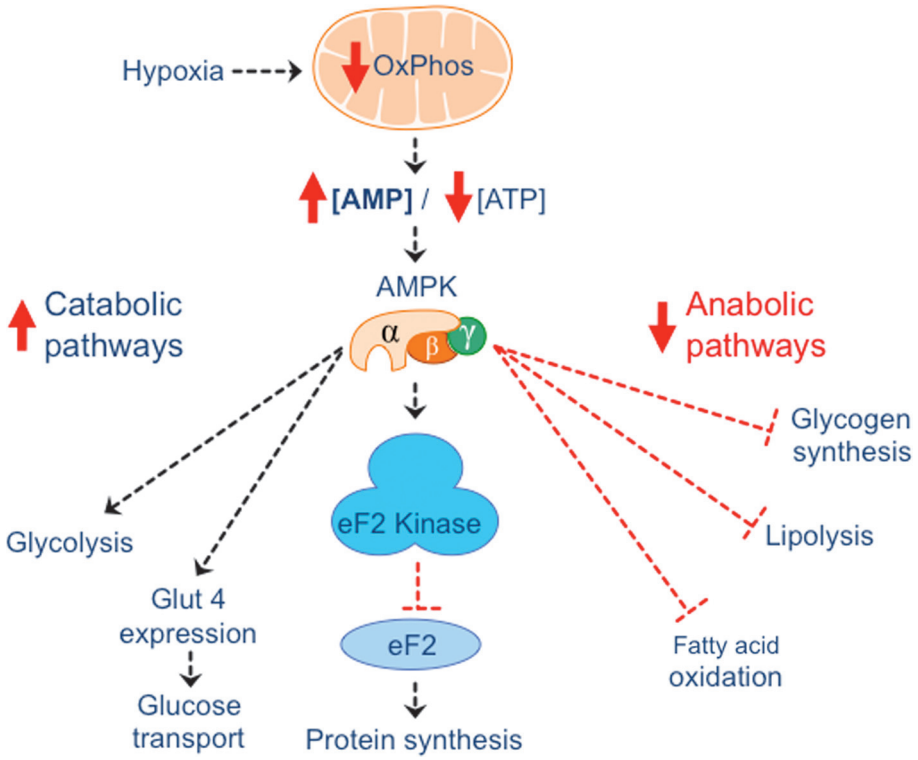
Opening of mitochondrial ion channels allows ions to flow across the mitochondrial inner membrane along their concentration gradient, which indirectly dissipates Ψ_m. In particular, activation of K^+ conductance leads to a depolarization of Ψ_m. This is because increased K^+ influx is partially countered by increasing the activity of the K^+/H^+ exchanger, which pumps K^+ ions out of the matrix at the expense of the H^+ gradient. The loss of available H^+ ions to compensate for the flux of K^+ ions results in a partial or "mild" uncoupling of the H^+ gradient and a depolarization of Ψ_m. Such mild uncoupling causes a small depolarization of Ψ_m that is not sufficient to abolish mitochondrial functions driven by Ψ_m, but is sufficient to alter their rate of activity. ATP production, mitochondrial Ca^{2+} buffering, and ROS generation are examples of mitochondrial functions significantly affected by mild uncoupling (O'Rourke 2000, 2007), and metabolites related to ATP, Ca^{2+}, and ROS are all important cellular and intracellular messengers. For example, (*i*) ATP and its metabolites (ADP, adenosine monophosphate (AMP), and adenosine) modulate purinergic receptors; (*ii*) by buffering Ca^{2+}, mitochondria indirectly modulate Ca^{2+}-mediated cellular signaling pathways, while Ca^{2+} influx into the mitochondria activates matrix dehydrogenases and thereby accelerates electron flow through the ETC and ATP synthesis; (*iii*) as discussed above, ROS directly act to modulate protein function, including stabilization of HIFs, modification of cytosolic proteins and enzymes, and direct modification of ion channels. Each of these mitochondrial functions are critical to a variety of adaptive cellular responses to hypoxia in endogenously hypoxia-tolerant facultative anaerobes and also to induced protection in hypoxia-intolerant species, and alterations of these second messengers by mild uncoupling makes the mitochondria a pivotal player in signaling mechanisms during periods of low oxygen stress. Examples of signaling roles for energetic metabolites (Ca^{2+} and ROS) in mediating both endogenous and inducible hypoxia tolerance will be addressed in the proceeding sections.

Effects of mild uncoupling

AMPK activation and inhibition of protein synthesis

5′-AMP-activated protein kinase (AMPK) is an enzyme that plays a central role in cellular energy homeostasis. AMPK is a heterotrimeric protein composed of a catalytic α subunit combined with regulatory β and γ subunits, which detects changes in the cellular AMP/ATP ratio with a high degree of sensitivity (Xiao et al. 2007). This sensitivity allows AMPK to function as a metabolic master switch that regulates a wide variety of cellular metabolic systems including glucose uptake, β-oxidation of fatty acids, and mitochondrial activity (Fig. 2) (Hardie 2003). During periods of hypoxia, mitochondrial oxidative phosphorylation decreases, leading to a drop in cellular ATP production. Simultaneously, as ATP is utilized in the cell, it creates an ADP molecule. ADP then acts to replenish cellular ATP by donating a phosphate group to another ADP, forming an AMP molecule in the process. Overall these processes result in an increase in the AMP/ATP ratio, leading to allosteric activation of AMPK (Carling et al. 2011). AMPKs then act to inhibit anabolic energy consuming pathways in the cell including protein synthesis via the phosphorylation of elongation factor 2 (eF2), while also stimulating energy production via catabolic pathways, such as glycolysis, fatty acid oxidation, and glucose uptake (Fig. 2) (Marsin et al. 2000; Ronnett et al. 2009). In hypoxia-tolerant species, energy consumption decreases as much as 90% within minutes to hours of the onset of hypoxia (Buck and Pamenter 2006), and large reductions in protein synthesis have been reported in multiple tissues of anoxia-tolerant turtles and crucian carp (Land et al. 1993; Smith et al. 1996; Fraser et al. 2001), among others. Furthermore, during periods of prolonged low oxygen stress, facultative anaerobes rely increasingly on oxygen-independent energy production pathways, which are typically modulated by AMPKs (Marsin et al. 2000).

Fig. 2. Mitochondria mediate metabolic signaling via AMPK activity. During low oxygen stress, mitochondrial oxidative phosphorylation is reduced or prevented, resulting in decreased ATP production and an increase in the cellular adenosine monophosphate to adenosine triphosphate (AMP/ATP) ratio. This activates AMP-activated protein kinase (AMPK). AMPK activation upregulates cellular catabolic pathways and downregulates anabolic pathways. For example, AMPK inhibits protein synthesis by stimulating elongation factor-2 (eF2) kinase, which inhibits eF2, a key modulator of protein synthesis. Black arrows indicate upregulated pathways. Broken red lines indicated downregulated pathways.



The first direct evidence of a role for AMPK in mechanisms of hypoxia tolerance in facultative anaerobes was presented by Jibb and Richards (2008), who found that severe hypoxic exposure for 1–12 h increases AMPK activity and decreases protein synthesis and eF2 expression in the liver (but not in muscle, heart, gill, or brain) of hypoxia-tolerant goldfish (*Carassius auratus* (L., 1758)) (Jibb and Richards 2008). These increases in AMPK activity and eF2 expression correlate closely with increases in the AMP/ATP ratio, suggesting that AMPK activation is due to a disruption of cellular energy homeostasis. More recently, the same group reported a similar role for AMPK in isolated goldfish hepatocytes in which pharmacological agonism of AMPK activity led to increased AMPK enzyme activity and phosphorylation, increased eF2 phosphorylation, and decreased protein synthesis rates and oxygen consumption (metabolic rate) (Lau and Richards 2011). It is worth noting that in vivo changes of AMPK and eF2 in the liver of goldfish are accompanied by an approximately 50% decrease in liver [ATP] that is sustained throughout the hypoxic episode (Jibb and Richards 2008). This change in ATP is similar to measurements from excitable tissues in other facultative anaerobes such as the brain of anoxia-tolerant turtles, in which in vivo ATP levels drop ~23% during prolonged anoxic exposure (Buck et al. 1998) suggesting that AMPK-mediated metabolic changes may also be activated in the brain of this organism. Unfortunately, AMPK activity in the brain of anoxia-tolerant turtle has not been measured; however, increases in AMPK and eF2 phosphorylation have been reported in white muscle of turtles following a 20 h dive (Rider et al. 2009). Although significant changes in AMPK activity are not observed in the brain of goldfish during hypoxia, the activity of this enzyme tends to increase with hypoxia and the failure to detect significant changes here may have been due to an insufficient sample size. Unfortunately, goldfish brain [ATP] was not also measured in these experiments, so comparison with measurements in turtles is not possible.

Another possible explanation for the lack of an increase in AMPK activity in excitable brain and heart tissue in goldfish may be the severity of hypoxic exposure. In the heart and brain of crucian carp exposed to severe hypoxia for 1–21 days, hypoxia does not affect the phosphorylation state of AMPK in either tissue (Stensløkken et al. 2008). Conversely, in carp exposed to total anoxia, large-scale increases in the phosphorylation of AMPK in brain and heart are detected within 1 day of anoxia and these changes persist through 21 days of anoxia in the brain and 7 days in heart. Importantly, blockade of AMPK in vivo increases ethanol secretion from anoxic carp indicating that AMPK acts to suppress metabolism during anoxia in this organism (Stensløkken et al. 2008). These findings suggest that in truly anoxia-tolerant facultative anaerobes, AMPK-mediated changes in metabolic activity are not needed until complete anoxia is achieved. A similar relationship may occur in goldfish such that with increased severity of hypoxia, changes in AMPK activity in the brain and other organs may reach significance. This question deserves further study.

Additional support for a critical role for AMPK in mediating adaptive responses to low oxygen stress in hypoxia-tolerant species comes from studies of anoxia-tolerant zebrafish (*Danio rerio* (Hamilton, 1822)) embryos in which development halts in response to anoxia. In this model, developmental arrest is accompanied by a large upregulation of AMPK pathways (Mendelsohn et al. 2008). Similarly, in the liver of the hypoxia-tolerant Perez's frog (*Pelophylax perezi* (López-Seoane, 1885)), which depresses its metabolic rate during periods of low oxygen stress to extend sur-

vival time, AMPK and eF2 phosphorylation both increase during 30–120 min of anoxic exposure (Bartrons et al. 2004).

Together, these studies provide a reasonable body of evidence in support of a key role for AMPK in mediating cellular and systemic metabolic responses to low oxygen stress in hypoxia-tolerant species. Such responses are likely indirectly coordinated by mitochondria since large-scale changes in the AMP/ATP ratio in these organisms primarily occur following a cessation of mitochondrial oxidative phosphorylation in the absence of oxygen (Egan et al. 2011). The fact that complete anoxia, and not severe hypoxia, is sufficient and necessary to initiate AMPK-mediated responses in some tissues of the most hypoxia-tolerant species is likely related to the ability of these organisms to reduce ATP demand in excitable cells via a variety of mechanisms such as channel arrest and spike arrest (see below) that can be switched on more rapidly than protein synthesis is switched off, along with the ability to preferentially shunt blood flow to at-risk organs (e.g., brain and heart), thereby increasing the depth and (or) duration of hypoxia that these organisms and their cells can tolerate before activation of AMPK-mediated metabolic adjustments becomes a necessity. In this fashion, protein synthesis and other anabolic pathways can continue to function during periods of hypoxia, which fish and turtles likely experience regularly in their daily activities, but such anabolic processes can also be switched off to provide greater degrees of energy savings during periods of prolonged anoxic stress, as signaled by a complete cessation of oxidative phosphorylation and controlled reduction in cellular [ATP]. Furthermore, enhanced sensitivity of AMPK-mediated metabolic responses in the liver of these organisms relative to brain and heart is also sensible, as this would serve to increase glycogen release from the liver to serve as a substrate for anaerobic pathways in more sensitive organs during prolonged anoxia, but also during routine periods of intermittent hypoxia.

Ca^{2+} buffering and regulation of channel arrest

In addition to mitochondria-related signaling of energetic state, this organelle also plays a central role in the regulation of $[Ca^{2+}]_c$, which is a key second messenger in a wide variety of cellular signaling pathways. Mitochondrial Ca^{2+} uptake is primarily a response to increasing $[Ca^{2+}]_c$, and during ischemia or low oxygen stress, increased $[Ca^{2+}]_c$ is an early trigger of mitochondria-mediated cell death. In hypoxia-intolerant organisms, low oxygen stress induces elevations in the excitatory amino acid glutamate (Bosley et al. 1983; Abele et al. 1990; Andiné et al. 1991), primarily by the reversed operation of glutamate transporters (Rossi et al. 2000). Increased glutamate chronically activates glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate receptors (NMDARs), which permit excessive Na^+ and Ca^{2+} influx that leads to neuronal depolarization and electrical hyperexcitability (Abele et al. 1990; Michaels and Rothman 1990; Andiné et al. 1991; Crépel et al. 1993; Lyubkin et al. 1997). These excitatory events permit significant ion movement that requires compensation by ATP-dependent pump activity to restore ionic gradients and neuronal homeostasis. However, as discussed above, the hypoxic or ischemic cell suffers an approximately 90% reduction in ATP availability due to reductions in oxidative phosphorylation. This energy-production deficit, combined with greatly increased ATP demand due to heightened neuronal excitability, results in a rapid depletion of cellular ATP stores (Kopp et al. 1984; Santos et al. 1996). Depletion of ATP results in the abolishment of ATP-dependent pump activity, triggering a further depolarization of V_m, which is irreversible upon reoxygenation (Lundberg and Oscarsson 1953; Anderson et al. 2005). Extended neuronal depolarization chronically overactivates voltage-sensitive channels and deleterious concentrations of Ca^{2+} and Na^+ continue to enter the cell, leading to further depolarization and acceleration of excitatory events. As $[Ca^{2+}]_c$ rises, mitochondria take up free cytosolic Ca^{2+} and $[Ca^{2+}]_m$ increases concomitantly, opposed by the activity of the mitochondrial Ca^{2+}/H^+ or Ca^{2+}/Na^+ exchangers (Pizzo et al. 2012). When $[Ca^{2+}]_c$ reaches approximately 4–500 nmol/L, the ability of these exchangers to oppose mitochondrial Ca^{2+} uptake is overwhelmed and $[Ca^{2+}]_m$ begins to rise rapidly. This is termed the 'set point' and $[Ca^{2+}]_m$ becomes overloaded at 1–3 μmol/L $[Ca^{2+}]_c$, although this threshold is modulated by a variety of cellular factors, most notably cellular and mitochondrial pH (Di Lisa and Bernardi 2009).

Excessive uptake of Ca^{2+} into the mitochondria induces the formation of the MPTP, a junctional complex that permits ions and solutes up to 1500 Da in size to readily pass out of the organelle and which enables the release of mitochondrial apoptotic factors that trigger cell death (Kannurpatti et al. 2004; Wang and Qin 2010). Many laboratories have shown that prevention of MPTP formation is critical to avoiding neuronal apoptosis and necrosis following ischemic damage. For example, in neonatal rat myocytes, ischemia–reperfusion results in apoptotic events that are abolished by cyclosporine A (CsA), an inhibitor of the MPTP (Xu et al. 2001). The MPTP is currently believed to be formed between the adenine nucleotide translocase protein of the inner mitochondrial membrane and an as yet unidentified protein of the outer mitochondrial membranes (for review see Crompton 2000; Halestrap 2010; Suh et al. 2013). However, there remains considerable debate regarding the identity of the core components of the MPTP, as genetic mutants lacking each of the individual proposed components of the MPTP (including adenine nucleotide translocase) still exhibit pore formation (Rasola and Bernardi 2007; Brenner and Moulin 2012). There is also some variability in the stage of a given stressor that induces MPTP formation, and in some pathologies and tissues, MPTP formation is induced upon reoxygenation (e.g., in cardiomyocytes; Assaly et al. 2012), while in others MPTP formation can occur within minutes of stress onset (e.g., in somatosensory cortex; Liu and Murphy 2009). Overall then, the maintenance of $[Ca^{2+}]_m$ during both hypoxia/ischemia and reoxygenation is critical to surviving low oxygen stresses, but this goal is not naturally achieved in hypoxia-intolerant animals and pharmacological interventions are required to provide neuroprotection. Study of the MPTP, its regulation, and its components are a large and highly contested field of research that is beyond the scope of the present review. Interested readers will find further information in several excellent recent reviews (Rasola and Bernardi 2007; Halestrap 2010; Brenner and Moulin 2012; Suh et al. 2013).

Unlike hypoxia-intolerant animals, limiting influx of Ca^{2+} into neurons during periods of low oxygen stress is a hallmark of facultative anaerobe brain. Although no measurements of changes in $[Ca^{2+}]_m$ during hypoxia have been made to date in hypoxia-tolerant species, many groups have demonstrated blunted increases in $[Ca^{2+}]_c$ in the brain of such species relative to hypoxia-intolerant species. For example, slower $[Ca^{2+}]_c$ accumulation during hypoxic or anoxic stresses have been reported in the brain of anoxia-tolerant freshwater turtles (Bickler 1992), and also in hypoxia-tolerant neonatal rats (Bickler et al. 2003) and in juvenile and adult naked mole-rats (Peterson et al. 2012*a*). Of these models, the mechanism mediating reduced Ca^{2+} accumulation during hypoxia is best understood in freshwater turtle brain, where moderate elevations of 10%–30% in brain $[Ca^{2+}]_c$ have been reported in Painted Turtle (*Chrysemys picta* (Schneider, 1783)) cortical slices during prolonged anoxic exposure (Bickler et al. 2000). This increase has been linked to channel arrest of glutamatergic AMPARs and NMDARs.

Channel arrest is a mechanism that has been reported in the brain and other tissues of numerous hypoxia-tolerant organisms wherein the activity and (or) expression of ion channels is reduced during periods of low oxygen stress to reduce passive ion leak and the associated workload on ion pumps, thus reducing ATP demand (Hochachka 1986). Several studies support the occurrence of channel arrest in the anoxic turtle brain, including observations of 50%–65% decreases in whole-cell AMPAR and NMDAR currents,

NMDAR open probability, NMDAR-mediated Ca^{2+} influx, K^+ conductance, and whole-cell K^+ leakage (Chih et al. 1989; Buck and Bickler 1995, 1998; Shin and Buck 2003; Pamenter et al. 2008*b*; Rodgers-Garlick et al. 2013). In addition to decreased ion conductance, both NMDAR and Na^+ channel density decrease during prolonged anoxia in turtle brain (Pérez-Pinzón et al. 1992*b*; Bickler et al. 2000). Channel arrest of NMDARs has also been demonstrated in hypoxia-tolerant goldfish brain (Wilkie et al. 2008) and NMDAR NR1 subunit gene expression in crucian carp brain decreases following 1 or 7 days of anoxic exposure (Ellefsen et al. 2008), while NMDAR expression and activity also decrease in the brain of hypoxia-tolerant hibernating arctic ground squirrels (*Spermophilus parryii* (Richardson, 1825)) (Ross et al. 2006; Zhao et al. 2006). Finally, the expression of NMDAR isoforms that have reduced permeability to Ca^{2+} are upregulated in the brain of naked mole-rats following prolonged hypoxia (Peterson et al. 2012*b*). Of these examples, the mechanism of glutamatergic receptor channel arrest in turtle brain has received the most attention and appears to be regulated by a mitochondrial-mediated change in $[Ca^{2+}]_c$ during low oxygen stress.

In turtle brain, the mitochondrial H^+ gradient is partially dissipated during anoxia due to the activation of mitochondrial ATP-sensitive K^+ (mK_{ATP}) channels (Pamenter et al. 2008*c*; Hawrysh and Buck 2013). mK_{ATP} are located on the inner membrane of the mitochondria and although their specific structure is unknown, it is thought to be similar to plasmalemmal K_{ATP} channels, which are composed of four pore-forming inward-rectifying K^+ channel subunits (KIR6.1, KIR6.2) and four modulatory sulfonylurea receptors (SUR-1, SUR-2) (Karschin et al. 1998; Aguilar-Bryan and Bryan 1999). Physiologically, several cellular messengers including protein kinase C (PKC), adenosine, $O_2^{\bullet}$, and nitric oxide can activate these channels (Sasaki et al. 2000; Korge et al. 2002). Mild mitochondrial uncoupling mediated by the activation of mK_{ATP} channels reduces the driving force on MCU-mediated mitochondrial Ca^{2+} uptake, and thereby reduces the rate of Ca^{2+} uptake into the mitochondria (Pamenter et al. 2008*c*). As mitochondrial Ca^{2+} buffering decreases, $[Ca^{2+}]_c$ rises and Ca^{2+} bind to calmodulin, which leads to the dephosphorylation of NMDARs and presumably AMPARs. This mechanism thus reduces excitatory Na^+ and Ca^{2+} influx and hyperexcitability during anoxia (Fig. 3A). Evidence in support of this mechanism includes the observations that anoxic increases in $[Ca^{2+}]_c$, channel arrest of AMPARs and NMDARs, and anoxic NMDAR-mediated Ca^{2+} influx is prevented by (*i*) intracellular Ca^{2+} chelation but not by chelation of extracellular Ca^{2+} or antagonism of endoplasmic reticulum (ER) dependent Ca^{2+} cycling and (*ii*) agonists of mK_{ATP} channels, while (*iii*) calmodulin inhibitors prevent channel arrest of NMDARs but presumably do not effect the anoxic increase in $[Ca^{2+}]_c$ (Bickler et al. 2000; Shin et al. 2005; Pamenter et al. 2008*c*; Zivkovic and Buck 2010). In addition, channel arrest and the anoxic increase in $[Ca^{2+}]_c$ can be mimicked in normoxia or restored in anoxia by agonists of (*iv*) mK_{ATP} channels or (*v*) the MCU, which would reduce mitochondrial buffering of Ca^{2+}, and importantly, these effects are all abolished by chelation of intracellular Ca^{2+} (Pamenter et al. 2008*c*, Zivkovic and Buck 2010).

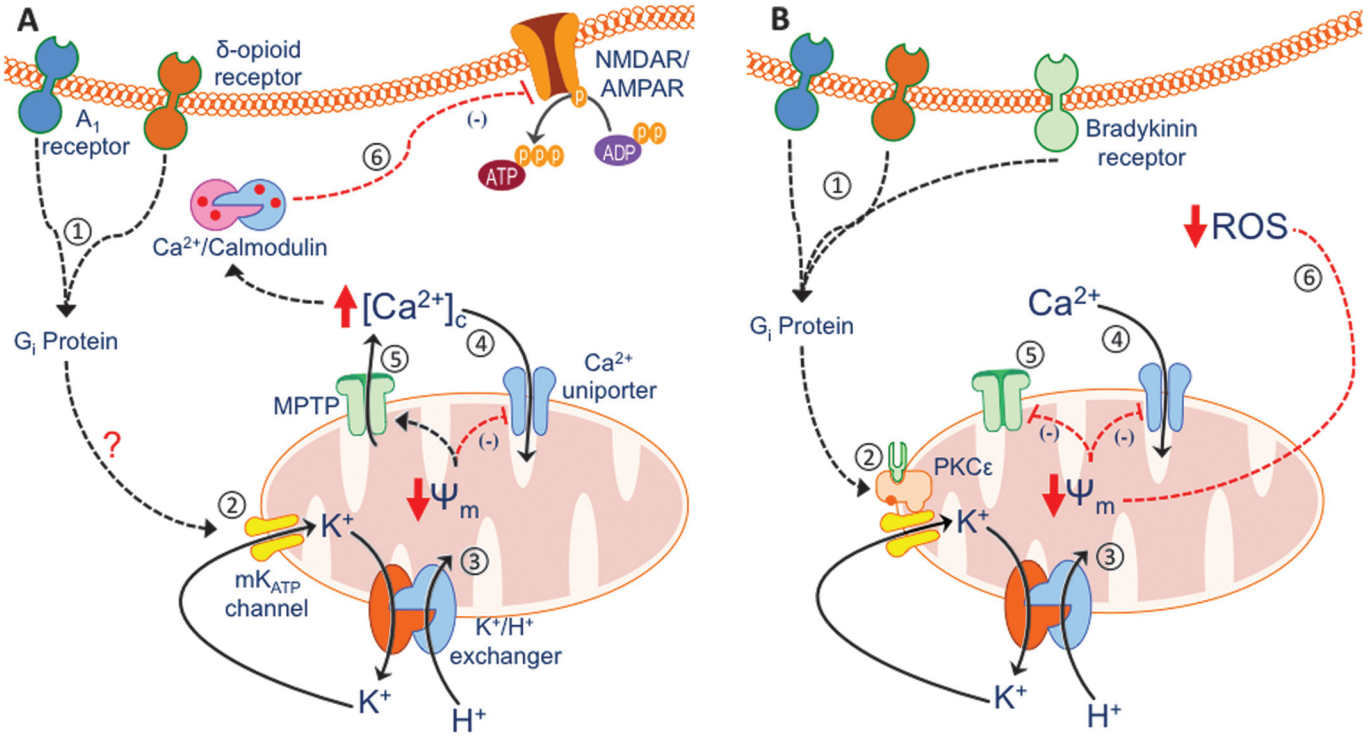
In addition to reducing the driving force behind MCU activity, mitochondrial uncoupling also directly induces Ca^{2+} release via the MPTP during anoxia. In a recent study, Hawrysh and Buck (2013) demonstrated that the specific MPTP inhibitor CsA reduces (but does not entirely abolish) mitochondrial Ca^{2+} release during anoxia and prevents channel arrest of NMDAR currents, while stimulation of the MPTP during normoxia causes an increase in $[Ca^{2+}]_c$ and a decrease in NMDAR currents that mimicks anoxia (Hawrysh and Buck 2013). The trigger mechanism behind MPTP opening is unclear but requires mild uncoupling of mitochondria, as it is initiated by activators of mK_{ATP} or mK_{Ca} channels and prevented by mK_{ATP} channel antagonists, which also prevent mitochondrial depolarization during anoxia. It is also possible that MPTP-mediated Ca^{2+} release is due to Ca^{2+} accumulation in the mitochondria, as this would tend to alkalinize the mitochondrial matrix (Pizzo et al. 2012), a condition that is associated with increased MPTP-mediated conductance (Halestrap 1991). Investigation of Ca^{2+}-mediated effects on mitochondrial pH, MPTP-mediated Ca^{2+} release and channel arrest would be a fascinating addition to this literature with implications far beyond comparative physiology.

Interestingly, although the channel arrest mechanism is critical to the anoxia tolerance of turtle neurons, it is not conserved in other excitable tissues within this organism. For example, channel arrest is not observed in turtle heart during anoxia (Stecyk et al. 2007) and heart mitochondria are not uncoupled following 2 weeks of anoxia (Galli et al. 2013). However, analysis of turtle heart mitochondria performed on tissue from cold-acclimated animals shows that markedly different pathways may be activated during prolonged anoxia relative to the acute anoxic exposure used to examine channel arrest in isolated brain tissues. Nonetheless, this difference is potentially fascinating as mechanisms of ischemic preconditioning involving mild uncoupling of mitochondria have been well described in mammalian heart as well as brain (see section "Ischemic preconditioning in hypoxia-intolerant species" below).

It is important to note that in the example of turtle brain, mitochondria function as a signaling intermediate that coordinates signals of low oxygen (input) with channel arrest of glutamatergic receptors (output). The extra-mitochondrial oxygen sensor in this system is poorly understood but appears to involve cell membrane-based proteins, which likely communicate changes in environmental oxygen to the mitochondria via poorly understood signaling pathways. There is considerable evidence in particular supporting roles for adenosine and to a lesser degree δ-opioid receptors in this mechanism, as activation of either receptor during normoxia has a similar effect as anoxia on NMDAR currents (Buck and Bickler 1995, 1998; Pamenter and Buck 2008; Pamenter et al. 2008*a*). In both cases, G-protein inhibitors and mK_{ATP} antagonists abolish the effects of agonizing these receptors, suggesting that adenosine and δ-opioid receptors mediate two redundant signaling mechanisms that converge on inhibitory G proteins as the intracellular signaling molecule that connects cellular detection of low oxygen and activation of the mitochondria-controlled activation of channel arrest. δ-Opioid receptors are purported oxygen sensors in mammalian brain (He et al. 2013) and adenosine has been proposed as a detector of reduced energy availability during low oxygen stress due to breakdown of ATP (Buck 2004). Thus, with regards to regulation of glutamatergic channel arrest, the role for mitochondria is as a transducer rather than a trigger.

Along with buffering bulk cytosolic Ca^{2+} intake and regulating channel arrest, mitochondria make additional contributions to local cellular Ca^{2+} dynamics that may prove to be important in the regulation of cytoprotective mechanisms during hypoxia. For example, mitochondria are typically found in close apposition to the ER and rapidly take up Ca^{2+} released from this organelle. Via this function, mitochondria are also intimately involved in ER-mediated cellular Ca^{2+} oscillations through IP_3-regulated pathways in all cell types studied (Grimm 2012). Potential roles for mitochondrial–ER Ca^{2+} dynamics in the regulation of adaptive responses to hypoxia have not been explored in any model, although manipulation of ER Ca^{2+} release has no effect on NMDAR channel arrest in anoxic turtle cortex (Pamenter et al. 2008*c*). Mitochondria are also found in close proximity to plasma membranes, and in some cell types, likely serve an important role in absorbing Ca^{2+} influx from outside the cell (Pizzo et al. 2012). However, in postsynaptic neurons, the distance between mitochondria and plasma membrane is likely too great to facilitate direct uptake of glutamate-receptor-mediated Ca^{2+} influx (>100 nm), and it is more likely that indirect mechanisms, such as those currently thought to underlie channel arrest in turtle cortex, explain links between mitochondrial Ca^{2+} handling and glutamate receptor activity in mammalian cells (Budd and Nicholls 1996).

Fig. 3. Mitochondria mediate glutamatergic receptor channel arrest and ischemic preconditioning (IPC). (A) In the anoxia-tolerant Painted Turtle (*Chrysemys picta*) brain, (1) anoxia activates adenosine (A_1) and δ-opioid receptors, which converge on a G-protein-mediated mechanism to (2) activate mitochondrial ATP-sensitive K^+ (mK_{ATP}) channels. (3) mK_{ATP} channel opening permits a futile cycling of K^+ ions across the mitochondrial membrane and reduces the mitochondrial H^+ gradient. This leads to "mild uncoupling" of the mitochondria, which manifest as decreased mitochondrial membrane potential ($\Delta\Psi_m$). (4) The activity of the mitochondrial Ca^{2+} uniporter (MCU) is dependent on $\Delta\Psi_m$ and uncoupling reduces the driving force powering this pump, which decreases the rate of Ca^{2+} uptake into the mitochondria. (5) Concomitantly, decreased $\Delta\Psi_m$ also activates Ca^{2+} release through the mitochondrial permeability transition pore (MPTP). (6) As Ca^{2+} is released from the mitochondria, cytosolic Ca^{2+} rises approximately 10%–30% and binds to calmodulin to dephosphorylate *N*-methyl-D-aspartate receptors (NMDARs), thereby preventing large-scale Ca^{2+} influx during low oxygen stress. A similar mechanism acts to inhibit α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), which further reduces neuronal electrical excitability by reducing excitatory Na^+ influx. (B) In hypoxia-intolerant species, a similar mechanism can be upregulated by preconditioning protocols. In this mechanism, preconditioning (1) activates adenosine (A_1), δ-opioid, or bradykinins receptors, which converge on a G-protein-mediated mechanism to (2) activate mK_{ATP} channels via the modulation of protein kinase C (PKC), (3) activating a similar futile cycling of K^+ ions across the mitochondrial membrane that reduces the mitochondrial H^+ gradient and thereby (4) reduces the driving force on the MCU and prevents toxic accumulation of Ca^{2+} in the mitochondria and (5) related formation of the MPTP. (6) Decreased Ψ_m also reduces reactive oxygen species (ROS) generation from mitochondria, preventing ROS-related reoxygenation injury. Black arrows indicate upregulated pathways. Broken red lines indicated downregulated pathways.



Ischemic preconditioning in hypoxia-intolerant species

Although most species are not tolerant of ischemia or low oxygen stress, mechanisms are beginning to emerge wherein a brief ischemic insult confers neuro- or cardio-protection against subsequent, otherwise lethal insults in the brain and heart of hypoxia-intolerant organisms (Murry et al. 1986), including humans (Koch and Gonzalez 2013). This phenomenon has been termed ischemic preconditioning (IPC), and beyond ischemia, IPC-like cytoprotection can be induced by a wide variety of stressors, including hypoxia (Shizukuda et al. 1992), anoxia (Cave et al. 1996), ionic imbalances (Ashraf et al. 1994), spreading depression (Matsushima et al. 1996), hyperbaric oxygen (Kim et al. 2001), hyperoxia (Petrosillo et al. 2011), inhaled nitric oxide (Shinbo et al. 2013), hypothermia or heat shock (Ning et al. 1998), and inflammation (Deplanque and Bordet 2000), among others. Furthermore, in addition to the brain and heart, preconditioning has been observed in a variety of other tissues as diverse as lungs and testes (Gidday 2006). Remarkably, the underlying mechanisms of most of these protective pathways are largely conserved. For example, a period of exposure to high levels of Ca^{2+} will confer protection against a subsequent hypoxic insult (Xu et al. 2001), and pathways that employ common mechanistic components will typically confer this protection. The field of preconditioning research has ballooned since IPC was first described in canine myocardium challenged with ischemic/reperfusion injury (Murry et al. 1986), and a full discussion of the pathways and mechanisms involved is beyond the scope of the present paper and these have been expertly reviewed elsewhere (Gidday 2006; Downey et al. 2007; Heusch et al. 2008).

The underlying signaling lynchpin of preconditioning in both brain and heart is generally accepted to be mitochondria, and appropriately, mitochondria have been dubbed the "gatekeepers of preconditioning" (Dirnagl and Meisel 2008). Given the similarly central role of mitochondria in mediating endogenous mechanisms of hypoxia tolerance in facultative anaerobes, it is perhaps not surprising that research has begun to elucidate remarkable similarities between the pathways that mediate IPC in hypoxia-intolerant organisms and those that underlie endogenous tolerance to low oxygen stress in the brain of facultative anaerobes. In particular, there is strong evidence supporting a central role for mild mitochondrial uncoupling and downstream Ca^{2+} or ROS-mediated signaling in the induction of preconditioned protection of both the brain and the heart of hypoxia-intolerant species. Similar to the regulation of endogenous neuroprotective mecha-

nisms in turtle brain, mK_{ATP} channels are presently favored as the mitochondrial uncoupling mechanism that underlies hypoxic or ischemic preconditioning in mammalian brain and heart (Auchampach et al. 1991; Oldenburg et al. 2003; Kis et al. 2004). As in turtle brain, activation of mK_{ATP} channels partially dissipates the mitochondrial H^+ gradient, reducing the driving force of the MCU and subsequently decreasing mitochondrial accumulation of Ca^{2+} during hypoxia, ischemia, or related stresses, along with subsequent MPTP formation and cytochrome *c* loss from the mitochondria (Fig. 3B). Such effects have been demonstrated in rat cortical neurons exposed to glutamate toxicity (Kis et al. 2003, 2004), following focal ischemia in rat brain (Shimizu et al. 2002), and following anoxia/reperfusion injuring in adult rat hippocampus and cortex (Heurteaux et al. 1995; Semenov et al. 2000), and in juvenile mouse brain stem (Müller et al. 2002). Conversely, blockade of mK_{ATP} channels abolishes preconditioning-mediated neuroprotection ubiquitously (Grover 1997, Takashi et al. 1999; Murata et al. 2001; Korge et al. 2002; Yoshida et al. 2004).

A similar mechanism has been described to underlie IPC-mediated cardioprotection in the hearts of hypoxia-intolerant species, and here again, mitochondrial K^+ channels are leading candidates to mildly uncouple mitochondria and provide preconditioning-based cytoprotection. In rat heart, mildly uncoupling mitochondria by activating mK_{ATP} is cardioprotective against subsequent hypoxic or ischemic insults and prevents MPTP formation (Holmuhamedov et al. 1999; Cao et al. 2005; Cuong et al. 2006). Mild uncoupling decreases the rate of Ca^{2+} uptake into isolated rat heart mitochondria and also increases the rate of Ca^{2+} release from isolated mitochondria that have been preloaded with Ca^{2+} (Holmuhamedov et al. 1998), and both of these responses to uncoupling have been linked to decreases in Ψ_m. Similarly, in intact cardiomyocytes, activation of mitochondrial K^+ channels decreases Ψ_m and reduces $[Ca^{2+}]_m$ accumulation during ischemia (Murata et al. 2001; Wang et al. 2001; Sato et al. 2005). Importantly, pharmacological stimulation of MPTP formation abolishes the protective effects of preconditioning, suggesting that cytoprotection induced by IPC opening prevents MPTP formation (Cao et al. 2005). Taken all together, these data suggest preconditioning-mediated cytoprotection against hypoxic and ischemic stresses in both the brain and the heart is due to prevention of mitochondrial Ca^{2+} accumulation resulting from mild mitochondrial uncoupling. The prevention of Ca^{2+} overload in the mitochondria theoretically prevents the rupture of mitochondrial membranes and release of pro-apoptotic signals via the MPTP (see section "Mitochondrial control of cell death pathways" below). Whether or not this change in Ca^{2+} handling also modulates additional cytoprotective mechanisms—such as glutamatergic channel arrest in the brain—as a signaling intermediate, remains to be determined.

Mitochondrial K_{ATP} channels are not the only mitochondrial ion channels that provide neuroprotection against ischemic insults upon activation. Similar to mK_{ATP} channel activation, Ca^{2+}-mediated increases in mitochondrial Ca^{2+}-sensitive K^+ (mK_{Ca}) channel activity dissipates the mitochondrial H^+ gradient, partially uncouples mitochondria, reduces MCU activity, and slows the cytotoxic accumulation of $[Ca^{2+}]_m$. Mitochondrial Ca^{2+}-sensitive K^+ channels are similar to plasmalemmal large-conductance K^+ channels: they are multiconductance state channels with an open probability that is both voltage and $[Ca^{2+}]$ dependent such that cellular membrane potential (or in the case of mK_{Ca} channels, Ψ_m) depolarization potentiates channel activity (Siemen et al. 1999). In mitoplast-attached patches, mK_{Ca} currents increase when $[Ca^{2+}]$ outside the pipette is increased, suggesting that the Ca^{2+} sensor of the channel is located on the matrix side of the mitochondrial membrane (Xu et al. 2002). Therefore, channel activity increases as $[Ca^{2+}]_m$ rises due to sequestration of $[Ca^{2+}]_c$. Such an accumulation of Ca^{2+} occurs during ischemia and mK_{Ca} channels are also activated by hypoxia (Gu et al. 2007). Since activation of either mK_{Ca} or mK_{ATP} channels should have the same effect on cells (increased mitochondrial K^+ conductance), it is not surprising that activation of mK_{Ca} channels in cardiac myocytes confers protection during global ischemia and reperfusion experiments that is similar in magnitude to the protection afforded by activation of mK_{ATP} channels or IPC (Xu et al. 2002; Cao et al. 2005). Furthermore, cytoprotection due to the activation of mK_{ATP} channels is not impaired by blockade of mK_{Ca} channels, or vice versa, suggesting that these two channels function independently, although their mechanism of action is similar—increasing mitochondrial K^+ conductance. Together, these data independently confirm the central role of K^+ influx into the mitochondrial matrix in IPC-mediated protection against ischemic injury that has been suggested by mK_{ATP} channel experiments. Interestingly, channel arrest of AMPARs and NMDARs in the anoxic turtle cortex can also be induced by mK_{Ca} channel activation, although blockade of mK_{Ca} channels does not prevent channel arrest, indicating they are not the primary mediators of this phenomenon (Pamenter et al. 2008*c*; Zivkovic and Buck 2010).

The mechanism via which mK_{ATP} channels are activated has not been directly elucidated in turtle brain and varies between systems and tissues in hypoxia-tolerant mammals; however, in mammals, this mechanism appears to commonly involve the activation of PKCε, which is functionally associated with mK_{ATP} in the mitochondrial inner membrane (Jabůrek et al. 2006), and whose activation leads to opening of mK_{ATP} channels (Raval et al. 2003; Costa and Garlid 2008). In mammalian brain and heart, PKCε activation is mediated primarily through the activation of adenosine A_1 receptors (Liu et al. 1991; Pérez-Pinzón et al. 1996), while redundant pathways mediated by bradykinins and δ-opioids also triggers IPC in brain (Wall et al. 1994; Schultz et al. 1995). All three of these pathways are G-protein coupled and converge on PKCε activation. The role of PKC in regulating mild uncoupling of turtle brain mitochondria and channel arrest remains to be studied, but it is likely that PKCε also plays a critical role in activating mK_{ATP} channels in turtle brain during anoxia.

Beyond mitochondrial Ca^{2+}-related signaling in preconditioning, there is also evidence that redox signaling plays a role in hypoxia and ischemia tolerance, since ROS production is increased during preconditioning protocols (but reduced during subsequent ischemic stresses). This mechanism is most prevalent in models of cardiac preconditioning and there is less evidence that such a mechanism occurs in the brain (Vanden Hoek et al. 1998). For example, in hypoxic rabbit heart or ischemic rat heart, preconditioning-mediated protection is abolished by inclusion of free-radical scavengers (Vanden Hoek et al. 1998; Garlid et al. 2013), suggesting that preconditioning triggers cardioprotection via the regulation of free-radical generation. The mechanism of ROS-mediated cardioprotection in preconditioning is similar to mechanisms of Ca^{2+}-mediated cytoprotection and involves the activation of mK_{ATP} channels in the heart; however, the trigger of mK_{ATP} activation is ROS in this system, which acts on PKCε to open mK_{ATP} channels (Garlid et al. 2013). ROS also form part of an automatic feed-forward loop in this model through which mK_{ATP} channel opening leads to $O_2^{\bullet}$ generation, which then enhances activation of PKCε that in turn maintains mK_{ATP} channels in an active state (Costa and Garlid 2008). This mechanism has been proposed as part of the "memory" component of preconditioning.

Overall, the parallels between the mitochondrial role in the mild uncoupling mechanism that endogenously mediates anoxic channel arrest in turtle brain and the mitochondrial role in inducible hypoxic or ischemic preconditioning in mammalian brain and heart are numerous and clear. Specifically, (*i*) activation of mK_{ATP} or mK_{Ca} channels or inhibition of the MCU induces channel arrest in turtle brain and IPC in mammalian brain; (*ii*) blockade of mK_{ATP} channels (but not mK_{Ca} channels; Gáspár et al. 2008, 2009; Pamenter et al. 2008*c*; Zivkovic and Buck 2010) or activation of the MCU prevents channel arrest in turtle brain and IPC in rat heart (Zhang et al. 2006; Pamenter et al. 2008*c*; Zivkovic and Buck 2010); (*iii*) mitochondria from turtles

and mammals are similarly uncoupled by 10%–20% through mK_{ATP} channel activation (Holmuhamedov et al. 1999; Murata et al. 2001; Pamenter et al. 2008*c*); (*iv*) in both the turtle and the preconditioned rat brain, small increases in cytoplasmic Ca^{2+} are associated with subsequent neuroprotection (Bickler et al. 2000; Bickler and Fahlman 2004); (*v*) pharmaceutical activation of mK_{ATP} channels increases cytosolic Ca^{2+} mildly by ~10% in turtle brain and 50% in rat heart, and these increases correlate with an approximately 80% reduction in mitochondrial Ca^{2+} accumulation during a subsequent ischemic stress in rat and also with cell survival in both species (Bickler et al. 2000; Wang et al. 2001; Pamenter et al. 2008*c*). Furthermore, (*vi*) upstream of mitochondrial induction of cytoprotective events, activation of adenosine A_1 receptors has been implicated as a key trigger mechanism in both ischemic preconditioning in hypoxia-intolerant species and in anoxic channel arrest in the brain of turtle (Pérez-Pinzón et al. 1996; Pamenter et al. 2008*a*), while (*vii*) additional roles for δ-opioid receptors as triggers of both mechanisms have also been reported (Schultz et al. 1995; Pamenter and Buck 2008), suggesting even greater overlap between these two phenomena. The mechanism through which preconditioning provides cytoprotection in hypoxia-intolerant organisms is poorly understood but is thought to involve prevention of mitochondrial Ca^{2+} overload, MPTP formation, and the activation of related cell death pathways during deleterious stress. Glutamatergic channel arrest in turtle neurons provides clear neuroprotection against low oxygen stress or glutamatergic excitotoxicity and examination of the effect of IPC protocols on AMPAR and NMDAR activity in mammalian neurons may reveal a related important neuroprotective mechanism, particularly given the myriad similarities between mitochondrial regulation of channel arrest and preconditioning in brain, and the important role of glutamate toxicity in ischemic or hypoxic mammalian brain.

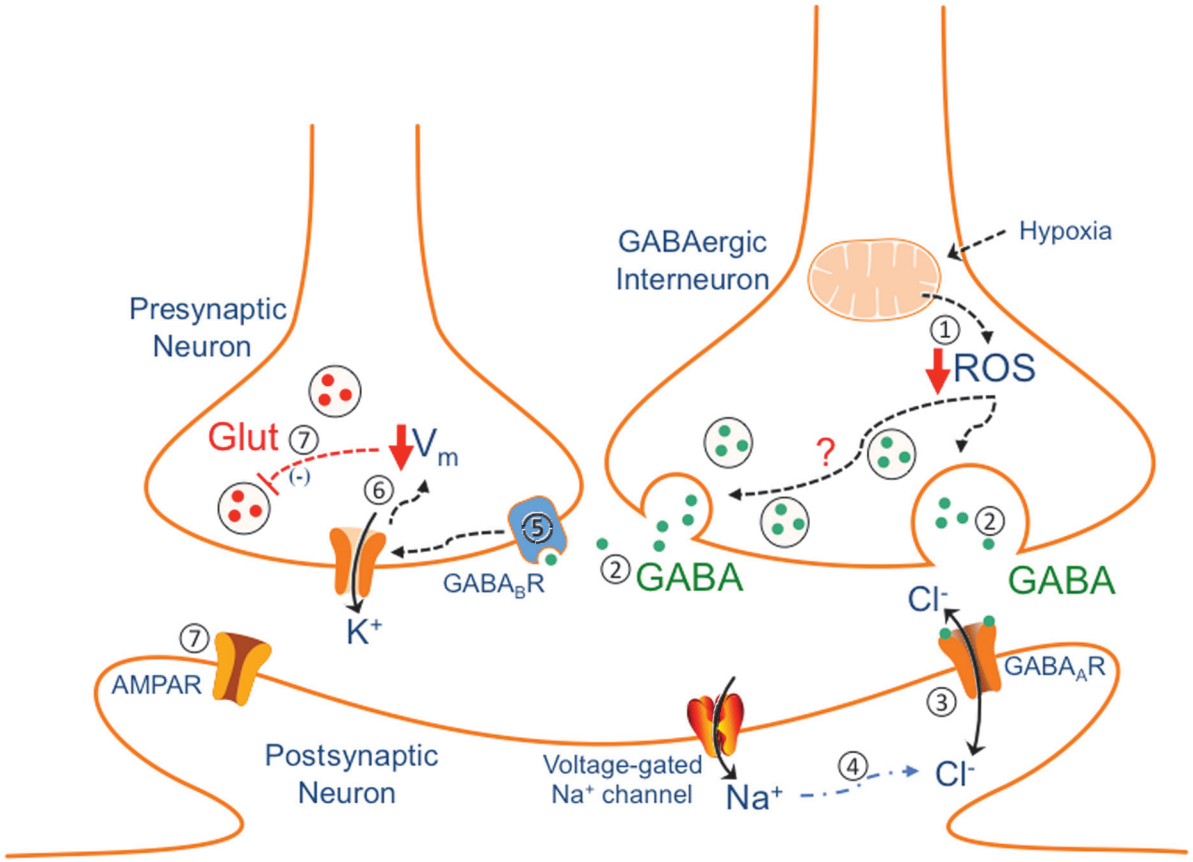
Importantly, preconditioning is not unique to hypoxia-intolerant organisms and was in fact first identified in anoxia-tolerant goldfish (Prosser et al. 1957). More recently, hypoxic preconditioning has been studied in the epaulette shark (*Hemiscyllium ocellatum* (Bonnaterre, 1788)), which is exposed to progressively longer bouts of hypoxia in the spring tidal season as tides become lower each night, exposing this organism to increasingly long daily periods of hypoxia or anoxia. Following such bouts of natural "hypoxic preconditioning", the sharks' oxygen consumption rate and critical oxygen tension decrease approximately 20%–30% via a mechanism that is, perhaps not surprisingly, mediated by adenosine receptors, and which conserves brain [ATP] (Renshaw et al. 2002; Nilsson and Renshaw 2004). Preconditioning mechanisms appear to be a general protective response to stress and the observation that such pathways are endogenously upregulated in hypoxia-tolerant species suggests that such organisms are naturally "primed" to respond to a variety of stresses. Along this line, it is interesting to note that hypoxia-tolerant organisms also constitutively express high basal levels of heat shock proteins (Prentice et al. 2004; Ramaglia and Buck 2004), which are normally only observed in hypoxia-intolerant species following a period of stress. Similarly, as discussed above, HIF-related pathways are generally more active in normoxic conditions in hypoxia-tolerant species. Thus, an increasingly common theme is that endogenous hypoxia tolerance involves the chronic or constitutive activation of pathways that are conserved in hypoxia-intolerant species, but that are not normally activated. This remarkable overlap highlights the utility of comparative model organisms in the study of stress responses that are relatable to clinical insults. Indeed the basic mechanism of mK_{ATP} channel-mediated cytoprotection (induced or endogenous) appears to be conserved in all animals studied, and hypoxic preconditioning mediated by mK_{ATP} channels has recently even been reported in *C. elegans* (Wojtovich et al. 2012).

Altered ROS production and the regulation of spike arrest

While the study of Ca^{2+} dynamics as a means of cellular signaling has been at the forefront of research for decades, exploration of the role of ROS as signaling intermediates is somewhat less advanced. As discussed above, mitochondria are a major source of ROS production under normal physiological conditions. ROS in large quantities are highly deleterious to the cell; however, ROS are constantly being produced by the mitochondria and alterations in the rate of radical formation act as a redox signaling mechanism, potentially regulating downstream messengers that may mediate deleterious or neuroprotective changes against low oxygen stresses. For example, recent studies have described H_2O_2-mediated inhibition of dopamine release (Avshalumov et al. 2007), $O_2^{\bullet}$-mediated inhibition of γ-amino butyric acid (GABA) release (Chen and Pan 2007), and H_2O_2-mediated regulation of synaptic plasticity (Kamsler and Segal 2003, 2004). The production of ATP by oxidative phosphorylation is regulated partially by Ψ_m and reverse electron flow in the ETC during oxidative phosphorylation results in incidental ROS generation (St-Pierre et al. 2002). The rate of ROS generation by the mitochondria is associated with the rate of ATP production via the ETC, which in turn is regulated by the mitochondrial H^+ gradient. Therefore, a partial uncoupling of mitochondrial respiration will alter the rate of ROS production (Moroney et al. 1984). Changes in ROS generation have been implicated as key triggers of ischemic preconditioning mechanisms in mammals (see section "Ischemic preconditioning in hypoxia-intolerant species" above); however, the role of ROS in regulating protective responses to low oxygen stress in facultative anaerobes has received considerably less attention. Reduced ROS production during periods of hypoxia or anoxia have been reported in many of the most hypoxia-tolerant species, including the brain of freshwater turtles *C. picta* and the Common Slider (*Trachemys scripta* (Schoepff, 1792)) (Milton et al. 2007; Pamenter et al. 2007), embryos of killifish (Cyprinodontiformes: Fundulidae) (Duerr and Podrabsky 2010), heart of epaulette shark (*Hemiscyllum ocellatum* (Bonnaterre, 1788)) (Hickey et al. 2012), and hypoxia-adapted *Drosophila* lines (Ali et al. 2012). The impact of diminished ROS production on cellular function and systemic survival is not well understood, although a recent study supports a critical role for decreased ROS production in the brain of freshwater turtles as the initial trigger of neuroprotective spike arrest mechanisms during acute anoxic stress (Hogg et al. 2012).

Spike arrest is a mechanism that was proposed to explain reductions in electrical activity in turtle brain, which is depressed by as much as 75%–95% during periods of anoxia (Feng et al. 1988; Pérez-Pinzón et al. 1992*a*; Fernandes et al. 1997; Pamenter et al. 2011). The spike arrest theory posits that in hypoxia-tolerant species, a mechanism exists that reduces excitatory electrical activity, such as excitatory postsynaptic potentials (EPSPs) and action potentials (APs), to reduce ATP demand and conserve neuronal energy supplies. Such a mechanism is particularly important in the brain of hypoxia-tolerant species because during normoxia, AP generation and propagation and EPSPs have been estimated to account for ~81% of neuronal energy expenditure (Attwell and Laughlin 2001). Since energy production decreases ~90% in the absence of oxidative phosphorylation during anoxia, severe reductions in such electrical activity and associated ion pumping must occur to successfully reduce the metabolic rate of brain below the anaerobic energy production threshold. Recent research has demonstrated that spike arrest in turtle brain is mediated by an inhibitory electrical shunt regulated by GABA receptors (Pamenter et al. 2011). In anoxic turtle brain, a large-scale release of GABA (Nilsson and Lutz 1991) chronically activates postsynaptic $GABA_A$ receptors and presynaptic $GABA_B$ receptors (Fig. 4). At the presynapse, $GABA_B$ receptor activation mediates an inhibitory K^+ current that opposes depolarization of the presynaptic cell and reduces the release of the excitatory neurotransmitter glutamate, leading to decreases in the frequency of EPSPs postsynaptically. Concomitantly, activation of postsynaptic $GABA_A$ receptors opens a large inhibitory conductance to Cl^-, which essentially clamps membrane potential at the Cl^- reversal potential (E_{GABA}) opposing further depolarization. These effectively prevent

Fig. 4. Mitochondria mediate GABAergic spike arrest. In the anoxia-tolerant Painted Turtle (*Chrysemys picta*) brain, synaptic inhibition is mediated by an inhibitory GABAergic shunt. This mechanism is triggered by (1) a decrease in mitochondrial reactive oxygen species (ROS) production in GABAergic interneurons, which (2) increases presynaptic γ-amino butyric acid (GABA) release. GABA activates ($GABA_A$) receptors in the postsynaptic neuron, which mediate a large conductance to inhibitory Cl^- ions. Subsequently, when (4) excitatory synaptic inputs activate voltage-gated Na^+ channels, depolarizing Na^+ influx is countered by a rapid and opposing charge shift of Cl^- ions. In this fashion, neuronal depolarization is prevented and action potentials are not generated. Concomitantly, increased synaptic GABA also (5) acts on G-protein-coupled $GABA_B$ receptors on presynaptic glutamatergic neurons. (6) $GABA_B$ receptor activation opens K^+ channels and increased K^+ efflux from the cell hyperpolarizes the presynaptic neuron, thereby (7) inhibiting depolarization-dependent glutamate (Glut) release and associated excitatory depolarization of the postsynaptic neuron, further suppressing electrical activity. Black arrows indicate upregulated pathways. Broken red lines indicated downregulated pathways.



AP firing, which preserves neuronal [ATP] and imbues tolerance against long-term anoxia (Pamenter et al. 2011). Although spike arrest has only been described in turtle brain to date, it is worthwhile to note that [GABA] increases in the brain of many other hypoxia-tolerant species, including crucian carp (Nilsson 1990), Northern Leopard Frog (*Rana pipiens* Shreber, 1782) (Milton et al. 2003), epaulette shark (Nilsson and Renshaw 2004; Renshaw et al. 2002), and green crab (*Carcinus maenas* (L., 1758)) (Nilsson and Winberg 1993), as well as in embryos of killifish (Podrabsky et al. 2007); blocking GABAergic transmission in carp abolishes metabolic suppression during anoxia and increases ethanol excretion (Nilsson 1992); the expression of GABA transporter 2 and 3 genes decreases after 1 and 7 days of anoxic exposure in crucian carp brain, which would result in synaptic accumulation of GABA due to slowed uptake (Ellefsen et al. 2009). Taken together, these results suggest that the inhibitory GABAergic spike arrest mechanism may be a conserved response to low oxygen stress in facultative anaerobes.

Remarkably, in turtle brain, this inhibitory shunt is also protective against harsher pathological stresses, including external high K^+ (64 mmol/L) and ischemia (Pamenter et al. 2012*a*), and turtle brain tolerates global ischemic insults in vivo (Belkin 1963). Conversely, although GABA is the primary inhibitory neurotransmitter in the mature mammalian central nervous system (Blaesse et al. 2009), GABAergic mechanisms are not strongly recruited in ischemic or hypoxic mammalian neurons. In fact, although [GABA] is elevated by ~30% in ischemic murine brain and endogenous synaptic GABA release in the first few minutes of ischemia slows the onset of deleterious membrane potential depolarization, this limited inhibition is insufficient to preserve [ATP] and maintain ion pumping for more than 2–3 min (Allen et al. 2004). Furthermore, $GABA_A$ receptor subunit mRNA expression is decreased by ~85%, and GABA-evoked currents and [ATP] run down rapidly, suggesting endogenous GABAergic neuroprotection is transient and largely ineffective (Erecińska et al. 1984; Li et al. 1993; Allen et al. 2004). Nonetheless, activating GABA receptors preinsult limits neuronal hyperexcitability in mammalian models of ischemic damage, and extensive membrane potential depolarization and cell death are not observed in the afflicted brain region (Galeffi et al. 2000; Costa et al. 2004).

The mechanism that senses changes in oxygen availability and translates this into increased GABA release in the brain of facultative anaerobes is poorly understood; however, a recent study implicates changes in mitochondrial ROS generation in this mechanism (Hogg and Buck 2011; Hogg et al. 2012). These authors showed that ROS scavenging or inhibition of mitochondrial ROS production with cyanide both decrease ROS generation and cause GABA release leading to electrical depression that mimics the

effects of anoxia, while addition of H_2O_2 abolishes anoxic GABA release and prevents spike arrest during acute anoxia. Furthermore, the anoxic increase in GABA is prevented by inhibition of voltage-gated Na^+ channels with tetrodotoxin, but not by inhibition of glutamatergic AMPARs and NMDARs, indicating that GABA release is dependent upon excitatory depolarizing events in GABAergic interneurons, and this event is triggered by changes in mitochondrial ROS generation in these cells. The exact mechanism through which changes in ROS mediate presynaptic GABA release is unclear and direct examination of interneurons is required to resolve this question.

H_2O_2 can mediate synaptic plasticity via the activation of protein phosphatases, which induces Ca^{2+} release from intracellular stores in rat hippocampus (Kamsler and Segal 2003, 2004). Such a mechanism may lead to vesicular GABA release at the presynapse. ROS may also directly induce Ca^{2+} influx through redox regulation of membrane or organelle ion channels, which could also induce vesicular GABA release. Alternatively, ROS have been shown to interact with a wide variety of signaling molecules including protein kinases A, C, and G, tyrosine kinase, MAPKs, tyrosine phosphatase 1B, protein phosphatases 2A and 2B, phospholipases A2, C, and D, guanalyl cyclase, JAK/STATs, and G proteins (Hancock et al. 2001). Despite the myriad of potential interactions, the study of the role of ROS in mediating synaptic events is in its infancy, although a clear link exists between mitochondrial ROS generation and regulation of inhibitory synaptic GABA activity in turtle cortex. Future studies are required to determine whether this relationship is conserved in the brain of other facultative anaerobes where increases in GABA release are observed during periods of low oxygen stress, and if modulating ROS production in mammalian brain can induce similar neuroprotective responses in hypoxia-intolerant species.

Mitochondrial control of cell death pathways

In addition to signaling pathways mediated by mitochondria-controlled second messenger cascades, mitochondria also directly regulate cell death pathways via a variety of well-understood transcription and protein interactions. In fact, it has been suggested that cell death pathways evolved with the incorporation of aerobic α-proteobacteria (mitochondrial precursors) into eukaryotic cells (Kroemer 1997). As such, mitochondria actively regulate cell death in most organisms, from *C. elegans* to humans (Hengartner et al. 1992; Kroemer et al. 2007). The key event in the initiation of most cell death pathways is the permeabilization of the mitochondrial outer membrane, which permits cell-death-pathway-associated proteins to either dissociate from the membrane, or to be released from inside the mitochondria, thus allowing them to interact with cellular components to mediate membrane and organelle dismantling involved in both apoptotic and programmed necrotic cell death pathways (Tait and Green 2010).

Considerable research has extensively described the role of mitochondria in cell death pathways and this topic has been expertly reviewed elsewhere (Kroemer et al. 2007; Tait and Green 2010). Briefly, in mitochondrial-regulated apoptosis, loss of Ψ_m (e.g., due to excessive Ca^{2+} accumulation during hypoxia or ischemia) is an early and irreversible step in the induction of the cell death pathway (Zamzami et al. 1995*a*, 1995*b*). Loss of Ψ_m leads to the generation of ROS and mitochondrial outer membrane permeabilization (MOMP) via alterations in the balance of the pro- and anti-apoptotic members of the B cell lymphoma 2 (Bcl-2) protein family (Chipuk and Green 2008). MOMP permits several mitochondrial components to be released into the cytoplasm where they act as pro-apoptotic intracellular signaling molecules to execute programmed cell death. For example, cytochrome *c*, a key component of the ETC, also activates caspase-9-dependant cell death when released into the cytoplasm (Liu et al. 1996). Similarly, apoptosis reducing factor (AIF), which is a component of complex I of the ETC, acts directly on the nucleus to induce chromatin condensation and fragmentation of the nuclear envelope in caspase-independent cell death (Susin et al. 1999).

In addition to apoptosis induced by alterations to intracellular homeostasis (intrinsic apoptosis, e.g., due to hypoxia), mitochondria also participate in apoptosis induced by extracellular stimuli (extrinsic apoptosis). For example, activation of death receptors on the cell membrane (such as CD95/FAS receptors) induces a separate caspase-8-dependent cell death pathway that leads to MOMP and release of pro-apoptotic agents (Kroemer et al. 2007; Tait and Green 2010). Similarly, mitochondria also control regulated necrosis mediated by both intrinsic and extrinsic signals, primarily via oxidative bursts (Vandenabeele et al. 2010).

In response to anoxia, most mammalian cells undergo intrinsic (mitochondrial) apoptotic cell death, mediated by cytochrome *c* release from mitochondria (McClintock et al. 2002). A key aspect of apoptosis is that, unlike necrosis, it permits the removal of excess or damaged cells without harming neighboring cells. This distinction is particularly important in hypoxia-tolerant species, where some degree of cell death may be expected during repeated or prolonged bouts of long oxygen stress. Indeed, where excess apoptosis is a hallmark of spreading cell death in mammalian pathologies related to hypoxia, the targeted activation of apoptosis to clean up damaged cells, in combination with increased neurogenesis to replace removed cells, is a sensible strategy in hypoxia-tolerant species.

Apoptosis is an ATP-dependent mechanism (Leist et al. 1997), and the ability of facultative anaerobes to maintain [ATP] during prolonged anoxia or ischemia may imbue them with the ability to utilize apoptotic mechanisms to target damaged cells for replacement in a manner that is not available in hypoxia-intolerant organisms, where ATP is rapidly depleted, leading to extensive necrotic cell death. In support of this, marked increases in the expression of the apoptotic marker annexin V but not extensive membrane degradation indicative of necrosis has been observed in turtle cortex following a 24 h ischemic insult (Pamenter et al. 2012*a*), and wide-spread neurogenesis has also been reported in this model 3 weeks after global ischemia (Kesaraju and Milton 2009). Conversely, in ischemia-afflicted mammalian neurons, both annexin V staining and membrane rupture are widespread following 24 h of ischemia (Pamenter et al. 2012*b*, 2012*c*), and neurogenesis is mostly centered on a few subregions of the adult brain.

The regulation of cell death pathways during anoxic/ischemic insult has received comparatively minimal attention in hypoxia-tolerant species. In anoxic turtle brain, the expression of both Bcl-2 and Bax decreases through the first 4 h of anoxia and then returns to baseline levels following 24 h of anoxic exposure (Kesaraju et al. 2009). The ratio of Bcl-2 to Bax decreases at 1 h of anoxia in vivo but recovers to control levels at 4 and 24 h of anoxia, indicating that the induction of apoptosis is dominant over its inhibition at this early time point. Opposing results were reported in vitro in cultured turtle brain neurons, in which Bcl-2 increases ~85% and the Bcl-2/Bax ratio also increases following 4 h of anoxia (Nayak et al. 2011). Similarly, increased Bcl-2 mRNA expression was reported in the liver of the common carp (*Cyprinus carpio* L., 1758) following a 4-day hypoxic exposure (Poon et al. 2007). In turtle brain, in vivo expression of the inactive zymogen procaspase-3 (which is mitochondrial in origin) also increases after 24 h of anoxia; however, this is not the case for expression of cleaved caspase 3 (the active form), which is not solely attributable to mitochondrial signaling (Kesaraju et al. 2009). Lastly, AIF increases 2-fold following 4 h in anoxic turtle brain, further supporting the occurrence of activation of mitochondrial apoptotic pathways in this model. During 4 h of normoxic reperfusion, the expression of Bcl-2, Bax, and procaspase 3 all increase further relative to anoxic levels, although caspase 3 and AIF expression remain unchanged. Interestingly, just as for channel arrest, the prevention of mitochondria-mediated apoptosis in turtle brain

may be partially mediated by adenosine receptor activation, since blocking these receptors in neuronally enriched primary turtle cell cultures prevents anoxia-mediated increases in anti-apoptotic Bcl-2, while agonizing these receptors increases Bcl-2 expression to the same degree as anoxia (~85%; Nayak et al. 2011). Furthermore, adenosine receptor antagonism has been shown to prevent anoxic decreases in ROS generation and to induce cell death in turtle brain (Milton et al. 2007), consistent with a role for adenosine in mediating cell death. Importantly, these authors also reported that neither anoxia or reperfusion resulted in the translocation of Bax from the cytosol to mitochondria, or of AIF from mitochondria to the cytosol (Kesaraju et al. 2009). This is an important distinction, as taken together, these results indicate that although apoptotic pathways are initiated in anoxia-tolerant turtle brain, the overall balance is in favor of cell survival pathways over cell death pathways. The eventual execution of cell death pathways is largely prevented in the turtle brain, possibly due to high endogenous expression of heat shock proteins, which in many cases is also a mitochondria-dependent process (Fenton et al. 1994; Nargund et al. 2012).

Differences in mitochondrial regulation of cell death pathways relative to hypoxia-intolerant species may constitute important adaptations to low oxygen stress in hypoxia-tolerant species. In particular, differences between responses to anoxia will likely be critical as in most hypoxia-intolerant mammals; hypoxia (0.5%–3% oxygen) activates a variety of cellular events that contribute to cell survival, whereas anoxia (<0.5% oxygen) activates intrinsic apoptotic cell death pathways (Snyder and Chandel 2009; however, [oxygen] >0.5% is also lethal with sufficient exposure duration). Clearly, in anoxia-tolerant species, this threshold must be shifted, while differences may also become apparent between hypoxia-tolerant and anoxia-tolerant species. Further study is warranted to sort out these questions.

Conclusions, significance, and future directions

Hypoxia-tolerant species exhibit a range of molecular and cellular responses to low oxygen stress that underlie metabolic depression from the cellular to the whole-animal level. These systems-wide energy-conserving adaptations enable survival during prolonged hypoxia. Key to this survival are mechanisms that (*i*) enable the detection of hypoxia at the cellular level, (*ii*) coordinate reductions in energy consumption to match decreased energy production in the absence of oxidative phosphorylation, yet (*iii*) still permit the maintenance of basic organ and organelle function, and (*iv*) prevent the activation of deleterious cell death cascades. Studies to date suggest that most hypoxia-tolerant species exhibit each of these characteristics. Furthermore, research examining the underlying mechanisms controlling these responses demonstrates a remarkable degree of overlap not only among responses to hypoxia between hypoxia-tolerant species, but also between endogenous mechanisms of naturally evolved hypoxia tolerance and inducible mechanisms of hypoxia tolerance in otherwise intolerant species. Amazingly, the occurrence of these related mechanisms spans a wide range of species and organisms that are not closely related evolutionarily (e.g., freshwater turtles vs. hamsters; Mabanta et al. 2006; Pamenter et al. 2008*c*), suggesting that similar cytoprotective strategies against low oxygen stress evolved repeatedly over time, or that the underlying mechanisms of hypoxia tolerance (or at least the cellular architecture thereof) are conserved from a common hypoxia-tolerant ancestor.

Given this similarity, it is perhaps not surprising that so many endogenous or inducible responses to low oxygen stress are initiated by or coordinated through mitochondria, since the structure and function of these organelles is highly conserved throughout natural history and between species. Indeed, the fact that, for example, inducible cytoprotective mechanisms in the heart and brain of hypoxia-intolerant species appear to utilize similar molecular and cellular pathways as those central to endogenous cytoprotective mechanisms in hypoxia-tolerant species argues for a common evolutionary origin of these traits, as hypoxia-intolerant species would not likely experience environmental hypoxia or other conditions that would presumably be required to drive the evolution of such traits.

Beyond questions of evolutionary origin, the overlap between the mechanisms that underlie endogenous versus inducible cytoprotection is particularly important when evaluating the impact and cross-applicability of research into such mechanisms on either side of the hypoxia research spectrum. For example, based on similarities identified to date, research aimed at elucidating endogenous mechanisms of cytoprotection in comparative species may be expected to not only increase our knowledge of basic physiological responses to environmental hypoxia, but also to inform the development of therapeutics targeted against pathologies related to low oxygen stress, such as heart attack and stroke, altitude-related illnesses, chronic obstructive pulmonary disease, etc. Conversely, more clinically oriented research into preconditioning mechanisms that protect against these pathological stresses related to hypoxia can likely be utilized as templates to inform explorations of endogenous mechanisms in naturally hypoxia-tolerant species. Given the magnitude of the time and money that is dedicated to biomedical research relative to comparative research, this overlap represents a potential goldmine of experimental hypotheses to test in hypoxia-tolerant comparative model organisms.

Lastly, although considerable progress has been made over the previous decades into understanding the pathways and evolution of endogenous mechanisms of hypoxia tolerance, many questions remain unanswered. In particular, pressing questions related to the role of some of the most basic cellular signals connected to hypoxia, such as HIF- or AMPK-related signaling, have received relatively minimal research attention in the comparative field and much more work is required in this area. Recently, considerable strides have been made towards understanding the cellular adaptations to hypoxia in the brain of freshwater turtles and also in naked mole-rats, two of the champions of hypoxia tolerance in their respective classes; however, many questions remain regarding the extent to which mechanisms described in these models are conserved among other hypoxia-tolerant species; how these relate to inducible cytoprotection in hypoxia-intolerant species; and how such mechanisms evolved between and among these species. With the advancement of research into inducible neuroprotection in hypoxia-intolerant species and the recent sequencing of the genomes of some of the most hypoxia-tolerant model organisms identified (Zhou et al. 2007; Kim et al. 2011; Qiu et al. 2012; Shaffer et al. 2013), we stand now at a very promising point in the field of hypoxia-related comparative research and should expect striking advances in our understanding of the mechanisms and evolution of natural hypoxia tolerance.

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