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Review

Turtle anoxia tolerance: Biochemistry and gene regulation

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ABSTRACT

Background: While oxygen limitation can be extremely damaging for many animals, some vertebrates have perfected anaerobic survival. Freshwater turtles belonging to the *Trachemys* and *Chrysemys* genera, for example, can survive many weeks without oxygen, and as such are commonly used as model animals for vertebrate anoxia tolerance.
Scope of review: In the present review we discuss the recent advances made in understanding the biochemical and molecular nature of natural anoxia tolerance of freshwater turtles.
Major conclusions: Research in recent years has shown that activation of several important pathways occurs in response to anoxia in turtles, including those that function in the stress response, cell cycle arrest, inhibition of gene expression and metabolism. These likely contribute to anoxia tolerance in turtle tissues by minimizing cell damage in response to anoxia, as well as facilitating metabolic rate depression.
General significance: The research discussed in the present review contributes to the understanding of how freshwater turtles can survive without oxygen for prolonged periods of time. This could also improve understanding of the molecular nature of hypoxic/ischemic injuries in mammalian tissues and suggest potential ways to avoid these.

1. Introduction

Many animals experience situations of interrupted oxygen supplies. These can occur either due to variations in environmental oxygen levels (e.g. ice-locking of lakes) or behaviours that interrupt oxygen supply (e.g. breathold diving). As a result, many species have developed mechanisms that allow them to compensate for episodes of low oxygen (hypoxia). These mechanisms typically act to improve oxygen delivery to tissues and/or to increase ATP production by oxygen-independent means (e.g. glycolysis) to compensate for the reduced ATP output by oxygen-dependent pathways in the mitochondria. However prolonged oxygen deprivation (severe hypoxia and anoxia) is still extremely damaging for most vertebrates. In contrast, some ectothermic vertebrates are extremely well-adapted for surviving oxygen limitation. For example, freshwater turtles living in the northern regions of the United States and southern Canada spend their winters underwater to escape the freezing temperatures. Lakes and ponds often become ice-locked, limiting the ability of lung-breathing animals to surface. While some turtle species compensate for this with a good capacity for extrapulmonary gas exchange across epithelia, others have perfected strategies that allow anaerobic survival [1]. For example, red-eared sliders (*Trachemys scripta elegans*) and painted turtles (*Chrysemys picta*) can survive without oxygen for up to two weeks at 16–18 °C and for 12–18 weeks at 3 °C [1]. Understanding the mechanisms that underlie natural anoxia tolerance is not only of interest from a comparative biochemistry and physiology point of view, but could also contribute to medical science by improving the understanding of the injuries caused by anoxia/ischemia during heart attack or stroke and the potential way to avoid these [2–5]. In addition, it has also been proposed that the mechanisms that allow turtles to survive anoxia can also contribute to their longevity, and as such these turtles could potentially be used as models for longevity [6,7].

Anoxia-tolerant turtle species employ various physiological and biochemical mechanisms to enable survival without oxygen. These include accumulation of large glycogen stores in the liver, strategies for buffering glycolytic end products to minimize acidosis, and a capacity to depress their metabolic rate to only 10–20% of the corresponding aerobic rate. Glycogen stores provide a fermentable fuel for glycolytic ATP production under anaerobiosis, while the buffering is provided by calcium and magnesium carbonates released from the shell and the skeletal system, as well as storage of lactate in the shell [8]. Metabolic rate depression has been hailed as the most important contributor to anoxia survival across phylogeny [9–12]. In turtles, it was demonstrated that the low metabolic rates during submergence in cold water could allow survival for as long as 3 months in anoxic water and 5 months

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in aerated water [13,14]. Metabolic rates for turtles submerged in cold water were only about 10% of those for animals in air at the same temperature. The turtle brain also undergoes several adjustments in response to anoxia and has been studied as a model for neuroprotection [15]. Key adaptations include channel arrest, decrease in N-methyl-D-aspartate (NMDA) receptor activity and γ-aminobutyric acid (GABA)-mediated suppression of neuronal excitation [16–19]. In the cardiovascular system, levels of nitric oxide (NO) rise in the blood and heart in response to decreased O_2 levels [20,21], which could rescue O_2 supplies via vasodilation and reduce mitochondrial O_2 consumption [22] during the hypoxic period, as well as have cytoprotective functions [20]. Similar changes have also been observed in another model for anoxia tolerance, the crucian carp [23]. Other cardiovascular adaptations include an ATP-independent increase in hemoglobin-O_2 affinity during acclimation to winter hibernation [24], along with a reduction in the heart rate [25] and peripheral vasoconstriction.

While initial studies of anoxia tolerance have focused on the physiological and enzymatic mechanisms involved, recent years have seen a rise in research related to gene expression mechanisms that contribute to anoxic survival and upregulation and/or activation of various genes and transcription factors associated with stress resistance, metabolism and metabolic arrest has been documented. In the present review we discuss some of the recent advances in understanding natural anoxia tolerance on the molecular level in the red-eared slider turtle, a common animal model for natural anoxia-tolerance.

2. Activation of stress-responsive transcription factors and proteins

Several studies have addressed the role of different stress-responsive pathways and transcription factors in anoxia tolerance and a number of these were shown to be anoxia-responsive. They are discussed in this section.

2.1. HSF and HSPs

One of the best-studied cytoprotective mechanisms known to respond to stress is the proliferation of chaperone proteins. These proteins are involved in the folding of nascent proteins, as well as aid in the refolding of malfolded or unfolded proteins that can often accumulate under cell stress conditions, and their action can allow the functional life of cellular proteins to be extended [26]. Heat shock proteins (HSPs) are the best-known group of chaperones and their increased expression is widespread in response to a variety of stress conditions [27]. The transcription of *hsp* genes is regulated by heat shock transcription factors (HSFs), which bind to the heat shock element in the promoter region of *hsp* genes in response to stress, resulting in increased expression of various HSPs [28,29]. While several HSF family members have been found in vertebrates, HSF-1 has emerged as the main mediator of the HSP stress response [30]. The classic mechanisms described for activation of HSF-1 is by hyperphosphorylation in response to stress. This modification allows it to trimerize and acquire DNA-binding activity. This is followed by nuclear translocation and upregulation of *hsp* transcription [31,32].

We have previously shown that HSF-1 is activated in several turtle tissue in response to anoxia [33]. This was accompanied by upregulation of several HSPs, including Hsp25, Hsp40, Hsp70, Hsc70, and Hsp90. Some of these have also been shown to be anoxia-responsive at the transcript and/or protein level in other turtle studies [34–36].

In addition to helping to sustain the correct folding of proteins under anoxic conditions, some HSPs have additional functions. For example, Hsp27 has been shown to possess antioxidant properties [37,38]. Moreover, Hsp27, as well as Hsp70 and Hsp90 have also been shown to play a role in the regulation of apoptosis by the binding and inhibition of members of the apoptotic cascade. Hsp27 has been shown to inhibit apoptosis by inhibiting the release of mitochondrial cytochrome c in response to stress [39], or by binding cytochrome c directly [40]. Hsp70 and Hsp90 have been shown to bind Apaf-1 and by doing so inhibit caspase activation [41,42]. Hsp70 can also bind Apoptosis-Inducing Factor (AIF) that is released from the mitochondria and by doing so prevent caspase-independent cell death [43]. In a recent study, it was shown that knockdown of Hsp72 (the inducible HSP70 family member) in neuronally enriched primary cell culture from turtles increased AIF during anoxia and reoxygenation, as well as resulting in a strong increase in hydrogen peroxide levels [44].

In addition to being anoxia-inducible in various turtle tissues, some HSPs are constitutively elevated in turtles. Levels of Hsp60 in the hearts of anoxia-tolerant painted turtles have been shown to be significantly higher compared to those in anoxia-intolerant softshell turtles, rabbits and rats [45]. This protein is a predominantly mitochondrial chaperone that is also known to have protective effects against oxidative stress [46]. High constitutive levels of Hsp72 have also been reported in the turtle brain [36] although it is generally found at very low levels under basal conditions in other organisms and is induced in response to stress. Therefore, constitutive expression of Hsp72 suggests a potentially important function in neuroprotection.

2.2. NF-κB

NF-κB is an oxygen-responsive transcription factor that is known to be activated in response to a variety of stress stimuli. While originally this transcription factor was characterized as a major mediator of the immune response, it has also been shown to control many other genes, including genes involved in the stress response, antioxidant defense, cell growth and differentiation, and apoptosis [47]. NF-κB can be a homo- or heterodimer, composed of proteins containing the Rel Homology domain, such as, p50 (also known as NF-κB1), p52 (also known as NF-κB2), p65 (also known as RelA), RelB, and c-Rel [48], with the 'classical' combination being between p50 and p65. Under basal conditions, the NF-κB transcription complexes are present in the cytosol in an inactive state through their interaction with the inhibitor protein IκB, which masks their nuclear localization signals. In response to various stimuli, IκB becomes phosphorylated, targeting it for ubiquitination and degradation, and freeing NF-κB to translocate to the nucleus and mediate expression of its target genes [49].

We have previously examined the potential role of NF-κB in turtle anoxia tolerance [50]. Elevated levels of IκB phosphorylation were detected in the liver of anoxic turtles. This coincided with increased expression of the major NF-κB subunits, p50 and p65, increased nuclear localization, and increased DNA-binding activity.

Several target genes of NF-κB involved in antioxidant defense and cell survival were also assessed. The antioxidant genes included the heavy chain of ferritin, copper/zinc superoxide dismutase (Cu/Zn SOD) and manganese superoxide dismutase (MnSOD). Ferritin protects against oxidative stress by sequestering redox-active iron inside its protein core, thereby reducing the potential for iron-catalyzed hydroxyl radical formation via the Fenton reaction [51]. Cu/Zn- and MnSOD protect against oxidative stress by catalyzing the dismutation of superoxide radicals into water and hydrogen peroxide. Transcript levels of all three proteins were significantly increased in response to anoxia in turtle liver, suggesting potential roles in cell protection during long-term anoxic hypometabolism and/or as a preparatory defense against a rapid increase in oxidative stress triggered by reoxygenation during aerobic recovery.

Other target genes of NF-κB include anti-apoptotic proteins. The promotion or inhibition of apoptosis is controlled by two conserved pathways, the death receptor pathway and the mitochondrial pathway, and the decision to initiate apoptosis depends on the relative levels of pro- vs. anti-apoptotic proteins [52]. NF-κB is known to control the expression of several anti-apoptotic genes, including Bcl-2 [53,54] and Bcl-xL [55,56]. These proteins reside in the outer mitochondrial membrane and function in preventing the loss of outer mitochondrial membrane integrity [57], thereby preventing apoptosis. Transcript

levels of both proteins were upregulated in turtle liver in response to anoxia, suggesting a potential function for these genes in preventing apoptotic cell death by counteracting potential apoptotic signals arising from the stress of oxygen limitation. In addition, it was recently shown that knockdown of Bcl-2 in neuronally enriched primary cell cultures from anoxia-tolerant turtles resulted in increases in apoptotic markers, as well as increased ROS levels [44].

In addition to being anoxia-responsive, NF-κB has also been studied in mammalian hibernation, another condition that is characterized by metabolic rate depression and variations in oxygen consumption. NF-κB was activated during entrance into torpor in hibernating ground squirrels [58]. Furthermore, protein levels of MnSOD and HO-1 rose during early torpor and early arousal, respectively [58]. This suggests that NF-κB might have a general adaptive role in vertebrate stress tolerance.

2.3. The unfolded protein response

The unfolded protein response (UPR) is an important stress-responsive pathway that can be activated in response to accumulation of unfolded proteins in the endoplasmic reticulum (ER). It consists of several signaling pathways that act to return the ER to its normal physiological state [59]. The UPR acts to reduce the number of unfolded proteins in the ER lumen by inhibition of protein translation [60–62], induction of chaperone proteins to increase the protein-folding capacity of the ER [63,64], and upregulation of the ER-associated degradation (ERAD) pathway to remove proteins that cannot be refolded (Fig. 1) [65–67].

Activation of the UPR is controlled by three sensor proteins: the inositol requiring kinase 1 (IRE1) [68,69], the double-stranded RNA-activated protein kinase-like ER kinase (PERK) [70], and the Activating Transcription Factor 6 (ATF6) [69,71]. Under basal conditions PERK is kept in an inactive state through its interaction with glucose-regulated protein 78 (GRP78; also known as BiP), an ER-resident chaperone. However, ER stress drives GRP78 to dissociate from PERK, allowing it to become activated through autophosphorylation and oligomerization [72]. PERK then phosphorylates the eukaryotic initiation factor 2 on its alpha subunit (eIF2α) at Ser 51, leading to its inactivation, thereby attenuating protein synthesis due to inhibition of eIF2α which brings the initiating methionine residue to the assembling ribosome [60,62]. Activation of PERK also induces the expression of many downstream genes [73–75]. Its downstream transcription factor, Activating Transcription Factor 4 (ATF4), is activated in response to phosphorylation of eIF2α [73–75] and mediates the transcription of several genes involved in amino acid biosynthesis and transport, antioxidant stress responses, and apoptosis [73–75]. In turtle tissues, the phosphorylation state of both PERK and eIF2α increased in response to anoxia in the heart, kidney and liver. ATF4 was also activated as shown by increased mRNA and protein levels, increased nuclear content, and DNA-binding activity [76].

Several UPR-responsive target genes were also examined in turtles in response to anoxia [76]. ATF3 is a transcription factor that is involved in the cellular adaptive response [77]. GADD34 is another target gene of ATF4. It becomes activated late in the UPR and is used as a feedback control mechanism by targeting the type 1 serine/threonine protein phosphatase to eIF2α and dephosphorylating it [78,79]. Both ATF3 and GADD34 showed elevated protein expression in response to anoxia in turtle heart and liver [76].

One of the most important functions of the UPR is the upregulation of molecular chaperones that can enhance the folding capacity of the ER. The anoxia-dependent expression of two chaperones, GRP78 and GRP94 was studied in turtle tissues. These proteins are upregulated by multiple branches of the UPR, and therefore are used as markers for UPR activation [80]. GRP78 expression is known to be up-regulated by all three ER sensors of the UPR, whereas GRP94 is mainly regulated by ATF6 and IRE1. Both proteins are involved in refolding protein substrates and, as such, function in relieving ER stress [80]. Both proteins responded positively to anoxia in turtles, with increases in both transcript and protein levels in the heart and liver for GRP94 and the heart, liver and kidney for GRP78 [76], suggesting that these proteins alleviate potential anoxia-induced protein folding stress in the ER.

2.4. FoxOs, p53 and cell-cycle arrest

The forkhead class O (FoxO) proteins are a family of transcription factors that have emerged as important regulators of cellular metabolism

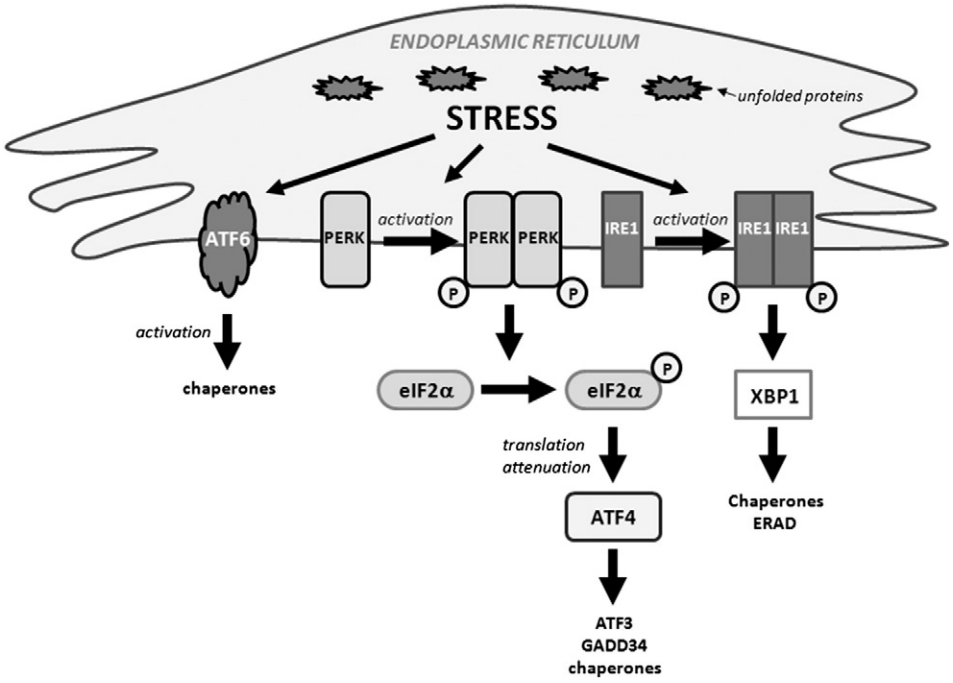

Fig. 1. Overview of the UPR. Accumulation of unfolded proteins in the ER lumen leads to activation of the ER-resident stress-sensors ATF6, PERK and IRE1. These in turn result in the upregulation of protein chaperones, translational attenuation, and ER-associated degradation (ERAD).

and differentiation, survival and apoptosis, cell cycle arrest, autophagy, stress resistance and lifespan. They are comprised of three main functionally-related proteins that are vertebrate orthologs of the *Caenorhabditis elegans* transcription factor DAF-16 [81,82] and include FoxO1 [83], FoxO3 [84,85], and FoxO4 [86]. A fourth member, FoxO6, has been identified by homology, and is expressed in the brain [87]. One of the key functions of the FoxO proteins is facilitating cellular quiescence by induction of cell cycle arrest. Progression through the cell cycle is regulated by the balance between the levels and activities of the cyclin–CDK (cyclin-dependent kinase) complexes and those of their inhibitors [88]. FoxOs are known to be involved in blocking cell cycle progression at the G1 phase by inducing the expression of *p27kip1*, an inhibitor of the cell cycle [89–92]. In addition, FoxOs have also been shown to promote the expression of the retinoblastoma family member p130 [93], a protein involved in the repression of the genes required for re-entry into the cell cycle, thereby promoting a quiescent state [94]. Furthermore, FoxOs have also been shown to induce the transcription of cyclin G2 [95], a cyclin that is highly expressed in quiescent cells [96,97].

In addition to promoting quiescence, FoxOs have been shown to enhance cellular resistance to oxidative stress. For example, in mammals, oxidative stress triggers the relocalization of FoxOs from the cytoplasm to the nucleus [98,99], resulting in induction of several antioxidant genes. FoxO3 upregulates the expression of sterol carrier protein x (SCPx) and SCP2 [100] that might be involved in protecting lipids against oxidative damage [100]. Another FoxO target is GADD45a, a protein that is involved in DNA-damage repair in response to a variety of stressful stimuli [101].

FoxO1 and FoxO3 responses to anoxia have been analyzed in turtle tissues [102]. FoxO3 was activated in turtle heart, kidney and liver in response to anoxia. FoxO1 was also activated in turtle liver under anoxic conditions, as evidenced by increased expression, decreased phosphorylation (which is associated with inhibition of FoxOs), increased nuclear translocation, and increased DNA-binding activity. In addition, transcript levels of two FoxO target genes, p27kip1 and catalase, were elevated in the liver in response to anoxia (Fig. 2) [102], suggesting that FoxOs play a role in the regulation of cell cycle arrest and stress resistance in turtles under anaerobiosis.

The p53 transcription factor is another transcription factor that has been shown to have major roles in regulating apoptosis, the cell cycle, DNA damage repair, and energy metabolism [103–105]. The activity of p53 is regulated by various post-translational modifications, including ubiquitination, phosphorylation and acetylation [106–108]. Phosphorylation of p53 prevents it from being targeted for ubiquitination by promoting conformational changes that conceal binding sites of the ubiquitin E3 ligase MDM2 [107]. Acetylation of p53 can also contribute to increased stability by preventing poly-ubiquitination and degradation. Many genes are under p53 control including *14-3-3σ*, *Growth Arrest and DNA-Damage-inducible α* (*Gadd45α*) and *phosphoglycerate mutase* (*Pgm*) that are involved in the arrest of cellular proliferation, DNA repair, and apoptosis [109–112]. In addition, p53 is also a transcriptional regulator of *miR-34a*, a microRNA that has been shown to have effects on the cell cycle by repressing E2F5, Cyclin E2, and CDK4/6 translation [110,113–118].

The activation status of p53 has been examined in turtle liver and muscle in response to anoxia [119]. The liver displayed activation of p53 in response to anoxia as well as differential regulation of several phosphorylation and acetylation sites on the protein. In addition, three p53 target genes, *14-3-3σ*, *Gadd45α* and *Pgm*, were examined. *14-3-3σ* is involved in cell cycle arrest by inhibiting Akt-dependent inactivation of $p27^{Kip1}$ [120,121]. *Gadd45α* is critical to cell cycle arrest at the G2 checkpoint [122] and can also increase the stability of p53 in the presence of DNA damage [123]. Transcript levels of both proteins increased in response to anoxia in turtle tissues [119]. Transcript levels of miR-34a were also elevated in both the liver and the muscle in response to anoxia [119]. In addition to promoting expression of the above proteins, p53 has a negative effect on the expression of *Pgm*, contributing to a decreased glycolytic capacity [124]. A significant decrease in the transcript levels of this enzyme were observed in both the liver and the muscle in response to anoxia, suggesting a negative effect of p53 on glycolysis. It is also possible that this inhibitory effect is related to inhibition of glycogen synthesis under anoxic conditions in order to prevent glucose from being stored into the glycogen stores. PGM is reversible and converts glucose 6-phosphate (G6P) to glucose 1-phosphate (G1P), which is the substrate for UDP-glucose formation that is in turn a substrate for glycogen synthase. Therefore, reduction of PGM capacity might promote G6P use by glycolysis rather than glycogen synthesis.

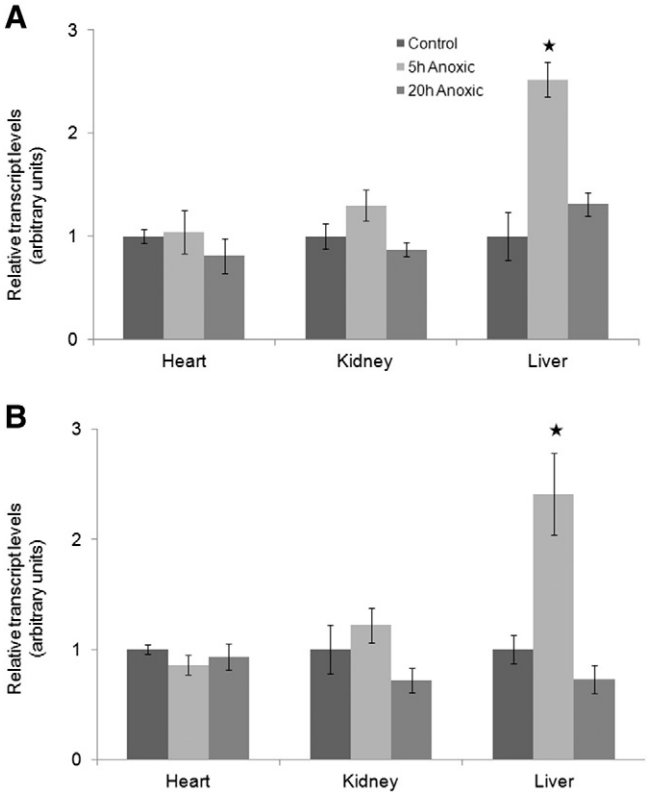

Fig. 2. Upregulation of the transcript levels of FoxO-responsive genes *p27kip1* (A) and *catalase* (B) in response to anoxia in tissues of *T. s. elegans*. ★Significantly different from the corresponding control, $P < 0.05$.
Data are from [102].

Another recent study has suggested a potential role for cyclin D1 inhibition in cell cycle suppression in response to anoxia [125]. Cyclin D1 is an important regulator of the G1 phase of the cell cycle and has a key role in initiation of cellular proliferation [126,127]. It was shown that the relative expression of cyclin D1 in both total protein, as well as nuclear fractions, was significantly decreased in response to anoxia in turtle liver and kidney [125]. However, both the phosphorylation state of cyclin D1, which controls its proteasomal degradation, as well as its transcript levels were unchanged in response to anoxia in turtle tissues. Inhibitory control over cyclin D1 expression might come from a different source since analysis of its 3′UTR showed a binding site for microRNA-16-1 and microRNA-15a which could potentially inhibit translation of the cyclin D1 transcript. Levels of both miRNAs increased in the liver and kidney in response to anoxia, suggesting that miRNA-based inhibition could play an important role in cell cycle suppression in response to anoxia [125].

3. Antioxidant systems in anoxia-tolerance

Antioxidant defenses are important for detoxifying reactive oxygen species (ROS) and/or reducing/repairing damage caused by oxidative

stress. They are usually composed of enzymatic defenses as well as small molecular weight antioxidant molecules. Well-developed antioxidant defenses appear to be important for biochemical adaptation of anoxia-tolerant species. These could be involved in minimizing oxidative damage to macromolecules caused by a rapid rise in oxygen concentrations when animals transition from hypoxic/anoxic states back to normoxia.

Glutathione is one of the primary low molecular weight thiol antioxidants in the cell. Reduced glutathione (GSH) is converted to the oxidized form (GSSG) when GSH is used in multiple processes such as to (a) maintain the redox state of different proteins, (b) directly detoxify oxygen radicals, and (c) act as a substrate for antioxidant enzymes such as glutathione peroxidase (GPX) and glutathione S-transferase (GST). It was previously shown that anoxia-tolerant turtles possess constitutively high antioxidant defenses [128,129]. Turtles have much higher constitutive activities of several antioxidant enzymes, including catalase, superoxide dismutase, and alkyl hydroperoxide reductase as compared to other ectothermic vertebrates [128]. These activities are more comparable to mammalian values even though the metabolic rate of turtles is significantly lower. In addition, levels of ascorbic acid (another low molecular weight antioxidant) were very high in the turtle brain [130] and tissue pools of glutathione were higher in turtles compared to other ectotherms [129]. Furthermore, the GSH/GSSG ratio increased in several turtle tissues during recovery from anoxia, suggesting that oxidative stress did not occur in these tissues [129]. This was also in agreement with measurements of several lipid peroxidation products, which revealed that only minimal changes in oxidative damage to lipids occurred during anoxia or recovery [128].

The structure of some turtle enzymes also appears to be suitable for anoxic survival. Turtle glutathione reductase (GR), which catalyzes the reduction of GSSG back to GSH, was shown to have very high affinity for GSSG [131], as well as a broad pH range, allowing for better enzyme efficiency under different conditions. Turtle GSTs also undergo modifications in response to changing oxygen that could potentially improve function under changing redox conditions [132].

In addition to maintaining high constitutive antioxidant defenses, turtles also show upregulation of several proteins associated with antioxidant defense in response to anoxia. This includes the heavy chain of ferritin and SOD as discussed above. Expression of the heme oxygenase-1 (HO-1) gene was also upregulated in the turtle brain under anoxic conditions [34]. This enzyme is involved in the degradation of heme, a major source of redox active iron in the cell whose induction is sometimes tied to simultaneous upregulation of ferritin [133]. Screening of cDNA arrays also showed elevated transcript levels of glutathione peroxidase (GPX) isozymes 1 and 4, GST isozymes M5 and A2, and peroxiredoxin 1 in response to anoxia in the heart and liver of hatchling painted turtles *Chrysemys picta marginata* [134]. In addition, the measured activities of selected antioxidant enzymes also increased under anoxia; for example, glutathione reductase activity rose by 52% in the liver of red-eared sliders in response to anoxia [129].

4. Molecular mechanisms affecting global gene expression

Gene transcription and protein synthesis typically consume a very large portion of a cell's energy budget [135]. Therefore, these processes must be suppressed to facilitate long term anoxia survival, a situation where the cell's capacity to generate ATP is compromised. Early studies showed that protein synthesis was reduced in response to anoxia in turtle tissues [136,137]. Furthermore, protein half-lives significantly increased under anoxia in painted turtle hepatocytes while proteolysis was suppressed [138]. Other studies have examined mechanisms that could potentially control protein synthesis in turtles in response to anoxia. Rider et al. [139] investigated the potential role of the AMP-activated protein kinase (AMPK) for the regulation of protein synthesis during anoxia. This protein acts as a sensor of the energy status of the cell and plays an important role in the maintenance of cellular energy homeostasis in mammals [140–142]. AMPK is activated by increases in the intracellular AMP:ATP ratio due to a drop in energy status, resulting from various stresses [140]. When energy is limiting, AMPK becomes activated and inhibits mTORC1 signaling that normally promotes translation [143]. AMPK showed a tissue-specific activation in turtles in response to anoxia. In addition, other proteins associated with promotion of translation, such as the p70 ribosomal protein S6 kinase (p70S6K), the 40 S ribosomal protein S6 (rpS6), and the eukaryotic initiation factor-4E-binding proteins-1 (4E-BP1) were all inhibited by dephosphorylation in response to anoxia [139].

Recently, a possible involvement of epigenetic mechanisms in the suppression of gene transcription in response to anoxia has been proposed [144]. The term epigenetics refers to various modifications to DNA and chromatin processes that can result in changes in genes expression without changing the DNA sequence [145]. Epigenetic modifications of chromatin proteins, for example, can alter their interaction with DNA and change accessibility to DNA by the transcriptional apparatus. These proteins include histones, which are subject to a variety of modifications that affect transcriptional activity [146,147]. Acetylation of histone 3 (H3) generally leads to transcriptional activation, whereas deacetylation leads to repression. Histone deacetylases (HDACs) are enzymes that remove acetyl groups from histones, and therefore, are associated with transcriptional silencing (Fig. 3) [148]. The expression of several different HDACs was examined in turtle tissues in response to anoxia [144]. Muscle was the most responsive tissue, with both transcript and protein levels of all HDACs examined rising in response to anoxic exposure (Fig. 4). This was accompanied by both a rise in HDAC activity and a decrease in the levels of H3 acetylation. Turtle liver also displayed increased amounts of selected HDACs, corresponding with a decrease in histone H3 acetylation. These results suggest that chromatin condensation occurs in response to anoxia in selected tissues, and implicate a role for epigenetic modifications in transcriptional silencing in response to anoxia.

5. Regulation of metabolism

Re-organization of metabolism is important for optimization of metabolic efficiency during anaerobiosis. For example, under aerobic conditions, ATP can be generated from multiple sources including carbohydrates, lipids, and amino acids whereas under anoxia anaerobic glycolysis is the only method of ATP production. Recently, it was shown that such metabolic reorganization can occur on the transcriptional level, through activation of the Carbohydrate Response Element Binding Protein (ChREBP) [149]. ChREBP is a glucose-responsive transcription factor that has emerged as an important regulator of glycolytic and lipogenic genes [150,151]. It is believed that in response to high concentration of glucose, ChREBP undergoes post-translational modifications that allow it to translocate to the nucleus and acquire DNA-binding activity [152,153]. One important target gene of ChREBP is the liver form of pyruvate kinase (LPK), a flux-controlling enzyme that catalyzes the formation of pyruvate and ATP from phosphoenolpyruvate and ADP. ChREBP was shown to be anoxia-responsive in the kidney and liver of turtles, increasing in expression as well as undergoing nuclear translocation. In the liver, ChREBP DNA-binding activity also increased, and transcript levels of LPK were elevated [149]. These results suggest that ChREBP could potentially be an important factor in anaerobic survival by contributing to enhancing glycolytic capacity during anaerobiosis. Earlier studies that examined the enzymatic properties of pyruvate kinase in turtle tissues showed that it acquired altered kinetic properties in response to anoxia including a 1.5-fold increase in the I_{50} for L-alanine and a 1.2-fold increase in maximal activity [154]. This suggests that posttranslational modifications of the pyruvate kinase protein also contribute to altering glycolytic capacity under anoxia.

Other glycolytic enzymes are also modified in response to anoxia. For example, phosphofructokinase (PFK) showed a 3-fold increase in its I_{50} value for citrate and a 1.5-fold increase in the K_m for ATP in

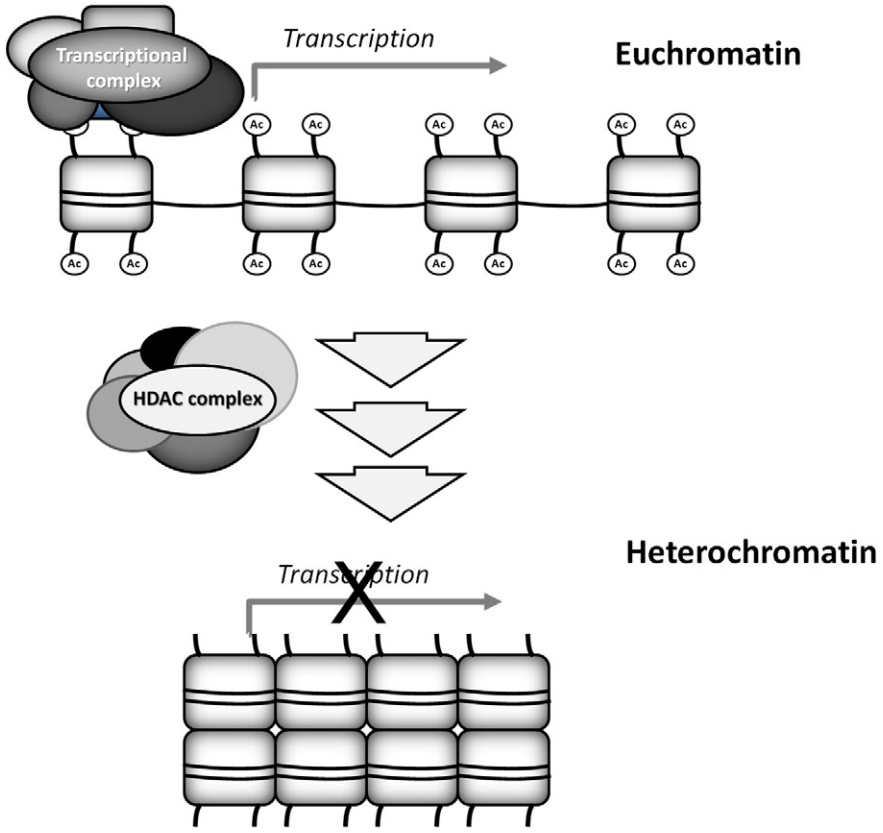

Fig. 3. Histone acetylation in transcriptional repression. Acetylation of histones leads to an open chromatin structure (euchromatin) allowing access of transcriptional machinery to DNA, facilitating transcription. Histone deacetylases remove acetyl groups from histones, leading to a closed chromatin structure (heterochromatin) and transcriptional repression.

response to anoxia [154]. Lactate dehydrogenase (LDH), which catalyzes the conversion of pyruvate to lactate and thereby recycles the NAD^+ needed to sustain glycolysis, is also anoxia-responsive. In turtle liver, LDH showed an increase in the K_m value for pyruvate, a decrease in the maximal activity in the lactate-producing direction and a decrease in the I_{50} for pyruvate in response to anoxia [155]. In addition, LDH isolated from anoxic liver showed greater phosphorylation and acetylation, as compared with the enzyme from aerobic liver [155]. In skeletal muscle, however, the K_m for lactate of the anoxic enzyme was significantly lower compared to normoxic conditions, whereas the Vmax was significantly higher, possibly due to a change in its phosphorylation status [156]. Recently, fructose-1,6-bisphosphate (FBP) aldolase, which cleaves FBP to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, was also shown to have increased protein levels and some modified enzymatic properties in the liver in response to anoxia [157].

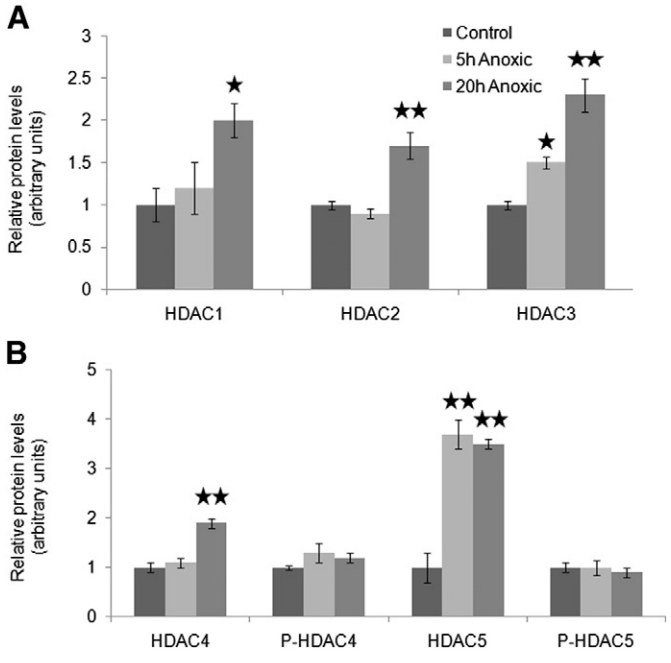

Fig. 4. Effect of anoxic submergence on protein levels of HDAC1-5 in white skeletal muscle of *T. s. elegans*. Representative (A) Class I HDACs, HDAC1-3. (B) Class II HDACs, HDAC4-5 and their phosphorylated forms p-HDAC4 (Ser632) and p-HDAC5 (Ser498). ★Significantly different from the corresponding control ($P < 0.05$). ★★Significantly different from the corresponding control ($P < 0.05$).
Data are from [144].

6. Concluding remarks

Recent years have seen advances in the understanding of the biochemical and gene-regulatory responses that underlie natural anoxia tolerance. In particular, pathways implicated in the stress response, hypometabolism and carbohydrate metabolism have been shown to be anoxia-responsive in turtle tissues, suggesting a crucial importance of these pathways for anoxia tolerance. A general picture of the molecular mechanisms of natural anoxia tolerance is emerging. This includes proliferation of protein chaperones to aid/sustain protein folding, induction of antioxidant defenses as a preparatory mechanism for high ROS generation during aerobic recovery, induction of pro-survival genes to counteract any apoptotic signals arising in response to anoxia, cell cycle arrest, and enhancement of mechanisms that facilitate global transcriptional and translational inhibition.

Understanding the molecular mechanisms associated with natural anoxia tolerance is not only interesting in itself, but could also be relevant from a medical point of view. The molecular mechanisms of ischemic injury in mammals are not yet completely understood, and

therefore studies of the responses of anoxia tolerant organisms could highlight mechanisms that are beneficial for survival. Indeed many of the pathways that are involved in anaerobic survival in turtles have also been shown to be involved in human disease, including NF-κB, the UPR, the HSP response, FoxOs and p53. However, much still remains to be understood about their specific roles under anoxic conditions. For example, many of these pathways are activated in a tissue-specific manner and it is not yet clear as to why a particular pathway would be activated in one tissue but not another. It is expected that as our understanding about the role of these pathways (and others) in natural anoxia tolerance increases, it will be possible to improve our understanding of the molecular nature of various mammalian pathological conditions associated with oxygen deprivation, allowing the identification of new treatments for these conditions.

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