Biochimica et Biophysica Acta 1850 (2015) 1188–1196

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Turtle anoxia tolerance: Biochemistry and gene regulation

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ARTICLE INFO

Article history:
Received 21 December 2014
Accepted 1 February 2015
Available online 7 February 2015

Keywords:
Heat shock response
NF-kappaB
Unfolded protein response
FoxO
p53
HDAC

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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http://dx.doi.org/10.1016/j.bbagen.2015.02.001
0304-4165/

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