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Anoxia-responsive regulation of the FoxO transcription factors in freshwater turtles, *Trachemys scripta elegans*

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ABSTRACT

Background: The forkhead class O (FoxO) transcription factors are important regulators of multiple aspects of cellular metabolism. We hypothesized that activation of these transcription factors could play crucial roles in low oxygen survival in the anoxia-tolerant turtle, *Trachemys scripta elegans*.
Methods: Two FoxOs, FoxO1 and FoxO3, were examined in turtle tissues in response to 5 and 20 h of anoxic submergence using techniques of RT-PCR, western immunoblotting and DNA-binding assays to assess activation. Transcript levels of FoxO-responsive genes were also quantified using RT-PCR.
Results: FoxO1 was anoxia-responsive in the liver, with increases in transcript levels, protein levels, nuclear levels and DNA-binding of 1.7–4.8 fold in response to anoxia. Levels of phosphorylated FoxO1 also decreased to 57% of control values in response to 5 h of anoxia, indicating activation. FoxO3 was activated in the heart, kidney and liver in response to anoxia, with nuclear levels increasing by 1.5–3.7 fold and DNA-binding activity increasing by 1.3–2.9 fold. Transcript levels of two FoxO-target genes, *p27kip1* and *catalase*, also rose by 2.4–2.5 fold in the turtle liver under anoxia.
Conclusions: The results suggest that the FoxO transcription factors are activated in response to anoxia in *T. scripta elegans*, potentially contributing to the regulation of stress resistance and metabolic depression.
General significance: This study provides the first demonstration of activation of FoxOs in a natural model for vertebrate anoxia tolerance, further improving understanding of how tissues can survive without oxygen.

1. Introduction

While most vertebrates are sensitive to oxygen limitation, some species have evolved the capacity to survive for extended periods of time without oxygen. This includes several turtle species such as red-eared sliders (*Trachemys scripta elegans*) and painted turtles (*Chrysemys picta*). These animals typically encounter anoxia during the winter, when they escape freezing air temperatures by hibernating underwater. These bodies of water can become ice-locked, limiting oxygen supply and imposing anoxia on the turtles. As a consequence, these species developed an ability to survive without oxygen for several months at cold temperatures [1]. Various physiological mechanisms contribute to anoxia-tolerance; chief among them is profound metabolic rate depression to levels as low as just 10–20% of the corresponding aerobic resting rate at the same temperature [2–6]. Several molecular mechanisms have also been identified, including various enzymatic processes, as well as changes in gene expression [6,7]. However, many of the molecular mechanisms involved in anoxia tolerance still remain unknown.

Further elucidation of the mechanisms that confer anoxia tolerance to vertebrate tissues could contribute to better understanding of the mechanisms involved in hypoxic/ischemic damage in oxygen-sensitive systems, and lead to better treatment and/or prevention of injuries resulting from oxygen deprivation. The present study addresses the responses of forkhead class O transcription factors to anoxia in turtle tissues. The forkhead proteins are a large family of evolutionary conserved transcription factors of multiple classes. The forkhead class O (FoxO) members have emerged as important regulators of cellular metabolism and differentiation, survival and apoptosis, cell cycle arrest, autophagy, stress resistance and lifespan. The FoxO class is comprised of three main functionally-related proteins: FoxO1 [8], FoxO3 [9,10], and FoxO4 [11] that are vertebrate orthologs of the *Caenorhabditis elegans* transcription factor DAF-16 [12,13]. A fourth member, FoxO6, has been identified by homology, and is expressed only in the brain [14].

While FoxOs mediate gene expression with respect to a variety of cellular needs, one of the key functions of the FoxO proteins is the induction of quiescence by facilitating cell cycle arrest. Progression through the cell cycle is a tightly controlled process that is regulated by the balance between the amounts and activities of the cyclin–CDK (cyclin-dependent kinase) complexes and those of their inhibitors [15]. FoxOs have been shown to block cell-cycle progression at the G1 phase by inducing the expression of the cell-cycle inhibitor, *p27kip1* [16–19]. FoxOs also contribute to regulating the exit from

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the cell cycle into a state of quiescence by inducing the expression of the retinoblastoma family member, p130 [20]. This protein is thought to be involved in the repression of genes required for re-entry into the cell cycle, and therefore, its induction allows maintenance of a quiescent state [21]. In addition, FoxOs can induce transcription of cyclin G2 [22], a cyclin that is highly expressed in quiescent cells but is under-expressed when cells enter the cell cycle [23,24].

Another important FoxO function is to enhance cellular stress resistance. In *C. elegans*, induction of DAF-16 (the FoxO homologue) is associated with increased resistance to oxidative stress [13,25], as well as extended lifespan [26]. DAF-16 was shown to undergo nuclear translocation in response to various types of oxidative stress [27]. Similarly in mammals, oxidative stress caused by H_2O_2, menadione, or heat shock triggers the relocalization of FoxOs from the cytoplasm to the nucleus [28,29] and FoxOs induce the expression of antioxidant enzymes such as manganese-dependent superoxide dismutase (MnSOD) and catalase. In addition, FoxO3 upregulates expression of sterol carrier protein x (SCPx) and SCP2 [30] that might be involved in protecting lipids against oxidative damage [30]. Another target gene of FoxO signaling is GADD45a, a protein that is induced in response to a variety of stressors, such as ionizing radiation, UV, and reactive oxygen species (ROS) and is involved in DNA-damage repair [31].

Because of the important roles that FoxO proteins play in anoxia-relevant processes such as hypometabolism and stress resistance, we examined their activation status in the tissues of the anoxia-tolerant turtle *T. scripta elegans* in response to anoxia exposure. This study evaluates the expression and activation of two important members of the FoxO family, FoxO1 and FoxO3, as well as two of their downstream target genes. The data show that FoxOs are activated in a tissue-specific manner in response to anoxia, suggesting that these proteins play a role in anoxia tolerance.

2. Materials and methods

2.1. Animals

Adult red eared slider turtles, *T. scripta elegans*, were purchased from Carolina Biological. Turtles were placed in a holding tank containing dechlorinated water at ~11 °C. To acclimate turtles to a temperature typically found in the natural environment during winter hibernation, the temperature was then lowered to ~4 °C over one week. Animals were then acclimated at 4 °C ± 1 °C for 2 weeks before use. Just prior to experimentation, turtles were moved to 4 °C fridges where they were held for 2 h before experiments began. Aerobic control turtles were sampled from this condition, euthanized by decapitation, and then tissue samples were quickly dissected and immediately frozen in liquid nitrogen. To impose anoxia, other turtles were moved into containers filled with dechlorinated water at 4 °C that had been previously bubbled with 100% nitrogen gas for at least 6 h beforehand. After turtles were placed in a tank, a wire mesh was fitted ~10 cm below the water line so that turtles could not surface during the anoxic episode; a low level of nitrogen bubbling continued throughout. Animals were exposed to 5 or 20 h of anoxia and were then rapidly sampled. All turtles survived the experimental treatments. Tissues obtained included the heart, kidney, liver and white skeletal muscle. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and all experimental procedures had the prior approval of the Carleton University Animal Care Committee.

2.2. RNA extraction and cDNA synthesis

All solutions and materials were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved prior to use. Total RNA was isolated from samples of frozen tissues using Trizol™ (Invitrogen). Briefly, 100 mg of tissue was homogenized in 1 mL Trizol using a Polytron homogenizer followed by the addition of 200 μL of chloroform and centrifugation at 10,000 ×*g* for 15 min at 4 °C. The upper aqueous phase (containing the RNA) was removed to a fresh tube and precipitated by the addition of 500 μL isopropanol followed by incubation for 10 min at room temperature (RT). The samples were then centrifuged again at 10,000 ×*g* for 15 min at 4 °C. The total RNA pellet was washed with 1 mL of 70% ethanol and centrifuged again as above. The resulting pellet was air-dried for 10–15 min and resuspended in 50 μL of DEPC-treated water. The quality of RNA was judged based on the ratio of absorbances at 260 and 280 nm as well as gel electrophoresis on a 1% agarose gel stained with ethidium bromide to check for the integrity of 18S and 28S ribosomal RNA bands.

2.3. Primers

Forward and reverse primers for *foxo1*, *foxo3*, *p27kip1* and *catalase* were designed from conserved regions in these genes based on DNA alignment of the sequences from other vertebrate species. After initial work to retrieve the species-specific sequence, perfect primers were then designed and used for quantification of transcript levels for turtle *foxo1*, *foxo3*, and *p27kip1*. Primer sets were as follows:

foxo1: original consensus primer set was forward 5′-AACCTGTCCTA CGCSGACCT-3′ and reverse 5′-GCAGGTGASGACTGKGTTGA-3′ whereas the perfect primer set was forward 5′-GGTGCAGAATGAAGGAA CAG-3′ and reverse 5′-GGTTGATACGGTCAGTGATG-3′.
foxo3: original primers were forward 5′-GAGCTGGATGCCTGG ACAGA-3′ and reverse 5′-GCSTGCTTGTTCTCCTGGAT-3′ whereas the perfect primer set was forward 5′-AATAGTCCATCAAGCAT GTC-3′ and reverse 5′-GAGAACCAAGTCCGGAACCT-3′.
p27kip1: original primers were forward 5′- GCCTGCAGRAACCT CTTCGG -3′ and reverse 5′- AATGCTACAT CCRAYGCTTT -3′ whereas the perfect primer set was forward 5′-CCTGGCTGGCAAGTTC GAGT-3′ and reverse 5′-ACCACCGAGGCATCCGATGA-3′.
catalase: the consensus primers were forward 5′- GAGAGMGGA TTCCTGARAGAG-3′ and reverse 5′- GATCCRTAKCCATTCATRTG -3′.
α-tubulin: perfect forward (5′- GGAAGATGCTGCCAATAACT′ -3′) and perfect reverse (5′-GTCTGGAACTCGGTCAGATC-3′) primers.

2.4. cDNA synthesis and RT-PCR

cDNA was prepared using a method previously described [32–34]. The PCR reaction of 25 μL final volume was composed by mixing 13.25 μL of sterile water, 5 μL of diluted cDNA, 1.25 μL of primer mixture (to a final concentration of 1.5 μM forward and 1.5 μM reverse), 2.5 μL of 10× PCR buffer (Invitrogen), 1.5 μL of 50 mM $MgCl_2$, 0.5 μL of dNTP mixture (10 mM each) and 1 μL of *Taq* Polymerase. The PCR program was as follows: 7 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at a predetermined annealing temperature (54 °C for *catalase* and *tubulin*, 62.5 °C for *foxo1*, *foxo3* and *p27kip1*), and 1.5 min at 72 °C. The final step was 72 °C for 10 min. PCR products were separated on a 1% agarose gel stained with ethidium bromide, visualized using the ChemiGenius imaging system (Syngene) under UV light and quantified using the GeneTools program. The bands from the most dilute cDNA sample that gave visible product were used for quantification to ensure that the products had not reached amplification saturation.

PCR products were sequenced by DNA Landmarks and sequences were verified as encoding the correct genes using the program BLASTN (http://www.ncbi.nlm.nih.gov/blast) at the NIH.

2.5. Protein extracts and western blotting

Protein extracts were prepared as previously described [34]. Equal amounts of protein (20–30 μg) were loaded into each lane of 10% SDS