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Hypoxia inducible factor-1α responds to freezing, anoxia and dehydration stresses in a freeze-tolerant frog

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ABSTRACT

The wood frog, *Rana sylvatica* (aka *Lithobates sylvaticus*) is the main model for studies of natural freeze tolerance among amphibians living in seasonally cold climates. During freezing, ~65% of total body water can be converted to extracellular ice and this imposes both dehydration and hypoxia/anoxia stresses on cells. The current study analyzed the responses of the alpha subunit of the hypoxia-inducible transcription factor (HIF-1), a crucial oxygen-sensitive regulator of gene expression, to freezing, anoxia or dehydration stresses, examining six tissues of wood frogs (liver, skeletal muscle, brain, heart, kidney, skin). RT-PCR revealed a rapid elevation *hif-1α* transcript levels within 2 h of freeze initiation in both liver and brain and elevated levels of both mRNA and protein in liver and muscle after 24 h frozen. However, both transcript and protein levels reverted to control values after thawing except for HIF-1 protein in liver that dropped to ~60% of control. Independent exposures of wood frogs to anoxia or dehydration stresses (two components of freezing) also triggered upregulation of *hif-1α* transcripts and/or HIF-1α protein in liver and kidney with variable responses in other tissues. The results show active modulation of HIF-1 in response to freezing, anoxia and dehydration stresses and implicate this transcription factor as a contributor to the regulation of metabolic adaptations needed for long term survival of wood frogs in the ischemic frozen state.

1. Introduction

Wood frogs, *Rana sylvatica* (recently renamed *Lithobates sylvaticus*), range across the boreal forests of northern Canada and Alaska and in these seasonally cold environments they survive for weeks or months frozen solid during the winter, with as much as 65–70% of total body water sequestered into extracellular and extra-organ ice. This species is the major model for studies of the physiological and biochemical adaptations that support vertebrate freeze tolerance [8,34,35]. One obvious consequence of whole-body freezing is the interruption of vital processes including breathing, heart beat and blood circulation and this imposes hypoxia/anoxia on cells, as evidenced by the accumulation of glycolytic end products (lactate, alanine) in tissues of frozen frogs (that is reversed upon thawing) [34,35]. Adaptations for freezing survival include both seasonal adjustments (e.g. accrual of high tissue glycogen levels in late summer/early autumn) and rapid freeze-stimulated responses, most notably the activation of liver glycogenolysis within 2–5 min of ice nucleation on the skin surface. The latter allows a quick synthesis and export of huge amounts of glucose from the liver that is delivered by the blood to all organs and taken up as a cryoprotectant (until extensive ice formation halts circulation) [31–33]. Supporting this, measurements of wood frog respiration (as CO_2 output) show a rapid spike in CO_2 production within minutes of freeze initiation [28] along with a doubling of heart rate within 1 min of ice nucleation on the skin surface [15]. These immediate physiological responses, likely all adrenaline-driven, help to deal with the emergency synthesis and distribution of glucose cryoprotectant to all organs as well as other cellular adjustments (e.g. increased synthesis of chaperone and other freeze-responsive proteins) before freezing halts all interorgan transport, shrinks and dehydrates cells, and "locks down" the frog, often for the whole winter [31–35].

The rise in CO_2 output immediately post-nucleation suggests that the early protective responses to freezing utilize oxygen-based metabolism but within the next 2–3 h, CO_2 output decreases as ice accumulates [29], bringing breathing and heartbeat to a halt, and placing a physical barrier on O_2 acquisition that remains in place for the duration of the freeze. One common response of animal cells to oxygen limitation is the activation of the hypoxia-inducible transcription factor (HIF-1) that triggers

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the upregulation of a large set of genes and the subsequent synthesis of specific proteins [3–5]. The best-known consequences of HIF-1 action are upregulation of (a) erythropoietin to enhance erythrocyte production (increasing oxygen-carrying capacity), (b) vascular endothelial growth factor to proliferate capillaries, (c) glycolytic enzymes to increase oxygen-independent ATP production, and (d) glucose transporters to provide cells with fermentable fuel [5,24]. Although erythropoiesis and angiogenesis are unlikely to be factors in wood frog freeze tolerance, the metabolic actions of HIF-1 that optimize/enhance the capacity for anaerobic ATP production or suppress highly ATP-expensive cell processes are likely crucial to prolonged survival in the frozen state. Hence, the focus of the present study was to determine the tissue-specific responses by HIF-1 during wood frog freezing and assess whether HIF-1 could play a significant role in mediating metabolic adjustments that deal with oxygen limitation in frozen frogs.

HIF-1 is a heterodimeric transcription factor composed of HIF-1α (120 kDa) and HIF-1β (91–94 kDa) subunits. HIF-1β (also known as ARNT) can dimerize with a number of transcription factor partners but HIF-1α is highly specific and has a very short half-life (~5 min) under normal oxygen conditions [18,24]. Hence, the alpha subunit is the determining factor in HIF-1 action. Under normal oxygen conditions, the alpha subunit is rapidly modified by O_2-dependent prolyl hydroxylases that attack two proline residues in the oxygen dependent domain (ODD) and set up the protein for ubiquitylation by an E3 ubiquitin-protein ligase followed by rapid degradation by the proteasome [26,27]. However, when oxygen levels are low, prolyl hydroxylase activity falls and HIF-1α is stabilized, allowing it to dimerize with HIF-1β, move into the nucleus and bind to the hypoxia responsive elements of target genes to stimulate their expression. The N termini of both subunits have basic helix-loop-helix (bHLH) domains that mediate DNA binding and downstream PAS domains that facilitate heterodimerization with ARNT to target gene specificity [9]. The C terminal end of HIF-1α contains a transactivation domain that is key to intracellular localization [18]. Overall, HIF-1 action in cells/tissues experiencing hypoxia depends on an elevation of HIF-1α due to suppression of proline hydroxylation, the action of various posttranslational modifications, nuclear translocation of the HIF-1 dimer, and the recruitment of transactivators [6,9,20].

For wood frogs, freezing is a hypoxic/anoxic event since ice formation within the body halts breathing and heartbeat, leading to depletion of cell oxygen that cannot be replaced until frogs thaw. Thus, metabolism in frozen wood frogs depends on anaerobic pathways for ATP production throughout the freeze with accumulation of glycolytic end products, lactate and L-alanine [8,32,33]. In the present study, we hypothesized that the oxygen limitation associated with freezing would trigger a change in HIF-1α subunit levels in wood frog tissues and stimulate HIF-1 mediated metabolic actions that are key for long term survival. Importantly, HIF-1 triggers upregulation of the glucose transporter, GLUT-1, the primary glucose uptake uniporter found in almost all vertebrate tissues [5,12,14]. GLUT-1 action is likely crucial to facilitate glucose uptake by all cells as freezing progresses and provide cells with both an anaerobic fuel and a cryoprotectant. Indeed, the widespread occurrence of GLUT-1 in diverse tissues coupled with elevated hypoxia signals as freezing progresses, and a copious and readily mobilized store of glycogen in liver may be the reason that wood frogs evolved the use of glucose as their cryoprotectant. GLUT-2, the bidirectional glucose transporter found mainly in liver, is also strongly upregulated during wood frog freezing [23] and can support both glucose export from liver when freezing is triggered and the reclamation of glucose back into liver glycogen stores after thawing. HIF-1 also upregulates glycolytic enzymes to increase the potential for anaerobic ATP production in tissues and, in addition, elevates pyruvate dehydrogenase kinase 1 (PDK1) that phosphorylates and inhibits pyruvate dehydrogenase (PDH), thereby suppressing the mitochondrial tricarboxylic acid cycle when oxygen is unavailable [13,14]. Indeed, a study by our lab showed a 3-fold increase in PDK1 protein in liver of 24 h frozen frogs (compared with 5 °C controls) along with a 2.5-fold increase in phosphorylation of PDH on serine residue 300 (an inhibitory site), providing further evidence for the proposal that HIF-1 action is crucial to the metabolic adjustments needed for freezing survival [2].

The current study focuses on *hif-1α* gene expression and HIF-1α protein levels in wood frog tissues, evaluating not only responses to freezing stress but also independent responses to two of the component stresses imposed by the frozen state: anoxia and cell/tissue dehydration [30,34,35].

2. Materials and methods

2.1. Animal experiments

Male wood frogs (5–7 g body mass), newly emerged from winter hibernation, were collected from woodland ephemeral ponds near Kemptville, Ontario on early April nights when breeding activity took place (frogs are still fully freeze tolerant at this time). Frogs were transported to Carleton University on ice where they were briefly washed in a tetracycline bath and then housed in plastic boxes (on a bed of damp sphagnum moss) and acclimated at 5 °C for ~2 weeks before use.

For freezing exposure, small groups of wood frogs were placed in closed rectangular plastic boxes lined with a sheet of damp paper towel and transferred to an incubator set at −4 °C. They were held at this temperature for 45 min to allow body temperature of the frogs to cool to subzero values and trigger transdermal nucleation of body fluids by contact with ice crystals forming on the paper towel (this occurs below about −1.5 °C, the freezing point of wood frog body fluids). Temperature was then raised to −2.5 °C and freezing exposure times of 30 min, 2 h, 6 h or 24 h were timed from this point. Other frogs were frozen for 24 h and then returned to 5 °C to thaw with sampling after 2 or 4 h of thawing. A second group of frogs was given 24 h anoxia exposure (under a N_2 gas atmosphere) at 5 °C or 24 h anoxia followed by 4 h recovery from anoxia in normal air. A third group of frogs was allowed to dehydrate in air at 5 °C until either 20% or 40% of total body water was lost followed by full rehydration overnight at 5 °C in a container with ~0.5 cm depth of dechloraminated water. Detailed experimental protocols have been previously published [30–33]. All frogs were euthanized by pithing and tissues (liver, heart, kidney, brain, ventral skin, hind leg thigh muscle) were rapidly dissected, flash frozen in liquid nitrogen and transferred to −80 °C for storage. Collection of male wood frogs was authorized by a Wildlife Scientific Collector's permit from the Ontario Ministry of Natural Resources. All experimental lab protocols had the prior approval of the Carleton University Animal Care Committee (protocol #106935) and followed the guidelines of the Canada Council on Animal Care.

2.2. RT-PCR analysis of hif-1a transcript levels

Nucleotide sequences for *hif-1a* were retrieved from the NCBI Genbank for human, mouse and African clawed frog (*Xenopus laevis*) (Genbank accession numbers U22431, NM010431 and AJ277829, respectively). Sequences were imported into DNAMAN 4.11 (Lynnon Biosoft) and a multiple sequence alignment was used to identify highly conserved regions. Consensus sequences were imported into Primer Designer (Scientific and Educational Software) and used to generate forward and reverse pairs of gene specific primers. The final *hif-a* primers used were: forward 5′-CCTTGCGATCATGAAGAG-3′ and reverse 5′-TCATATCCAGGCTGTGACGACTGAG-3'. These primers were expected to retrieve a cDNA sequence of ~350 bp from wood frog tissues. Tubulin primers were forward 5′-AAGGAAGATGCTGCCAATAA-3′ and reverse 5′-GGTCACATTTCACCATCTG-3′, and were expected to retrieve a tubulin cDNA sequence of ~616 bp. Primers were synthesized by Sigma Genosys and were aliquoted into 300 pmol/μl stocks in DEPC-treated water and stored at −20 °C.

Total RNA was isolated from wood frog tissues using Trizol reagent

(GIBCO BRL, Grand Island, N.Y.) with the concentration and purity of RNA was quantified spectrophotometrically at 260 and 280 nm; the ratio A_{260}/A_{280} ratio was ~1.6. For cDNA first strand synthesis a 10 μl volume containing 0.5 μg of mRNA was mixed with 1 μl 200 ng/μl oligo (dT), followed by incubation in a 68 °C water bath for 5 min, chilling on ice for 5 min and then a rapid spin. To obtain a cDNA:mRNA hybrid, 8 μl of first stand mixture was added containing 4 μl of 5X first strand buffer (Gibco-BRL), 2 μl 0.1 M DTT (Gibco-BRL), 1 μl 10 mM dNTP mix, and 1 μl superscript reverse transcriptase (200 U/μl). This 19 μl final volume was incubated at 40 °C for 45 min and then stored at 4 °C. PCR amplification of both *hif-1*α and *tubulin* was carried out for 35 cycles using an iCycler PCR machine (Bio-Rad) with an annealing T_m of 44.2 °C. Electrophoresis of PCR products on 1.0% TAE agarose gels, coupled with visualization with ethidium bromide, showed amplification of a single product using either primers designed for wood frog hif-1α or commercial primers for α-*tubulin*. Serial dilutions also established that a 10^{-3} dilution gave subsaturating results that were readily quantifiable when visualized on the agarose gels.

Quantification of band intensities used a Chemi-Genius Bioimaging system with GeneTools software (Syngene, Frederick, MD). Band intensities for hif-1α in each sample were expressed relative to the corresponding α-tubulin band density for that sample. PCR products of both hif-1α and α-tubulin were sequenced by Canadian Molecular Research Services (Orleans, ON) and confirmed their identities.

2.3. Western blotting analysis of HIF-1 protein levels

Samples of frozen tissue were weighed and immediately homogenized 1:10 w:v with a Polytron PT10 in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% w:v sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40), 0.5% w:v sodium deoxycholate, 1 mM EGTA, and 1 mM EDTA with freshly prepared protease inhibitors added immediately before homogenization (0.1 mM phenylmethysulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Samples were centrifuged at 13,000 rpm for 15 min at 4 °C and supernatants were removed. Soluble protein concentrations were determined by the Coomassie blue dye-binding method using the Bio-Rad prepared reagent and bovine serum albumin as the standard. All samples were then standardized to a common concentration via addition of small aliquots of homogenization buffer. Aliquots of protein samples were then mixed 1:1 v:v with SDS-PAGE loading buffer containing 100 mM Tris-HCl (pH 6.8), 4% w:v SDS, 20% v:v glycerol, 5% v:v 2-mercaptoethanol and 0.2% w:v bromophenol blue and boiled for 5 min to denature protein. Samples were stored at −80 °C until use.

Equal amounts of protein (15 μg) were loaded into wells of SDS-PAGE gels and electrophoresis was carried out on a Bio-Rad mini-gel apparatus, run at 180 V for 30–45 min at 21 °C with running buffer (25 mM Tris-base, 190 mM glycine, 0.1% w/v SDS, pH 8.3). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membrane, Millipore corp. Bedford, MA) at 350 mA for 1.5 h at 4 °C with transfer buffer containing 25 mM Tris (pH 8.5), 192 mM glycine and 10% v/v methanol. PVDF membranes were blocked with 2.5% nonfat milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 25 min at 21 °C. Blots were then rinsed with TBST and incubated with primary antibody to human HIF-1α (Promega, 1:500 dilution) in TBST on a rocking platform. Blots were washed twice with TBST and incubated with secondary antibody for 1.5 h at 21 °C, followed by final washes of 3 × 10 min in TBST. Bands were visualized by adding 2 ml of enhanced chemiluminescence reagent (NEN Life Science Products, Inc. Boston, MA) with detection and quantification using a Chemi-Genius Bioimaging system with GeneTools software (Syngene, Frederick, MD). Preliminary testing of wood frog protein extracts using 2-dimensional electrophoresis showed that the HIF-1α antibody detected a single spot that corresponded to a pI of 5.17 and a molecular weight of ~120 kDa, consistent with HIF-1α from other sources and with data on the ExPASy.org website. One-dimensional gels run with a molecular weight ladder, also showed HIF-1α bands at ~120 kDa.

2.4. Data and statistics

Mean values (±SEM, n = 4–5 independent samples) for band intensities from control and experimental samples were tested for significant differences using analysis of variance (ANOVA) and a Tukey post-hoc test. Subsequently, data for experimental and recovery groups were plotted relative to the mean control value that was set to 1.0.

3. Results

3.1. Gene and protein hif-1α sequences

Primers designed from the consensus sequences of *hif-1*α from three species (mouse, human, *X. laevis*) were used to retrieve a 340 nucleotide segment of cDNA from wood frog liver. Analysis of the sequence using the Genbank database (www.ncbi.nlm.nih.gov) identified this sequence as *hif-1α*. The amplified segment of the sequence was bounded by the PAS-a and PAS-b domains.

3.2. Hif-1α mRNA expression in wood frog tissues

The effect of experimental freezing or thawing on hif-1α transcript levels in tissues was evaluated using RT-PCR. Fig. 1A shows the responses to 24 h freezing at −2.5 °C and 4 h thawing recovery (at 5 °C) by *hif-1*α mRNA in six organs of wood frogs. After 24 h freezing at −2.5 °C (a time when ice content has been at maximum for several hours), *hif-1*α transcript levels were elevated significantly in liver and skeletal muscle (by 1.50 ± 0.13 and 1.25 ± 0.05-fold, respectively; mean ± SEM), as compared with control values from 5 °C acclimated frogs. However, levels were unchanged in brain, heart and kidney, during freezing but decreased significantly to 0.83 ± 0.04 in ventral skin. After 4 h of thawing recovery at 5 °C, *hif-1*α transcript levels had decreased significantly in five organs, returning to control levels in liver and muscle and falling below controls values in brain, heart and skin to just to 57 ± 3.6%, 81 ± 2% and 67 ± 2% % of controls, respectively (Fig. 1A). *Hif-1*α transcript levels were unaffected by freeze-thaw in kidney.

To further investigate tissue-specific responses to freeze/thaw, *hif-1*α mRNA levels were assessed over a time course of freezing and thawing in wood frog heart, brain and liver (Fig. 2). In brain, a highly oxygen-sensitive organ, mean relative *hif-1*α mRNA levels showed a sharp peak of 2.78 ± 0.14-fold higher than controls when assessed after 2 h freezing exposure at −2.5 °C but levels had fallen again by 6 h (0.72 ± 0.11) and returned to near control levels (0.95 ± 0.06) in 24 h frozen frogs. However, thawing triggered a second peak of *hif-1*α expression in brain with transcript levels rising to 1.75 ± 0.13-fold over controls after 2 h thawing followed by a return to near control values after 4 h thawed (0.91 ± 0.1). Liver showed a similar pattern with peaks in *hif-1*α transcripts after both 2 h freezing and 2 h thawing. Liver *hif-1*α transcript levels were significantly elevated by 1.31 ± 0.12-fold within 30 min of ice nucleation, peaked after 2 h at 2.3 ± 0.1-fold over controls and then declined to 1.53 ± 0.12-fold higher than controls after 24 h frozen. Liver transcript levels rose again to 2.0 ± 0.14-fold over controls after 2 h of thawing at 2.5 °C and then declined to 1.54 ± 0.02-fold over controls after 4 h thawed. Heart showed no early changes in *hif-1*α transcripts but levels fell to 0.41 ± 0.07 after 6 h frozen, as compared with controls. During thawing transcript levels in heart rose to a peak of 1.46 ± 0.09-fold over controls after 2 h before returning to control values after 4 h thawed (1.09 ± 0.04).

3.3. Hif-1α protein expression during freeze/thaw

Fig. 1B shows the comparable effects of 24 h freezing and 4 h thaw on HIF-1α protein levels in six wood frog organs. After 24 h freezing, HIF-1α levels were elevated significantly in liver (1.4 ± 0.05-fold), and skeletal

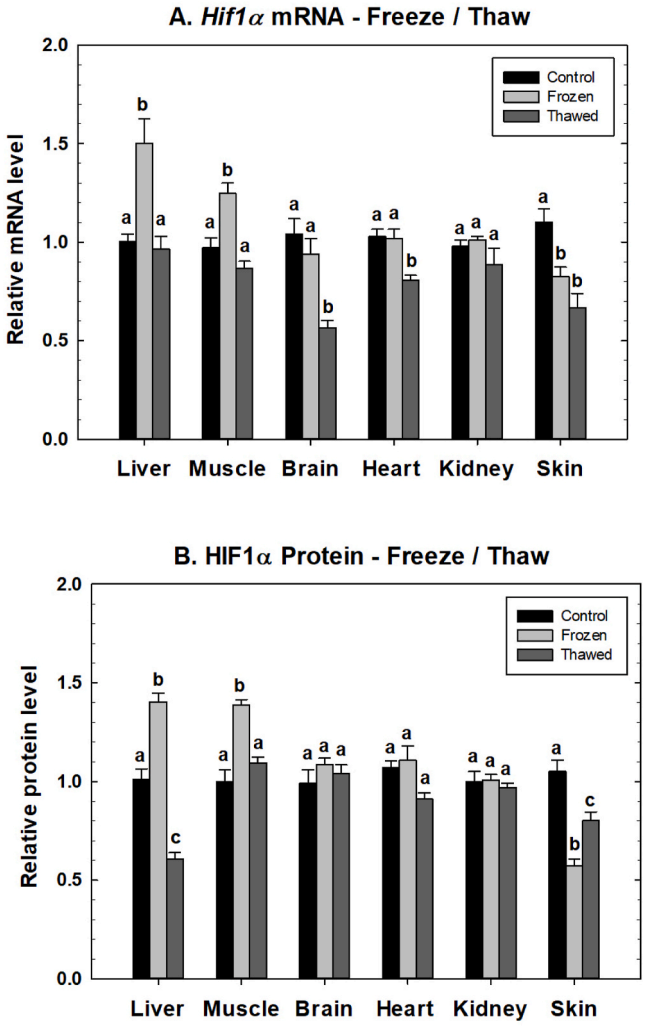

Fig. 1. Effect of 24 h freezing at -2.5°C and 4 h thawed recovery at 5°C, as compared with 5°C controls, on the relative levels of (A) *hif-1α* mRNA transcripts and (B) HIF-1α protein from six tissues of wood frogs. Histograms show mean ± SEM, n = 4 independent determinations on tissue extracts. Due to very small masses of brain, heart and kidney from individual frogs (~20–30 mg), tissue samples from two or more frogs were pooled to make each extract. Statistical analysis used analysis of variance (ANOVA) followed by the Tukey post-hoc test with $P < 0.05$ accepted as a significant difference. Within each triplet of histogram bars, values with different letter notations (a, b) are significantly different from one another. Muscle samples were from hind leg thigh and skin was sampled from the ventral abdomen.

muscle (1.38 ± 0.02-fold), paralleling the increases in *hif-1α* transcripts in these tissues. By contrast HIF-1α protein content fell in skin of frozen frogs (to 0.57 ± 0.03 compared with controls) and was unchanged in brain, heart and kidney. After a 4 h thaw, HIF-1α protein had returned to control values in muscle but liver levels were low at just 0.61 ± 0.03 of control values. Skin showed an increase in HIF-1α protein after 4 h thawing rising to 0.80 ± 0.10 as compared with controls. HIF-1α levels were again unchanged in brain, heart and kidney, as compared with controls.

3.4. Hif-1α mRNA and HIF-1α protein responses to anoxia and dehydration stresses

Two of the component stresses imposed upon tissues during freezing are (a) anoxia/ischemia because freezing halts breathing and blood circulation and, thereby, cuts off inter-organ delivery of oxygen and nutrients, and (b) dehydration due to water outflow from cells to join

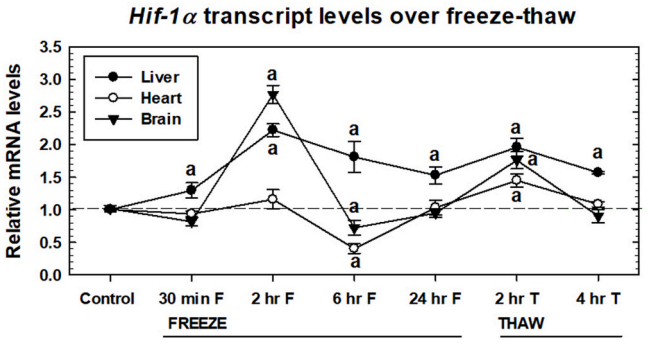

Fig. 2. Relative mRNA transcript levels of *hif-1α* in liver, heart and brain over a time course of freezing at -2.5°C and thawing at 5°C. Data for each tissue were standardized and plotted in comparison with 5 °C controls. Statistical testing used ANOVA followed by a Tukey post-hoc test. Data are mean ± SEM, n = 4–5 independent samples; (a) indicates a significant difference as compared with the control value for the same tissue, $P < 0.05$.

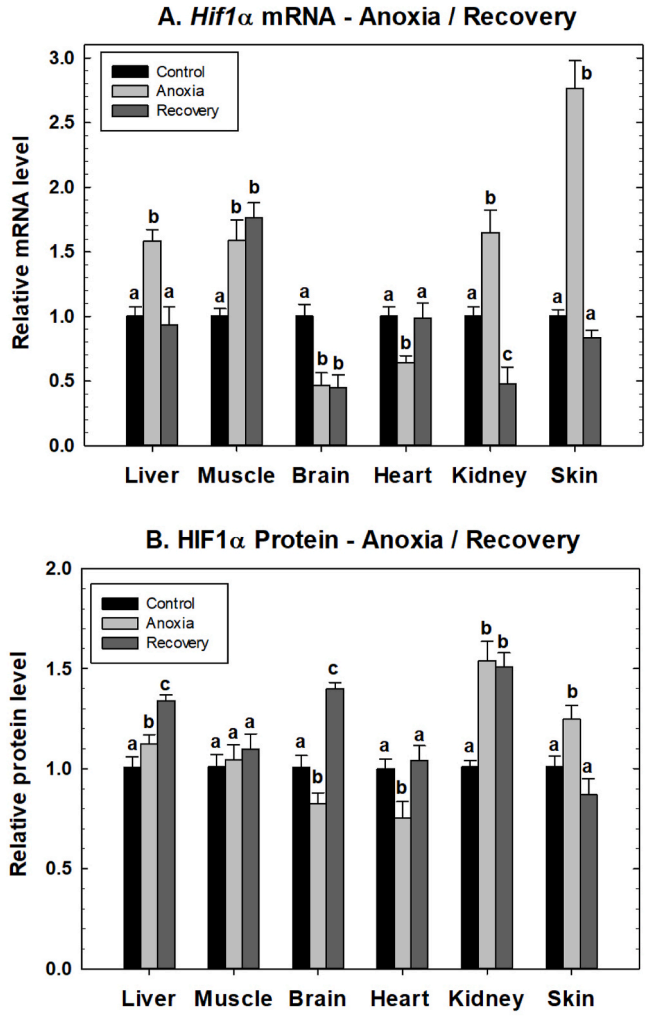

Fig. 3. Effect of 24 h anoxia exposure at 5°C and 4 h aerobic recovery at 5°C on the relative levels of *hif1* transcripts and HIF-1 protein in wood frog tissues. Experimental data for each tissue were standardized and plotted in comparison with 5 °C controls and provide the relative levels of (A) *hif-1α* mRNA transcripts, and (B) HIF-1α protein from six tissues of wood frogs. Other information as in Fig. 1.

extracellular ice masses. Both of these stresses can have crucial and independent consequences for cells and tissues of frozen frogs. Fig. 3 shows the effects of 24 h anoxia exposure at 5 °C on *hif-1*α mRNA (A) and HIF-1α protein (B) in the six organs. Transcript levels of *hif-1*α were significantly up-regulated after 24 h anoxia (under a nitrogen gas atmosphere at 5 °C) in liver, muscle, kidney and skin by 1.6 ± 0.07, 1.6 ± 0.16, 1.65 ± 0.16 and 2.7 ± 0.21-fold, respectively (Fig. 3A). By contrast, *hif-1*α transcripts decreased significantly by about one-half in brain and heart under anoxia. After 24 h recovery from anoxia, *hif-1*α transcripts had returned to near control levels in liver, heart and skin but remained elevated in skeletal muscle and were suppressed in brain and kidney.

Anoxia exposure for 24 h stimulated small but significant increases in HIF-1α protein levels in liver, kidney and skin (by 1.12 ± 0.05, 1.54 ± 0.10, and 1.25 ± 0.07-fold, respectively) whereas HIF-1α levels fell significantly in brain and heart (Fig. 3B). During recovery from anoxia HIF-1α protein remained high in liver and kidney but fell back to control levels in skin. HIF-1α levels in heart rebounded to control values and brain HIF-1α levels rose significantly to 1.4 ± 0.08-fold higher than controls.

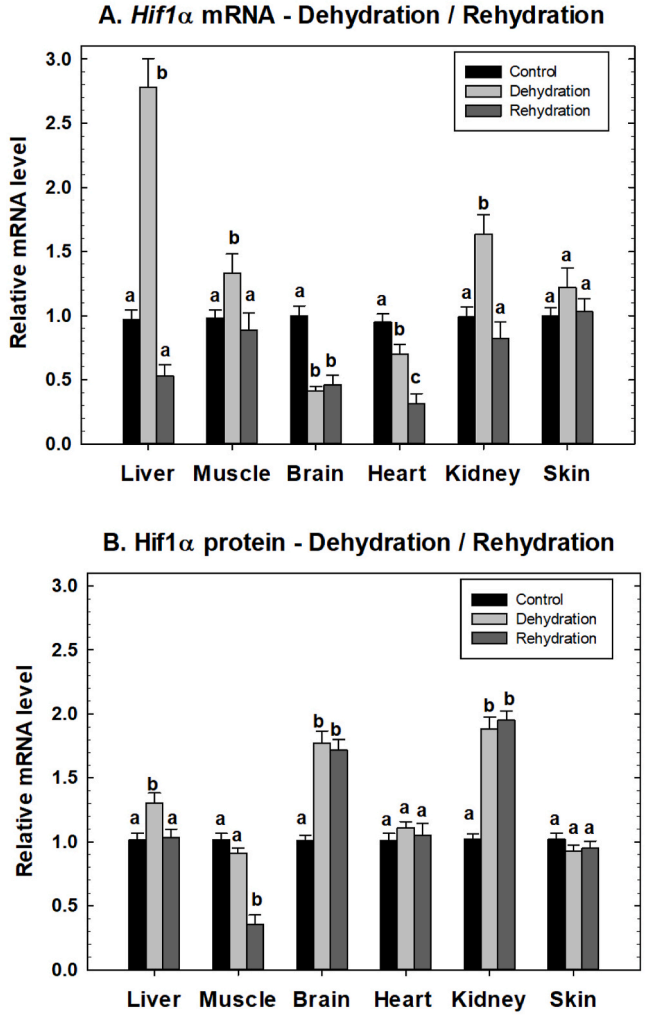

Fig. 4. Effect of dehydration and subsequent rehydration at 5°C on the relative levels of *hif1* transcripts and HIF-1 protein in wood frog tissues. Experimental data for each tissue were standardized and plotted in comparison with 5 °C controls. (A) *hif-1*α mRNA transcript levels in frogs that underwent the loss of 20% of total body water, and (B) HIF-1α protein levels in frogs that underwent the loss of 40% of total body water and subsequent rehydration. Data for six tissues are presented, standardized in comparison with 5 °C controls. Other information as in Fig. 1.

Dehydration to 20% of total body water lost also stimulated a significant increase in *hif-1*α transcripts in liver, muscle, and kidney by 2.8 ± 0.18, 1.34 ± 0.15 and 1.6 ± 0.16 over control levels, respectively (Fig. 4A). By contrast, transcript levels fell significantly in brain (to 0.41 ± 0.04) and heart (to 0.70 ± 0.08) and were unchanged in skin. After full rehydration, *hif-1*α transcript levels returned to near control values in muscle, kidney, and skin whereas liver values remained low (0.53 ± 0.09% of controls). Transcript levels remained suppressed in brain but fell further to 31 ± 0.08% of control values in heart.

Dehydration effects on HIF-1α protein levels were evaluated in frogs that had lost 40% of total body water. HIF-1α protein content in liver, brain and kidney increased significantly by 1.3 ± 0.07, 1.8 ± 0.08 and 1.9 ± 0.2 fold, respectively, after 40% dehydration but body water loss did not affect protein content in other tissues (Fig. 4B). After full rehydration, HIF-1α protein had returned to control levels in liver whereas levels remained high in brain and kidney but fell in skeletal muscle to just 0.39 ± 0.08 of the control value.

4. Discussion

Relatively little has been reported about HIF-1 in anurans despite the fact that this transcription factor is known to mediate multiple responses to low oxygen. A study of *Xenopus laevis* showed that gene and protein sequences and the organization of protein domains are highly conserved as compared to mammalian HIF-1 [3,29]. Other work documented HIF-1 involvement in embryonic development of tadpoles, particularly during the transition from water to aerial environments (a gill to lung transition) [20,29]. Elevated levels of HIF-1 have also been associated with tail regeneration in both lizards and tadpoles, presumably because the energy demands for regeneration are best met by aerobic ATP production [21,22]. Amphibians actually have three modes of respiration: via gills as tadpoles, lungs as adults, and skin at all times (although mainly for species that are aquatic or semi-aquatic) [37]. HIF-1 action can be involved in all of these.

Whole body freezing places multiple stresses on the cells and tissues of animals; these include (a) hypoxia/anoxia due the interruption of breathing, heartbeat and blood flow by extracellular ice formation, (b) the cessation of inter-organ transport of all molecules and cells normally circulated via the bloodstream, (c) cell/tissue dehydration and cell volume reduction due to water loss into extracellular and extra-organ ice masses, (d) a halt to all muscle movement, and (e) the potential for physical damage by ice to delicate tissues [34]. For most animals, whole body freezing is lethal but various species living at high latitudes or altitudes have mastered the art of freeze tolerance. Successful freezing survival requires a multi-faceted metabolic response that must also be fast-acting; for example, when exposed to −2.5 °C, maximum ice content by 5–7 g wood frogs (typical sizes in southeastern Canada) is reached in only a few hours and disruption of physiological functions occurs even sooner. Indeed, a respirometry analysis of freeze-thaw in wood frogs reported that breathing ceased at a mean of just 70 min (range 24–150 min) after detection of the freezing exotherm [28] and this would soon lead to tissue hypoxia and then anoxia. Not surprisingly, therefore, analysis of *hif-1*α transcript levels in tissues of freezing wood frogs showed a sharp increase at 2 h post-nucleation in both brain and liver (Fig. 2) suggesting that this was a crucial time for tissues to be making hypoxia-responsive adjustments to their metabolism. Given that liver is the main organ producing and exporting glucose and that brain of all vertebrates is highly dependent on glucose as its primary substrate (much of it imported from liver), it is not surprising that *hif-1*α transcript levels are quickly elevated in these two tissues when freezing begins. By contrast, a more muted response by heart over the course of freeze/thaw may reflect a substantial supply of endogenous glycogen and/or an ability to continue to take up glucose from the blood for an extended time since heart and liver are the last organs to freeze [25]. Indeed, for liver, previous studies have shown that glycogenolysis in support of cryoprotectant glucose production and anaerobic metabolism is well

underway within a 2 h time frame and so is glucose export to other tissues [32,33]. A range of proteins are also quickly upregulated to aid tissue survival in the frozen state including production of chaperones as well as several novel freeze-responsive proteins made by liver such as FR10 that appears to have a function in inhibiting or managing ice recrystallization [17,36]. Production of proteins that aid freeze tolerance (and distribution in the case of secreted proteins) would benefit from fast metabolic responses while aerobic metabolism is still possible.

Falling tissue oxygen levels are well-known to trigger the action of HIF-1 in vertebrate organs and HIF-1 upregulates at least 70 known target genes (many of them organ-specific) with a core of ~17 targets that are consistently upregulated in all tissues in response to hypoxia [9]. These latter include proteins/enzymes involved in metabolic reprogramming in the face of low oxygen including glucose transporter (GLUT) proteins to enhance uptake of fermentable fuel (glucose) into tissues, most glycolytic enzymes, and several inhibitors of mitochondrial function. It is not surprising, then, that the oxygen restriction imposed by freezing also triggers HIF-1 action in frog tissues and this can aid the transition to anaerobic metabolism as well as promote freeze-specific uptake (via GLUT-1 and GLUT-3) of copious amounts of glucose into peripheral tissues, this sugar being produced and exported from liver (via GLUT-2) as the cryoprotectant [32–35].

The present study shows a rapid elevation of *hif-1*α transcripts in liver and brain within the first 2 h of freezing exposure (2.3- and 2.8-fold, respectively) (Fig. 2) and sustained elevated *hif-1*α transcripts and HIF-1α protein in liver after 24 h frozen as well as in skeletal muscle (Fig. 1). Other organs showed more modest responses to freezing and thawing by *hif-1*α transcripts and HIF-1α protein. Based on what is known about *hif-1*α in other systems, it is likely that sustained elevation of *hif-1*α is due to stabilization of constitutively expressed *hif-1*α transcripts in frog tissues. This is the response to hypoxia in most species [4,6,27], rather than a sole dependence on up-regulation of hif-1α gene transcription. The strong peak of hif-1α transcripts in brain after 2 h freezing is highly suggestive that this is the time when brain is experiencing serious hypoxia and rapidly implementing HIF-1 mediated protective measures, consistent with a progressive isolation of brain as freezing shuts down blood circulation. A more sustained HIF-1 elevation in liver would support the longer continued metabolic function of liver to make and export both glucose cryoprotectant and freeze-responsive proteins for as long as possible. Indeed, ^{1}H magnetic resonance imaging of frogs over a freeze/thaw cycle showed that wood frog liver was the last internal organ to freeze as well as the first to thaw (probably due to the colligative action of extremely high glucose content) [25]. A second smaller peak of hif-1α transcripts occurred early (2 h) during thawing of wood frogs (Fig. 2) and may be associated with hypoxia signals arising as tissues are rehydrating and reactivating metabolic functions but are still without oxygen delivery from circulating blood. Indeed, assessment of the return of physiological functions in wood frogs during thawing at 6–8 °C showed a slow heartbeat after ~1 h (reaching a constant value after 2 h) but first breathing was not recorded until 3 h into the thaw [16].

In previous studies, our lab has found that various of the biochemical responses to freezing can also be triggered or mimicked by exposures of frogs to individual stresses, either anoxia or dehydration [e.g. 1,11,30,34,36. This suggests that the HIF-1 responses to freezing probably arose from pre-existing anuran responses to these stresses. In the present study we found, not surprisingly, that hif-1α transcript levels increased strongly in response to exposure to anoxia (nitrogen gas atmosphere) in four tissues (liver, muscle, kidney, and skin) (Fig. 3A) and the hif-1α response to anoxia by liver and skeletal muscle showed strong parallels with the responses to freezing (Fig. 1A). However, the protein response to anoxia exposure was more muted in liver and muscle when assessed after 24 h, as compared with freezing, possibly related to the continuation of circulation among anoxia-exposed frogs at 5 °C.

The strong 2.8-fold increase in hif-1α transcripts and 1.3-fold increase in HIF-1α protein in skin in response to anoxia, but not during dehydration or freezing, is very interesting. We propose that this may be related to skin as a respiratory surface in frogs, in addition to lungs. Many frog species can shift between these two options when in aerial (lung) versus aquatic (skin) environments but in aquatic situations, the oxygen content of water can vary widely, both diurnally and seasonally. Indeed, it has been reported that frogs in oxygen-poor water can even lose oxygen from capillaries close to the skin surface [19]. As a result, a phenomenon called hypoxic vasoconstriction (HVC) has been described for frog skin and limits cutaneous blood flow at the skin surface when Po_2 is low [19]. This innate response might also be triggered in wood frog skin when exposed to a nitrogen gas atmosphere, leading to the strong hif-1α and HIF-1α responses by skin under anoxic conditions (Fig. 3A and B) that did not occur under either freezing or dehydration stresses. Related to this, the hif-1α mRNA and HIF-1α protein responses by kidney under anoxia may be linked with the role of vertebrate kidney in sensing blood oxygen levels and triggering HIF-mediated production of the hormone, erythropoietin (EPO), that stimulates red blood cell production to increase oxygen-carrying capacity [10].

A similar rise in hif-1α mRNA and HIF-1α protein occurred in kidney of frogs experiencing dehydration (Fig. 4), a stress that leads to reduced blood volume, increased blood viscosity and a slowing of circulation, all contributing to an increase in tissue hypoxia. All amphibians are highly susceptible to dehydration due to the high permeability of their skin, but they also have a much higher tolerance for variations in body water content than do most other vertebrates. Hence, counter measures that deal with dehydration-induced stress are integral to amphibian survival. This may account for HIF-1α upregulation at both gene and protein levels in kidney of frogs under anoxia or dehydration stresses although targets of HIF-1 action may be different under the two stresses. Indeed, our previous work with wood frogs showed that dehydration triggered hyperglycemia in this species and elevated tissue and blood glucose to levels that were almost as high as during freezing [7]. This hyperglycemic response has the same function under both freezing and dehydration stresses: ie. to act as a colligative cryo/osmo-protectant to limit cell volume reduction. The elevation of hif-1α transcripts in liver under both dehydration and freezing (2.8- and 1.5-fold, respectively) and corresponding elevation of HIF-1α protein, shows a strong correlation between HIF-1 and glucose production that indicates a regulatory relationship.

In conclusion, the current study provides strong evidence of an important role for the HIF-1 transcription factor in mediating organ-specific responses to environmental stresses by wood frogs: freezing, hypoxia/anoxia, and dehydration. Multiple adaptations that support freeze tolerance may be derived from pre-existing HIF-1 mediated gene/protein responses to anoxia or dehydration that more generally aid amphibian species in enduring diverse environmental stress conditions.

Declaration of competing interest

The authors have no competing interests to declare.

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