Stress-induced antioxidant defense and protein chaperone response in the freeze-tolerant wood frog *Rana sylvatica*

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Abstract

Freeze tolerance is an adaptive response utilized by the wood frog *Rana sylvatica* to endure the sub-zero temperatures of winter. Survival of whole body freezing requires wood frogs to trigger cryoprotective mechanisms to deal with potential injuries associated with conversion of 65–70% of total body water into ice, including multiple consequences of ice formation such as cessation of blood flow and cell dehydration caused by water loss into ice masses. To understand how wood frogs defend against these stressors, we measured the expression of proteins known to be involved in the antioxidant defense and protein chaperone stress responses in brain and heart of wood frogs comparing freezing, anoxia, and dehydration stress. Our results showed that most stress proteins were regulated in a tissue- and stress-specific manner. Notably, protein levels of the cytosolic superoxide dismutase (SOD1) were upregulated by 1.37 ± 0.11 -fold in frozen brain, whereas the mitochondrial SOD2 isoform rose by 1.38 ± 0.37 -fold in the heart during freezing. Catalase protein levels were upregulated by 3.01 ± 0.47 -fold in the brain under anoxia stress, but remained unchanged in the heart. Similar context-specific regulatory patterns were also observed for the heat shock protein (Hsp) molecular chaperones. Hsp27 protein was down-regulated in the brain across the three stress conditions, whereas the mitochondrial Hsp60 was upregulated in anoxic brain by 1.73 ± 0.38 -fold and by 2.13 ± 0.57 -fold in the frozen heart. Overall, our study provides a snapshot of the regulatory expression of stress proteins in wood frogs under harsh environment conditions and shows that they are controlled in a tissue- and stress-specific manner.

Keywords Oxidative stress · Redox · Anoxia · Metabolic depression · Dehydration · Cryoprotection

Introduction

Environmental surroundings are major factors that influence the physiology and lifespan of an organism. When confronted with unfavorable habitat changes (e.g., temperature fluctuation, drought), animals in the wild must make physiological adjustments in order to adapt and survive (Wu and Storey 2016). One of the most extreme examples of environmental

stress adaptation is used by the North American wood frog *Rana sylvatica* (recently reclassified as *Lithobates sylvaticus*) that undergoes whole body freezing in their terrestrial wintering sites (Schmid 1982). The ability of wood frogs to tolerate freezing is thought to be a versatile survival strategy that allow these amphibians to rapidly respond to fluctuations in ambient temperature, and presents a unique adaptive mechanism to endure the harsh conditions of winter (Storey and Storey 2017). Once frozen, this continuous state of frozen dormancy can be maintained for days or months at a time with approximately 65–70% of their total body water frozen in extracellular compartments (Storey and Storey 1988; Storey and Storey 1992). In the wild, wood frogs have been predicted to undergo up to 23 freeze-thaw cycles in a single winter based on microclimate recordings and have been recorded to be naturally frozen for up to 193 consecutive days (Sinclair et al. 2013; Larson et al. 2014).

While frozen, sweeping physiological changes are observed in the wood frog, including complete arrest of the animal's heartbeat, blood circulation, breathing, detectable brain

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activity, and muscle movement (Storey and Storey 2017). At the cellular level, wood frogs initiate many stress protective mechanisms in order to defend against the harmful effects of freezing. Potential damage can include the following: (1) structural damage to cell caused by ice (Storey and Storey 2017), (2) ischemia and reperfusion-related damage caused by the oscillation of cardiac and respiratory functions between freeze-thaw cycles (Joanisse and Storey 1996), and (3) onset of dehydration/osmotic stress triggered by the formation of extracellular ice drawn from water in the cells (Costanzo et al. 2013; Storey and Storey 2017). To date, many biochemical adaptations utilized by wood frogs to protect against the detrimental effects of freezing have been documented; these include metabolic rate depression to reduce ATP consumption and conserve energy (Sinclair et al. 2013; Storey and Storey 2017), accumulation of low-molecular weight organic compounds as cryoprotectants (Costanzo et al. 1993), and activation of stress protective pathways to maintain cellular stability (Hermes-Lima and Zenteno-Savín 2002; Rider et al. 2006; Rosendale et al. 2012) (for a comprehensive review, please see (Storey and Storey 2017)).

Due to periods of ischemia/reperfusion-related stress endured by wood frogs over freeze-thaw cycles, it is proposed that intrinsic mechanisms that defend against oxidative stress would be enhanced during freezing to increase the animal's resistance toward deleterious effects of reactive oxygen species (ROS). Evidence of oxidative stress has previously been reported in wood frogs during freezing, with the ratio of glutathione disulfide (GSSG) to glutathione (GSH), an indicator of oxidative stress, increased by 33% in the brain of wood frogs during freezing (Joanisse and Storey 1996). In metazoans, defense against ROS and oxidative stress involves proteins that include the superoxide dismutase (SOD) and catalase enzymes which act to detoxify ROS into water and oxygen molecules (Halliwell 1996). SOD is an important enzyme that catalyzes the conversion of highly reactive superoxide radicals into hydrogen peroxide or oxygen molecules; the dimeric cytosolic SOD1 requires metalation with copper/zinc in the enzymatic reactive center whereas the tetrameric mitochondrial SOD2 uses manganese (Fig. 1a) (Fukui and Zhu 2010). Working in conjunction with SOD, the catalase enzyme further processes the hydrogen peroxide produced into water and oxygen molecules. Additional small redox proteins such as thioredoxin (Trx) and peroxiredoxin (Prx) can also function as antioxidants that directly facilitate the reduction of proteins and neutralize ROS (Collet and Messens 2010; Perkins et al. 2015). Whereas antioxidant proteins are the first line of defense against oxidative stress, another class of stress-inducible proteins called heat shock proteins (Hsp) can also contribute to protecting cells from ROS (Kalmar and Greensmith 2009). Heat shock proteins are molecular chaperones that stabilize the

proteome by maintaining proper protein folding during cellular stress, while also guiding the refolding of misfolded proteins or directing proteins to degradation to prevent accumulation of protein aggregates (Feder and Hofmann 1999). The oxidative stress response and the heat shock protein chaperone pathways are both fundamental stress mechanisms that are evolutionarily conserved from yeast to humans (Liu et al. 1997; Herrero et al. 2008), with homologies of these proteins indicating very high levels of conservation across vertebrate animals.

In this study, we set out to understand how the oxidative stress response and heat shock protein chaperone pathways are regulated in the brain and heart of wood frogs during periods of freezing. As mentioned earlier, freezing can also introduce other physiological constraints that include ischemia and osmotic stress, and thus, we also tested the singular effects of anoxia or dehydration stresses on the frogs to determine if we could delineate the cellular responses facilitated during freezing to individual stressors. Here, we provide new biochemical evidence that outlines the expression levels of several key proteins involved in the oxidative stress and heat shock protein responses in the wood frog under various environmental stresses.

Materials and methods

Animal preparations

Male wood frogs were captured in the Ottawa area (Oxford Mills, Ontario, Canada) during breeding season (late March to mid-April) and transported to Carleton University. Wood frogs captured had a mean body mass of 6.42 ± 0.89 g and a mean snout-vent length of 42.70 ± 2.37 mm. Frogs were washed in tetracycline bath and housed in containers with damp sphagnum moss at 5 °C for 1–2 weeks before use; control animals were sampled from this (Dawson and Storey 2016). Experimental protocols used to induce the three environmental stresses were previously described in detail (Dawson and Storey 2016; Hoyeck et al. 2017) and briefly outlined here. For freezing experiments, wood frogs were transferred to a closed box lined the damp paper towel and placed in an incubator set at -3 °C. After periods of 4 or 24 h of freezing, wood frogs were quickly euthanized and tissues were rapidly dissected and frozen in liquid nitrogen. For anoxia experiments, wood frogs were transferred into closed plastic jars (held in crushed ice) fitted with syringe port that was connected to a nitrogen gas line. Nitrogen gas was introduced into the closed jar for 30 min; then, ports were closed and the lid was sealed with Parafilm. Frogs were returned to 5 °C. After anoxia exposure of 4 or 24 h, nitrogen lines were reconnected to the jars and the wood frogs were quickly euthanized and tissues

Fig. 1 Protein expression of superoxide dismutase enzymes in wood frogs under environmental stress. **a** Superoxide dismutase catalyzes the enzymatic conversion of superoxide free radicals into hydrogen peroxide and oxygen molecules. **b** Relative protein levels of SOD1 in wood frog brain and heart under freezing, anoxia, or dehydration stress. **c** Amino acid alignment of cytoplasmic Cu/Zn SOD1 sequence from zebrafish, chicken, human, clawed frog, and mouse.

Color bar above sequence indicates amino acid similarities between species. **d** Relative protein levels of the mitochondrial Mn SOD2 in wood frog brain and heart under freezing, anoxia, or dehydration stress. **e** Amino acid alignment of the SOD2 sequences from zebrafish, chicken, human, clawed frog, and mouse. * $P < 0.05$, ** $P < 0.01$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

collected and frozen. In dehydration experiments, wood frogs were weighted and placed in the dry buckets (5–6 animals per bucket) and held in 5 °C incubator. Frogs were weighed at 8–12 h intervals and euthanized for tissue collection when either 20 or 40% of total body water loss was attained. The percentage water loss was calculated with the following equation: percentage water loss = $[(M_i - M_d) / (M_i \times BMC_i)] \times 100$, where M_i is the initial body mass of the frog, M_d is the dehydrated frog body mass, and BMC_i is the initial body water content of the frogs before dehydration in grams H₂O per gram body mass (mean $BWC_i = 80.8 \pm 1.2\%$). All wood frogs were sacrificed by pithing followed by rapid tissue dissection and flash freezing in liquid nitrogen. Four animals were used for each of the control and experimental conditions. Tissues were stored in a -80 °C freezer until use. All animal handling and experimental protocols performed were approved by the Carleton University Animal Care Committee (approval protocol #13683), in accordance with the Canadian Council on Animal Care guidelines. Frogs were collected under the permit #1085726 issued by Ontario Ministry of Natural Resources.

Protein extraction and Luminex assay

Approximately 50 mg of wood frog brain or heart tissues were manually homogenized with a Dounce homogenizer in protein lysis buffer supplemented with 2 mmol/L phenylmethylsulfonyl fluoride. Soluble protein lysates were extracted after centrifuging the homogenates at 4000g for 10 min and normalized to a concentration of 10 mg/mL with lysis buffer after protein quantification by the Bradford assay. To assess protein expression, two Luminex multiplex assay kits were used: Oxidative Stress Magnetic Bead Panel (#H0XSTMAG-18K) and 5-Plex Heat Shock Protein Panel Magnetic Bead kit (#48-615MAG). The Oxidative Stress panel contained antibodies for the following proteins: catalase, peroxiredoxin 2 (Prx2), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and thioredoxin 1 (Trx1). The 5-plex Heat Shock Protein panel contained antibodies for the following proteins: Hsp27, phosphorylated Hsp27 (pS78/pS82), Hsp60, Hsp70, and Hsp90- α . Both Luminex assays were carried out according to the manufacturer protocols and measured with a Luminex 200 machine; detailed assay protocols and machine settings were described previously (Biggar et al. 2015). All proteins measured in this study show high degree of conservation across multiple vertebrate species compared to the human sequences, indicating that the antibodies were capable of detecting the same proteins in the frogs (see “Results” section for protein sequence comparisons). Expression levels of the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured for each lysate with a GAPDH Single Plex assay kit (#46-667MAG) in the Luminex 200 machine and used for standardization of the protein data.

Protein sequence alignment

Amino acid sequences for each proteins measured in this study were retrieved for humans (*Homo sapiens*), mouse (*Mus musculus*), zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), and chicken (*Gallus gallus*) from the NCBI protein database. Amino acid sequences were aligned using Geneious software to illustrate the level of protein conservation across species.

Data statistical analysis

Luminex expression data expressed as median fluorescent intensities (MFI) were standardized to the appropriate GAPDH MFI values for each sample. Standardized MFI values were then normalized to the value of the first control sample that was then arbitrarily set to one. All data presented are as mean $\pm$ SEM for tissues from $n = 4$ independent animals for each condition. Statistical analysis was performed using a one-way ANOVA with a Tukey's post hoc test using the SigmaPlot 12.0 statistical software; * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$, compared to the control.

Results

Antioxidant proteins are differentially expressed under various environmental stressors

To understand how the oxidative stress response is regulated in the wood frog brain and heart during periods of environmental stress, we measured the protein expression of three major antioxidant enzymes SOD1, SOD2, and catalase in response to freezing, anoxia, and dehydration stresses. The amino acid sequences of SOD1 and 2 are both highly similar across vertebrate species (as exemplified by the monomer sequences shown in Fig. 1c, e), suggesting high evolutionary conservation of their protein function. Protein expression of SOD1 was significantly elevated by 1.37 ± 0.11 -fold in wood frog brain after 24 h of freezing exposure, as compared with control values in 5 °C acclimated frogs, but significantly reduced in response to 24 h anoxia to 0.73 ± 0.09 -fold or 20% dehydration to 0.67 ± 0.18 -fold (Fig. 1b). In the heart, SOD1 protein expression decreased significantly in response to freezing or dehydration conditions with reductions observed after 24 h freezing to 0.75 ± 0.09 -fold, after 20% dehydration to 0.78 ± 0.05 -fold, and after 40% dehydration to 0.74 ± 0.07 -fold, as compared with controls (Fig. 1b). Protein levels of SOD2 were significantly reduced to 0.66 ± 0.08 -fold in wood frog's brain in response to 20% dehydration, whereas short-term freezing (4 h) upregulated SOD2 in the heart to 1.38 ± 0.37 -fold over control values (Fig. 1d).

Next to the SOD enzymatic reaction, the hydrogen peroxide produced can be further processed into water and oxygen molecules by the enzyme catalase (Fig. 2a). Protein levels of catalase were highly upregulated by 3.01 ± 0.47 -fold in wood frog's brain after 24 h anoxia stress, but remained unchanged in the heart under all stress conditions (Fig. 2b). The catalase monomer shows high sequence conservation (81.7%) between human, zebrafish, chicken, clawed frog, and mouse (Fig. 2c).

Small redox protein expression varies in a tissue-specific manner during stress

Small redox proteins such as thioredoxin-1 and peroxiredoxin-2 can together participate in detoxification reactions; thioredoxins facilitates the cysteine thiol-disulfide exchange with proteins such as peroxiredoxins that then directly reduce

hydrogen peroxide into water molecules (Fig. 3a). Both Trx-1 and Prx-2 show high levels of protein conservation across species (Fig. 3c, e). Interestingly, protein levels of Trx1 were uniformly reduced across all stress conditions in the wood frog brain with expressions ranging from 0.19 ± 0.02 to 0.36 ± 0.12 -fold compared with controls, while remaining unchanged in the heart (Fig. 3b). Conversely, Prx-2 protein levels were elevated by 1.39 ± 0.17 -fold in the brain after 24 h of anoxia and down-regulated to 0.65 ± 0.01 -fold at 20% dehydration stress in the heart (Fig. 3d).

Regulation of heat shock proteins in wood frogs during environmental stress

Another facet of the cellular stress response is the induction of heat shock chaperone proteins that assist in maintaining

Fig. 2 Protein expression of catalase in wood frogs under environmental stress. **a** Catalase mediates the enzymatic conversion of hydrogen peroxide into water and oxygen molecules. **b** Relative protein levels of catalase in wood frog brain and heart under

freezing, anoxia, or dehydration stress. **c** Amino acid alignment of catalase sequences from zebrafish, chicken, human, clawed frog, and mouse. *** $P < 0.001$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

Fig. 3 Expression levels of antioxidant proteins thioredoxin-1 and peroxiredoxin-2 in the wood frogs under environmental stress. **a** Thioredoxin-1 proteins mediate the cysteine thiol-disulfide exchange with other proteins such as peroxiredoxin-2 which can then function to detoxify hydrogen peroxide into water molecules. **b** Relative protein levels of Trx-1 in the brain and heart of wood frogs under freezing, anoxia, or dehydration stress. **c** Amino acid alignment

of the Trx-1 monomeric protein from zebrafish, chicken, human, clawed frog, and mouse. **d** Relative protein levels of Prx-2 in the brain and heart of wood frogs under freezing, anoxia, or dehydration stress. **e** Amino acid alignment of the Prx-2 protein from zebrafish, chicken, human, clawed frog, and mouse. ** $P < 0.01$, *** $P < 0.001$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

proper protein folding to stabilize the cell proteome (Kalmar and Greensmith 2009). While induction of Hsps was first described in cells under heat stress, this protective response is also triggered by many other types of cellular stress including inflammation, infection, UV radiation, or oxidative stress (Feder and Hofmann 1999; Zügel and Kaufmann 1999). In the brain of wood frogs, the protein levels of Hsp27 were suppressed after 24 h of freezing to 0.48 ± 0.11 -fold, as compared with controls, as well as after short and long periods of anoxia (0.48 ± 0.10 -fold at 4 h and 0.56 ± 0.12 -fold at 24 h) and dehydration (0.32 ± 0.07 -fold at 20% dehydration and 0.46 ± 0.07 -fold at 40% dehydration) (Fig. 4a). By contrast, expression of Hsp27 was upregulated to 2.06 ± 0.63 -fold above

controls in the heart after 4 h of freezing exposure (Fig. 4a). Hsp27 can also be regulated via post-translational phosphorylation at the conserved Ser-78/Ser-82 residues (Fig. 4c), with phosphorylation at these amino acids previously associated with stressors such as heat shock (Landry et al. 1992). Levels of p-Hsp27 (S78/S82) were significantly reduced in response to 20% dehydration to 0.67 ± 0.11 -fold in the brain of the wood frog, whereas p-Hsp27 in the heart increased by 1.36 ± 0.31 -fold after 4 h of freezing followed by a reduction to 0.63 ± 0.08 -fold at 24 h (Fig. 4b). The mitochondrial Hsp60 protein was upregulated to 1.73 ± 0.38 -fold and 1.59 ± 0.45 -fold over control levels after 4 and 24 h of anoxia, respectively, in wood frog brain, as well as increasing by 2.13 ± 0.57 -

Fig. 4 Regulation of Hsp27 in the wood frogs during environmental stress. **a** Relative protein expression and **b** phosphorylation (S78/S82) levels of Hsp27 protein in wood frog brain and heart during freezing, anoxia, or dehydration stress. **c** Protein alignment of the Hsp27 amino

acid sequences from zebrafish, chicken, human, clawed frog, and mouse. Arrows indicate the phosphorylation residues (S78/S82) on the Hsp27 protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

fold after 4-h freezing in the heart (Fig. 5a). Meanwhile, Hsp70 protein levels were significantly reduced to 0.70 ± 0.09 -fold after 20% dehydration in the brain but remained unchanged in wood frog heart (Fig. 6a). Finally, Hsp90 protein levels did not change in the brain in response to any of the stresses but were reduced to 0.62 ± 0.07 -fold after 24-h freezing in wood frog heart and to 0.62 ± 0.01 -fold after 24 h anoxia and 0.64 ± 0.05 after 20% dehydration (Fig. 7a). The sequences of Hsp60, Hsp70, and Hsp90 proteins were all highly conserved across species, showing amino acid similarities of 90.2, 84.7, and 90.1%, respectively, from fish to human (Figs. 5b, 6b, and 7b).

Discussion

Freeze tolerance is utilized by the wood frog to survive when ambient temperatures drop below freezing in their winter habitat under the leaf litter on the forest floor (Storey and Storey 2017). In the frozen state, wood frogs experience multiple perturbations that include anoxia due to the interruption of oxygen delivery caused by the

freezing of blood and dehydration as water is drawn out of cells to join extracellular ice masses (Storey and Storey 2017). While normal physiological functions are paused in the frogs during freezing, they are quickly restored when the animals thaw as the ambient temperature rises in the spring. This process of thawing can also be quite stressful, perhaps especially due to the rapid rise in tissue oxygenation when respiration is restored. Such a rapid re-oxygenation and reperfusion of previously ischemic tissues can trigger a rapid and large increase in ROS generation leading to oxidative damage, not unlike that seen during reperfusion of ischemic tissues in human heart attack or stroke (Hermes-Lima and Zenteno-Savín 2002; Granger and Kviety 2015). As such, it is postulated that wood frogs need to activate cellular protective mechanisms that provide both cytoprotection while frozen (e.g., chaperone proteins) and defense against oxidative stress during thawing (Joanisse and Storey 1996).

In this study, we sought to determine if the antioxidant defense and protein chaperone responses were enhanced in wood frogs during freezing and to investigate whether one (or both) of the known component stresses of freezing

Fig. 5 Regulation of Hsp60 in the wood frogs during environmental stress. **a** Relative protein levels of Hsp60 in wood frog brain and heart under freezing, anoxia, or dehydration stress. **b** Protein alignment of the

Hsp60 amino acid sequences from zebrafish, chicken, human, clawed frog, and mouse. * $P < 0.05$, *** $P < 0.001$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

Fig. 6 Regulation of Hsp70 in the wood frogs during environmental stress. **a** Relative protein levels of Hsp70 in wood frog brain and heart under freezing, anoxia, or dehydration stress. **b** Protein alignment of the

Hsp70 amino acid sequences from zebrafish, chicken, human, clawed frog, and mouse. * $P < 0.05$, *** $P < 0.001$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

(e.g., anoxia, cell dehydration) was a trigger of the observed responses. In general, we found that there was no consensus regulatory pattern in the expression profile of these stress proteins in the wood frogs under freezing, anoxia, or dehydration stresses. Rather, our data indicated that changes in these stress responsive proteins were controlled in a tissue- and stress-specific manner. One possible explanation is that during freezing, wood frogs produce significant amount of glucose as cryoprotectants, which is not normally observed in animals exposed to anoxia or dehydration stress (Costanzo et al. 1993; Larson et al. 2014). High concentration of glucose can drastically alter the physiology of the cells and may be a potential contributing factor in different types of cellular stress responses observed under freezing, anoxia, and dehydration stress in the wood frogs.

Antioxidant defense is not globally upregulated in wood frogs under stress

A common indicator of oxidative stress is an increase in the ratio of oxidized (GSSG) to reduced (GSH) forms of the antioxidant metabolite glutathione. Previous work showed that the GSSG/GSH increased significantly in wood frog brain during freezing, providing evidence of increased oxidative stress (Joanisse and Storey 1996). However, measures of oxidative damage to wood frog brain did not change significantly during freezing or thawing, suggesting that protective mechanisms may be in place to combat against the damages to cellular macromolecules. In our study, the expression of the antioxidant protein SOD1 was elevated after prolonged periods of freezing. SOD1 is the cytosol isozyme, and the increase in SOD1 protein expression

Fig. 7 Regulation of Hsp90 in the wood frogs during environmental stress. **a** Relative protein levels of Hsp90 in wood frog brain and heart under freezing, anoxia, or dehydration stress. **b** Protein alignment of the

(Fig. 1b) suggests that it may function to protect against oxidative stress in the wood frog's brain during freezing. This upregulation of SOD1 may partially explain why there was no evidence of increased oxidative damage in wood frog brain despite an elevation of GSSG/GSH ratio during freezing (Joanisse and Storey 1996). The protective role of SOD1 during freezing has also been previously demonstrated in wood frog skeletal muscle, where it was shown that SOD1 maximal activity increased significantly by 1.52-fold in frozen frogs (Dawson et al. 2015). However, the SOD1 protein expression did not change in skeletal muscle, and this suggested that SOD1 activity may be subject to different regulatory mechanisms that enhance the protein's antioxidant action during freezing (Dawson et al. 2015). Interestingly, the protein expression of SOD2 that is

Hsp90 amino acid sequences from zebrafish, chicken, human, clawed frog, and mouse. * $P < 0.05$, *** $P < 0.001$ compared to control as determined by a one-way ANOVA with a Tukey post hoc test

typically found in mitochondria or peroxisomes was upregulated in the heart during freezing but not in the brain (Fig. 1d). This isoform-specific upregulation of the SOD protein in different tissues suggests that the frog heart may experience higher levels of mitochondrial ROS generation during freezing than the brain.

Next to SOD proteins, catalase functions as a hydrogen peroxide scavenger that detoxifies ROS into water and oxygen molecules. Protein expression of catalase was upregulated by ~3-fold in wood frog brain under prolonged periods of anoxia (Fig. 2b), but interestingly not during freezing. Whereas wood frogs when frozen also experience a degree of anoxia stress, it is possible that the relative difference in the ambient and body temperatures (-3°C in freezing vs. 5°C in anoxia) of the animals under these two stresses may contribute to this

difference in regulatory pattern. Catalase has previously been shown to be an important protein in protection against hypoxia-related ischemia injuries and may have a similar role in defending against chronic anoxia stress in the wood frog's brain (Undyala et al. 2011).

While tissue-specific upregulation of SOD and catalase proteins support an enhanced redox system to alleviate potential ROS damage during stress, we found that the small antioxidant protein Trx-1 was significantly down-regulated in wood frog brain under all three conditions tested (Fig. 3b). Interestingly, the protein levels of Prx-2, which can function together with Trx-1 to detoxify hydrogen peroxide, did not undergo a similar reduction (Fig. 3d). In general, overexpression of Trx-1 can reduce the levels of oxidative stress in cells (Koháryová and Kolárová 2008; Umekawa et al. 2008); thus, it is puzzling that the expression of Trx-1 is down-regulated in wood frog brain during freezing. One possible explanation is that Trx-1 has also been implicated in regulating the cell cycle (Mochizuki et al. 2009), with knockdown of Trx-1 shown to induce cell cycle arrest at the G₁ stage (Mochizuki et al. 2009). We have previously reported that the cell cycle is arrested in wood frogs during freezing as part of a regulatory mechanism to support metabolic rate depression (Zhang and Storey 2012) and that the down-regulation of Trx-1 may function in a similar capacity to support a state of cell cycle arrest in wood frog's brain under freezing, albeit at the expense of a compromised antioxidant system.

The lack of a global increase in antioxidant proteins in wood frog tissues during stress suggests that while oxidative stress is evident during periods of freezing, an effective protective mechanism may only require specific proteins to be upregulated in a tissue- and context-dependent manner. Similarly, antioxidant enzymes are not globally upregulated in ground squirrels during hibernation, another state of metabolic rate depression similar to freezing that encompass both temperature stress and ischemia/reperfusion-related insults (Page et al. 2009; Wu and Storey 2012; Wu and Storey 2014).

Chaperone protein stress response in wood frogs

Heat shock proteins are well known to play essential roles in maintaining proteome stability in response to various types of stress (Feder and Hofmann 1999; Kalmar and Greensmith 2009). While the canonical role of Hsps is to facilitate the folding or refolding of proteins into their native confirmation, chaperones such as Hsp27 also play protective roles against oxidative damage and apoptosis (Vidyasagar et al. 2012). Protein expression of Hsp27 was upregulated by approximately 2-fold in the heart of the wood frogs in response to freezing, suggesting a cardioprotective role of this protein during stress. However, it was surprising to us that Hsp27 protein levels were significantly reduced in wood frog brain under all

three environmental stress conditions (Fig. 4a). However, interestingly, the phosphorylation levels of Hsp27 (S78/S82) were mostly unchanged in the brain (Fig. 4b), suggesting that the relative ratio of phosphorylated to total Hsp27 protein may actually be higher under stress conditions in the brain. Hsp27 can be directly phosphorylated by the p38 mitogen-activated protein kinase, and phosphorylation of Hsp27 has been positively associated with antagonizing apoptosis to promote cell survival (Coulthard et al. 2009). We previously showed that the p38 kinase is strongly activated in wood frog brain during freezing (Greenway and Storey 2000), and this enhanced p38 kinase activity could then lead to increased phosphorylation of Hsp27 proteins in the brain under stress in order to trigger pro-survival effects. A comparable situation appears to occur in the wood frog heart during early hours of freezing where both Hsp27 protein and Hsp27 phosphorylation were significantly increased during the early hours of freezing. This correlates with previous data that showed that the p38 kinase is also strongly activated in wood frog heart during freezing (Greenway and Storey 2000). Interestingly, the observed changes in Hsp27 regulation are absent in wood frog heart under either anoxia or dehydration stresses, and the study by Greenway and Storey also reported that the p38 kinase remained unchanged in either anoxia or dehydrated frogs. Together, these results provide a regulatory connection between p38 kinase and Hsp27 in wood frog heart and suggest that this response may be unique to addressing freezing-induced stresses and are unrelated to problems associated with oxygen or osmotic perturbations (Greenway and Storey 2000).

Next to Hsp27, the expression pattern of Hsp60, 70, and 90 all displayed tissue- and stress-specific expression profiles, suggesting the absence of a uniformly activated Hsp stress response. Hsp60 for example was up-regulated in wood frog brain in response to anoxia and in the heart during freezing (Fig. 6a). Hsp60 is predominantly a mitochondrial chaperone (Pellegrino et al. 2013), and its upregulation in the heart during freezing is particularly interesting as this is also when mitochondrial SOD2 protein was upregulated (Fig. 1d). This enhanced antioxidant and chaperone protection in the mitochondria suggest that this subcellular compartment may be particularly susceptible to high levels of stress in the wood frog's heart during freezing. While Hsp70 remained mostly stable in the wood frogs under stress, Hsp90 was generally down-regulated (Fig. 7a). Hsp90 is a highly conserved protein chaperone (Fig. 7b). In addition to Hsp90's canonical role as a stress protein, it also functions as a regulatory protein of many non-stress-related cellular processes (Taipale et al. 2010). One such role of interest is the Hsp90's control over cardiac cell growth, where it has been previously shown that Hsp90 positively promotes the angiotensin II-induced cardiac cell hypertrophy (Lee et al.

2010). As such, the down-regulation of Hsp90 in the wood frog's heart could function as a potential mechanism to suppress unnecessary cell growth during periods of environmental stress, as a mean to conserve cellular energy and maintain a hypometabolic state to prolong survival.

Conclusions

In this study, we show that the antioxidant and protein chaperone stress response mechanisms are selectively activated in both a tissue- and stress-specific manner in the wood frog. Whereas the hypothesized overall increase in antioxidant defenses and protein chaperone stress resistance are often viewed as general mechanisms to promote environmental stress survival, our study here illustrates a more complex and context-dependent manner for how these stress pathways are activated. By studying expression level of proteins with known roles in specific cellular compartments, we have gained valuable insights into the potential importance of mitochondrial protection in the heart under freezing stress, as well as the possible non-canonical role of Hsp90 in controlling of cardiac cell growth in wood frogs under different environmental stress conditions. In summary, our current study provides new molecular insights into the regulatory profiles of the oxidative stress response and protein chaperone systems in wood frog heart and brain in response to changing environmental conditions and implicates the relative importance of different protective proteins in a context- and stress-dependent manner.

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Compliance with ethical standards

All animal handling and experimental protocols performed were approved by the Carleton University Animal Care Committee (approval protocol #13683), in accordance with the Canadian Council on Animal Care guidelines. Frogs were collected under the permit #1085726 issued by Ontario Ministry of Natural Resources.

Conflict of interest The authors declare that they have no conflict of interest.

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