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**GENE EXPRESSION SUPPORTING FREEZE TOLERANCE
IN THE WOOD FROG, *RANA SYLVATICA***

by

LI, ZHENHONG

**A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FUFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

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ABSTRACT

Natural freezing survival by the wood frog, *Rana sylvatica*, is supported by multiple metabolic and gene expression adaptations. The present study explored the responses by protein chaperones, both heat shock proteins (Hsps) and glucose-regulated proteins (Grps), as well as the hypoxia-inducible transcription factor (HIF-1 α), to freezing and two component stresses of freezing (anoxia, dehydration) in seven organs of wood frogs. Sequence analysis of Grp78, Grp94 and HIF-1 α found high identity between the frog proteins and other species but HIF-1 α showed unique amino acid substitutions that might aid low temperature function. RT-PCR and Western blotting were used to monitor mRNA transcript levels and protein levels, respectively. All three stresses stimulated up-regulation of HIF-1 α at transcriptional and translational levels in liver. Other organs showed stress-specific responses and time course studies showed maximum *hif-1 α* mRNA levels by 2 hours of freezing which suggests that hypoxia-induced proteins may contribute to cryoprotection. Levels of all seven shock proteins (Grp78, Grp94, Hsp110, Hsc70, Hsp60, Hsp40, Hsp10) rose during freezing in liver; six responded in skeletal muscle and kidney, and four in heart. The distribution of shock proteins and HIF-1 α between liver cytosolic and nuclear fractions also changed during freezing. Most shock proteins responded to anoxia stress in liver and to dehydration stress in skin but other organs showed mixed responses to these stresses. The mitochondrial chaperones Hsp60 and Hsp10 were consistently elevated during freezing and/or anoxia in most organs. Up-regulation of chaperones during freezing suggests that they play a cryoprotective role in stabilizing proteins against the multiple stresses on cells imposed by extracellular freezing.

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List of Abbreviations

2D-PAGE	Two-Dimensional Poly-Acrylamide Gel Electrophoresis
AFPs	antifreeze proteins
ANP	atrial natriuretic peptide
ARNT	aryl hydrocarbon receptor nuclear translocator
ATF	activating transcription factor
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BZIP	basic leucine zipper
CBP	CREB-binding protein
cDNA	complementary DNA
CHOP	CCAAT/enhancer-binding protein homologous protein
C-TAD	C-terminal transactivation domain
DEPC	diethylpyrocarbonate
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylenebis(oxyethylenitrilo)tetraacetic acid
eIF2 α	α subunit of eukaryotic initiation factor 2
EPO	erythropoietin
ER	endoplasmic reticulum
ERSE	ER stress-response element
EST	expressed sequence tag

EtBr	ethidium bromide
FIH1	factor-inhibiting HIF1
GLUT1	Glucose transporter 1
GRP78	78 kDa glucose-regulated protein
GRP94	94 kDa glucose-regulated protein
GTP	guanosine triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIF-1 α	Hypoxia-inducible factor 1 α
Hip	Hsp70-interacting protein
HSF	Heat shock factor
HSE	Heat shock element
HSP	Heat shock protein
INPs	ice nucleating proteins
IP ₃	1,4,5-trisphosphate
kb	kilobase
kDa	kilo Dalton
MAPK	mitogen-activated protein kinase
MOPS	2-[N-morpholine] proanesulfonic acid
MRI	magnetic resonance imaging
mRNA	messenger RNA
mtDNA	mitochondrial DNA
N-TAD	N-terminal transactivation domain
ODD	oxygen-dependent degradation domain

ORF	open reading frame
PAS	Per/Arnt/Sim
PCR	polymerase chain reaction
pfu	plaque forming unit
PHD	prolyl hydroxylase domain
pI	isoelectric point
PMSF	phenylmethanesulfonyl fluoride
RACE	rapid amplification of cDNA ends
Rbx1	Ring box protein 1
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
UPR	unfolded protein response
UPRE	UPR element
UTP	uridine triphosphate
XBP	X-box-binding protein

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CHAPTER 1

General Introduction

General introduction:

During the winter, wood frogs (*Rana sylvatica*) are challenged with multiple environmental stresses including food shortage, water limitation and subzero temperatures in their natural hibernation sites on the forest floor. This species is one of just a few cold-blooded vertebrate animals that can survive whole body freezing (Storey and Storey, 1992). Freezing can cause many forms of physical and cellular damage including physical damage due to ice expansion in tissue capillaries, a long interruption of heart beat, blood flow and breathing so that organs are ischemic, anoxic and energy-deprived, and cellular and organ dehydration caused by water outflow from cells into extra-organ ice masses (Storey and Storey, 1988; 2004). Data from magnetic resonance imaging have illustrated the process of ice permeation through wood frog organs (Rubinsky *et al.*, 1994). Ice formation begins at peripheral sites on frog skin and then spreads inward through the muscles, the abdominal cavity and the brain cavity. During this time the liver synthesizes large amounts of glucose which are exported into the blood to be pumped by the heart to other organs to act as a cell cryoprotectant. Due to their high glucose contents, the heart and liver are the last organs to freeze and also first to thaw when the frog rewarms. This allows blood circulation to be restored to each organ as soon as it thaws.

To survive freezing, frogs must have defenses against a variety of stresses including cold stress, anoxia/ischemia stress, oxidative stress during thawing, and osmotic/dehydration stress (Storey and Storey, 1988; 2004). In response to these stresses, evolutionary changes in body biochemistry have developed that help to (a) sustain metabolic homeostasis in cells and (b) regulate the necessary defensive changes in organ

metabolism to endure long periods of natural freezing. To endure freezing various stress-responsive mechanisms are turned on whereas many normal metabolic functions of cells and organs are strongly depressed. Studies by our lab have documented many metabolic adaptations that allow freezing survival by wood frogs. These include the synthesis and accumulation of high levels of glucose as a cryoprotectant (including enhanced activities of the enzymes that synthesize the sugar), altered regulatory mechanisms for controlling glycogenolysis, the presence of ice nucleating proteins, metabolic rate depression, enhanced antioxidant defenses, and freeze-induced changes in gene expression, including the synthesis of some novel proteins (Storey and Storey, 2004). During freezing, glucose levels in wood frog plasma and central body organs (liver, heart) can rise to 200-300 $\mu\text{mol/g}$, more than 50-fold higher than control values ($\sim 3\text{-}5 \mu\text{mol/gm}$) (Storey and Storey, 1986). Studies have shown that the activities of glycogen phosphorylase and glucose transporters play important roles in glucose production and distribution during freeze-thaw cycles (Storey and Storey, 1992). Recent studies have also shown that the up-regulation of selected genes and proteins occurs during freezing in wood frogs organs (Storey, 2004). The up-regulated genes/proteins known to date include fibrinogen, the ATP-ADP translocase and the inorganic phosphate transporter as well as three novel genes (FR10, FR47, Li16) whose protein functions are still unknown (Cai and Storey, 1997a,b; Cai et al., 1997; McNally et al., 2002, 2003). Other genes/proteins undoubtedly have important roles to play in regulating or minimizing the various types of damage that could occur during freezing including physical damage from ice, the consequences of cell water outflux/influx during freeze/thaw (changes in osmolality, ionic strength, cell

volume, and strain on membranes), and ischemia/anoxia caused by the freezing of blood plasma.

One of the common responses of cells to stresses of many kinds is the synthesis of a variety of shock proteins. In response to a stress, the cellular protein synthesis machinery interrupts its normal production of proteins and produces high levels of shock proteins (Hightower and Hendershot, 1997). Most shock proteins are now known to be chaperones that assist in the proper folding of other proteins as they are synthesized and/or in stabilizing protein conformation in response to the denaturing effects of the stress. The first shock proteins to be discovered responded to heating stress and were therefore named heat shock proteins (Hsps) (Hightower and Hendershot, 1997). However, the synthesis of heat shock proteins is now known to be triggered by a wide variety of stresses including heat, hypoxia/ischemia, osmotic stress, heavy metals, xenobiotics and pathogens (Basu et al., 2002). Several families of Hsps are now known, differentiated by size: small Hsps (10-30 kDa), 40-60 kDa Hsps, 70-90 kDa Hsps, and Hsps greater than 100 kDa. Another family of shock proteins is the glucose-regulated proteins (Grps), so called because they were first identified as responses by cells to glucose deprivation (Lee, 2001). They are produced in response to stresses that affect biosynthesis on the endoplasmic reticulum (ER). Stresses including the accumulation of unfolded or misfolded proteins, depletion of oxygen, glucose and ATP limitation trigger the production of ER specific chaperones such as Grp78 and Grp94 (Lee, 2001; Pelham, 1986).

Hypoxia/anoxia (low/no oxygen) and ischemia (interruption of blood flow) are known stresses that occur during freezing and must be endured by freeze tolerant

animals. Previous studies have shown that glycolytic end products (lactate and alanine) accumulate during freezing in wood frog organs and that a slow depletion of cellular ATP levels also occurs (Storey and Storey, 1986). Hence, organs in frozen animals are oxygen-limited and switch to oxygen-independent methods of ATP generation, e.g. glycolysis, to supply energy metabolism in the frozen state. In many animals, low oxygen stress stimulates the up-regulation of a variety of genes whose protein products address two general needs: (1) improved oxygen delivery to tissues (e.g. by stimulating capillary growth or the synthesis of red blood cells) and (2) improved energy production from oxygen-independent pathways (e.g. elevating activities of glycolytic enzymes) (Bunn and Poyton, 1996; Iyer et al., 1998; Fedele et al., 2002). These effects are mediated via the action of the hypoxia-inducible transcription factor 1 (HIF-1). The alpha subunit of HIF-1 is highly sensitive to oxygen. It is rapidly destroyed as a consequence of oxygen-dependent proline hydroxylation when oxygen is high but is stabilized when oxygen is low and can then migrate to the nucleus, combine with the beta subunit, and activate genes whose protein products aid hypoxia survival.

Stress response mechanisms such as the production of shock protein chaperones and HIF-1 mediated gene up-regulation have been extensively explored in mammalian systems and in various other animals. However, nothing is known to date about whether these stress response mechanisms contribute to freezing survival by freeze tolerant animals. It would be predicted that multiple changes in the intracellular environment that occur during freezing, including water loss, a great increase in osmolality and ionic strength of the cytoplasm, and strong compression stress on membranes would require the action of shock proteins to help protect and preserve protein structure and function.

Furthermore, HIF-1 mediated changes in gene expression could help cells to adjust their metabolism for long term survival under the low oxygen conditions of the frozen state. In addition, expression of this transcription factor can also act as a marker for confirming that freezing causes hypoxia stress to wood frog organs. In this thesis I have tested two hypotheses:

- (1) Freezing will stimulate increased production of shock proteins (Hsps, Grps) to help stabilize cellular proteins against stresses associated with freezing.
- (2) Freezing will increase levels of the HIF-1 alpha transcription factor in response to the low oxygen stress during freezing.

Within cells, the levels of proteins can be controlled at multiple levels. Very commonly, protein expression reflects the level of gene expression and increases or decreases in the steady-state levels of mRNA often lead to proportional changes in protein levels. Control can also be applied by RNA modification at a post-transcriptional level. Protein levels are also controlled at the translational level by regulation of both the initiation and elongation phase of protein synthesis. In addition, after proteins are synthesized, multiple forms of post-translational modification can alter protein function or subcellular location in response to various stresses. Finally, controls on proteolysis can regulate the rate of degradation of different proteins. The data in this thesis focus mainly on two parameters. Measurements of changes in mRNA levels are used to indicate changes in gene expression in response to freezing and other stresses whereas immunoblotting analysis examines the net changes in protein levels in tissues. Overall, these two measurements give a good picture of the responses by HIF-1 α and shock proteins to freezing and associated stresses in wood frog organs.

Chapter 3 examines the responses by HIF-1 α to freezing and thawing at both the transcriptional level (changes in mRNA levels assessed by RT-PCR) and the translational levels (immunoblotting to measure protein content). Sequence analysis of HIF-1 α was also used to determine whether known functional groups found in HIF-1 α in other species are conserved in the wood frog sequence and to see the wood frog sequence contains any substitutions that might be important for low temperature function of the protein. The organ-specific responses by HIF-1 α to two other stresses were also examined: anoxia and dehydration. Each of these mimics one of the components of freezing: the anoxia/ischemia stress on cells caused by the freezing of blood plasma and the cell volume shrinkage caused by water loss into extracellular ice masses. In previous studies our lab has found that most of the gene responses to freezing are also triggered by either anoxia or dehydration (Storey and Storey, 2004) which suggests that the observed gene/protein changes help the frog cells to endure either the anoxia or the dehydration associated with freezing.

Chapter 4 analyzes the responses by Grp 78 and Grp94 to freezing, thawing, anoxia and dehydration and reports changes in the mRNA and protein levels of these ER stress proteins. This suggests that increased Grp levels support freeze tolerance in some organs. Sequence analysis also evaluated the degree of conservation of these shock proteins in wood frogs compared with other species. Chapter 5 examines Hsps from several different families (hsp110, hsc70, hsp60, hsp40 and hsp10) and shows coordinated increases in the expression of several hsp in response to freezing, anoxia or dehydration stresses. The data indicates an important role of hsp in cell survival of freezing.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal experiments

Male wood frogs, *Rana sylvatica*, were collected from breeding ponds in the Ottawa area during April 2002. Frogs were washed in a tetracycline bath and held at 5°C in a container with sphagnum moss for 1-2 weeks before use. Control animals were directly sampled from this condition. For freezing exposure, wood frogs were placed in closed plastic containers lined with damp paper towels and transferred to another incubator at -2.5°C. Under this condition, frogs cool below 0°C and start to freeze within ~45 min (Storey and Storey, 1985a). After this period of chilling, the length of freezing exposure was timed and frogs were sampled after 2, 6, 12 and 24 h of freezing at -2.5°C. In order to sample frogs at shorter times, the body temperature of individual animals was monitored during cooling with a thermistor taped to the abdomen; the exotherm produced by the nucleation event was set as time zero and frogs were sampled at 30 min or 1 h post-nucleation (Storey and Storey, 1985a). Other frogs were frozen for 24 h at -2.5°C and then returned to 5°C and allowed to thaw with sampling of these recovered frogs at 1 h, 2 h, 4 h and 8 h. All frogs were killed by pithing and each was dissected within 30-90 sec with the group cooperation of 3-5 persons. Tissues were flash frozen in liquid nitrogen and then transferred for storage to a -80°C freezer. Tissues sampled included brain, heart, liver, kidney, gut (stomach and intestine), hind leg thigh skeletal muscle, skin (from frog abdomen) and blood (separated in to plasma and cells).

For anoxia exposure, frogs were treated essentially as described previously (Holden and Storey, 1997). After 1 week acclimation at 5°C, groups of 5-6 frogs were transferred into closed plastic jars sitting on crushed ice; the jars were lined with damp paper toweling (towels wetted with deoxygenated distilled water) and had been

flushed with 100% nitrogen gas for 20 min through syringe ports in the caps. After the frogs were added the jars were flushed with N₂ for a further 20 min and then the ports were plugged and the jar lids were sealed all around with three layers of parafilm. Jars were returned to the 5°C incubator and frogs were sampled after 4 or 24 h of anoxia exposure. During sampling, the jars were again held on ice and the N₂ gassing was reconnected. A third group of frogs was given 24 h anoxia exposure and were then transferred back to aerobic conditions and sampled after 4 h recovery from anoxia.

Dehydration experimental protocols were described by Churchill and Storey (1994). Frogs were individually tagged for identification and weighed. They were then placed in closed jars (5-6 frogs per jar) with a layer of silica gel desiccant on the bottom separated from the frogs by a sponge. Jars were held 5°C. At intervals, animals were removed and quickly weighed. The change in mass was used to calculate the percentage of body water lost from the following equation: $(M_i - M_d) / (M_i \times \%H_2O)$ where M_i is the initial mass of an individual, M_d is the mass at each weighing, and % H₂O is the percentage of total body mass that is water in control frogs; this value is 80.8 ± 1.2 % for *R. sylvatica* (Churchill and Storey, 1994). The mean rate of body water loss under this dehydration protocol was 0.5% of total body water lost per hour. Animals were sampled after they reached 20% or 40% dehydration. Other frogs were first dehydrated to 40% of total body water lost and then transferred into jars containing 1cm distilled water and allow to fully rehydrate at 5°C; they were sampled after 24 h as rehydrated frogs.

2.2 RNA isolation

All solutions and materials used during RNA handling were treated overnight with 1% v:v diethylpyrocarbonate (DEPC) and later autoclaved. Total RNA was isolated from frog tissues using Trizol reagent (GIBCO BRL, Grand Island, N.Y.). Samples of frozen tissue (50-150mg) were quickly weighed, placed in 2 ml Eppendorf tubes, allow to thaw for 0.5-1 min and then 0.8-1 ml Trizol reagent was added. Samples were homogenized, allowed to sit for 5 min at 21°C (room temperature) and then 0.35 ml chloroform was added and mixed for 10 sec. After a further 2-3min incubation at 21°C, samples were centrifuged at 13,000 x g for 15 min at 4°C. The supernatant was transferred to 1.5 ml Eppendorf tubes containing 800 µl isopropanol to allow RNA precipitation. After 10 min at 21°C, the samples were centrifuged for 15 min at 12,000 x g at 4°C. The supernatant was discarded and 0.35 ml of 70% ethanol was added to the pelleted RNA to remove the lingering chemicals in the tubes. After washing for 1.5 min at 21°C, the pellets were recentrifuged for 5 min at 12,000x g at 4°C. The supernatant was discarded and the RNA pellet was air-dried at 21°C and then dissolved in 100µl DEPC-treated water. Aliquots of 20 µl were transferred to other tubes and all were stored at -80°C until use. RNA concentration in samples was quantified spectrophotometrically at 260nm and 280nm; at 260nm, an optical density of 1.0 is equivalent to an RNA concentration of 40 µg/mL. The ratio A_{260}/A_{280} assesses RNA quality with a value of 1.6 or higher indicating high purity.

2.3 Testing RNA purity

RNA purity (and lack of genomic DNA contamination) was assessed by formaldehyde RNA agarose gel electrophoresis (FRAGE). The apparatus used (e.g. gel tray, comb, beaker) was treated with 3% hydrogen peroxide for 30 min, then washed with DEPC water and dried at 21°C; DEPC-treated pipetter tips were used in all cases. For 125 ml gel preparation, 1.5 g agarose (GIBCO BRL) was melted in 107.5 ml DEPC-treated distilled water and then 12.5ml of 10X formaldehyde gel running buffer (FGRB)(containing 0.2 M 3-N-morpholino propanesulfonic acid [MOPS], 50 mM sodium acetate, 10 mM EDTA, pH7.0) and 7 ml of 37% v:v formaldehyde solution were added. The mixture was cooled to 50-60°C and then 1.5 µl of ethidium bromide (EtBr) solution was mixed in and the gel was poured. After the gel solidified for 20-30 min at 21°C, it was pre-run for 5-10 min at 70 V in 1X FGRB. RNA samples were prepared by mixing 20 µg of total RNA with 21.5 µl FGRB loading solution that contained 12.5 µl formamide solution, 2.5 µl 10X MOPS buffer (1 M MOPS, 0.1 M sodium acetate and 1 mM EDTA, pH 7.0), 4 µl 37% formaldehyde, and 2.5 µl of 10X RNA loading buffer (0.9 ml of 50% glycerol, 2 µl of 0.5M EDTA pH 8.0, 50 µl of 0.25% w:v bromophenol blue, 50 µl of 0.25% xylene cyanol FF) in a 0.5 ml RNAase-free Eppendorf tube. The mixture was heated for 5 min at 65°C, cold-snapped on ice for 5 min, quickly centrifuge, and then loaded onto the gel. In some cases a 7 µg aliquot of RNA ladder was also loaded in one lane as a size marker. The gel was run at 70 V for 1 h until the blue dye migrated 5 cm away from the gel wells. The gel was photographed on a UV light transilluminator or using the SynGene. When needed, the bands of RNA ladder as well as ribosomal RNA could be visualized by staining gels with methylene blue; gels were treated in 5% v:v acetic acid for 10 min,

then with 0.05% w:v methylene blue (in 0.5 M sodium acetate, pH 5.2) for 15 min and destained with distilled water for 20 min.

2.4 Gene specific primer design and sequencing

Oligonucleotide primers were designed and synthesized for each gene. Primer design began with the retrieval of nucleotide sequences for the gene of interest from NCBI Genbank for 3-5 other animal species, as closely related to frogs as possible. The sequences were imported into DNAMAN 4.11 (Lynnon Biosoft) and a multiple sequence alignment was used to find highly conserved regions and generate one or more consensus sequences that could be used for primer design. Consensus sequences were imported into Primer Designer (Scientific and Educational Software) and used to generate both forward and reverse gene specific primers. Primers were synthesized by Sigma Genosys and were aliquoted into 300 pmol/ μ l stocks in DEPC-treated water and stored at -20°C. Table 2.2 lists the forward and reverse primers that were made.

2.5 First strand synthesis:

For cDNA first strand synthesis a 10 μ l volume containing 0.5 μ g of mRNA was mixed with 1 μ l 200 ng/ μ l oligo(dT). The mixture was incubated in a 68°C water bath for 5 min, then chilled on ice for 5 min and given a rapid spin. To obtain cDNA:mRNA hybrid, 8 μ l of first stand mixture was added containing 4 μ l of 5X first strand buffer (Gibco-BRL), 2 μ l 0.1 M DTT (Gibco-BRL), 1 μ l 10mM dNTP mix, and 1 μ l superscript reverse transcriptase (200 U/ μ l). This 19 μ l final volume was incubated at 40°C for 45 min and then stored at 4°C.

2.6 Quantifying cDNA:mRNA hybrid by RT-PCR

Reverse transcription and polymerase chain reaction amplification (RT-PCR) was used to amplify cDNA and quantify the relative levels of selected mRNA species in tissue extracts of control versus experimental frogs. A 5 μ l aliquot of first strand cDNA product was mixed with 45 μ l DEPC-treated water to produce a 10^{-1} dilution; this procedure was then repeated to produce serial dilutions of 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . The PCR reaction mixture contained 15.5 μ l distilled water, 2.5 μ l 10X PCR buffer, 1.25 μ l 50 mM $MgCl_2$, 0.5 μ l 10 mM dNTP mix, 0.125 μ l Taq DNA polymerase (5 U/ μ l, Gibco-BRL) and 1.25 μ l primer mixture (1:10 dilution of 300 pmol stock). Each PCR reaction contains 5 μ l of serial diluted cDNA and 21.125 μ l PCR reaction mixture. After mixing well and a quick spin, PCR reactions were performed in the thermocycler with the following steps: (a) 95°C pre-heat for 3 min, (b) 37 cycles of running: 95°C for 30 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 30 sec (elongation), (c) final elongation at 72°C for 7 min and (d) hold at 22°C. The total running time was 2 h, 16 min.

PCR products were mixed with DNA loading buffer containing 40% w:v sucrose, 0.25% w:v bromophenol blue and 0.25% w:v xylene cyanol FF. Samples were loaded onto 1% agarose TAE gels (1% w/v agarose, 40 mM Tris-acetate, 1 mM EDTA, pH 8.3, 0.0005% v:v EtBr) with 1% TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) and separated by electrophoresis at 130 V for the 22-well gel tray or at 90 V for the 16-well gel tray. DNA was visualized using the UV transilluminator function of the SynGene (SynGene Company, California) and band intensities were analyzed with the GeneTools program on ChemiGenius Bio Imaging System.

To compare PCR product levels of target genes between control and experimental states, confirmation that the starting samples contained equal amounts of mRNA is needed. This is provided by running parallel RT-PCR reactions using primers for a housekeeping gene (alpha-tubulin). Analysis of tubulin band intensities were used to normalize the band intensities for the PCR product of interest and experimental samples were then expressed relative to control values that were set to 1.0. This analysis by the GeneTools used the following equation:

$$W = ([Ep]/[Et]) / ([Cp]/[Ct])$$

[Ep] is the concentration (band intensity) of stress PCR products from gene specific primers; [Cp] is the concentration (band intensity) of control PCR products from gene specific primers; [Et] is the concentration (band intensity) of stress PCR products from housekeeper primers; and [Ct] is the concentration (band intensity) of control PCR products from housekeeper primers. If $W > 1$ and the t-test comparing control and experimental shows a significance at $P < 0.05$, then the gene is designated as up-regulated gene; oppositely $W < 1$ with $P < 0.05$ indicates down-regulation.

PCR products were sequenced to confirm that they encoded the target gene. Automated sequencing was performed by Canadian Molecular Research Services (Orleans, ON) or by CORTEC (Kingston, ON) using the dideoxy method. PCR product sequences were verified as the sequence expected using the program BLASTN (<http://www.ncbi.nlm.nih.gov/blast>) at the National Institutes of Health, Bethesda, MD. Deduced amino acid sequences of frog proteins were analyzed by BLASTP to determine their percent identity compared to the sequences from other animals.

2.7 RACE: (5'&3' rapid amplification of cDNA ends)

For first strand cDNA synthesis, 1-5 µg mRNA and 1 µl of gene specific primers (10 µM) were mixed in DEPC water with total volume adjusted to 15.5 µl. The mixture was heated for 10 min at 70°C to denature RNA and then cooled for 1 min on ice. To this was added 2.5 µl of 10X first strand PCR buffer, 2.5 µl 0.1mM DTT, 2.5 µl 25 mM MgCl₂ and 1 µl of dNTP mix (10 mM). After preheating to 42°C for 1 min, 1 µl of AMV reverse transcriptase (20 U/µl) was added followed by incubation at 42°C for 35 min. The reaction was terminated by heating at 70°C for 15 min. Samples were then transferred to a 37°C water bath and 0.5 µl of RNase H mix was added followed by incubation for 30 min at 37°C. Samples were then stored on ice for immediate use or at -20°C.

Next the cDNA was purified to remove unincorporated nucleotides and primers. First strand cDNA samples were treated with addition of 2.5 µl 3 M sodium acetate pH 5.2 and 25 µl isopropanol, mixed well and placed at -20°C for 2 h. Following centrifugation at 13,000 rpm for 20 min at room temperature, the supernatant was removed and the pellet was resuspended in 10 µl DEPC water. Poly(dC) tails were then added to the purified cDNA samples at the 5' end by incubating the cDNA with terminal deoxynucleotidyl transferase (TdT) tailing solution containing 5 µl 5X tailing buffer, 2.5 µl 2mM dCTP, 6.5 µl DEPC water and 1 µl TdT. After terminating the tailing reaction at 65°C for 10 min, the dC-tailed cDNA was used for PCR.

To amplify the dC-tailed target cDNA, gene specific nested primers and anchor primers (AAP: 5'-GGCCACGCGTCGACTAGTACGGGG//GGG//GGG-3' or AUAP: 5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGG//GGG//GGG-3') were used. A 5 µl aliquot of dC-tailed cDNA was added to a PCR reaction containing 5 µl 10X

PCR buffer (200 mM Tris-HCl, 500 mM KCl), 3 μ l 25 mM MgCl₂, 1 μ l 10mM dNTP mix, 2 μ l of 10 μ M GSP, 2 μ l of 10 μ M AAP/ AUAP or UAP, 31.5 μ l distilled water and 0.5 μ l Taq DNA polymerase (5 unit/ μ l). The sample was placed in the thermal cycler at 95°C for a hot start for 2 min, and then run for 35 repeat cycles of 95°C for 1 min, gradient T_m for 1 min and 72°C for 2 min. Final extension was at 72°C for 7 min and then PCR products were loaded onto 1% agarose TAE gels to detect the expected band.

2.8 Western blotting:

2.8.1 Isolation of total protein from frog tissues

Total soluble protein was extracted from samples of frog tissues from control, 24 h frozen and 4 h thawed experimental groups. A sample of frozen tissue was weighed and then quickly homogenized 1:10 w:v in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% w:v SDS, 1% Nonidet P-40 (NP-40), 0.5% w:v sodium deoxycholate, 1 mM EGTA, and 1 mM EDTA with fresh prepared protease inhibitors (0.1mM phenylmethanesulfonyl fluoride [PMSF], 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) added just prior to homogenization with a Polytron PT10. Samples were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant were stored at -80°C.

2.8.2 Isolation of nuclear extracts from frozen tissues

Frozen tissue samples (0.5 g) were weighed and added to 500 μ l of homogenization buffer (1:1 w:v) containing 10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM DTT, and 10 μ l of protease inhibitor cocktail (1 mM PMSF, 0.015% w/v aprotinin, 10 μ M leupeptin, 1 μ M pepstatin, 1 mM NaF). Tissue was disrupted with 20 piston strokes

of a teflon-glass homogenizer and then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant representing the cytoplasmic extract was removed into a sterile Eppendorf tube and frozen at -80°C. The pellet was resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% v/v glycerol, 0.1 mM DTT and 1.5 µl of protease inhibitor cocktail. This resuspension was held on ice and shaken for 1 h followed by centrifuging at 10,000 x g for 10 min at 4°C. The second supernatant was saved as the nuclear extract and stored at -80°C.

2.8.3 Measurement of protein concentration

Protein concentrations were determined by the Coomassie blue dye-binding method (Bradford, 1976) with the Bio-Rad prepared reagent and bovine serum albumin as the standard. The reagent was diluted 5-fold with distilled water and protein extracts of tissues were diluted 1:20 v:v. For assay, 10 µl protein samples were added 190 µl of dye reagent in microplate wells followed by mixing and a 10 min incubation at 21°C. Absorbance at 595 nm was read and protein concentration was determined from a preprogrammed standard curve using BioLinx 2.0 software which used the equation:

$$\text{protein concentration (mg/ml)} = (\text{Abs} - 0.393) \times 20 / 1.074$$

2.8.4 SDS polyacrylamide gel electrophoresis

Protein samples were mixed 1:1 v:v with SDS-PAGE sample 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. Aliquots containing 15-20 µg protein were loaded into sample wells. On any given gel, the same amount of protein was in each

well of 8%-12% acrylamide gels (acrylamide:bis-acrylamide ratio 29.2:0.8; the percentage varied depending on the size of the target protein to be analyzed). Electrophoresis was carried out on a Bio-Rad mini-gel apparatus, run at 180V for 30-45 min at 21°C with 1x running buffer containing 3.03 g Tris base, 14.4g glycine, and 1 g SDS, pH 8.3. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membrane, Millipore corp. Bedford, MA) at 350 mA or 70 V for 1.5 h at 4°C with transfer buffer containing 25 mM Tris (pH 8.5), 192 mM glycine and 20% v/v methanol. PVDF membranes were then 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. The blot was rinsed with TBST and incubated with primary antibody in TBST on a shaking platform, either overnight at 4°C or for 1 h at 21°C. Dilutions of stock primary antibodies that were used are given in Table 2.1. Blots were then washed twice with TBST and incubated with secondary antibody at the appropriate dilution (Table 2.1) in TBST according to the manufacturer's instructions for 1.5 h at 21°C. Blots were washed for 3 x 10 min in TBST and then bands were visualized by adding 2 ml of enhanced chemiluminescence (ECL) reagent (NEN Life Science Products, Inc. Boston, MA) with detection and quantification using a Bio-Rad SynGene and Gene Tools software.

As appropriate, membranes were stripped and re-probed with different antibodies up to 3 times. For stripping, membranes were placed in Falcon tubes with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% w:v SDS, 100 mM 2-mercaptoethanol) and rotated at 50°C for 30 min. Membranes were then washed with distilled water, then TBST, and then re-probed with another antibody as described above beginning with blocking the membrane with nonfat milk.

Total proteins were then visualized on the PVDF membrane by staining for 30 min with Coomassie blue (0.25% w:v Coomassie Brilliant Blue R, 50% v:v methanol, 7.5% v:v acetic acid) followed by destaining for 5 min with destain solution (50 ml distilled water, 50 ml acetic acid, 150 ml methanol). An appropriate band (or bands) was chosen on each blot and band intensities in each lane were quantified using the SynGene. This was used to confirm equal loading of protein into each lane and immunoblot band intensities in each lane were normalized against the corresponding Coomassie blue “standard” band in that lane.

2.9 Two dimensional protein gel electrophoresis (2D-PAGE)

Total soluble protein was extracted from tissue samples by homogenizing 1:10 w/v in buffer containing 250 mM sucrose, 25 mM HEPES, 25 mM KCl and 1 mM EDTA/EGTA. Samples were centrifuged at 2,500 rpm for 10 min and the supernatant was transferred into 1.5 ml Eppendorf tubes and stored at -80°C .

First dimension gels were prepared in capillary gels with a composition of 9.2 M urea, 4% acrylamide, 1.6% pH 5-8 ampholytes, 0.4% pH 3.5-10 ampholytes, 0.01% ammonium persulfate and 0.1% TEMED. The gels were pre-electrophoresed at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min with 100 mM NaOH as the upper chamber solution and 10 mM H_3PO_4 as the lower chamber solution using the Modular Mini-Protean II System (Bio-Rad). Protein samples were mixed 1:1 v:v with first dimension sample buffer containing 9.5 M urea, 5% 2-mercaptoethanol, 1.6% pH 4-6 ampholytes and 0.4% pH 3.5-10 ampholytes. After incubation for 10-15 min at 21°C , aliquots of 15-20 μg protein were loaded into the capillary sample reservoirs and overlaid

with first dimension overlay buffer (9 M urea, 0.8% pH 5-8 ampholytes, 0.2% pH 3.5-10 ampholytes, 0.005% w:v bromophenol blue). Gels were run at 500 v for 10 min and then 750 v for 3 h or 300 v overnight.

After isoelectric focusing, gels were removed from the capillary tubes and incubated in SDS equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% w:v SDS, 5% 2-mercaptoethanol, 10% v/v glycerol and 0.00125% w:v bromophenol blue) for 10 min at 21°C. Capillary gels were then positioned onto a 10% SDS-PAGE gel and overlaid with overlay buffer containing 1% v:v agarose, 62.5 mM Tris-HCl, pH 6.8, 2.3% w:v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol and 0.00125% w:v bromophenol blue. SDS-PAGE was run at 180V for 30-45 min at 21°C in 1x running buffer (3.03 g Tris base, 14.4g glycine, 1 g SDS, pH 8.3 per liter). Proteins were then transferred to PVDF membranes and immunoblotting, detection with ECL and stripping/reprobing were carried out as described above for Western blotting.

Before proteins were transferred to PVDF membranes, protein profiles on the two-dimensional gels were visualized by silver staining. SDS-PAGE gels were placed in fix solution (50% v:v methanol and 10% v:v acetic acid) for 30min and then washed in distilled water for 2 x 15 min. Gels were then incubated in sensitive solution containing 0.01% w/v sodium thiosulphate for 1 min and following 2 x 30 sec washing with distilled water, the gels were placed in pre-chilled 0.2% w/v silver nitrate solution for 30 min at 4°C. After a quick rinse with distilled water, gels were incubated with 100 ml developing solution containing 12 g sodium carbonate, 100 µl of 37% formaldehyde solution and 8 µl of 10% w/v sodium thiosulphate. Development was terminated with 5% acetic acid and gels were stored in distilled water.

Table 2.1 Antibodies used in western blotting

	Primary antibody & dilution (v/v) used	Secondary antibody & dilution (v/v) used	Company
Hif-1a	Anti-Hif-1 α (1:500)	Anti-chicken IgY-HRP (1:1000)	Promega
Grp94	Grp94 (C-19) P (1:500)	Bovine anti-goat Ig- HRP (1:1,000)	Santa Cruz Biotechnology
Grp78	Anti- grp78 (1:10,000)	Goat-anti-rabbit Ig- HRP (1:1,000)	Stressgen
Hsp110	Anti-hsp110/70 polyclonal antibody (1:2,000)	Goat-anti-rabbit IgG- HRP (1:1,000)	Stressgen
Hsc70	Anti-hsc70 monoclonal antibody (1:10,000)	Anti-rat IgG:HRP (1:1,000)	Stressgen
Hsp60	Anti-hsp60 polyclonal antibody (1:1,000)	Goat-anti-rabbit IgG- HRP (1:1,000)	Stressgen
Hsp40	Anti-hsp40 polyclonal antibody (1:10,000)	Goat-anti-rabbit IgG- HRP (1:1,000)	Stressgen
Cnp10 (HSP10)	Anti-Cpn10 polyclonal antibody (1:2,000)	Goat-anti-rabbit IgG- HRP (1:1,000)	Stressgen

Table 2.2: Gene-specific primers used in RT-PCR

Genes	Primers	Gene specific sequence	Length
Hif-1 α	Forward:	5'-ccttgcatcatgaagag-3'	18 bp
	Reverse:	5'-tcatatccaggctgtgacgactgag-3'	25 bp
Grp94	Forward:	5'-tccactgcagcttcat-3'	16 bp
	Reverse:	5'-ctgctgcatgtcacagacacag-3'	21 bp
Grp78	Forward:	5'-accgctcacgccgtttat-3'	20 bp
	Reverse:	5'-ggctggtacagtaacaac-3'	18 bp
Alpha-Tubulin	Forward:	5'-aaggaagatgctgccaataa-3'	20 bp
	Reverse:	5'-ggtcacatttcacatctg-3'	19 bp

CHAPTER 3

Hypoxia Inducible Factor-1 α (HIF-1 α)

3.1 Introduction:

Tissue hypoxia can be caused by reduced blood oxygenation, by altered oxygen release from hemoglobin, or by impaired blood delivery to tissues as a result of low cardiac output or vascular obstruction (Safran et al., 2003). Freezing stress in wood frog tissues causes hypoxia mainly due to vascular obstruction caused by the freezing of blood plasma which halts circulation. Tissue adaptation to these conditions involves both physiological and cellular adaptive mechanisms.

One common response of many animal cells to hypoxia is the triggering of a set of gene transcriptional and translational activities including erythropoietin (EPO) (Goldberg, 1988), vascular endothelial growth factor (VEGF) (Shweiki, 1992), and glycolytic enzymes (Ebert, 1995). These responses enhance the delivery of O₂ to cells and facilitate the production of ATP by glycolysis to augment a shortfall of ATP output from oxidative phosphorylation (Semenza et al., 1994). At the cellular level, gene activation results because under hypoxic conditions the alpha subunit of the hypoxia-inducible transcription factor (HIF-1 α) is stabilized, allowing increased translocation of HIF-1 α into the nucleus where it forms a dimer with HIF-1 β and then a complex with the hypoxia-responsive element (HRE) of target genes (Ebert, 1995; Semenza et al., 1994). We knew that freezing cut off oxygen delivery to tissues in wood frogs and then caused ischemia in tissues. Hence, I hypothesized that an activation of HIF-1 α could help frogs to survive under conditions of low oxygen and energy depletion during freezing. Turtles can survive total anoxia for weeks to months when they are submerged in lakes and rivers during winter hibernation and HIF-1 α was demonstrated to play an important role in this process (Boutilier, 2001).

The actions of HIF-1 in cells are determined by the specific structure of its two subunits. HIF-1 is a basic helix-loop-helix/PAS heterodimer transcription factor consisting of a 120 kDa HIF-1 α subunit and a 91-94 kDa HIF-1 β subunit (Wang, 1995). The alpha subunit is unique to HIF-1 whereas HIF-1 β (ARNT) can also dimerize with the arylhydrocarbon receptor (Gleadle et al., 1995; Wang et al., 1995; Huang et al., 1996). The N terminus of both subunits has a basic helix-loop-helix (bHLH) domain that is known to be necessary for DNA binding. Downstream PAS domains on HIF-1 α are required for heterodimerization with ARNT and target gene specificity (e.g. oxygen-dependent genes and other oxygen homeostasis regulators) (Fandrey, 2004). The C-terminus contains various transactivation domains interacting with basal transcription machinery and critical for intracellular localization of HIF-1 α (Ruas *et al.*, 2002). Therefore, regulated nuclear translocation requires the presence of the PAS domain and a C terminal nuclear localization signal (Fandrey, 2004).

Changes in HIF-1 levels and activity are primarily determined by the stability of the HIF-1 α subunit (Huang *et al.*, 1999). When oxygen is high, HIF-1 is modified by O₂-dependent prolyl hydroxylases that catalyze hydroxylation on two specific proline residues in the oxygen dependent domain (ODD) of HIF-1 α (Semenza, 2002). Hydroxylation targets the protein for ubiquitylation by an E3 ubiquitin-protein ligase. A specific protein, the von Hippel–Lindau tumour-suppressor protein (VHL), is the recognition component of the ligase complex and binds to hydroxylated HIF-1 α (Semenza, 2002). Binding by VHL sets up HIF-1 α for ubiquitylation and then ubiquitylated HIF-1 α is rapidly degraded by the proteasome. Under hypoxic conditions, prolyl hydroxylase activity is reduced due to reduced levels of its substrate, oxygen, and

therefore HIF-1 α is stabilized long enough to undergo transfer to the nucleus, heterodimerization with ARNT, and binding to the hypoxia responsive elements of target genes.

Current studies have demonstrated that in hypoxia HIF-1 α activation includes an elevation of *hif-1 α* mRNA, an increase of nuclear translocation of HIF-1 α protein, and recruitment of transactivators (Semenza, 2002). The responses of HIF to different stresses and the regulatory controls on HIF have been well studied in multiple mammalian systems but relatively little is known to date about HIF in lower vertebrates and the potential role of HIF in dealing with hypoxia/anoxia stresses associated with freeze tolerance is completely unknown. I hypothesized that HIF-1 would be activated during freezing in wood frog tissues and that one of the mechanisms HIF-1 α control during freezing would be transcriptional control on *hif-1 α* gene expression. This chapter presents results from studies of *hif-1 α* gene expression and HIF-1 α protein levels in wood frog tissues as well as the characterization of nucleotide and amino acid sequences of the frog gene/protein. The aims of the study were to determine: 1) the effect of freezing on the expression levels of the HIF-1 α gene and protein, and 2) the comparable responses by this transcription factor to two component stresses of freezing: anoxia or dehydration.

3.2 Materials and methods:

3.2.1 Animal and tissue samples:

For freezing exposure, wood frogs were treated for 30 min, 2 h, 6 h or 24 h at -2.5°C, respectively. To sample thawed frogs, animals were first frozen for 24 h at -2.5°C and then allowed to thaw for 2 or 4 h at 5°C. A second group of wood frogs was given 24 h anoxia exposure at 5°C or 24 h anoxia followed by 4 h recovery from anoxia. A third group of wood frogs was dehydrated to 20% or 40% of total body water lost at 5°C or 40% dehydrated followed by full rehydration at 5°C. The detailed protocols were described in section 2.1. All dissected tissues were stored at -80°C until analysis.

3.2.2 Primer Design:

Primer design for *hif-1α* was performed using Primer Designer as described in Chapter 2.2 and 2.4. The gene specific primers were created by evaluating the melting temperature, GC %, and minimizing the possibility of formation of dimers or hairpins between primer pairs and/or within a single primer. Multiple nucleotide sequences were designed based on the consensus sequences given in Genbank for human (Accession numbers: U22431), mouse (NM010431) and African clawed frog (*Xenopus laevis*) (AJ277829) *hif-1α* mRNA sequences. The forward and reverse primers designed for *hif-1α* were 5'-CCTTGCGATCATGAAGAG-3' and 5'-TCATATCCAGGCTGTGACGACTGAG-3', respectively. These primers were expected to retrieve an approximately 350 bp cDNA sequence from wood frog tissues. Tubulin primers were: forward 5'-AAGGAAGATGCTGCCAATAA-3' and reverse 5'-

GGTCACATTTACCATCTG-3'. These primers were expected to retrieve an approximately 616 bp cDNA sequence from wood frog tissues. The Sigma Genosys Company located at Winston Park Drive, ON synthesized these primers.

3.2.3 RT-PCR amplification of *hif-1α*:

Total RNA was isolated from tissues of control, frozen, anoxic and dehydrated *R. sylvatica* and quantified spectrophotometrically as described in section 2.2. The ratio A_{260}/A_{280} assesses RNA quality with a value of 1.6 or higher. First strand synthesis of cDNA and PCR amplification were performed as described in section 2.5 and 2.6, respectively. The PCR amplification for both genes was carried out for 35 cycles, with an annealing T_m of 44.2°C in an iCycler PCR machine (Bio-Rad). PCR products were visualized via electrophoresis on a 1.0% TAE agarose gel. The band intensities were measured on a SynGene machine as described in section 2.6. The PCR products of expected sizes for each gene were sequenced by Canadian Molecular Research Services (CMRS, Orleans, ON). *Hif-1α* sequence analysis using DNAMAN and the BLAST database located on the NCBI was used to align the wood frog *hif-1α* sequences with other genes and proteins obtained from Genbank. The Prosite blocks and prints programs were used to search the translated amino acid sequence for recognizable domains (details as described in section 2.4 and 2.6.3).

3.2.4 Western blotting and 2-dimensional protein electrophoresis:

Total soluble protein and cytoplasmic/nuclear proteins were extracted from *R. sylvatica* tissues as described in section 2.8 and 2.9, respectively. Samples were prepared from

wood frogs exposed to different experimental conditions and protein concentrations were measured by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (15 μ g) were loaded onto each lane of SDS-PAGE gels and, after electrophoresis, were transferred onto Hybond-ECL nitrocellulose membranes (PVDF) (Amersham, Buckinghamshire, England). For 2-D gels, isolated gel slices from the first dimension were placed directly on the stacking gel of the second dimension 10% SDS-polyacrylamide gel and following electrophoresis in the second dimension, proteins were transferred to PVDF membrane. Membranes were blotted with HIF-1 α antibodies (described as table 2.1), and visualized using ECL (Amersham, Buckinghamshire, England). The gels were calibrated with high range molecular weight standards or prestained molecular weight markers (Bio-Rad). Protein band intensities were quantified with Genetools as described in section 2.6. Coomassie blue and silver staining were used to visualize protein patterns and to analyze differences between experimental groups. Silver staining was performed with silver nitrate as described in section 2.9. Molecular weight estimation for HIF-1 α polypeptides was based on SDS-polyacrylamide gels. The isoelectric point of wood frog HIF-1 α protein was compared with that of mammal published sequences of HIF-1 α . Each experiment was repeated four to five times. Statistical significance of changes was determined by the Student's t-test in Excel. The ratio of relative protein levels in experimental versus control situations and their significance levels ($p < 0.05$) were used to assess the up-regulation of protein expression.

3.3 Results:

3.3.1 Analysis of *hif-1α* sequences

Primers designed from the consensus sequences of *hif-1α* from three species (mouse, human, *X. laevis*) were used to retrieve a 343 nucleotide segment of cDNA from wood frog liver. Comparison of the sequence to the Genbank database located on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) identified the sequence as belonging to *hif-1α*. Nucleotide and deduced amino acid sequences for the wood frog liver protein are shown in Figures 3.1(A) and 3.2(A), respectively, and homology trees comparing the wood frog sequences with other species are shown in Figure 3.1(B) and Figure 3.2(B). The single open reading frame of *hif-1α* sequence from wood frog liver contained 343 nucleotides that encoded 114 amino acids. This represents a partial sequence of *hif-1α* as compared with sequences from *X. laevis* (Genbank accession number BC043769), mouse (NM010431) and human (U22431) *hif-1α* that have full length sequences of 3710, 3973 and 3678 nucleotides. The homology tree showed that the segment of wood frog *hif-1α* nucleotide sequence shared 68% identity with the *X. laevis*, human or mouse *hif-1α* (Figure 3.1B), whereas the comparison of amino acid sequences showed that wood frog HIF-1α also shared more than 68% identity with *X. laevis*, human and mouse (Figure 3.2B).

The HIF-1α protein contains several domains that are required for protein function including the basic helix-loop-helix (bHLH) domain and two PAS domains (named for the first three genes shown to contain this domain: Period, ARNT, single-minded) that are the binding sites for heterodimerization with ARNT. Figure 3.3 shows a

comparison of the PAS domains in wood frog *hif-1α* with the sequences of the African clawed frog, mouse and human PAS domains (published in website: www.pnas.org/cgi/content/full/98/14/7916). It can be seen that there is high identity (74%) between these functional groups in the different species (the two PAS domains are highlighted in Figure 3.2).

3.3.2 RT-PCR analysis of *hif-1α* mRNA expression and Western blot analysis of HIF-1α in wood frog tissues

Total RNA was isolated from tissues of wood frogs using Trizol reagent as described in section 2.2. The effect of experimental freezing or thawing on *hif-1α* transcript levels in tissues was evaluated using RT-PCR. *Hif-1α* transcripts were amplified in serial dilutions of wood frog RNA using the primers designed for wood frog *hif-1α*. Equivalent runs amplified the mRNA of a housekeeping gene (alpha-tubulin) from the same samples using specific commercial α-tubulin primers. PCR products were then separated on 1% TAE agarose gels and visualized with ethidium bromide. Figure 3.4(A) shows the typical pattern of bands resulting from RT-PCR of serial dilutions of wood frog RNA using *hif-1α* primers for amplification. Figure 3.4(B) shows the comparable bands of RT-PCR products resulting from amplification using α-tubulin primers. The decreasing intensity of the bands with increasing dilution confirmed that suitable mRNA concentrations were used for PCR amplification. A visual inspection of the band intensities of the [10⁻³] dilution from the *hif-1α* amplification indicates a substantially higher *hif-1α* mRNA content in liver extracts from 24 h frozen wood frogs than in 5°C acclimated control frog. Band intensities were measured and each *hif-1α* band was expressed relative to its

corresponding α -tubulin band. Mean values for normalized band intensities for control and experimental samples from multiple runs were then subjected to statistical testing.

Figure 3.5 shows Western blots used to assess Hif-1 α protein levels in wood frog tissues. HIF-1 α in frog tissue extracts was detected via cross-reaction with commercial human anti-Hif-1 α antibody (Promega). Figure 3.5B shows a 2D-PAGE gel loaded with 20 μ g of soluble protein from liver of 2 h frozen frogs and blotted with anti-HIF-1 α antibodies. The antibodies crossreacted with a single spot on the 2D gel showing specificity of the antibody reaction. The frog HIF-1 α protein was ~120 kDa in molecular weight with an isoelectric point (pI) of ~5.17, in good agreement with values reported for mammalian HIF-1 α in ExPASy website. Therefore, I am confident that the antibodies cross-reacted with the frog HIF-1 α protein. Figure 3.5A shows representative one-dimensional gel separations of soluble protein extracts from skeletal muscle of control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (4 h recovery at 5°C) frogs. The upper panel shows Coomassie blue staining of a prominent protein band on the gels and confirms equal loading of lanes. The lower panel (Figure 3.5A-ii) shows the cross-reaction of anti-HIF-1 α antibodies with frog HIF-1 α protein; this band was at the appropriate molecular weight (~120 kDa) for HIF-1 α as determined in preliminary runs that included lanes loaded with molecular weight protein standards.

3.3.3 Analysis of *hif-1 α* mRNA and HIF-1 α protein expression during freezing and thawing

Figure 3.6(A) shows the effect of 24 h freezing and 4 h thawing recovery on *hif-1 α* mRNA levels in seven organs of wood frogs (also see Appendix 3.1). Transcript levels

rose significantly during freezing in liver, skeletal muscle and gut (by 1.5-, 1.25- and 1.34- fold, respectively) but were unchanged in brain, heart and kidney and decreased slightly in skin. During thawing after freezing, *hif-1α* transcript levels fell in six organs, dropping back to near control levels in liver, muscle and gut and showing significant reductions compared with control values in brain, heart and skin (levels fell to 57, 80 and 67% of control values).

Figure 3.6(B) shows the comparable effects of freezing and thawing on HIF-1α protein levels (also see Appendix 3.1). During freezing HIF-1α protein rose in liver, muscle and gut (by 1.4-, 1.4- and 1.2-fold, respectively), paralleling the increases in *hif-1α* transcripts. By contrast HIF-1α protein content fell in frozen skin and was unchanged in brain, heart and kidney. After a 4 h thaw, HIF-1α had dropped to near control levels in muscle and gut but liver levels were strongly reduced to just 61% of control values. Skin showed an increase in HIF-1α protein back towards control values.

Figure 3.7 shows the changes in *hif-1α* mRNA levels over a time course of freezing and thawing in wood frog heart, brain and liver. Mean relative *hif-1α* mRNA levels in brain were 0.78 ± 0.06 , 2.78 ± 0.10 , 0.69 ± 0.05 , 0.96 ± 0.08 , 1.7 ± 0.09 and 0.91 ± 0.08 as compared with corresponding control values (set at 1.0) after 30 min, 2 h, 6 h and 24 h freezing as well as 2h and 4h thawing, respectively. Comparable values in liver were 1.31 ± 0.11 , 2.31 ± 0.17 , 1.85 ± 0.21 , 1.53 ± 0.012 , 1.97 ± 0.11 and 1.54 ± 0.04 and in heart were 0.98 ± 0.07 , 1.15 ± 0.08 , 0.41 ± 0.05 , 1.08 ± 0.07 , 1.46 ± 0.08 and 0.95 ± 0.06 respectively. These data show that *hif-1α* transcript levels rose quickly in all three organs during early freezing to reach peak values after 2 h of freezing exposure (the effect was most pronounced in brain and liver). With longer freezing exposure *hif-1α*

transcripts fell back to near control levels in brain and heart but remained elevated in liver after 24 h frozen. During thawing transcript levels in all three organs rose again early in the thaw (2 h) but declined again in all cases by 4 h thawed.

3.3.4 Analysis of *hif-1 α* mRNA and HIF-1 α protein expression under anoxia and dehydration stresses

Two of the components of freezing stress on cells and tissues are (a) anoxia/ischemia because freezing of blood plasma cuts off the delivery of oxygen and nutrients, and (b) dehydration due to water outflow from cells into extracellular ice masses. In previous work, our lab has found that several of the biochemical responses to freezing can also be triggered by either anoxia or dehydration stress and this suggests that the observed response to freezing addresses a problem that is caused by either anoxia or dehydration effects on cells. I hypothesized that the up-regulation of HIF-1 α during freezing would be a response to anoxia stress on frog tissues during freezing and to test this, I compared the effects of the individual stresses of anoxia and dehydration on the expression of HIF-1 α in wood frog tissues. Figure 3.8(A-i) and (B-i) shows the effects of 24 h anoxia exposure and dehydration to 20% of total body water lost, as well as the effects of recovery from these stresses, on wood frog *hif-1 α* transcript levels in seven organs. The results show that *hif-1 α* mRNA levels are up-regulated after 24 h anoxia (under a nitrogen gas atmosphere at 5°C) in liver, muscle, skin, and kidney (by 1.6-, 1.6-, 2.7- and 1.6-fold, respectively). By contrast, *hif-1 α* transcripts decreased significantly in brain and heart under anoxia and were unaltered in gut. After 24 h recovery from anoxia, *hif-1 α* transcripts returned to near control levels in most cases but remained elevated in muscle

and suppressed in brain and kidney (42-47% of control values) as well as skin (78% of control values). Dehydration to 20% of total body water lost also stimulated an increase in *hif-1 α* transcripts in liver muscle, and kidney (by 2.8-, 1.34- and 1.6-fold, respectively). Transcripts in heart, gut and skin did not change but levels fell significantly in brain of dehydrated frogs. After rehydration, *hif-1 α* transcript levels fell back to control levels in kidney and muscle and to about 50% of control levels in liver. Transcripts remained suppressed in brain and fell to 30% of control values in heart.

Figure 3.8(A-ii) and (B-ii) show the effects of anoxia and dehydration on HIF-1 α protein levels. Anoxia stimulated increased levels in HIF-1 α protein in liver, skin and kidney (by 1.12-, 1.25-, and 1.54-fold, respectively) whereas HIF-1 α levels fell significantly in brain and heart. During recovery from anoxia HIF-1 α remained high in liver and kidney but fell again in skin. Heart HIF-1 α rebounded to control values and brain HIF-1 α levels rose to 1.4-fold higher than control. Dehydration to 40% of total body water lost elevated HIF-1 α protein in liver, brain and kidney by 1.3-, 1.8- and 1.9-fold, respectively, but did not affect the protein in other tissues. After rehydration, HIF-1 α protein returned to control levels in liver whereas the levels remained high in brain and kidney and rose in gut.

3.3.5 Analysis of HIF-1 α protein distribution in frog liver cytoplasmic and nuclear fractions

Western blotting was used to examine HIF-1 α distribution between cytoplasmic and nuclear fractions in wood frog liver (Figure 3.9). In liver from control (5°C acclimated) frogs HIF-1 α was evenly distributed between the subcellular fractions. In liver from 24 h

frozen frogs the distribution had altered and a higher percentage was in the nucleus; the ratio was 58:42 nuclear:cytoplasmic. The elevated HIF-1 α in the nuclear fraction suggests that freezing stimulates the up-regulation of various hypoxia-responsive genes that are under the control of the HIF-1 transcription factor.

3.4 Discussion

3.4.1 HIF-1 α expression in frozen wood frogs

Freezing causes hypoxia in wood frog tissues due to a disruption of blood flow by ice formation within blood vessels. In vertebrates, hypoxia stress typically stimulates the increased production of red blood cells through erythropoiesis (Goldberg, 1988), increases the supply of blood to affected areas by angiogenesis (Shweiki, 1992), or adapts cells for a greater reliance on ATP generation by glycolysis by increasing glucose transporter and glycolytic enzyme expression (Ebert, 1995; Semenza, *et al.*, 1994). Adaptive increases in gene expression stimulated by hypoxia are mediated by an oxygen-sensitive transcription factor, the hypoxia-inducible factor (HIF-1). The active form of HIF-1 is a heterodimer, made up of HIF-1 α and HIF-1 β (or ARNT) subunits. HIF-1 β is quite stable in cells but HIF-1 α is rapidly degraded when oxygen levels are high due to oxygen-dependent hydroxylation of two proline residues that set up the protein for proteolytic degradation. In mammalian systems HIF-1 α is constitutively expressed but its net level is very low due to constant degradation (half-life of HIF-1 α is ~5 min) (Iyer *et al.*, 1998; Fedele *et al.*, 2002). However, when oxygen levels are low, HIF-1 α is stabilized (half-life rises to ~30 min) and the protein accumulates because the rate of degradation is reduced. Thus, HIF-1 α levels, and therefore the activity of the HIF-1 transcription factor,

are mainly controlled at the posttranslational level. In systems studied to date, which are mostly mammalian, there is relatively little evidence that changes in the rate of transcription of the HIF-1 α gene contributes to changing the overall levels HIF-1 α .

The data in this chapter disagree with this last point and suggest that transcriptional control is a significant mechanism for altering HIF-1 α levels in wood frog organs in response to low oxygen stress. Levels of *hif-1 α* transcripts changed significantly (either up or down) in response to freezing, anoxia or dehydration stresses in selected organs. For example, *hif-1 α* transcript levels increased in liver in response to all three of these treatments, levels rose in skeletal muscle under both freezing and anoxia, in kidney under anoxia and dehydration, in skin under anoxia and in gut during freezing. In other instances, *hif-1 α* transcripts were reduced, most notably in brain under anoxia and dehydration. In general, transcript levels recovered to near control values when the stress was removed during thawing, return to an oxygenated atmosphere, or rehydration. Thus, it appears that transcriptional level regulation of *hif-1 α* occurs in response to low oxygen stresses in wood frogs. Although this does not seem to be a regulatory mechanism used extensively in mammalian systems, it may prove to be common in lower animals. There are two main methods of transcriptional regulation: control of the rate of transcription of the gene (the most common method) and control of the stability of mRNA transcripts after transcription. Additional studies will be needed to confirm the mechanism that applies to *hif-1 α* mRNA transcripts. For example, changes in transcription rate under different experimental conditions could be assessed with a nuclear run-off assay.

Freezing, anoxia, and dehydration stresses also increased the levels of HIF-1 α protein in wood frog tissues. For example, protein content rose in liver in response to all

three stresses, increased in kidney under anoxia and dehydration, and rose selectively in other tissues. In general, tissues that showed elevated *hif-1 α* transcripts also showed increased HIF-1 α protein, except for skeletal muscle where transcripts increased under all three stresses but protein only increased during freezing. The correlation between *hif-1 α* transcripts and HIF-1 α protein suggests that net HIF-1 α levels in wood frog tissues may result from both regulation of HIF-1 α synthesis and oxygen-dependent proteolysis. Why both mechanisms would be used is not known. It is interesting, however, that substantial levels of HIF-1 α protein were found in control tissues of frogs compared with the reports in the mammalian literature that HIF-1 α is nearly absent in well oxygenated tissues. Perhaps the oxygen-dependent proteolysis mechanism is not as well developed in amphibians as in mammals, especially at low body temperatures (control frogs were at 5°C) so that another mechanism for controlling the levels of the protein may also be needed.

Both *hif-1 α* transcripts and HIF-1 α protein expression were increased in selected wood frog tissues (e.g. liver, muscle, gut) after 24 h freezing exposure (Figure 3.6). However, the figure also shows that HIF-1 α levels were not elevated in other tissues which indicates that HIF-1 α expression has a tissue-specific character. This suggests that freezing survival in some tissues is aided by the synthesis of protein products of genes that are regulated by HIF-1. Which genes are stimulated by HIF-1 during freezing is not yet known. It is unlikely that genes involved in angiogenesis or erythropoiesis would be up-regulated during freezing and previous studies by our lab have not found changes in the activities of glycolytic enzyme activities during freezing (Storey and Storey, 1984). A key future direction will be to identify the target genes of HIF-1 activation during natural

freezing. It is possible that the glucose transporter isoform GLUT-1 may be one of these. GLUT-1 responds to HIF-1 in mammals and it is possible that the transcription factor may be stimulating an increase in the capacity of tissues for the uptake of the cryoprotectant glucose that is produced and exported by liver. However, this remains to be tested.

Tissue specific differences in the time course of *hif-1 α* transcript expression were also seen (Figure 3.7). All three tissues tested (liver, brain, heart) showed highest levels of transcripts after 2 h of freezing exposure. After 2 h at -2.5°C frogs have considerable ice in their skin and skeletal muscle but the internal organs are not yet frozen. In this situation *hif-1 α* is probably responding to the increasing hypoxia as breathing becomes impaired. With longer freezing, however, the *hif-1 α* response declined in all tissues although remaining significantly elevated in liver (Figure 3.7). This suggests that frog organs become hypoxic quite early during freezing (within 2 h) and that this triggers a response by *hif-1 α* that probably activates some tissue-specific gene responses to hypoxia. With longer freezing *hif-1 α* transcript levels may decline for one of two reasons: (1) the protein products stimulated by *hif-1 α* reach optimal levels, or (2) *hif-1 α* mediated responses to low oxygen are replaced by other metabolic responses when full anoxia develops. For example, hypoxia often stimulates an increase in glycolytic rate that helps to compensate for a shortfall in ATP output by mitochondrial oxidative phosphorylation but in anoxia many animals show a strong suppression of metabolic rate that helps them to survive for a long time without oxygen. Therefore, the data in Figure 3.7 suggest that the *hif-1 α* response is one of the early responses to freezing and is stimulated by declining oxygen tension as ice formation begins to inhibit breathing and tissues become hypoxic.

The results for 2 and 4 hour thawed frogs are also interesting. After 2 h thawing at 5°C some ice still remains in the frogs's body. The core organs (e.g. heart, liver) are melted but heart beat and breathing have not resumed. Hence, low oxygen levels could stimulate the increase in *hif-1α* transcripts seen in all three organs. However, after 4 h thawing at 5°C, all ice is gone and heart beat has resumed. Hypoxic conditions are alleviated and hence *hif-1α* levels fall again.

A redistribution of HIF-1α protein between cytoplasm and nucleus occurred in response to freezing stress in frog liver (Figure 3.9). Approximately one-half of HIF-1α was present in the nucleus in the control situation. Stroka *et al.* (2002) reported that HIF-1 has an important role in tissue homeostasis in normoxic mice and displays an organ specific regulation under systemic hypoxia. A greater percentage of total HIF-1α was found in the nuclear fraction after 24 h of freezing. Since freezing is a low oxygen stress, this indicates that HIF-1α in wood frogs behaves as it does in other animals systems and moves into the nucleus when oxygen is low in order to up-regulate hypoxia-responsive genes. This analysis of HIF-1α protein distribution also demonstrated that HIF-1α protein was present in the nucleus under control conditions in frog liver. This finding agrees with the observations of Jiang *et al.* (1996) that reported that HIF-1α protein was detected in the nucleus under normoxic conditions (Jiang, *et al.*, 1996; Jewell, *et al.*, 2001).

3.4.2 HIF-1α expression in wood frog tissues exposed to anoxia and dehydration

Western blotting and PCR amplification analysis of HIF-1α mRNA and protein levels in wood frog tissues in response to anoxia and dehydration stresses provide other important information about HIF-1α expression patterns in each tissue. Transcript levels of *hif-1α*

were elevated in liver, muscle, skin and kidney of 24 h anoxic frogs and protein was also elevated except in muscle. HIF-1 α protein was also increased in liver, brain and kidney of 40% dehydrated frogs. At this level of dehydration, tissue oxygenation is often affected due to low blood volume and high blood viscosity so the increased HIF-1 α levels in some dehydrating tissues suggests that they are experiencing reduced oxygen availability. The responses to both anoxia and dehydration suggest that HIF-1 mediated changes in gene expression are probably aiding the adaptive responses of frog tissues to low oxygen. Interestingly, both *hif-1 α* transcripts and HIF-1 α protein were reduced in wood frog brain and heart after 24 h anoxia which contrast with the observations of elevated HIF-1 α expression in mammal brain and heart during anoxia (Bunn *et al.*, 1998). Furthermore, we found that HIF-1 α protein was present in very low levels in frog brain and heart during 40% dehydration. The reasons for these low levels are not yet known but might include: (1) HIF-1 α in these organs may up-regulated mainly during hypoxia but not during long term anoxia when other mechanisms such as metabolic rate depression may be more important, or (2) a related hypoxia-inducible factor, HIF-2 α , may be important in these tissues. HIF-1 α and HIF-2 α share 48% amino acid identity (Ema *et al.*, 1997), and both are stabilized and transcriptionally activated by hypoxia (Makino *et al.*, 2001). During hypoxia, HIF-1 α and HIF-2 α are translocated from the cytoplasm into the nucleus (Kallio *et al.*, 1998) and heterodimerize with the constitutively nuclear ARNT to bind to the cognate HRE DNA sequence and induce transcription of target genes containing the core HRE consensus sequence 5' ACGTGC 3' (Guillemin 1997; Epstein *et al.*, 2001; O'Rourke *et al.*, 1999). Therefore, in order to gain further insight into freeze responsive

sensing and signaling mechanisms, we should undertake further studies to analyze HIF-2 activation and stability in frozen wood frog.

3.4.3 Wood frog HIF-1 α sequence structure analysis

The segment of wood frog *hif-1 α* sequence that was retrieved from RT-PCR showed good similarity (68%) with the sequences from *X. laevis*, human and mouse *hif-1 α* . The percent identity was similar for the amino acid sequence, 68 %. Both *X. laevis* and wood frogs are anuran amphibians but the HIF-1 α sequence from *X. laevis* showed a higher identity (~85%) with the mammalian sequences than did the wood frog and this may suggest that the wood frog sequence is modified for some specific circumstances. For example, it may be that some substitutions in the wood frog sequence, compared with the others, may help to stabilize HIF-1 α function at near 0°C, since wood frogs in their natural habitat would spend about half of the year at low body temperatures. On the other hand, both the mammalian and the African clawed frog sequences probably produce proteins that have optimal functionality at higher temperatures (e.g. about 37°C for mammals). The wood frog HIF-1 α sequence showed 20 instances where an amino acid substitution replaced an amino acid that was conserved in all three of the other sequences (Figure 3.2) and some of these substitutions may aid the wood frog protein in functioning at low temperature.

The action of HIF-1 α in cells is determined by a number of specific functional groups within its structure. The degradation of HIF-1 α depends primarily on the hydroxylation of two proline residues in an oxygen-dependent degradation (ODD) domain. A C-terminal transactivation domain (CAD) and N-terminal transactivation

domains regulate HIF-1 α heterodimerization, DNA binding and transactivation under normoxia and stresses (Wang *et al.*, 1995). The bHLH domain located near the N terminus of HIF-1 α is known to be necessary for DNA binding and the two PAS domains are the targets of gene specificity and the regions of ARNT heterodimerization (Fandrey, 2004).

Analysis of the segment of wood frog sequence that was retrieved showed that it was found near the N terminal of the protein; for example, the segment corresponded with amino acids 138 to 250 of the *X. laevis* sequence. The segment contained the two PAS domains that occur near the N-terminal of the full length protein (Figure 3.3). Within these PAS domains, a comparison of the percent identity of amino acid sequences showed a much higher conservation of amino acid residues between wood frog and mammalian sequences than for the protein as a whole. In general, frog HIF-1 α amino acid sequence showed ~85% identity with the region occupied by the PAS domains in the mammal sequences. This high identity confirms the importance of these domains for protein function and suggests that they must be conserved in HIF-1 α in all species for proper function of the protein.

Regulation of the activation of HIF-1 α includes protein stability, phosphorylation (Wang *et al.*, 1995; Salceda *et al.*, 1997), hydroxylation and nuclear localization (Kallio *et al.*, 1998), the interaction with adjacent transcription factors, and coactivator proteins. These regulating activations mainly depend on the specific binding domains and the interactive sites located on HIF-1 α specific structure. Therefore, to further study the role of HIF-1 α in regulating mechanisms that contribute to freezing survival, future studies will be necessary to further discover the full genomic sequence of frog HIF-1 α .

Figure 3.1

A. Multiple nucleotide sequence alignment of *hif-1 α* from wood frogs (*Rana sylvatica*) compared with the sequences from the African clawed frog (*Xenopus laevis*), mouse (*Mus musculus*) and human (*Homo sapiens*), produced using the DNAMAN program. The full wood frog sequence is shown whereas in other sequences dashes (-) replace base pairs that are shared with the wood frog sequence. Periods represent bases that are missing in the alignment. The alignment covers a 340-343 nucleotide segment of the clawed frog, mouse and human cDNA that have full open reading frames of 3710, 3973 and 3678 nucleotides, respectively; Genbank accession numbers are BC043769, NM010431 and U22431 for clawed frog, mouse and human, respectively.

(A)

<i>R. sylvatica</i>	CCTTGCGATCATGAAGAGATTAGAGAGAATCTCAGTTTCA	40
<i>X. laevis</i>	-----c-a-g---tg-a-c-----	40
Mouse	--a--t--c-----g--a--g-----a-tg--t-caca--	40
Human	t-a--t--c-----g--a--g-----a-tg--t-caca--	40
<i>R. sylvatica</i>	GAAATGGTGCAGGAAAAAAGAAGAACAAAGACGCAAGTAC	80
<i>X. laevis</i>	-----gc---c-----agga...-----aca--tc--	77
Mouse	-----cc---tg-g---agg...-----act--ac--	77
Human	-----cctt-tg-----ggt...-----aca--ac--	77
<i>R. sylvatica</i>	AGAACGGGATTTTTTCATGAGGATGAAATGCACCGTCACC	120
<i>X. laevis</i>	---ga-aag---c---c-tc-t-----t--at-a---	117
Mouse	-c-g---agc-----tc-c--a-----g-----c-a--a	117
Human	-c-g--aagc-----tc-c--a-----g--t---c-a--t	117
<i>R. sylvatica</i>	AACAGAGGCCGAACCGTGAATCTCAAATCTGCTACCTGGA	160
<i>X. laevis</i>	-gtc-t--aa-----a-a-g--a--c--a-----	157
Mouse	-g-c-g--ga-g--ga---ca---g--a--a-g----	157
Human	-g-c---aa-----ta---ca-a-g-----a--a-----	157
<i>R. sylvatica</i>	AGGTACTTCACTGCACCTGGCCACGTGAAGGTGTACAACGC	200
<i>X. laevis</i>	----c-----t--a--a---a--cgt--a--tg-taa	197
Mouse	----g-----g-----ta-tc-t--c--tg-ta-	197
Human	----t-g-----a-----a-tc-c--a--tg-ta-	197
<i>R. sylvatica</i>	CTACAATCCGCATGCACTCTGCGGCTACAAAGAACCTCTG	240
<i>X. laevis</i>	tgca--caac--gaac-at--t-----ga-----cc	237
Mouse	-a---g-aac--ac-t-ag--t--g-----ga---a--cc	237
Human	-a---g-aac--ac-t-ag--t--g--t--ga---a--ct	237
<i>R. sylvatica</i>	CTGAATTGCCTGGTGATGATGTGTCAGCCAATCCAGCACC	280
<i>X. laevis</i>	a---ca---a---tg-a--c---g-a--t--t-ct--t-	277
Mouse	a---cg---t-----c---t---g-a--c--t-ct--t-	277
Human	a---cc---t-----c---t---g-a--c--t-ct----	277
<i>R. sylvatica</i>	CCTCCAACATCGACATCCCCTGGACAGCAAGACCTTCCT	320
<i>X. laevis</i>	-a--g--t--t--gt---ct-----	317
Mouse	-g--a--t--t--a--t--tt-a--t-----a--t--	317
Human	-a--a--t--t--a--t--tt-a--t-----t-----	317
<i>R. sylvatica</i>	CAGTCGTCACAGCCTGGATATGA	343
<i>X. laevis</i>	t-----c-----t--c----	340
Mouse	-----a-----	340
Human	-----a-----	340

(B)

Figure 3.2

Multiple amino acid sequence alignment (A) and homology tree (B) for HIF-1 α protein from wood frog (*Rana sylvatica*) compared with African clawed frog (*Xenopus laevis*), mouse (*Mus musculus*), and human (*Homo sapiens*) sequences, produced using the DNAMAN program. Genbank accession numbers are BC043769, NM010431 and U22431 for clawed frog, mouse and human, respectively. The wood frog sequence is shown whereas in other sequences dashes (-) replace amino acids that are shared with the wood frog sequence. Periods represent amino acids that are missing in the alignment. The alignment covers a 113-114 amino acid segment of the proteins that have full lengths of 1226-1324 amino acids. The segments of the Pas-A and Pas-B domains that are present in the wood frog sequence are highlighted.

Figure 3.2(A)

	(PAS-a)	
<i>R. sylvatica</i>	PCDHEEIRENLSFRNGAGKKKNKDASTERDFFMRMKCTVT	40
<i>X. laevis</i>	-----l--m-t---pa--gkeqi.---s--l-----l-	39
Mouse	-----m--m-th---pvr-gkeln.-q-s--l-----l-	39
Human	-----m--m-th---lv--gkeqn.-q-s--l-----l-	39
<i>R. sylvatica</i>	NRGRTVNLKSATWKVLHCTGHVKVYNAYNPHALCGYKEPL	80
<i>X. laevis</i>	s-----i-----mr--dna-nqnh----k-p	79
Mouse	s---m-i-----ih--dtnsnqpq----k-p	79
Human	s---m-i-----ih--dtnsnqpq----k-p	79
	(PAS-b)	
<i>R. sylvatica</i>	LNCLVMMCQPIQHPSNIDIPLDSKTFLSRHSLDM	114
<i>X. laevis</i>	mt-m-vi-e--p-----ef-----	113
Mouse	mt---li-e--p-----e-----	113
Human	mt---li-e--p-----e-----	113

(B)

Figure 3.3

Homology tree prepared with DNAMAN showing the percent identities between the amino acid sequences of the PAS domains of HIF-1a from wood frog, *X. laevis*, mouse and human sequences. The sequences compared were two recognized PAS domains in the frog HIF-1a sequence as highlighted in Figure 3.2 (A).

Figure 3.3

Figure 3.4

Analysis of *hif-1α* mRNA transcript levels in wood frog liver by RT-PCR.

(A) Representative gel showing RT-PCR products from the amplification of *hif-1α* mRNA in extracts from liver of control versus frozen frogs at three different dilutions. Bands were visualized with ethidium bromide.

(B) Representative gel showing RT-PCR products from the amplification of the mRNA of alpha-tubulin, a constitutive gene, from the same extracts.

Histograms shown in subsequent figures present the relative levels of *hif-1α* mRNA in experimental versus control situations, calculated by the equation: $W = ([E_p]/[E_t]) / ([C_p]/[C_t])$ (more details given in section 2.6.).

(A)

Control liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$

24 h frozen liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$

(B)

Control liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

24 h frozen liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

Figures 3.5

(A) Representative Western blot shows protein levels in extracts from skeletal muscle of control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (4 h recovery at 5°C) frogs; equal amounts of protein (15 µg) were loaded in each lane. (A-i) shows the PVDF membrane stained with Coomassie blue; (A-ii) shows HIF-1α immunoblotting. N-1 and N-2 present proteins isolated from two independent experiments.

(B) Representative two-dimensional Poly-Acrylamide Gel Electrophoresis (2D-PAGE) of HIF-1α proteins in liver extracts from frozen (2 h at -2.5°C) wood frogs. Each gel was loaded with 20 µg of protein. Separation in the first dimension was via isoelectrofocusing and the second dimension was SDS-PAGE; proteins were transferred to PVDF membranes before immunoblotting with anti-HIF-1α antibodies. The cross-reacting spot is circled and corresponds to a pI of 5.17 and a molecular weight of approximately 120 kDa, consistent with HIF-1α from other sources.

(B) 2D-PAGE of 2 h frozen frog liver
pI: 3.5→10

M.W (120kDa) →

Figure 3.6.

Effect of 24 h freezing and 4 h thawed recovery on the levels of *hif-1 α* mRNA transcripts and HIF-1 α protein in different tissues of wood frogs. Bands intensities on individual gels and blots (see representative data in Figure 3.4 and 3.5) were quantified using GeneTools. Data for RT-PCR was first normalized against the corresponding tubulin bands for the same samples. Mean values for controls versus frozen groups were tested for significant differences using the Student's t-test and then the ratio frozen:control was plotted. Histograms show mean $\pm$ SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A) Effect of 24 h freezing and 4 h thawing recovery on *hif-1 α* mRNA expression in different frog tissues.

(B) Effect of 24 h freezing and 4h thawing recovery on HIF-1 α protein expression in different frog tissues.

(A)

(B)

Figure 3.7

Relative mean mRNA levels of *hif-1 α* in heart, brain and liver over a time course of freeze (-2.5°C) and thaw exposure ($\pm$ SEM, n=4-5 independent experiments). Band intensities on individual blots were quantified using Genetools and the ratio frozen:control was plotted. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

Figure 3.7

Figure 3.8

(A) Effect of 24 h anoxia exposure at 5°C and 4 h aerobic recovery from anoxia on *hif-1α* mRNA (A-i) and protein (A-ii) expression in different tissues of wood frogs.

(B) Effect of dehydration to 20% of total body water lost at 5°C and full rehydration on *hif-1α* mRNA expression in different tissues of wood frogs (B-i). Effect of 40% dehydration exposure at 5°C and full rehydration on HIF-1α protein expression in different tissues of wood frogs (B-ii).

Bands intensities on individual gels and blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean $\pm$ SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A-i)

(A-ii)

(B-i)

(B-ii)

Figure 3.9

Analysis of HIF-1 α protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots for detection of HIF-1 α proteins in nuclear and cytoplasmic fractions of liver from control (5°C) and 24 h frozen (-2.5°C) wood frogs.

(B) Histogram shows protein level of HIF-1 α in the two fractions, relative to the value for control cytoplasmic HIF-1 α content. Data are n=1. Bands intensities on blots were measured using Genetools, and are relative to control cytoplasmic protein, set to 1.

(A)

(B)

CHAPTER 4

Glucose Regulated Proteins

4.1 Introduction:

This chapter focuses on freezing-induced changes in stress-related proteins of the endoplasmic reticulum (ER). These proteins have never before been examined to determine whether they make contributions to the mechanisms of cellular defense during natural freezing. The ER is an important site of biosynthesis for sterols, lipids, membrane-bound and secreted proteins, and glycoproteins. Approximately one-third of all cellular proteins are synthesized on the membranes of the rough ER (Matlack *et al.*, 1999). Disruption of ER stabilization can directly stimulate apoptosis or cell death. This damage is induced by “ER stresses” including accumulation of unfolded or misfolded proteins, depletion of oxygen, glucose and ATP limitation (Lee, 2001). In mammals, the ER responds to the accumulation of unfolding proteins by triggering the activities of ER specific chaperones that include glucose-regulated proteins (Grps) and other glycoproteins. Grps are so called because they were first identified as being strongly induced when chicken embryo fibroblasts were cultured in glucose free medium (Shiu *et al.*, 1977). The most abundant chaperones on the ER are Grp78 (also called BiP) and Grp94. They play important roles in protein folding and secretion, and can confer protection against cell death by regulating signal transduction and protein translation under a variety of stress conditions (Gazit *et al.*, 1995; Lee, 1987; Yoshida *et al.*, 1998). These proteins have also proven to be markers for diseases that result from misfolding of secretory and transmembrane proteins (Hamman *et al.*, 1998).

The wood frog, *Rana sylvatica*, can survive various environmental stresses including freezing, anoxia and dehydration. All of these cause major metabolic disruption

to cells; for example, extracellular freezing cuts off cell access to both oxygen and blood-borne fuel supplies. Studies in our lab have demonstrated that one aspect of this stress survival is the up-regulation of selected genes and proteins including several unidentified proteins that may address novel functions in freeze tolerance (e.g. FR47, FR10, Li16) (Cai and Storey, 1997; McNally *et al.*, 2002, 2003). Glucose has been identified as the primary cryoprotectant in wood frogs and it plays an important role in preventing intracellular water loss during extracellular ice formation. The very high concentrations of glucose (200-300 mM) accumulated in the frozen state can also help to protect subcellular macromolecules from freeze-associated damage (Storey and Storey, 1983). We wondered whether stress-responsive proteins such as the Grps are also a part of the natural response to freezing stress in wood frogs. In their role as chaperones these could act to minimize the accumulation of unfolded/misfolded proteins under the ischemia and dehydration stresses that accompany freezing and aid the correct production of specific freeze-induced proteins. In this chapter Grp78 and Grp94 were studied. My aim was to quantify the organ-specific responses of these proteins to freezing and related stresses, analyzing both mRNA and protein expression.

4.2 Material and Methods

4.2.1 Primer Design:

Primer design for *grp78* and *grp94* was conducted using Primer Designer as described in Chapter 2. Gene specific primers were created based on the melting temperature, %GC content, and potential for forming dimers or hairpins between primer pairs and/or single primers. Primers were synthesized by Sigma Genosys at Winston Park Drive, ON.

4.2.2 *grp78* and *grp94* primers:

The primers for *grp78* (Forward: 5'-ACCGCATCACGCCGTCTTAT-3' and reverse: 5'-GGCTGGTACAGTAACAAC-3') were created by identifying appropriate consensus sequences from the *grp78* sequences of other species as reported in Genbank (accession numbers in brackets): human (AF188611), mouse (AJ002387) and African clawed frog, *Xenopus laevis* (U62807). These primers were expected to retrieve an approximately 350 bp cDNA sequence in wood frog.

The *grp94* primers (Forward: 5'-CTGTGTCTGTGACATGCAGCAG-3' and reverse: 5'-ATGAAGCTGCAGTGGA-3') were similarly designed from human (AY040226), mouse (J03297) and *X. laevis* (AY187545) nucleotide sequences. The primers were expected to retrieve an approximately 520 bp cDNA sequence.

4.2.3 RT-PCR amplification of *grp78* and *grp94*:

Total RNA was isolated from tissues of control, frozen, anoxic and dehydrated *R. sylvatica*, qualified spectrophotometrically (a ratio A_{260}/A_{280} of 1.6 or higher showed good

quality) and then used for first strand synthesis of cDNA followed by PCR amplification (methods described in Chapter 2). The PCR amplification of both genes was conducted over 37 cycles, with an annealing temperature T_m of 48°C for *grp78* and a T_m of 55°C for *grp94*. PCR products were visualized via electrophoresis on a 1.0% TAE agarose gel. Band intensities were quantified using the SynGene as described in section 2.6. PCR products of the appropriate sizes for each gene were sequenced by Canadian Molecular Research Services (CMRS, Orleans, ON) and analyzed using BLAST and DNAMAN as described in section 2.4 and 2.6.

4.2.4 Western analysis and two-dimensional protein electrophoresis:

Total soluble protein and cytoplasmic vs. nuclear fractions were extracted from *R. sylvatica* tissues as described in section 2.8 and 2.9, respectively. Samples were prepared from wood frogs exposed to different conditions: control (5°C acclimated), frozen (24 h and 2 h at -2.5°C) and thawed (4 h, 5°C), 24 h anoxia (5°C), 4 h aerobic recovery after 24 h anoxia (5°C), 40% dehydration (5°C) and rehydration after 40% dehydration (5°C). Aliquots of 10-20 ug protein were loaded into each lane of SDS gels (protein amount was the same in all wells of any given gel) and electrophoresis and immunoblotting were carried out with Grp78 and Grp94 antibodies, as described as Table 2.1. Coomassie blue and silver staining were used to visualize overall protein patterns and analyze differences between experimental groups. The Student's t-test was used to test for significant differences between stress versus control conditions and then the ratios stress:control were plotted.

4.3 Results:

4.3.1 Analysis of *grp* sequences:

The PCR products derived using *grp78* and *grp94* specific primers were sequenced and compared to the Genbank database located on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The nucleotide and deduced amino acid sequences are shown in Figures 4.1 and 4.2 for *grp78* and Figure 4.11 and 4.12 for *grp94*. The *R. sylvatica* *grp78* sequence that was retrieved from frog liver contained 346 nucleotides and encoded 115 amino acids which is approximately 17-18% of the full length sequence of 639, 655 and 658 amino acids in human, mouse and *Xenopus laevis*, respectively. Both nucleotide and deduced amino acid sequence comparisons showed a high degree of identity with other species and confirmed that the amplified products encoded Grp78. The homology tree for *grp78* showed that the segment of wood frog sequence that was amplified shared 80% identity of nucleotides and 91% identity of amino acids compared with the other three sequences (Figure 4.1 and 4.2).

Amplification using primers for *grp94* produced a sequence from wood frog liver containing 512 nucleotides and encoding 179 amino acids. This represented about 19-22% of full sequence length of 2552, 2759, and 2349 nucleotides in *X. laevis* (AY187545), mouse (J03297), and human (AY040226), respectively. A comparison of nucleotide sequences showed 84% sequence identity between wood frog and *X. laevis* *grp94* and 77% identity with human and mouse sequences (Figure 4.11). Amino acid sequence comparison showed that wood frog Grp94 shared 90% identity with *X. laevis* and 85% identity with human and mouse Grp94 (Figure 4.12). These comparisons

revealed that both wood frog Grp78 and Grp94 share high identity with the proteins of other vertebrate species.

4.3.2. PCR analysis of *grp* mRNA levels:

Total RNA was isolated from different tissues of control (5°C acclimated) versus 24 h frozen wood frogs, using Trizol reagent. The relative quantity of *grp* mRNA present in tissues was assessed with RT-PCR and standardized by monitoring the comparable amplification of a housekeeping gene (alpha-tubulin). Figure 4.3 for *grp78* and Figure 4.13 for *grp94* show the serial dilutions of first strand cDNA and their relative bands of RT-PCR amplification on 1% TAE agarose gels. Figure (A) shows the bands of PCR products using *grp* specific primers, while figure (B) shows the results for alpha-tubulin primers. These serial dilutions confirm that suitable mRNA concentrations were used for PCR amplification. A comparison of gradient intensities of bands on gels shows that mRNA expression levels are different between 24 h frozen and control tissues. The ratio $W = ([Fp]/[Cp]) / ([Ft]/[Ct])$ was used to calculate the relative mRNA levels in frozen versus control tissues (Details shown in section 2.6).

4.3.3 Immunoblot analysis of Grp protein levels:

Two-dimensional polyacrylamide gel electrophoresis was used to confirm the specificity of the Grp78 and Grp94 antibodies and show that each crossreacted with only a single frog protein. For Grp78 testing, total soluble protein was extracted from liver of control (5°C) and 2 h frozen (at -2.5°C) frogs and separated by 2D-PAGE followed by immunoblotting with anti-Grp78 antibodies (Stressgen). Figure 4.4(B) shows Grp78

protein expression in control and 2 h frozen frogs. In both cases, the anti-Grp78 antibody reacted with only one specific spot that corresponded with an estimated molecular weight of ~78 kDa and an isoelectric point (pI) of 5.0, in agreement with published values for the mammal protein on the Pubmed website. A comparison of the intensities of the specific spots in liver extracts from control and 2 h frozen wood frogs suggested that Grp78 protein expression was up-regulated during the 2 h freezing exposure. Figure 4.14(B) shows the comparable analysis of Grp94 protein using extracts from liver of 2 h frozen frogs. The anti-Grp94 antibody crossreacted with a single protein of ~94 kDa and with a pI of 4.7.

Figure 4.4(A) and 4.14(A) show representative SDS-PAGE gels analyzing protein levels of Grp78 in liver and Grp94 in heart of control, 24 h frozen and 4 h thawed frogs. Total protein was isolated from tissues and equal amounts of protein (measured by the Bio-Rad assay) were loaded into each lane. Representative Western blots are shown in Figure 4.4(A-ii) for Grp78 and in Figure 4.14(A-ii) for Grp94, whereas Figures 4.4(A-i) and 4.14(A-i) show Coomassie blue staining of a prominent band on the gels that was used to confirm equal loading of the lanes.

4.3.4. Effect of freezing on *grp* mRNA and Grp protein in wood frog tissues:

The effect of 24 h freezing on *grp78* mRNA levels, as determined from RT-PCR, is shown in Figure 4.5(A) for seven wood frog organs. Band intensities for *grp78* in both control and 24 h frozen situations were first normalized against their corresponding alpha-tubulin bands and then the ratio frozen:control was calculated. Transcript levels were significantly higher ($P < 0.05$) in three organs of 24 h frozen frogs; the ratio

frozen:control was 1.09 ± 0.03 in liver, 1.24 ± 0.14 in brain, and 1.2 ± 0.09 in kidney. However, *grp78* mRNA levels of muscle, gut, heart and skin were unchanged. The effect of freezing on *grp94* transcript levels is shown in Figure 4.15(A). Levels changed significantly ($P < 0.05$) in four organs, the ratio frozen:control transcript levels being 1.21 ± 0.03 in liver, 2.21 ± 0.18 in brain, 0.68 ± 0.07 in heart and 0.74 ± 0.07 in skin. Other tissues showed stable levels of *grp94* mRNA levels over the 24 h freezing.

The effect of 24 h freezing on Grp78 protein levels in wood frog organs is shown in figure 4.5(B). Protein content increased significantly ($P < 0.05$) in three organs during freezing; the ratios frozen: control were 1.45 ± 0.15 in liver, 1.7 ± 0.09 in muscle, and 1.76 ± 0.16 in kidney. Grp78 levels in other organs were unchanged during freezing. Changes in Grp94 protein levels in wood frog organs are shown in Figure 4.15(B). Levels increased significantly ($P < 0.05$) during freezing in two organs; the ratio frozen:control was 1.8 ± 0.18 in liver and 1.38 ± 0.03 in skeletal muscle. Grp94 protein levels also decreased significantly in heart and skin in frozen animals; ratios were 0.54 ± 0.03 for heart and 0.38 ± 0.09 for skin. Grp94 levels remained constant in other organs.

4.3.5. Effect of thawing on *grp* mRNA and Grp protein levels in wood frog tissues:

RT-PCR and Western blot analyses were also used to assess changes in Grps during thawing. Relative mRNA levels in four tissues were assessed in 24 h frozen and 4 h thawed frogs (Figure 4.6A). Levels of *grp78* mRNA decreased significantly ($P < 0.05$) in liver, muscle and heart during thawing recovery but were unchanged in brain. Grp78 protein levels also decreased significantly over 4 h of thawing with significantly reduced levels seen in liver, brain and muscle during thawing recovery, but unchanged levels in

heart (Figure 4.6B). Figure 4.16(A) shows the comparable effects of thawing on relative *grp94* transcript levels and Grp94 protein levels in six organs. During thawing, *grp94* transcript levels decreased significantly in brain and heart compared with levels in 24 h frozen animals while transcripts in other tissues remained stable. Western blot analysis (Figure 4.16B) showed that Grp94 protein levels decreased significantly ($P < 0.05$) in liver, muscle, heart and skin during 4 h of thawing recovery as compared with the frozen value, while protein levels in other tissues including brain and gut were stable.

4.3.6 Analysis of *grp94* mRNA over a time course of freezing:

Figure 4.17 illustrates the changes in *grp94* transcript levels over a time course of freezing for heart, brain and liver. Following standardization against corresponding controls, the mean frozen:control ratio of mRNA levels in heart from animals frozen for 30 min, 2 h, 6 h and 24 h were 0.70 ± 0.11 , 0.59 ± 0.07 , 0.58 ± 0.1 , and 0.68 ± 0.07 compared with controls ($n=4$ independent experiments). In frog brain samples the ratios frozen:control were 2.3 ± 0.16 , 2.44 ± 0.08 , 1.37 ± 0.09 , and 2.21 ± 0.18 , respectively, while in liver the ratios were 0.98 ± 0.18 , 1.96 ± 0.2 , 1.26 ± 0.12 , and 1.21 ± 0.03 , respectively. Hence, the data suggest that *grp94* mRNA expression is rapidly up-regulated in brain and liver during freezing with maximal mRNA levels in 2 h frozen animals.

4.3.7 Studies of protein expression during anoxia and dehydration:

Figure 4.7 shows the effects of 24 h anoxia exposure on *grp78* mRNA and Grp78 protein expression in wood frog liver and muscle. Neither mRNA nor protein levels were

affected by anoxia exposure in either organ. However, Western blotting analysis showed that Grp78 protein levels rose during anoxia exposure in heart and skin (Figure 4.8). Following standardization against the corresponding control and confirmation of equal loading of total protein, the mean increase in Grp78 protein levels during 24 h anoxia was 3.32 ± 0.23 fold (N=4) in heart and 2.09 ± 0.15 fold (N=4) in skin. During aerobic recovery after anoxia exposure Grp78 protein decreased again in both organs and, in addition, Grp78 protein was also strongly reduced in kidney during recovery (Figure 4.8).

Figure 4.18A shows the effects of 24 h anoxia exposure on *grp94* mRNA and Grp 94 protein levels in wood frog organs. Anoxia exposure led to a significant increase in *grp94* mRNA in five organs; the ratio anoxia:control was 1.91 ± 0.08 in brain, 1.81 ± 0.17 in liver, 1.63 ± 0.16 in kidney, 1.74 ± 0.17 in skin and 1.55 ± 0.13 in muscle (Figure 4.18A-1). By contrast, anoxia exposure significantly suppressed *grp94* transcript levels in heart (ratio was 0.54 ± 0.07) and gut (0.59 ± 0.07). Western blotting showed increased levels of Grp94 protein in liver, muscle, brain, heart, skin and kidney of anoxia-exposed frogs (Figure 4.18A-2). Grp94 protein levels increased further during aerobic recovery after anoxia in liver, muscle, skin and brain. Grp94 protein levels remained high in heart during recovery but decreased significantly in kidney when frogs were returned to aerobic conditions.

Cellular dehydration is one of the major consequences when ice forms in extracellular spaces. As a consequence, wood frogs have a high tolerance for wide variations in body water contents and can endure the loss of 50% or more of total body water. Figure 4.8B shows the effects of dehydration to 40% of total body water lost on Grp78 protein levels in wood frog organs. Dehydration affected Grp78 content in only

two organs; levels rose by 3.26 fold (± 0.18 , N=4) in heart and by 2.15 fold (± 0.12 , N=4) in skin. After rehydration Grp78 levels remained high in heart, 3.82 fold ± 0.19 (N=4) higher than controls, but significantly decreased back to control levels in skin.

Figure 4.18B shows the comparable effects of dehydration stress on *grp94* transcripts in wood frog organs. Dehydration to 20% of total body water lost resulted in a significant up-regulation of *grp94* message levels in liver, skin and muscle; ratios of dehydrated:control were 2.24 ± 0.31 , 2.73 ± 0.32 and 1.97 ± 0.22 , respectively. In heart and brain, however, message levels were suppressed during dehydration with ratios of 0.49 ± 0.09 for heart and 0.36 ± 0.06 for brain. Effects of 40% dehydration or rehydration (full water content restored after 40% dehydration) on Grp94 protein levels are shown in Figure 4.18C. Dehydration led to increased protein levels in muscle (1.8 ± 0.12 fold over control), brain (1.42 ± 0.1), heart (1.44 ± 0.09) and skin (1.22 ± 0.02). Muscle Grp94 levels increased further during rehydration and heart levels remained elevated but Grp94 fell in skin, brain and liver during rehydration.

4.3.8 Analysis of Grp expression in frog cytoplasmic and nuclear fractions:

Western blotting was also used to analyze the effect of freezing on Grp78 protein distribution between cytoplasmic and nuclear fractions in frog liver. Figure 4.9A shows representative Western blots and Figure 4.9B shows the relative changes in Grp78 distribution. Freezing stimulated a distinct redistribution of the protein. In the control situation, the relative distribution was about 100:60 cytoplasmic:nuclear. In the 24 h frozen condition, the ratio was about 180:40 cytoplasmic: nuclear. Thus, after 24h freezing, the percentage of Grp78 that was cytoplasmic increased substantially by 80%.

4.4 Discussion

4.4.1 Structural analysis of Grp sequences in *R. sylvatica*

The data from analysis of wood frog *grp* nucleotide and Grp putative amino acid sequences show that wood frog grps (Grp78 and Grp94) share high identity with the sequences of other species, both mammalian and amphibian. Indeed, the proteins are known to be highly conserved across phylogeny. In particular, about 80% nucleotide identity and 90% amino acid identity was seen with the African clawed frog *Xenopus laevis*. This suggests that wood frog grps probably contain the same functional domain(s) as the protein from other species and undoubtedly carry out the same roles *in vivo* in protecting proteins from various stresses that affect the ER.

In mammals, all *grp* promoters contain multiple copies of highly conserved ER stress response elements (ERSEs) with a consensus sequence of 5'-CCAAT(N₉)CCACG-3', a tripartite structure, and with N₉ being a strikingly GC-rich region of 9 bp (Yoshida *et al.*, 1998). The genes for *grp78* and *grp94* in wood frogs probably also contain such consensus ER stress response elements (ERSEs) in the transcriptional binding domains that allow them to respond to transcription factors that signal ER stress. It remains to be determined whether *grp* gene up-regulation during freezing is due to direct activation by a freeze-specific transcription factor or is a consequence of a freeze-associated stress signal (such as hypoxia) that triggers one of the transcription factors that commonly regulate vertebrate *grps*.

Previous studies have reported that Grp78 (alternate names: BiP, p78 or Grp80) has an ATPase catalytic domain in its amino terminus and a peptide-binding domain in its

carboxyl terminus that have been modeled on the crystal structures of the heat-shock protein family members Hsc70 and DnaK, respectively (Flaherty *et al.*, 1990). These domains communicate to regulate the affinity and duration of (poly)peptide binding. Thus, Grp78 can undergo cycles of binding and release, whose duration depends on the rate at which Grp78 undergoes adenine nucleotide exchange (of ATP for ADP) and ATP hydrolysis (Knarr *et al.*, 1995). Grp78 uses the energy from ATP hydrolysis to facilitate protein folding by preventing aggregation of proteins within the ER. Only those polypeptides that are properly folded and assembled in the ER can transit to the Golgi. Other misfolded proteins are transported into the cytosol for degradation by the 26S proteasome (Tsai *et al.*, 2002). The high degree of Grp78 sequence conservation between wood frog and mammals suggests that wood frog Grp78 may have these same functional groups and a similar mode of action as its mammalian relatives.

Grp94 is now the accepted name for a protein that was initially given a wide variety of names in different studies including Grp96/endoplasmic (the ER paralog of Hsp90), Grp90, Grp100, Erp99 and ERP100. In mammalian studies it has been shown that the N-terminal domain of Grp94 contains a nucleotide-binding site and a binding site for geldanamycin, which affects the function of Grp94 in cells. Basu *et al.* (1999) indicated that accessory proteins that bind to these sites on Grp94 regulate its peptide-binding activity through ATP hydrolysis. Wood frog Grp94 nucleotide and amino acid sequences show high identities with other mammalian and amphibian sequences and this suggests that Grp94 binding activities are probably similarly regulated by ATPase activities in frozen cells. Moreover, the C-terminal domain of Grp94 of mammals contains the segment responsible for dimer formation and a C-terminal KDEL sequence,

which is the common retention signal for ER proteins. It is possible that wood frog GRP94 sequences also contain a KDEL retention domain in C terminus.

Under various ER stresses, Grp78 and Grp94 have been shown to have a cooperative relationship. Both can be isolated in a ternary complex with newly synthesized immunoglobulin chains. However, Grp78 preferentially binds an early disulphide intermediate of the Ig light chain and dissociates within a few minutes, whereas Grp94 exclusively binds to fully oxidized molecules and dissociates with a half time of 50 min (Melnick *et al.*, 1994). Lihue *et al.* (1991) have shown that the regulation of Grp94 expression is highly similar and linked to the regulation of Grp78 expression. Deficiency in Grp94 induction also impairs induction of Grp78, while both overexpression of Grp78 and an antisense Grp78 fragment reduce the induction of Grp94 (Lihue *et al.*, 1991). This suggests that Grp78 and Grp94 may also be coordinately expressed in frozen frogs and, indeed, the data for both mRNA and protein up-regulation in response to freezing support this idea for liver, skeletal muscle and brain (Figure 4.5 and 4.15). The coordinated production of these ER molecular chaperones in wood frog may assist in the biosynthesis and processing of freeze-responsive proteins or in the general stabilization of cellular proteins under the stress conditions that occur during freezing (e.g. hypoxia/anoxia, cell volume reduction, elevated ionic strength, energy stress, etc.).

4.4.2 Analysis of grp mRNA and protein levels in different frog tissues under freezing stress:

To characterize the Grp response to freezing stress in wood frogs, *grp* transcript levels and Grp protein expression were analyzed over freeze-thaw courses by RT-PCR and Western blotting. Initial trials using 2D-PAGE confirmed that the mammalian antibodies to Grp78 and Grp94 crossreacted with single frog proteins of appropriate molecular mass and isoelectric point. Therefore, we are confident that the antibodies crossreacted correctly with frog Grp78 and Grp94. The high amino acid sequence similarity seen between frog and mammalian proteins also supports this conclusion. Analysis of *grp78* mRNA levels in frozen tissues showed up-regulation in liver, brain and kidney, while protein also rose in liver and kidney and also in skeletal muscle after 24 h freezing (Figure 4.5). The same pattern of results was seen for Grp94; *grp94* mRNA rose in liver and brain during 24 h freezing and Grp94 protein content was elevated in liver and muscle (Figure 4.15). In both cases muscle Grp protein levels rose without seeing a corresponding increase in mRNA levels which suggests that a post-transcriptional mechanism is involved in controlling Grp protein levels in skeletal muscle.

The coordinated increase in both mRNA and protein levels of both Grps in liver suggests a special importance of these proteins in liver during freezing. Liver is of major importance for the freezing survival of the whole animal. Liver synthesizes and exports the cryoprotectant (glucose) that is taken up by all other organs to protect their cells. Liver also synthesizes a variety of freeze-induced proteins including some that are released into the blood (e.g. fibrinogen) for freeze protection of the whole animal. Because of its high glucose content, liver is the last organ to freeze in the frog and this allows it to continue the synthesis of freeze protective molecules for the longest possible time. Magnetic resonance imaging has also shown that liver is the first organ to thaw

(Rubinsky et al., 1994) and therefore it can rapidly begin metabolic and protein responses that aid cell recovery after freezing stress. The important role of freeze induced protein synthesis by liver suggests that the protein synthesizing machinery of the hepatocytes must be protected and preserved during freeze/thaw from stresses that accompany freezing (e.g. hypoxia, major changes in cell volume and ionic strength). The up-regulation of Grp78 and Grp94 in liver may provide this protection via their actions as chaperones to guide the correct folding of freeze-responsive proteins under the unusual physical and metabolic conditions during freezing.

Apart from liver, other organs showed more limited (or no) responses by Grps to freezing stress. In muscle, Grp78 and Grp94 protein levels increased during freezing but, as mentioned above, this was not accompanied by the elevated mRNA. Oppositely, brain *grp* transcript levels rose during freezing but protein did not. This finding suggests that Grp78 and Grp94 expression in muscle and brain might be mediated at translational levels. Studies have shown that expression of *grp78* mRNA in brain was well correlated with that of vascular endothelial growth factor (VEGF) mRNA under all conditions (Abcouwer *et al.*, 2002) controlled by oxygen tension (Minchenko *et al.*, 1994). Hence, up-regulation of *grp78* expression in frog brain during freezing may be triggered by low oxygen conditions. Moreover, our data on Grp78 protein up-regulation in heart during 24 h anoxia (Figure 4.8A) also support this idea that Grp78 is regulated by oxygen tension. Our additional comparison of the Grp78 protein expression during 24 h anoxia and 40% dehydration in each tissue (Figure 4.8 A & B) showed a coordinated response to both stresses suggests that *grp78* may respond to a common signal. In kidney Grp78 mRNA and protein increased during freezing but Grp94 did not which suggests that the two

proteins may not be coordinately regulated in this organ. The same may be true in heart and skin since Grp78 was unaffected in these two organs but Grp94 protein levels decreased by 50% or more in these organs during freezing.

When frogs were thawed the freeze-induced changes in Grps were generally reversed. In liver of 4 h thawed frogs, both mRNA transcripts and protein levels fell back to or below control levels (Figure 4.6 and 4.16). This further confirms the importance of the Grp response to freezing survival since it was reversed so quickly during thawing. Grp mRNA and protein levels were reduced in muscle during thawing and Grp94 protein was further reduced in heart of thawing frogs. The strong freeze-induced increase in *grp94* mRNA in brain was also reversed during thawing.

Two of the major stresses of freezing on cells are anoxia/ischemia stress because blood flow is cut off to organs by freezing and dehydration stress caused by water flowing out of cells to join extracellular ice masses. Studies in our lab have shown that several of the metabolic and gene expression responses to freezing are actually triggered by either the anoxia or the dehydration stresses. For example, the synthesis of glucose as a cryoprotectant in wood frog liver is triggered by both freezing and dehydration but not by anoxia. Up-regulation of some freeze responsive genes in liver is also triggered by dehydration (e.g. fibrinogen, FR10) whereas other genes respond to both freezing and anoxia. By determining whether Grp genes respond to anoxia or dehydration stresses, we can get an indication of why they are up-regulated during freezing.

The results for Grp78 showed that anoxia did not stimulate either mRNA or protein expression in liver and muscle (Figure 4.7 and 4.8). This suggests that low oxygen is not the major trigger for the freeze up-regulation of Grp78 in these organs.

Similarly, dehydration to 40% of total body water lost did not increase Grp78 protein in these organs. This suggests that the response by Grp78 to freezing in these organs is not mediated by either anoxia or dehydration stresses but has another trigger. Oppositely, both anoxia and dehydration stresses caused strong increases in Grp78 protein in heart and skin although freezing did not affect Grp78 in these organs.

Both anoxia and dehydration exposures increased *grp94* transcript levels in liver but protein did not increase significantly while under these stresses (Figure 4.18) although Grp94 levels rose during recovery from anoxia exposure. This suggests that an additional factor must be involved in promoting Grp94 protein synthesis during freezing. Transcript levels of *grp94* responded strongly to anoxia in four other organs (brain, kidney, skin and muscle) and to dehydration in skin and muscle and this resulted in increased Grp94 protein levels under the stress and/or recovery conditions. The increase in *grp94* transcripts during anoxia in brain but suppression during dehydration suggests that the strong *grp94* transcript response to freezing in brain is stimulated by low oxygen but whereas Grp94 protein accumulated under anoxic conditions in brain, it did not during freezing. Similarly, anoxia stimulated an increase in *grp94* transcripts and protein in kidney but neither freezing nor dehydration affected transcript or protein levels which indicates that Grp94 is under control by oxygen levels in kidney. Both stresses resulted in Grp94 accumulation in muscle and skin suggesting that either stress might trigger the accumulation of Grp94 in muscle during freezing.

4.4.3 Analysis of the relationship between *grp78* and *grp94* in transcription and translation levels:

Analysis of the relationship between *grp78* and *grp94* transcription and translation levels in frog tissues under stresses (freezing, anoxia and dehydration) demonstrated that the changes of frog mRNA levels also generally led to changes in protein levels. For example, in frog liver, freeze exposure resulted in increased *grp78* mRNA levels and protein also rose. During anoxia, however, liver *grp78* transcript levels were unchanged and so was its protein. This suggests that transcriptional activation is responsible for changes in the translation status of ER-resident chaperones. Increase of transcriptional activation is typically achieved by stimulation from transcription factors, proteins that bind specific DNA sequences in the promoter or enhancer regions of ER-genes (Pahl, 1999). The candidate transcription factors that may control *grp* expression in wood frog organs are ATF4, ATF6 (a bZIP transcription factor), XBP-1 (an X box binding protein), and nuclear factor κ B (NFkB). These transcription factors promote *grp* transcription in other systems. The genes for *grp78* and *grp94* share some common binding domains in their promotor regions (Li *et al.*, 2000). Yoshida *et al.* (1998) proposed that ATF6 could constitutively induce the *grp* promoter in an ERSE-dependent manner whereas ATF4, a transcription factor whose translation is up-regulated by the PERK-eIF2 α pathway, could activate the *grp78* promoter independent of the ERSE because the ATF4-activating site is localized to an ATF/CRE sequence upstream of the ERSEs (Luo *et al.*, 2003; Lee, 2001). In addition, current studies have demonstrated that a SREBP pathway also regulates mammal *grps* (Haze *et al.*, 1999). SREBPs are broken at the two sites in a sequential manner, allowing entrance of the N-terminal fragment into the nucleus. This released N-terminal fragment contains all of the functional domains necessary for active transcription factors such as DNA-binding (basic helix-loop-helix), dimerization

(leucine zipper), and transactivation (acid blob) domains and thus enhances the transcription of target genes in the nucleus (Haze *et al.*, 1999). Obviously, one goal of our future studies will be to determine which signal transduction pathways are the most important in the activation of *grp* transcription in wood frog organs in response to freezing stress.

4.4.4 Conclusions

Our lab has previously demonstrated that overall transcription rate decreases in freeze-exposed frogs, thus, leading to a reduction of overall protein translation and a stable reduction in the activity of the transcriptional/ translational machinery of cells is one of keys to long term survival under energy-stressed states such as freezing (Storey 2001). However, despite an overall decrease in protein biosynthesis, our data show that the synthesis of selected proteins, such as Grp78 and Grp94 in some organs, is enhanced during freezing.

Two important questions remain about Grps in freeze tolerance: (a) what factors cause Grp induction, and (2) why are Grps induced under freezing, anoxia and dehydration stresses. Mammalian cells experience a stress response resulting in the specific activation of the *grp* promoter (Gazit *et al.*, 1999). Several transcription factors are known that can activate *grp* activities including ATF6, ATF4, XBP-1 and NFkB. Moreover, mammalian cells have gained versatility over the control of *grp* expression by evolving three different *cis*-acting elements (ERSE, UPRE, AARE) and three different signaling pathways (ATF6, IRE1/XBP1 and PERK/ATF4) (Haze *et al.*, 1999). However,

which of these systems mainly responds to freezing in wood frog remains to be determined.

Our future studies will focus on discovering the regulation of the *grp* genes including the identification of the ERSE domains located on their promoters, Grp interactions with other ER chaperones, and their protein functions with respect to cell survival during freezing stress.

Figure 4.1

- A. Multiple nucleotide sequence alignment of *grp78* from wood frog compared with the sequences from the African clawed frog (*Xenopus laevis*), mouse (*Mus musculus*) and human (*homo sapiens*), produced using the DNAMAN program. The full wood frog sequence is shown whereas in other sequences dashes (-) replace nucleotides that are shared with the wood frog sequence. Periods represent bases that are missing in the alignment. The alignment covers a 344-347 nucleotide segment of the clawed frog, mouse and human cDNAs that have full open reading frames of 2470, 2282 and 1917 nucleotides, respectively; Genbank accession numbers of the *grp78* sequences of these species are U62807, AJ002387 and AF188611.
- B. Homology tree prepared with DNAMAN showing the percent identities between the nucleotide sequences of *grp78* from wood frog, *X. laevis*, mouse and human.

Figure 4.1. (A)

<i>R. sylvatica</i>	ACCGCATCACGCCGTCTTATGTAGCTTTTACTCCAGAAGG	40
<i>X. laevis</i>	----t-----a--c--g-----g--a-----c-----	40
Mouse	-----g-----g--c--c-----t-----	40
Human	-----c-----c--c--c-----t-----	40
<i>R. sylvatica</i>	TGAACGTTTGATTGGAGATGCTGCCAAGAATCAGTTGACT	80
<i>X. laevis</i>	---g---c-c-----a--a--c---c-t--a	80
Mouse	g--g---c-----c-----g-----c--ac-c--g	80
Human	g-----c-----c-----c-----c--c--c	80
<i>R. sylvatica</i>	TCCAATCCTGAAAATACAGTGTGTTGATGCCAAGCGTCTGA	120
<i>X. laevis</i>	--t-----a--g--c--c-----a-----	120
Mouse	-----c--c--g--c--g--c--c-----c--c--	120
Human	-----c--c--g--c--g--c-----c-----g--c--	120
<i>R. sylvatica</i>	TTGGCCGCATTTGGAATGACCCATCTGTACAACAGGATGT	160
<i>X. laevis</i>	-----ca-----t-----t--g---ca-	160
Mouse	-c--a---c-----t--g--g--g---ca-	160
Human	-c-----cg-----g-----g--g---ca-	160
<i>R. sylvatica</i>	CAAGTATCTGCCTTTTAAGGTTGTTGACAAGAAGACCAAA	200
<i>X. laevis</i>	a-----c-----a-----aa-----g-----a--t---	200
Mouse	-----tct---a--c---g---a---a--t---	200
Human	-----tct---g--c---g---a---a--t---	200
<i>R. sylvatica</i>	CCCTATATAGAAGTTGATGTT...GGTGATCAGAGAAAGA	237
<i>R. sylvatica</i>	--t--c--t-----c--a--gga-a-...--tg--a-	237
Mouse	--g--c--tc-----a--gga---gg--a--cc---	240
Human	--a--c--tc-----a--gga---gg--a--c-----	240
<i>R. sylvatica</i>	CCTTTGCTCCAGAAGAAATCTCTGCTATGGTTCTCACTAA	277
<i>X. laevis</i>	-t-----c-----g--t-----g--agtg--	277
Mouse	-a-----c-----t-----c-----	280
Human	-a-----t-----t-----c-----	280
<i>R. sylvatica</i>	GATGAAAGAGACTGCTGAGGCGTATCTTGCCAAATATAGG	317
<i>X. laevis</i>	-----g--a-----a-----t--ct-g--..c-g-a---	315
Mouse	a-----g-----t-g--..--aga---	318
Human	a-----a--c-----t--t-g--..--aga---	318
<i>R. sylvatica</i>	TTACACATGCTGTTGTTACTGTACCAGCC	346
<i>X. laevis</i>	---t-----c-----t--t	344
Mouse	---c-----a-----t	347
Human	---c-----a-----	347

(B)

Figure 4.2

Multiple amino acid sequence alignment (A) and homology tree (B) for GRP78 protein from wood frog compared with mouse (*Mus musculus*), human (*Homo sapiens*) and African clawed frog (*Xenopus laevis*) sequences produced using the DNAMAN program. Genbank accession numbers are BAA11462, AAF13605, and AAB08760 for mouse, human and *X. laevis*, respectively. The wood frog sequence is shown whereas in other sequences dashes (-) replace amino acids that are shared with the wood frog sequence. Periods represent amino acids that are missing in the alignment. The alignment covers 115-116 amino acid segments of the proteins that have full lengths of 639-658 amino acids.

Figure 4.2(A)

<i>R. sylvatica</i>	DRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDKRL	40
<i>X. laevis</i>	n-----	40
Mouse	n-----	40
Human	n-----	40
<i>R. sylvatica</i>	IGRIWNDPSVQQDVKYLPFKVVDKKTTPYIEVDVGDQR.K	79
<i>X. laevis</i>	---t-----i-----ie-----i---m.-	79
Mouse	---t-----i-f-----e-----q--i-ggqt-	80
Human	---t-----i-f-----e-----q--i-ggqt-	80
<i>R. sylvatica</i>	TFAPEEISAMVLTKMKETAAYLGKYVTHAVVTVPA	115
<i>X. laevis</i>	-----v-----rk-----	115
Mouse	-----k-----	116
Human	-----k-----	116

Figure 4.3

Analysis of *grp78* mRNA transcript levels in wood frog liver by RT-PCR

(A) Representative gels showing RT-PCR products from amplification of *grp78* mRNA in extracts of liver from control versus frozen frogs. Amplification was done with four different dilutions. Bands were visualized with ethidium bromide.

(B) Representative gel showing RT-PCR products of the amplification of the mRNA for alpha-tubulin, a constitutively active gene, from the same extracts.

The relative *grp78* mRNA transcript levels of frozen versus control were calculated by the equation: $W = ([Ep]/[Et])/([Cp]/[Ct])$ using the band intensities of the $[10^{-2}]$ cDNA dilution (more details explained in section 2.6).

(A)

Control liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

24h frozen liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

(B)

control liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

24h frozen liver

$[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

Figures 4.4

(A) Representative Western blots show Grp78 protein levels in tissue extracts from control (5°C acclimated), frozen (24 h at -2.5°C) and recovered (4 h thaw at 5°C) frog liver; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane was stained with Coomassie blue; (A-ii) shows Grp78 immunoblotting. N-1, n-2 and n-3 represent proteins isolated from three independent experiments.

(B) Representative two-dimensional PolyAcrylamide Gel Electrophoresis (2D-PAGE) of Grp78 proteins in liver extracts from control (5°C)(B-i) and frozen (2 h at -2.5°C)(B-ii) wood frogs. Each gel was loaded with 20 µg of protein. Separation in the first dimension was via isoelectrofocusing and the second dimension was SDS-PAGE; proteins were transferred to PVDF membranes before immunoblotting with anti-Grp78 antibodies. The cross-reacting spot is circled and corresponds to a pI of 5.0 and a molecular weight of 78 kDa, consistent with Grp78 from other sources.

(A-i) Control 24h frozen 4h thawing
 n-1 n-2 n-3 n-1 n-2 n-3 n-1 n-2 n-3

(A-ii) Control 24h frozen 4h thawing
 n-1 n-2 n-3 n-1 n-2 n-3 n-1 n-2 n-3

(B-i) 2D-PAGE of liver extract from control frog

pI: 10→3.5

pI: 5.0

(B-ii) 2D-PAGE of liver extract from 2 h frozen frog

pI: 10→3.5

pI: 5.0

Figure 4.5.

Effect of 24 h freezing exposure on the levels of *grp78* mRNA transcripts and Grp78 protein in different tissues of wood frogs. Bands intensities on individual gels and blots (see representative data in Figure 4.3 and 4.4) were quantified using GeneTools. Data for RT-PCR was first normalized against the corresponding tubulin bands for the same samples. Mean values for control versus frozen groups were tested for significant differences using the Student's t-test and then the ratios frozen:control were plotted. Histograms show mean $\pm$ SEM, n=4-5 independent determinations. Stars show frozen values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A)

(B)

Figure 4.6.

Effect of 24 h freezing and 4h thawing (recover) exposure on the levels of *grp78* mRNA transcripts and Grp78 protein in different tissues of wood frogs. Bands intensities on individual gels and blots were quantified using GeneTools. Data for RT-PCR was first normalized against the corresponding tubulin bands for the same samples. Histograms show mean $\pm$ SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A) Effect of 24 h freezing and 4 h thawing recovery frog on *grp78* mRNA expression in different frog tissues.

(B) Effect of 24 h freezing and 4 h thawing recovery frog on Grp78 protein expression in different frog tissues.

(A)

(B)

Figure 4.7

Effect of 24 h anoxia exposure at 5°C on liver and muscle *grp78* mRNA (A) and Grp78 protein (B) expression of wood frogs.

Bands intensities on individual gels and blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean $\pm$ SEM, n=4-5 independent determinations.

(A)

(B)

Figure 4.8

(A) Effect of 24 h anoxia exposure at 5°C and 4 h aerobic recovery after anoxia on Grp78 protein expression in different organs of wood frogs.

(B) Effect of 40% dehydration at 5°C and full rehydration on Grp78 protein expression in different organs of wood frogs.

Bands intensities on individual blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean $\pm$ SEM, n=4 independent determinations. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A)

(B)

Figure 4.9

Analysis of Grp78 protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots for detection of Grp78 protein in nuclear and cytoplasmic fractions of liver from control (5°C) and 24 h frozen wood frogs.

(B) Histogram shows protein level of Grp78 in the two fractions, relative to the value for control cytoplasmic Grp78 content. Data are n=1. Bands intensities on individual blots were measured using Genetools, and are relative to control cytoplasmic protein, set to 1.

(A)

(B)

Figure 4.11

A. Multiple nucleotide sequence alignment of *grp94* from wood frog compared with the sequences from the African clawed frog (*Xenopus laevis*), mouse (*Mus musculus*) and human (*Homo sapiens*), produced using the DNAMAN program. The full wood frog sequence is shown whereas in other sequences dashes (-) replace base pairs that are shared with the wood frog sequence. Periods represent bases that are missing in the alignment. The alignment covers a 506-512 nucleotide segment of the clawed frog, mouse and human cDNA that have full open reading frames of 2552, 2759 and 2349 nucleotides, respectively. Genbank accession numbers are AY187545, J03297 and AY040226 for clawed frog, mouse and human, respectively.

B. Homology tree prepared with DNAMAN showing the percent identities between the nucleotide sequences of *grp94* from wood frog, *X. laevis*, mouse and human.

Figure 4.11. (A)

<i>R. sylvatica</i>	CTGCTGCATGTCACAGACACAGGCATTGGCATGACAAAGG	40
<i>X. laevis</i>	a-----a-a--t--t--t-----t-----	40
Mouse	-----g--tg-a--a----t-ga-	40
Human	-----c--tg-a--a----c-ga-	40
<i>R. sylvatica</i>	AGGAGCTAGTGAAGAATTTGGGAACGATTGCTAAATCTGG	80
<i>X. laevis</i>	----a-----a-----t-----c--g----	80
Mouse	-----t-g--t--a--c-c--c--c--a--c-----	80
Human	-a---t-g--t--a--cc-t--t--c--a--c-----	80
<i>R. sylvatica</i>	AACAAGTGAATTTTGAACAAGTTTACAGAAGTCCAGGAT	120
<i>X. laevis</i>	---t-----c---g---c---t---c---a--a	120
Mouse	-----c--g-----a-----aa-g-----ct--a--a	120
Human	g-----c--g-----a-----aa-g--t---ca-----a	120
<i>R. sylvatica</i>	TCTGCCCAGTCTACCTCTGAGCTGATTGGACAGTTTGGTG	160
<i>X. laevis</i>	ga--g-----a-----c-----	160
Mouse	ga--gt-----a-----a-----c-----	160
Human	ga--g-----gt-t-----at-----c-----	160
<i>R. sylvatica</i>	TTGGCTTCTATTCTGCCTTCCTTGTGCGACCGTGTAAT	200
<i>X. laevis</i>	---t-----t---a-g-----	200
Mouse	-c--t--t-----a--a--taag--c--	200
Human	-c--t-----c-----a--a--taag--t--	200
<i>R. sylvatica</i>	TGTGACCTCAAAACACAACAATGGCACACAGCACATCTGG	240
<i>X. laevis</i>	-----t-----g-----t--c-at-----	240
Mouse	---c--a--g-----at--c-----	240
Human	---c--t-----c-at--c-----	240
<i>R. sylvatica</i>	GAATCTGATTCCAATGAATTCTTTGTGACTGAAGATCCTC	280
<i>X. laevis</i>	-----c-----a-----g-----c-----t--c---	280
Mouse	-----a--c-----c---a-t--ct--c--aa	280
Human	--g-----c-----t-c---a-t--ct--c--aa	280
<i>R. sylvatica</i>	GAGGTGACACATTGGGAAGAGGAACAACAATAACCTTGGT	320
<i>X. laevis</i>	-t-----c-----cc-t---t---c--t--ac---	320
Mouse	---aa-----c-a--tc-t-----t--tc-t--	320
Human	---aa-----tc-a---c-g-----g-----t---c-t--	320
<i>R. sylvatica</i>	TTTGAAAGAAGAGGCAACTGATTATCTTGAATTGGAAACC	360
<i>X. laevis</i>	gc-c-----g-----c-----gc-t-----	360
Mouse	c--a-----a--t-----c-----c--a	360
Human	c--a-----a--t-----c-----t--a	360
<i>R. sylvatica</i>	ATTAAGAACCTGGTCCGGAATATCTCAGTTTATCAACT	400
<i>X. laevis</i>	g---a-----a--ga-----c-----t--	400
Mouse	-----a--t--t--a---g--c-----c-----	400
Human	-----a--t--c---aaa-----a-----c--a---	400
<i>R. sylvatica</i>	TCCCTATTTATGTTTGGAGTAGCAAGACTGAGACTGTAGA	440
<i>X. laevis</i>	-----c--g-----c-----a-----	440
Mouse	-t--c--c--c--g-----a-----t--	440
Human	-t-----a-----c-----a-----t--	440
<i>R. sylvatica</i>	AGAGCCCATCGAT...GAAGAGGATGCTAAAGAGGAGGAA	477
<i>X. laevis</i>	g-----c-t---...-----a-----g	471
Mouse	g-----t-g--agaa--t--a-ca--a-----a--a--	480
Human	g-----g--ggaa-----a-ca--c-----a--a--	480
<i>R. sylvatica</i>	AAAGATGAGGATGCTGATGATGAAGCTGCAGTGGA	512
<i>X. laevis</i>	-----a--ca-----a-----t--	506
Mouse	g---at.....-----a--	509
Human	g---at.....-----a--	509

Figure 4.11 (B)

Figure 4.12

Multiple amino acid sequence alignment (A) and homology tree (B) for Grp94 protein from wood frog compared with mouse (*Mus musculus*), human (*Homo sapiens*) and African clawed frog (*Xenopus laevis*) sequences, produced using the DNAMAN program. Genbank accession numbers are AY187545, J03297 and AY040226 for clawed frog, mouse and human, respectively. The wood frog sequence is shown whereas in other sequences dashes (-) replace amino acids that are shared with the wood frog sequence. Periods represent amino acids that are missing in the alignment. The alignment covers 168-170 amino acid segments of the proteins that have full lengths of 783-919 amino acids. The *R. sylvatica* Grp94 protein showed 90% amino acid identity with the *X. laevis* protein.

Figure 4.12(A)

<i>R. sylvatica</i>	LLHVTDTGIGMTKEELVKNLGTIAKSGTSEFLNKFTEVQD	40
<i>X. laevis</i>	m--i-----s-l--a-e	40
Mouse	-----v--r-----m--a-e	40
Human	-----v--r-----m--a-e	40
<i>R. sylvatica</i>	SAQSTSELIGQFGVGFYSAFLVADRVIVTSKHNNGTQHIW	80
<i>X. laevis</i>	dg-----d----	80
Mouse	dg-----k-----d----	80
Human	dg--s-----k-----d----	80
<i>R. sylvatica</i>	ESDSNEFFVTEDPRGDTLGRGTTITLVLKEEATDYLELET	120
<i>X. laevis</i>	-----d-----s-----	120
Mouse	-----s-ia---n-----s---d-	120
Human	-----s-ia---n-----s---d-	120
<i>R. sylvatica</i>	IKNLVRKYSQFINFPIYVWSSKTETVEEPIDEEDAKEEEK	160
<i>X. laevis</i>	v-----l---e---.-	158
Mouse	-----l---eaak-e	159
Human	----k-----m.--eeak-e	159
<i>R. sylvatica</i>	DEDADDEAAV	170
<i>X. laevis</i>	---t-e----	168
Mouse	k-es-----	169
Human	k-es-----	169

(B)

Figure 4.13

Analysis of *grp94* mRNA transcript levels in wood frog liver by RT-PCR

- (A) Representative gels showing RT-PCR products from amplification of *grp94* mRNA in liver extracts at different dilutions, comparing control versus 24 h frozen frogs. Bands were visualized with ethidium bromide.
- (B) Representative gel showing RT-PCR products of the amplification of mRNA of alpha-tubulin, a constitutive gene, from the same extracts.

The relative *grp94* mRNA transcript levels of frozen versus control were calculated by the equation: $W = ([Ep]/[Et])/([Cp]/[Ct])$ using the band intensities of the $[10^{-2}]$ cDNA dilution (details explained in section 2.6).

(A)**Control liver** $[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$ **24 h frozen liver** $[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$ **(B)****Control liver** $[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$ **24 h frozen liver** $[10^{-1}]$ $[10^{-2}]$ $[10^{-3}]$ $[10^{-4}]$

Figures 4.14

(A) Representative Western blot shows protein levels in tissue extracts from heart of control (5°C acclimated), frozen (24 h at -2.5°C) and recovered (4 h thawed at 5°C) wood frogs; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane stained with Coomassie blue; (A-ii) shows Grp94 immunoblots. N-1 and N-2 represent proteins isolated from two independent experiments.

(B) Representative 2D-PAGE of liver extract from a 2 h frozen frog immunoblotted with anti-Grp94 antibody. The gel was loaded 20 µg of protein. Separation in the first dimension was via isoelectrofocusing and the second dimension was SDS-PAGE; proteins were transferred to PVDF membranes before immunoblotting with anti-Grp94 antibodies. The cross-reacting spot is circled and corresponds to a pI of 4.7 and a molecular weight of ~96 kDa, consistent with Grp94 from other sources.

Figure 4.14

(B) 2D-PAGE of liver extract from 2 h frozen frog
 pI: 3.5→10

M.W (94kDa)

Figure 4.15.

Effect of 24 h freezing exposure on the levels of *grp94* mRNA transcripts and Grp94 protein in different tissues of wood frogs. Bands intensities on individual gels and blots (see representative data in Figure 4.13 and 4.14) were quantified using GeneTools. Data for RT-PCR was first normalized against the corresponding tubulin bands for the same samples. Values for experimental (frozen) groups are expressed relative to controls that are set to 1. Histograms show mean $\pm$ SEM, n=4-5 independent determinations. Stars show frozen values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A)

(B)

Figure 4.16.

Effect of 24 h freezing and 4 h thawing recovery on the levels of *grp94* mRNA transcripts and Grp94 protein in different tissues of wood frogs. Bands intensities on individual gels and blots were quantified using GeneTools. Data for RT-PCR was first normalized against the corresponding tubulin bands for the same samples. Mean values for controls versus experimental groups were tested for significant differences using the Student's t-test. Histograms show mean $\pm$ SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A) Effect of 24 h freezing and 4h thawing recovery frog on *grp94* mRNA expression in different frog tissues.

(B) Effect of 24 h freezing and 4h thawing recovery frog on Grp94 protein expression in different frog tissues.

(A)

(B)

Figure 4.17

Histogram shows the relative mean mRNA level of *grp94* in heart, brain and liver over a time course freeze exposure (mean $\pm$ SEM, n = 4-5 independent determinations). Bands intensities on individual blots were measured using Genetools, and the ratio frozen:control was plotted. Stars show frozen values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

Figure 4.17

Figure 4.18

(A) Effect of 24 h anoxia exposure at 5°C on *grp94* mRNA (A-1) and Grp94 protein (A-2) expression multiple tissues of wood frogs.

(B) Effect of 20% dehydration at 5°C on *grp94* mRNA expression in tissues of wood frogs.

(C) Effect of 40% dehydration at 5°C and rehydration recovery on Grp94 protein expression in tissues of wood frogs.

Histograms show mean $\pm$ SEM, n = 4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, as determined by the Student's t-test, $P < 0.05$.

(A-1)

(A-2)

(B)

(C)

CHAPTER 5

Heat Shock Proteins (HSPs)

5.1 Introduction:

Diverse stresses including heat, cold, hemodynamics, mutant proteins, and oxidative stress can produce multiple injuries to cells that can ultimately affect protein structure and function. Cells typically respond to such stresses with a rapid shutdown of the synthesis of most proteins and with a dramatic transient increase in the synthesis of a small set of proteins. Current studies have identified these stress-induced proteins as heat shock proteins (Hsps), glucose regulated proteins (Grps) and transcription factors (TFs), which are mainly located on mitochondria, endoplasmic reticulum (ER) and nuclei in cells, respectively. Hsps as molecular chaperones, alone or in cooperation with "partner" chaperones (e.g. other ER chaperones and mitochondrial chaperones), actively participate in the rearrangement of cellular processes including cytoprotection, correct folding and assembly of proteins, transport of proteins to specific intracellular locations, and protein degradation. These cytoprotective stress proteins decrease or neutralize the deleterious effects of various stresses by preventing inappropriate intra- and intermolecular interactions of polypeptide chains that can lead to protein denaturation. Accumulating evidence has indicated that the main functions of molecular chaperones (Hsps) in cells are to maintain polypeptide chains in an appropriate conformation suitable for translocation across organelle membranes, actively trigger stress-regulated mechanisms and other cytoprotective stress proteins; and to assist in degrading toxic metabolites by promoting ubiquitination and proteasome lysis (Chiang *et al.*, 1989; Knowlton, 1995) .

The Hsps are a large family of proteins that are grouped by their different sizes, including (1) the small Hsps of 10-30 kDa, (2) the 40-60 kDa Hsps, (3) the 70-90 kDa Hsps, and (4) the Hsps greater than 100 kDa. Currently, using cDNA array and 2D-

PAGE technology, our lab has identified and demonstrated that many Hsps (e.g. Hsp27) and their associated co-chaperones (Grp78, Grp94 discussed in Chapter 4) are induced and play an important role in supporting natural freeze tolerance in wood frog tissues during freezing stress. Therefore, we predict that other heat shock proteins (Hsps) such as hsp110, hsc70, hsp60, hsp40 and hsp10 may play multiple roles in cellular survival in wood frogs during severe freezing, anoxia and dehydration stresses. Other studies have demonstrated that hsp60 and hsp72/73 play a cardioprotective role in the heart of painted turtles during hypoxia or ischemia and recovery from these stresses (Chang *et al.*, 2000). They may interact with other Hsps such as Hsp10, Hsp40 and Hsp110. Therefore, I hypothesized that wood frog tissues might have high expression levels of Hsps and that these might be further up-regulated under stress. These Hsps alone or in cooperation with other chaperones may represent a major stress-adaptive mechanism in wood frogs. The major functions of these Hsps have been identified in mammals. However, their roles in wood frog tolerance of stresses associated with winter low temperature survival are not yet known.

The present chapter assesses the changes in protein expression of heat shock proteins Hsp10 (Cnp10), Hsp40, Hsp60, Hsp72/73(Hsc70) and Hsp110 under multiple physiological stresses (24 h freezing, 24 h anoxia, 40% dehydration) and recovery from these stresses using western blotting. Furthermore, the distribution of Hsp expression in wood frog liver cytoplasm and nuclear fractions was also assessed.

5.2 Materials and methods:

5.2.1 Animal and tissue samples:

The experimental groups of frogs used in the studies reported in this chapter are: (a) controls acclimated to 5°C, (b) frozen for 24 h at -2.5°C, (c) thawed for 4 h at 5°C after 24 h frozen, (d) 24 h anoxia exposure at 5°C, (e) 4 h aerobic recovery from anoxia after 24 h anoxia, (f) frogs dehydrated to 40% of total body water lost at 5°C, and (g) frogs fully rehydrated at 5°C after 40% dehydration. The detailed experimental protocols were described in section 2.1. All excised tissues were frozen in liquid nitrogen and then stored at -80°C before experiments.

5.2.2 Western blotting and 2-dimensional protein electrophoresis:

Total soluble proteins and cytoplasm/nuclear proteins were extracted from wood frog tissues as described in section 2.8 and 2.9, respectively. Protein concentrations in each sample were measured spectrophotometrically as described in section 2.8.3. Protein samples were mixed with sample buffer (1:1 v:v) 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. Aliquots of 10 ug soluble protein were used in each lane for western blotting and 20 ug was used for 2D-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and probed with Hsps antibodies (see Table 2.1). The band intensities on membranes were quantified using the Genetools program, described in section 2.6. Coomassie blue or silver staining were used to visualize total protein patterns. Protein levels in stress versus control samples were tested for significant differences using the Student's t-test and then ratios stressed:control were plotted as histograms showing mean $\pm$ SEM, n=4-5 independent determinations.

5.3 Results:

5.3.1 Hsp110 and stress survival in wood frogs:

Two-dimensional PAGE followed by immunoblotting showed that the mammalian antibodies used crossreacted with a frog liver protein of the correct molecular weight (~105 kDa) and the appropriate pI value of 5.3 (Figure 5.1B). Therefore, I am confident that the antibodies were cross-reacting with the frog Hsp110 protein. The Hsp110 cross-reaction showed a very interesting doublet pattern unlike any of the other Hsps (see below). The two spots differed slightly in pI value and may represent phosphorylated versus dephosphorylated forms of Hsp110; further studies will be required to investigate this. Figure 5-1A shows representative Western blots analyzing Hsp110 levels in heart from control, 24 h frozen and 4 h thawed frogs. The upper panel stained with Coomassie Blue (Figure 5.1A-i) demonstrates equal loading of samples in the different lanes whereas the lower panel shows immunoblotting with anti-Hsp110 antibodies (Figure 5.1A-ii).

Changes in Hsp110 protein expression in frog tissues in response to stresses and recovery are shown in Figure 5.2 (also see Appendix 5.1). Panel A shows responses to 24 h freezing and 4 h thawing, panel B shows responses to 24 h anoxia exposure and 4 h aerobic recovery, and panel C shows responses to the loss of 40% of total body water and rehydration. The relative Hsp110 levels in frog liver were up-regulated strongly in response to 24 h freezing (1.6-fold) or 24 h anoxia (2.6-fold) but were unaltered during 40% dehydration. During recovery from these stresses, Hsp110 levels decreased again, returning to control levels in thawed frogs and falling significantly below the control value during aerobic recovery after anoxia (levels were 56% of control values); Hsp110

remained unaltered in rehydrated animals. Heart showed a similar response of Hsp110 elevation in response to freezing and anoxia (levels increased by 1.9 and 1.5 fold, respectively) but also increased by 1.4-fold during dehydration. During thawing, Hsp110 levels in heart returned to control values but levels were largely unchanged during recovery from the other stresses. Kidney also showed increased Hsp110 levels during freezing (1.24-fold) and strongly reduced levels during thawing but protein levels did not respond to the other two stresses. However, Hsp110 decreased below control values during aerobic recovery after anoxia in kidney. In skeletal muscle Hsp110 levels were unaffected during freeze/thaw or during anoxia but rose by 1.5-fold during 40% dehydration. Hsp110 levels also rose during recovery after anoxia in muscle. Hsp110 protein levels were also unaffected by freezing in brain but were up-regulated in brain during anoxia and dehydration (by 1.5- and 1.6-fold, respectively) and remained elevated during recovery. Hsp110 was also elevated under dehydration stress in skin but was not affected in gut under any condition.

Figure 5.3 shows the distribution of Hsp110 in liver cytoplasmic and nuclear fractions from control versus 24 h frozen frogs. Western blotting showed that Hsp110 protein was approximately equally distributed between cytoplasmic and nuclear fractions in control liver. After 24 h freezing Hsp110 levels were higher overall in both cytoplasmic and nuclear fractions (by 25-26%) but the distribution between the two fractions was unaltered.

5.3.2 Hsc70

Figure 5.4 show representative results from Western blotting to detect levels of Hsc70, the constitutive heat shock protein 70, in frog tissues. Panel A-i confirms equal loading of lanes via Coomassie blue staining of a prominent protein band and Panel A-ii shows the relative levels of Hsc70 expression in skin of control, 24 h frozen and 4 h thawed frogs. Figure 5.4 B shows 2D-PAGE analysis of mammalian Hsc70 antibody cross-reaction with frog proteins. The antibody cross-reacts with a major spot with a molecular weight of ~73 kDa and a pI of 5.5, consistent with Hsc70 from other sources.

Figure 5.5 shows changes in the level of Hsc70 expression in frog tissues in response to stresses (24 h freezing, 24 h anoxia, 40% dehydration) and recovery after stress (also see Appendix 5.1). Hsc70 levels increased significantly in response to freezing in 5 out of 7 tissues tested, decreased significantly in brain (to 62% of control values) and were unchanged in skin (Figure 5.5A). The increases in Hsc70 levels during freezing were 1.4, 1.9, 2.0, 1.7 and 1.5 fold in liver, muscle, heart, gut and kidney, respectively. During thawing Hsc70 levels fell again to near control values in liver, heart and gut but remained high in muscle and kidney after the 4 h thaw. Anoxia exposure stimulated increases in Hsc70 levels in skeletal muscle and heart (1.6- and 1.2-fold, respectively) but levels fell in anoxic liver to 50% of control values. During aerobic recovery, levels returned to near control values in liver and heart but remained high in muscle, and fell in brain to 63% of control values. The response to 40% dehydration was increased Hsc70 levels in skeletal muscle, heart and kidney (1.3-, 1.4, and 1.3-fold, respectively), but reduced levels in brain and skin (about 65% of control values). During rehydration, Hsc70 levels returned to control values in muscle and kidney, remained

elevated in heart, remained suppressed in brain and skin, and decreased significantly in liver to 54 % of control values.

Figure 5.6 shows the distribution of Hsc70 between cytoplasmic and nuclear fractions in wood frog liver. In control frogs, the relative distribution was about 100:60 cytoplasmic:nuclear. In liver of frozen frogs, the increase in Hsc70 content appears to be largely directed into the cytoplasmic fraction whereas the nuclear content decreased; the ratio was about 140:40 cytoplasmic:nuclear.

5.3.3 Hsp60

Figure 5.7 shows representative results from Western blotting to detect levels of Hsp60 in frog tissues. Analysis via 2D-PAGE showed cross-reaction of the mammalian antibody with a single spot on the frog liver gel, corresponding with molecular weight of 60 kDa and a pI of 4.6, consistent with Hsp60 from other sources (Figure 5.7B). Figure 5.7A-i confirms equal loading of lanes via Coomassie blue staining of a prominent protein band whereas Figure 5.7A-ii shows the relative levels of Hsp60 in skin of control, 24 h frozen and 4 h thawed frogs.

The effects of freezing, anoxia and dehydration exposures on Hsp60 levels in seven frog tissues are shown in Figure 5.8 (also see Appendix 5.1). Freezing for 24 h resulted in significant increases in Hsp60 in all organs except heart (a decrease to 48% of control); levels increased by 1.6-, 1.7-, 2.2-, 2.0-, 1.5- and 1.5-fold, respectively, in liver, muscle, brain, gut, skin and kidney. After the 4 h thaw, Hsp60 remained high in liver, muscle, brain and gut but was reduced to control levels (kidney) or below (skin). Heart Hsp60 remained low after thawing at 45% of the control value. Anoxia exposure also

generally elevated Hsp60 levels in wood frog organs (Figure 5.8B); levels increased significantly in liver, muscle, brain, heart and skin after 24 h anoxia by 1.6-, 1.7-, 1.2-, 1.5-, and 1.3-fold, respectively. Anoxia exposure suppressed Hsp60 levels in gut. During recovery after anoxia, Hsp60 levels returned to control levels in liver, muscle, heart and gut but remained elevated in brain and skin. Dehydration resulted in increased Hsp60 levels in liver, skin and kidney (by 1.4-, 1.7-, 1.6-fold, respectively) but levels fell in gut. During rehydration Hsp60 levels fell again in skin and kidney but they continued to increase in liver, reaching 2.3-fold higher than controls. Oppositely, muscle showed reduced Hsp60 during rehydration.

Figure 5.9 shows the distribution of Hsp60 between cytoplasmic and nuclear fractions of frog liver. In control liver Hsp60 was nearly equally distributed between the two fractions (ratio 100:90) whereas during freezing the proportion of the enzyme in the cytoplasmic fraction rose substantially whereas the amount in the nuclear fraction decreased (ratio 170:70).

5.3.4 Hsp40

Figure 5.10 shows representative results from Western blotting to detect levels of Hsp40 in wood frog tissues. The 2D-PAGE showed cross-reaction of the mammalian antibody with a single spot on the frog liver gel, corresponding with a molecular weight of ~40 kDa and a pI of 9.0, consistent with Hsp40 from other sources (Figure 5.10B). Figure 5.10A-i confirms equal loading of lanes via Coomassie blue staining of a prominent protein band whereas Figure 5.10A-ii shows the relative levels of Hsp40 in heart of control, 24 h frozen and 4 h thawed frogs.

Figure 5.11 shows the effects of freezing, anoxia and dehydration exposures on Hsp40 levels in seven frog organs (also see Appendix 5.1). Freezing for 24 h stimulated Hsp40 levels in liver, muscle, heart and kidney with amounts rising by 1.9-, 3.4-, 1.5- and 1.5-fold, respectively. Levels were unchanged in brain and gut but fell to 40% of control levels in skin. After 4 h of thawing, Hsp40 levels remained the same in liver, brain, gut, skin and kidney but had decreased significantly and returned to near control levels in muscle and heart. Anoxia exposure elevated Hsp40 in muscle, brain, heart and kidney by 1.9-, 1.6-, 1.9- and 6.5-fold, respectively, the effect in kidney being particularly large. However, gut Hsp40 was strongly reduced to just 25% of the control value during anoxia. During recovery after anoxia, Hsp40 remained elevated in brain and kidney but decreased back to control levels in muscle and heart. Gut Hsp40 remained suppressed during recovery whereas liver and skin both showed increased Hsp40 during the aerobic recovery period only (by 2.5- and 2.6-fold, respectively). Dehydration of frogs stimulated an increase in Hsp40 levels in five organs, again with a particularly strong response by kidney; levels rose by 1.4-, 1.4-, 1.6-, 1.9-, and 4.2-fold in liver, muscle, brain, skin and kidney, respectively. Oppositely, dehydration reduced Hsp40 levels in gut. During recovery after dehydration, Hsp40 remained high in liver but returned to near control levels in other organs except kidney where Hsp40 content rose even more to 6.6-fold higher than control values.

The distribution of Hsp40 between cytoplasmic and nuclear fractions is shown in Figure 5.12. In the liver of control frogs the distribution in cytoplasmic vs nuclear fractions was 100:55. The amount of Hsp40 increased in both fractions of frozen frogs

with an apparent relative increase in the amount of Hsp40 in the nuclear fraction (ratio about 120:80).

5.3.5 Hsp10

Figure 5.13 shows representative results from Western blotting to detect levels of Hsp10, also known as Cnp10. The 2D-PAGE showed cross-reaction of the mammalian antibody with a single spot on the frog liver gel, corresponding with a molecular weight of ~10 kDa and a pI of 4.5, consistent with Hsp10 from other sources (Figure 5.13B). Figure 5.13A-i confirms equal loading of lanes via Coomassie blue staining of a prominent protein band whereas Figure 5.13A-ii shows the relative levels of Hsp10 in heart of control, 24 h frozen and 4 h thawed frogs.

Figure 5.14 shows the effects of freezing, anoxia or dehydration exposure and recovery after these stresses on Hsp10 levels in seven organs of wood frogs (also see Appendix 5.1). Hsp10 levels rose significantly during freezing in five organs by 1.5-fold in liver, 1.7-fold in skeletal muscle, and 1.6-fold in heart, skin and kidney. Levels in brain and gut were unaltered but Hsp10 increased during thawing in gut. Thawing resulted in a reduction in Hsp10 levels in four organs but the amount of this protein in liver continued to increase to a value in 4 h thawed frogs of 2-fold higher than controls. Anoxia exposure stimulated an increase in Hsp10 levels in liver, brain, gut and kidney by 1.5-, 2.3-, 1.5-, and 1.4-fold, respectively (Figure 5.14B). In muscle and heart, however, Hsp10 fell under anoxia to values that were 40 and 65% of control levels. Hsp10 returned to control levels during aerobic recovery in all organs except brain where levels remained 2-fold higher. Dehydration also stimulated an increase in organ Hsp10 levels in five organs with no

change in muscle or gut. Hsp10 content rose by 1.2-, 1.9-, 1.9-, 1.3- and 1.3-fold in liver, brain, heart, skin and kidney, respectively. After rehydration, levels returned to control values in brain and skin but remained elevated in liver, heart and kidney. Hsp10 rose strongly in gut by 2.6-fold during rehydration.

The relative distribution of Hsp10 between cytoplasmic and nuclear fractions is shown in Figure 5.15. In both control and frozen liver, the protein was present in higher amounts in the nuclear fraction. The ratio in control liver was about 100:230 whereas in 24 h frozen liver the ratio was 200:320.

5.4 Discussion:

Studies in our lab have shown that freeze tolerant wood frogs also have well developed tolerances for anoxia and dehydration (Storey and Storey, 2004). Both anoxia and dehydration are components of freezing stress. Anoxia occurs when the freezing of blood plasma cuts off the delivery of oxygen to organs. Cellular dehydration occurs when water leaves cells to freeze in extracellular ice masses. In the present studies, I tested the hypothesis that shock proteins aid stress survival by wood frogs by monitoring the responses of five different Hsps to stress and recovery from freezing, anoxia, and dehydration.

5.4.1 Hsp110

Hsp110, a constitutive heat shock protein, is found in both cytoplasmic and nuclear fractions of the cell and in frog liver is approximately equally distributed between the two fractions (Figure 5.3). Hsp110 (also known as APG-1 and Osp94) is known to be synthesized under stress conditions in cells and plays an important role in protein refolding and thermal tolerance (Hatayama *et al.*, 1998; Kojima *et al.*, 1996; Muller *et al.*, 1996). Hsp110 was identified as an osmotic stress protein (Osp94) in kidney; it is plentiful in the medulla and is increased by water restriction and hyperosmotic stress in rat kidney (Kojima *et al.*, 1996; Rauchman *et al.*, 1997). Kaneko *et al.* (1997) also demonstrated that APG-1 is abundantly expressed in testicular germ cells and responds to a sharp temperature increase from 32 to 39°C.

Hsp110 responded positively to freezing in three organs of wood frogs (liver, heart, and kidney), responded to anoxia stress in liver, brain and heart and was increased

in muscle, brain, heart and skin of dehydrated frogs. Hence, levels of Hsp110 were elevated in heart under all three-stress conditions and in brain, liver and muscle under two out of three stresses. This demonstrates that Hsp110 is responsive to environmental stresses that are naturally encountered by wood frogs and suggests that Hsp110 has a role to play in the adaptive response to stress, particularly freezing, in selected organs of wood frogs. In many cases, Hsp110 levels in frog organs fell again during the recovery after freezing, anoxia or dehydration returning to control levels within 4 h of thawing or the return to aerobic conditions or after rehydration. This quick return to control levels further supports the idea that Hsp110 has an important job to do when frogs are under stress.

5.4.2 Hsc70

Hsc70, also called Hsp73, HspA8 and Ly-hsc73, is the constitutive form of the 70 kDa stress protein family. It is known to be essential for cell growth and viability (Agsteribbe *et al.*, 1993). Many recent studies on Hsc70 expression have indicated that under normal conditions, Hsc70 resides in the cytoplasm, peroxisomes and nucleus. However, under stress most Hsc70 translocates into the nucleus where it binds to extended polypeptides, guides protein synthesis, prevents aggregation of unfolded peptides, and dissociates some oligomers (Chang *et al.*, 2000). The protein also binds ATP and shows ATPase activity. Therefore, Hsc70 expression is believed to promote cellular adaptation to stress and protect against apoptosis during anoxia and ischemia (Chang *et al.*, 2000; Gabai *et al.*, 1997; Saleh *et al.*, 2000). Hsc70 has a function during stress in mammals but as the constitutive form of the Hsp70 family, its levels do not typically change under stress in

mammals (Dean *et al.*, 1999). However, the situation is different in wood frogs where Hsc70 protein levels increased in response to freezing in five organs. Furthermore, Hsc70 levels increased in muscles, both skeletal muscle and heart, under all three stresses (freezing, anoxia, dehydration) which suggests that Hsc70 has an important role in muscle adaptation to stress. Analysis of Hsc70 protein distribution between cytoplasm and nuclear fractions of liver after 24 h freezing also showed that Hsc70 protein is redistributed during freezing with a greatly increased fraction of the total protein found in the cytoplasm under stress. The present data demonstrating that Hsc70 is up-regulated in five organs during freezing (an ischemic stress) as well as heart and muscle under anoxia stress agrees with the recent results of Chang *et al.* (2000) who reported that anoxia induced a significant increase in myocardial HSP72/73 in painted turtles. Hence, in lower vertebrates it appears that Hsc70 is inducible and that its levels are adjusted in response to various environmental stresses. Interestingly, wood frog Hsc70 levels are stable in frog brain and reduced in liver during 24 h anoxia. These results do not agree with the Hsc70 patterns seen in painted turtle and African clawed toad (*Xenopus laevis*). Studies from Phang *et al.* (1999) indicated that Hsc70 expression is increased in *X. laevis* liver during anoxia whereas Scott *et al.* (2003) reported that Hsp73 expression was unchanged in liver following a 24 h dive in the western painted turtles (although it increased twofold in brain and threefold in heart and skeletal muscle). Our data suggest that the hsc70 response in wood frogs is similar to that of painted turtles with respect to heart and muscle, but different in liver and brain.

Hsc70 levels also rose in frog heart, muscle and kidney in response to 40% dehydration which suggests that in addition to anoxia/ischemic stress, changes in cell

volume may also trigger Hsc70 expression. In the normal rat kidney, the tissue content of HSP73 increases steeply along the corticopapillary axis of kidney in a situation of exacerbated hypotonic stress (Cowley *et al.*, 1995; Müller *et al.*, 1996; Gaudio *et al.*, 1998; Ohno *et al.*, 1997). Enhancement of this HSP73 expression in cultured renal epithelial cells is associated with significantly improved cytoprotection against subsequent heat stress or ATP depletion (Wang *et al.*, 1996). Hence, our observation of increased Hsc70 expression in wood frog kidney during freezing and dehydration (both are volume and osmotic stresses on cells) is consistent with the responses of HSP73 to osmotic stresses in mammals. This suggests that the high Hsc70 contents observed in wood frog kidney are used for protection of these cells against damage caused by osmotic or volume stress associated with freezing or dehydration conditions. Further research can continue in two directions: (a) to identify the function(s) of Hsc70 protein in frog tissues during freezing, and (b) to determine whether freeze stimulated expression of Hsc70 is due to gene up-regulation (altered mRNA expression) and analyze the regulatory mechanisms (e.g. transcription factors) that control Hsc70 expression under stress.

5.4.3 Hsp60 and Hsp10

Hsp60 and hsp10 are predominantly constitutive mitochondrial stress proteins and reside in the mitochondrial matrix. Hsp60 is also known as Hsp58, Hsp65 or GroEL and Hsp10 has alternate names of Cnp10 and GroES. In eukaryotic cells, they assemble into multimeric complexes and bind to partially folded polypeptides to assist correct folding (Xanthoudakis *et al.*, 1999). In addition, Hsp60 plays a function in stabilizing mitochondrial oxidative enzymes and biogenesis (Agsteribbe *et al.*, 1993; Cheng *et al.*,

1989). Hsp60 acts as a critical factor for normal protein import into and/or retranslocation to the mitochondria (Cheng *et al.*, 1989) in response to brief ischemia in rabbit heart and muscle (Marber *et al.*, 1993). Hsp60 contributes to the high anoxia tolerance in painted turtle heart (Cheng *et al.*, 1989).

In our studies, Hsp60 levels increased in six wood frog organs during freezing by 1.5 to 2.2 fold, in five organs during anoxia by 1.2 to 1.7 fold, and in three organs during dehydration by 1.4 to 1.7 fold. Hsp60 was consistently elevated in liver and skin under all three conditions. This suggests that high levels of Hsp60 may provide an adaptive advantage for wood frogs to survive environmental stresses, particularly freezing. Ohashi *et al.* (2000) demonstrated that Hsp60 expression is protective against ischemia/reperfusion damage in rat skin and may have a similar role in frog skin.

Hsp10 was similarly elevated in five organs during freezing, four organs during anoxia, and five organs during dehydration. Hsp10 was also consistently elevated in liver under all three stresses as well as in kidney and it was elevated under two stresses in skin, heart and brain. The correlation between Hsp60 and Hsp10 in liver is particularly interesting because liver is very active metabolically during freezing. It is the center for biosynthesis of cryoprotectant and many freeze up-regulated proteins.

Recent studies by Afshin *et al.* (1999) have shown that there are interactions among Hsp60, Hsp10 and Hsc70. In *in vitro* systems, the mitochondrial heat-shock proteins HSP60 and HSP10 form a mitochondrial chaperonin complex. This complex of Hsp60 and Hsp10 accelerated the activation of pro-caspase-3 by cytochrome *c* and dATP in an ATP-dependent manner. Hsp10 promotes substrate release in conjunction with Hsp60, whereas Hsp60 guides mitochondrial protein import in conjunction with Hsp70.

Hsc70 binds ATP and shows ATPase activity while ATP hydrolysis and substrate release were stimulated by Hsp10 (Afshin *et al.*, 1999; Chang *et al.*, 2000). Furthermore, the functions of Hsp60 are strongly dependent upon Hsp10, which binds to the Hsp60 molecule to regulate its substrate binding and ATPase activity (Lindquist *et al.*, 1988; Ryan *et al.*, 1997; Bukau *et al.*, 1998). Their increased expression in cardiac myocytes exerts a protective effect against ischemic injury (Xanthoudakis *et al.*, 1999) and prevents apoptotic cell death induced by simulated ischemia-reoxygenation (Kurt *et al.*, 2001). These studies suggest that the mitochondrial chaperonins Hsp60 and Hsp10 in combination or individually play an important role in maintaining mitochondrial integrity and capacity for ATP generation. Thus, they act as crucial factors in determining survival of cardiac myocytes undergoing ischemia/reperfusion injury (Kurt *et al.*, 2001; Lau *et al.*, 1997).

Martin and Hartl (1997) have illustrated one of the typical pathways among chaperones (Hsp40, Hsp70, Hsp60, Hsp10) for nascent chain folding in *Escherichia coli*. DnaJ (an Hsp40) and DnaK (an Hsp70) have access to the growing polypeptide. The nascent chain is passed from DnaJ/DnaK into the lumen of a double-ring complex formed by GroEL (an Hsp60) and GroES (an Hsp10), transiently enclosing the nascent polypeptide within the GroEL cavity. DnaK and GroEL interactions with the incompletely folded substrate are ATP-dependent and regulated by ATP hydrolysis (Martin and Hartl, 1997). The present data demonstrate that Hsp60, Hsp10, Hsp40 and Hsc70 all show parallel up-regulation in three wood frog tissues (liver, muscle, kidney) in response to 24 h freezing. This suggests the importance of the coordinated function of these four proteins in controlling the correct folding of proteins that are up-regulated

during freezing. Because freezing puts multiple stresses on cells including ischemia and dehydration, it is probable that cells must respond by enhancing the levels of the chaperonin proteins that aid proper protein folding during biosynthesis.

5.4.4 Hsp40

Hsp40, also known as DnaJ, is a co-chaperone for Hsp70 and plays a major catalytic function in loading target substrates onto the Hsc70 binding/release cycle and increases cellular protein folding activities during stresses (Freeman *et al.*, 1995; Cyr, 1997). Hsp40-stimulated Hsc70 ATPase activity enhanced the amount of hydrolysis of ATP in stress cells (Yamagishi *et al.*, 2000, Yaohui *et al.*, 1999). The J domain of Hsp40 is the region of the protein responsible for mediating the binding of Hsp40 to Hsc70 (Hartl, 1996). These interactions of cytosolic Hsc70 and protein substrates are mediated by cycles of ATP binding and hydrolysis (Hightower *et al.*, 1997). Several molecular chaperones including an Hsp70-interacting protein (Hip) and Hsp40 interact with cytosolic Hsc70 and modulate its ATPase activity and the stability of the Hsc70-polypeptide substrate complex (Frydman *et al.*, 1997). Hip stimulates the assembly of Hsc70 with Hsp40 and the polypeptide substrate (Hohfeld *et al.*, 1995), whereas Hsp40 stimulates the ATPase activity of Hsc70 (Suh *et al.*, 1999) leading to increased rates of binding and release of polypeptides. In addition, Hsp40 promotes the multimerization of Hsc70 (King *et al.*, 1999). If several molecules of Hsc70 are present in each complex of chaperones and co-chaperones, they could interact with substrate proteins in a cooperative manner.

Such an interaction pattern is suggested by the responses of Hsp40 and Hsc70 in wood frog organs during freezing. Western analysis showed that Hsp40 levels were elevated in liver, muscle, heart and kidney during freezing whereas Hsc70 was increased in the same tissues. By contrast, both Hsp40 and Hsc70 were reduced in brain during freezing. During dehydration, Hsp40 increased in the same tissues (liver, muscle, brain, kidney) as during freezing as well as in skin whereas in anoxia Hsp40 rose in muscle, kidney and heart. The expression of various freeze responsive genes is often paralleled by the response of the same genes to either anoxia or dehydration stress, suggesting that one of these component stresses is the actual trigger of the freezing response (Storey and Storey, 2004). However, this does not appear to be the case for Hsp40 which suggests that increased Hsp40 expression may be a general response to cellular stress. In most cases Hsp40 levels declined again during the recovery after stress and this further supports the idea that general stress conditions increase Hsp40 expression. An interesting exception was the response of kidney where Hsp40 levels increased strongly under all three stresses and remained elevated during recovery after stress.

5.4.5 Heat-shock transcription factor (HSF) & Heat shock element (HSE)

Transcriptional activators are believed to play a major role responsible for induction of HSPs. Hsps are encoded by nuclear genes, synthesized on free ribosomes in the cytoplasm, and subsequently transported into other subcellular compartments. *Hsp* gene regulation is controlled by the heat shock factors (HSFs) (Sorger, 1991; Morimoto *et al.*, 1997; Chang *et al.*, 2001). The regulatory heat shock element (HSE) consists of three inverted repeats of the sequence nnGAAn that are conserved in all *hsp* genes in all

species (Klemenz *et al.*, 1991). The heat-shock transcription factor (HSF) in its trimeric form binds to the HSE. Both are activated by hypertonic stress (Alfieri *et al.*, 1996). In response to heat or other stresses, inactive HSF monomers, constitutively present in the cytoplasm, are converted into DNA-binding homotrimers that translocate to the nucleus. HSF in its trimeric state binds to a consensus target sequence, the HSE, present in the promoter region of heat shock genes (Sorger, 1991; Xiao *et al.*, 1988). This could allow for coordinate expression of multiple types of Hsps in response to a single stress. For example, the present data show increased amounts of all five Hsps in wood frog kidney in response to freezing. Other authors have reported that HSPs may fulfill specific, kidney-related functions such as participation in the maturation of steroid hormone receptors, in the contractility of mesangial cells, and in the adaptation of renal medullar cells to stress-caused damages (Zhong *et al.*, 1999; Xia *et al.*, 1997).

Rapid induction of stress protein expression is accomplished through mechanisms of transcriptional activation and preferential translation. HSFs (HSF1 through HSF4) regulate the inducible synthesis of HSPs during development, growth, and adaptation (Morimoto *et al.*, 1994). Hsp expression in wood frogs during freezing is undoubtedly mediated by one or more of these HSFs and identification of the HSF(s) involved would be an important next step that would provide leads into the signal transduction pathway(s) involved in mediating the Hsp response to freezing. That more than one HSF may be involved is suggested by the different organ-specific patterns of response by each Hsp to anoxia versus dehydration (frequently an individual organ responded to only one of these stresses) and the fact that, in general, more organs showed elevated Hsp levels in

response to freezing (that combines both anoxia and dehydration stresses) than to either of anoxia or dehydration stress alone.

5.4.6 Conclusions

In general, the results show that levels of heat shock proteins are elevated in response to freezing and other stresses in wood frogs with stress-specific and organ-specific changes seen for each of the Hsps. Recovery after stress typically resulted in a full or partial reversal of the stress effect. These data strongly support the hypothesis that Hsps have roles to play in the stabilization of intracellular proteins under stress conditions and are one component of natural freezing survival.

Figures 5-1

(A) Representative Western blot shows Hsp110 protein levels in heart extracts from control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (4 h at 5°C) frogs; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane stained with Coomassie blue; (A-ii) shows Hsp110 protein levels in control, 24 h frozen and recovered heart. N-1 and N-2 show two independent experiments.

(B) Representative 2D-PAGE showing Hsp110 protein in a liver extract from frozen (2 h at -2.5°C) frogs. Each gel was loaded 20 µg of protein and PVDF membranes were blotted with anti-Hsp110 antibodies. The cross-reacting spots are circled (2 spots shown) and correspond to a pI of ~5.3 and a molecular weight of approximately 105 kDa, consistent with Hsp110 from other sources.

(A-i) Control 24h frozen 4h thaw
 n-1 n-2 n-1 n-2 n-1 n-2

(A-ii) Control 24h frozen 4h thaw
 n-1 n-2 n-1 n-2 n-1 n-2

(B) 2D-PAGE of 2h frozen frog liver

pI: 10→3.5

pI: 5.28

M.W (105kDa)

Figure 5-2.

Effect of stresses (freezing, anoxia, dehydration) and recovery on Hsp110 protein expression in different tissues of wood frogs:

- (A) .Histogram shows Hsp110 protein levels in tissue extracts from control (5°C acclimated), frozen (24 h at -2.5°C), and thawed (4 h at 5°C) frogs.
- (B) Histogram shows Hsp110 protein levels in tissue extracts from control (5°C acclimated), anoxic (24 h at 5°C), and recovered (4 h at 5°C) frogs.
- (C) Histogram shows Hsp110 protein levels in tissue extracts from control (5°C acclimated), 40% dehydrated (at 5°C), and rehydrated (at 5°C) frogs.

Western blots were run with equal amounts of protein (10 µg) in each lane. Bands intensities on individual blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean ± SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, using the Student's t-test, $P \leq 0.05$.

(A)

(B)

(C)

Figure 5-3

Analysis of Hsp110 protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots for detection of Hsp110 protein in nuclear and cytoplasmic fractions of liver in control (5°C) and 24 h frozen wood frogs.

(B) Histogram showing Hsp110 protein levels in the two fractions, relative to the value for control cytoplasmic Hsp110 content. Data are n=1.

(A)

(B)

Figure 5-4

(A) Representative Western blot shows Hsc70 protein levels in skin extracts from control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (4 h at 5°C) frogs; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane stained with Coomassie blue; (A-ii) shows Hsc70 protein levels in control, 24 h frozen and recovered skin. N-1 and N-2 show two independent experiments.

(B) Representative 2D-PAGE showing Hsc70 protein in a liver extract from frozen (2 h at -2.5°C) frogs. Each gel was loaded 20 µg of protein and PVDF membranes were blotted with anti-Hsc70 antibodies. The cross-reacting spot is circled and corresponds to a pI of 5.5 and a molecular weight of approximately 73 kDa, consistent with Hsc70 from other sources.

(B) 2D-PAGE of 2h frozen frog liver

pI: 10→3.5

pI: 5.5

M.W (73kDa)

Figure 5.5.

Effect of stresses (freezing, anoxia, dehydration) and recovery on Hsc70 protein expression in different tissues of wood frogs:

(A) Histogram shows Hsc70 protein levels in tissue extracts from control (5°C acclimated), frozen (24 h at -2.5°C), and thawed (4 h at 5°C) frogs.

(B) Histogram shows Hsc70 protein levels in tissue extracts from control (5°C acclimated), anoxic (24 h at 5°C), and aerobic recovered (4 h at 5°C) frogs.

(C) Histogram shows Hsc70 protein levels in tissue extracts from control (5°C acclimated), 40% dehydrated (at 5°C), and rehydrated (at 5°C) frogs.

Western blots were run with equal amounts of protein (10 µg) in each lane. Bands intensities on individual blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean ± SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, using the Student's t-test, $P \leq 0.05$.

(A)

(B)

(C)

Figure 5.6

Analysis of Hsc70 protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots showing detection of Hsc70 proteins in nuclear and cytoplasmic fractions of liver from control (5°C) and 24 h frozen wood frogs.

(B) Histogram shows protein level of Hsc70 in the two fractions, relative to the value for control cytoplasmic Hsc70 content. Data are n=1.

(A)

(B)

Figure 5.7

(A) Immunoblotting of Hsp60 protein levels in skin extracts from control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (4 h at 5°C) frogs; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane was stained with Coomassie blue; (A-ii) shows Hsp60 protein levels detected by immunoblotting. N-1 and N-2 show two independent experiments.

(B) Representative 2D-PAGE of Hsp60 protein in a liver extract from freeze-exposed frogs (2 h at -2.5°C); each gel was loaded 20 µg of protein and PVDF membranes were blotted with anti-Hsp60 antibodies. The cross-reacting spot is circled and corresponds to a pI of 4.6 and a molecular weight of approximately 60 kDa, consistent with Hsp60 from other sources.

(B) 2D-PAGE of 2 h frozen frog liver

pI: 3.5→10
pI:4.6

M.W (60kDa)

Figure 5.8.

Effect of stresses (freezing, anoxia, dehydration) and recovery on Hsp60 protein expression in different tissues of wood frogs:

(A) Histogram shows Hsp60 protein levels in tissue extracts from control (5°C acclimated), frozen (24 h at -2.5°C), and thawed (4 h at 5°C) frogs.

(B) Histogram shows Hsp60 protein levels in tissue extracts from control (5°C acclimated), anoxic (24 h at 5°C), and recovered (4 h at 5°C) frogs.

(C) Histogram shows Hsp60 protein levels in tissue extracts from control (5°C acclimated), 40% dehydrated (at 5°C), and rehydrated (at 5°C) frogs.

Western blots were run with equal amounts of protein (10 µg) in each lane. Bands intensities on individual blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean ± SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, using the Student's t-test, $P \leq 0.05$.

(A)

(B)

(C)

Figure 5.9

Analysis of Hsp60 protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots showing detection of Hsp60 proteins in nuclear and cytoplasmic fractions of control (5°C) and 24 h frozen wood frog liver.

(B) Histogram shows Hsp60 protein level in the two fractions, relative to the value for control cytoplasmic Hsp60 content. Data are n=1.

(A)

(B)

Figure 5.10

(A) Representative Western blot shows protein levels in heart extracts from control (5°C acclimated), frozen (24 h at -2.5°C) and thawed recovery (4 h at 5°C) frogs; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane was stained with Coomassie blue; (A-ii) shows immunoblots of Hsp40 protein levels. N-1 and N-2 show two independent experiments.

(B) Representative 2D-PAGE of Hsp40 protein in a liver extract from freeze exposed wood frogs (2 h at -2.5°C); 20 µg of protein was loaded and the PVDF membrane was blotted with anti-Hsp40 antibody. The cross-reacting spot is circled and corresponds to a pI of 9.0 and a molecular weight of ~40 kDa, consistent with Hsp40 from other sources.

(B) 2D-PAGE of 2h frozen frog liver

pI: 3.5→10

pI:9.0

M.W (40kDa)

Figure 5.11.

Effect of stress (freezing, anoxia and dehydration) and recovery on Hsp40 protein expression in different tissues of wood frogs.

(A) Hsp40 protein levels in tissue extracts from control (5°C acclimated), frozen (24 h at -2.5°C), and thawed (4 h at 5°C) frogs.

(B) Hsp40 protein levels in tissue extracts from control (5°C acclimated), anoxic (24 h at 5°C), and aerobic recovery (4 h at 5°C) frogs.

(C) Hsp40 protein levels in tissue extracts from control (5°C acclimated), 40% dehydrated (at 5°C), and rehydrated (at 5°C) frogs.

Data are derived from Western blots with equal amounts of protein (10 µg) loaded in each lane. Bands intensities on individual gels and blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean ± SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, using the Student's t-test, $P \leq 0.05$.

(A)

(B)

(C)

Figure 5.12

Analysis of Hsp40 protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots for detection of Hsp40 proteins in nuclear and cytoplasmic fractions of control (5°C) and 24 h frozen wood frog liver.

(B) Histogram showing protein level of Hsp40 in the two fractions, relative to the value for control cytoplasmic Hsp40 content. Data are n=1. Bands intensities on individual blots were measured on Genetools, and are relative to control cytoplasm protein, set to 1.

(A)

(B)

Figure 5.13

(A) Representative Western blot shows Hsp10 protein levels in heart extracts from control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (4h at 5°C) wood frogs; equal amounts of protein (10 µg) were loaded in each lane. (A-i) shows the PVDF membrane was stained with Coomassie blue; (A-ii) shows immunoblots of Hsp10 protein. N-1 and N-2 present proteins isolated from two independent experiments.

(B) Representative 2D-PAGE of Hsp10 protein in a liver extract from frozen frogs (2 h at -2.5°C). Each gel was loaded 20 µg of protein and PVDF membranes were blotted with anti-Hsp10 antibodies. The cross-reacting spot is circled and corresponds to a pI of 4.5 and a molecular weight of ~10 kDa, consistent with Hsp10 from other sources.

(B) 2D-PAGE of 2h frozen frog liver

pI: 10→3.5

pI: 4.5

M.W (10kDa)

Figure 5.14.

Effect of stresses (freezing, anoxia and dehydration) and their recovery frog on Hsp10 protein expression in different tissues.

(A) Hsp10 protein levels in tissue extracts from control (5°C acclimated), frozen (24 h at -2.5°C), and thawed (4 h at 5°C) frogs.

(B) Hsp10 protein levels in tissue extracts from control (5°C acclimated), anoxic (24 h at 5°C), and recovered (4 h at 5°C) frogs.

(C) Hsp10 protein levels in tissue extracts from control (5°C acclimated), 40% dehydrated (5°C), and rehydrated (at 5°C) frogs.

Data are from Western blots with equal amounts of protein (10 µg) loaded in each lane. Bands intensities on individual gels and blots were quantified using GeneTools; values for experimental groups are expressed relative to controls that were set to 1. Histograms show mean ± SEM, n=4-5 independent determinations. Stars show experimental values that are significantly different from corresponding controls, using the Student's t-test, $P \leq 0.05$.

(A)

(B)

(C)

Figure 5.15

Analysis of Hsp10 protein distribution between cytoplasmic and nuclear fractions of liver in control versus 24 h frozen wood frogs.

(A) Western blots show detection of Hsp10 proteins in nuclear and cytoplasmic fractions of control (5°C) and 24 h frozen wood frogs.

(B) Histogram shows protein level of Hsp10 in the two fractions, relative to the value for control cytoplasmic Hsp10 content, set to 1. Data are n=1.

(A)

Control: 24h Frozen:

Cytoplasm Nuclear Cytoplasm Nuclear

(B)

frog liver Hsp10

CHAPTER 6

General Discussion

The wood frog, *Rana sylvatica*, is one of the primary model species used for studies of natural freeze tolerance and the main model for vertebrate freeze tolerance (Storey and Storey, 1992; 2004). Frogs can survive the freezing of 65-70% of total body water in extracellular and extra-organ ice masses, and endure several freeze-thaw events over the winter. Freezing halts all vital physiological functions, imposes ischemia on organs, and cell water loss into extracellular ice causes a large decrease in cell volume as well as elevated intracellular osmolality and ionic strength. Multiple biochemical adaptations help cells to survive these stresses as well as to minimize physical damage to tissues from ice crystals. Work in our lab has identified a large number of adaptations that aid freeze tolerance by wood frogs. These include the accumulation of high concentrations of glucose as a cryoprotectant, the use of ice nucleators to trigger and regulate ice growth, good anoxia tolerance, high antioxidant defenses, and the up-regulation of a variety of genes (Storey, 2004; Storey and Storey, 2004).

Stresses that disrupt normal cellular metabolism often trigger the synthesis of stress proteins. Many of these act as chaperones to stabilize the conformations of other proteins against the damaging effects of the stress. Many studies have documented the production of stress proteins in response to heat, cold, heavy metals, toxins, radiation, and osmotic stresses, among others (Knowlton, 1995; Hightower and Hendershot, 1997; Lindquist and Craig, 1998; Morimoto et al., 1997). However, the possible role for stress proteins in vertebrate freeze tolerance have not previously been assessed. The present study examined the responses by two kinds of stress proteins (heat shock proteins, glucose regulated proteins) to freezing and thawing as well as to two of the components of freezing – anoxia and dehydration. The results show that these proteins are

ubiquitously present in wood frog cells and that their synthesis is increased in response to freezing with a general reversal of the stress protein response after thawing. Organ specific differences in stress protein synthesis were seen as well as different responses to freezing, anoxia and dehydration. The organ-specific differences suggest that different tissues may require different levels of protection during freezing as well as different combinations of stress proteins to protect their cells. This suggests that a complex network of regulators and cell protective mechanisms may be required for freezing protection and recovery of normal functions after thawing. In addition, increased levels of *hif-1 α* transcripts and HIF-1 α protein in some frog organs in response to all three stresses indicates that wood frogs are detecting and responding to low oxygen stress via the same mechanism as occurs in other vertebrates and suggest that freezing survival may be aided by the up-regulation of various proteins that are under HIF-1 regulatory control.

6.1. Shock proteins contribute to cryoprotection:

Previous studies in our lab have demonstrated that glucose, the most abundant monosaccharide in nature, acts as an important cryoprotectant during freezing (Storey and Storey, 1988). Glucose acts as a colligative protectant to raise the osmolality of intracellular fluids and thereby limit the decrease in cell volume that occurs when water is drawn out of cells to freeze in extracellular spaces. This colligative action of glucose helps to ensure that cell volume does not drop below a critical minimum that can cause irreversible damage to cell membranes. High glucose may also have other actions; for example, sugars can stabilize the structure of proteins against stresses that could denature proteins such as low water or high salt. Thousands of studies have shown that the

accumulation of high levels of sugars or polyhydric alcohols (e.g. glycerol) is a very important adaptation for subzero temperature survival in many types of cold hardy organisms (Storey and Storey, 1991).

Protective proteins also aid cold hardiness for many organisms. Some have specific functions such as the antifreeze proteins that block ice formation in polar fish and cold-hardy insects or the ice nucleating proteins that act in freeze tolerant species to stimulate and guide ice formation in extracellular compartments (Storey and Storey, 1996). Cold shock proteins (Csps) have been identified in bacteria and a variety of cold responsive (COR) proteins are produced by plants (Bae et al., 2000; Thomashow, 1999). The major Csps in bacteria are apparently RNA chaperones, which are thought to facilitate translation at low temperatures (Bae et al., 2000) whereas many of the COR proteins in plants act to stabilize membranes during freezing (Thomashow, 1999). Less is known about protein responses to cold shock in animals. In insects, changes in heat shock proteins occur during entry into diapause (a state of arrested development); in pupae of flesh flies, *Sarcophaga crassipalpis*, HSP23 and HSP70 are up-regulated while HSP90 is down-regulated (Denlinger, 2002). In this species cold hardiness and diapause are firmly linked and hence, the elevated levels of Hsp23 and Hsp70 may have a cryoprotective role for survival of winter cold temperatures (Denlinger, 2002). This idea is supported by studies on the gypsy moth, *Lymantria dispar*, where diapausing embryos are not initially cold hardy, but become cold hardy after chilling for a few weeks. Their development of cold hardiness coincides with the acquisition of the ability to synthesize heat shock proteins (Denlinger et al., 1992). However, heat shock proteins may also have other roles in overwintering animals. Hsp23 and Hsp70 are up-regulated by desiccation in

nondiapausing pupae of *S. crassipalpis* but not in diapausing pupae which already have high levels of these shock proteins (Hayward et al., 2004); this suggests that hsps may aid desiccation resistance in overwintering pupae. Heat shock proteins may also contribute to cell cycle arrest over the winter months; this has been linked to expression of both small heat shock proteins and Hsp70. Development is retarded or halted when high levels of stress proteins are present (Denlinger 2002). Interestingly, Hsp90 and Hsc70 were unresponsive to desiccation in both nondiapausing and diapausing pupae but both were up-regulated when pupae were rehydrated which shows that the different Hsps may have different protective actions in cell stress and recovery (Hayward et al., 2004).

My data on Hsp expression during freezing in wood frogs fits well with the information on Hsps and cold hardiness in insects and shows that the up-regulation of selected Hsps may be a common feature of winter survival for cold hardy animals. Hsps could help to prevent cell damage due to ischemia and dehydration during freezing and possibly also help to maintain a hypometabolic state over the winter months. Hence, the selected HSPs including Hsp110, Hsc70, Hsp60, Hsp40, Hsp10 as well as the glucose regulated proteins (Grp78 and Grp94) appear to have cryoprotective functions in frozen cells. Indeed, protein levels of the five HSPs and two GRPs were all up-regulated in liver of frozen frogs. Six out of the seven were elevated in muscle (except Hsp110) and kidney (Grp94 was not measured) during freezing and four in heart (Hsp10, 40, 70 and 110). By contrast, only one HSP and no GRPs increased during freezing in brain (Hsp60), gut (Hsp60) and skin (Hsp10). This argues for organ-specific functions for stress proteins during freezing with some organs that have greater needs for the actions of these proteins to protect their cells. Among the shock proteins the one that may be most important for

freezing survival is Hsp60 which showed increased levels in six organs (not heart) during freezing. Its mitochondrial partner Hsp10 was also elevated in five organs during freezing. This suggests that these two Hsps may have useful roles in protecting some aspect of mitochondrial metabolism during freezing. Hsc70 also increased in five organs during freezing, Hsp40 in four and Hsp110 in three. Freezing stimulated increased levels of Grp78 in three organs and Grp94 in just two which suggests that Grps have a role during freezing in only selected organs (liver and muscle increased both).

The up-regulation of all the shock proteins in liver may be for one of two reasons: (1) liver may be very sensitive to the stresses associated with freezing, or (2) liver may need these chaperones to support a high level of protein biosynthesis during freezing. In support of the first idea, Flanagan et al. (1995) demonstrated that the gut and liver are the first organs to accumulate HSPs following whole body hyperthermia because these tissues are the most sensitive to heat damage. In support of the second idea, liver shows up-regulation of many genes during freezing (Storey and Storey, 2004) and liver synthesizes proteins that are exported into the blood during freezing (e.g. fibrinogen) (Cai and Storey, 1997a) to contribute to the protection of all other organs. A high demand on liver for protein biosynthesis during freezing may require that chaperone proteins are elevated in order to help correctly fold, stabilize and guide new proteins into their correct place under the stressful conditions in frozen cells. Some other organs might do relatively little protein biosynthesis during freezing and mainly rely instead on the import of cryoprotectant (glucose) to protect their cells during freezing.

In support of this idea, Dorner et al. (1992) have demonstrated that Grp78 protects cells from death under various stresses. HSPs were first discovered in 1962 (Ritossa,

1962) and described as a set of proteins that have strong cytoprotective effects in stress cells (Moseley, 1997). Hsps are rapidly synthesized after the onset of a stress and have a dynamic, specific intracellular localization. They can interact with other chaperones or cofactors to assist in correct protein assembly and transporting. In addition, their interactions also enhance inter-organelle communication and cellular signaling transductions. The high degree of conservation of these proteins across species suggests that HSPs in wood frog are critical for both normal cellular function and survival after a stress. Several important cytoprotective functions have been attributed to HSPs, Grp78 and Grp94 including ensuring the correct folding, refolding, transportation or degradation of proteins in various intracellular compartments (e.g. ER, lysosome, proteasome, mitochondria, Golgi and nucleus), and maintaining structural proteins in organelle membranes (Pelham 1986; Knowlton, 1995; Hightower and Hendershot, 1997; Lindquist and Craig, 1998; Morimoto et al., 1997; Li, 2001). Therefore, these proteins may play a key role in the development of freeze tolerance and protection from cellular damage associated with stresses such as ischemia, dehydration and energy depletion.

Although HSPs and GRPs can be induced separately, simultaneously, or reciprocally (Dill et al., 1997; Lee, 1987) and have specific functions in cellular protective mechanisms by themselves, evidence from in vitro studies suggests the Hsp chaperones and chaperonin systems generally function cooperatively in protein folding and assembly in eukaryotes (Garrido et al, 2001). These stress proteins also represent a novel class of apoptosis regulators that when expressed at high levels can protect the host cell against death (Zhu et al., 1996). For example, mitochondrial chaperonins Hsp60 and Hsp10 constitute a separate system for folding a subset of proteins in vivo. TRiC

chaperonin is considered to be the functional equivalent of Hsp60/Hsp10 chaperonin in the eukaryotic cytosol. TRiC requires additional components, such as Hsp40, which stimulates the Hsc70 ATPase, for protein folding in the cytosol. Protein cooperative interactions and overexpression enhance the speed of physiological recovery after stresses in heart (Layan and Dirst, 1991). The shock proteins probably also mediate physiological recovery in frog organs during recovery from stress. Rapid thawing and resumption of frog heart function is advantageous for it allows oxygen to be delivered as soon as possible to organs that have been ischemic for the duration of the freeze. Hence, cardioprotection during freezing would be very important. Overall, then, my data indicate that up-regulation of Hsp and Grp expression may have an important role in cell and organelle protection during freezing.

6.2 A rapid synthesis of shock proteins:

Glucose synthesis by wood frog liver glycogen is triggered within 2 minutes after the skin begins to freeze and glucose is exported so that cryoprotectant levels rise quickly in all tissues over the first few hours of freezing (Storey and Storey, 1988). Analysis of the time course of *hif-1α* and *grp94* transcript levels showed that the mRNA for these stress responsive proteins also responded very rapidly after freezing began. Transcript levels for both genes peaked in liver and brain within 2 hours after freezing began. For *hif-1α* this suggests that the organs were experiencing hypoxia stress within this time; indeed, liver *hif-1α* was significantly increased within 30 minutes. This suggests that the genes that are under *hif-1α* control would also be quickly induced over the early minutes/hours of freezing. Brain *grp94* mRNA was also strongly increased within 30 minutes. Given that

Grp94 protein levels were also elevated by anoxia exposure in both organs, it is possible that both *hif-1a* and *grp94* genes were responding to the decreasing tissue oxygen levels as ice grows through the body fluids. This rapid increase of gene expression in frog cells/tissues may provide a quick protective mechanism for organs.

6.3 A rapid protective system in the vital tissues such as heart and liver:

(A). Protection in the cardiovascular system. Amrani et al. (1997) demonstrated that heat shock proteins are present and can be induced in all cell types in the blood vessel wall, which protects both nuclear and cytoplasmic structures from stress caused injury. Currie et al. (1988) reported that heat shock proteins in rats are associated with improvement of cardiac functional recovery after a global ischemic insult. Moreover, Mehta et al (1997) demonstrated that high levels of Hsp70 and Hsc70 expression were found in transplanted rat hearts after a long-lasting hypothermia. Therefore, the relationship between Hsp induction and cardiac transplantation has drawn the attention of some investigators. Our data showing that up-regulation of Hsc70 expression occurred in wood frog heart under all three stresses (freezing, anoxia, dehydration) supports the idea that Hsps may play an important role in protecting frog cardiovascular systems from stress damages. Genetic studies provided further proof in different phyla that overexpression of stress proteins is a powerful means of cytoprotection in the intact heart (Thomas et al., 1995).

(B). Protection in the hepatic system. As discussed in previous chapters, all HSPs and GRPs analyzed were up-regulated in wood frog liver. This suggests that these proteins may provide specific protective mechanisms to decrease or neutralize the deleterious effects of stress on frog liver. Multiple other studies have demonstrated that

HSP synthesis is associated with protection against stress-induced damage in liver (Cairo et al., 1985; Cabin et al., 1990; Buchman et al., 1989, Bernelli et al., 1992).

6.4 Rearrangement of subcellular location of proteins during stress:

Analysis of the subcellular location of HIF-1 α , HSPs and GRPs in wood frog liver showed a redistribution of some of these proteins during freezing between cytoplasmic and nuclear fractions that suggests altered functions for the proteins during freezing. The amount of HIF-1 α present in the nucleus increased in frozen frogs and this is consistent with the well-known mechanism of HIF-1 activation and suggests that activation of various HIF-1-mediated genes (Semenza et al., 1994; Guillemin and Krasnow, 1997) may occur during freezing. By contrast, Grp78 showed reduced protein levels in the nucleus and a very large increase in cytosolic protein content which could be consistent with a greater need for Grp78 in the ER during freezing in order to correctly guide the synthesis of freeze-responsive proteins under the stress conditions (low oxygen, higher ionic strength, etc.) of the frozen state. Hsp60 and Hsc70 also showed redistributions that resulted in much higher levels in the cytosolic fraction and a lower percentage in the nucleus after 24 h freezing. For Hsp60 this is consistent with a function as a constitutive stress protein that resides in the mitochondrial matrix (Chang et al., 2000). Under the cell fractionation methods used, the mitochondria would be found in the cytosolic fraction and hence the greatly increased fraction of HSP60 in the cytosolic fraction is likely consistent with increased levels of the protein in mitochondria during freezing. Chang et al. (2000) showed that HSP60 levels in 4 species correlated with their anoxia tolerance with the highest constitutive levels in good anaerobes; hence, elevated HSP60 in wood

frog tissues during freezing may aid anoxia/ischemia resistance. However, HSP10, 40 and 110 showed little or no change in their distribution patterns between cytoplasmic and nuclear fractions during freezing even though overall levels increased.

6.5 Responses of stress proteins to anoxia and dehydration:

Amphibians in general show a high tolerance for wide variation in body water content and ionic strength of body fluids (Shoemaker, 1992) and many species also show high tolerance for prolonged oxygen deprivation (Jackson, 2001). Both of these capacities are well developed in wood frogs where they aid freeze tolerance (Storey and Storey, 1989). In previous studies of freeze responsive proteins in wood frogs, our lab has generally found that such proteins also respond to either anoxia or dehydration stresses which indicates that their function during freezing may be related to either ischemia protection or dehydration resistance. For example, fibrinogen up-regulation responded to freezing and dehydration whereas up-regulation of the ADP-ATP translocase responded to freezing and anoxia (Cai and Storey 1997a; Cai et al., 1997). Of the novel proteins that are freeze responsive in liver, FR10 responded to dehydration, FR47 to anoxia and Li16 were up-regulated under both stresses (Storey, 2004).

The data for the shock proteins is interesting. In liver, Hsp60, Hsp 10, Hsp110 and Grp94 responded strongly to anoxia stress whereas a large response to dehydration was seen only for Hsp60. This implies that the freeze-responsive increases in Hsps and Grps in liver may be triggered by low oxygen stress or possibly by the disruption of cellular energetics that would be caused by low oxygen. In heart, six of the shock proteins (except Hsp 10) increased under anoxia and five increased under dehydration so it is difficult to

link freeze-responsive shock protein expression with one of the alternate stresses. A similar situation was seen in skeletal muscle. In kidney, six shock proteins were elevated during freezing and four of these were also elevated under dehydration (Hsp60, Hsp40, Hsp10, Hsc70) whereas only Hsp40 and Hsp10 increased to anoxia. With its important role in cellular water balance, it is perhaps not surprising that shock proteins would be elevated in response to dehydration stress in kidney and this might also be the trigger for their up-regulation during freezing. Brain was interesting because only Hsp60 rose during freezing whereas anoxia exposure triggered elevated levels of four Hsps (including Hsp60) and Grp94; similarly, dehydration up-regulated three Hsps and Grp94 in brain. Thus, brain showed little response by shock proteins to freezing although the responses to the other stresses indicate that the organ was fully capable of synthesizing Hsps and Grp94 under either anoxia or dehydration stress. Skin showed very strong responses to dehydration, with increased levels of all the shock proteins except Hsc70. By contrast, freezing elevated only Hsp60 and Hsp10 and anoxia only Hsp60 and Grp78. The strong response by skin to dehydration is not unexpected as this organ is in direct contact with the environment and undoubtedly undergoes the most extreme dehydration of any organ when the animal is under desiccation stress. Gut showed up-regulation of stress proteins in only a few instances and no correlation between the responses to freezing and the response to either anoxia or dehydration. Hence, it would seem that there are multiple patterns of response by shock proteins to the three stresses (freezing, anoxia, dehydration) and only in a couple of organs is there reasonable evidence that freeze-responsive changes in shock proteins can be associated with either anoxia (liver) or dehydration (kidney) stresses.

6.6 Future studies:

Our future studies need to further determine whether these molecular chaperones, either alone or in combination, alleviate freezing damage and accelerate physiological recovery in the intact organism by identifying chaperone specific protective actions involved in cellular proliferation, intracellular signaling, cytoskeletal rearrangement and the cellular apoptosis activities in cells. In addition, cell adaptive regulation largely depends on the activities of transcription factors. We have demonstrated that up-regulation of HSPs and GRPs play an important role in freezing survival but how these proteins are regulated by transcription factors and upstream kinases remains unknown in wood frogs. Therefore, our future studies will also include the investigation of the responses to freezing by transcription factors that are known to regulate these proteins in other systems (e.g. ATF binding to ERSEs, HSF binding to HSE, and others) to try to determine how the freeze signal is transmitted into the up-regulation of shock proteins.

Cellular adaptations to environmental oxygen deprivation constitute an important protective pathway for cells. HIF-1 α , acting as a master regulator of oxygen homeostasis, mediates adaptive responses to changes in tissue oxygenation (Semenza, 2003). Multiple genes are up-regulated by HIF-1 (Semenza et al., 1994; Guillemin and Krasnow, 1997) but several of the commonly known ones (e.g. VEGF, erythropoietin) seem to have no obvious connection with freeze tolerance. Hence, it will be of great interest to identify the proteins that are up-regulated by HIF-1 during freezing in wood frog organs and identify their functions. This could lead to a deeper understanding of the molecular adaptations that support freezing survival. Furthermore, the primary mechanism for regulating HIF-1 α levels in mammalian cells is oxygen-dependent degradation by an ubiquitin-

proteasome pathway that is inhibited by hypoxia (Huang et al., 1998). However, the data for wood frogs suggest that transcriptional control of *hif-1 α* mRNA may also be important in the response to stress. Further studies should explore this to determine whether *hif-1 α* transcriptional control is an important mode of regulation of this transcription factor in lower vertebrates. Studies should also explore the responses to freezing by transcription factors that could up-regulate *hif-1 α* gene expression.

References:

- Abcouwer, S.F., Marjon, P.L., Loper, R.K. and Vander Jagt, D.L. (2002) Response of VEGF expression to amino acid deprivation and inducers of endoplasmic reticulum stress. *Invest. Ophthalm. Vis. Sci.* **43**: 2791-2798.
- Agsteribbe, E., A. Huckriede, M. Veenhuis, M. H. J. Ruiters, K. E. Niezen-Koning, O. H. Skjeldal, K. Skullerud, R. S. Gupta, R. L. Hallberg, O. P. van Diggelen, and H. R. Scholte. (1993) A fatal, systemic mitochondrial disease with decreased mitochondrial enzyme activities, abnormal ultrastructure of the mitochondria and deficiency of heat shock protein 60. *Biochem. Biophys. Res. Commun.* **193**: 146-154.
- Agarraberes, F.A. and Dice. J.F. (2001) A molecular chaperone complex at the lysosomal membrane is required for protein translocation. *J. Cell Sci.* **114**: 2491-2499.
- Agarraberes, F., Terlecky, S. and Dice, J.F. (1997) An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. *J. Cell Biol.* **137**: 825-834.
- Alfieri, R., Petronini P.G., Urbani S., and Borghetti, A.F. (1996) Activation of heat-shock transcription factor 1 by hypertonic shock in 3T3 cells. *Biochem. J.* **319**: 601-606.
- Amrani, M., Latif, N., Morrison, K., Gray, C.C., Jayakumar, J., Corbett, J., Goodwin, A.T., Dunn, M.J., and Yacoub, M.H. (1997) Relative induction of heat shock protein in coronary endothelial cells and cardiomyocytes: implications for myocardial protection. *J. Thorac. Cardiovasc. Surg.* **115**: 200-209.
- Bae W, Xia B, Inouye M, Severinov K 2000. Escherichia coli CspA-family RNA chaperones are transcription antiterminators. *Proc Natl Acad Sci USA.* **97**: 7784-7789.
- Basu, S. and Srivastava, P.K. (1999) Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J. Exp. Med.* **189**: 797-802.
- Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M. and Iwama, G.K. (2002) Heat shock protein genes and their functional significance in fish. *Gene* **295**: 173-183.
- Beck, I., Weinmann, R., and Caro, J. (1993) Characterization of hypoxia responsive enhancer in the human erythropoietin gene shows presence of hypoxia inducible 120-kD nuclear DNA binding protein in erythropoietin producing and nonproducing cells. *Blood* **82**: 704-711.

- Beck, F.X., Neuhofer, W. and Müller, E. (2000) Molecular chaperones in the kidney: distribution, putative roles, and regulation. *Am. J. Physiol.* **279**: F203-F215.
- Benjamin, I.J. and McMillan, D.R. (1998) Stress (heat shock) proteins molecular chaperones in cardiovascular biology and disease. *Circ. Res.* **83**:117-132.
- Bernelli-Zazzera, A., Cairo, G., Schiaffonati, L., and Tacchini, L. (1992) Stress proteins and reperfusion stress in the liver. *Ann. NY Acad. Sci.* **663**: 120-124.
- Brewer, J.W., Cleveland, J.L. and Hendershot, L.M. (1997) A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells. *EMBO J.* **16**: 7207-7216.
- Boutillier, R.G. (2001) Mechanisms of cell survival in hypoxia and hypothermia. *J. Exp. Biol.* **204**: 3171-3181.
- Brostrom, M.A., Cade, C., Prostko, C.R., Gmitter-Yellen, D. and Brostrom, C.O. (1990). Accommodation of protein synthesis to chronic deprivation of intracellular sequestered calcium. A putative role for GRP78. *J. Biol. Chem.* **265**: 20539-20546.
- Buchman, T.G., Cabin, D.E., Porter, J.M. and Bulkley, G.B. (1989) Change in hepatic gene expression after shock/resuscitation. *Surgery* **106**: 283-291.
- Bunn, H.F. and Poyton, R.O. (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**: 839-885.
- Buzzard, K.A., Giaccia, A.J., Killender, M. and Anderson, R.L. (1998) Heat shock protein 72 modulates pathways of stress-induced apoptosis. *J. Biol. Chem.* **273**: 17147-17153.
- Cabin, D.E. and Buchmann, T.G. (1990) Molecular biology circulatory shock. Part III. Human hepatoblastoma (HepG2) cells demonstrate two patterns of shock-induced gene expression that are independent, exclusive, and prioritized. *Surgery* **108**: 902-912.
- Cai, Q. and Storey, K.B. (1997a) Freezing-induced genes in the wood frog *Rana sylvatica*: fibrinogen upregulation by freezing and dehydration. *Am. J. Physiol.* **272**: R1480-R1492.
- Cai, Q. and Storey, K.B. (1996) Anoxia induced gene expression in turtle heart: up-regulation of mitochondrial genes for NADH-ubiquinone oxidoreductase subunit 5 and cytochrome C oxidase subunit 1. *Eur. J. Biochem.* **241**: 83-92.
- Cai, Q. and Storey, K.B. (1997b) Upregulation of a novel gene by freezing exposure in the freeze-tolerant wood frog (*Rana sylvatica*) *Gene* **198**: 305-312.

- Cai, Q., Greenway, S.C. and Storey, K.B. (1997). Differential regulation of the mitochondrial ADP/ATP translocase gene in wood frogs under freezing stress. *Biochim. Biophys. Acta* **1343**: 69-78.
- Cairo, G., Bardella, L., Schiaffonati, L. and Bernelli-Zazzera, A. (1985) Synthesis of heat shock proteins in rat liver after ischemia and hyperthermia. *Hepatology* **5**: 357-361.
- Chai, Y., Koppenhafer, S.L., Bonini, N.M. and Paulson, H.L. (1999) Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. *J. Neurosci.* **19**: 10338–10347.28.
- Chang J., Knowlton A.A. and Wasser J.S. (2000) Expression of heat shock proteins in turtle and mammal hearts: relationship to anoxia tolerance. *Am. J. Physiol.* **278**: R209-R214.
- Chang, J., Wasser, J.S., Cornelussen, R.N.M., Knowlton, A.A. (2001) Activation of heat-shock factor by stretch-activated channels in rat hearts. *Circulation* **104**: 209.
- Chappell, T.G., Welch, W.J., Schlossman, D.M., Palter, K.B., Schlesinger, M.J. and Rothman, J.E. (1986) Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* **45**: 3-13.
- Cheng, M.Y., Hartl, F.U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989) Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature* **337**: 620-625.
- Chiang, H.L., Terlecky, S.R., Plant, C.P. and Dice, J.F. (1989) A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* **246**: 382–385.
- Ciavarra, R.P., Goldman, C., Wen, K.-K., Tedeshi, B. and Castora, F.J. (1994) Heat stress induced hsc70/nuclear topoisomerase I complex formation in vivo: evidence for hsc70-mediated ATP-independent reactivation in vitro. *Proc. Natl. Acad. Sci. USA* **91**: 1751-1755.
- Collier, N.C. and Schlesinger, M.J. (1986) The dynamic state of heat shock proteins in chicken embryo fibroblasts. *J Cell Biol* **103**: 1495-1507.
- Cowan, K.J. and Storey, K.B. (2003) Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *J. Exp. Biol.* **206**: 1107-1115.

- Cowley, B.D., Muessel, M.J., Douglass, D. and Wilkins, W. (1995) In vivo and in vitro osmotic regulation of HSP-70 and prostaglandin synthase gene expression in kidney cells. *Am. J. Physiol.* **269**: F854-F862.
- Currie, R.W., Karmazyn, M., Kloc, M. and Mailer, K. (1988) Heat-shock response is associated with enhanced post-ischemic recovery. *Circ. Res.* **63**: 543-549.
- Dean, D.O., Kent, C.R. and Tytell, M. (1999) Constitutive and inducible heat shock protein 70 immunoreactivity in the normal rat eye. *Invest. Ophthalmol. Visual Sci.* **40**: 2952-2962.
- Denlinger DL. 2002. Regulation of diapause. *Annu Rev Entomol.* **47**: 93-122.
- Denlinger DL, Lee RE, Yocum GD, Kukal O. 1992. Role of chilling in the acquisition of cold tolerance and the capacitation to express stress proteins in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* **21**:271-80
- Dhahbi, J.M., Cao, S.X., Mote, P.L., Rowley, B.C., Wingo, J.E. and Spindler, S.R. (2002) Postprandial induction of chaperone gene expression is rapid in mice. *J. Nutr.* **132**: 31-37.
- Dill, K.A. and H.S. Chan. (1997). From Levinthal to pathways to funnels. *Nat. Struct. Biol.* **4**: 10-19.
- Dorner, A.J., Wasley, L.C. and Kaufman, R.J. (1992) Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. *EMBO J.* **11**: 1563-1571.
- Ebert, B.L., Firth, J.D. and Ratcliffe, P.J. (1995) Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter 1 via distinct cis acting sequences. *J. Biol. Chem.* **270**: 29083-29089.
- Ema, M., Taya, S., Tokotani, N., Sogowa, K., Matsuda, Y. and Fujii-Kuriyama, Y. (1997) A novel bHLH-PAS factor with close sequence similarity to hypoxia inducible factor 1 α regulates VEGF expression and is potentially involved in lung and vascular development. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 4273-4278.
- Emerson, F., Malyshev, I.Y. and Zamotrinsky, A.V. (1992) Phenomenon of the adaptive stabilization of structures and protection of the heart. *Can. J. Cardiol.* **8**: 965-974.
- Epstein, A.C.R., Gleadle, J.M. and McNeill, L.A. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**: 43-54.

- Fandrey, J. (2004) Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. *Am. J. Physiol.* **286**: R977-R988.
- Fedele, A.O., Whitelaw, M.L. and Peet, D.J. (2002) Regulation of gene expression by hypoxia-inducible factors. *Mol. Interventions* **2**: 229-243.
- Flanagan, S.W., Ryan, A.J., Gisolfi, C.V. and Moseley, P.L. (1995) Tissue-specific HSP70 response in animals undergoing heat stress. *Am. J. Physiol.* **268**: R28-R32.
- Flaherty, K.M., Flaherty, C.D. and McKay, D.B. (1990). Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* **346**: 623-628.
- Freeman, B.C., Myers, M.P., Schumacher, R. and Morimoto, R.I. (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO J.* **14**: 2281-2292.
- Frydman, J. and Hohfeld, J. (1997) Chaperones get in touch: the Hip-Hop connection. *Trends Biochem. Sci.* **22**: 87-92.
- Gabai, V.L., Meriin, A.B., Mosser, D.D., Caron, A.W., Rits, S., Shifrin, V.I. and Sherman, M.Y. (1997) Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *J.Biol. Chem.* **272**: 18033-18037.
- Garrido, C., Gurbuxani, S., Ravagnan, L. and Kroemer, G. (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Commun.* **286**: 433-442.
- Gaudio, K.M., Thulin, G., Mann, A., Kashgarian, M. and Siegel, N.J. (1998) Role of heat stress response in the tolerance of immature renal tubules to anoxia. *Am. J. Physiol.* **274**: F1029-F1036.
- Gaut, J.R. (1997) In vivo threonine phosphorylation of immunoglobulin binding protein (BiP) maps to its protein binding domains. *Cell Stress Chaperones* **2**: 252-262.
- Gazit, G., Kane, S.E., Nichols, P. and Lee, A.S. (1995) Use of the stress-inducible grp78/BiP promoter in targeting high level gene expression in fibrosarcoma *in vivo*. *Cancer Res.* **55**: 1660-1663.
- Gazit, G., Hung, G., Chen, X., Anderson, W.F. and Lee, A.S. (1999) Use of the glucose starvation-inducible glucose-regulated protein 78 promoter in suicide gene therapy of murine fibrosarcoma. *Cancer Res.* **59**: 3100-3106.
- Gething, M.J. (1999) Role and regulation of the ER chaperone BiP. *Cell Dev. Biol.* **10**: 465-472.
- Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**: 33-45.

- Gleadle, J.M., Ebert, B.L., Firth, J.D. and Ratcliffe, P.J. (1995) Regulation of angiogenic growth factor expression by hypoxia, transition metals, and chelating agents. *Am. J. Physiol.* **268**: C1362-C1368.
- Gomer, C.J., Ferrario, A., Rucker, N., Wong, S. and Lee, A.S. (1991) Glucose regulated protein induction and cellular resistance to oxidative stress mediated by porphyrin photosensitization. *Cancer Res.* **51**: 6574-6579.
- Goldberg, M.A., Dunning, P. and Bunn, H.F. (1988) Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. *Science* **242**: 1412-1415.
- Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J.S., Dalrymple, B., Kuramitsu, H., Shiroza, T., Foster, T., Clark, W.P., Ross, B., Squires, C.L. and Maurizi M.R. (1999) Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **87**: 3513-3517.
- Guillemin, K. and Krasnow, M.A. (1997) The hypoxic response: Huffing and HIFing. *Cell* **89**: 9-12.
- Hai, T.W., Liu, F., Coukos, W.J. and Green, M.R. (1989) Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* **3**: 2083-2090.
- Hamman, B.D., Hendershot, I.M. and Johnson, A.E. (1998) BiP maintains the permeability barrier of ER membrane by sealing the luminal end of the translocon pore before and early in translocation. *Cell* **92**: 747-758.
- Hartl, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature* **381**: 571-580.
- Hatayama, T., Yasuda, K. and Yasuda, K. (1998) Association of HSP105 with HSC70 in high molecular mass complexes in mouse FM3A cells. *Biochem. Biophys. Res. Commun.* **248**: 395-401.
- Hatayama, T., Yamagishi, N., Minobe, E. and Sakai, K. (2001) Role of hsp105 in protection against stress-induced apoptosis in neuronal PC12 cells. *Biochem. Biophys. Res. Commun.* **288**: 528-534.
- Hatayama, T., Nishiyama, E., and Yasuda, K. (1994) Cellular localization of high-molecular-mass heat shock proteins in murine cells. *Biochem. Biophys. Res. Commun.* **200**: 1367-1373.

- Hayward SA, Rinehart JP, Denlinger DL 2004 Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae. *J Exp Biol.* **207**: 963-971.
- Haze, K., Yoshida, H. Yanagi, H., Yura, T. and Mori, K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol. Biol. Cell* **10**: 3787-3799.
- Hermes-Lima, M., Storey, J.M. and Storey, K.B. (2001). Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. *In: Cell and Molecular Responses to Stress* (Storey, K.B. and Storey, J.M., eds.), Elsevier Press, Amsterdam, Vol. 2, pp. 263-287.
- Hightower, L. and Leung, S.M. (1997) Mammalian Hsc70 and Hsp70. *In: Guidebook to Molecular Chaperones and Protein-Folding Catalysis* (Gething, M.J., ed), London: Oxford University Press, pp. 53-58.
- Hohfeld, J., Minami, Y. and Hartl, F.U. (1995) Hip, a novel cochaperone involved in the eukaryotic hsc70/hsp40 reaction cycle. *Cell* **83**: 589-598.
- Hightower, L.E. and Hendershot, L.M. (1997) Molecular chaperones and the heat shock response at Cold Spring Harbor. *Cell Stress Chaperones* **2**: 1-11.
- Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M. and Pavietich, N.P. (1999) Structure of an E6-AP-Ubc H7 complex insight into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**: 1321-1326.
- Huang, L.E., Arany, Z., Livingston, D.M. and Bunn, H.F. (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. *J Biol Chem.* **271**: 32253-32259.
- Hurtley, S.M. and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell. Biol.* **5**: 277-307.
- Iwaki, K., Chi, S.H., Dilmann, W.H. and Mestril, R. (1993) Induction of HSP70 in cultured rat neonatal cardiomyocytes by hypoxia and metabolic stress. *Circulation* **87**: 2023-2032.
- Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., Semenza, G.L. (1998) Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor 1 α . *Genes Dev.* **12**: 149-162.

- Jackson, D.C. (2001) Anoxia survival and metabolic arrest in the turtle. *In: Metabolic mechanisms of metabolic arrest* (Storey, K.B., ed), Oxford: BIOS Scientific Publishers, pp.103-114.
- Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H., Gassmann, M. (2001) Induction of HIF-1 α in response to hypoxia is instantaneous. *FASEB J* **15**: 1312-1314.
- Jiang, B.H., Semenza, G.L., Bauer, C. and Marti, H.H. (1996) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O₂ tension. *Am. J. Physiol.* **271**: C1172-C1180.
- Jiang, B.H., Zheng, J.Z., Leung, S.W., Roe, R. and Semenza, G.L. (1997) Transactivation and inhibitory domains of hypoxia inducible factor 1 α : Modulation of transcriptional activity by oxygen tension. *J. Biol. Chem.* **272**: 19253–19260.
- Kaelin, W.G. (2002) How oxygen makes its presence felt. *Genes Dev.* **16**: 1441–1445.
- Kallio, P.J., Pongratz, I., Gradin, K., McGuire, J. and Poellinger, L. (1997) Activation of hypoxia-inducible factor 1 α : posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 5667-5672.
- Kallio, P.J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L. (1998) Signal transduction in hypoxic cells: Inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia inducible factor-1 α . *EMBO J.* **17**: 6573–6586.
- Kaneko, Y., Nishiyama, H., Nonoguchi, K., Higashitsuji, H., Kishishita, M. and Fujita, J. (1997) A novel hsp110-related gene, *apg-1*, that is abundantly expressed in the testis responds to a low temperature heat shock rather than the traditional elevated temperatures. *J. Biol. Chem.* **272**: 2640–2645.
- Kietzmann, T., Cornesse, Y., Brechtel, K., Modaressi, S. and Jungermann, K. (2001) Perivenous expression of the mRNA of the three hypoxia-inducible factor α -subunits, HIF1 α , HIF2 α and HIF3 α , in rat liver. *Biochem. J.* **354**: 531-537.
- King, C., Eisenberg, E. and Greene, L.E. (1999) Interaction between Hsc70 and DnaJ homologues: relationship between Hsc70 polymerization and ATPase activity. *Biochemistry* **38**: 12452-12459.
- Klemenz, R., Fröhli, E., Steiger, R.H., Schäfer, R. and Aoyama, A. (1991) α B-crystallin is a small heat shock protein. *Proc. Natl. Acad. Sci. USA* **88**: 3652-3656.
- Knowlton, A.A. (1995) The role of heat shock proteins in the heart. *J. Mol. Cell. Cardiol.* **27**: 121-131.

- Knarr, G., Gething, M.J., Modrow, S. and Buchner, J. (1995) BiP-binding sequences in antibodies. *J. Biol. Chem.* **270**: 27589-27694.
- Kojima, R., Randall, J., Brenner, B.M. and Gullans, S.R.. (1996) Osmotic stress protein 94 (Osp94). *J. Biol. Chem.* **271**: 12327-12332.
- Koong, A.C., Chen, E.Y., Lee, A.S., Brown, J.M. and Giaccia, A.J. (1994) Increased cytotoxicity of chronic hypoxic cells by molecular inhibition of GRP78 induction. *Int. J. Radiat. Oncol. Biol. Phys.* **28**: 661-666.
- Kozak, M. (1991) An analysis of vertebrate mRNA sequences: Intimations of translational control. *J. Cell. Biol.* **115**: 887-903.
- Krebs, R. and Bettencourt, B. (1999) Evolution of thermotolerance and variation in the heat shock protein. *Am. Zool.* **39**: 910-919.
- Lau, S., Patnaik, N. and Sayen, M.R. (1997) Simultaneous overexpression of two stress proteins in rat cardiomyocytes and myogenic cells confers protection against ischemia- induced injury. *Circulation* **96**: 2287-2294.
- Lee, A.S. (1987). Coordinated regulation of a set of genes by glucose and calcium ionophore in mammalian cells. *Trends Biochem. Sci.* **12**: 20-24.
- Lee, A.S. (2001) The glucose-regulated proteins: stress induction and clinical applications. *Trends Biol. Sci.* **26**: 504 – 510.
- Leustek, T., Toledo, H., Brot, X. and Weissbach, H. (1991) Calcium-dependent autophosphorylation of the glucose-regulated protein, grp78. *Arch. Biochem. Biophys.* **289**: 256-261.
- Lindquist, S. and Craig, E. A. (1998) The heat-shock proteins. *Ann. Rev. Gen.* **22**: 631 – 677.
- Lin, K.M., Lin, B., Lian, I.Y., Mestril, R., Scheffler, I.E. and Dillmann, W.H. (2001) Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia-reoxygenation. *Circulation* **103**: 1787.
- Lipton, P. (1999) Ischemic cell death in brain neurons. *Physiol. Rev.*, **79**: 1431-2568.
- Li, L.-J., Li, X., Ferrario, A., Rucker, N., Liu, E.S., Wong, S., Gomer, C.J. and Lee, A.S. (1992) Establishment of a Chinese hamster ovary cell line that expresses GRP78 antisense transcripts and suppresses A23817 induction of both GRP78 and GRP94. *J. Cell Physiol.* **153**: 575-582.

- Li, W.W., Hsiung, Y., Zhou, Y., Roy, B. and Lee, A.S. (1997) Induction of the mammalian GRP78/BiP gene by Ca^{2+} depletion and formation of aberrant proteins: activation of the conserved stress-inducible grp core promoter element by the human nuclear factor YY1. *Mol. Cell. Biol.* **17**: 54-60.
- Li, M., Baumeister, P., Roy, B., Phan, T., Foti, D., Luo, S. and Lee, A.S. (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Mol. Cell. Biol.* **20**: 5096-5106.
- Liu, E.S. and Lee, A.S. (1991) Common sets of nuclear factors binding to the conserved promoter sequence motif of two coordinately regulated ER protein genes, GRP78 and GRP94. *Nucl. Acids Res.* **19**: 5425-5431.
- Luo, S., Baumeister, P., Yang, S., Abcouwer, S.F. and Lee, A.S. (2003) Induction of Grp78/BiP by translational block activation of the Grp78 promoter by ATF4 through an upstream ATF/CRE site independent of the endoplasmic reticulum stress elements. *J. Biol. Chem.* **278**: 37375-37385.
- Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Åsman, M., Tanaka, H., Cao, Y., Berkenstam, A. and Poellinger, L. (2001) Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature* **414**: 550-554.
- Marber, M.S., Latchman, D.S., Walker, J.M. and Yellon, D.M. (1993) Cardiac stress protein elevation 24 h after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* **88**: 1264-1272.
- Marber, M.S., Mestril, R., Chi, S.H., Sayen, M.R., Yellon, D.M. and Dillmann, W.H. (1995) Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J. Clin. Invest.* **95**: 1446-1456.
- Matlack, K.E., Misselwitz, B., Plath, K. and Rapoport, T.A. (1999) BiP acts as a molecular ratchet during posttranslational transport of prepro- α factor across the ER membrane. *Cell* **97**: 553-564.
- Maxwell, P.H., Wiesener, M.S., Chang, G.-W., Clifford, S.C., Vaus, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R. and Ratcliffe, P.J. (1999) The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen dependent proteolysis. *Nature* **399**: 271-275.
- McNally, J.D., Wu, S., Sturgeon, C.M. and Storey, K.B. (2002) Identification and characterization of novel freezing inducible gene, Li16, in the wood frog, *Rana sylvatica*. *FASEB J.* **16**: 902-904 or online at FASEB J. 10.1096/fj.02-0017fje.

- McNally, J.D., Sturgeon, C.M. and Storey, K.B. (2003) Freeze induced expression of a novel gene, *fr47*, in the liver of the freeze tolerant wood frog, *Rana sylvatica*. *Biochim. Biophys. Acta* **1625**: 183-191.
- McMillan, D.R., Gething, M.J. and Sambrook, J. (1994) The cellular response to unfolded proteins: intercompartmental signaling. *Curr. Opin. Biotechnol.* **5**: 540-545.
- Melnick, J., Dul, J.L. and Argon, Y. (1994) Sequential interaction of the chaperones Bip and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature* **370**: 373-375.
- Mehta, N.K., Carroll, M., Sykes, D.E., Tan, Z., Bergsland, J., Canty, J., Bhayana, J.N., Hoover, E.L. and Salerno, T.A. (1997) Heat shock protein 70 expression in native and heterotopically transplanted hearts. *J. Surg. Res.* **70**: 151-155.
- Minchenko, A., Bauer, T., Salceda, S. and Caro, J. (1994) Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab. Invest.* **71**: 374-379.
- Minchenko, A., Leschinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V. and Caro, J. (2002) Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (*PFKFB3*) gene. *J. Biol. Chem.* **277**: 6183-6187.
- Morris, J.A., Dorner, A.J., Edwards, C.A., Hendershot, L.M. and Kaufman, R.J. (1997) Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. *J. Biol. Chem.* **272**: 4327-4334.
- Morimoto, R.I., Kline, M.P., Bimston, D.N. and Cotto, J.J. (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem.* **32**: 17-29.
- Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (1994) Progress and perspectives on the biology of heat shock proteins and molecular chaperones. *In: The Biology of Heat Shock Proteins and Molecular Chaperones.* (Morimoto, R.I., Tissieres, A. and Georgopoulos, C, eds.) Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Moseley, P.L. (1997) Heat shock proteins and heat adaptation of the whole organism. *J. Appl. Physiol.* **83**: 1413-1417.
- Muller, E., Neuhofer, W., Ohno, A., Rucker, S., Thureau, K. and Beck, F.X. (1996) Heat shock proteins HSP25, HSP60, HSP72, and HSP73 in isoosmotic cortex and hyperosmotic medulla of rat kidney. *Pflügers Arch.* **431**: 608-617.

- Muresan, Z. and Arvan, P. (1997) Thyroglobulin transport along the secretory pathway. Investigation of the role of molecular chaperone, GRP94, in protein export from the endoplasmic reticulum. *J. Biol. Chem.* **272**: 26095-26102.
- Nakano, M., Mann, D.L. and Knowlton, A.A. (1997) Blocking the endogenous increase in HSP72 increases susceptibility to hypoxia and reoxygenation in isolated adult feline cardiocytes. *Circulation* **95**: 1523–1531.
- Neuhöfer, W., Müller, E., Thürau, K. and Beck, F.-X. (1999) Inhibition of NaCl-induced HSP72 expression causes apoptosis in MDCK cells exposed to high urea concentrations (Abstract). *Pflügers Arch* **437**: R99.
- Ohashi, K., Burkhardt, V. and Flohé, S. (2002) Cutting edge: heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. *J Immunol.* **164**: 558–561.
- Ohno, A., Müller, E., Fraek, M.L., Thürau, K., and Beck, F.-X. (1997) Solute composition and heat shock proteins in rat renal medulla. *Pflügers Arch.* **434**: 117-122.
- Okada, T., Yoshida, H., Akazawa, R., Negishi, M. and Mori, K. (2002) Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response *Biochem. J.* **366**: 585-594.
- O'Rourke, J.F., Tian, Y.M., Ratcliffe, P.J. and Pugh, C.W. (1999) Oxygen regulated and transactivation domains in endothelial PAS protein 1: Comparison with hypoxia inducible factor 1 α . *J. Biol. Chem.* **274**: 2060–2071.
- Ozawa, K., Kuwabara, F., Tamatani, M., Takatsuji, K., Tsukamoto, Y., Kaneda, S., Yanagi, H., Stern, D.M., Eguchi, Y., Tsujimoto, Y., Ogawa, S. and Tohyama, M. (1999) 150-kDa Oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. *J. Biol. Chem.* **274**: 6397-6404.
- Pahl, H.L. (1999) Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol. Rev.* **79**: 683-701.
- Palmer, L.A., Gaston, B. and Johns, R.A. (2000) Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides. *Mol. Pharm.* **58**: 1197-1203.
- Pelham, H.R. (1986) Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell* **46**:959-961.
- Phang, D., Joyce, E.M. and Heikkilä, J.J. (1999) Heat shock-induced acquisition of thermotolerance at the levels of cell survival and translation in *Xenopus* A6 kidney epithelial cells. *Biochem. Cell Biol.* **77**: 141-151.

- Piacentini, L. and Karliner, J.S. (1999) Altered gene expression during hypoxia and reoxygenation of the heart. *Pharm. Ther.* **83**: 21-37.
- Qu, D., Mazzarella, R.A. and Green, M. (1994) Analysis of the structure and synthesis of GRP94, an abundant stress protein of the endoplasmic reticulum. *DNA Cell Biol.* **13**: 117-124.
- Rauchman, M.I., Pullman, J. and Gullans, S.R. (1997) Induction of molecular chaperones by hyperosmotic stress in mouse inner medullary collecting duct cells. *Am. J. Physiol.* **273**: F9-F17.
- Ritossa, F. (1962) A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia* **18**: 571-573.
- Roy, B. and Lee, A.S. (1995) Transduction of calcium stress through interaction of the human transcription factor CBF with the proximal CCAAT regulatory element of the *grp78/BiP* promoter. *Mol. Cell. Biol.* **15**: 2263-2274.
- Ruas, J.L., Poellinger, L. and Pereira, T. (2002) Functional analysis of hypoxia-inducible factor-1 α -mediated transactivation – identification of amino acid residues critical for transcriptional activation and/or interaction with CBP. *J. Biol. Chem.* **10**: 1074.
- Rubinsky, B., Wong, S.T.S., Hong, J.S., Gilbert, J., Roos, M. and Storey, K.B. (1994) ^1H magnetic resonance imaging of freezing and thawing in freeze-tolerant frogs. *Am. J. Physiol.* **266**: R1771-R1777.
- Ryan, M.T., Naylor, D.J., Hoj, P.B., Clark, M.S. and Hoogenraad, N.J. (1997) The role of molecular chaperones in mitochondrial protein import and folding. *Int. Rev. Cytol.* **174**: 127-193.
- Sachs, A.B., Sarnow, P. and Hentze, M.W. (1997) Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell* **89**: 831-838.
- Sadis, S. and Hightower, L.E. (1992) Unfolded proteins stimulate nucleolar chaperone Hsc70 ATPase by accelerating ADP/ATP exchange. *Biochemistry* **31**: 9406-9412.
- Safran, M. and Kaelin, M.G. (2003) HIF hydroxylation and the mammalian oxygen-sensing pathway. *J. Clin. Invest.* **111**: 779-783.
- Saleh, A., Srinivasula, S.M., Balkir, L., Robbins, P.D. and Alnemri, E.S. (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat. Cell Biol.* **2**: 476-483.

- Santos, B.C., Chevaile, A., Kojima, R. and Gullans, S.R. (1998) Characterization of the Hsp110/SSE gene family response to hyperosmolality and other stresses. *Am. J. Physiol.* **274**: F1054-F1061.
- Salceda, S., Beck, I., Srinivas, V. and Caro, J. (1997) Complex role of protein phosphorylation in gene activation by hypoxia. *Kidney Int.* **51**: 556.
- Salceda, S. and Caro, J. (1997) Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. *J. Biol. Chem.* **272**: 22642-22647.
- Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P. and Orrenius, S. (1999) Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. *EMBO J.* **18**: 2040-2048.
- Schmidt, E. and Schibler, U. (1995) Cell size regulation, a mechanism that controls cellular RNA accumulation: consequences on regulation of the ubiquitous transcription factors Oct1 and NF-Y and the liver-enriched transcription factor DBP. *J. Cell Biol.* **128**: 467-483.
- Scholz, H. (2002) Adaptational responses to hypoxia. *Am. J. Physiol.* **282**: R1541-R1543.
- Scott, M.A., Locke, M. and Buck, L.T. (2003) Tissue-specific expression of inducible and constitutive Hsp70 isoforms in the western painted turtle. *J. Exp. Biol.* **206**: 303-311.
- Semenza, G.L. (2002) HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol. Med.* **8**: S62-67.
- Semenza, G.L., Roth, P.H., Fang, H.M. and Wang, G.L. (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia inducible factor 1. *J. Biol. Chem.* **269**: 23757-23763.
- Shipley, J., Qian, Y., Levasseur, J. and Kukreja, R. (1995) Expression of the stress proteins HSP-27 and HSP-72 in rat hearts does not correlate with ischemic tolerance after heat-shock. *Circulation* **92**: 3139-3142.
- Shiu, R.P., Pouyssegur, J. and Pastan, I. (1977) Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in rous sarcoma virus-transformed chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **74**: 3840-3844.
- Shoemaker, V.H. (1992) Exchange of water, ions, and respiratory gases in terrestrial amphibians. *In* *Environmental physiology of the amphibians*. (Feder, M.E. and Burggren, W.W., eds). Chicago: University of Chicago Press, pp.125-159.

- Shweiki, D.A., Itin, A., Soffer, D. and Keshet, E. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**: 843–845.
- Smith, D.F., Whitesell, L. and Katsanis, E. (1998) Molecular Chaperones: Biology and Prospects for Pharmacological Intervention. **50**(4): 493-514.
- Snoeckx, L.H.E.H., Cornelussen, R.N., Van Nieuwenhoven, F.A., Reneman, R.S. and van der Vusse, G.J. (2001) Heat shock proteins and cardiovascular pathophysiology. *Physiol. Rev.* **81**: 1461-1497.
- Soitamo, A.J., Christina, M. I., Sistonen, L. and Nikinmaa, M. (2001) Characterization of a hypoxia-inducible factor (HIF-1 α) from rainbow trout. *J. Biol. Chem.* **276**: 19699-19705.
- Sorger, PK. (1991) Heat shock factor and the heat shock response. *Cell* **65**: 363-366.
- Srivastava, P. (1993) Peptide-binding heat shock proteins in the endoplasmic reticulum: role in immune response to cancer and in antigen presentation. *Adv. Cancer Res.* **62**: 153-177.
- Storey, J.M. and Storey, K.B. (1996b) β -Adrenergic, hormonal, and nervous influences on cryoprotectant synthesis by liver of the freeze tolerant wood frog *Rana sylvatica*. *Cryobiology* **33**: 186-195.
- Storey, J.M. and Storey, K.B. (1985). Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*. *J. Comp. Physiol. B* **155**: 333-337.
- Storey, K.B. (1987). Glycolysis and the regulation of cryoprotectant synthesis in liver of the freeze tolerant wood frog. *J. Comp. Physiol. B* **157**: 373-380.
- Storey, K.B. (2001) Turning down the fires of life: metabolic regulation of hibernation and estivation. *In: Molecular Mechanisms of Metabolic Arrest* (Storey, K.B., ed.). BIOS Scientific Publishers, Oxford, pp.1-21.
- Storey, K.B. 2004. Strategies for exploration of freeze responsive gene expression: advances in vertebrate freeze tolerance. *Cryobiology* **48**:134-45.
- Storey, K.B. and Storey, J.M. (1983) Biochemistry of freeze tolerance in terrestrial insects. *Trends Biochem. Sci.* **8**: 242-245.
- Storey, K.B. and Storey, J.M. (1986). Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze/thaw cycles. *Can. J. Zool.* **64**: 49-56.

- Storey, K.B. and Storey, J.M. (1988) Freeze tolerance in animals. *Physiol. Rev.* **68**: 27-84.
- Storey, K.B. and Storey, J.M. (1989). Freeze tolerance and freeze avoidance in ectotherms. *In: Animal Adaptation to Cold* (Wang, L.C.H., ed.), Springer-Verlag, Heidelberg, pp. 51-82.
- Storey, K.B. and Storey J.M. (1991) Biochemistry of cryoprotectants. *In: Insects at Low Temperature* (Denlinger, D. and Lee, R.E., eds.), Chapman and Hall, New York, pp. 64-93.
- Storey, K.B. and Storey, J.M. (1992). Natural freeze tolerance in ectothermic vertebrates. *Ann. Rev. Physiol.* **54**: 619-637.
- Storey, K.B. and Storey, J.M. (1996). Natural freezing survival in animals. *Ann. Rev. Ecol. Syst.* **27**: 365-386.
- Storey, K.B. and Storey, J.M. (1999). Gene expression and cold hardiness in animals. *II: Cold-adapted Organisms - Ecology, Physiology, Enzymology and Molecular Biology* (Margesin, R. and Schinner, F., eds.), Springer, Heidelberg, pp. 385-407.
- Storey, K.B. and Storey, J.M. (2001) Signal transduction and gene expression in the regulation of natural freezing survival. *Cell and Molecular Responses to Stress* (Storey, K.B. and Storey, J.M., eds.) Elsevier Press, Amsterdam, Vol., 2, pp. 1-19.
- Storey, K.B. and Storey, J.M. (2002). Editors: *Cell and Molecular Responses to Stress, Vol. 3: Sensing, signaling and cell adaptation*. Elsevier Press, Amsterdam.
- Storey, K.B. and Storey, J.M. (2004) Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: the wood frog. *In: Life in the Frozen State* (Benson, E., Fuller, B., and Lane, N., eds.) CRC Press, Boca Raton, pp. 243-274.
- Storey, K.B., Bischof, J. and Rubinsky, B. (1992) Cryomicroscopic analysis of freezing in liver of the freeze tolerant wood frog. *Am. J. Physiol.* **263**: R185-R194.
- Storey, K.B., Storey, J.M. and Churchill, T.A. (1997) De novo protein biosynthesis responses to water stresses in wood frogs: freeze-thaw and dehydration-rehydration. *Cryobiology* **34**: 200-213.
- Storey, K.B., Storey, J.M., Brooks, S.P.J., Churchill, T.A. and Brooks, R.J. (1988) Hatchling turtles survive freezing during winter hibernation. *Proc. Natl. Acad. USA* **85**: 8350-8354
- Stroka D.M., Burkhardt, T., Wenger R.H., Desley A.H., Desley A. H. (2002) *Candina D.* HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia *The FASEB Journal*. **15**:2445-2453.

- Subjectek, J.R., Shyy, T., Shen, J. and Johnson, R.J. (1983) Association between the mammalian 110.000 dalton heat shock protein and nucleoli. *J. Cell. Biol.* **97**: 1389-1395.
- Sugawara, S., Takeda, K., Lee, A. and Dennert, G. (1993) Suppression of stress protein GRP78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. *Cancer Res.* **53**: 6001-6005.
- Suh, W.C., Lu, C.Z. and Gross, C.A. (1999) Structural features required for the interaction of the Hsp70 molecular chaperone DnaK with its cochaperone DnaJ. *J. Biol. Chem.* **274**: 30534-30539.
- Talks, K.L., Turley, H., Gatter, K.C., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J. and Harris, A.L. (2000) The expression and distribution of the hypoxia-inducible factors HIF-1 α and HIF-2 α in normal human tissues, cancers, and tumor-associated macrophages. *Am. J. Pathol.* **157**: 411-421.
- Thomas, P.J., Qu, B.H. and Pedersen, P.L. (1995) Defective protein folding as a basis of human disease. *Trends Biochem. Sci.* **20**: 456-459.
- Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Mol. Biol.*, **50**, 571-599.
- Thuerauf, D.J., Arnold, N.D., Zechner, D., Hanford, D.S., DeMartin, K.M. McDonough, P.M., Prywes, R. and Glembotski, C.C. (1998) p38 Mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element. A potential role for the transcription factor ATF6. *J. Biol. Chem.* **273**: 20636-20643.
- Tian, H., McKnight, S.L. and Russell, D.W. (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11**: 72-82.
- Tsai, B., Ye, Y. and Rapoport, T.A. (2002) Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat. Rev. Mol. Cell Biol.* **3**: 246-255.
- Turman, M.A. and Rosenfeld, S.L. (1999) Heat shock protein 70 overexpression protects LLC-PK₁ tubular cells from heat shock but not hypoxia. *Kidney Int.* **55**: 189-197.
- Van Why, S.K., Hildebrandt, F., Ardito, T., Mann, A.S., Siegel, N.J. and Kashgarian, M. (1992) Induction and intracellular localization of HSP-72 after renal ischemia. *Am. J. Physiol.* **263**: F769-F775.

- Wang, G.L., Jiang, B.-H. and Semenza, G.L. (1995) Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1. *Biochem. Biophys. Res. Commun.* **216**: 669.
- Wang, Y.H. and Borkan, S.C. (1996) Prior heat stress enhances survival of renal epithelial cells after ATP depletion. *Am. J. Physiol.* **270**: F1057-F1065.
- Wang, G.L. and Semenza, G.L. (1995) Purification and characterization of hypoxia-inducible factor 1. *J. Biol. Chem.* **270**: 1230-1237.
- Warters, R.L., Chu, G.L., Wong, R.S.L., Lyons, B.W. and Dewey, W.C. (1993) Nuclear protein redistribution in heat-shocked cells. *J. Cell Physiol.* **154**: 402-409.
- Welch, W.J. and Suhan, J.P. (1985) Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelle, cytoskeleton, and nucleoli, and the appearance of intranuclear actin filaments in rat fibroblasts after heat shock treatment. *J. Biol. Chem.* **101**: 1198-1211.
- Welch, W.W. and Mizzen, L.A. (1988) Characterization of the thermotolerant cell. Effects of the intracellular distribution of heat-shock protein 70, intermediate filaments, and small nuclear ribonucleoprotein complexes. *J. Cell Biol.* **106**: 1117-1130.
- Wiener, C.M., Booth, G. and Semenza, G.L. (1996) In vivo expression of mRNAs encoding hypoxia-inducible factor 1. *Biochem. Biophys. Res. Commun.* **225**: 485-488.
- Willmore, W.G., English, T.E. and Storey K.B. (2001). Mitochondrial gene responses to low oxygen stress in turtle organs. *Copeia* 2001: 628-637.
- Wojtaszek, P.A., Heasley, L.E. and Berl, T. (1988) In vivo regulation of MAP kinases in *Rattus norvegicus* renal papilla by water loading and restriction. *J. Clin. Invest.* **102**: 1874-1881.
- Welch, W.J. (1992) Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol. Rev.* **72**: 1063-1081.
- Xanthoudakis, S., Roy, S., Rasper, D., Hennessey, T., Aubin, Y., Cassady, R., Tawa, P., Ruel, R., Rosen, A. and Nicholson, D.W. (1999) Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. *EMBO J.* **18**: 2049-2056.
- Xiao H, Lis JT. (1988) Germline transformation used to define key features of heat-shock response elements. *Science.* **239**:1139-1142.

- Xia, W, and Voellmy R. (1997) Hyperphosphorylation of heat shock transcription factor 1 is correlated with transcriptional competence and slow dissociation of active factor trimers. *J Biol Chem* **272**: 4094-4102.
- Xu, Y. and Lindquist, S. (1993) Heat-shock protein hsp90 governs the activity of pp60v-src kinase. *Proc. Natl. Acad. Sci. USA* **90**: 7074 –7078.
- Yamagishi, N., Saito, Y., Ishihara, K. and Hatayama. T. (2002) Enhancement of oxidative stress-induced apoptosis by Hsp105 α in mouse embryonal F9 cells. *Eur. J. Biochem.* **269**, 4143-4151.
- Yamagishi, N., Nishihori, H., Ishihara, K., Ohtsuka, K. and Hatayama, T. (2000) Modulation of the chaperone activities of Hsc70/Hsp40 by Hsp105 α and Hsp105 β . *Biochem. Biophys. Res. Commun.* **272**, 850–855.
- Yenari, M.A., Giffard, R.G., Sapolsky, R.M. and Steinberg, G.K. (1999) The neuroprotective potential of heat shock protein 70 (HSP70). *Mol. Med. Today* **5**, 525-531.
- Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T. and Mori, K. (2000) ATF6 activated by proteolysis directly binds in the presence of NF-Y (CBF) to the *cis*-acting element responsible for the mammalian unfolded protein response. *Mol. Cell. Biol.* **20**, 6755–6767.
- Yoshida, H., Haze, K., Yanagi, H., Yura, T. and Mori, K. (1998) Identification of the *cis*-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins; involvement of basic-leucine zipper transcription factors. *J. Biol. Chem.* **273**: 33741–33749.
- Yu, A.Y. Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL. (1998) Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *Am. J. Physiol.* **275**:L818-L826.
- Zhang J, Furukawa RD, and Fremes SE. (1996) The beneficial effects of heat shock for prolonged hypothermic storage. *J Surg Res* **63**: 314-319.
- Zhong, M, Kim SJ, and Wu C. (1999) Sensitivity of *Drosophila* heat shock transcription factor to low pH. *J Biol Chem* **274**: 3135-3140.
- Zhu, X., X. Zhao, W.F. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman, and W.A. Hendrickson. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**: 1606-1614.

Table 3.2 Summary of the changes in HIF-1 α protein levels in wood frog tissues during stress and recovery (see Figures 3.6 and 3.8)

Tissues	Liver	Muscle	Brain	Heart	Gut	Skin	Kidney
24 h frozen	▲ (1.4±0.04)	▲ (1.38±0.03)	—	—	▲ (1.22±0.05)	▼ (0.57±0.02)	—
24 h anoxia	▲ (1.12±0.07)	—	▼ (0.81±0.08)	▼ (0.75±0.05)	—	▲ (1.25±0.06)	▲ (1.54±0.04)
40% dehydration	▲ (1.28±0.09)	—	▲ (1.78±0.09)	—	—	—	▲ (1.87±0.10)
Recover from 24 h frozen: Relative to 24 h frozen:	▼	▼	—	▼	▼	▲	—
Relative to control:	▼ (0.61±0.04)	—	—	—	—	▼ (0.80±0.04)	—
Recover from 24 h anoxia: Relative to 24 h anoxia:	▲	—	▲	▲	—	▼	—
Relative to control:	▲ (1.31±0.07)	—	▲ (1.4±0.08)	—	—	—	▲ (1.5±0.09)
Recover from 40% dehydration: Relative to 40% dehydration:	—	▼	—	—	▲	—	—
Relative to control:	—	▼ (0.39±0.08)	▲ (1.72±0.08)	—	▲ (1.38±0.08)	—	▲ (1.93±0.19)

“—” represents relative stable between the compared data, $p>0.05$; “▲” represents relative up-regulation, $p<0.05$; “▼” represents relative down regulation, $p<0.05$. The relative values represent Mean $\pm$ SEM (n=4,5 independent experiments).

Tissues	Liver	Muscle	Brain	Heart	Gut	Skin	Kidney
24h frozen	▲ (1.61±0.08)	—	—	▲ (1.88±0.16)	—	—	▲ (1.24±0.10)
24h anoxia	▲ (2.61±0.15)	—	▲ (1.46±0.08)	▲ (1.51±0.09)	—	—	▲ (1.15±0.09)
40%dehydration	—	▲ (1.47±0.08)	▲ (1.63±0.08)	▲ (1.42±0.09)	—	▲ (1.33±0.06)	—
Recover from 24h frozen: Relative to 24h frozen:	▼	▲	▼	▼	▼	—	▼
Relative to control:	—	—	▼ (0.47±0.07)	—	—	—	—
Recover from 24h anoxia: Relative to 24h anoxia:	▼	▲	—	—	—	—	▼
Relative to control:	▼ (0.56±0.08)	▲ (1.61±0.06)	▲ (1.58±0.10)	▲ (1.66±0.05)	—	▲ (1.20±0.08)	▼ (0.63±0.09)
Recover from 40%dehydration: Relative to 40% dehydration:	—	▼	▲	▼	—	▲	▼
Relative to control:	—	—	▲ (1.93±0.12)	▲ (1.32±0.09)	—	—	▼ (0.67±0.03)

“—” represents relative stable between the compared data, p>0.05; “▲” represents relative up-regulation, p<0.05; “▼” represents relative down regulation, p<0.05. The relative values represent Mean ± SEM (n=4,5 independent experiments).

(B) Hsc70 protein expression

Tissues	Liver	Muscle	Brain	Heart	Gut	Skin	kidney
24h frozen	▲ (1.39±0.08)	▲ (1.85±0.06)	▼ (0.62±0.05)	▲ (1.98±0.12)	▲ (1.71±0.12)	—	▲ (1.53±0.09)
24h anoxia	▼ (0.5±0.08)	▲ (1.64±0.05)	—	▲ (1.21±0.05)	—	—	▲ (1.10±0.05)
40%dehydration	—	▲ (1.30±0.07)	▼ (0.63±0.04)	▲ (1.36±0.02)	—	▼ (0.65±0.04)	▲ (1.34±0.07)
Recover from 24h frozen: Relative to 24h frozen:	▼	—	—	▼	▼	▼	—
Relative to control:	—	▲ (1.98±0.11)	▼ (0.75±0.07)	▲ (1.37±0.06)	—	▼ (0.59±0.08)	▲ (1.64±0.14)
Recover from 24h anoxia: Relative to 24h anoxia:	▲	▼	▼	▼	—	▼	▼
Relative to control:	▼ (0.72±0.08)	▲ (1.51±0.05)	▼ (0.63±0.09)	—	—	▼ (0.81±0.07)	—
Recover from 40%dehydration: Relative to 40% dehydration:	▼	▼	—	▼	▼	—	▼
Relative to control:	▼ (0.54±0.07)	—	▼ (0.64±0.04)	▲ (1.20±0.09)	—	▼ (0.57±0.06)	—

“—” represents relative stable between the compared data, $p>0.05$; “▲” represents relative up-regulation, $p<0.05$; “▼” represents relative down regulation, $p<0.05$. The relative values represent Mean $\pm$ SEM (n=4,5 independent experiments).

(C) Hsp60 protein expression

Tissues	Liver	Muscle	Brain	Heart	Gut	Skin	Kidney
24h frozen	▲ (1.62±0.06)	▲ (1.67±0.09)	▲ (2.20±0.11)	▼ (0.48±0.05)	▲ (1.97±0.07)	▲ (1.48±0.10)	▲ (1.46±0.10)
24h anoxia	▲ (1.57±0.12)	▲ (1.69±0.18)	▲ (1.22±0.07)	▲ (1.46±0.05)	▼ (0.45±0.06)	▲ (1.31±0.09)	—
40%dehydration	▲ (1.41±0.07)	▼ (0.76±0.05)	—	—	▼ (0.67±0.08)	▲ (1.67±0.03)	▲ (1.56±0.09)
Recover from 24h frozen: Relative to 24h frozen:	—	—	▼	—	▲	▼	▼
Relative to control:	▲ (1.56±0.11)	▲ (1.64±0.06)	▲ (1.81±0.06)	▼ (0.45±0.04)	▲ (2.52±0.12)	▼ (0.63±0.07)	—
Recover from 24h anoxia: Relative to 24h anoxia:	▼	▼	—	▼	▲	▲	—
Relative to control:	—	—	▲ (1.27±0.06)	—	—	▲ (1.42±0.03)	—
Recover from 40%dehydration: Relative to 40% dehydration:	▲	▼	—	—	—	▼	▼
Relative to control:	▲ (2.26±0.20)	▼ (0.56±0.02)	—	—	▼ (0.7±0.03)	▲ (1.38±0.03)	—

“—” represents relative stable between the compared data, $p>0.05$; “▲” represents relative up-regulation, $p<0.05$; “▼” represents relative down regulation, $p<0.05$. The relative values represent Mean $\pm$ SEM (n=4,5 independent experiments).

(D) Hsp40 protein expression

Tissues	Liver	Muscle	Brain	Heart	Gut	Skin	Kidney
24h frozen	▲ (1.87±0.06)	▲ (3.4±0.20)	▼ (0.78±0.09)	▲ (1.48±0.06)	—	▼ (0.40±0.06)	▲ (1.51±0.11)
24h anoxia	—	▲ (1.89±0.08)	▲ (1.60±0.08)	▲ (1.87±0.13)	▼ (0.25±0.08)	—	▲ (6.50±0.09)
40%dehydration	▲ (1.44±0.09)	▲ (1.37±0.06)	▲ (1.56±0.11)	—	▼ (0.13±0.02)	▲ (1.91±0.07)	▲ (4.17±0.19)
Recover from 24h frozen: Relative to 24h frozen:	—	▼	▲	▼	—	—	—
Relative to control:	▲ (1.75±0.15)	—	—	—	—	▼ (0.48±0.03)	▲ (1.51±0.07)
Recover from 24h anoxia: Relative to 24h anoxia:	▲	▼	—	▼	—	▲	▼
Relative to control:	▲ (2.45±0.20)	—	▲ (1.54±0.13)	—	▼ (0.29±0.06)	▲ (2.63±0.15)	▲ (4.91±0.17)
Recover from 40%dehydration: Relative to 40% dehydration:	—	▼	▼	▼	▲	▼	▲
Relative to control:	▲ (1.57±0.12)	—	—	▼ (0.69±0.08)	▼ (0.53±0.04)	▲ (1.31±0.08)	▲ (6.61±0.13)

“—” represents relative stable between the compared data, $p>0.05$; “▲” represents relative up-regulation, $p<0.05$; “▼” represents relative down regulation, $p<0.05$. The relative values represent Mean $\pm$ SEM (n=4,5 independent experiments).

(E) Hsp10 protein expression

Tissues	Liver	Muscle	Brain	Heart	Gut	Skin	Kidney
24h frozen	▲ (1.48±0.06)	▲ (1.67±0.11)	—	▲ (1.55±0.11)	—	▲ (1.60±0.12)	▲ (1.62±0.07)
24h anoxia	▲ (1.48±0.07)	▼ (0.40±0.02)	▲ (2.30±0.16)	▼ (0.65±0.08)	▲ (1.47±0.09)	—	▲ (1.39±0.09)
40%dehydration	▲ (1.23±0.06)	—	▲ (1.89±0.11)	▲ (1.86±0.17)	—	▲ (1.32±0.07)	▲ (1.28±0.07)
Recover from 24h frozen: Relative to 24h frozen:	▲	▼	—	▼	▲	▼	▼
Relative to control:	▲ (1.98±0.10)	▲ (1.24±0.06)	—	—	▲ (1.45±0.05)	—	—
Recover from 24h anoxia: Relative to 24h anoxia:	▼	▲	▼	▲	▼	▲	▼
Relative to control:	—	—	▲ (1.99±0.09)	—	—	▲ (1.46±0.08)	—
Recover from 40%dehydration: Relative to 40% dehydration:	▲	—	▼	—	▲	▼	—
Relative to control:	▲ (1.4±0.09)	—	—	▲ (1.76±0.15)	▲ (2.55±0.14)	—	▲ (1.35±0.08)

“—” represents relative stable between the compared data, $p>0.05$; “▲” represents relative up-regulation, $p<0.05$; “▼” represents relative down regulation, $p<0.05$. The relative values represent Mean $\pm$ SEM (n=4,5 independent experiments).