FREEZE TOLERANT FROGS: EXPRESSION AND REGULATION OF TRANSCRIPTION FACTORS OF THE UNFOLDED PROTEIN RESPONSE AND THE ER-ASSOCIATED DEGRADATION

By

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Abstract

Wood frogs (*Rana sylvatica*) are the primary model used in the study of freeze tolerance in vertebrates and much is known about the adaptations of physiology, biochemistry and gene expression that support winter freezing survival, particularly their natural cryoprotective processes. Freezing and/or its components (anoxia and dehydration) places multiple stresses on cells; one of these is endoplasmic reticulum (ER) stress, a condition caused by accumulation of unfolded or misfolded proteins in the ER. The regulated expression of selected transcription factors, such as ATF4, that trigger genes that protect against ER stress is important for cell survival of freezing. During ER stress, the unfolded protein response (UPR) and the ER-associated degradation (ERAD) pathway are triggered, which can potentially lead to apoptosis. Western blots were used to evaluate the responses by key protein components of the UPR and the ERAD under freezing, anoxia and dehydration stresses in two major organs of wood frogs (skeletal muscle and liver). The proteins analyzed included the activating transcription factors (ATF3, ATF4, ATF6), the growth arrest and DNA damage proteins (GADD34, GADD153), and the EDEM and XBP1 proteins. Stress-induced redistribution of transcription factors between cytoplasmic and nuclear fractions was also evaluated. All three stresses triggered the UPR in both tissues but only freezing of skeletal muscle seemed to trigger the ERAD. Only anoxic treated skeletal muscle showed metabolic signs of potential apoptosis. It was concluded that wood frog organs activate the UPR as a means of stabilizing cellular proteins and shutting down global protein synthesis in order to survive freezing exposures without irreparable injury.

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Table of Contents

List of Abbreviations

AFP	Anti-Freeze Protein
ATF	Activating transcription factor
CHOP	C/EBP-homologous protein
CREB1	cAMP response element binding protein
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDEM	ER degradation enhancing α-mannosidase-like protein
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene(oxyethylenenitrilo)tetraacetic acid
eIF2α	Eukaryotic initiation factor 2, α subunit
ER	Endoplasmic Reticulum
ERAD	Endoplasmic reticulum associated degradation
GADD	Growth arrest and DNA damage
GLS	Golgi localization sequence
GRP	Glucose regulated protein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
INP	Ice Nucleating Proteins
IRE1	Inositol requiring kinase 1
kDa	kilodalton
mRNA	messenger RNA
PAGE	Polyacrylamide gel electrophoresis
P-eIF2α	Phosphorylated eukaryotic initiation factor 2 alpha

PERK	PKR-like endoplasmic reticulum kinase
PP1	Protein phosphatase 1
P^{58IPK}	Protein kinase inhibitor p58
PVDF	Polyvinylidene difluoride
S1P, S2P	Site-1 protease, Site-2 protease
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
TBST	Tris-buffered saline Tween-20
Tris	Tris(hydroxymethyl)aminomethane
UPR	Unfolded protein response
XBP1	X-box binding protein 1

List of Tables

List of Figures

Chapter 1

General Introduction

The wood frog, *Rana sylvatica*, has been widely used as the main model animal for studying the mechanisms of whole body freezing survival in vertebrates. Numerous studies have analyzed physiological, biochemical and gene expression adaptations that confer freeze tolerance to wood frogs, many of them conducted in our laboratory. The focus of this thesis is a phenomenon that has not previously been analyzed in any freeze tolerant species – the unfolded protein response (UPR). Cell stress can frequently disrupt the folding of proteins into their correct functional conformation, either disrupting the initial folding of nascent proteins or destabilizing existing proteins. It is known that ~20% of proteins are synthesized or associated with the endoplasmic reticulum (Becker et al., 2003). The UPR encompasses a variety of responses that address the problems that result from an accumulation of unfolded proteins in cells. This thesis examines how the UPR functions in a freeze tolerant species.

1.1 Natural Stress survival

Every living organism on the earth must deal with a multitude of environmental stresses such as fluctuation in oxygen levels, the availability of food, and variation in temperature, to name a few. Organisms must be flexible in order to survive the range of variability of their environment. Extreme changes outside of the normal range can cause structural or metabolic damage to cells/tissues or, ultimately, even death of the organism.

For terrestrial ectotherms, temperature change is a very important variable; extremes of both heat and cold can be damaging or lethal. In particular, ectotherms living in northern environments have to deal with ambient temperatures that can drop far below 0°C during the winter months. For most animals, freezing is synonymous with death for

several reasons including: (a) physical damage caused by ice crystals, (b) extreme cellular dehydration due to water loss into extracellular ice masses and causing irreversible compression damage to cell membranes (Figure 1.1), and (c) cut off of oxygen and fuel supplies to tissues when blood freezes. Hence, adaptive strategies are needed to survive below 0°C. Some animals prevent freezing by moving to warmer environments; this includes migration (short or long distances), retreating underground below the frost line, or spending the winter underwater (Storey and Storey, 2004b). However, others cannot avoid subzero exposure and once temperatures decrease below 0°C, the organisms have two options in order to survive: (a) freeze avoidance through deep supercooling, or (b) freeze tolerance using mechanisms that allow animals to survive when as much as 65-70% of body water turns to ice (Storey, 2004).

The wood frog is one of the species that has developed freeze tolerance. These frogs are found throughout most of Canada and their range extends north to the treeline (Figure 1.2). *R. sylvatica* is known to spend the winter on the forest floor under layers of leaf detritus and snow. Here they are insulated from the lowest air temperatures, but under the snow the temperatures will still fall to -5°C or lower in midwinter (Storey and Storey, 2004b). The wood frog's skin is no barrier to either water or ice and therefore, once ice begins to form on the skin's surface, it quickly seeds body fluids and ice propagates through the body (Storey and Storey, 2004b). As the animals freeze, vital signs including muscle movement, heart beat, breathing and brain activity are progressively lost but after thawing, their vital functions are fully recovered (Storey and Storey, 1993).

1.2 Freeze Tolerance

Freeze tolerance in nature involves the controlled freezing of the extracellular water in an organism, while preserving the cytoplasm in a liquid state. Some reptiles, amphibians and many kinds of invertebrates are capable of tolerating freezing by making use of four general adaptive processes. The first is the addition of ice nucleating proteins to the blood in order to trigger ice formation in extracellular compartments and allow ice growth to be slow and controlled (Storey and Storey, 1992; Storey and Storey, 2004b). Secondly, organisms alter their metabolism to synthesize high concentrations of cryoprotectants (e.g. glycerol, glucose) that are packed into cells and help to minimize cell water loss and volume reduction by colligative action (Storey, 1997; Storey and Storey, 1996; Storey and Storey, 2004b) (Figure 1.1). Thirdly, because plasma freezing cuts off supplies of oxygen, cells must reduce their energy demands through metabolic rate depression and switch to a reliance on carbohydrate fuels while frozen (Storey and Storey, 2004b). Finally, freeze-responsive up-regulation of specific genes produces selected proteins that provide protection to cells while frozen.

One of the key components of the controlled ice propagation in wood frogs are Ice Nucleating Proteins (INPs). These proteins induce controlled freezing of extracellular water at multiple sites which, in turn, ensures that ice formation is confined to extracellular spaces (Storey et al., 1992; Wolanczyk et al., 1990). The presence of INPs will trigger the ice formation at temperatures that are usually well above -10°C (-0.5°C in wood frogs on wet substrate), thus preventing extensive supercooling and allowing for slow formation of the crystals (Storey and Storey, 2004b). INP triggered ice formation

produces small crystals at a controlled rate which reduces their chance of causing physical damage to cells. However, such small ice crystals are thermodynamically less stable and susceptible to recrystallization into much larger crystals that would be physically damaging to cells (Storey and Storey, 2004b; Storey et al., 1992; Wolanczyk et al., 1990). In insects, it was found that ice management is also aided by Anti-Freeze Proteins (AFPs) that are capable of inhibiting extracellular ice recrystallization, but previous studies have failed to detect anti-freeze activity in the blood of *R. sylvatica* (Storey and Storey, 2004b; Storey et al., 1992; Wolanczyk et al., 1990).

For intracellular cryoprotection, wood frogs use glucose. As soon as freezing begins, the levels of glucose rise rapidly from about 5 mM in unfrozen frogs to 150-300 mM in core organs once the animal is fully frozen (Storey and Storey, 2004b; Storey and Storey, 1986). High glucose in cells acts in a colligative manner to slow down or minimize the reduction in cell volume caused by water loss into extracellular ice (Figure 1.1) and because of this also minimizes the total body ice content (Layne and Lee, 1995; Costanzo et al., 1993).

Freezing is a complicated stress that actually causes several different problems for cells. Freezing can cause physical damage by ice crystal formation and when wood frogs freeze their liver up-regulates its production of fibrinogen, a blood clotting protein, so that the animals can deal rapidly with any internal bleeding that is detected after thawing (Cai and Storey, 1997). Freezing also cuts off blood flow, causing ischemia, and cutting off the delivery of oxygen and nutrients to tissues. Therefore, frog tissues are under low oxygen (hypoxia) or even anoxia stress while they are frozen. Furthermore, during thawing, the rapid re-introduction of oxygen to tissues could cause oxidative stress due to

a rapid rise in reactive oxygen species generation during reperfusion (Joanisse and Storey, 1996). Damage due to oxidative stress is common in many systems that undergo ischemia/reperfusion. The cells of frozen frogs must also contend with extensive dehydration that is caused by cellular water loss into extracellular ice (Storey and Storey, 2004b; Joanisse and Storey, 1996). All of these stresses have potentially negative consequences for intracellular metabolism.

It is well known that proteins can denature when exposed to both high and low temperatures and that the change in conformation of these proteins could cause a perturbation in the homeostasis of the endoplasmic reticulum. Unfolded and malfolded proteins can also accumulate under other stress conditions such as oxygen limitation. In previous studies done in our lab it has been shown that under freezing, anoxia and dehydration stresses in wood frogs, increased levels of glucose regulated proteins (GRPs) are observed (Zhenhong, 2004). Since these proteins are involved in the protein folding machinery, increased GRP levels would then suggest that the cells of frozen frogs are under endoplasmic reticulum (ER) stress caused by unfolded or malfolded proteins present in the organelle. Several mechanisms are available to cells in order to deal with ER stress; collectively these mechanisms constitute the UPR. They include: (a) halting general protein synthesis by inhibiting ribosomal translation factors (commonly by phosphorylation of the initiation factor, eIF2α), (b) enhancing the folding capacity of the ER, and (c) increasing the rate of degradation of malfolded proteins. Activation of the UPR includes a cascade of transcription factors centered around the activating transcription factor 4 (ATF4).

Hypothesis: Freezing along with its two associated stresses (anoxia,

dehydration) activates the unfolded protein response in wood frog tissues to limit or prevent the accumulation of malfolded and unfolded proteins in the endoplasmic reticulum.

The experiments described in the following chapters tested this hypothesis for skeletal muscle and liver of wood frogs. These two major organs have different characteristics during freezing. Being a peripheral organ, skeletal muscle is one of the earliest organs to freeze (along with skin) and has low cryoprotectant levels. By contrast, magnetic resonance imaging showed that liver is the last organ to freeze in the frogs (Rubinsky et al., 1994) and during the freezing process, liver is highly active in the synthesis of glucose cryoprotectants and in the production of protective proteins. To determine the cellular responses to ER stress during freezing, a range of transcription factors and other proteins associated with the UPR were analyzed in skeletal muscle (Chapter 3) and liver (Chapter 4).

Figure 1.1: Cellular responses to freezing in wood frogs. The figure shows the effects of freezing on both protected and unprotected cells. INP, ice nucleating protein; MP, membrane protectant; AFP, antifreeze protein; g, glucose. From Storey and Storey (2004b).

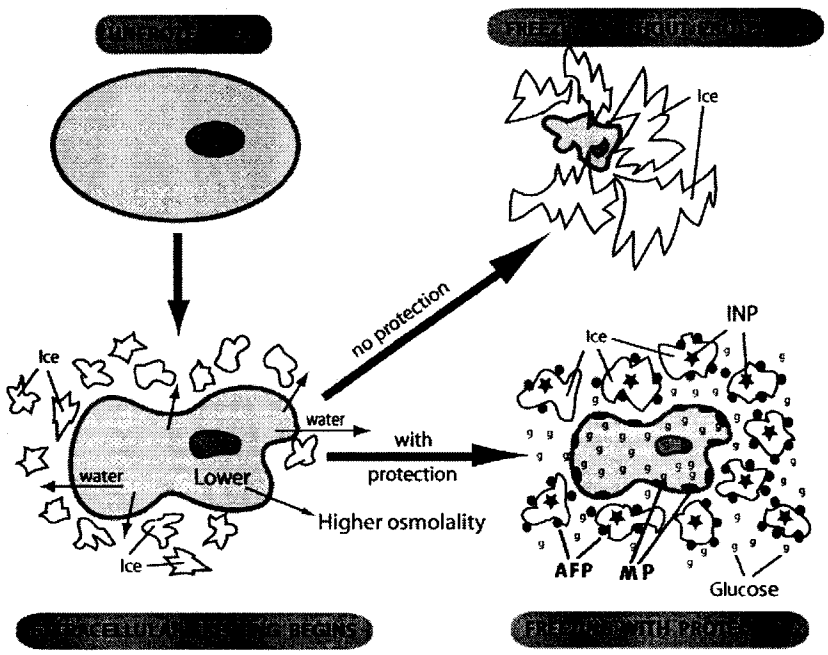
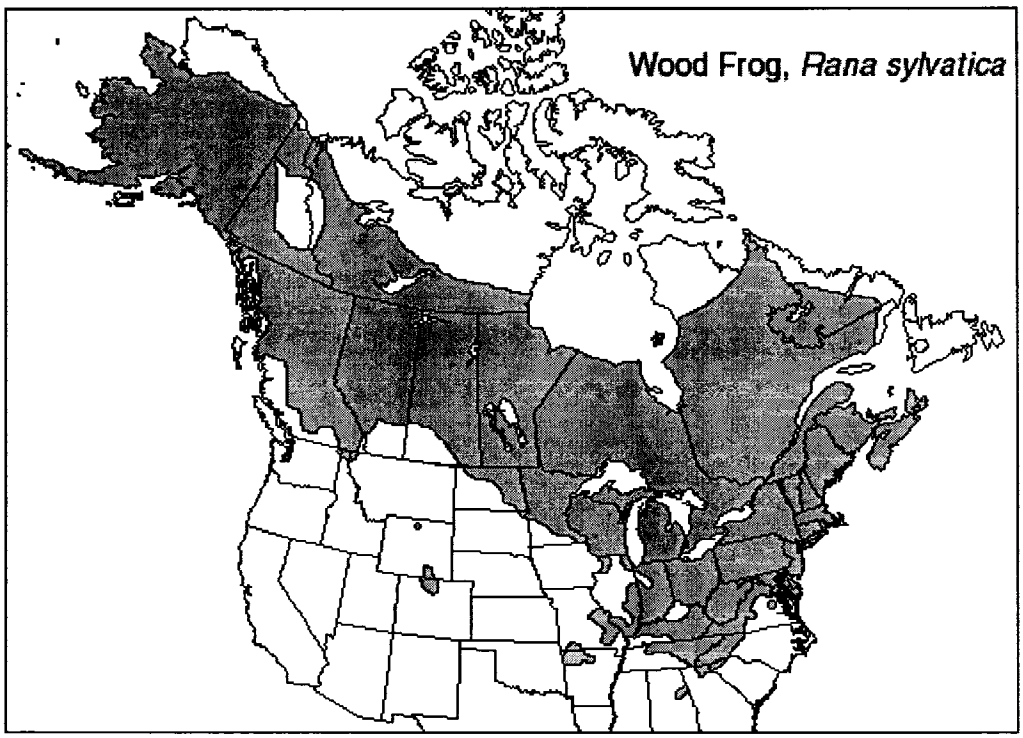


Figure 1.2: The distribution of wood frogs, *Rana sylvatica*, in North America. Copied from the Northern Prairie wildlife Research Center, 2007.



Chapter 2

Materials and methods

2.1 Animal experiments

Male wood frogs, *Rana sylvatica*, were collected from Ottawa area breeding ponds during the spring of 2003. The captured frogs were washed in a tetracycline bath and then held in a plastic box containing damp sphagnum moss at 5°C for 1 to 2 weeks before use. The control frogs were killed by pithing and the organs of interest were quickly excised and flash frozen in liquid nitrogen before being stored at -80°C until used. For the freezing exposure wood frogs were placed in plastic containers that had been lined with dampened paper towels, closed and placed in a -2.5°C incubator. The dampened paper towels allowed for rapid seeding of tissue freezing when ice formed on the substrate. The frogs cooled below 0°C and within 45 min started to freeze, as shown from initial trials using frogs with thermistors taped onto their abdomens (Storey and Storey, 1985). After the initial 45 min of cooling, a 24 h freezing exposure was initiated. Some frogs sampled after 24 h while still frozen, whereas others were returned to 5°C and sampled after 8 h thawing. Frogs were killed by pithing and then each was dissected within 30 to 90 seconds with the help of 4-6 people. Tissues were flash frozen in liquid nitrogen. Tissues sampled were brain, liver, heart, kidney, gut (stomach and intestine), hind leg thigh skeletal muscle and abdominal skin.

For the anoxia exposure experiments the protocol of Holden and Storey (1997) was used. The frogs were first acclimatized for a week at 5°C. Plastic jars (700 ml) were lined with wet paper towelling that had been wetted with distilled water that was previously bubbled with 100% nitrogen gas. The jars were set in crushed ice and then flushed for 20 min with nitrogen gas via a syringe port in the lid (another port vented gas). Following this, frogs were transferred into the jars (5 to 6 frogs in each). The jars

were then capped and parafilm was used to seal the top of the jars. Nitrogen gassing was continued through the syringe port for a further 30 min before the gas lines were removed and the ports were sealed. Jars were then placed in the 5°C incubator for a period of 24 h. After this time, half of the frogs were sampled whereas the others were transferred to back to aerobic conditions and sampled after a 4 h recovery period.

For dehydration experiments, the frogs were treated according to the protocol described by Churchill and Storey (1994). Each frog subjected to the experiment was weighed and individually tagged for identification. Approximately 5 to 6 frogs were placed in a large dessicator jars containing a layer of silica gel desiccant on the bottom. A 1 cm thick sponge separated the frogs from the silica. The jars were kept at 5°C. The frogs were quickly removed and weighed at intervals during the experiment to determine the amount of body water lost using the equation: (Mi-Md) / (Mi x $\%H_2O$), where Mi is the initial mass of the animal, Md is the mass at each weighing and $\%\ H_2O$ is the percentage of total body mass that is water. For control wood frogs, $\%\ H_2O$ is 80.8 ± 1.2 % (Churchill and Storey, 1994). The mean rate of water loss during the experiment was 0.5% of total body water lost per hour. Experimental dehydration was continued until the animals lost 40% of total body water. Half of the frogs were then sampled whereas the others were allowed to recover to full rehydration over 24 h after transferring the animals to a container with 1 cm of distilled water on the bottom.

Protocols for the care, experimentation and euthanasia of the animals were approved by the Carleton University Animal Care Committee in accordance with the guidelines put forth by the Canadian Council on Animal Care.

2.2 Western Blotting

2.2.1 Isolation of total protein from frog tissues

Total soluble proteins were extracted independently from tissue samples of control, frozen (24 h), thawed (8 h), anoxic (24 h), aerobic recovery (6 h), 40 % dehydrated and rehydrated frogs. For cell free extracts ~500 mg of frozen tissue was placed in 1 ml of homogenization buffer (20 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 10 mM ß-glycerophosphate, 1% v/v Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) and homogenized independently using a Polytron homogenizer. Samples were then centrifuged for 15 min at 10,000 x g and 4°C and supernatants were transferred to clean tubes. Soluble protein concentration was then measured. This was done for four independent samples of each treatment.

2.2.2 Isolation of nuclear extracts from frozen tissues:

Nuclear extracts were made using a modified version of the method described by Dignam *et al.* (1983). Aliquots of ~0.5 g frozen tissue were weighed, then 500 µl of homogenization buffer was added [10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM DTT and 10 µl of protease inhibitor cocktail (Sigma)] and the tissue was independently disrupted using a Dounce homogenizer. A 10 min centrifugation at 8000 x g was used to pellet the nuclei and then the cytoplasmic extract in the supernatant was transferred to a new clean tube. The pellets were then independently washed with the same buffer as above, then resuspended in 150 µl of extraction buffer (20 mM HEPES, pH7.9, 400 mM NaCl, 1 mM EDTA, 10% v/v glycerol, 10 mM DTT, and 1.5 µl protease inhibitor cocktail) and placed on a rocker at 4°C for 1 h at a slow speed. After

centrifugation at 10,000 x g for 10 min, the supernatant (nuclear extract) was collected. This was done for four independent samples for controls and stressed experiments. Protein concentrations in both cytoplasmic and nuclear fractions were determined using the Bio-Rad assay. Since these extracts are used to study the migration of transcription factors into the nucleus, it is important that the separation of cytoplasmic and nuclear fractions be confirmed. This was done by running 10 µg aliquots of both fractions on a SDS-PAGE gel, followed by Western blotting using an anti-histone H3 antibody (Cell Signaling) at a dilution of 1:1000, followed by anti-rabbit secondary antibody for 1.5 h at 1:2000 dilution. Bands for histone H3 were found only in the nuclear extracts and not in cytoplasmic extracts, thus confirming the integrity of the nuclei (data not shown).

2.2.3 Measurement and dilution of proteins:

The Coomassie blue dye binding method was used to determine protein concentrations using the Bio-Rad prepared reagent with bovine serum albumin as the standard (BioRad, Hercules, CA). The reagent was diluted 5-fold and typically protein extracts were diluted 20-fold, both with distilled water, before assay. Microplate wells typically contained 10 µl of diluted protein and 190 µl of reagent. Color development occurred over 10 min and then samples were read at 595 nm using a microplate reader.

Aliquots of standard tissue extracts were then diluted 1:1 v/v with 2X SDS-PAGE sample buffer (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.4% w/v bromophenol blue) with 10% v/v fresh 2-mercaptoethanol added. These were then boiled for 5 min and stored at -20°C. The final protein concentration in samples for standard Western blotting was 2 µg/µl.

2.2.4 SDS polyacrylamide gel electrophoresis:

SDS polyacrylamide gels (10% acrylamide, 0.4 M Tris pH 8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED) were prepared with 5% upper stacking gels (5% acrylamide, 0.13 M Tris pH6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED). For each gel, an equal amount of soluble protein (ranging from 10-20 µg depending on the protein to be detected) was loaded into each well and then proteins were separated at 180 V for 45 min. In order to estimate the size of the proteins, an aliquot of Kaleidoscope prestained molecular mass ladder (Bio-Rad) was loaded in one well. After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore) by wet transfer with pre-chilled transfer solution (25 mM Tris pH8.5, 192 mM glycine, 20% methanol) at 4°C for 1 h and 30 min 160 mA. The blots were then blocked with 2.5% non-fat dried milk dissolved in Tris buffered saline containing Tween-20 (TBST: 20 mM Tris base, 140 mM NaCl, 0.05% v/v Tween-20), followed by an overnight incubation at 4°C with primary antibody (Santa Cruz Biotechnology Inc.) diluted with TBST with a little bit of sodium azide added; different primary antibodies had different working dilutions (Table 2.1). The next day the blots were washed multiple times with TBST and then incubated with secondary antibody, either anti-rabbit, anti-goat or anti-mouse conjugated to horseradish peroxidase (Cell Signaling) for 2 h at 1:2000 v:v dilution in TBST. Detection of the signal used the enhanced chemiluminescence (ECL) system as recommended by the manufacturer (Millipore). The membrane was then scanned with the Syngene (Chemiluminescence digital camera system) and the band densities were analyzed using Gene tools software (Syngene, MD, USA). To confirm equal loading of the lanes, the blots were then stained with Coomassie blue.

2.3 Statistical analysis

Statistical significance of the differences between normalized control (n=4, data from 4 independent samples), normalized experimental values (n=4, data from 4 independent samples) and normalized recovered values was evaluated using the Mynova program using Analysis of Variance followed by the Student-Newman-Keuls test with $P<0.05$ accepted as significantly different. The statistical significance of the cytoplasmic and nuclear normalized experimental values (n= 4, data from 4 independent samples) and normalized control values (n=4, data from 4 independent samples) was evaluated with the Student's t-test, $P<0.05$.

2.4 Variability between samples

In order to check if there was any variability between aliquots of the same sample as well as the variability between a sample run on multiple gels, former student from the lab performed experiments. Different aliquots of the same sample were run on a single blot. The variability was calculated as being <2%. One aliquot of a sample was run on many different gels and the variability was also calculated to be <2%.

For a more in depth look at the methods see attached technical bulletins in Appendices A and B.

Table 2.1. List of primary antibodies used with their source, their corresponding secondary antibody, the dilution factors used (in brackets) and the expected molecular size of the protein detected by each primary antibody.

Primary Antibody	Secondary Antibody	Size	Company
Anti-ATF 3 (1:200)	Anti-Rabbit (1:2000)	~ 21 kDa	Santa Cruz
Anti-ATF 4 (1:1000)	Anti-Rabbit (1:2000)	~ 40 kDa	Santa Cruz
Anti-ATF 6 (1:1000)	Anti-Goat (1:2000)	~ 90 kDa ~ 50 kDa	Santa Cruz
Anti-GADD34 (1:200)	Anti-Rabbit (1:2000)	~ 80 kDa	Santa Cruz
Anti-GADD 153 (1:200)	Anti-Mouse (1:2000)	~ 30 kDa	Santa Cruz
Anti-XBP1 (un) (1:1000)	Anti-Goat (1:2000)	~ 31 kDa	Santa Cruz
Anti-XBP1 (sp) (1:1000)	Anti-Rabbit (1:2000)	~ 50 kDa	From Dr. Glimcher
Anti-EDEM (1:200)	Anti-Goat (1:2000)	~75 kDa	Santa Cruz

Chapter 3

Coping with ER stress in Skeletal Muscle

Introduction

This chapter deals with the ability of skeletal muscle to cope with the stress of freezing and with two stresses that are components of freezing: anoxia and dehydration. As discussed in Chapter 1, all three stresses analyzed in this thesis have some things in common. It is known that in muscles undergoing long periods of hypoxic stress that the numbers of mitochondria in the tissue decrease and that metabolism shifts towards carbohydrate reliance. During freezing, muscle activity is halted along with subsequent losses (23-36%) of water (Storey and Storey, 2004). Stresses that reduce/halt muscle activity have previously been shown to drastically reduce the amount of protein synthesis in muscle and are also associated with malfolding of some of the synthesized protein (Paddon-Jones et al., 2006) which is a precursor to endoplasmic reticulum (ER) stress (Schröder and Kaufman, 2005a).

The ER is the initial compartment in a vast membranous secretory pathway in eukaryotic cells. Proteins are synthesized, modified, transported and exported via this pathway (Harding et al., 2002). This organelle is very important to the well-being of the cell and, in addition to protein production, the ER has a vital role as a quality control center (Becker et al., 2003). The signaling pathways of quality control are known to be upregulated during ER stress.

ER stress is, in the simplest words, a perturbation of cell functions that triggers two cascade pathways, the unfolded protein response (UPR) and the endoplasmic reticulum associated degradation (ERAD) pathway. ER stress is caused by the accumulation of unfolded or malfolded proteins (Schröder and Kaufman, 2005a). Physiological conditions that can induce ER stress generally fall into five major

categories: (a) development of secretory cells (plasma cells, pancreatic β cells), (b) altered metabolism (e.g. glucose deprivation, ischemia), (c) genetic mutation (DNA damage, mutated secretory proteins), (d) pathogenesis (viral infection), and (e) chemical insult (e.g. inhibition of N-linked glycosylation, disruption of Ca^{2+} homeostasis) (Rutkowski and Kaufman, 2004). These conditions impart a high folding demand on the ER and its resident protein folding machinery, and can exceed the capacity of the machinery resulting in a build up of malfolded protein that triggers the ER stress response (Schröder and Kaufman, 2005 b).

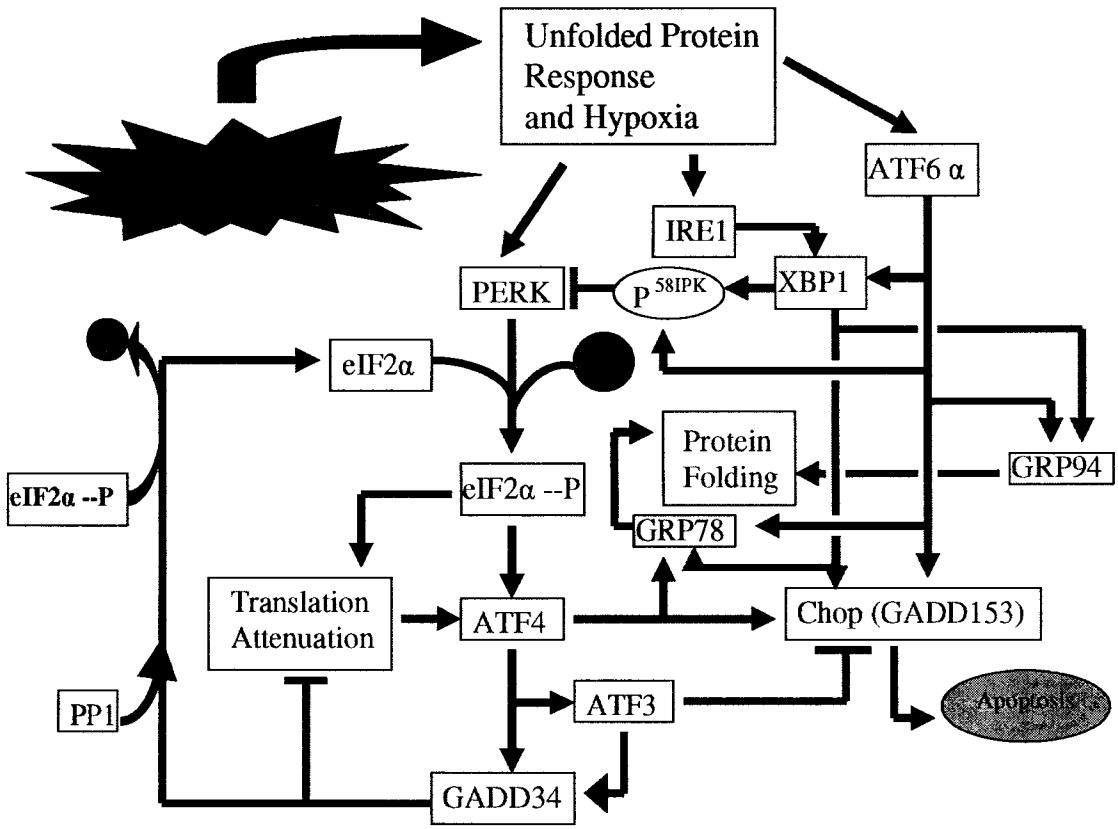
The UPR is comprised of three major inducers (ATF6, IRE1 and PERK) that are all regulated by one master protein named BiP (also known as GRP78) (Zhang and Kaufman, 2004). These important proteins induce three major signaling pathways that play a major role in cell survival. These pathways are known as the PERK/eIF2α/ATF4, IRE1/XBP1 and ATF6 pathways (Kim et al., 2006), which act in concert to aid in (a) the reduction of misfolded protein translation, (b) increased translation of ER chaperone proteins such as BiP, and (c) induction of the ERAD pathway (Kim et al., 2006). A model of the UPR signaling cascade is presented in Figure 3.1.

PERK is a type I ER transmembrane protein known as the ER protein kinase that phosphorylates eIF2α, thereby attenuating protein translation by targeting this key ribosomal initiation factor (Ron, 2002). This, in turn, increases the translation of a transcription factor called activating transcription factor 4 (ATF4), a member of the ATF/CREB family of transcription factors (Jiang et al., 2004; Ron, 2002). ATF4 is an important mediator of the UPR as it activates a number of pro-survival as well as pro-apoptotic genes that participate in the UPR (Blais et al., 2004). According to Mamady

and Storey (2006), ATF4 interacts with phosphorylated CREB1 to enhance the transcription of the pro-survival gene *grp78,* whose function is to promote folding of misfolded proteins during ER stress. ATF4 also enhances the transcription of GADD34, another pro-survival protein, in order to reinitiate translation by interacting with protein phosphatase 1 (PP1) to dephosphorylate eIF2α (Wouters et al., 2005; Conner et al., 2001). Among pro-apoptotic genes, ATF4 increases the expression of *gadd153* (Hayashi et al., 2005; Fawcett et al., 1999). GADD153 or C/EBP-homologous protein (CHOP) is normally undetectable, but is produced in high amounts during a perturbation of the ER protein folding machinery (Wang et al., 1998).

Another gene downstream of ATF4 is the Activating Transcription Factor 3 (ATF3). There have been many debates in the past few years as to whether ATF3 in humans induces or represses GADD153 as well as itself (Wolgang et al., 1997; Fawcett et al., 1999; Hashimoto et al., 2002). This is said to be possible due to the occurrence of an alternatively spliced isoform of ATF3 named ATF3Δzip (Chen et al., 1994). Most recently it has been suggested that ATF3 can induce GADD153 during nutritional stress, but acts as a potential repressor during ER stress, the difference in roles being attributed to different binding partners of ATF3 (Jiang et al., 2004). The role of ATF3 in wood frogs during ER stress has yet to be discovered and will be discussed further.

Figure 3.1: Model of UPR signaling in cells undergoing stress, depicting the three major inducers as well as their corresponding cascades. Upon ER stress the GRP78 protein dissociates from IRE1, PERK and ATF6, allowing these to undergo post-translational modification. The phosphorylated form of PERK (pPERK) inhibits ribosomal translation via the phosphorylation of eIF2α. This phosphorylation enhances the expression of the ATF4 protein and its downstream genes.



Adapted From Hapsatou (2006)

Results

The levels of proteins involved in the ER stress response were evaluated in wood frog skeletal muscle using the Western blotting techniques described in Chapter 2 and assessing protein responses to three stresses: freezing, anoxia, and dehydration.

Expression levels of ATF4 and GADD34

Freezing

Western Blotting was used to measure the levels of these two proteins. Probing the membranes with anti-ATF4 antibodies yielded a single bright band at ~ 40 kDa for all wood frog skeletal muscle extracts analyzed. This is the expected molecular weight based on the known protein size in other systems (Karpinski et al., 1992). ATF4 levels increased significantly by 1.3 fold after 24 hours of freezing (Fig. 3.2 A). However, after 8 hours thaw ATF4 protein content was strongly suppressed with respect to both the control and frozen states; levels were reduced by 50% compared with controls.

Analysis of GADD34 in wood frog muscle extracts similarly showed crossreaction of the antibody with a single protein band of ~80 kDa, again consistent with the know size of the protein in other systems (Hollander et al., 1997). GADD 34 protein levels did not change during freezing or thawing (Fig. 3.2 B).

Anoxia

Figure 3.2 shows comparable results for the effects of anoxia exposure on ATF4 and GADD34 in wood frog muscle. ATF4 levels increased significantly (by 1.5 fold) when frogs were exposed to anoxia for 24 hours but were strongly suppressed again when the stress was relieved. ATF4 levels were reduced to just 21% of the control values after

4 hours of recovery from anoxia or to just 13% when compared with the peak value in anoxia (Fig. 3.2 A).

GADD34 expression levels responded differently to anoxia than they did during freezing. Anoxia exposure for 24 hours produced a significant 2.2 fold increase in GADD34. After frogs were allowed to recover for 8 hours, levels fell somewhat (~30%) below anoxia levels, but remained elevated compared with control levels (by 1.5 fold) (Fig. 3.2 B).

Dehydration

Dehydration to 40% of total body water lost produced a different pattern of ATF4 response as compared with freezing or anoxia. Dehydration resulted in a strong significant decrease in ATF4 in muscle; levels were reduced to just 31% of the control value and remained low after animals were rehydrated (Fig. 3.2 A).

Dehydration stress did not affect GADD34 levels in muscle but the protein responded during rehydration. The levels of expression for GADD34 increased above the previous levels by 1.4 fold (Fig. 3.2 B).

Expression level of ATF3 and GADD153

Freezing

The rabbit polyclonal anti-ATF3 antibody used cross-reacted with a single 21 kDa protein band in wood frog muscle extracts, consistent with the known size of ATF3 in other systems. ATF3 protein levels increased significantly by 1.4 fold during 24 hours freezing exposure and remained at this elevated level during thawing (Fig. 3.3 A).

By contrast, the 30 kDa protein GADD153, the interaction partner of ATF3, virtually disappeared during freezing. Levels fell to only about 3% of the corresponding control value and remained at this very low level after thawing (Fig. 3.3 B).

Anoxia

ATF3 levels responded strongly to anoxia, increasing significantly by 3.5 fold after 24 hours of anoxia stress. Levels remained elevated after 4 hours of aerobic recovery (Fig. 3.3 A).

GADD153 protein levels increase significantly during anoxia (by 1.3 fold) but then fell during aerobic recovery (Fig. 3.3 B). Levels in recovery were reduced to ~50% of control values.

Dehydration

ATF3 protein levels were suppressed during dehydration to about one-third of control values. These levels were maintained even after the animals were fully rehydrated (Fig. 3.3 A).

GADD153 levels also decreased significantly in muscle samples from dehydrated frogs; levels were reduced to about 45% of control values. However, protein levels returned to control levels when the animals were rehydrated (Fig. 3.3 B).

Expression level and subsequent splicing of ATF6

Freezing

The goat polyclonal anti-ATF6 antibody cross-reacted with two protein bands representing the 90 kDa (inactive) and the 50 kDa (active) forms of ATF6. Levels of the inactive 90 kDa form of ATF6 did not change after 24 hour freezing or 8 hour thawing

(Fig. 3.4 A). However, levels of the 50 kDa, active form of the protein rose significantly by 1.6 fold during freezing. Levels remained elevated after thawing (Fig. 3.4 B).

Anoxia

During anoxia there were no significant changes in the protein levels of either the inactive 90 kDa protein or the active 50 kDa ATF6. However, the amount of inactive ATF6 increased approximately 2 fold after 4 hours of aerobic recovery (Fig. 3.4 A) but levels of the active protein were still unchanged (Fig. 3.4 B).

Dehydration

During dehydration and rehydration the responses of the inactive and active forms of ATF6 were very similar. Neither form was affected by the dehydration stress but in both cases levels rose significantly during rehydration. Inactive 90 kDa ATF6 rose by 2.2 fold during rehydration (Fig. 3.4 A) whereas the active 50 kDa form increased by 1.3 fold (Fig. 3.4 B).

Protein levels of both active and inactive XBP1

Similarly to ATF6, the X-box binding protein (XBP1) exists in two forms. In order to detect both forms two different antibodies were used. A goat polyclonal antibody cross-reacted with a single 30 kDa band, which corresponds to the inactive form, whereas a rabbit polyclonal antibody cross-reacted with both the inactive and 50 kDa active form of the XBP1 protein, but was only used to quantify the active form.

Freezing

The levels of inactive XBP1 remained unchanged during freezing and the subsequent 8 hour thawed period (Fig. 3.5 A). On the other hand, levels of the active

form of the transcription factor decreased significantly by 2.5 fold during the 24 hour freeze. After thawing, levels returned to near control values (a 2 fold increase compared with levels in frozen muscle) (Fig. 3.5 B).

Anoxia

Levels of the inactive form of XBP1 had decreased significantly (to 33% of the control value) after 24 hours of anoxia exposure. Levels partially rebounded during the recovery period, rising by 2 fold compared with the anoxic condition (Fig. 3.5 A). By contrast, the amount of active 50 kDa XBP1 did not change under anoxia. However, data gathered from the 4 hour recovered experimental point showed that levels fell during recovery to about 45% of the control value (Fig. 3.5 B).

Dehydration

Inactive and active XBP1 responded to dehydration and rehydration in the same way. Inactive XBP1 decreased significantly by 45% during dehydration (Fig. 3.5 A) whereas active 50 kDa XBP1 decreased by 33% (Fig. 3.5 B). Levels of both the 30 and 50 kDa forms remained low after animals were fully rehydrated.

Expression levels of EDEM

Cross-reaction of goat anti-EDEM in muscle yielded a single protein band with wood frog samples at an approximate molecular weight of 75 kDa, consistent with the mammalian protein. Levels increased significantly during freezing (by 3.2 fold) and rose further (to 5.8 fold over control levels and 1.8 fold higher than frozen) after 8 hours thawed (Fig. 3.6).

Anoxia exposure for 24 hours had the opposite effect on EDEM levels which decreased significantly to 43% of the control values. Levels remained low during the 4 hours of recovery (Fig. 3.6). However, dehydration and rehydration had no significant effect on EDEM levels.

Movement of transcription factors into the nucleus

The action of transcription factors takes place in the nucleus and one effect of various stimuli / stresses is to alter the distribution of transcription factors between the nucleus and cytoplasm compartments. Hence, it is important to gain information on the relative amounts of transcription factors in the nucleus under the various stress conditions. The data in this section analyzed the effects of freezing, anoxia and dehydration on the cytoplasmic versus nuclear distributions of ATF4, ATF3, ATF6 (active) and XBP1 (active). Cytoplasmic and nuclear fractions of wood frog muscle were separated by well-established methods and then proteins were separated on SDS-PAGE gels and used for immunoblotting.

ATF4

Data for a short time of freezing exposure (6 hours) were also included here since many gene expression changes would be expected to occur within the early hours in order to trigger gene/protein changes that set up the animal for long term freezing survival. Figure 3.7 A shows ATF4 levels in cytoplasmic and nuclear fractions after 6 and 24 hours of freezing, 24 hours of anoxia exposure, and 40% dehydration. The cytoplasmic fraction isolated from muscle after 6 hours of freezing showed a large, 2.2 fold increase in ATF4 protein levels, which remained high (2.4 fold higher than mean control values)

after 24 hours of freezing. Anoxia exposure also strongly stimulated ATF4 content in the cytoplasmic fraction, levels increasing significantly by 2.7 fold over controls. However, dehydration had the opposite effect and cytoplasmic ATF4 content actually decreased significantly (to 67% of control values).

Changes in the nuclear contents of ATF4 were somewhat different (Fig. 3.7 A). After 6 hours of freezing exposure ATF4 levels had increased significantly (by 1.5 fold), but levels were reduced again after 24 hours frozen. Nuclear levels of ATF4 decreased significantly (by 17%) during anoxia, but were not affected by dehydration.

ATF3

Cytoplasmic levels of ATF3 rose significantly during short term freezing by ~1.4 fold as compared with controls. However, after freezing for 24 hours, levels had fallen again to ~50% of control values (Fig. 3.7 B). Both anoxia and dehydration exposures similarly suppressed cytoplasmic ATF3 levels to 45 and 43%, respectively, of control values.

Nuclear ATF3 levels behaved differently (Fig. 3.7 B). Levels were unchanged after 6 hours of freezing but showed a small but significant increase (1.2 fold) after 24 hours. However, anoxia exposure for 24 hours had a very strong effect on nuclear ATF3 with levels increased by ~4.2 fold. Nuclear ATF3 content also rose by 1.4 fold when frogs were dehydrated.

Active ATF6 (50 kDa)

Only the active, 50 kDa, ATF6 protein was measured in the cytoplasmic and nuclear fractions of frog muscle. The content of active ATF6 in cytoplasmic fractions increased significantly by 3.2 and 1.5 fold after 6 and 24 hours of freezing, respectively

(Fig. 3.8 A) and was also elevated by 1.3 fold after 24 hours of anoxia. There was no significant change in cytoplasmic ATF6 levels in muscle of 40% dehydrated frogs.

The nuclear ATF6 content increased significantly under all experimental conditions (Fig. 3.8 A). After 6 and 24 hours of freezing protein levels were elevated significantly in the nucleus by 3.4 and 2.7 fold respectively, whereas levels were elevated significantly by 2.2 fold after 24 hours and by 2.5 fold in 40% dehydrated frogs.

Active XBP1

Cytoplasmic levels of active 50 kDa XBP1 did not change after either 6 or 24 hours of freezing but rose significantly by 1.7 fold higher in anoxia. However, cytoplasmic XBP1 decreased significantly by 57% in muscle of 40% dehydrated frogs (Fig. 3.8 B).

The nuclear levels of XBP1 significantly increased in samples from frozen frogs by 1.6 folds after 6 hours and 1.8 fold after 24 hours. Levels also increased significantly by 1.3 fold in muscle of dehydrated animals (Fig. 3.8 B). However, anoxia exposure had no significant effect on nuclear XBP1 content.

Figure 3.2:

Western blot analysis showing the effects of freezing (**F**), anoxia (**A**) and dehydration (**D**) on ATF4 and GADD34 protein content in skeletal muscle of wood frogs.

(A) ATF4 protein levels in wood frog muscle.

i) Representative Western blots showing ATF4 protein levels in muscle under control (5°C acclimated), stressed and recovery conditions. Stress/recovery pairs are: (**F**) 24 h freezing at -2.5°C, 8 h thawed at 5°C, (**A**) 24 h anoxia under a nitrogen gas atmosphere at 5°C, 4 h aerobic recovery at 5°C, and (**D**) dehydration to 40% of total body water lost, 24 h rehydration at 5°C. Equal amounts of soluble protein (20 μg) were loaded into each lane.

ii) Histograms showing normalized mean values (± SEM, n=4 independent determinations) for ATF4 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery).

a- Significantly different from the corresponding control value as assessed via analysis of variance followed by the Student-Newman-Keuls test, $P<0.05$;

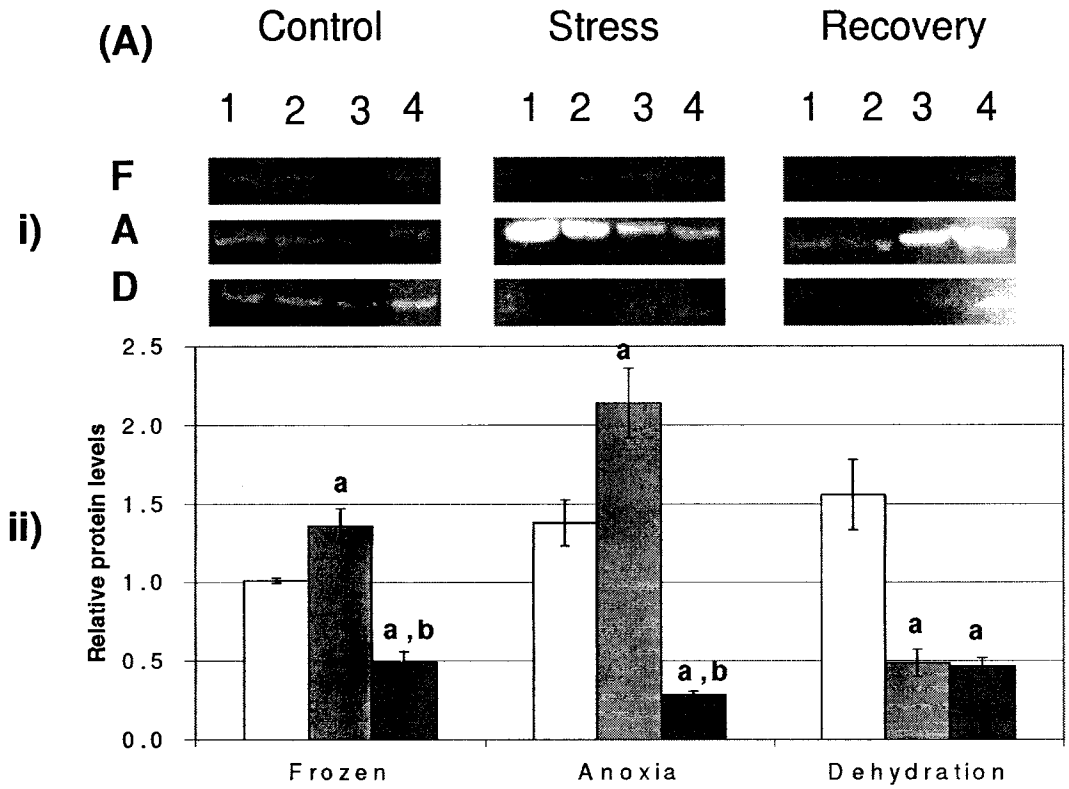
b- Significantly different from the corresponding stress value, $P<0.05$.

(B) GADD34 protein levels in skeletal muscle of wood frogs.

i) Representative Western blots showing GADD34 protein levels

ii) Histograms showing normalized mean protein levels

Other information as in (A) above.



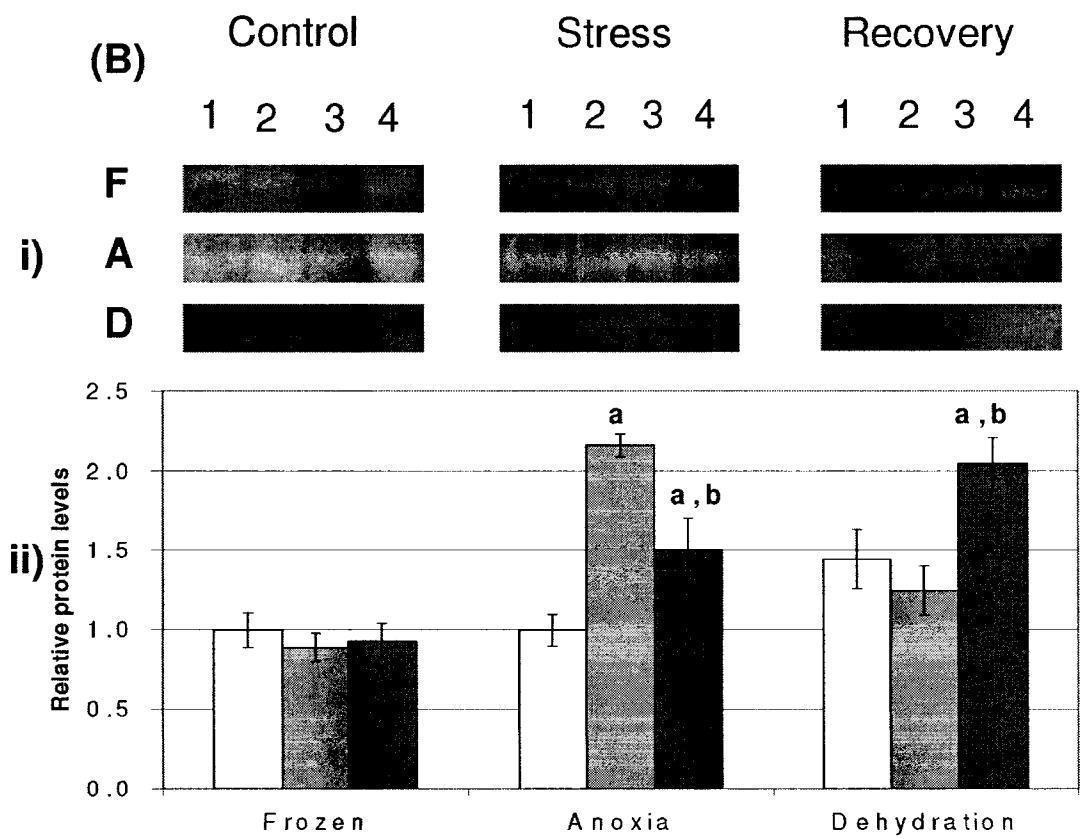


Figure 3.3:

Western blot analysis showing the effects of freezing (**F**), anoxia (**A**) and dehydration (**D**) on ATF3 and GADD153 protein in skeletal muscle of wood frogs.

(A) ATF3 protein levels in muscle of wood frogs.

i) Representative Western blots show ATF3 protein levels in muscle comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

ii) Histograms showing normalized mean values ($\pm$ SEM, n=4 independent determinations) for ATF3 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery).

a- Significantly different from corresponding control values, $P<0.05$;

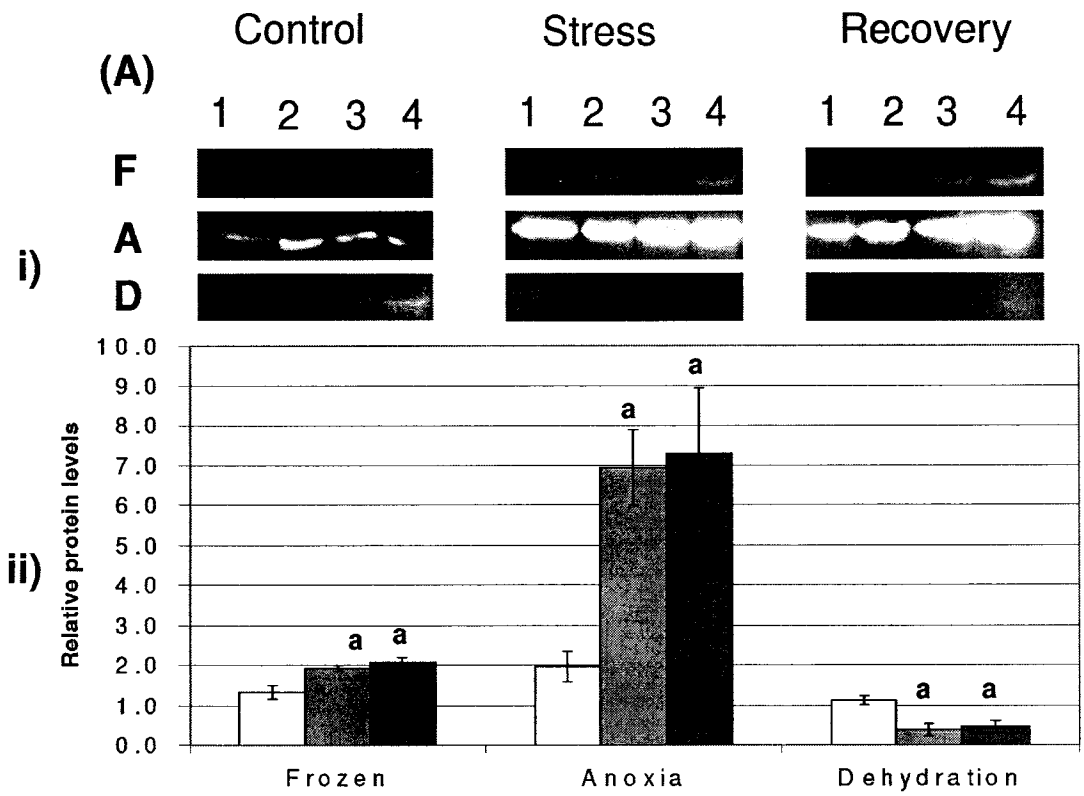
b- Significantly different from corresponding stressed values.

(B) GADD153 protein levels in muscle of wood frogs during all three stresses.

i) Representative Western blots showing GADD153 protein levels

ii) Histograms showing normalized mean protein levels

Other information as in (A) above.



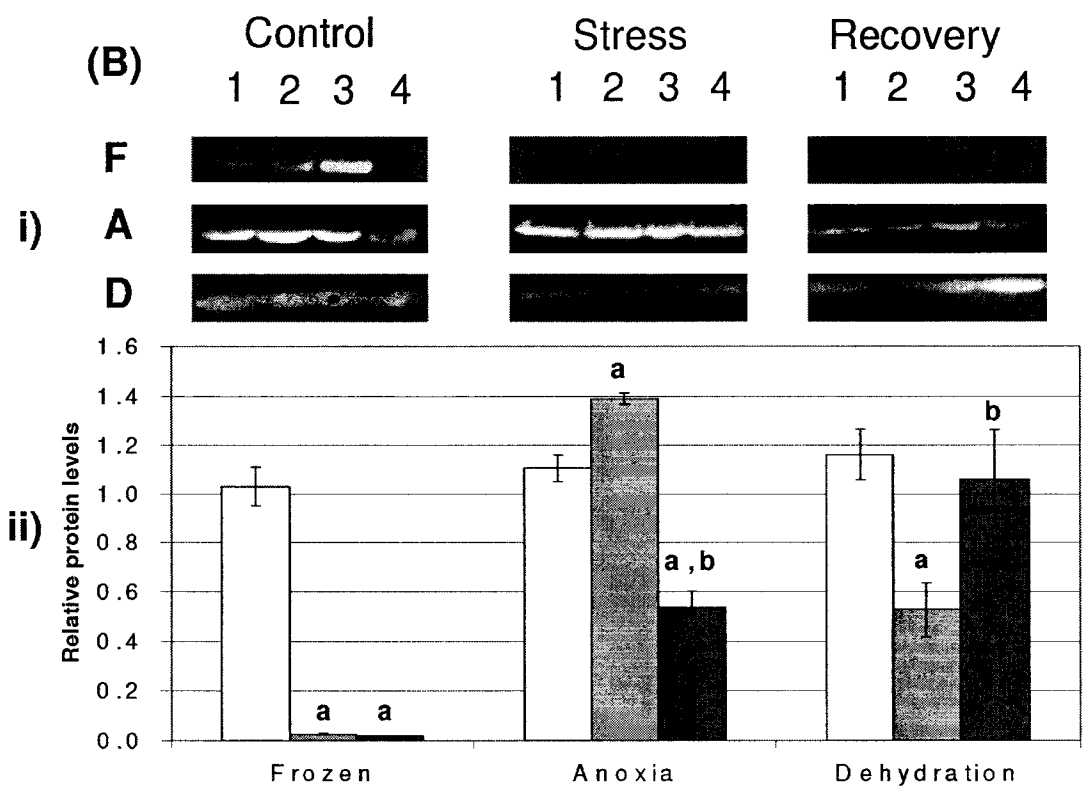


Figure 3.4:

Western blot analysis showing the effects of freezing (**F**), anoxia (**A**) and dehydration (**D**) on the inactive 90 kDa and active 50 kDa forms of ATF6 in muscle of wood frogs.

(A) Inactive 90 kDa ATF6 protein levels.

i) Representative Western blots show inactive ATF6 protein levels in muscle comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

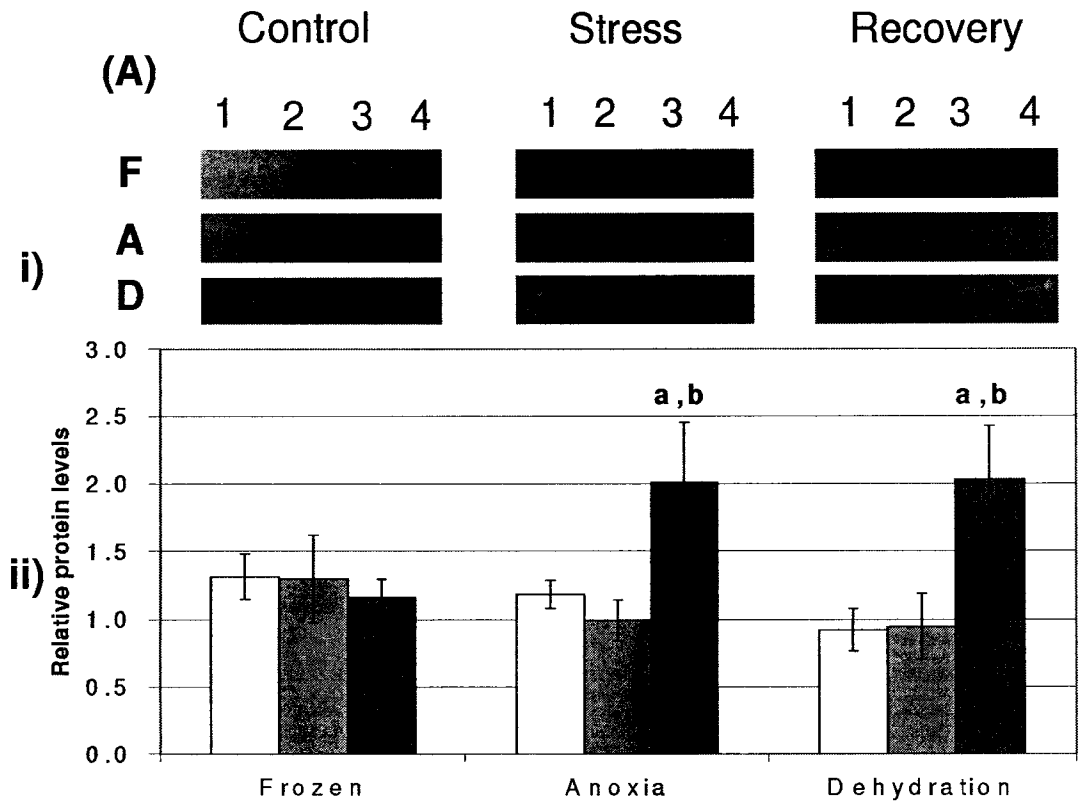
ii) Histograms showing normalized mean values ($\pm$ SEM, n=4 independent determinations) for inactive ATF6 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery). **a**- Significantly different from corresponding control values, $P<0.05$; **b**- Significantly different from corresponding stressed values.

(B) Active 50 kDa ATF6 protein levels in muscle of wood frogs during all three stresses.

i) Representative Western blots showing active ATF6 protein levels

ii) Histograms showing normalized mean protein levels

Other information as in (A) above.



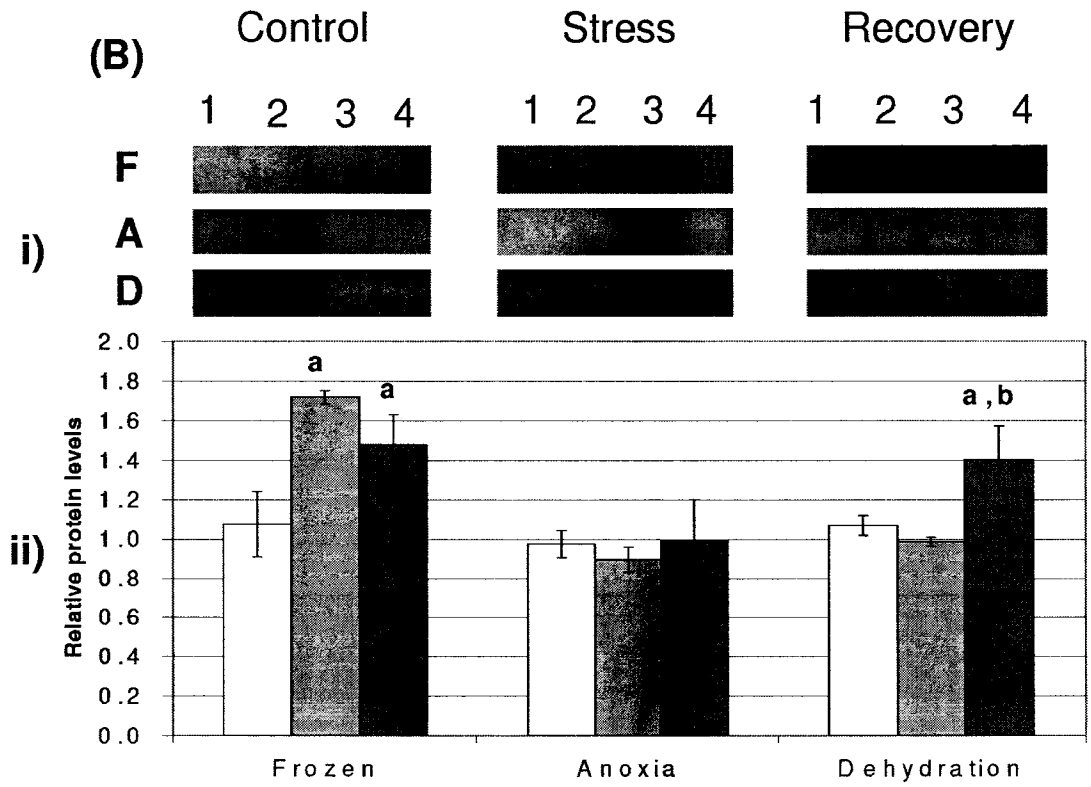


Figure 3.5:

Western blot analysis showing the effects of freezing (**F**), anoxia (**A**) and dehydration (**D**) on the levels of inactive 30 kDa XBP1 and active 50 kDa XBP1 proteins in wood frog muscle.

(A) Inactive 30 kDa XBP1 protein levels in wood frog muscle.

i) Representative Western blots show inactive XBP1 protein levels in muscle comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

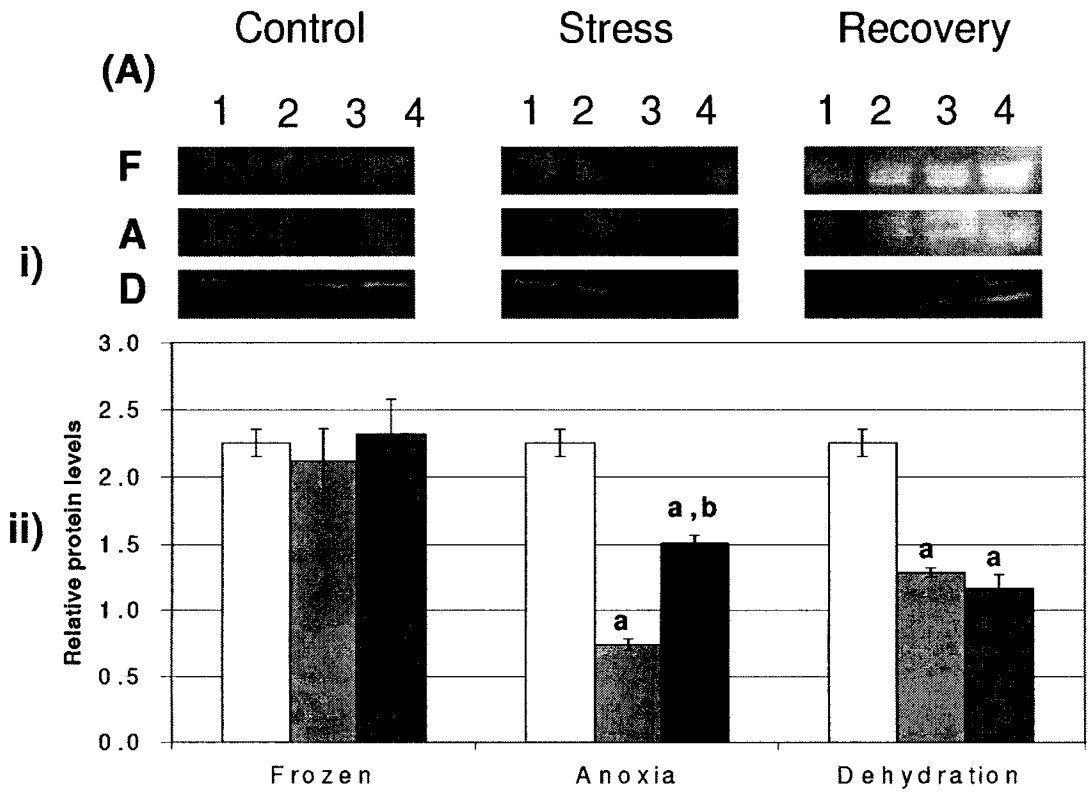
ii) Histograms showing normalized mean values (± SEM, n=4 independent determinations) for inactive XBP1 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery). **a**- Significantly different from corresponding control values, $P<0.05$; **b**- Significantly different from corresponding stressed values.

(B) Active 50 kDa XBP1 protein levels in muscle of wood frogs under all three stresses.

i) Representative Western blots showing active XBP1 protein levels

ii) Histograms showing normalized mean protein levels

Other information as in (A) above.



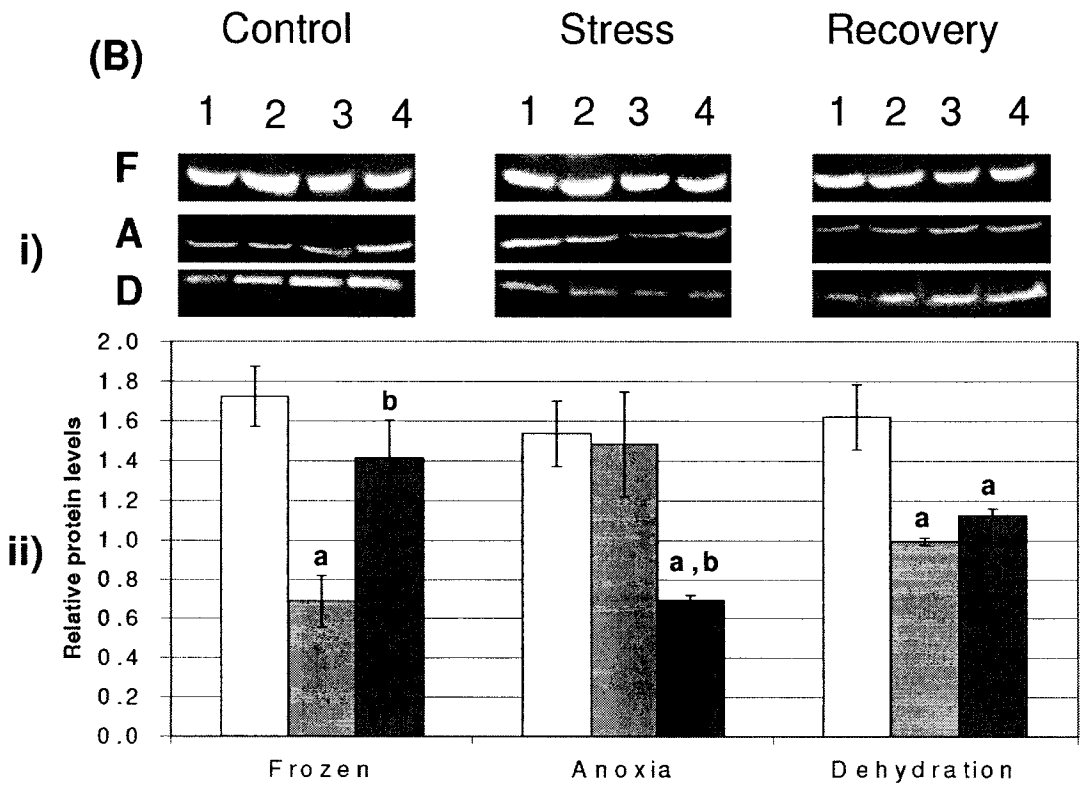


Figure 3.6:

Western blot analysis showing the effects of freezing (**F**), anoxia (**A**) and dehydration (**D**) on EDEM protein in skeletal muscle of wood frogs.

EDEM protein levels in muscle of wood frogs during all three stresses.

i) Representative Western blots show EDEM protein levels in muscle comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

ii) Histograms showing normalized mean values (± SEM, n=4 independent determinations) for EDEM levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery). **a**- Significantly different from corresponding control values, $P<0.05$; **b**- Significantly different from corresponding stressed values.

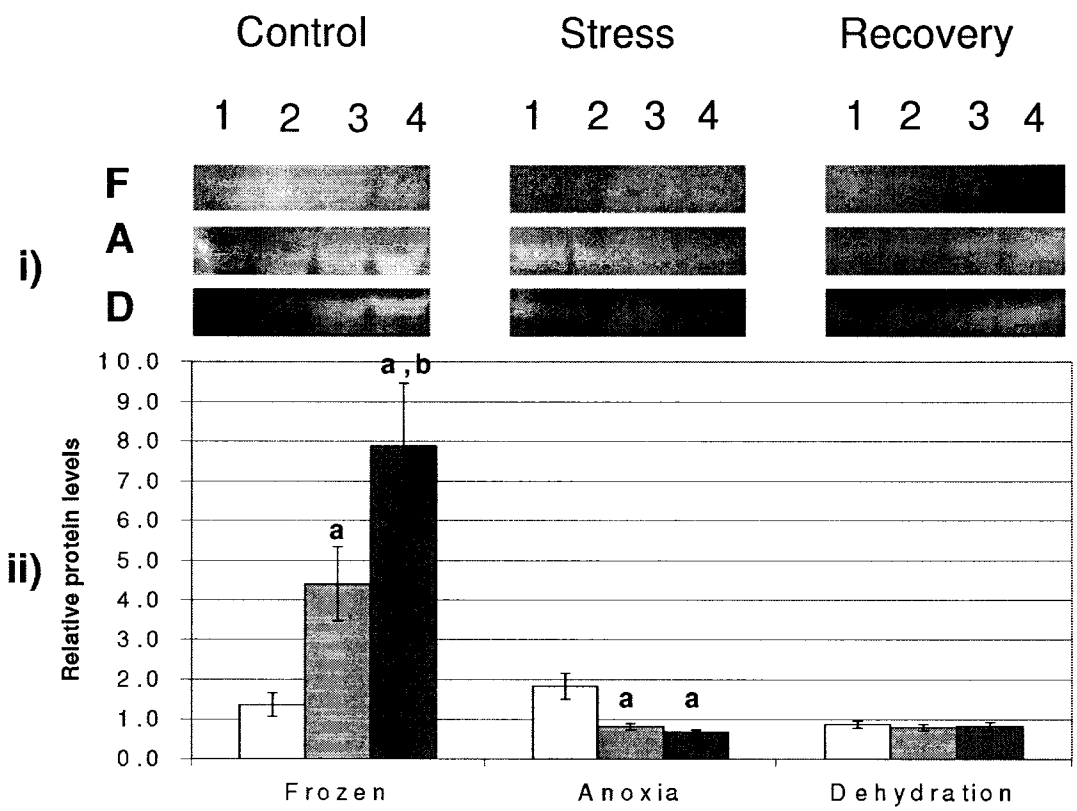


Figure 3.7:

Western blot analysis showing the distribution of ATF4 (**A**) and ATF3 (**B**) between cytoplasmic and nuclear fractions during freezing, anoxia and dehydration stresses.

(**A**) Distribution of ATF4 protein in wood frog skeletal muscle under all three stresses.

i) Representative western blots showing ATF4 protein levels in cytoplasmic and nuclear fractions of skeletal muscle under the following conditions: (**C**) control at 5°C, (**6F**) 6 h freezing at -2.5°C, (**F**) 24 h freezing -2.5°C, (**A**) 24 h Anoxia exposure at 5°C, and (**D**) 40% dehydrated at 5°C. The gels were loaded with 20 μg of protein from each cytoplasmic and nuclear.

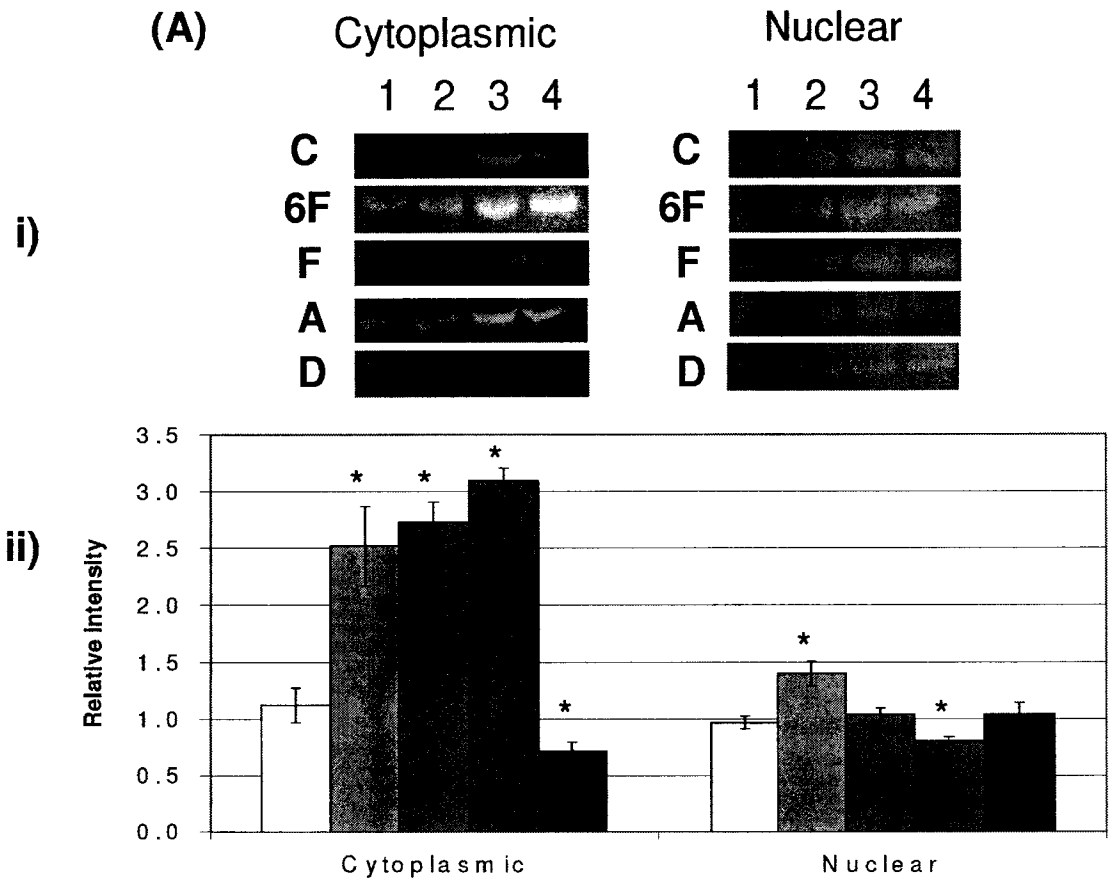
ii) Histograms show normalized mean values (± SEM, n=4 independent determinations) for the five conditions. *- Significantly different from control values using the Student's t-test, $P<0.05$.

(**B**) Distribution of ATF3 protein in wood frog skeletal muscle under the three stresses.

i) Representative Western blots showing ATF3 protein levels in cytoplasmic and nuclear fractions of skeletal muscle.

ii) Histograms showing normalized mean protein levels

Other information as in (A) above.



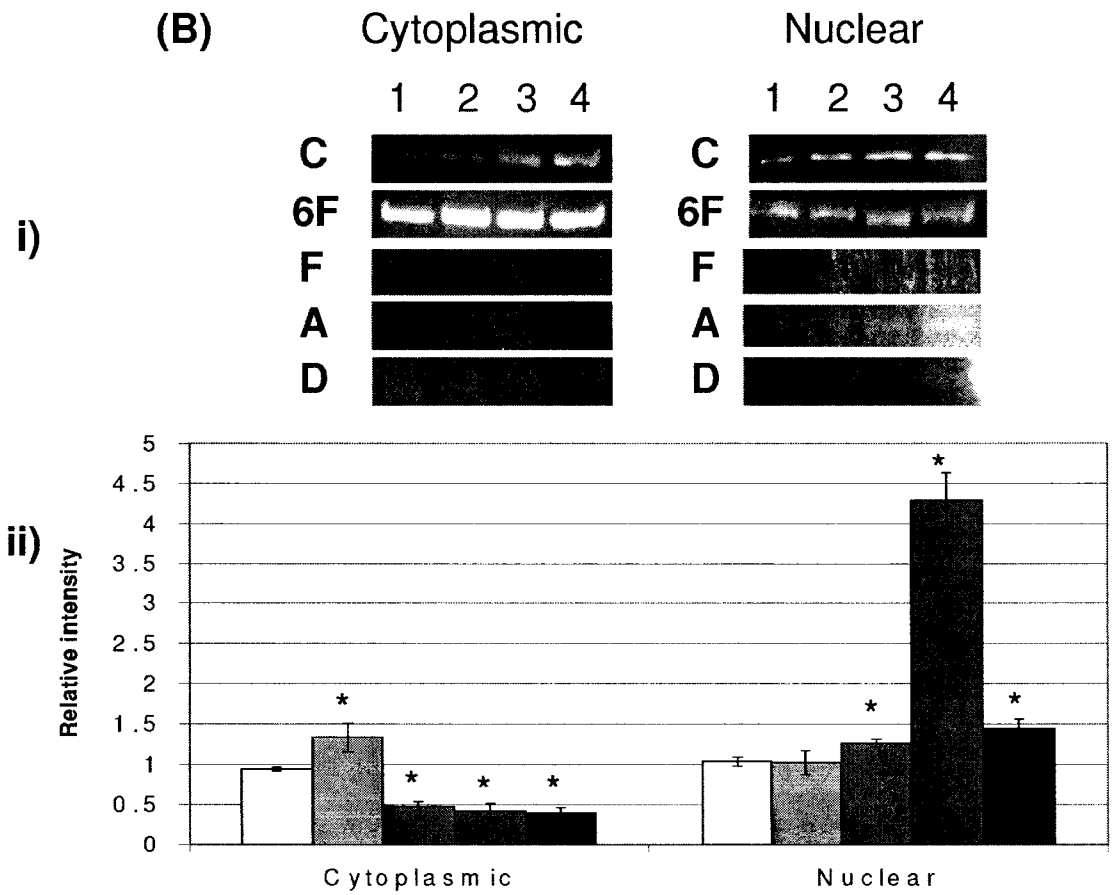


Figure 3.8:

Western blot analysis showing the distribution of active 50 kDa ATF6 (**A**) and active 50 kDa XBP1 (**B**) between cytoplasmic and nuclear fractions under freezing, anoxia and dehydration stresses in skeletal muscle of wood frogs.

(**A**) Distribution of active 50 kDa ATF6 protein levels in wood frog skeletal muscle.

i) Representative western blots showing ATF6 protein levels in cytoplasmic and nuclear fractions of skeletal muscle under the following conditions: (**C**) control at 5°C, (**6F**) 6 h freezing at -2.5°C, (**F**) 24 h freezing -2.5°C, (**A**) 24 h Anoxia exposure at 5°C, and (**D**) 40% dehydrated at 5°C. The gels were loaded with 20 μg of protein of each cytoplasmic and nuclear.

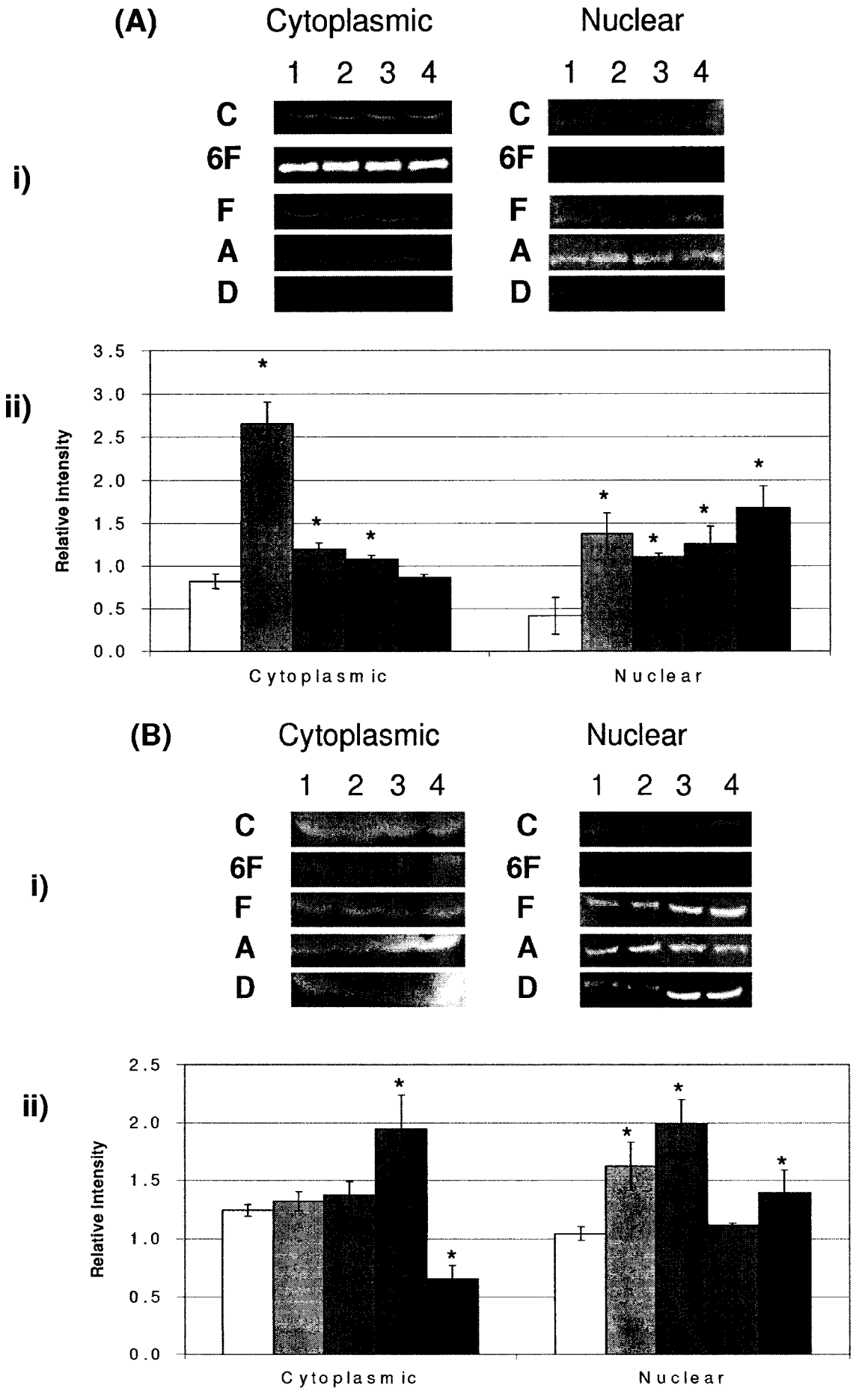
ii) Histograms show normalized mean values (± SEM, n=4 independent determinations) for the four conditions. *- Significantly different from control values, $P<0.05$.

(**B**) Distribution of active 50 kDa XBP1 protein levels in wood frog skeletal muscle.

i) Representative western blots showing XBP1 protein levels in cytoplasmic and nuclear fractions of skeletal muscle.

ii) Histograms showing normalized mean protein levels

Other information as in (A) above.



Discussion

Much research has been undertaken on the affects of unfolded proteins on cellular metabolism as well as the defenses employed by cells in order to survive. Unfolded and malfolded proteins are known to be disruptive to cells and contribute to some metabolic diseases including diabetes and Alzheimer's disease (Schröder and Kaufman, 2005b). As mentioned in the introduction to this chapter, cells have two pathways in particular that they use to deal with and regulate the accumulation of unfolded proteins: the UPR and ERAD (Schröder and Kaufman, 2005a).

The UPR and ERAD pathways help the cells to return to homeostasis by using two simple mechanisms that adapt to ER stress. The first adaptive method is to increase the protein folding capacity, while simultaneously reducing the folding load in the ER (Schröder and Kaufman, 2005b). The second adaptive response is to increase the cell's ability to dispose of misfolded, unassembled and unfolded proteins (Schröder and Kaufman, 2005b; Meusser et al., 2005). The present study examines the major proteins involved in the initiation and regulation of these two adaptive mechanisms.

The up-regulation of glucose-regulated chaperone proteins, either GRP 78 or GRP 94, is a key indicator of the onset of the UPR during ER stress in cells (Mamady and Storey, 2006). Previous work on *Rana sylvatica* found that GRP 78 protein levels increased during freezing in muscle, but were unchanged under both anoxia and dehydration stresses, whereas GRP 94 protein levels increased significantly under all three stresses (Zhenhong, 2004). As mentioned before, the three main signaling pathways that are involved in the unfolded protein response to ER stress are the PERK/eIF2α/ATF4, the IRE1/XBP1, and the ATF6 pathways. In order to understand

and potentially map out both the UPR and ERAD responses to stresses (freezing, anoxia, dehydration) in wood frogs, I evaluated the responses of a number of key proteins chosen from the three survival pathways. These included ATF3, ATF4, ATF6, GADD34, GADD153, XBP1 and EDEM.

One mechanism by which cells can alleviate the amount of malfolded protein is to slow or stop the translation of proteins. The phosphorylation of eIF2α, by PERK and/or GCN2, inhibits the translation of most mRNA transcripts, with the exception of the translation of ATF4 mRNA (Harding et al., 2000). In wood frog muscle, the phosphorylation of eIF2α increased during freezing, was unchanged in anoxia, and significantly decreased in the dehydrated animals (data not shown). The continued translation of ATF4 can, in turn, increase the transcription of some of its downstream genes involved in the survival of cells such as GADD34 and GRP 78, which are the major proteins of the PERK/eIF2α/ATF4 pathway.

PERK/eIF2α/ATF4 signaling pathway

Freezing

The protein levels of ATF4 increased significantly during freezing. The data also shows that more ATF4 is present in the nucleus during freezing and this suggests the probability of increased transcription of downstream genes under ATF4 control. Indeed, ATF4 is known to activate the transcription of ATF3, GADD34, GADD153 and GRP78 (Schröder and Kaufman, 2005b). Of these four, ATF3 (Fig. 3.3A) and GRP78 (Zhenhong, 2005) showed significant increases in protein levels in response to freezing.

GADD34 levels remained relatively unchanged (Fig. 3.2B) and GADD153 was very strongly suppressed (Fig. 3.3B).

The GADD153 transcription factor, also known as CHOP, is a component of the UPR that is involved in ER stress-related cell death (Kim et al., 2006), whereas ATF3 is a repressor of both itself and CHOP (Jiang et al., 2004). The data in Figure 3.3 B could suggest, therefore, that apoptosis is suppressed in muscle during freeze/thaw. This could function to help extend the viability of cells while the animal is frozen, perhaps postponing "decisions" on damage repair versus apoptosis until after thawing. However, this interpretation must also be made cautiously since apoptosis is a large and complex pathway and although GADD153 mediated functions are strongly suppressed during freezing; there are still other routes to regulate apoptosis that could be active in the frozen animal.

GADD34 protein levels did not change during freezing and thawing. This observation correlates with previous studies involving hibernating squirrels, where it was found that an increase in the levels of the ATF4 transcription factor does not necessarily lead to an increase in GADD34 protein (Mamady, 2006).

Anoxia

The responses by ATF4 in wood frog muscle to anoxia stress were similar to those seen during freezing. Under anoxia stress the levels of GADD34 significantly increased compared to the control, this was somewhat of a surprise considering that the ATF4 protein did not seem to migrate into the nucleus. The lack of migration of the ATF4 protein supported the previous data suggesting that the GRP78 levels were not

significantly different during anoxia (Zhenhong, 2004). Thus, this could suggest that GADD34 transcription is being upregulated by another transcription factor.

A possible candidate for this job could be ATF3 whose levels were very strongly increased under anoxia. Furthermore, ATF3 clearly migrated into the nucleus with a greater than 4-fold increase in nuclear ATF3 content under anoxia as compared with aerobic conditions. By contrast, the lack of migration of ATF4 protein into the nucleus would suggest that downstream genes under its control would not increase under anoxia. According to Liang et al. (1996), although ATF4 can up-regulate ATF3 expression (Figure 3.1), increased transcription of ATF3 can also result from ATF2 and c-Jun binding to the promoter region of the *atf3* gene.

ATF3 is known to be a repressor of GADD153 (Wolfgang et al., 1997), yet the data did not show a decrease in GADD153 protein levels even though total and nuclear ATF3 were very high during anoxia. However, GADD153 levels were reduced somewhat during the aerobic recovery period after anoxia exposure, a time when ATF3 levels still remained high. It is possible then that the increased expression of GADD153 under anoxia may be responding to a different transcription factor. This increase in total GADD153 protein levels would suggest that, unlike freezing, the muscle cells could undergo apoptosis under anoxic conditions, as it is well known that GADD153 induces apoptosis (Oyadomari et al., 2002).

Dehydration

During dehydration ATF4 in frog muscle responded differently than it did to freezing or anoxia stresses. The levels of the transcription factor decreased significantly and no migration into the nucleus was observed. These findings correlate with the

previous finding that the levels of GRP78 did not change significantly during dehydration (Zhenhong, 2004). Furthermore, GADD34 levels did not change after dehydration, which again correlates with the ATF4 data. Dehydration also resulted in a decrease in ATF3 protein levels and a decrease in GADD153 levels. Since it is known that ATF3 is a repressor of both GADD153 as well as itself, the migration of the transcription factor would then explain the reduced levels of both proteins (Wolfgang et al., 1997; Wolfgang et al., 2000; Jiang et al., 2004). The reduced levels of most of the major protein components of the PERK pathway in response to dehydration suggests one of two things: 1) there is no ER stress present in the muscle cells during dehydration, or 2) another signaling pathway is involved in helping cells to cope with ER stress. According to Zhenhong (2004), the levels of GRP94, an ER chaperone, increased significantly during dehydration, thus supporting the idea that there is another signaling pathway involved in coping with ER stress.

ATF6 signaling pathway

Freezing

This signaling pathway is induced by the accumulation of unfolded proteins inside the lumen of the ER. In response to ER stress, GRP78 releases ATF6 thereby exposing localization signals for the Golgi apparatus, that then mediate the export of ATF6 from the ER lumen (Shen et al., 2005). Once inside the Golgi the transcription factor is then cleaved by two proteases, site-1 (S1P) and site-2 (S2P) (Shen and Prywes, 2005). This cleavage produces the active 50 kDa protein.

The data showed no change in the levels of inactive 90 kDa ATF6 in wood frog muscle during freezing, whereas there was a significant increase in the levels of 50 kDa active ATF6. There was also an increase in the nuclear levels of the ATF6. In turn, the rise in nuclear ATF6 suggests activation of various downstream genes under its control such as XBP1, glucose regulated proteins (GRP78, GRP94), GADD153, EDEM and P^{58IPK}. According to Zhenhong (2004), *grp* transcript levels were elevated during freezing, thus suggesting an emphasis on GRP activation through the ATF6 signaling pathway in frozen frogs. The levels of EDEM (ER degradation enhancing α-mannosidase-like protein) also increased significantly during freezing. The enhanced migration of active ATF6 into the nucleus did not translate to an increase of GADD153 protein, thus supporting the previous suggestion that muscle cells do not undergo apoptosis when exposed to 24 hour freezing.

Anoxia

It has already been eluded to that the nature of the stress can possibly determine which pathway is enhanced to deal with the increased of ER stress factors. ATF6 (both active and inactive) levels did not change significantly under anoxia stress, but the data presented in this study do show increased migration of the active protein into the nucleus. These results are supported by an increase in the levels of *grp94* transcripts documented by Zhenhong (2004). As mentioned with regard to the PERK/eIF2α/ATF4 signaling pathway, ATF6 can also stimulate GADD153 expression. In the description of the previous pathway it was shown that even with an increase of ATF3, a repressor of GADD153 (or CHOP), the levels of this protein significantly increased under anoxia. Coupled with a lack of significant change in the amount of active XBP1 protein in anoxia

and a significant decrease in inactive XBP1, it can be suggested that ATF6 signaling up-regulates GADD153 in anoxic muscle.

Dehydration

Similar to the effects of anoxia, the levels of ATF6 did not change significantly under dehydration stress, but levels of the transcription factor did increase in the nucleus. Unlike under anoxia, but similar to the effects of freezing, there was no increase in the levels of GADD153 in dehydrated muscle, again suggesting a lack of apoptosis in this situation. However, like the other stresses, GRP94 levels did rise, most likely due to ATF6 enhancement (Zhenhong, 2004). Levels of XBP1 decreased significantly whereas the levels of EDEM did not show significant change. This would suggest that during dehydration the ATF6 signaling pathway does not play a major role in cell survival or death, but does increase the folding capacity of the cell during ER stress.

IRE1/XBP1 signaling pathway

Freezing

IRE1 is a transmembrane protein that encompasses three distinct domains: 1) a stress-sensing/BiP binding domain, which not only senses the onset of ER stress but also binds to free BiP proteins, 2) an autophosphorylation domain, and 3) an endonuclease domain that is capable of splicing XBP1 mRNA (Urano et al., 2000). The transcription of the XBP1 mRNA is known to be increased under ATF6 control and spliced during ER stress by IRE1 (Yoshida et al., 2001). The data for XBP1 showed that there was a significant decrease in the levels of the active form (50 kDa) of the transcription factor in muscle from frozen frogs, whereas levels of the inactive form remained unchanged.

Even with the decrease in the total amount of active XBP1, the data showed that the protein did migrate into the nucleus, thus making it a candidate for triggering transcriptional up-regulation of target genes during freezing. The very strong increase in EDEM protein levels, known to be downstream of XBP1, during freezing correlated with the increased migration of XBP1 into the nucleus.

Anoxia

Under anoxia the levels of unspliced XBP1 decreased significantly, but the levels of the spliced or active form of XBP1 did not change, as compared with controls. This would suggest that splicing of the XBP1 mRNA by IRE1 is ongoing to maintain the levels of the active protein. This pathway does not seem to be of great importance in anoxia stressed cells. The data showed no significant migration of XBP1 into the nucleus under anoxic conditions nor did it show an increase in the levels of the downstream gene EDEM. Although it was shown earlier in the chapter that the levels one of the downstream genes controlled by XBP1 (GADD153) did increase in anoxia, the current data indicate that it is unlikely that the up-regulation is caused by the XBP1 transcription factor.

Dehydration

The levels of both forms of XBP1 decreased when frogs were dehydrated, but the data for the active form showed elevated levels in the nucleus. This did not result in an increase of either of the downstream genes, but EDEM levels did not decrease and thus it could be postulated that the levels of this particular protein are simply maintained and not increased under dehydration stress.

Conclusion

The data gathered for the responses by wood frog skeletal muscle to all three stresses suggested that the type of stress mediates which pathway is utilized. In frozen frogs it was clear that the PERK/eIF2α/ATF4 and the ATF6 signaling pathways were active in cell survival. There was no indication of apoptosis, but rather an increase in the folding capacity of the cells mediated by up-regulation of GRPs (GRP78 and GRP94), under the direction of both ATF4 and ATF6. This upregulation in conjunction with the maintained levels of GADD 34 would also suggest that the continued phosphorylation of eIF2α is needed to help lighten the load of unfolded protein. It could also be concluded that the ATF6 signaling pathway helps to alleviate the folding load in the ER by induction of ERAD, detected here by the increased levels of the EDEM protein. This degradation pathway degrades slowly folding or misfolded proteins (Römisch, 2005).

Under anoxic stress a different pathway seems to take the lead in wood frog muscle. All of the proteins involved in the PERK pathway showed increased levels during anoxia and even though ATF4 levels did not increase in the nucleus, up-regulation of GADD34 was observed. ATF3 is another known up-regulator of the GADD34 protein and its increased levels in the nucleus could suggest that it is the cause of the up-regulation. ATF6 (increased nuclear levels) can up-regulate the GADD153 protein as well as the previously mentioned GRP94. The IRE1 pathway did not seem to be activated under anoxic conditions, and coupled with increased GADD153 levels in anoxia; it is possible that there may be some apoptosis occurring in muscle under this stress.

The third and final stress examined during this study was dehydration. When examining dehydration of skeletal muscle, there was no significant up-regulation of any transcription factors important for the UPR or the ERAD. Migration of ATF6 protein into the nucleus was seen, but the only downstream response apparent was elevated levels of GRP94 (Zhenhong, 2004). There is a decrease in the phosphorylated form of eIF2α (unpublished data) which would suggest that there is no attenuation in translation during dehydration. The combined data would suggest that there is no ER stress in skeletal muscle during bouts of dehydration in wood frogs.

Chapter 4

Coping with ER stress in the Liver

Introduction

This chapter looks at how the frog liver deals with ER stress under all three conditions: freezing, anoxia and dehydration. The liver is a large organ in the frog's body which plays many important roles including the interconversion and storage of nutrients (e.g. carbohydrate, lipid), the detoxification of wastes and xenobiotics, the synthesis and excretion of a wide variety of plasma proteins, the excretion of bile, as well as important metabolic pathways (Arias et al., 1994). The liver also has very important roles in cryoprotection. In order to protect cells and organs during freezing, the liver stores a massive amount of glycogen (~ 840 μmol/g wet weight) and, when freezing begins, converts it into glucose (~390 μmol/g wet weight) that is distributed to all other organs as a cryoprotectant (Storey and Storey, 1984; Storey and Storey, 2004a). The liver also synthesizes at least three novel proteins that are induced by freezing and that appear to have cryoprotective roles. These proteins are called FR10 (Cai and Storey, 1997b), Li16 (McNally et al., 2002) and FR47 (McNally et al., 2003).

As mentioned previously (Chapter 3), ER stress is divided into two main pathways, the UPR and ERAD. The first is a pathway that allows the endoplasmic reticulum to alleviate the unfolded protein stress by folding and ER expansion. The second pathway, ERAD, allows for the destruction of misfolded proteins or even slowly folding proteins, thus alleviating the folding load of the ER during bouts of stress (Schröder and Kaufman, 2005a). Unlike the UPR that has three main signaling pathways, the ERAD is dependent on the IRE1/XBP1 pathway, but is also stimulated by ATF6 (Schröder and Kaufman, 2005a) (Fig. 4.1). It is important to note that the ERAD

pathway is not a stress dependent pathway, but rather a quality control one where damaged and unfolded proteins are marked for degradation (Hampton, 2002).

The ATF6 signaling pathway is much simpler than the other two signaling pathways of the UPR. ATF6 is a type II ER membrane bound transcription factor that has its C-terminus in the ER lumen, while the DNA binding N-terminus faces the cytosol (Shen and Prywes, 2005). ATF6 contains two Golgi localization sequences (GLS1 and GLS2) in the luminal domain of the transcription factor that were determined to be independent of each other. These two sequences are important in both the retention of ATF6 in the ER (GLS1) and its translocation into the Golgi (GLS2) (Schröder and Kaufman, 2005a). BiP/GRP78 can only bind to GLS1 and holds the ATF6 protein in the ER.

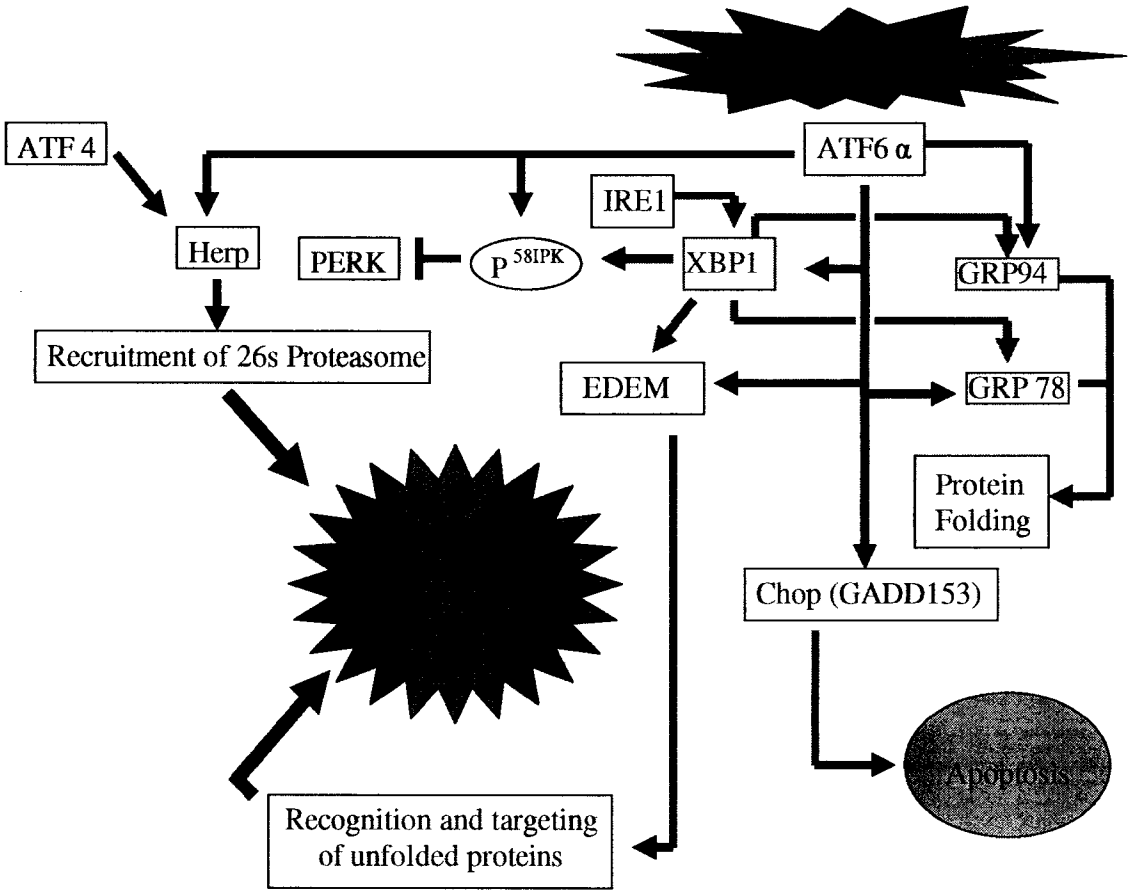
During ER stress BiP/GRP78 releases itself from the transcription factor, thus revealing the GLS2, which is dominant over GLS1 (Shen and Prywes, 2005; Schröder and Kaufman, 2005a). The GLS2 mediates the exit of ATF6 through the Golgi (Shen et al., 2002). The protein then undergoes two subsequent cleavage steps by site-1 protease (S1P) and site-2 protease (S2P) (Sommer and Jarosch, 2002; Shen et al., 2002; Schröder and Kaufman, 2005a; Schröder and Kaufman, 2005b). The serine protease S1P cleaves at the luminal domain, whereas the metalloprotease S2P cleaves the N-terminal within the lipid bilayer (Schröder and Kaufman, 2005a). The active ATF6 can then enter the nucleus to upregulate the transcription of the *XBP1* (X-box binding protein) gene, which can become a potent UPR activator (Lee et al, 2002; Yoshida et al., 2001).

XBP1 mRNA must undergo modifications before it can be translated into the potent transcription factor. As discussed in Chapter 3, IRE1 has an endonuclease domain

that splices the mRNA (Urano, et al., 2000). This endonuclease unconventionally cuts out a 26 nucleotide intron that a) increases the length of the translated protein from 30 kDa to 50 kDa, and b) increases its transactivation potential (Lee et al., 2002). The spliced or active form of XBP1 is a very important part of the ER stress response, because it's different downstream genes are involved in both the UPR and ERAD. XBP1 is known to increase the transcription of ER chaperones (GRP 78 and GRP94) along with ERAD proteins such as EDEM (Schröder and Kaufman, 2005a).

One of the key genes that is downstream of XBP1 is EDEM. This mannosidase-like protein has been shown to accelerate the degradation of proteins associated with the ER (Hosokawa et al, 2001). It has been shown that EDEM does not have any enzymatic activity, but rather it links the ER misfolding of proteins (glycoproteins) to retranslocation and thus proteosomal degradation in the cytosol (Hosokawa et al., 2001). The role of this relatively new protein has been compared to that of the immature glycoprotein binding to calnexin, except that the EDEM interaction with protein does not lead to folding, but rather to degradation (Hosokawa et al., 2001).

Figure 4.1: Model of ERAD signaling in cells undergoing stress, depicting the dependence on IRE1/XBP1 signaling for induction, as well as the stimulation by ATF6.



Results

The levels of the following proteins in liver were all measured using the Western blotting technique as described in Chapter 2.

Expression level of ATF4 and GADD34

Freezing

Western Blotting was used to measure the activation of these two proteins in the liver of wood frogs. Probing and size was the same as the previous chapter. There was a significant increase in ATF4 levels (3.4 fold) during 24 hours of freezing (Fig. 4.2 A). However, after thawing for ~8 hours ATF4 levels decreased again to near-control levels.

The single 80 kDa protein band of GADD34 was quantified and revealed a similar strong (2 fold) increase in the protein over 24 h of freezing (Fig. 4.2 B). The subsequent 8 h of thawing saw the protein levels of GADD34 decrease significantly from the 24 h frozen (83 %) value although levels remained elevated by 1.4-fold as compared with controls.

Anoxia

Different results were found during anoxia. ATF4 protein levels decreased significantly over 24 h of anoxia exposure, falling to 48% of control values, and remained low during aerobic recovery (Fig. 4.2 A).

GADD34 expression levels in anoxia/recovery followed the same pattern as was seen during freeze/thaw. The levels rose significantly above control values (by 1.4 fold) over 24 h of anoxia. However, once the frogs were allowed to recover for 4 hours, GADD34 levels fell below anoxia levels (to 71 % of anoxic values) and were not

significantly different from control levels (Fig. 4.2 B).

Dehydration

The protein levels of ATF4 increased significantly above control levels during 40% dehydration (by 2.4 fold) and remained high (2.1 fold over control) after rehydration (Fig. 4.2 A). GADD34 protein levels were unchanged in both the dehydrated (45%) and recovered samples as compared with the control values (Fig. 4.2 B).

Expression levels of ATF3 and GADD153

Freezing

Protein levels of ATF3 in liver increased strongly and significantly over 24 h of freezing by ~5-fold. After thawing, however, levels were reduced again to near control values (Fig. 4.3 A).

The levels of GADD153 in wood frog liver decreased to ~ 17 % of control values during freezing and remained at approximately this level after 8 h of thawing (25 % of control levels) (Fig. 4.3 B).

Anoxia

During 24 hours of anoxia ATF3 protein levels decreased significantly by approximately 2-fold. Levels remained low after 4 h aerobic recovery (Fig. 4.3 A).

GADD153 protein levels showed a similar pattern of response to 24 h anoxia and 4 h aerobic recovery. Protein levels decreased significantly in anoxia (to 43 % of control values) and remained low (48 % of control) during aerobic recovery (Fig. 4.3 B).

Dehydration

ATF3 levels in wood frog liver did not change significantly during dehydration or

rehydration (Fig. 4.3 A). GADD153 levels were similarly unchanged by dehydration or rehydration (Fig. 4.3 B).

Expression level and subsequent splicing of ATF6

Freezing

The relative protein levels of the 90 kDa (inactive) ATF 6 transcription factor increased significantly in liver of wood frogs after 24 h freezing exposure and remained high after 8 h thawing (values were 4.6- and 4.5-fold higher than controls, respectively) (Fig. 4.4 A). However, this did not correlate with an increase in the active form of the protein (50 kDa). The relative protein levels of active ATF 6 remained unchanged under all three conditions (control, 24 h freezing and 8 h thawed) (Fig. 4.4 B).

Anoxia

During anoxia exposure, 90 kDa ATF6 protein levels increased significantly and levels remained high after 4 h aerobic recovery (1.7 and 1.67 folds respectively) (Fig. 4.4 A). The quantification of the active form yielded very different results than the frozen data. Levels of the 50 kDa protein significantly increased (by 2.7 fold) during anoxia, but levels decreased again and returned to near-control values during aerobic recovery (Fig. 4.4 B).

Dehydration

During dehydration and rehydration the inactive forms of ATF 6 responded similarly to what was seen with the frozen and anoxic stress data. Levels of the inactive protein increased significantly over control levels by 1.5 fold during dehydration and remained 1.3 fold higher than control values during rehydration (Fig. 4.4 A). However,

levels of the active form, 50 kDa, did not change significantly during dehydration or rehydration (Fig. 4.4 B).

Protein levels of both active and inactive XBP1

Freezing

Levels of the inactive 30 kDa form of XBP1 in wood frog liver increased significantly over control values during 24 h of freezing (by 1.5 fold) but returned to near control values after thawing (Fig. 4.5 A). Levels of the active 50 kDa form of the transcription factor increased markedly during freezing and remained high after 8 h thawed (values were 3.4 and 4.2 fold higher than controls, respectively). Indeed, levels in 8 h thawed liver were significantly higher than frozen values (by 1.2 fold) (Fig. 4.5 B).

Anoxia

Levels of the inactive form of the transcription factor decreased significantly to 43 % of control values during 24 hours of anoxia exposure and remained low during aerobic recovery (Fig. 4.5 A). However, active 50 kDa XBP1 content in liver responded similarly to what was seen in the freezing experiments with a significant increase in the levels for both the 24 h anoxia (2.3 fold) and 4 h recovery (2.1 fold) data points. Data gathered from the 4 h aerobic recovery experimental point also showed a 1.7 fold rise in expression levels of active XBP1 compared with the 24 h anoxic point (Fig. 4.5 B).

Dehydration

Quantified data from the liver of 40% dehydrated frogs showed a 1.6 fold increase over control levels for inactive 30 kDa XBP1. However, expression levels decreased again to near control levels after full rehydration (Fig. 4.5 A). Similarly, levels of the

active 50 kDa form of XBP1 increased significantly (by 2.6 fold) during 40% dehydration only to decrease again to 1.7 fold above the control levels after full rehydration (Fig. 4.5 B).

Expression levels of EDEM

The protein EDEM was also assessed under all three experimental stresses. Levels of EDEM decreased significantly during 24 h of freezing (to 62 % of control values) and remained low after 8 h of thawing (55 % of control levels) (Fig. 4.6).

During anoxia exposure, EDEM levels responded similarly, decreasing significantly by 50 % as compared with initial control levels. EDEM levels remained low after 4 h of aerobic recovery (43 % of control levels) (Fig. 4.6).

Dehydration to 40 % of total body water lost had a strong effect on the relative protein levels of EDEM in liver. EDEM was reduced to only ~16% of control values after dehydration. Levels increased again after rehydration, rising significantly as compared with dehydrated levels (by 2.7 fold), while still being lower than the initial control levels (43 % decrease from control) (Fig. 4.6).

Movement of transcription factors into nucleus

The data for this section was gathered as outlined in Chapter 2 using the same antibodies for ATF4, ATF3, ATF6 (active) and XBP1 (active) as described above.

ATF4

The distribution of ATF4 between cytoplasmic and nuclear fractions in liver is shown in Fig. 4.7 A. Freezing exposure for 24 hours resulted in a significant decrease in

cytoplasmic ATF4 content to 67 % of the control value. Cytoplasmic ATF4 also decreased by 50 % under 24 h anoxia treatment. However, 40% dehydration had no effect on the cytoplasmic levels of ATF4.

ATF4 content in nuclear fractions showed a different pattern of response (Fig. 4.7 A). Both 24 h freezing and dehydration resulted in a significant increase in ATF4 levels in the nucleus; levels rose by 2 and 1.7 fold, respectively. By contrast, anoxia exposure had no effect on the nuclear content of ATF4.

ATF3

The cytoplasmic content of ATF3 decreased significantly (to 71 % of control levels) during 24 h of freezing. Levels were also significantly reduced during anoxia to ~63 % of control values, but were unchanged in liver of 40% dehydrated frogs (Fig. 4.7 B).

The nuclear ATF3 levels increased significantly during both freezing and anoxia exposures, by 1.7 and 2 fold, respectively. However, the nuclear content of ATF3 was unaltered under dehydration stress (Fig. 4.7 B).

Active ATF6 (50 kDa)

Levels of the active form of ATF 6 protein increased significantly (by 1.9 fold) in the cytoplasmic fractions of liver during freezing (Fig. 4.8 A). However, neither anoxia or dehydration affected cytoplasmic ATF 6 content.

ATF 6 levels in nuclear fractions increased under all three stress conditions. After 24 h freezing, nuclear ATF 6 protein levels were elevated by ~1.6 fold over control

values whereas the increases were ~1.9 fold under anoxia and ~2.3 fold in liver from 40% dehydration frogs (Fig. 4.8 A).

Active XBP1

Cytoplasmic levels of XBP1 were unchanged in liver from 24 h frozen frogs but levels increased strongly during both anoxia and dehydration exposures. The anoxic levels of XBP1 in the cytoplasm were 3.4 fold higher than control values, whereas in livers of dehydrated frogs a 3 fold increase was seen (Fig. 4.8 B).

Nuclear levels of XBP1 showed a similar trend to the cytoplasmic response. There was no significant change in nuclear protein levels of XBP1 during 24 h freezing. However, levels increased significantly by 1.7 and 2 fold in nuclear fractions from anoxic and dehydrated liver, respectively (Fig. 4.8 B).

Figure 4.2:

Western blot analysis showing the effects of freezing, anoxia and dehydration on ATF4 and GADD34 protein content in liver of wood frogs.

(A) ATF4 protein levels in wood frog liver.

i) Representative Western blots showing ATF4 protein levels in liver under control (5°C acclimated), stressed and recovery conditions. Stress/recovery pairs are: (**F**) 24 h freezing at -2.5°C, 8 h thawed at 5°C, (**A**) 24 h anoxia under a nitrogen gas atmosphere at 5°C, 4 h aerobic recovery at 5°C, and (**D**) dehydration to 40% of total body water lost, 24 h rehydration at 5°C. Equal amounts of soluble protein (20 μg) were loaded into each lane.

ii) Histograms showing normalized mean values (± SEM, n=4 independent determinations) for ATF4 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery).

a- Significantly different from the corresponding control value as assessed via analysis of variance followed by the Student-Newman-Keuls test, $P<0.05$;

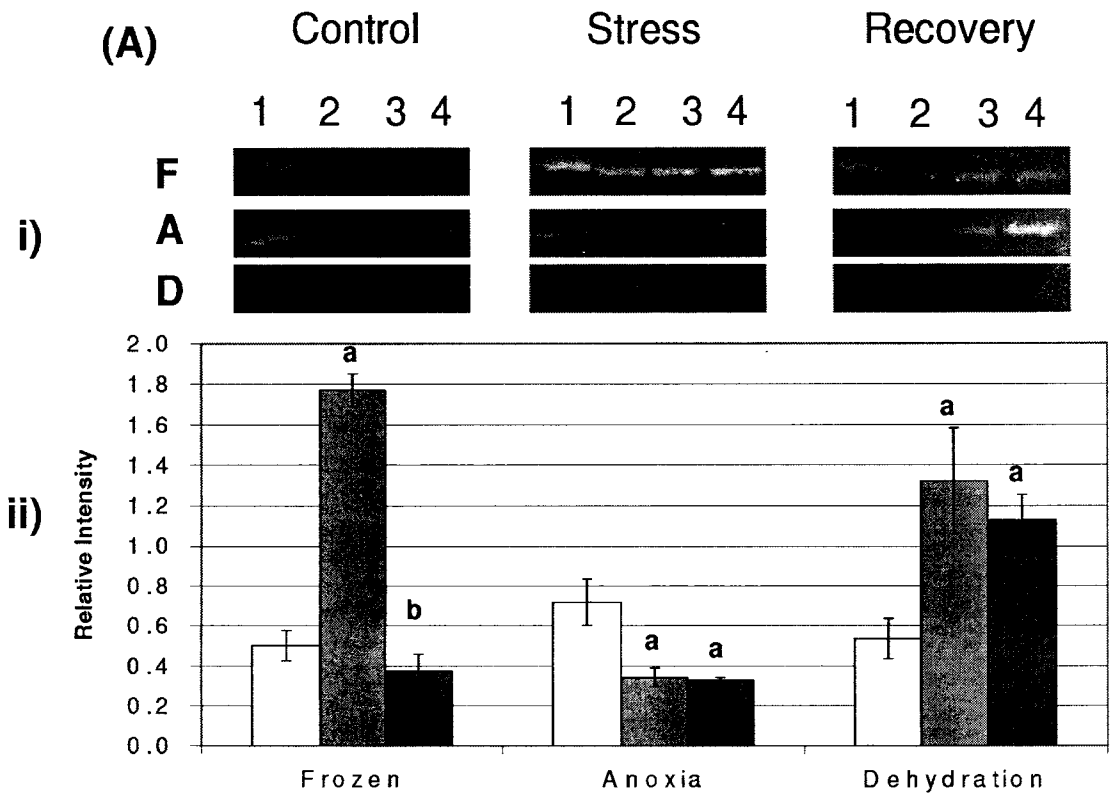
b- Significantly different from the corresponding stress value, $P<0.05$.

(B) GADD34 protein levels in liver of wood frogs.

i) Representative Western blots showing GADD34 protein levels.

ii) Histograms showing normalized mean protein levels.

Other information as in (A) above.



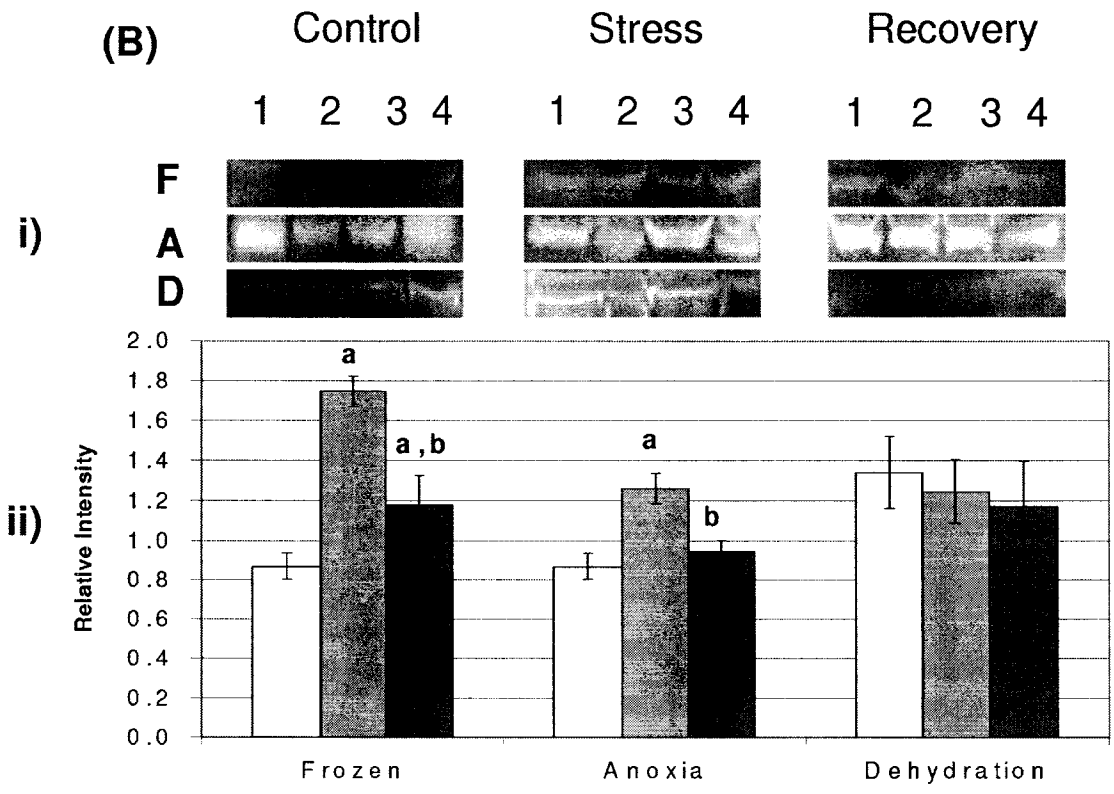


Figure 4.3:

Western blot analysis showing the effects of freezing, anoxia and dehydration on ATF3 and GADD153 protein in liver of wood frogs.

(A) ATF3 protein levels in liver of wood frogs.

i) Representative Western blots show ATF3 protein levels in liver comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

ii) Histograms showing normalized mean values (± SEM, n=4 independent determinations) for ATF3 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery).

a- Significantly different from corresponding control values, $P<0.05$;

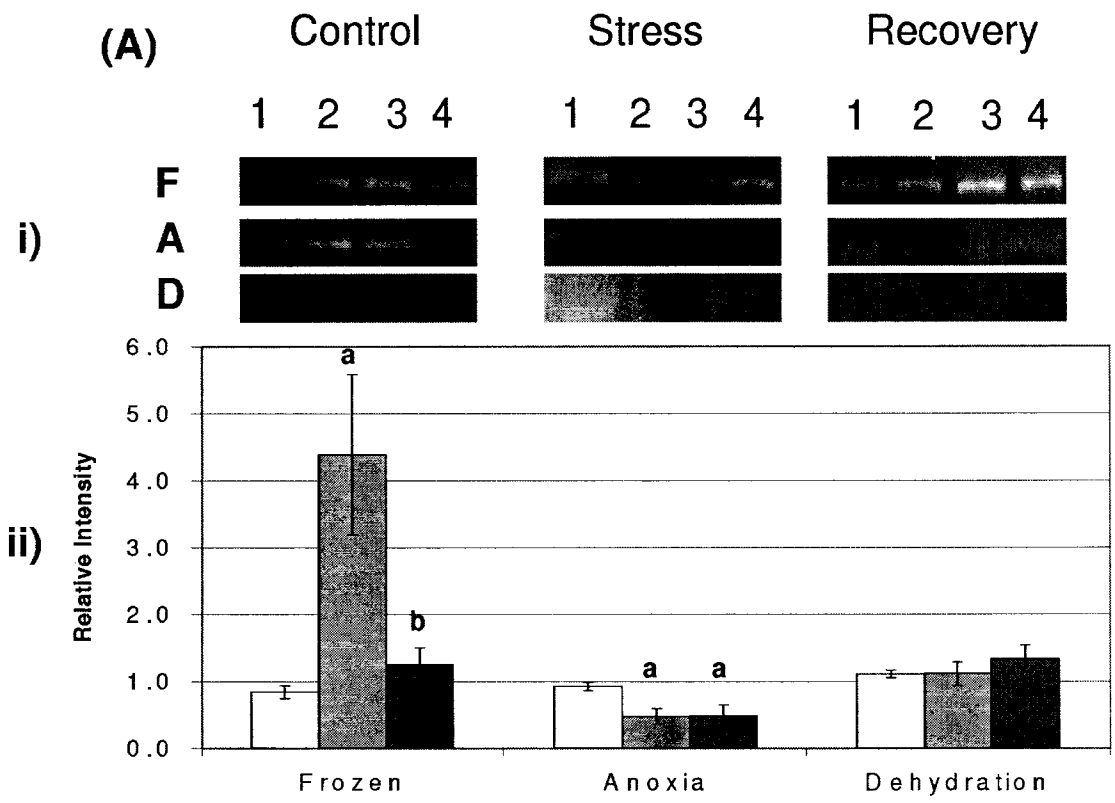
b- Significantly different from corresponding stressed values, $P<0.05$.

(B) GADD153 protein levels in liver of wood frogs during all three stresses.

i) Representative Western blots showing GADD153 protein levels.

ii) Histograms showing normalized mean protein levels.

Other information as in (A) above.



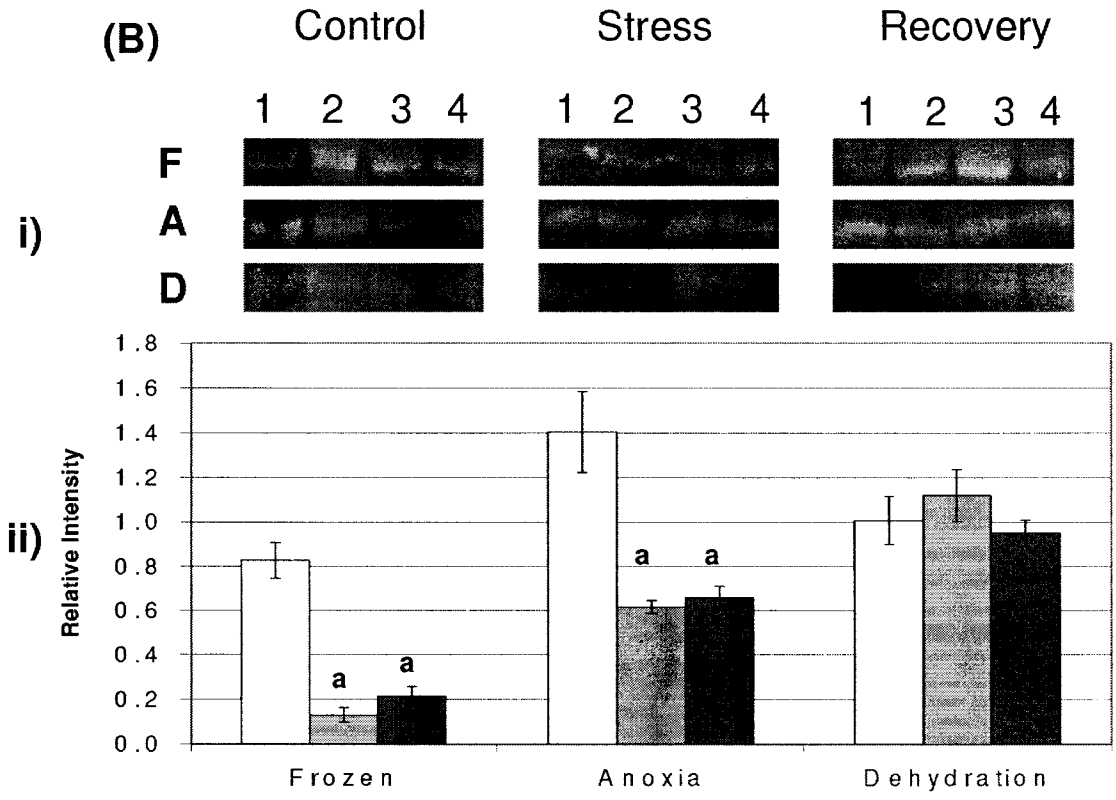


Figure 4.4:

Western blot analysis showing the effects of freezing, anoxia and dehydration on the inactive 90 kDa and active 50 kDa forms of ATF6 in liver of wood frogs.

(A) Inactive 90 kDa ATF6 protein levels.

i) Representative Western blots show inactive ATF6 protein levels in liver comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

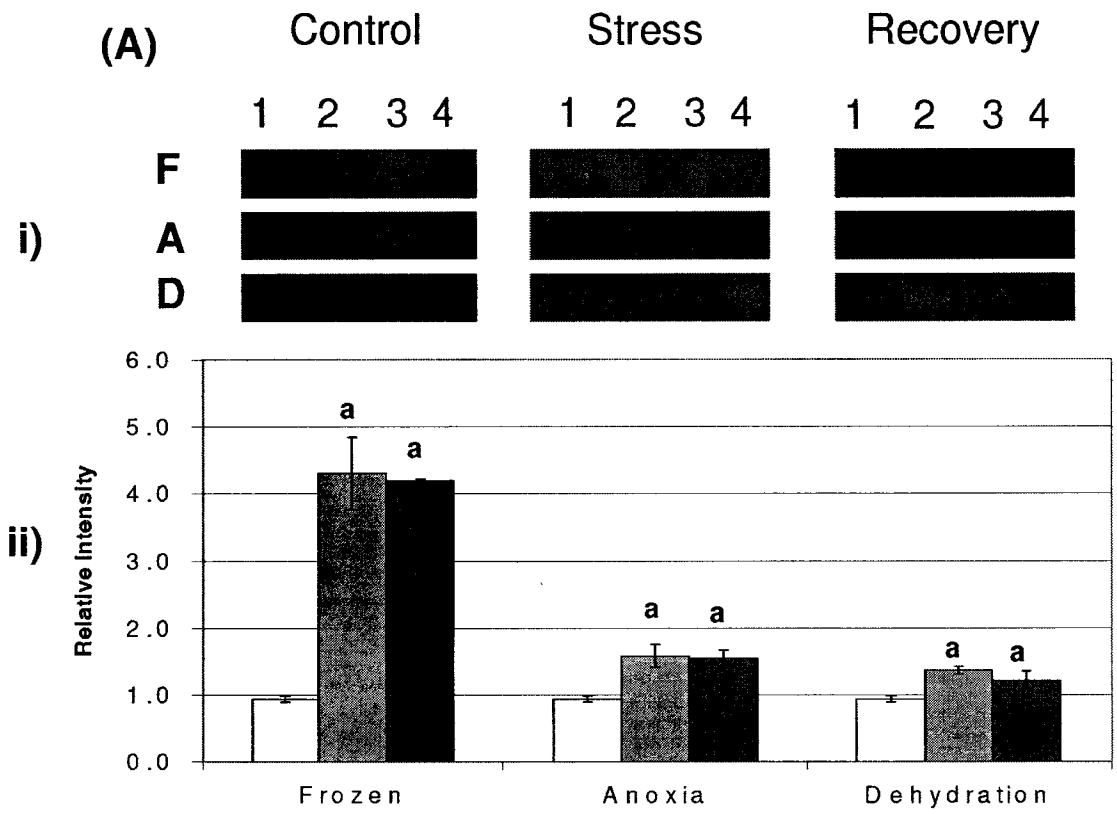
ii) Histograms showing normalized mean values (± SEM, n=4 independent determinations) for inactive ATF6 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery). **a**- Significantly different from corresponding control values, $P<0.05$; **b**- Significantly different from corresponding stressed values.

(B) Active 50 kDa ATF6 protein levels in liver of wood frogs under all three stresses.

i) Representative Western blots showing active ATF6 protein levels.

ii) Histograms showing normalized mean protein levels.

Other information as in (A) above.



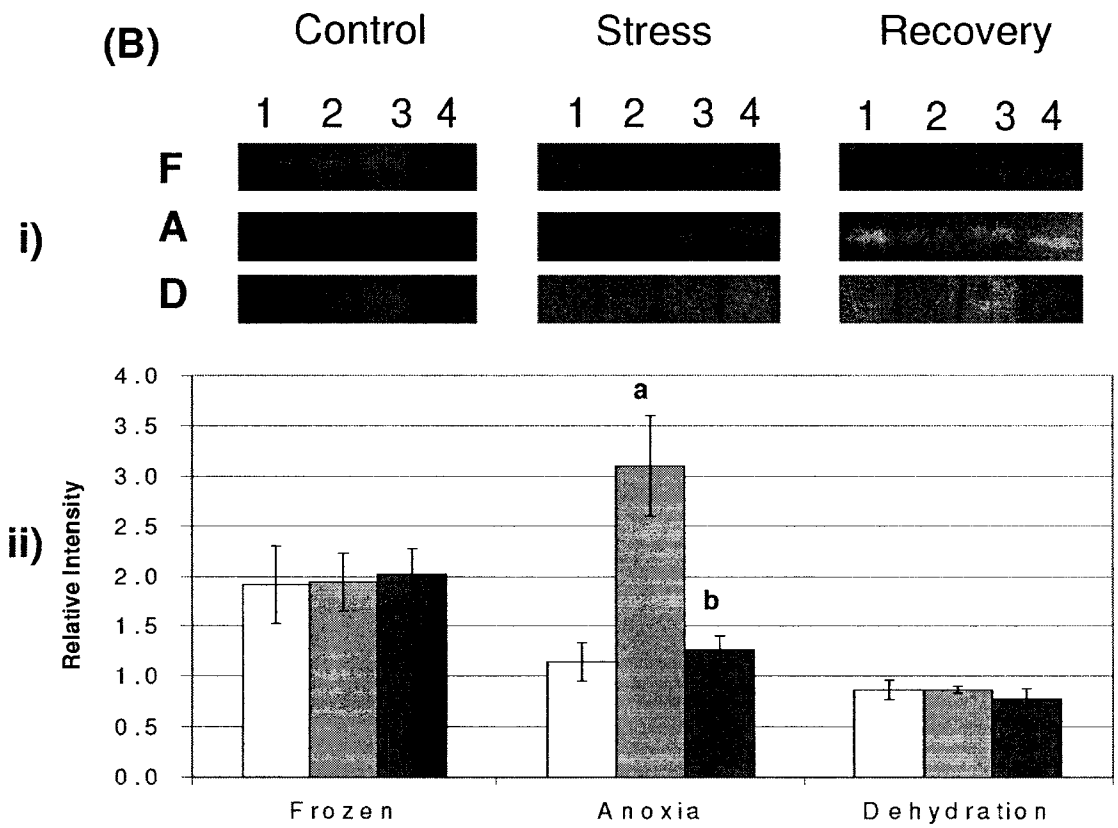


Figure 4.5:

Western blot analysis showing the effects of freezing, anoxia and dehydration on the levels of inactive 30 kDa XBP1 and active 50 kDa XBP1 proteins in wood frog liver.

(A) Inactive 30 kDa XBP1 protein levels in wood frog liver.

i) Representative western blots showing inactive XBP1 protein levels in liver comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

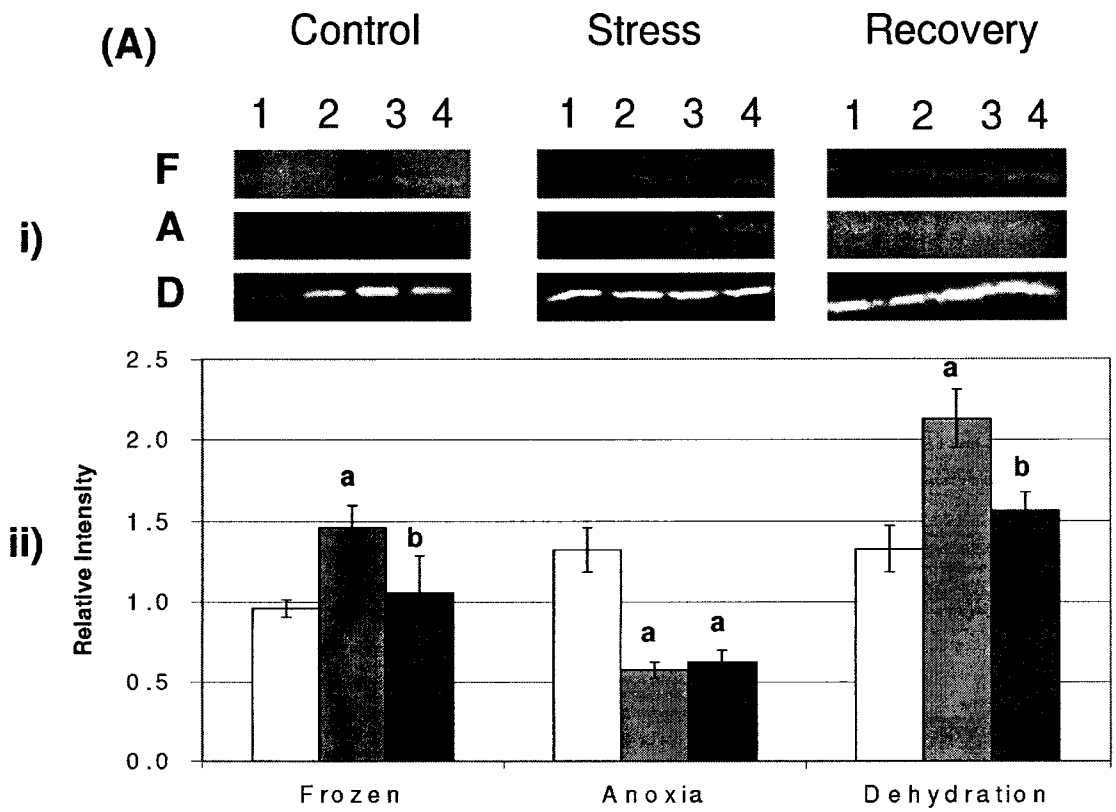
ii) Histograms showing normalized mean values ($\pm$ SEM, n=4 independent determinations) for inactive XBP1 levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery). **a**- Significantly different from corresponding control values, $P<0.05$; **b**- Significantly different from corresponding stressed values.

(B) Active 50 kDa XBP1 protein levels in liver of wood frogs under all three stresses.

i) Representative Western blots showing inactive XBP1 protein levels.

ii) Histograms showing normalized mean protein levels.

Other information as in (A) above.



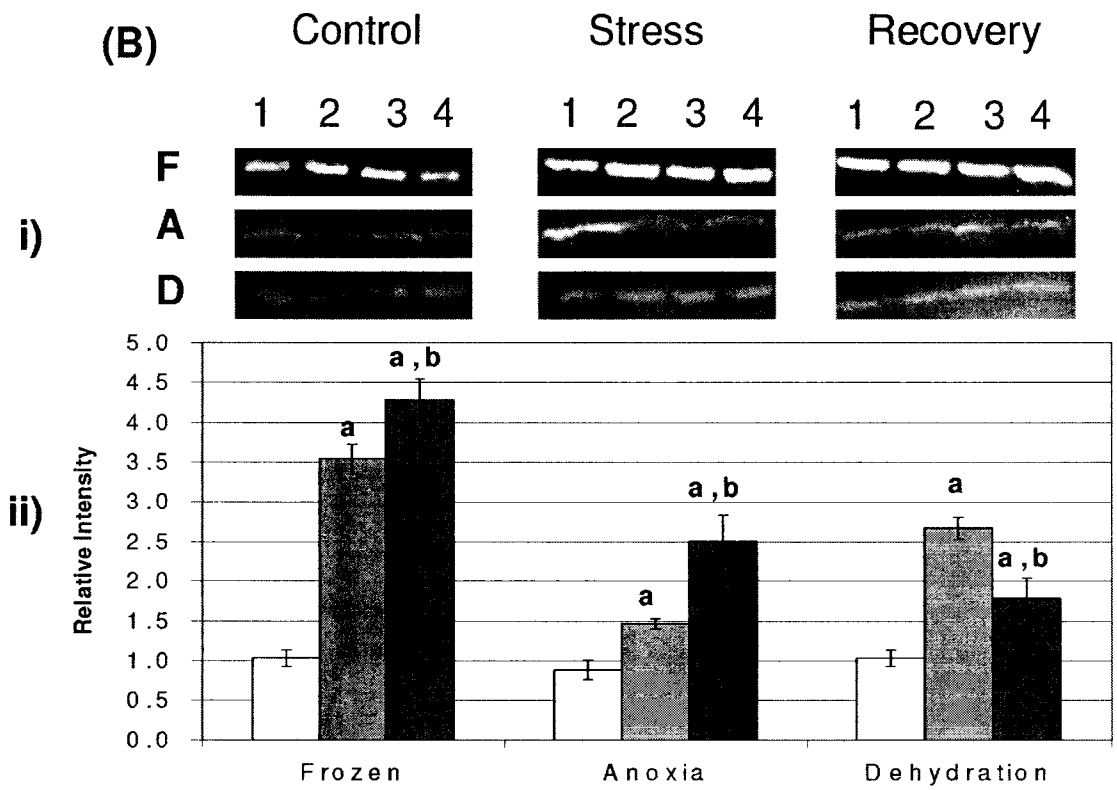


Figure 4.6:

Western blot analysis showing the effects of freezing, anoxia and dehydration on EDEM protein in liver of wood frogs.

EDEM protein levels in liver of wood frogs during all three stresses.

i) Representative western blots showing EDEM protein levels in liver comparing (**F**) control, 24 h frozen, 8 h thawed; (**A**) control, 24 h anoxia, 4 h aerobic recovery, and (**D**) control, 40% dehydrated, 24 h full rehydration.

ii) Histograms showing normalized mean values ($\pm$ SEM, n=4 independent determinations) for EDEM levels under the different experimental conditions. Bars are: white (control), light shading (stress), dark shading (recovery). **a**- Significantly different from corresponding control values, $P<0.05$; **b**- Significantly different from corresponding stressed values.

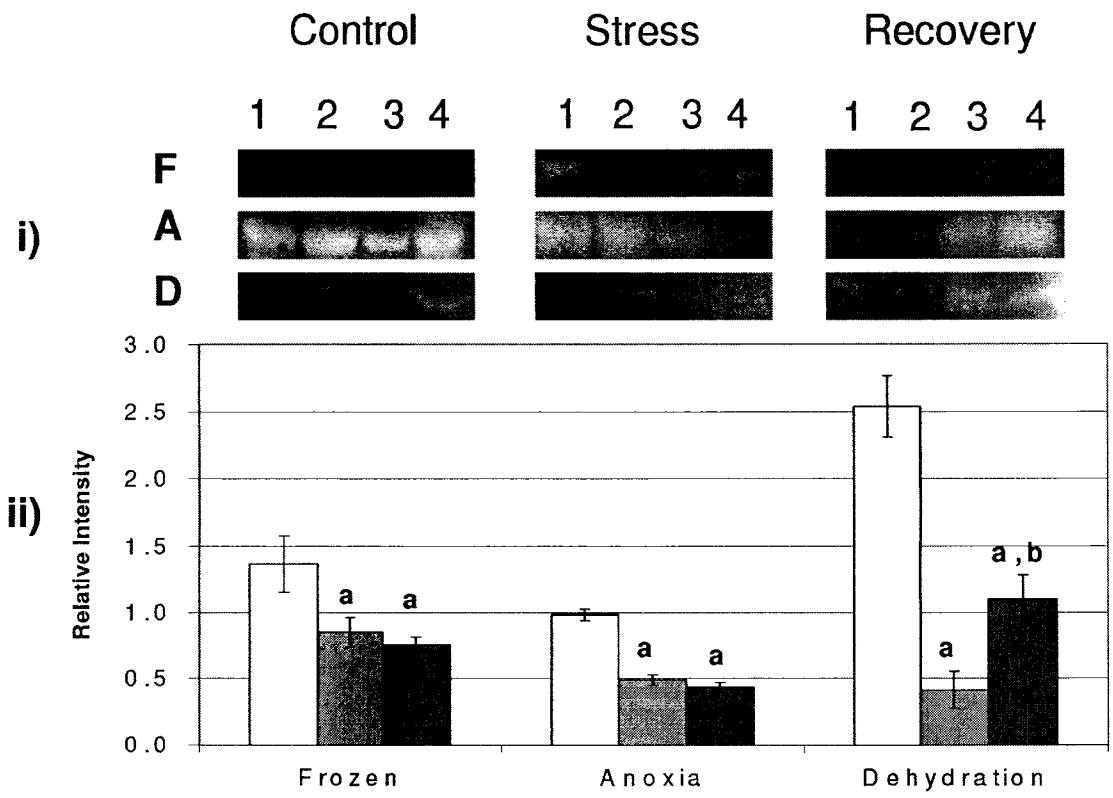


Figure 4.7:

Western blot analysis showing the distribution of ATF4 (**A**) and ATF3 (**B**) between cytoplasmic and nuclear fractions under freezing, anoxia and dehydration stresses.

(**A**) Distribution of ATF4 protein in wood frog liver under all three stresses.

i) Representative western blots showing ATF4 protein levels in cytoplasmic and nuclear fractions of liver under the following conditions: (**C**) control at 5°C, (**F**) 24 h freezing -2.5°C, (**A**) 24 h anoxia exposure at 5°C, and (**D**) 40% dehydrated at 5°C. The gels were loaded with 20 μg of each cytoplasmic and nuclear fraction.

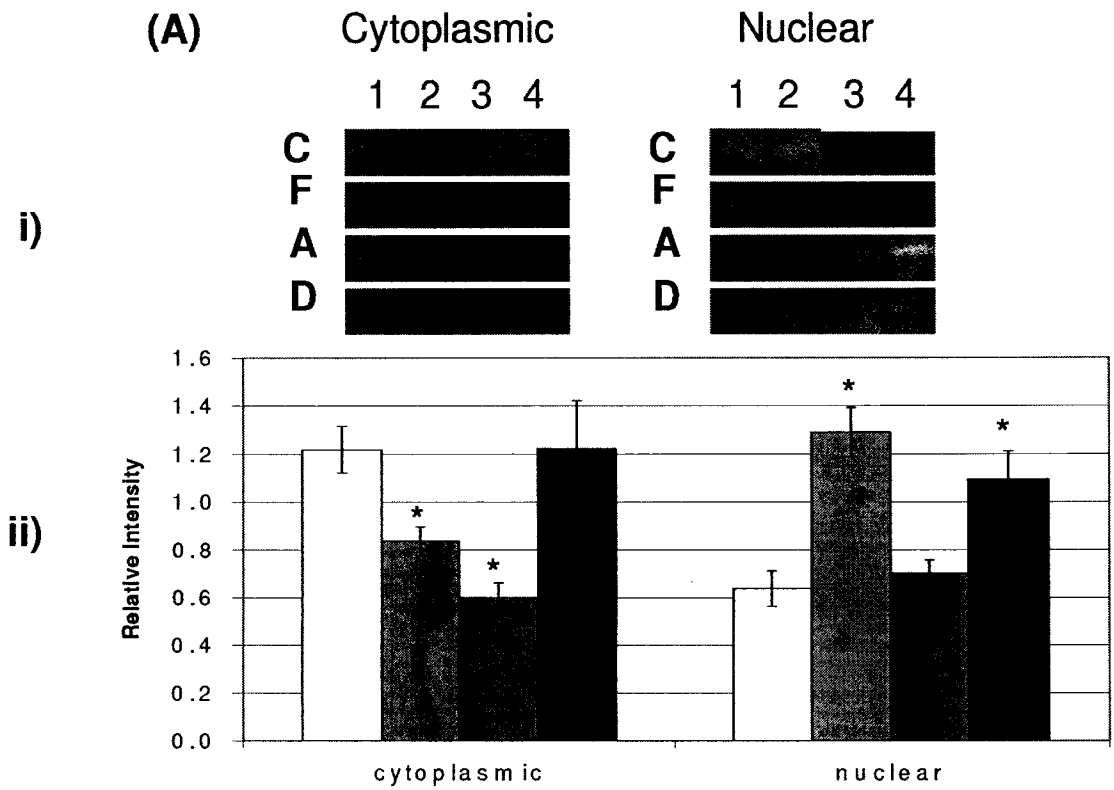
ii) Histograms show normalized mean values (± SEM, n=4 independent determinations) for the five conditions. *- Significantly different from corresponding control values, $P<0.05$.

(**B**) Distribution of ATF3 protein in wood frog liver under the three stresses.

i) Representative Western blots showing ATF3 protein levels in cytoplasmic and nuclear fractions of liver.

ii) Histograms showing normalized mean protein levels.

Other information as in (A) above.



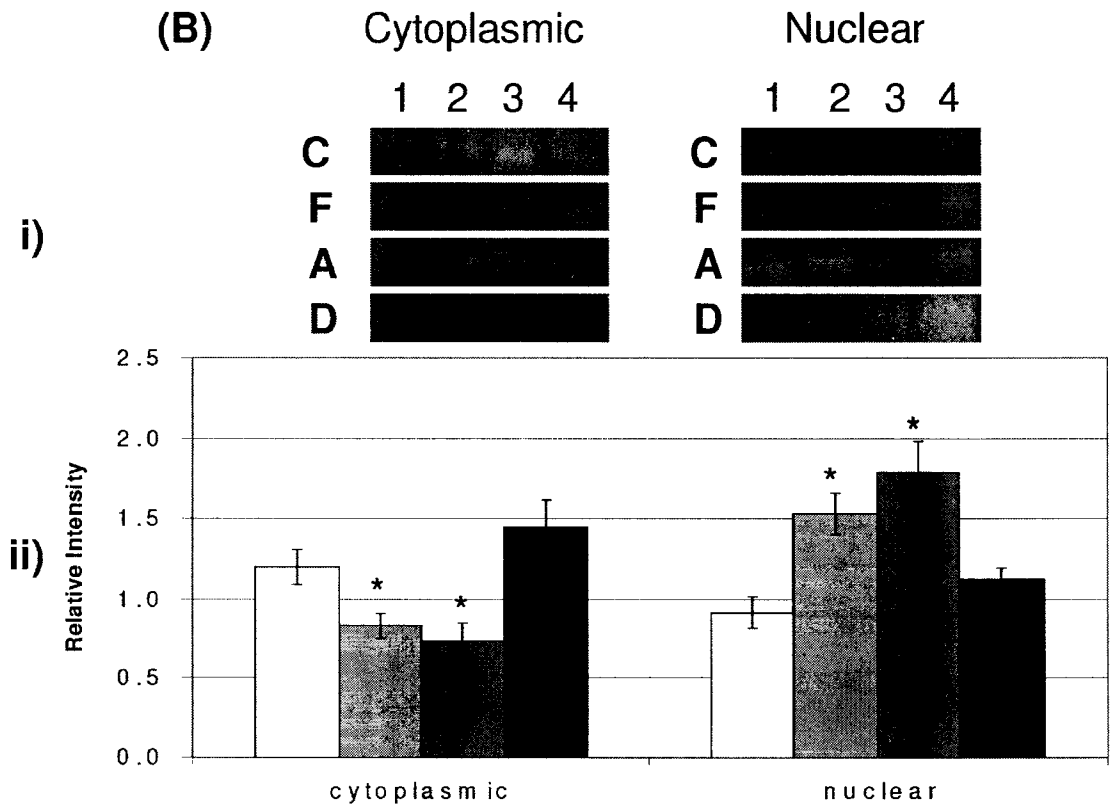


Figure 4.8:

Western blot analysis showing the distribution of active 50 kDa ATF6 (**A**) and active 50 kDa XBP1 (**B**) between cytoplasmic and nuclear fractions under freezing, anoxia and dehydration stresses in liver of wood frogs.

(**A**) Distribution of active 50 kDa ATF6 protein levels in wood frog liver.

i) Representative western blots showing ATF6 protein levels in cytoplasmic and nuclear fractions of liver under the following conditions: (**C**) control at 5°C, (**F**) 24 h freezing -2.5°C, (**A**) 24 h anoxia exposure at 5°C, and (**D**) 40% dehydrated at 5°C. The gels were loaded with 20 μg of each cytoplasmic and nuclear fraction.

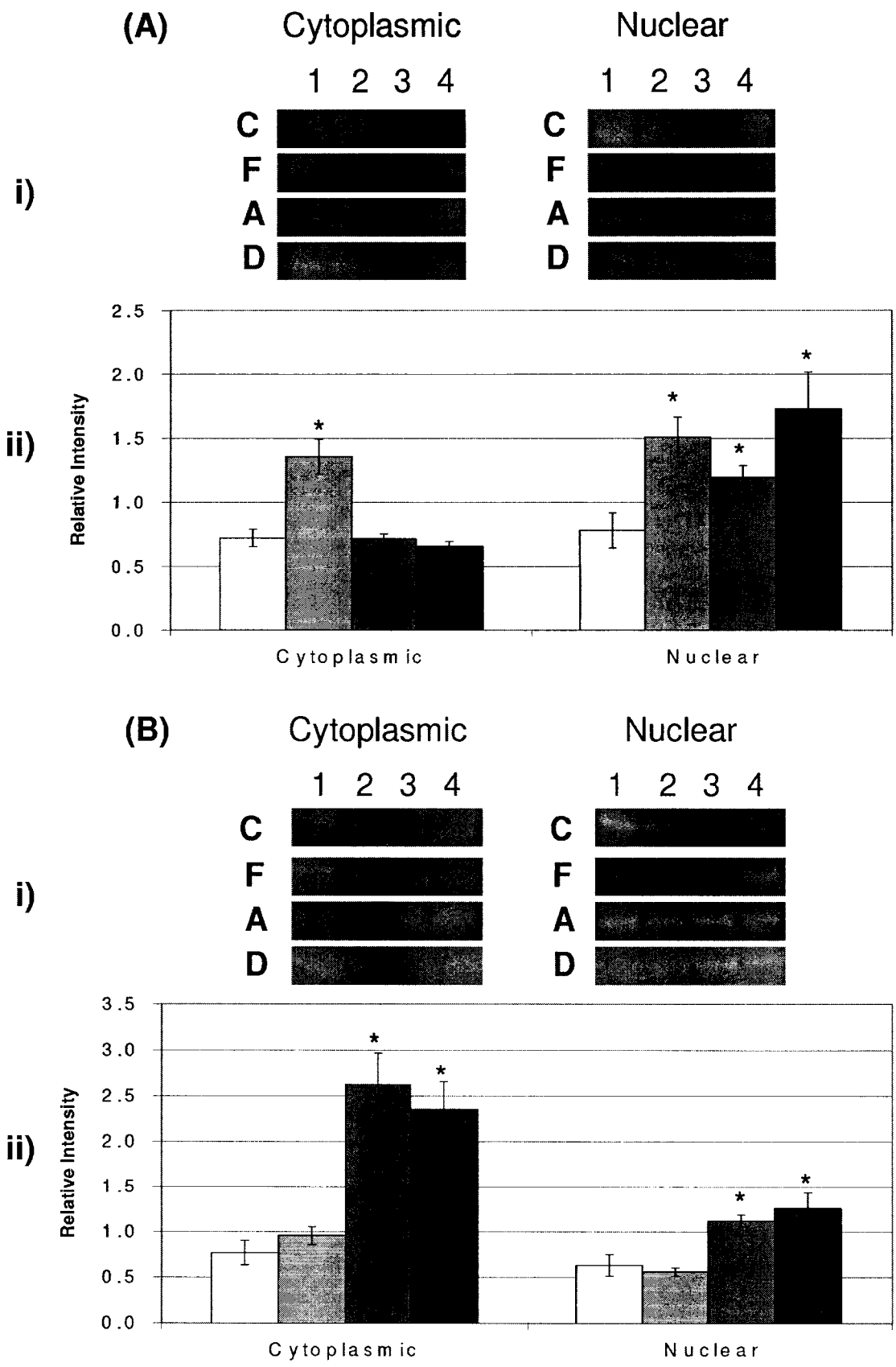
ii) Histograms show normalized mean values (± SEM, n=4 independent determinations) for the four conditions. *- Significantly different from corresponding control values, $P<0.05$.

(**B**) Distribution of active 50 kDa XBP1 protein levels in wood frog liver.

i) Representative western blots showing XBP1 protein levels in cytoplasmic and nuclear fractions of liver.

ii) Histograms showing normalized mean protein levels.

Other information as in (A) above.



Discussion

The previous chapter dealt with the unfolded protein response in skeletal muscle of *Rana sylvatica* and the defenses employed to protect muscle cells during times of stress. As mentioned in the introduction of this chapter, liver plays many important roles in the metabolism of organisms under both control and stressed conditions, and therefore it is highly important that the liver is well-prepared to survive these bouts of stress.

As discussed in the previous chapter, three different parts of the UPR contribute to cell survival – an increase in protein folding capacity, a simultaneous reduction in the folding load in the ER, and an increase in the cell's ability to dispose of misfolded, unassembled and unfolded proteins (Schröder and Kaufman, 2005b; Meusser et al., 2005). The present chapter focuses on some of the major proteins involved in these functions in liver.

Similar to skeletal muscle, previous work on wood frogs showed an increase in the protein levels of the key UPR indicator, GRP78, in liver during freezing whereas levels again remained unchanged under both anoxic and dehydration stresses (Zhenhong, 2004). The other indicator of ER stress, GRP94, rose under both freezing and anoxia in liver, but remained unchanged during dehydration (Zhenhong, 2004). The up-regulation of these glucose-regulated proteins suggests that ER stress is triggered in wood frog liver under all three experimental conditions (freezing, anoxia and dehydration). Similarly to the previous results for wood frog muscle, I evaluated the proteins levels of ATF3, ATF4, ATF6, GADD34, GADD153, XBP1 and EDEM and assessed their implication for the three main signaling pathways (the PERK/eIF2α/ATF4, the IRE1/XBP1, and the ATF6 pathways) of the UPR.

One key factor in reducing the folding load is to decrease the amount of new protein being translated; this is typically accomplished by inhibitory controls on ribosomal initiation and elongation factors, the phosphorylation eIF2α being of major importance (Harding et al., 2000). The phosphorylation of eIF2α inhibits the translation of most mRNA transcripts, with ATF4 (a key component PERK/eIF2α/ATF4 pathway) being one of the major exceptions. In liver of *Rana sylvatica* there was no significant change in the levels of the phosphorylated form of eIF2α during freezing, but phospho-eIF2α was elevated during both anoxia and dehydration (Data not shown).

PERK/eIF2α/ATF4 signaling pathway

Freezing:

The major protein of this pathway is ATF4 and as previously mentioned it is known to regulate ATF3, GADD34, GADD153 and GRP78 (Schröder and Kaufman, 2005b). In the liver ATF4 increased significantly during freezing and, furthermore, increased amounts of the transcription factor migrated into the nucleus (Fig. 4.2, 4.6). The data also showed an increase in GADD34 (Fig. 4.2), ATF3 (Fig. 4.3) and GRP78 (Zhenhong, 2004) levels during freezing. The levels of GADD153, on the other hand, were strongly suppressed.

GADD34 is known to interact with protein phosphatase 1 (PP1) and with human inhibitor 1 (I-1) to dephosphorylate eIF2α, thus reinitiating translation of mRNA transcripts (Connor et al., 2001). In response to freezing GADD 34 protein levels were significantly elevated (Fig. 4.2), which correlates with the unchanged levels of the

phosphorylated form of eIF2α during freezing (data not shown). This may help to promote the biosynthesis of cryoprotective proteins that are induced during freezing.

Similar to the results seen in muscle, GADD153, an apoptosis transcription factor (Kim et al., 2006), decreased dramatically in liver during 24 h freezing (Fig. 4.3). These results coupled with the significant increase in the protein levels of ATF3, a known repressor of GADD 153 (Jiang et al., 2004), suggest strong suppression of apoptosis during freeze/thaw (Fig.4.3). This is also supported by the enhanced migration of ATF3 into liver nucleus during freezing (Fig. 4.7). These data again suggest that active repression of apoptosis is a key factor in maintaining cell viability in the face of a stress (freezing) that has very great structural and metabolic effects on cells.

Anoxia:

The protein levels of the ATF4 transcription factor along with ATF3 and GADD 153 all decreased significantly in response to anoxia in wood frog liver (Fig. 4.2, 4.3) and GRP78 did not change from control levels (Zhenhong, 2004). However, GADD34 levels increased during anoxia, again suggesting an increase in the dephosphorylation of eIF2α (Fig. 4.2). There was no change in the nuclear content of ATF4 under anoxia, thus supporting the previous data that showed no change in GRP78 levels in this state (Zhenhong, 2004). However, the lack of ATF4 migration did not agree with the significant increase in GADD 34 levels, and therefore suggests that another factor must be involved in the up-regulation of GADD34 under anoxia (Fig. 4.2 B).

ATF3, unlike ATF4, did show increased migration into the nucleus during anoxia even though its total levels actually decreased (Fig. 4.3, 4.7). Studies have also shown that ATF3 can potentially up-regulate GADD34 (Schröder and Kaufman, 2005b).

Therefore, it could be suggested that ATF3 plays a dual role during anoxia, the first would be of up-regulation of GADD34 and the second as a repressor of GADD 153. The second role could be supported with the data in Figure 4.3, where the protein levels of GADD153 decreased during anoxia. With a decrease in the levels of GADD 153, also known as an apoptotic transcription factor that modulates the Bcl2 family (pro and anti-apoptotic), it could be postulated that reduced GADD153 would suppress/prevent anoxia-induced apoptosis in an anoxia tolerant species (Corazzari et al., 2003). Another possibility for the suppressed levels of GADD153 could be a lack of ATF4 migration into the nucleus, and thus no anoxia-induced up-regulation of *gadd153* transcripts (Jiang et al., 2004).

Dehydration:

ATF4 levels in liver of 40% dehydrated frogs were strongly elevated as in freezing. Both total ATF4 protein content in liver and nuclear levels of the transcription factor increased during dehydration. However, unlike the situation with freezing, this did not lead to increases in the levels of proteins that are downstream ATF4 (Fig. 4.2, 4.3). However, other genes are also controlled by ATF4; for example, ATF4 can up-regulate Herp, which is known to recruit the 26s proteasome for degradation of malfolded proteins (Schröder and Kaufman, 2005b). One could then interpret the data as if the liver does not undergo ER stress during dehydration, because as mentioned all through this chapter, glucose regulated proteins (GRP78 and GRP94) and ATF4 are key indicators of ER stress and the data shows that only ATF4 changed significantly (Mamady, 2006; Zhenhong, 2004).

ATF6 signaling pathway

Freezing:

As mentioned in the introduction, ER stress causes GRP78 to dissociate from inactive, ER membrane bound ATF6 allowing it to be spliced and released as its active form (Shen et al., 2005). During freezing, the levels of inactive 90 kDa ATF6 transcription factor increased significantly in wood frog liver, whereas the amount of the smaller 50 kDa active form did not change (Fig.4.4). However, increased nuclear translocation of active ATF6 occurred during freezing and, thus, this could increase the activation of genes that are downstream of ATF6, such as the GRPs, XBP1, GADD153 and EDEM (Fig.4.8 A). According to the data gathered, increased nuclear localization of active ATF6 did not result in increased protein levels of either EDEM or GADD153; indeed, both were suppressed during freezing. However, other downstream genes of ATF6 such as glucose regulated proteins (GRP78, GRP94) (Zhenhong, 2004) and XBP1 were elevated during freezing (Fig. 4.5).

Reduced levels of GADD153, as mentioned previously, would suggest that wood frog liver does not undergo apoptosis during freezing, whereas the reduced levels of the EDEM protein could be an indicator of either reduced or no protein degradation in the frozen state. These findings, combined with elevated levels of GRPs, suggest a preference in enhanced protein folding as the means to reduce ER stress in the frozen state rather than an increase in protein degradation or programmed cell death.

Anoxia:

The levels of inactive ATF6 increased strongly and significantly in wood frog liver during 24 hours of anoxic exposure and, unlike freezing stress, the levels of the

active 50 kDa form of the transcription factor also rose substantially (Fig.4.4). The increase in active ATF6 content is an indicator of a higher splicing occurrence that is associated with high levels of unfolded or malfolded proteins. The increase in spliced levels is also seen in the nucleus where the amount of active 50 kDa ATF6 was elevated under anoxia. This suggests that the ATF6 transcription factor would increase the transcription of its downstream genes under anoxic conditions. Indeed, Zhenhong (2004) demonstrated that the levels of *grp94* transcript, a downstream gene of ATF6, were elevated during anoxia whereas levels of *grp78* did not significantly change from control values. An increase in the amount of active XBP1 was also observed but the protein levels of other genes that are downstream of ATF6 such as EDEM and the inactive form of XBP1 did not increase, suggesting that the increase of ATF6 splicing did result in up-regulation of these two particular downstream genes. The increase in ATF6 splicing, *grp 94* transcripts and active XBP1 (a known activator of GRP94), coupled with the decrease in EDEM and GADD153 levels, suggests that frog liver cells again preferentially reduce the effects of ER stress by increasing the protein folding capacity and not decreasing the folding load during anoxia.

Dehydration:

Levels of the membrane bound 90 kDa inactive ATF6 increased slightly during dehydration, but there was no increase in the amount of the spliced active (50 kDa) form of the transcription factor (Fig.4.4). However, a significant increase in the content of active 50 kDa ATF6 was measured in the nuclear fraction, indicating a migration of the active transcription factor into the nucleus during dehydration (Fig.4.8 A).

This migration also correlated with an increase in the content of both the inactive and active forms of XBP1. According to previous studies (Zhenhong, 2004), *grp94* mRNA levels increased during dehydration, which is similar to freezing and anoxic treatments. Levels of the apoptotic transcription factor GADD153 did not change during dehydration (Fig.4.3 B) and the ER degradation enhancing α-mannosidase-like protein (EDEM) significantly decreased (Fig.4.6), once again suggesting that increased protein folding is preferentially selected during ER stress in wood frog liver.

IRE1/XBP1 signaling pathway

Freezing:

As mentioned in the previous chapter, IRE1 is a transmembrane protein with three distinct domains, one of which is an endonuclease domain involved in the splicing of the *XBP1* transcript to produce the highly active 50 kDa XBP1 transcription factor. During freezing, the levels of inactive XBP1 increased significantly along with a very large 3.5 fold increase in the content of the spliced active form in liver (Fig. 4.5). The significant increase of both forms of the transcription factor indicates that ATF6 is a strong activator of XBP1. The increase in the spliced form also indicates higher splicing occurrences during freezing/thaw.

However, despite the large increase in 50 kDa active XBP1 in liver during freezing, there was no increase in the nuclear content of this transcription factor (Fig. 4.8 B), and this may explain the decrease in EDEM levels (Fig.4.6). It has been suggested that the inactive XBP1 protein could be involved in the recovery stage of ER stress by acting as an inhibitor of the XBP1 pathway (Tirosh et al, 2006; Yoshida et al, 2006).

This theory has yet to be proven, but it would be fair to suggest that in this case the unspliced form of XBP1 protein might inhibit the migration of the spliced XBP1 into the nucleus during freezing.

Anoxia:

During anoxia exposure, the levels of unspliced XBP1 decreased below the control levels, whereas the amount of the highly active spliced form significantly increased (Fig. 4.5). Elevated levels of active XBP1 are caused by increased endonucleic activity of IRE1. It was also observed that the active form of the XPB-1 did indeed increase in the nucleus, thus showing an increased migration through the nuclear membrane. The results did not correlate with the decrease in EDEM levels (Fig. 4.6), but could contribute to the increased levels of the GRP94 (a downstream gene of XBP1) during anoxia.

The data gathered about this pathway during anoxia supports the previous suggestion that liver cells preferentially rely on increasing their folding capacity to deal with stress that affects ER function. The activation of glucose regulated proteins is possible through both the ATF6 (GRP 78 & 94) and IRE1/XBP1 pathways (GRP94), whereas the downstream gene involved in the increased degradation of unfolded proteins is suppressed.

Dehydration:

The data on the effects of dehydration on wood frog liver XBP1 were very similar to those observed during freezing. Both the inactive and active forms of XBP1 increased significantly in 40% dehydrated frogs (Fig. 4.5). However, unlike the results seen during freezing, the active form of XBP1 did migrate into the nucleus during dehydration, but

once again did not trigger an increase in the levels of EDEM (Fig. 4.6). Again, like the other two stresses, the UPR seems to be preferentially enhanced during dehydration rather than the ERAD pathway.

Similar to the results for freezing, it can be postulated that the unspliced form of the transcription factor could inhibit the active form, but it could also be suggested that the active from of XBP1 preferentially activates GRP94 rather than the degradation pathway.

Conclusion

The data gathered about the responses by wood frog liver to freezing, anoxia and dehydration stresses suggested once again that the type of stress mediates which pathway is expressed. Much like the data gathered for skeletal muscle, it was clear that both the PERK/eIF2α/ATF4 and ATF6 pathways were most active in the survival of cells during freezing. There was a clear indication of increased folding capacity while not necessarily reducing the folding load. The increased expression of GADD34 protein in conjunction with PP1 would dephosphorylate eIF2α in order to maintain translation, thus not reducing the protein folding load. It would also seem that during freezing ATF4 and ATF6 both activate the GRPs in order to increase the folding capacity. Unlike the data for frozen muscle in the previous chapter, it does not appear that the ATF6 pathway induces the ERAD pathway in order to alleviate the folding load. Therefore, it could be concluded that during freezing the liver cells depend solely on increasing the folding capacity in order to survive ER stress, whereas folding load is unchanged.

Under anoxia stress, ATF6 seemed to be the major activator of stress survival. The levels of ATF4 decreased during anoxia and there was no increase in migration of this transcription factor into the nucleus; thus, it was determined not to be an activator of GADD34. On the other hand, total ATF6 content increased under anoxia and active ATF6 levels increased in the nucleus. These findings suggested that ATF6 enhanced the expression of *grp94* as well as the active form of XBP1. The increased levels of GADD34 could be explained by the increased content of ATF3 in the nucleus, a known activator of GADD 34. The decrease in EDEM expression levels also suggest that, similar to the frozen situation, liver cells under anoxia do not enhance the ERAD pathway, but simply rely on increased folding capacity to survive ER stress.

Similar to the freezing and anoxia stresses the levels of EDEM in liver decreased during dehydration suggesting that there is no enhancement of the degradation pathway under these conditions. This again leaves the reduction of the folding load and the increased folding capacity as the primary, if not the only means of survival for the cells. To reduce the folding load in the ER, the translation of new proteins must be reduced or even completely attenuated. During dehydration the levels of GADD34 remained virtually unchanged, thus contributing to the elevated levels of phosphorylated eIF2α and attenuation of newly synthesized proteins. The levels of ATF4 increased in the nucleus of dehydrated liver, but did not seem to enhance transcription of any of its downstream genes. On the other hand, the enhanced levels of active ATF6 in the nucleus would be a good candidate to trigger genes that enhance the folding capacity of the endoplasmic reticulum. The increased expression of XBP1, coupled with elevated ATF6 levels, and with elevated nuclear contents of both in dehydrated liver would activate *grp94*

transcription and thus allow liver cells to increase the folding activity of the ER under stress.

Chapter 5

General Discussion

The wood frog is the major model for studies of natural freeze tolerance by vertebrate animals (Storey and Storey, 2004a). Our laboratory has uncovered many of the biochemical and physiological adaptations used by wood frogs to survive winter freezing. Previous studies showed that glucose-regulated proteins, GRP 78 and GRP 94, were elevated during freezing at both the transcript and protein levels (Zhenhong, 2004). These findings suggested that ER stress was occurring and that wood frogs cells might be triggering the UPR response as an adaptive measure to protect themselves during freezing. The studies in this thesis examined these ideas by analyzing markers of ER stress and of the associated survival responses: the unfolded protein response (UPR) and the endoplasmic reticulum associated degradation (ERAD). My thesis focused on three main response pathways in liver and skeletal muscle of wood frogs: (1) PERK/eIF2α/ ATF4, (2) ATF6, and (3) IRE1/XBP1 (Fig. 5.1).

As mentioned in the previous chapters, the two main mechanisms for cell survival of ER stress are the UPR and ERAD signaling cascades. These two cascades are intertwined with each other and not mutually exclusive (Fig. 3.1 and 4.1). There is overlap between the three different response pathways and my studies with wood frogs also show overlap in how these pathways respond to the three stresses of freezing, anoxia and dehydration. For the purpose of simplicity, the following discussion is subdivided to deal with the three different pathways and under each I discuss how they respond to the three environmental stresses with a further comparison of liver and muscle responses.

5.1 PERK/eIF2α/ATF4 pathway

The activating transcription factor 4 (ATF4) is a central regulator of ER stress

(Mamady and Storey, 2006). When cells are under ER stress, one of the first responses is to shut off ATP-expensive protein synthesis which is accomplished by phosphorylation of the ribosomal initiation factor, eIF2α. Translation of most proteins is reduced or halted, except for the production of a small number of proteins, ATF4 being one of these (Jiang and Wek, 2005; Luo et al., 2003; Blais et al., 2004). Experiments showed that the levels of phosphorylated eIF2α increased substantially in skeletal muscle during freezing while remaining almost stable in liver of the wood frog (data not shown). Notably, this response by liver probably reflects the need by the liver to not only protect itself from ER stress but also to still synthesize a number of cryoprotective proteins, some of which are exported into the plasma (Storey and Storey, 2004a). ATF4 protein levels increased significantly in both tissues indicating potential ER stress during the 24 hour freeze. Furthermore, the ATF4 nuclear content increased significantly during freezing in both muscle and liver, with a fast response seen in muscle (6 h data point). This fast response correlates with much faster freezing in muscle (a peripheral tissue) compared to the liver (a core organ). An increase of ATF4 levels can cause activation of ATF3, GRP78, GADD34 and GADD153 (Fig. 5.1A). Three of these were assessed.

The downstream factor of ATF4, ATF3, increased in protein levels during freezing in both tissues. This transcription factor is known to repress the transcription of GADD153, a known apoptosis transcription factor, as well as being a potential activator of GADD34 protein. The effect on GADD153 was very pronounced with levels of this protein strongly reduced in liver and nearly disappearing in muscle. This was also supported by the increased nuclear content of ATF3 during both freezing and anoxia for both tissues. Unlike the ATF4 nuclear content, the ATF3 nuclear content increases in the

later stages of freezing. In conjunction with previous data by Du (2005), who assessed other markers of apoptosis, this suggests that there is little or no apoptosis in these two very important organs during natural freezing exposures. Even though both the liver and skeletal muscle have regenerative capabilities, it would be unwise to let these organs lose cellular mass as this could prove costly after thawing, when the frog needs to resume normal physiological functions. Furthermore, since multiple freeze/thaw events could occur over a winter, the benefit of inhibiting apoptosis during each freeze is obvious.

GADD34 levels were increased in liver during freezing but did not change in muscle. It is known that GADD34 associates with protein phosphatase 1 (PP1) in order to dephosphorylate eIF2α (Connor et al, 2001). This enables the reinitiation of protein translation. The fact that GADD34 was elevated only in liver could be related to the production of a variety of cryoprotective proteins during freezing which would require protein synthesis to remain active. The data showed an increase in GADD34 protein levels in liver, whereas the levels in skeletal muscle did not show any significant change.

The data for GADD34 can be correlated with the changes in P-eIF2α levels. The unchanged protein levels of GADD34 in skeletal muscle are likely unable to prevent the rise in P-eIF2α levels that may be triggered by other stimuli during freezing. In liver, however, increased levels of GADD34, by interacting with PP1, could counteract other stimuli that would trigger eIF2α phosphorylation in order to maintain P-eIF2α at low levels, sustain protein synthesis capacity at near-normal levels, and allow the synthesis of cryoprotective proteins by this biosynthetic organ.

Various cryoprotective and metabolic responses to freezing in wood frog organs are also triggered by one of two component parts of freezing (anoxia/ischemia and

cellular dehydration) (Storey and Storey, 2004a). For example, glucose accumulation is triggered by both freezing and dehydration but not by anoxia. This has led to the idea that a number of the adaptive responses to freezing grew out of pre-existing metabolic responses to either dehydration or anoxia with the further addition of selected freeze-specific adaptations. It seemed possible, therefore, that the responses to freezing by ATF4, ATF3, GADD34 and GADD153 might mimic the responses of these proteins to either anoxia or dehydration stress. Overall, however, there were few patterns seen. In skeletal muscle ATF4 and ATF3 responded similarly to freezing and anoxia exposures (elevated) whereas levels of both were reduced during dehydration. In liver, ATF4 responses were also similar in freezing and dehydration (elevation) but levels decreased during anoxia. However, ATF3 responded differently under all three stresses in liver, but levels of both ATF4 and ATF3 were reduced under anoxia. Levels of GADD34 and GADD153 both increased in muscle under anoxia but only GADD34 increased in liver under the same stress. Dehydration reduced levels of ATF4, ATF3 and GADD153 in muscle except for a rise in ATF4 levels in liver, the other proteins did not respond to dehydration in liver. Dehydration has a greater physical effect on peripheral tissues (skin, muscle) than it does on core organs whose water content is maintained for as long as possible. This is different from the effect of freezing since ice growth throughout the body affects the hydration state of all organs. Hence, the general lack of response by proteins to dehydration (except for ATF4) might be because the liver was still relatively unstressed in the dehydrated frogs.

5.2 ATF6 pathway

In this second pathway, the importance is not placed on the levels of proteins per say, but on the splicing of ATF6 to create the active form of the transcription factor. The full length ATF6 protein is a transmembrane protein that under ER stress is spliced into a smaller-sized active protein (Shen et al., 2002; Schröder and Kaufman, 2005a; Shen et al., 2005; Shen and Prywes, 2005). In skeletal muscle the splice occurrence increased during freezing and thus potentially increases transcription of the downstream genes under ATF6 control (Fig. 5.1B). However, stress exposure did not alter ATF6 during anoxia or dehydration but muscle responded during recovery with elevated levels of 90 kD inactive ATF6 which led to enhanced 50 kD active protein during recovery from dehydration. The ATF6 response in liver was very different. Although the amount of 90 kD inactive ATF6 rose strongly during freezing, the amount of active 50 kD protein remained constant. This response deserves further attention. It is possible that unspliced 90 kD ATF6 may have a function of its own in freezing. Notably both anoxia and dehydration stress increased 90 kD content by a low amount (1.5-fold or less) but during freezing the increase was >4-fold.

When looking at Figure 5.1B it is clear that signaling from ATF6 can radiate in three ways: 1) an apoptotic route, 2) the ER associated degradation route, and 3) the protein folding route. The data gathered for GADD153 showed a decrease in levels for both skeletal muscle and liver, thus removing the notion that apoptosis occurred. To determine the possibility that the endoplasmic reticulum associated degradation (ERAD) pathway was activated, the responses of EDEM protein to stress were assessed. In liver EDEM levels uniformly decreased in response to all three stresses, clearly suggesting that

ERAD is not the route taken to deal with the any unfolded proteins in this organ in response to environmental stress. The situation in muscle was much the same under anoxia or dehydration exposure but a uniquely different response was seen during freezing where EDEM protein levels were strongly elevated during freezing (by >4-fold) and remained high during thawing (~8-fold over controls). This suggests that in muscle during freezing, malfolded proteins are targeted to the proteasome for degradation. A study by Woods and Storey (2006) found that the amount of proteins damaged by oxidation (measured as carbonyl protein content) increased during freezing in muscle whereas the activity of the multicatalytic proteasome (MCP) that preferentially degrades such proteins was suppressed. However, MCP activity rapidly rebounded during thawing rising to levels that were >2-fold higher than controls and carbonyl protein levels correspondingly dropped. Taken together with my results, this suggests that significant protein damage occurs during freezing in skeletal muscle. This may be tolerated in the frozen ischemic state but after thawing the data indicate that protein degradation pathways go into high gear to clear both unfolded and proteins damaged by oxidation. The amino acids generated could then be recycled to resynthesize new muscle protein after thawing.

The last protein that I measured that is associated with the ATF6 pathway was XBP1. This protein can be associated with all three branches of ATF6 signaling as mentioned in the literature (Schröder and Kaufman, 2005a; Schröder and Kaufman, 2005b). In my experiments XBP1 seems to be associated with the third branch of ATF6 signaling, the unfolded protein route, which is discussed in the following section.

5.3 IRE1/XBP1 pathway

XBP1 is also activated by splicing but in this case the splicing happens at the mRNA level and not at the protein level. I conducted preliminary studies to quantify XBP1 mRNA by RT-PCR but encountered difficulty with primer design and thus was unable to assess stress-induced changes in XBP1 transcription. Therefore, levels of both 30 kD inactive and 50 kD active XBP1 protein were determined to assess splicing occurrence. Levels of 50 kD active XBP1 are particularly crucial and in liver a consistent increase in this protein was found in all three stress situations and levels remained high during recovery. The particularly strong response to freezing (>3.5 fold increase) suggests the importance of XBP1 signaling to dealing with freezing stress in liver. Furthermore, since both anoxia and dehydration triggered XBP1 elevation in liver, the enhanced response to freezing may come from an additive effect of stimulation by both anoxia and dehydration signals on XBP1 splicing and synthesis of the active protein. As discussed previously, the spliced or active form of XBP1 is a very important part of the ER stress response and is known to increase the transcription of ER chaperones (GRP 78 and GRP94) along with ERAD proteins such as EDEM (Schröder and Kaufman, 2005a). As mentioned above, the need to synthesize cryoprotective proteins by liver and to maintain/enhance proteins involved in cryoprotectant glucose synthesis probably requires the liver to aggressively deal with problems of stress-induced protein malfolding or unfolding. Responses triggered by XBP1 may be key to achieving this. In muscle, however, the patterns of 50 kD XBP1 changes were different. In particular, active XBP1 levels fell during both freezing and dehydration suggesting that XBP1 control in muscle may be linked with changes in cell hydration. Furthermore, suppressed XBP1 levels may

indicate that the correction of unfolded proteins in muscle is of lesser importance than it is in liver. These results also fit with the conclusions drawn above for EDEM changes which suggested that degradation of unfolded/malfolded proteins was a major response to freezing in muscle.

In skeletal muscle, active XBP1 transcription factor levels decreased significantly during freezing and dehydration and yet EDEM levels were strongly stimulated in frozen muscle. Since both the ATF6 and IRE/XBP1 pathways can control EDEM protein levels (Figure 5.1), these results indicate that it is ATF6 signaling that triggers EDEM up-regulation during freezing. This proposal is also supported by the observed increase in the nuclear translocation of ATF6 during freezing, although the XBP1 route cannot be completely ruled out because nuclear levels of XBP1 also increased during freezing. In liver there were also oppositely-directed responses by XBP1 and EDEM: active XBP1 increased strongly whereas EDEM drops. This again suggests that XBP1 is not regulating EDEM with respect to the response to the three environmental stresses. This is also supported by the unchanged levels of active XBP1 in the nuclear fraction during freezing whereas nuclear ATF6 in liver rises sharply. Elevated XBP1 in liver may also have other actions, a very likely one being the up-regulation of *grp94*. This is also supported by the relatively unchanged levels of ATF6 in liver which is the other major influence on GRP94 levels.

Summary

In skeletal muscle, the decreased levels of GADD153 suggest that the cells are not undergoing apoptosis during natural freezing exposures. This left an increase in folding

capacity and the reduction of folding load as the potential mechanisms for addressing ER stress in muscle. The increased levels of active ATF6 and its nuclear translocation correlated with the previously observed release of the GRP78 protein, which in turn increases the folding capacity of the ER. In previous studies, it has been shown that the mRNA of both GRP78 and GRP94 do not change significantly, but that their protein levels are significantly increased during freezing (Zhenhong, 2004). Under freezing conditions in muscle, an strong increase in EDEM protein was also seen and this suggested an enhancement of the ERAD pathway. Hence, the present study suggests that during freezing, skeletal muscle cells are unlikely to undergo apoptosis, but instead respond with an increase in protein folding capacity (by stimulating GRP production) coupled with a reduction in folding load (ERAD) as the solutions for dealing with stress-induced increases in unfolded or malfolded proteins.

Magnetic resonance imaging has shown that the liver is one of the last organs to freeze in the frog and while the rest of the body is freezing, the liver remains very active in the output of cryoprotectants (Storey and Storey, 2004a). Just like skeletal muscle, the current results for liver show little or no evidence of the occurrence of apoptosis during bouts of freezing. Unlike the skeletal muscle the splicing occurrence of ATF6 did not increase during stress, but an increase in the active form of XBP1 correlated with the increase of both mRNA and protein levels of GRP78 and GRP94 (Zhenhong, 2004). Unlike in muscle, there was also a decrease in EDEM protein levels in liver which suggests that the response to misfolded or unfolded protein by liver during freezing is not to degrade them. The data suggest that liver cells place most of their resources into increasing the folding capacity which is consistent with a role for liver during freezing in

the synthesis of cryoprotective proteins.

Future Directions

Future studies involving the freeze tolerance of *Rana sylvatica* should no doubt include further research into the responses and regulation of the protein folding process and the ER associated degradation during natural freezing. There are many factors that decides the fate of a malfolded proteins and thus, many proteins involved in this decision. In the present study only EDEM was measured as a representative of the ERAD and, as shown in Figure 5.1, the folding pathways are extremely complex.

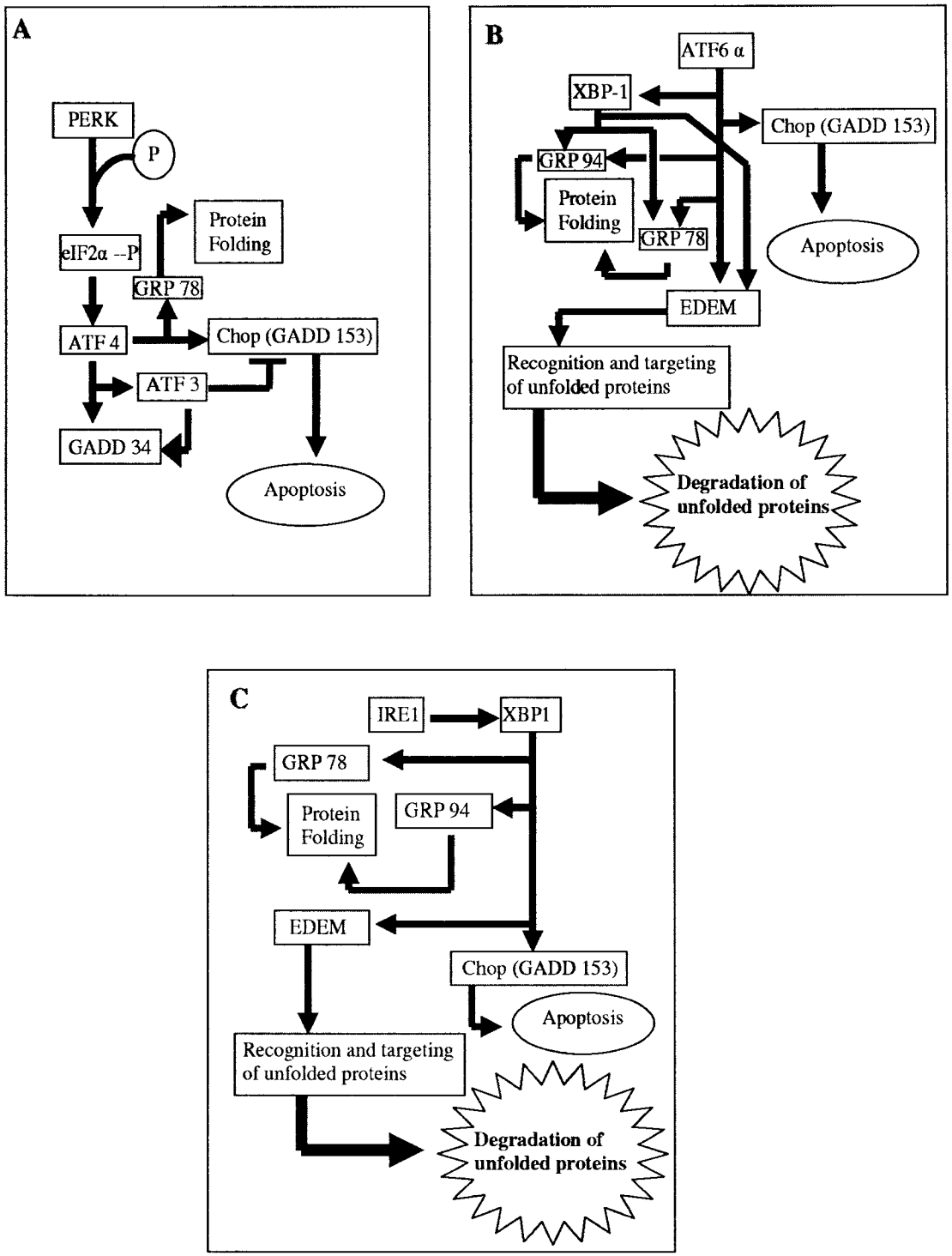
Brodsky (2007) suggested that glucose regulated proteins such as GRP78 (BiP), could have destructive roles during ERAD. It was suggested that BiP could bind to its co-chaperones along with lectins (calnexin) in order designate proteins for degradation (Brodsky, 2007). There has also been speculation that CHIP (C-terminus of the Hsc70-interacting protein) associates with Hsc70 and Hsp90 to ubiquitinate newly synthesized cystic fibrosis transmembrane conductance regulator (CFTR). Experiments involving immunoprecipitation, would help determine the interaction of the chaperones to ubiquitin ligases present in the ER.

Uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase (UGGT) and Calnexin are two other proteins involved in the recognition of unfolded and malfolded proteins. UGGT recognizes the folding defects in proteins and thus reglucosylates the single N-glycan near the misfolded area; this flags the protein for recycling through calnexin binding (Schröder and Kaufman 2005a). It is known that calnexin is a membrane protein that interacts with newly synthesized glycoproteins and is

involved in the assembly of these proteins as well as the retention of unassembled proteins in the ER. According to Schröder and Kaufman (2005a), the interaction between EDEM and calnexin bound to improper folded proteins, causes a retranslocation of the protein to the cytoplasm where it is degraded by the proteasome (Brodsky, 2007). A study of the calnexin protein levels along with its interaction with EDEM would give an idea of degradation instances during freezing. These two findings would help to map the quality control mechanism during periods of cellular stress.

Another pathway that is involved in protein regulation and that has not yet been studied in freeze tolerant frogs is autophagy. This pathway is a catabolic process in which the cell degrades its own components in order to continue the vital processes for survival (Ferraro and Cecconi, 2007; Yorimitsu and Klionsky, 2005; Yorimitsu and Klionsky, 2007). New information about autophagy indicates that it is closely related to ER stress and could be a viable alternative to apoptosis (Ferraro and Cecconi, 2007; Yorimitsu and Klionsky, 2005; Yorimitsu and Klionsky, 2007).

Figure 5.1: Schematic representation of the three main pathways involved in the unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD). (A) the PERK/eIF2α/ATF4 pathway, (B) the ATF6 pathway, and (C) the IRE1/XBP1 pathway, highlighting the key components of each.



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APPENDIX A

TECHNICAL BULLETIN EB07

THE COMPLETE WESTERN BLOTTING PROCEDURE

By: Joann Tye
(Modified from D. Douglas, S.P.J. Brooks, Pier Morin, and H. Mamady)
June 2005

TOTAL PROTEIN ISOLATION FOR WESTERNS

This section describes a detailed step-by-step foolproof procedure for the extraction of total soluble protein from tissue samples. If the instructions are followed carefully, your protein extracts will be of the highest quality.

1. Obtain:
   a) a pair of sharp tweezers for picking up tissues and puncturing holes on the test tube cover.
   b) a styrofoam box filled with ice.
   c) enough 2 ml micro-test tubes or falcon tubes for your samples. If you are making large volumes of protein isolations (>1.5 ml), use the falcon tubes. Label each tube with a Sharpie. Use the sharp end of the tweezers to puncture one hole in the cover of the test tube. This prevents the tube from exploding when the liquid nitrogen is poured into it. This step is very important to conserve tissue. Put the test tubes in the box of ice.
   d) 2 microspatulas – one for scraping the mortar and pestle and one for PMSF
   e) a mortar and pestle. It's a good idea to use a medium sized mortar and pestle. If you use a small one, the weight of the pestle is not heavy enough to crush the tissue, so you'll have to exert more strength to crush it. If it is too big, you lose more tissue when it gets stuck to the walls of the mortar.
   f) a mini ladle (in the buffer room) to scoop liquid nitrogen into the mortar.
   g) a bottle of PMSF (phenylmethyl sulfonyl fluoride – a protease inhibitor. Poisonous. NOTE that PMSF is rapidly inactivated when dissolved in water so we typically add a small amount of solid to each sample immediately prior to homogenization)
   h) a bottle of Sigma protease inhibitor cocktail (a mixture of several proteases)
   i) some paper towels
   j) sheets of weighing paper
   k) liquid nitrogen thermal gloves to protect your hands from the cold and and ear plugs to protect your hearing from the noise of the homogenizer.

   ***After you have obtained all these, bring them to the buffer room and set up a work place around a homogenizer. Use the small Polytron PT 1200 if you have 2 ml micro-test tubes and the bigger one if you have falcon tubes.

l) a styrofoam box with enough liquid nitrogen to keep your tissues samples frozen the whole time that you are making your homogenates.

***Now that you have obtained your tissue and secured it in liquid nitrogen, proceed to the buffer room.

2. Put the tip of one of the microspatulas in liquid nitrogen to cool it. Leave it in there until it is needed.

3. Put the pestle and tweezers in the mortar and, using the mini liquid scoop, fill the mortar with liquid nitrogen to bring the temperature down.

4. Weigh tissue on a piece of weighing paper on the scale, taking it out with the cold tweezers. MAKE SURE THE TISSUE IS FROZEN AT ALL TIMES. That means that you don't have much time to weigh it, so you will have to act fast. Aim to make homogenates that are 1:2 w:v (e.g. 0.5 g tissue + 1 ml buffer). These are concentrated samples but it is better to have concentrated samples that you can dilute later rather than samples that are too dilute to show any bands.
**<u>NOTE</u>: Some tissues are in critically small amounts (e.g. one wood frog heart may only be 20 mg). Please check with Ken or other senior researchers before using up all or most of any given sample so that you do not screw up the projects of other people

5. Put the tissue in the cold mortar and add more liquid nitrogen, as needed to keep the tissue frozen.

6. Crush the tissue to a powder. It's best to tap the tissue lightly until the tissue shatters into smaller pieces while covering the majority of the mortar with your other hand to prevent tissue from flying out. Then, grind up the tissue by twisting the pestle down on the mortar. Add liquid nitrogen, as needed.

7. Pour the powdered tissues with the liquid nitrogen into your tubes. Be careful not to pour too much at a time because the sample will spray out of the tube. Use more liquid nitrogen, if necessary. Scrape any excess tissue from the mortar and pestle into your tube with the cooled microspatula.

8. Put the tissue-filled tube into liquid nitrogen.

<u>**NOTE:**</u> There is another method to ensure that all of the ground tissue is transferred into the tube. The following method may be more labour intensive, but is effective if you are trying to be as accurate as possible.

   a. Preweigh the prechilled tubes and record the reading.
   b. Ground the appropriate amount of tissue sample into powder. You can determine the appropriate amount by weighing the tissue first.

c. Transfer the powdered sample into the appropriate prechilled preweighed tube.
d. Do a second weight of tissue+tube and then get the weight of the tissue by subtraction.

9. Clean the frozen mortar and pestle with a kimwipe or paper towel to remove any residual tissue powder. Then, bring the temperature of the mortar, pestle, and tweezers down again with more liquid nitrogen. Do NOT try to wash/dry the mortar between each use – water hitting a frozen mortar is often a great way to make it crack. When you have finished all samples, let the mortar/pestle warm up and then wash them for the next person.

10. Repeat steps 3 to 9 until you are done crushing your tissues.

    YOU MUST **ALWAYS** KEEP YOUR TISSUE FROZEN AT ALL TIMES. NEVER LET IT THAW!!

11. After all tissue samples are crushed and transferred to their labeled tubes, return unused tissues to their appropriate place in the freezer.

12. For each tissue sample in turn, removed the crushed sample from the liquid nitrogen and thaw slightly for ~1 minute.

13. Add a few crystals of PMSF with a microspatula, 1 ul of Sigma protease inhibitor, and the appropriate amount of homogenizing buffer. Calculate the amount of buffer needed as tissue weight x 2.5 (e.g. for 0.4 g use 1 mL in a 2 mL Eppendorf microtube; for 1.2 g use 4.8 mL in a Falcon tube).

14. Homogenize the tissue in the buffer with the Polytron homogenizer at high for 15 seconds or until the solution is homogeneous. Put the test tube in ice.

15. Rinse the homogenizer with ddH_2O between samples and suck off excess water by drying with a Kimwipe. Look at the homogenizer blades and make sure that no tissue "lumps" remains.

16. Sonicate your sample for 30 seconds each.

17. Repeat steps 12 to 15 until all tissues are homogenized.

18. Clean up.

    ***After you have homogenized all your samples and cleaned up, proceed to the enzyme assay room on the fifth floor to spin down your homogenates in the Eppendorf centrifuge that is in the cold box (if using the 2 mL microtubes) (there is also one in Rm 431) or use the Sorvall or Hermle centrifuges in Rm 424 if

using the 15 mL Falcon tubes. Ask for help if you have not used the centrifuge before.

19. Centrifuge your samples at 4°C for 15 minutes at 12,000 rpm. Make sure your centrifuge is balanced.

20. Pipette off the clear supernatant. If there is a floating fat layer, work your pipette tip down under the fat. Transfer the supernatant to a new clean, labeled tube and store on ice. These are your "white" homogenates.

NORMALIZING YOUR SAMPLES USING THE BIORAD PROTEIN ASSAY

The protein concentration of your white homogenates must be determined using the BioRad Protein Assay, the microplate reader and the Biolinx program. This section gives a detailed procedure on the operation of the Biolinx program for the BioRad Protein Assay. Before loading anything on your gel, you have to make sure that you are loading a known and equal amount of protein into each well.

1. Carefully dilute a small aliquot of each original supernatant in distilled water – a 20-fold dilution is usually good. Prepare 40 ul of diluted protein sample (ie. 2 uL original + 38 uL water) in order to assay n=3. (**note: some people prefer to assay 1 uL of undiluted sample directly but if you work with such small volumes, be sure to do n=3 at least for each sample to account for pipetting error**)

2. Dilute the BioRad reagent in distilled water, 1 volume of concentrate plus 4 volumes of water.

3. Put 10 ul of diluted sample into each microplate well and mix your sample by pipetting up and down a few times or by stirring it with a pipet tip. Run n=3 wells for each sample. You can set up your replicates however you like (ie. 3 across or 3 down). Make sure you keep track of your wells.

4. Put 190 ul of the diluted BioRad reagent into each of the microplate wells that contains your diluted sample.

5. Also prepare blanks (n=3) that have 10 ul distilled water added instead of diluted protein samples. Make sure the reagent does not turn blue – if it does, that indicates that your microplate was not cleaned properly.

6. Let stand for about 5 minutes for maximum color development.

PREPARING YOUR BLUE LOADING SAMPLES

Our common way of now doing this (as per 2007) is this. We move directly from white homogenates to blues on the same day. That is – NO FREEZING OF WHITES. This eliminates problems that we had in the past of whites precipitating out when frozen.

We also want the most concentrated final blue samples possible – preferably 5 ug/ul (or 4 or 2.5 ug/ul but 1 ug/ul is almost useless except for highly abundant proteins). Based on the protein concentrations of your samples, calculated as above, figure out the highest concentration that all samples could be adjusted to. For example, if concentrations for 5 samples were 8.2, 9.0, 9.8, 8.4 and 8.6 ug/ul (mg/ml), then all samples could be adjusted to a highest common concentration of 8.0 ug/ul. Using the equation

C1 x V1 = C2 x V2

figure out how much more white homogenizing buffer you have to add to each sample to dilute each to the final constant concentration (in this case 8 ug/ul).

Add the white buffer and mix the sample well.

Then mix each adjusted sample 1:1 v:v with 2x SDS blue buffer, mix and then boil.

Most people aliquot 250 ul of white sample into each of several Eppendorf tubes, then add 250 ul of blue buffer, cap, mix and boil. This keeps the volume low so you get good heating.

With large volume white samples, you could mix white and blue 1:1 v:v in 15 mL tubes but in that case, increase the boiling time to 10 min (from the 5 min suggested below) and make sure that the whole volume of sample is submerged. Then aliquot.

Freeze samples after boiling if not using right away.

For the above example, the 8 ug/ul concentration would become 4 ug/ul in the final blue extract. You would then load an equal volume of extract from each condition onto your gels.

STEPS

1. Combine appropriately adjusted white homogenates (see above) 1:1 v:v with 2X SDS buffer.

2. Put the samples in boiling water for 5 minutes to denature the proteins.

3. After boiling, cold-snap the samples on ice immediately.

4. When working with a new set of samples (or for a rookie), it is good to check out your new samples to be sure that they are properly normalized before running dozens of gels and finding, for example, that one particular sample never works. So load equal protein amounts from each onto a gel, run the SDS-gel and Coomassie stain it. Staining of gels is outlined in section XIV of this Technical Bulletin. All your bands should have the same intensity since you have loaded the same amount of protein in every lane. This step is optional but strongly suggested for the rookie.

   **As a routine procedure, you will also be staining your blots with

Coomassie after you finish your immunoblotting and using the constant Coomassie stained bands to normalize your immunoblotting data.

5. Take a Syngene picture of your gel.

6. Now that you have quantified your protein concentration via the BioRad protein assay and confirmed that you get equal loading of your lanes using Coomassie blue staining of the gel, you are ready to do your Western blotting.

ECL DETECTION USING SYNGENE

Note: for citation of this instrument in papers or thesis, it is correctly called the ChemiGenius bio-imaging system (Syngene, Frederick, MD).

1. Open up the Syngene and make sure the UV filter is not in front of the camera.

2. Make sure you select Chemiluminescence (ChemiSample 2) from the options and that you turn the light off (No light).

3. Pipet 1 mL of solution from the white ECL bottle and 1 mL from the brown ECL bottle into the Western box, over the membrane, and mix.

4. Place the membrane on a plastic tray inside the Syngene using tweezers.

5. Adjust the camera so that the image of the membrane fills the entire screen and is focused.

6. Pour the rest of the ECL solution (2 mL) from the Western box on top of the membrane and close the Syngene door. This prevents the membrane from drying out.

7. Select Series Exposure and set the different times of exposure you want.

8. To visualize your blot, you will have to select the Histogram option and modify the red lines to adjust the contrast.

9. Once your blot looks like a perfect Western blot, save it as a syngene file (.sgd file) in your directory. You will be able to open this file in Gene Tools for quantification.

QUANTIFICATION AND DATA ANALYSIS

To quantify your blots, you will use the program GeneTools that is installed on the Syngene computer and on the computer right next to Ken's desk in Rm 508. For details on running the Syngene programs see Technical Bulletins EQ10a and EQ10b.

You will need to normalize your immunoblot bands against the protein band(s) that does not change in concentration between samples. To do this, you will first gather your immunoblot band densities as described below and then you will stain the

membrane with Coomassie blue and gather band intensity data for the Coomassie stained bands. See **section XIV** below for how to Coomassie stain your membrane after immunoblotting.

A) Quantification

You must have a minimum of n=4 independent determinations for each experimental group. It is preferable that you gather n=5-6 so that you then have the option of discarding selected samples that do not work.

For situations with two groups (control vs. stress)

Please note that the following method is good for situations where you have one control situation (e.g. euthermic) and one stress situation (e.g. hibernating). There is another method (outlined next) to use when you are working with systems where there are multiple experimental situations, e.g. control, stress, recovery. This is as follows:

1. Start with your Western blot, i.e. the one with the antibody bands on it.

2. Using the GeneTools program, draw rectangles around all your bands (control and stressed).

3. Make sure to select the automatic background correction located in the program options.

4. Set your first control band as 1.00 and the values for the densities of the other bands will change automatically.

5. Here is an example of a data set from a Western blot:
   Control lanes: 1.00, 0.83, 0.96
   Stressed lanes: 1.65, 1.54, 1.73

6. Next, open up the picture of your Coomassie stained blot in Gene Tools

7. Draw a larger box around multiple bands of similar intensities in each lane (some people prefer drawing three or more boxes per lane and this is also acceptable). Again, set the boxed bands in the first lane to 1.00 and you will get the relative values for the same boxed bands in other lanes. Here is an example of a Coomassie stain blot of the same membrane:
   Control: 1.00, 0.94, 0.92
   Stressed: 0.89, 0.97, 0.93

8. Now, divide the values from your Western blot data by the values for the Coomassie stained blot (ie. Western blot/Coomassie stain blot) and you will get the following normalized values:
   Control: 1.00, 0.88, 1.04

Stressed: 1.85, 1.59, 1.86

9. Import these data (stressed vs control) into Excel (or Sigmaplot) where you can calculate the mean and SEM (or SD) for the two data sets and plot the data as histograms – one bar for control and one for stressed.

10. In some cases, you may prefer to plot only the mean fold up-regulation or down-regulation which comes from dividing the mean stressed value by the mean control. In this example, the gene would be up-regulated by a mean 1.82 fold.

11. ** This process of getting normalized band intensities and the different options for statistical testing and graphing are explained fully in **Tech Bull C01**. **READ IT ** Ask for help with understanding the testing and/or how to alter your procedures to improve data and demonstrate statistical differences correctly**

For situations with more than two groups (control vs. stress vs. recovery)

Please note that the following method is good for situations where you have more than two groups to compare. This is as follows:

1. Get your intensities for all conditions (e.g. control, stressed, recovered) as outlined above.

2. Divide these values by the intensities of the corresponding Coomassie stained band(s) as previously described to get normalized band intensities. You will then have one set of values for your control, another for your stress, and another for your recovered samples.

3. Calculate the mean and SEM of the values in each of the groups.

4. Plot the values as histograms or as line graphs in Excel or Sigmaplot.

5. A t-test to compare any 2 groups of data can be used for getting preliminary estimates of significance between groups and to help you decide if you need to increase your sample size or toss out the odd outrider point in order get data that "looks significant" to actually be significant.

6. However, for publication, if you have 3 or more groups of data that you are comparing, you need to also analyze your data using an Analysis of Variance (commonly called ANOVA) which determines if there are significant differences among groups. This is followed by one of the post-hoc tests (e.g. SNK, Tukey's, Dunnett's) to actually show which groups are different from which. In our lab this testing with 3 or more groups is done by using Steve Brooks's computer program called Mynova.

B) Significance testing – Excel and Mynova

The next step is to see if your data is significantly different; you need to perform a t-test. You can use two different programs to do your t-test: Excel or Mynova, in both cases, you will get similar values.

Excel

Excel is a good program since your data is usually in an Excel spreadsheet.

1. Open up Excel, put your two sets of data, control and stressed, in the spreadsheet. To do statistical testing you MUST have a minimum of n=3 for control and n=3 for experimental values; it is better to have n=4 or n=5 or even higher values. The greater the n values, the greater the probability of detecting significant differences between the two groups.

2. Click on the "=" sign and click on the "more functions" option.

3. Choose the "statistical" option and select "t-test".

4. The program will ask you to plug in your values for control (array 1) and for stressed (array 2) samples. You can also select the columns of data by clicking and selecting them.

5. Then select the option "2-tailed distribution" and click "enter"

6. Then select the option "2 sample unequal variance" which is entered as option 3. Do NOT choose "paired" – paired means that the control number in column 1 row 1 is matched with and compared to the experimental number in column 2 row 1, as is column 1 row 2 with column 2 row 2, etc. Since all of our samples are independent samples from a larger pool, there is NO special relationship between any control value and the experimental value that happens to be typed in the adjacent column so DO NOT pair your data.

7. You will now get your P value. To be acceptable as significantly different, control and experimental values should be different with 95% or greater confidence. Hence, your P value should be 0.05 or smaller (e.g. 0.01 = 99% confidence, 0.005 = 99.5% confidence).

8. If you believe that your experimental data should be significantly different from the control but is not different at $P<0.05$, then try these things:
    a) Recheck your calculations of mean $\pm$ SEM and your data entry for the statistical testing,
    b) Re-examine you data for obvious "outriders" that are greatly affecting your error bars (e.g. for these 5 values of 1.00, 1.15, 1.12, 1.17 and 1.99, your may be justified in dropping the final value as simply being wrong – remember, however, that you MUST have at least n=3),

c) Re-run additional gels to increase the n values for your samples (e.g. if you have 3 values of 1.0, 1.4 and 1.9, you get a mean of 1.43 $\pm$ 0.26 with a very high SEM, 18% of the mean, but add in another 3 values of 1.1, 1.25, 1.32 and drop your outrider of 1.9 and you get 1.21 $\pm$ 0.073, the SEM in this case being only 6% of the mean)

Mynova

The other option you have for your t-test is to use the program Mynova which is on the shared U and V drives.

1. Open up the program and select "add/edit data" -- use the ALT key and type "ALT a". (Note you can add you data as raw numbers or as means and SEM – your choice – if you add raw data, the program will calculate means, SD and SEM for you)

2. If entering means and SEM values, select "add/edit means" – use the down arrow key or type "m".

3. Plug in your values for n, mean, and SD or SEM (either SD or SEM, not both).

4. Then select "perform tests" (ALT t) and select "normal student t-test" using the down arrow key or type "n".

5. You will next see a screen of the calculated mean, n and SEM values.

6. Hit the space bar to move to the next screen where you are asked to type in the letter corresponding to the first group of data to be in the test and then the second group. As soon as you are finished, values for significance in both 1- or 2-tailed tests appear. Use the information for a 2-tailed test.

XV. STAINING/DESTAINING GEL/MEMBRANE

This section covers the common techniques (Coomassie and Silver) used on a day-to-day basis in the lab. Variations on these themes and more esoteric staining methods are covered by B. Thatcher in TB#9D.

All steps outlined below should be carried out with gentle shaking. Do not stain with silver stain if you are going to do autoradiography. Silver stain interferes with the fluors in Enhancing solutions so that gels stained with silver cannot be exposed to X-ray film. Coomassie can be used if you are using NEN products but not with Amersham products (see autoradiography bulletin). Staining is best carried out using cleaned glass casserole dishes. If a glass casserole dish is not available or there is no room for the dish on the rocker, you may use a smaller cleaned plastic tupperware box that can fit your gel or membrane. Cover the gel/membrane in the dish/box with saran wrap at all times. If you are staining the gel, NEVER, NEVER touch the gel with bare hands. If you are silver staining you cannot even use gloves. The easiest way to change solutions is to pour the

solution out through the corner of the dish using the saran wrap to keep the gel from falling out. Use about 250 ml of the following staining/fixing solutions per gel. Keep each gel separate in its own casserole dish.

A. Coomassie (Membrane):

After viewing the membrane in the SynGene, you must stain your membrane to further check if there are any discrepancies in the amount of sample loaded into each lane.

1. Place the membrane in a dish/box and pour more than enough staining solution to completely cover your membrane and stain 30 minutes. If the membrane is not dark after 30 minutes, allow the membrane to stain some more.

2. Pour off the staining solution. Remember that you can reuse it if you filter the solution.

3. Destain the membrane 30 minutes or until sharp blue bands appear and is easily visible on the membrane. To help sharpen the contrast, you may want to put a ball of tissue paper (ie. Kleenex) at the corner of the dish/box. Make sure you have enough destaining solution covering your membrane. You may initially have to change it once or twice after the first 5 to 10 minutes of destaining if the colourless solution becomes too dark.

4. After destaining has completed, remove the wet membrane and place it on a filter paper to air dry.

5. Take a picture of the membrane using the SynGene and quantify the band intensities in each lane using GeneTools. The instructions can be found in a TechBull in the Shared V drive. For normalization purposes you need to pick a good sharp band that appears to be of equal density in all lanes; sometimes people quantify a group of closely spaced bands instead.

i) <u>Gel Destaining Protocol:</u>

1. Destain with the destaining solution until no bands are visible; the gel will have a yellow hue.

2. Wash the gel with milli-Q water 4 – 5 times for 15 min until gel is transparent and has no background color.

3. You can stain the gel again starting with sodium thiosulfate solution (step 4 of the Staining procedure).

APPENDIX B
Technical Bulletin GT18
Isolation of Nuclear Extracts from Frozen Tissue

(By Pier Morin, modified by Mamady Hapsatou 2006)

- Prepare buffers as listed at the end of this technical bulletin.

- Be sure to pre-chill all the buffers and the Douce homogenizer before use.

- Break up frozen tissue with a mortar and pestle in liquid nitrogen.

- Homogenize your tissue using a Douce homogenizer (1 mL of Homogenization buffer per 0.5 gram of tissue, ie. a 0.5:1 w:v homogenate). **Note: you will probably need at least 1 g of tissue in order to isolate sufficient nuclear material for your work so design your animal experiments accordingly and realize that this technique will not be appropriate for many of the tiny tissues of some of our smaller animals. **Note: never use a homogenizer with blades for subcellular fractionation.

- Homogenize your samples with 20 piston strokes.

- Centrifuge the samples at about 10,000 rpm for 10 min at 4°C.

- Following centrifugation, pipet the supernatants into clean tubes; these are the cytoplasmic extracts.

- Resuspend the pellet in extraction buffer using a spatula (use about 150 uL of Extraction buffer per gram of starting material). **Note: Extraction buffer has high salt in it to lyse the nuclei isolated in the first step.

- Put the tubes horizontally on a bucket of ice sitting on top of a rocking platform for 1 h with gentle rocking (you should flick the tubes every 10 min).

- Centrifuge the samples at 10,000 x g for 10 min at 4°C.

- Remove the supernatants and put in sterile Eppendorf tubes; these are the nuclear extracts.

- Quantify the amount of protein in both fractions using the BioRad assay (Dilute the samples 60-80 fold before quantifying the same way as in Techbull EB07-Western Blotting, Section III).

- To validate the efficiency of your nuclear extract isolation, you should Western blot (EB07) your nucleic and cytosolic fractions with a protein that you know is only in the nuclei (ie. Histone H3 using anti-Histone H3 antibody) or cytoplasm (ie. creatine kinase using anti- creatine kinase antibody). Other cytoplasmic antibodies that could be used would include glucose-6-phosphate dehydrogenase or lactate dehydrogenase (**but see Ken). Once this is done, you can probe for your own protein.

*Note: You can combine this protocol with Western blots and assess the distribution of your enzyme or protein in cytosol vs nucleus to determine whether distribution changes in control vs stressed conditions.

*Note: Your subsequent analysis of nuclear vs cytoplasmic fractions - e.g. enzyme activities or protein amounts on gels - need to be expressed/standardized against the total protein contents in each fraction.

Buffers and reagents:

10X Buffer A
100 mM HEPES, pH 7.9; 100 mM KCl; 100 mM EDTA

5X Buffer B
100 mM HEPES, pH 7.9; 2 M NaCl; 5 mM EDTA; 50% v/v glycerol

Homogenization Buffer
Prepare 1X Buffer A (1 mL) and add to it 10 uL of 100 mM DTT stock and 10 uL of the lab's standard Protease Inhibitor Cocktail (cat # P8340)

Extraction Buffer
Prepare 1X Buffer B (147 uL) and add to it 1.5 uL of 100 mM DTT and 1.5 uL of Protease Inhibitor Cocktail.

If you don't have the prepared Protease Inhibitor Cocktail (Sigma), you can make your own by directly adding the following reagents at the indicated final concentrations in both the homogenizing and extraction buffers immediately as you are using them (ie. do not put them in the buffers ahead of time – add them right as you are homogenizing or extracting). PMSF cannot be made up in a water solution.

- PMSF 1 mM
- Aprotinin 0.15 units/ml
- Leupeptin 5 ug/ml
- Pepstatin 1 ug/ml