INVOLVEMENT OF FOXO TRANSCRIPTION FACTORS AND GLYCOGEN SYNTHASE KINASE 3 IN THE FREEZE TOLERANCE CAPABILITY OF THE WOOD FROG, *RANA SYLVATICA*

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B.Sc. Carleton University, 2005

A Thesis Submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Masters of Science
Department of Chemistry

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Ottawa, Ontario, Canada

Your file Votre référence
ISBN: 978-0-494-33691-5
Our file Notre référence
ISBN: 978-0-494-33691-5

Abstract

Animals cope with the subzero temperatures of winter in different ways. The wood frog, *Rana sylvatica*, endures whole body freezing and is able to survive weeks completely frozen. Organisms that endure extreme environmental stress on a periodic or seasonal basis have developed ways to strongly suppress their metabolic rate and enter a hypometabolic state to survive. Forkhead box 'other' (FOXO) transcription factors have important roles in various cellular processes such as metabolism, cellular proliferation, stress tolerance and lifespan. Immunoblotting was used to assess total and phosphorylated amounts of FOXO proteins in wood frog organs. Active FOXO1 increased in brain during freezing and thawing, possibly due to a need for gluconeogenesis during this stress. The levels of active FOXO3 increased in frog brain, kidney and liver during freezing and thawing and also during anoxia and aerobic recovery after anoxia, which could be due to the need to maintain or enhance antioxidant defenses under these stresses. Glycogen synthase kinase-3 (GSK3) is a protein kinase known to inhibit glycogen synthesis, cell growth and differentiation and protein translation. The amount of active GSK3 increased in the frozen state in brain, heart, kidney, liver and muscle of wood frogs. Furthermore, kinetic analysis of GSK3 showed that the skeletal muscle of frozen frogs appears to have a higher affinity for its substrate when compared to control GSK3. Allosteric effectors of GSK3 were also identified: glucose-6-phosphate activated the enzyme whereas AMP inhibited. The data expand our understanding of metabolic regulation during natural freeze tolerance.

Acknowledgements

First and foremost, I would like to thank my thesis supervisor, Dr. Kenneth Storey, for giving me the opportunity to do this research and also for his help and encouragement along the way. I would also sincerely like to thank Janet Storey for editing help with my thesis and also for sharing her vast knowledge of this research. I would also like to thank the Storey lab members for teaching me and helping me along the way.

I would also like to thank my parents, Kenneth and Lise-Anne Boal, and my brothers and sisters (Emilie, Justin, Mireille, Emma, Luke and Steven) for all their encouragement throughout all of my post-secondary studies.

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List of Abbreviations

Akt	Protein kinase B
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	Cyclic 3',5'-adenosine monophosphate
CK1	Casein Kinase 1
DNA	Deoxyribonucleic acid
DYRK1A	Dual-specificity regulated kinase 1A
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol bis(β-aminoethyl ether) tetra-acetic acid
eIF-2B	Eukaryotic initiation factor 2B
FOXO	Forkhead box 'other'
GADD	Growth arrest and DNA damage
GP	Glycogen phosphatase
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3
IGFBP-1	Insulin-like growth factor binding protein 1
IRS	Insulin receptor substrate
kDa	Kilodalton
K_m	Michaelis-Menten constant
MAPK	Mitogen-activated protein kinase
MnSOD	Manganese superoxide dismutase
mRNA	messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PEP	Phosphoenolpyruvate
PI3K	Phosphoinositide-3 kinase
PDK1	Phosphoinositide-dependent protein kinase
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethysulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SGK	Serum and glucocorticoid-regulated kinase
TBST	Tris-buffered saline Tween-20
Tris	Tris(hydroxymethyl)aminomethane
V_{max}	Maximum reaction velocity

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CHAPTER 1:

General Introduction

Adaptations to Cold

During the winter months, when temperatures fall to below 0°C, ectothermic animals cope with the cold in different ways. Some animals are able to keep their body temperatures above 0°C, and they do this by migrating to a warmer climate or wintering in sheltered environments either under water or underground below the frost line. Others allow their body temperature to drop below 0°C and deal with the potential for freezing using one of two strategies: freeze avoidance or freeze tolerance. Freeze avoidance involves the deep supercooling of body fluids and is achieved with the use of multiple kinds of antifreezes. Freeze tolerance is the controlled freezing of body water in extracellular fluid spaces while preserving the liquid state of the cytoplasm (Storey and Storey, 2004).

The Wood Frog

The wood frog, *Rana sylvatica*, is a freeze-tolerant vertebrate commonly found in North America (Figure 1.1) (Behler and King, 1979). This species is the primary model animal that has been used for studies of vertebrate freeze tolerance and is able to survive for weeks completely frozen. They spend the winter months near the soil surface, covered with layers of leaf litter and snow. Although these layers do provide insulation and protection from the low ambient air temperatures above the snowpack, the temperature of the soil surface during the winter months can fall as low as -6°C.

Vertebrate Freeze Tolerance

Many factors contribute to the injury and mortality that is associated with tissue

freezing in most organism (including man), and freeze tolerant animals have to find ways to overcome all of these factors to survive freezing. One of these factors is the formation of ice in restricted extracellular spaces. For example, ice expansion in small capillaries of organs can rupture vessel walls so that when the ice thaws, the integrity of the vascular system is lost causing extensive internal bleeding. Indeed, this has been one of the critical limitations in developing methods for human organ cryopreservation (Rubinsky *et al.*, 1987). Furthermore, the withdrawal of pure water into extracellular ice crystals increases the osmotic concentration of the remaining extracellular fluid and sets up a powerful osmotic gradient which sucks water out of cells, causing extensive dehydration and cell volume shrinkage. The wood frog deals with these problems in multiple ways so that ultimately 65-70% of total body water can freeze out as extracellular ice. One mechanism is to promote ice formation in extra-organ spaces such as the abdominal cavity where large masses of ice can accumulate but do little physical harm to organs. Plasma clotting factors, such as fibrinogen, are also up-regulated when frogs freeze so that when the animals subsequently thaw, any bleeding injuries can be dealt with quickly (Storey and Storey, 2004a). When freezing begins, frogs also synthesize and distribute huge amounts of glucose, a low molecular-weight cryoprotectant, to all tissues. Glucose can reach concentrations of 150-300 mM (compared with 1-5 mM normally) (Storey and Storey, 1984). Cryoprotectants are used to prevent cells from shrinking below a critical minimum cell volume since extreme shrinkage causes irreversible damage to cell membranes.. Freeze tolerant animals also manage the freezing process by initiating freezing close to the equilibrium freezing point (about -0.5°C for wood frogs) so that the rate of ice formation is slow and there is plenty of time to initiate metabolic adaptations and cell

volume adjustments. In freeze tolerant species, freezing is usually triggered in one of two ways: (a) by contact with environmental ice which seeds freezing across the skin or (b) by the action of endogenous nucleators, which can be specific ice-nucleating proteins that are synthesized and added to the blood of the organism, or bacteria (in the skin or gut) with ice-nucleating capabilities (Duman, 2001; Storey and Storey, 2004b). Wood frogs in a damp environment are typically seeded when their body temperature falls below the freezing point of their blood (about -0.5°C) whereas in a dry environment, bacteria trigger nucleation at -2 to -3°C. Wood frogs have blood proteins with ice nucleating abilities but it seems that these may act more guiding ice formation that in actually triggering it (Storey and Storey, 2004).

When wood frogs are nucleated at -2°C, the crystallization, an exothermic reaction, causes an increase in body temperature to just below the freezing point. The body temperature then holds at this value for several hours while ice slowly forms (generally the rates is <5% of total body water per hour) and then the body temperature gradually drops back down to the ambient temperature (Layne and Lee, 1987). Because the rate of freezing is so slow, frogs may only show a slight stiffness in some parts of their skin and limbs for the first hour or more and the maximal ice content may not be reached for 12-24 hours. Freezing in wood frogs begins at some peripheral point on the skin and ice propagates inwards asymmetrically through the body (Rubinsky *et al.*, 1994). Freezing stops blood circulation and so tissues are deprived of oxygen and blood-borne nutrients for the duration of the freeze and are also deprived of the way to dispose of accumulating waste products. This condition is called ischemia. Freeze-tolerant animals also show well-developed ischemia/anoxia resistance, which includes pathways

of fermentative ATP generation, regulated metabolic rate depression and antioxidant defenses (to provide protection against damage caused by the reperfusion with oxygen during thawing). Freezing also terminates all vital signs, including heart beat, breathing, muscle movement, and nerve transmission (Storey and Storey, 2004). All are gradually reactivated during/after thawing (heart beat being the first to be reactivated) but the molecular mechanisms underlying the reactivations are still unknown.

Metabolic Rate Depression

Organisms that have to endure extreme environmental stress on a periodic or seasonal basis have developed ways to strongly suppress their metabolic rate and enter a hypometabolic state (e.g. dormancy, torpor) in order to survive. When organisms encounter environmental extremes that threaten normal life, limit food availability or impose severe challenges to their physiology, they need to use this conservation strategy which allows them to remain alive until conditions are more favourable for active life (Storey and Storey, 2005).

Metabolic rate depression has three main principles: (a) both intrinsic and extrinsic mechanisms are involved, (b) the rates of energy-producing and energy-consuming cellular processes are suppressed in a coordinated manner so that a new lower net rate of ATP turnover can be sustained over the long term, and (c) cellular priorities are reorganized to give preference to key functions (e.g. maintenance of membrane potential differences) and more strongly suppress or halt functions that are less essential under energy-restricted conditions (e.g. protein synthesis) (Storey and Storey, 1990; Storey and Storey, 2004b). Extrinsic influences affect metabolic rate depression in

several ways, including a suppression of physiological functions (heart rate, breathing, digestion, muscle movement) in the hypometabolic state and a reduced cytosolic pH which usually accompanies entry into hypometabolism (often caused by CO_2 retention to acidify blood). Intrinsic mechanisms of metabolic rate depression account for at least half of the total metabolic rate depression. They involve biochemical adjustments that coordinate the suppression of many cellular processes and pathways and reorganize the priorities for ATP use.

Reversible Phosphorylation

The most important molecular mechanism of metabolic rate depression that has been identified to date is reversible protein phosphorylation involving the covalent addition or removal of phosphate groups to enzymes and functional proteins. This is carried out by ATP-dependent protein kinases (that add phosphate groups) or protein phosphatases (that cleave phosphate groups) (MacDonald and Storey, 2002). There are many advantages of reversible protein phosphorylation as a regulatory mechanism. Reversible protein phosphorylation can change the activity state of enzymes and functional proteins, sometimes resulting in fully on-off control, can trigger the association or dissociation of regulatory subunits or proteins, and can alter the microcompartmentation of proteins within cells (Storey, 1993). Thousands of proteins are susceptible to reversible protein phosphorylation, which makes this mechanism an excellent way of coordinating the responses by many cell functions.

Another benefit of reversible protein phosphorylation is that signal transduction cascades involving protein kinases and protein phosphatases are fast and allow rapid

inhibition of multiple ATP-utilizing functions and an equally fast reversal to re-establish normal cell functions during arousal from the hypometabolic state. Major changes in the activity states of enzymes and pathways are achieved without the need to change the overall amounts of proteins by synthesis or degradation, which is another factor that benefits fast recovery from the hypometabolic state (Storey and Storey, 2005).

Anoxia/Ischemia

When blood plasma freezes, the delivery of oxygen and nutrients to organs, the removal of wastes, and the inter-organ communication via hormones and other signals are all interrupted. Every cell is left in isolation to survive throughout the freeze using only its own internal reserves. The subzero body temperature of a frozen organism means that the metabolic rate will be very low, but because freezing may be prolonged for many weeks, each cell has to have enough fermentative fuel reserves and the ability to support basal metabolic needs over the long term using only the ATP generated from anaerobic pathways. Freeze-tolerant wood frogs show a good capacity to endure long term oxygen deprivation. Wood frogs can survive several weeks on continuous freezing (Layne et al., 1998) and easily endure 2 days of exposure to a nitrogen gas atmosphere at 5°C (Holden and Storey, 1997). Over the course of a freezing event, wood frog organs show the expected vertebrate response to oxygen limitation which is a depletion of ATP and an accumulation of glycolytic end products (lactate and alanine) (Storey, 1987). These parameters are normalized 3-11 days post-thaw.

All organisms have to deal with a constant assault to cellular metabolism by reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals and

peroxynitrite. These highly reactive species cause serious damage to cellular lipids, proteins and DNA. Because of this all organisms have antioxidant defenses consisting both of enzymes and of metabolites (e.g. glutathione, ascorbate, thioredoxin) that prevent, minimize or repair damage by reactive oxygen species. Oxidative damage is an inescapable part of an aerobic lifestyle but is also enhanced under many environmental stress conditions and in various disorders including three that are of direct relevance to vertebrate freeze tolerance – ischaemic heart disease, stroke and diabetes (Ahmad, 1995). It is now known that metabolic damage caused by ischemia arises both from oxygen deprivation during the period of restricted/halted blood flow and from the rapid reintroduction of oxygen during the reperfusion phase. Reoxygenation results in a burst of oxyradical production that can temporarily overwhelm the cell's antioxidant defenses and cause extensive damage to cellular macromolecules (Benson and Bremner, 2004).

Natural freeze-thaw by freeze tolerant organisms is an ischaemia-reperfusion event that has the potential to cause oxidative damage to organs when tissue oxygenation is restored during thawing. In the case of freeze-tolerant frogs that use glucose as a cryoprotectant, reactive oxygen species can also be enhanced by high glucose and glucose-mediated oxidative damage is another type of oxidative stress that must be dealt with (Woods and Storey, 2006). Reactive oxygen species are well known to play a role in tissue damage in human diabetes (Kristal and Yu, 1992) and several modes of glucose-related oxidative damage are known. For example, free glucose is prone to autooxidation in the presence of transition metals (iron, copper) to form protein-reactive dicarbonyl compounds and hydrogen peroxide (leading to hydroxyl radical formation) (Wolff et al., 1991).

Studies with wood frogs indicate that at least two different strategies exist which contribute to the defense against oxidative stress associated with freeze-thaw. The first is the maintenance of constantly high activities of antioxidant enzymes in frog organs. Activities of six antioxidant enzymes (superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase, and total and Se-dependent glutathione peroxidases) were assessed in wood frogs versus leopard frogs and they were found to be uniformly higher in the liver of freeze tolerant wood frogs as compared with freeze intolerant leopard frogs (Joanisse and Storey, 1996). The high antioxidant defenses of the wood frog correlates with the absence of oxidative damage to cellular lipids over a course of freeze-thaw (Joanisse and Storey, 1996). The second strategy for antioxidant defense in wood frogs is selective changes to the activities of the antioxidant enzymes in different tissues in response to freezing. For example, total glutathione peroxidase activity increased significantly (by 20-150%) in all five organs tested (brain, heart, kidney, liver, muscle) and selenium-dependent glutathione peroxidase activity rose by ~2-fold in heart, kidney and skeletal muscle (Joanisse and Storey, 1996). In most cases, activities returned to near control values after 24 h thawing, which suggests that the need for enhanced defenses against peroxidative damage is greatest during freezing or immediately after thawing, a time when glucose is also very high. Selective changes to the activities of these enzymes could minimize the potential for glucose-mediated oxidative damage to macromolecules, a significant problem associated with sustained high glucose levels in human diabetes (Wolff et al., 1991).

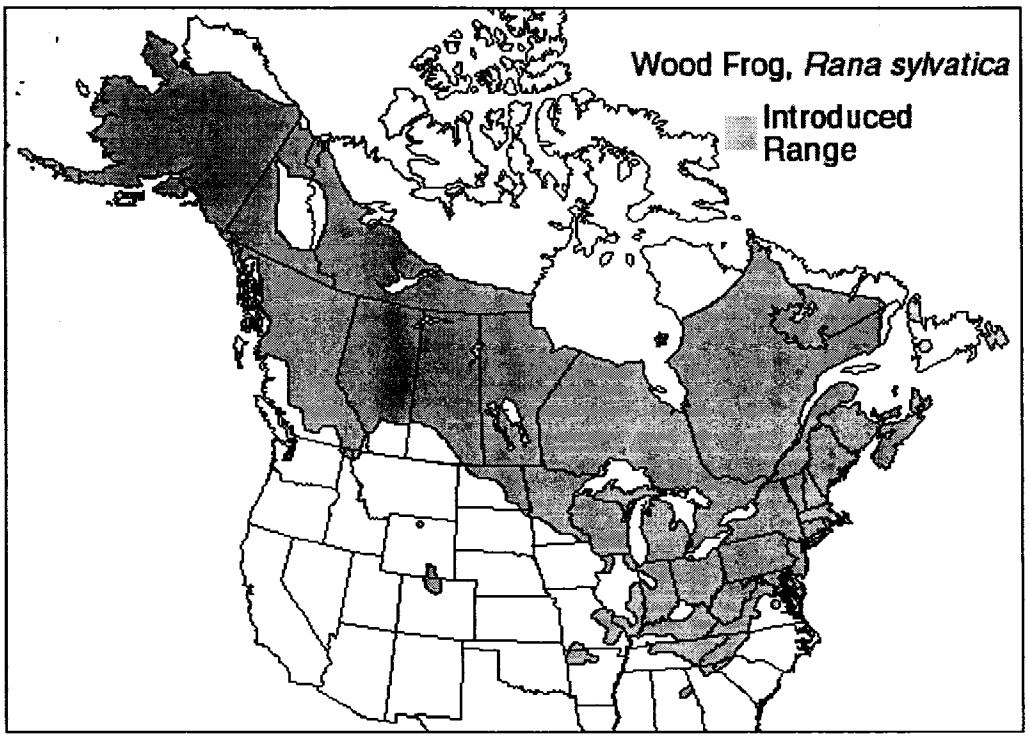
Hypothesis and Experimental Outline

Hypothesis: Freezing survival by wood frogs involves the up-regulation and activation of proteins involved in antioxidant defense and metabolic rate depression.

Experimental Outline

To test this hypothesis, two types of studies were undertaken. In Chapter 2 Western blotting was used to analyze organ-specific changes in the levels of Forkhead box other (FOXO) proteins during freeze/thaw and anoxia/recovery in wood frogs. FOXO proteins are important transcription factors that regulate the expression of multiple genes involved in cell proliferation, protection from oxidative stress and regulation of metabolism. By understanding how this family of transcription factors responds to freezing and anoxia stresses we gain valuable insights into the regulation and responses of genes under their control. In Chapter 3, enzymatic studies of an important cellular protein kinase, glycogen synthase kinase 3 (GSK3), are presented. GSK3 was first discovered as an important regulator of carbohydrate metabolism in animal cells helping to mediate glycogen storage versus glucose release. It is now known that substrates phosphorylated by GSK3 have roles in a wide spectrum of cellular processes that are influenced by fuel availability, including glycogen metabolism, gene transcription, protein translation, cytoskeletal regulation, intracellular vesicular transport, cell cycle progression and apoptosis. Studies of wood frog muscle GSK3 from control versus frozen animals were conducted using a radioactive assay and reveal important information about the activity, regulation, and actions of this kinase in an animal with a highly unusually carbohydrate (glucose) metabolism.

Figure 1.1 : Range distribution of the wood frog. From: Northern Prairie Wildlife Research Center (http://www.npwrc.usgs.gov/resource/herps/amphibid/species/wood1.htm)



CHAPTER 2:

Involvement of FOXO Transcription Factors during Freezing and Anoxia

Introduction

Forkhead Box Transcription Factors

The Forkhead family of transcription factors comprises more than 100 different members that have been shown to play important roles in cell proliferation, differentiation, tissue-specific gene expression and embryogenesis (Carlsson and Mahlapuu, 2002). Forkhead transcription factors are characterized by the presence of a highly conserved, monomeric DNA-binding domain, which is also known as the Forkhead box (Weigel and Jackle, 1990). The Forkhead box is made up of 110 amino acids that form a butterfly-shaped structure made up of three tightly packed N-terminal α-helices, three β-sheets, and two loop regions located at the C-terminal end that shape the wings of the structure (Clark *et al.*, 1993). The Forkhead box is sometimes referred to as the winged-helix motif as a result of its structural characteristics.

DNA binding of Forkhead proteins relies on interactions between the third helix of the Forkhead box (called H3) and DNA bases within the major groove of double-stranded DNA. The residues present in the two loops make additional contacts with the DNA-binding element which contribute to binding-site selectivity of the different Forkhead proteins (Clark *et al.*, 1993). A core recognition motif of 7 base pairs has been identified (T-(G/A)-T-T-(G/T)-(G/A)-(C/T)) which is necessary for Forkhead binding, whereas bases immediately flanking the core contribute to binding specificity of the different family members (Pierrou *et al.*, 1994).

Forkhead Box "Other" (Foxo) transcription Factors

Forkhead proteins have been assigned to 17 subfamilies ranging from FOXA to

FOXQ (Kaestner *et al.*, 2000). Of the 17 subfamilies, FOXO (also known as Forkhead Box "Other" protein) factors are the only ones known to date to be regulated by the PKB/Akt signal transduction pathway (Figure 2.1). They contain a unique insert of five amino acids within the region of the DNA binding domain (α-helix 3) that is directly involved in the sequence-specific interaction with DNA binding sites (Barthel *et al.*, 2005). The consensus binding sequence for FOXO proteins (TTGTTTAC) diverges from that of other Forkhead proteins, which provides a mechanism by which FOXO proteins can preferentially interact with a distinct set of target sites in the genome. Three FOXO proteins (FOXO1, FOXO3, FOXO4) are known to be phosphorylated at three highly conserved predicted PKB phosphorylation sites (corresponding to Thr24, Ser 256 and Ser 319 in human FOXO1) not found in other Forkhead transcription factors. Phosphorylation of these sites by PKB and/or related kinases results in inactivation and nuclear exclusion of FOXO proteins through multiple mechanisms (Biggs *et al.*, 1999). Inhibition of the PKB/Akt pathway causes FOXO to localize almost exclusively to the nucleus, and activation of PKB/Akt results in the retention of FOXO in the cytoplasm and a consequent inhibition of FOXO-mediated transcription (Brownawell *et al.*, 2001).

Even though FOXO proteins are mainly regulated by the Akt/PKB pathway, they are also "fine-tuned" and phosphorylated by other protein kinases such as Casein Kinase 1 (CK1) and the dual-specificity regulated kinase 1A (DYRK1A) (Figure 2.1 B). These kinases regulate the intracellular localization and function of FOXO proteins by phosphorylating FOXO factors within several different intramolecular domains (Van der Heide *et al.*, 2004).

FOXO sequences are highly similar, but there are significant variations between

members. Consequently, some genes display response elements that will bind multiple FOXO proteins, but other genes show selective sensitivity to one or another member (Czech, 2003). FOXO transcription factors are involved in regulation of cell proliferation, protection from oxidative stress and regulation of metabolism. Overexpression of FOXO factors in a large variety of mammalian cell types causes a strong inhibition of cell proliferation. FOXO factors can oppose the growth-stimulating effects of PKB/Akt when expressed at sufficiently high levels (Kops *et al.*, 2002a). FOXO members increase the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$, a protein that is linked to a cell-cycle arrest in G_0/G_1 (Nakamura *et al.*, 2000). FOXO proteins also induce cell-cycle arrest through repression of D-type cyclins which are required for cell-cycle progression in G1 (Schmidt *et al.*, 2002). FOXO transcription factors are also involved in protection from oxidative stress. The expression of manganese superoxide dismutase (MnSOD) and catalase were shown to be regulated by FOXO3 during oxidative stress (Kops *et al.*, 2002b; Nemoto and Finkel, 2002). FOXOs also control the expression of a protein called growth arrest and DNA damage (GADD)45, which is involved in DNA repair (Tran et al., 2002). FOXO proteins also stimulate the expression of pyruvate dehydrogenase kinase-4, which limits oxidative metabolism of glucose and conserves glucose for utilization in other tissues (Furuyama *et al.*, 2003). FOXO1 also binds to the insulin-like growth factor binding protein-1 (IGFBP-1) which binds to insulin growth factors and inhibits their activities (Durham *et al.*, 1999).

Since FOXO proteins are involved in cell-cycle arrest, DNA damage control and oxidative stress protection, I hypothesized that these transcription factors will be more active under freezing and anoxia stresses in the wood frog, *Rana sylvatica*.

Materials and Methods

Animal experiments

Male wood frogs, *Rana sylvatica*, were collected from breeding ponds in the Ottawa area during April 2003. Frogs were washed in a tetracycline bath and held at 5°C in a container with damp sphagnum moss for 1-2 weeks before use. Control animals were directly sampled from this condition. For freezing exposure, wood frogs were placed in closed plastic containers lined with damp paper towels and transferred to another incubator at –4°C. Under this condition, frogs cool below 0°C and start to freeze within ~45 min (Storey and Storey, 1985a). After this period of chilling, temperature was raised to -2.5°C and the length of freezing exposure was timed from this point. Frogs were sampled after 24 h of freezing at -2.5°C. Other frogs were frozen for 24 h at -2.5°C and then returned to 5°C and allowed to thaw with sampling of these recovered frogs after 8 h. All frogs were killed by pithing and each was dissected within 30-90 sec with the group cooperation of 3-6 persons. Tissues were flash frozen in liquid nitrogen and then transferred for storage to a -80°C freezer. The tissues sampled included brain, heart, liver, kidney and hind leg thigh skeletal muscle.

For anoxia exposure, frogs were treated essentially as described previously (Holden and Storey, 1997). After 1 week acclimation at 5°C, groups of 5-6 frogs were transferred into closed plastic jars sitting on crushed ice. The jars were lined with damp paper toweling (towels wetted with deoxygenated distilled water) and had been flushed with 100% nitrogen gas for 20 min through syringe ports in the caps. After the frogs were added the jars were flushed with N_2 for a further 20 min and then the ports were plugged

and the jar lids were sealed all around with parafilm. Jars were returned to the 5°C incubator and frogs were sampled after 24 h of anoxia exposure. During sampling, the jars were again held on ice and the N_2 gassing was reconnected. A third group of frogs was given 24 h anoxia exposure and were then transferred back to aerobic conditions at 5°C and sampled after 4 h recovery from anoxia.

Western blotting

Isolation of total protein from frog tissues

Total soluble protein was extracted from samples of frog tissues from control, 24 h frozen, 8 h thawed, 24 hour anoxic and 4 hour recovered (from anoxia) experimental groups. A sample of frozen tissue was weighed and then quickly homogenized 1:5 w:v in buffer containing 20 mM Hepes, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM ß-glycerophosphate, with fresh prepared protease inhibitors (0.1 mM phenylmethysulfonyl fluoride [PMSF], 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin) added just prior to homogenization with a Polytron PT10. Samples were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was removed and kept.

Measurement of protein concentration

Protein concentrations were determined by the Coomassie blue dye-binding method (Bradford, 1976) with the Bio-Rad prepared reagent and bovine serum albumin as the standard. The reagent was diluted 5-fold with distilled water and protein extracts of tissues were diluted 1:50 v:v. For assay, 10 µl of diluted protein samples were added 190 µl of dye reagent in microplate wells followed by mixing and a 10 min incubation at

21°C. Absorbance at 595 nm was read using a microplate spectrophotometer running BioLinx 2.0 software and protein concentration was determined from a standard curve.

SDS polyacrylamide gel electrophoresis

Protein samples were mixed 1:1 v:v with SDS-PAGE sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% w:v SDS, 20% v:v glycerol, 5% v:v 2-mercaptoethanol and 0.2% w:v bromophenol blue and boiled for 5 min. Aliquots containing 20-40 μg protein were loaded into sample wells. On any given gel, the same amount of protein was loaded into each well of 12% acrylamide gels (acrylamide:bis-acrylamide ratio 29.2:0.8; w/w). Electrophoresis was carried out on a Bio-Rad mini-gel apparatus, run at 180 V for 30-60 min at 21°C with 1x running buffer containing 3.03 g Tris base, 14.4g glycine, and 1 g SDS, pH 8.3. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membrane, Millipore corp. Bedford, MA) at 320 mA or 70 V for 1.5 h at 4°C with transfer buffer containing 25 mM Tris (pH 8.5), 192 mM glycine and 20% v/v methanol. PVDF membranes were then blocked with 1-2.5% nonfat milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 5-10 min at 21°C (see Table 2.1). Blots were rinsed with TBST and incubated with primary antibody in TBST on a shaking platform either overnight or for 2 days at 4°C. The dilution of stock primary antibodies used was 1:1000 and these were obtained from Cell Signaling Technology (Beverly, MA). Blots were then washed twice with TBST and incubated with anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA) diluted 1:2000 in TBST for 1.5 h at 21°C. Blots were washed for 3 x 10 min in TBST and then bands were visualized by adding 1.4 ml of enhanced chemiluminescence (ECL) reagent (Pierce)

with detection and quantification using the Chemi-Genius Bio-Imaging system and Gene Tools software (Syngene, MD, USA).

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

Table 2.1: Experimental conditions for Western blotting

Proteins	Amount of protein / lane (μg)	Percent milk used for blocking	Blocking time	Incubation time with primary antibody (at 4°C)
FOXO1	10	1.5 %	5 min	2 days
Phospho-FOXO1 (ser 256)	10	1.5 %	5 min	2 days
FOXO3	20	1 %	10 min	Overnight
Phospho-FOXO3 (ser 254)	20	2.5 %	10 min	Overnight
Phospho-FOXO3 (ser 318/321)	20	2.5 %	10 min	Overnight

Results

Several family members of the Forkhead Box Other transcription factors were detected in wood frog tissues: FOXO1, phospho-FOXO1 (Ser 253), FOXO3, phospho-FOXO3 (ser 254) and phospho-FOXO3 (ser 318/321). The tissues scanned included brain, heart, kidney, liver and skeletal muscle. FOXO1 was detected in frog brain, kidney and liver but not in cardiac or skeletal muscles. FOXO3 was detected in frog brain, heart, kidney and liver, but not in skeletal muscle.

FOXO1

Total FOXO1 protein levels was quantified using Western blotting in wood frog tissues under three experimental conditions: control (acclimated at 5°C), 24 h frozen (at -2.5°C) and 8 h thawed recovery (at 5°C) (Figure 2.2). The FOXO1 antibody crossreacted with a single band at the expected molecular weight of 80 kDa. A representative of a typical western blot is shown in Figure 2.2 C. FOXO1 protein was detected in the frog brain, kidney and liver. The levels of FOXO1 protein did not change during freezing and thawing in frog brain or liver. In the kidney, the levels of FOXO1 protein decreased significantly ($P<0.05$) to just 8.5% of the control value when frogs were frozen but had increased again to 38.7% of the control value when animals were thawed for 8 h at 5°C.

Phospho-FOXO1 (ser253)

FOXO1 can be phosphorylated on serine residue 253 and Western blotting was also used to determine the relative amounts of the phosphorylated form of FOXO1

(ser253) in control, frozen and thawed frogs (Figure 2.3). The phospho-FOXO1 antibody crossreacted with a single band at the expected molecular weight of 80 kDa. Phospho-FOXO1 (ser253) was again detected just in brain, kidney and liver. The levels of phospho-FOXO1 (ser253) in the liver did not change significantly during freezing and thawing. However, in brain, the levels of this phosphorylated form of FOXO1 decreased significantly ($P<0.05$) to 19.7% of the control value during freezing and then increased again to 42.6% of the control value ($P<0.05$) when thawed for 8 hours. Levels in the kidney showed a similar pattern, decreasing significantly ($P<0.05$) to 2.6% of the control value when frozen and increasing to 89.4% of the control value when thawed.

FOXO3 –Freeze/Thaw

The levels of FOXO3 were also examined during freezing and thawing in the wood frog (Figure 2.4). The antibody crossreacted with a single band on the blot at the expected molecular weight (97 kDa) of the protein. Five tissues were tested (brain, liver, heart, kidney, muscle) and the protein was detected in all but skeletal muscle. Total FOXO3 protein levels did not change significantly in the heart and kidney. However, levels increased significantly ($P<0.05$) in the brain and liver by 1.6-fold and 2.2-fold, respectively, when frogs were frozen for 24 h. Levels decreased again after 8 h thawing, returning to control levels in brain and to 1.7-fold above control levels in liver.

Phospho-FOXO3 (ser253) – Freeze/Thaw

FOXO3 can be phosphorylated on serine 253 and levels of FOXO3 (ser253) were determined in tissues from control, frozen and thawed frogs (Figure 2.5). The antibody

also crossreacted with a single band on the blot at the expected molecular weight (97 kDa) of the protein. Levels in heart and liver did not change significantly during stress/recovery but both brain and kidney showed reduced FOXO3 (ser253) content during freezing. In the brain, the levels of FOXO3 (ser253) dropped significantly ($P<0.05$) to 65.1% and 60.0% of control values for frozen and thawed tissues, respectively. In the kidney, levels were very strongly suppressed during freezing, decreasing to just 0.7% of the control value ($P<0.05$) and recovering substantially (53.3% of control values, $P<0.05$) after 8 h thawed.

Phospho-FOXO3 (ser 318/321) – Freeze/Thaw

Antibodies detecting phosphorylation of FOXO3 on serine 318/321 residues were used to quantify the effects of freeze/thaw on these alternate phosphorylation sites on FOXO3 (Figure 2.6). As with the other FOXO3 antibodies, phospho-FOXO3 (ser318/321) only crossreacted with one band at the expected molecular weight of 97 kDa. A significant ($P<0.05$) decrease in the content of phosphorylated FOXO3 protein (ser318/321) was observed in all the tissues during freezing. In the brain, phospho-FOXO3 (ser318/321) content decreased significantly to 68.9% of the control value ($P<0.05$) and to 64.7% of controls when thawed. In the kidney, levels of the phosphorylated transcription factor decreased significantly ($P<0.05$) during freezing to 39.6% of the control value but rose again to 64.9% of the control value when thawed. In the heart, phospho-FOXO3 (ser318/321) content decreased significantly ($P<0.05$) to 55.1% of the control value during freezing and rose to 88.9% of the control value when thawed. In the liver, the protein levels decreased significantly ($P<0.05$) during freezing to

36.4% of control values and remained suppressed (at 39.4% of control) when the animals had thawed.

FOXO3- Anoxia/Recovered

Anoxia/ischemia is one of the components of freezing since when the blood plasma freezes it cuts off oxygenation of the tissues. The levels of FOXO3 were examined in tissues of frogs that were given anoxia exposure for 24 h (at 5°C) followed by aerobic recovery for 4 h (also at 5°C) (Figure 2.7). FOXO3 was detected in brain, heart, kidney and liver these animals. The levels of FOXO3 did not change significantly during anoxia or recovery from anoxia in any of the tissues when compared to control values.

Phospho-FOXO3 (ser 318/321) –Anoxia/Recovered

The levels of phospho-FOXO3 (ser318/321) were also measured in the tissues of the frogs that were given 24 h anoxia exposure and 4 h aerobic recovery (Figure 2.8). The results were similar to those seen for the effects of freezing. Phospho-FOXO3 (ser318/321) levels did not change in heart but a significant decrease was seen in brain, kidney and liver. In the brain, the levels dropped significantly ($P<0.05$) to 18.3% of the control value during anoxia and remained low at16.5% of the control values during recovery. In the kidney, the levels were very strongly reduced during anoxia to 7.5% of the control value and again remained low (6.0% of control) during aerobic recovery ($P<0.05$). In liver tissues, the levels also dropped significantly ($P<0.05$) to 4.7% and 3.1% in anoxia exposed and aerobic recovery, respectively, compared to control values.

Discussion

A change in the expression of Forkhead box other (FOXO) proteins 1 and 3 was hypothesized during the freeze/thaw process of the wood frog *Rana sylvatica*. A change in the expression of FOXO3 was also hypothesized to be involved in the anoxia/recovery process of the wood frog. The data presented here evaluates these hypotheses.

FOXO1

FOXO transcription factors are under the control of insulin and/or insulin-like signaling factors. FOXO factors are phosphorylated by various protein kinases, one of the main ones being Protein Kinase B (PKB), a downstream mediator of phosphoinositide-3 kinase (PI3K) signaling. Phosphorylation of FOXOs results in their inactivation and nuclear exclusion (Biggs *et al.*, 1999). All FOXO proteins contain three highly conserved putative PKB recognition motifs (Alessi *et al.*, 1996a). They have also been shown to require the consensus N-terminal PKB site and the PKB site located in the forkhead domain to translocate from the nucleus to the cytosol (Brownawell *et al.*, 2001). Serum and glucocorticoid-regulated kinase (SGK), a structurally related family member of PKB, also phosphorylates FOXO factors. PKB and SGK are both activated by the PI3K / PDK1 cascade and are both able to phosphorylate identical substrate motifs, but with different preferences. SGK prefers the third C-terminal PKB motif (Brunet *et al.*, 2001). Casein kinase 1 (CK1) recognizes and phosphorylates FOXO motifs that have been primed (motifs that already contain a phosphorylated serine or threonine residue) (Flotow *et al.*, 1990). Dual-specificity tyrosine-phosphorylated and –regulated kinase 1A (DYRK1A)

also phosphorylates FOXO factors and is involved in the subcellular localization of FOXO proteins (Woods *et al.*, 2001).

FOXO transcription factors have important roles in various cellular processes such as metabolism, cellular proliferation, stress tolerance and lifespan (van der Horst and Burgering, 2007). FOXOs regulate the expression of genes coding for enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, which are essential regulators of gluconeogenesis (Nakae *et al.*, 2001). They can also suppress the expression of genes that are involved in glycolysis, the pentose shunt and lipogenesis, which implies that they induce a metabolic switch similar to low glucose and fasting conditions (Zhang *et al.*, 2006). In the absence of insulin signaling, FOXOs also induce cell-cycle inhibition, through regulation of cell-cycle arrest genes such as $p27^{kip1}$ and cyclin D (Kops *et al.*, 2002a).

In the current study, the levels of FOXO1 and a phosphorylated form of FOXO1 were assessed in control (acclimated at 5°C), 24 h frozen (at -2.5°C) and 8 h thawed (at 5°C) wood frogs in 5 tissues (brain, heart, kidney, liver, muscle). FOXO1 was not detected in heart or muscle, which was initially unexpected, since FOXO1 was found to be active in mammalian skeletal muscle during energy deprivation (Furuyama *et al.*, 2003). An explanation for this result could be that the levels of FOXO1 in frog muscle and heart are too low to be detected, or that the wood frog is not really in an energy deprived state when frozen. Although energy production slows to 5% of normal, energy utilization slows to 2% of normal which implies that energy deprivation is not a factor (Storey and Storey, 2004a). FOXO1 levels in frog brain did not change significantly. Since the antibody used to detect the amount of FOXO1 does not distinguish between the

unphosphorylated form and the phosphorylated forms of FOXO1, this result shows the total amount of FOXO1 (phosphorylated and unphosphorylated) does not change significantly when the wood frog freezes. In other words, FOXO1 is not degraded in the brain during freezing. However, the amount of the phosphorylated form of FOXO1 (ser256) decreased significantly when the wood frog freezes and the levels slowly increased again when the frogs thawed. When FOXOs are phosphorylated, they are inactivated (Biggs *et al.*, 1999). Hence, the lower amount of the phosphorylated form of FOXO1 in the brain implies that this transcription factor is more active in the brain during freezing. In the kidney, total FOXO1 protein decreased during freezing to low levels, and then increased again during thawing process. The same trend was observed in the amount of phospho-FOXO1 (ser256) detected in the kidney when frozen and thawed. These results indicate that FOXO1 is degraded in the kidney when frogs freeze. It also implies that FOXO1 is not more active in the kidney during freezing. The levels of FOXO1 and phospho-FOXO1 (ser256) in the frog liver during freezing and thawing did not change significantly. It appears as if FOXO1 is not important for freeze tolerance in the frog liver.

FOXO1 is involved in the upregulation of genes, such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), that are involved in gluconeogenesis, a process where glucose is generated from non-sugar substrates. PEPCK is the first committed step in gluconeogenesis, while G6Pase removes the phosphate group from glucose 6-phosphate and is the last step in gluconeogenesis (Hers and Hue, 1983). Glucose is the sole energy source for the brain. During freezing in wood frogs, glucose is used as a cryoprotectant and is synthesized by the liver and quickly

distributed to other organs, to levels that rise into the 200 mM range (from control values of ~5 mM) (Storey, 1987). Since the glucose available is needed as a cryoprotectant, another way to generate glucose needed for energy in the brain might be needed: gluconeogenesis. If gluconeogenesis is used to produce energy in the brain during freezing, an activation of FOXO1 in the brain is expected since FOXO1 upregulates genes involved in gluconeogenesis. The very low amounts of total FOXO1 protein detected during freezing in the kidney and the increasing amount of FOXO1 protein in the kidney when thawing implies that FOXO1 is not needed for freeze-tolerance in the frog kidney. The liver did not show any significant change of total FOXO1 and phospho-FOXO1 (ser256) which also implies the insignificance of this protein in the freeze-tolerance of the liver in the wood frog.

FOXO3 in Anoxia-tolerance

The levels of FOXO3 and a phosphorylated form of FOXO3 (ser 318/321) were assessed in control (acclimated at 5°C), 24 hour anoxic (at 5°C) and 4 hour recovered from anoxia (also at 5°C). One of the components of total body freezing is anoxia/ischemia caused by the freezing of blood plasma. This means that each cell in each organ is isolated and cut off from the delivery of oxygen (and nutrients) for the duration of the freeze. The involvement of FOXO3 in anoxia tolerance in wood frogs was evaluated because FOXO3 is well-known to be involved in antioxidant defense (Kops *et al.*, 2002b). In mammalian systems of anoxia/ischemia, a large portion of the metabolic damage is not actually caused by oxygen deprivation itself but by the rapid production of high amounts of reactive oxygen species when oxygen is suddenly reintroduced by

organs. Hence, good antioxidant defenses are needed to counter this reperfusion injury. The levels of FOXO3 and its phosphorylated form were determined in the brain, heart, kidney, liver and muscle. There was no expression in muscle, probably due to the inability to detect low enough levels of the transcription factor in muscle. FOXO3 was found in all other tissues, and total levels of FOXO3 did not change during anoxia and recovery in any of these tissues. Since the antibody used does not distinguish between unphosphorylated FOXO3 and phosphorylated FOXO3, this result implies that there is no change in the amount of total FOXO3 in the three states. A different result was observed when specific phosphorylation of the transcription factor at the serine 318/321 sites was examined. There was no significant change in the amount of phospho-FOXO3 (ser318/321) during anoxia and recovery in the heart but in brain, kidney and liver a strong and significant ($P<0.05$) reduction in the amount of phospho-FOXO3 (ser318/321) was seen during anoxia and recovery. Since the phosphorylated form of FOXO3 is the inactive form, this result means that there is less inactive FOXO3 in the brain, kidney and liver during anoxia and recovery. If the unphosphorylated FOXO3 and the phosphorylated FOXO3 data for anoxia and recovery are taken together, the lack of change in FOXO3 and the large reduction in phosphorylated FOXO3 (in brain, kidney and liver) implies that FOXO3 is more active during anoxia and recovery compared to control frogs. FOXO3 is involved in antioxidant defense by controlling the expression of the antioxidant enzymes such as MnSOD and catalase (Kops *et al.*, 2002b and Nemoto and Finkel, 2002). The data suggest that antioxidant defenses are present, and potentially enhanced, in the brain, kidney and liver during anoxia and recovery in the wood frog.

FOXO3 in freeze-tolerance

FOXO3 levels were also assessed in control (acclimated to 5°C), 24 h frozen (at -2.5°C) and 8 h thawed (at 5°C) frogs. The total content of FOXO3 in kidney and heart did not change significantly ($P<0.05$) during freezing and thawing, but rose substantially (1.5-2 fold) in the brain and liver. Two different phosphorylation states of FOXO3 were also evaluated, one at ser253 and the second at ser318/321. For phospho-FOXO (ser 253), the amount of this phosphorylated form did not change in the heart or liver, but did decreased significantly in the brain and kidney when the frogs were frozen and when they were thawing. These results imply that FOXO3 is more active in the brain and kidney during freezing and thawing. The response by phospho-FOXO3 (ser318/321) was slightly different: the phosphorylated content decreased significantly in all frozen tissues and remained low after 8 h thawed. The reduced levels of phospho-FOXO3 (ser318/321) were expected in the brain and kidney during freezing, but the decreased levels in the liver and heart were unexpected since the levels did not change when assessing the levels of phospho-FOXO3 (ser253). An explanation for this differential expression of phosphorylated FOXO3 forms is that the two phosphorylation sites of FOXO3 that were assessed are phosphorylated by (and under the control of) different protein kinase enzymes. The serine 253 position is phosphorylated by PKB, a main regulator of FOXO function (van der Heide *et al.*, 2004), whereas the serine 318/321 position is phosphorylated by CK1. It has been shown that CK1 phosphorylation of the serine 318/321 position is dependent on initial phosphorylation of the C-terminal PKB site (serine 253) (Woods *et al.*, 2002). FOXOs have also been described as being "fine-tuned" by CK1, which implies a differential regulation of FOXO3 in the liver compared to the

kidney and brain (van der Heide *et al.*, 2004). The reduced level of phosphorylated FOXO3 in the brain, kidney and liver, equaling a more active FOXO3 in these tissues, implies that this transcription factor is important in survival of the wood frog during freezing.

Conclusion

FOXO1 was found to be more active (lower amounts of phosphorylated FOXO1) in the brain of 24 h frozen and 8 hour thawed wood frogs, when compared to controls. FOXO3 was also more active in the brain, kidney and liver during 24 h anoxia exposure and when recovered from anoxia for 4 hours, and it was also more active in higher quantity in the brain, kidney and liver during freezing and thawing. The increase in active FOXO1 in the brain could be due to a need for gluconeogenesis in this tissue during freezing. FOXO1 has been shown to upregulate the gene expression of enzymes such as PEPCK and G6Pase which are essential regulators of gluconeogenesis (Barthel *et al.*, 2001). The increase in active FOXO3 in brain, heart, kidney and liver during freezing and thawing, and the increase in the brain, kidney and liver during anoxia and recovery from anoxia could be due to a need to maintain or enhance antioxidant defenses during freezing or anoxia. The reperfusion of blood creates a large amount of oxygen free radicals, and FOXO3 upregulates enzymes such as MnSOD and catalase which protect cells from damage caused by oxygen free radicals (Kops *et al.*, 2002b; Nemoto and Finkel, 2002). FOXO3 was also found to upregulate fatty acyl-CoA carriers sterol carrier protein-x (SCPx) and SCP2 which protect unsaturated fatty acids from oxidative damage (Dansen *et al.*, 2004). Another explanation for the activation of FOXO transcription

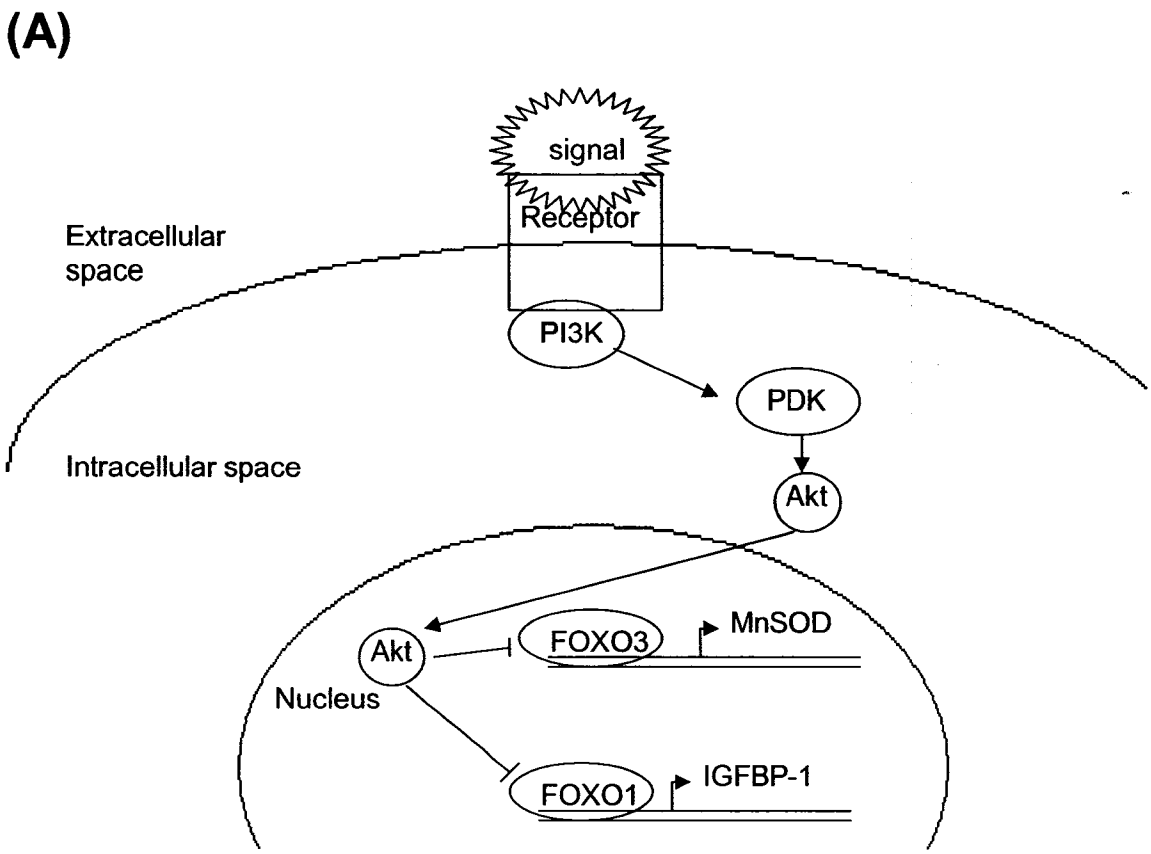
factors is that FOXO factors induce cell-cycle inhibition by regulating factors such as $p27^{kip1}$, p130Rb2, cyclin D and cyclin G2 which stimulate cells to enter a quiescent state (Kops *et al.*, 2002a). This quiescent state enables survival (extends viability) under fasting or other stressful conditions. It is difficult to determine the exact function of FOXO factors during freezing or anoxia, but since they are more active during these stresses, when many aspects of cell function are suppressed or shut down, this shows the importance of gene regulation by FOXO transcription factors when the wood frog is frozen or anoxic.

Figure 2.1

(A) PKB/Akt signal transduction pathway regulating FOXO transcription factors.

(B) Kinases that phosphorylate FOXOs (murine)and their phosphorylation sites

Figure 2.1



(Van der Heide *et al.*, 2004)

Figure 2.2

Western blot analysis of FOXO1 protein content in brain, kidney and liver of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen)

(A) Representative Western blots showing FOXO1 protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.

(B) Relative levels of FOXO1 protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

(C) Typical Western blot showing the antibody (FOXO1) crossreacting with a single band on the blot at the expected molecular weight (80 kDa).

(D) Typical Coomassie blue stained gel to determine relative protein levels.

Figure 2.2

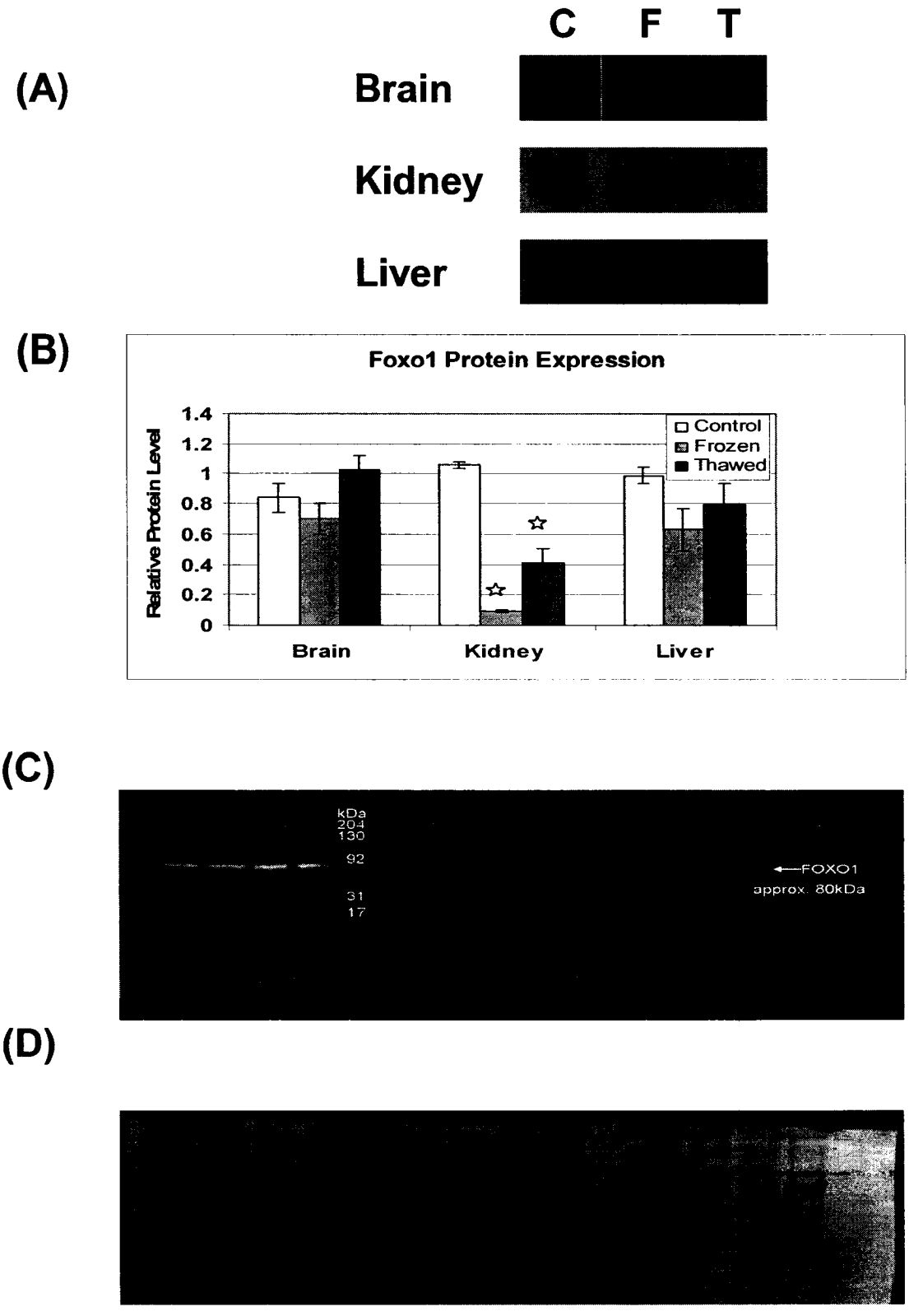


Figure 2.3

Western blot analysis of phospho-FOXO1 (ser 256) protein content in brain, kidney and liver of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen)

(A) Representative Western blots showing phospho-FOXO1 (ser256) protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.
(B) Relative levels of phospho-FOXO1 (ser256) protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 2.3

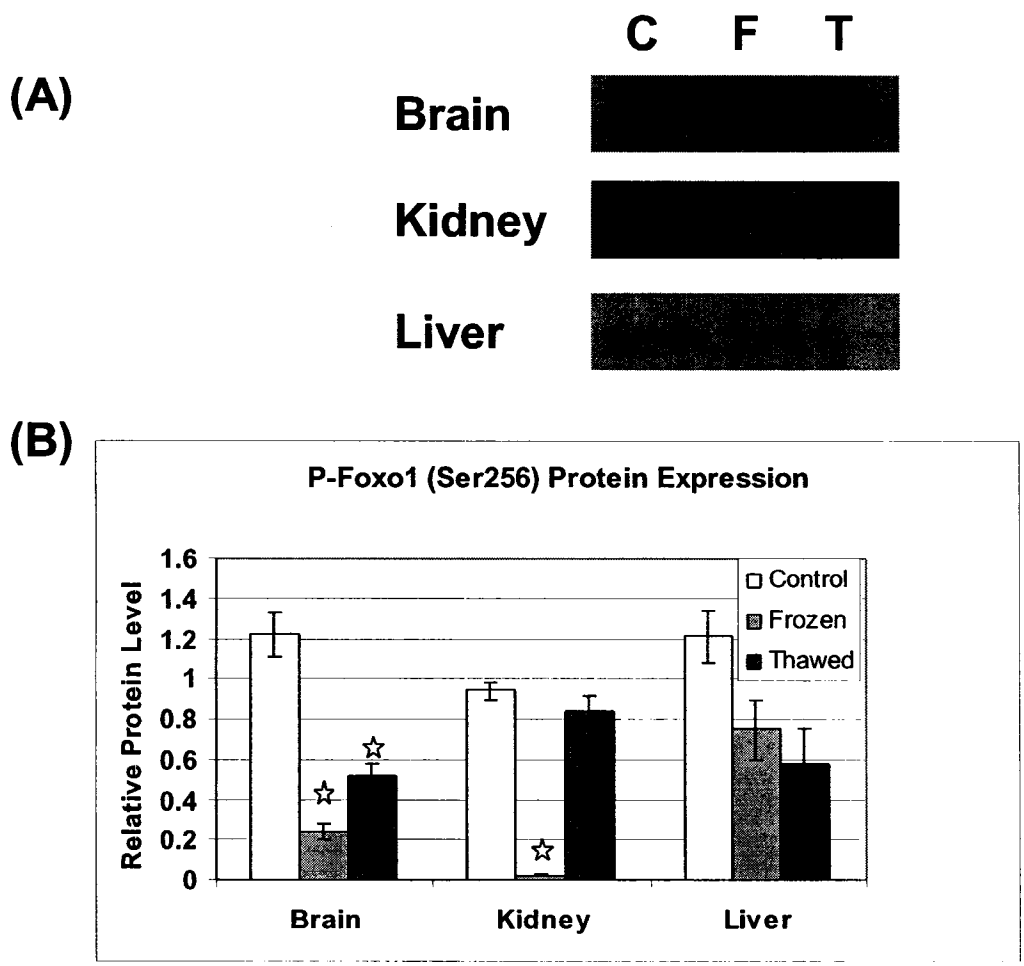


Figure 2.4

Western blot analysis of FOXO3 protein content in brain, heart, kidney and liver of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen)

(A) Representative Western blots showing FOXO3 protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.
(B) Relative levels of FOXO3 protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 2.4

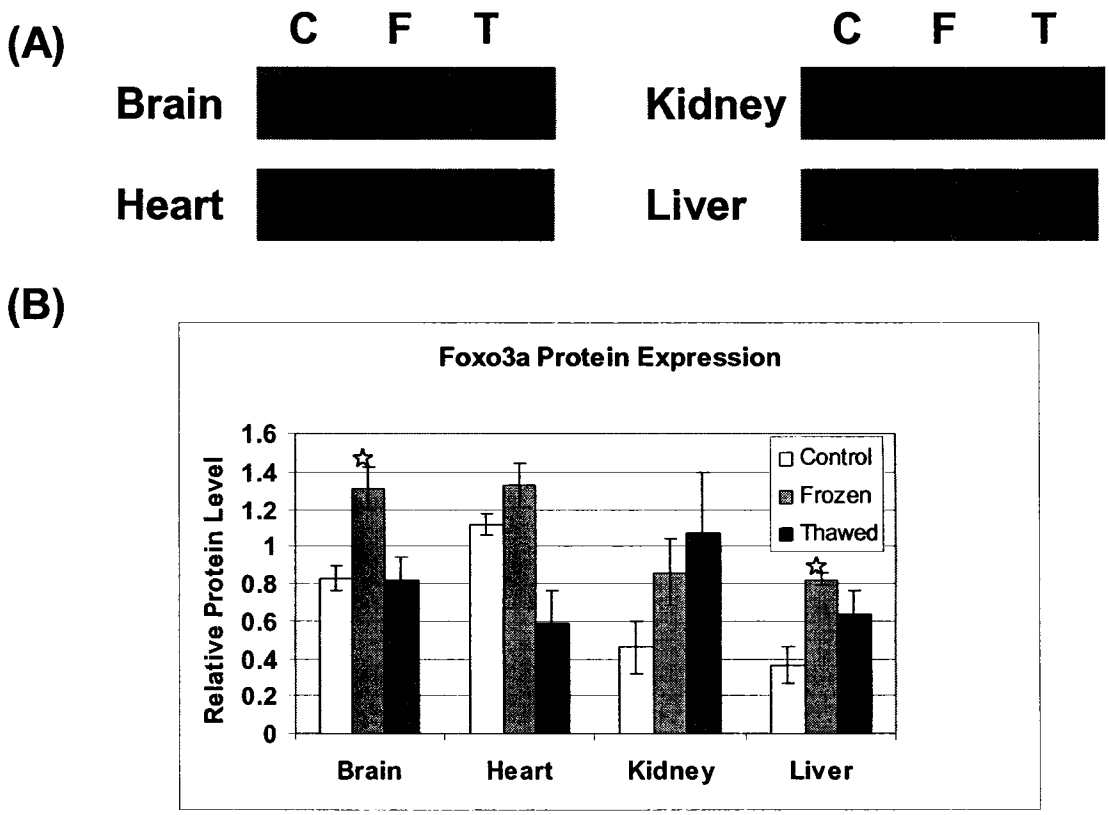


Figure 2.5

Western blot analysis of phospho-FOXO3 (ser 253) protein content in brain, heart, kidney and liver of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen).

(A) Representative Western blots showing phospho-FOXO3 (ser253) protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.
(B) Relative levels of phospho-FOXO3 (ser253) protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 2.5

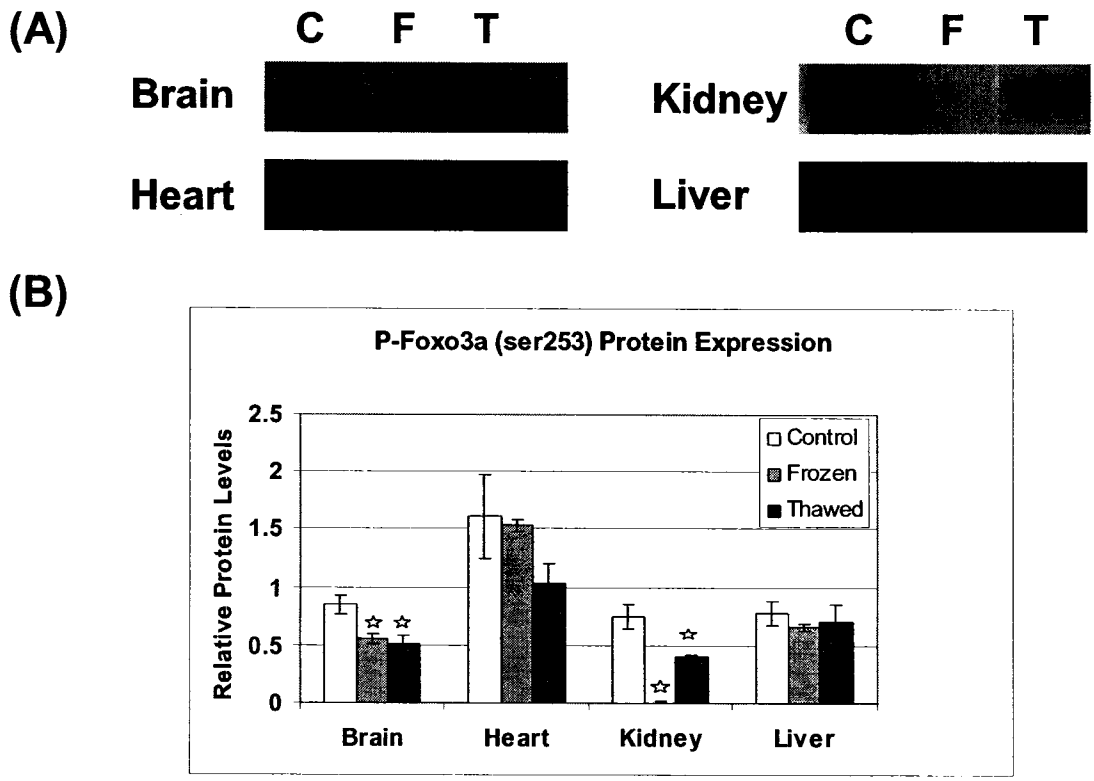


Figure 2.6

Western blot analysis of phospho-FOXO3 (ser 318/321) protein content in brain, heart, kidney and liver of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen)

(A) Representative Western blots showing phospho-FOXO3 (ser318/321) protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.
(B) Relative levels of phospho-FOXO3 (ser318/321) protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 2.6

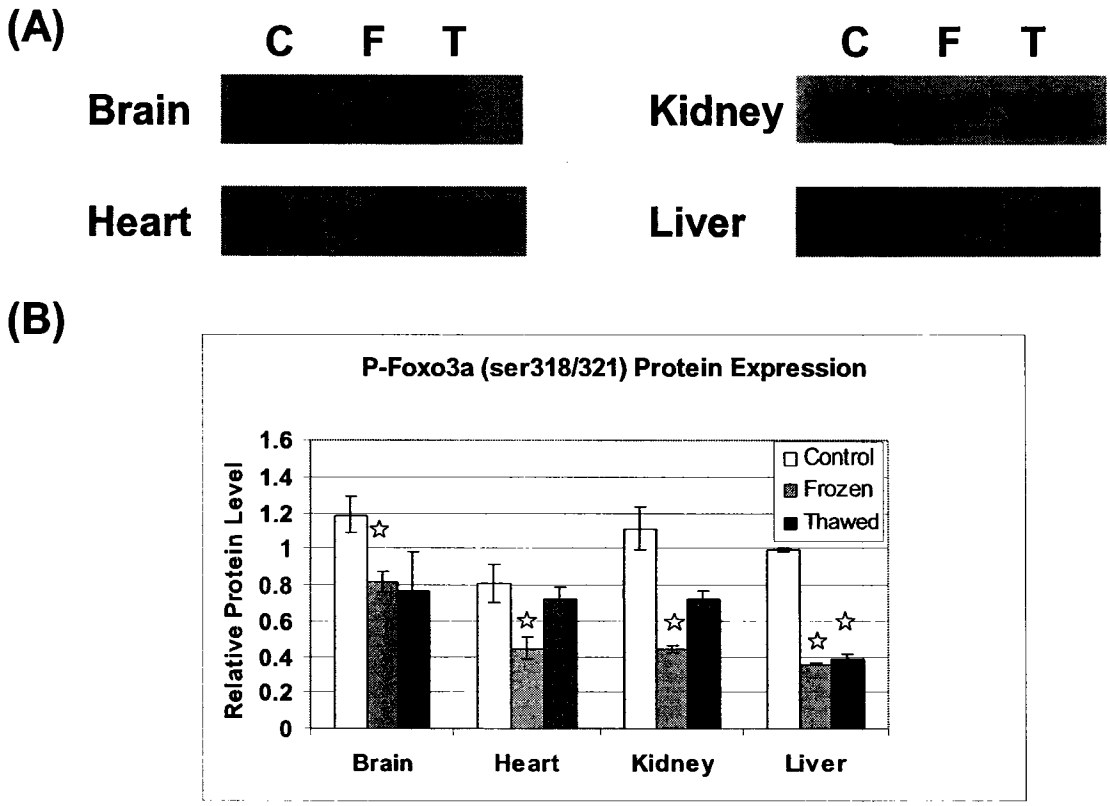


Figure 2.7

Western blot analysis of FOXO3 protein content in brain, heart, kidney and liver of wood frogs from three conditions: control (5°C acclimated), anoxic (24 h at 5°C) and aerobic recovery (4 h at 5°C after 24 h anoxic)

(A) Representative Western blots showing FOXO3 protein levels in tissues from control (C), anoxic (A) and recovered (R) frogs.
(B) Relative levels of FOXO3 protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 2.7

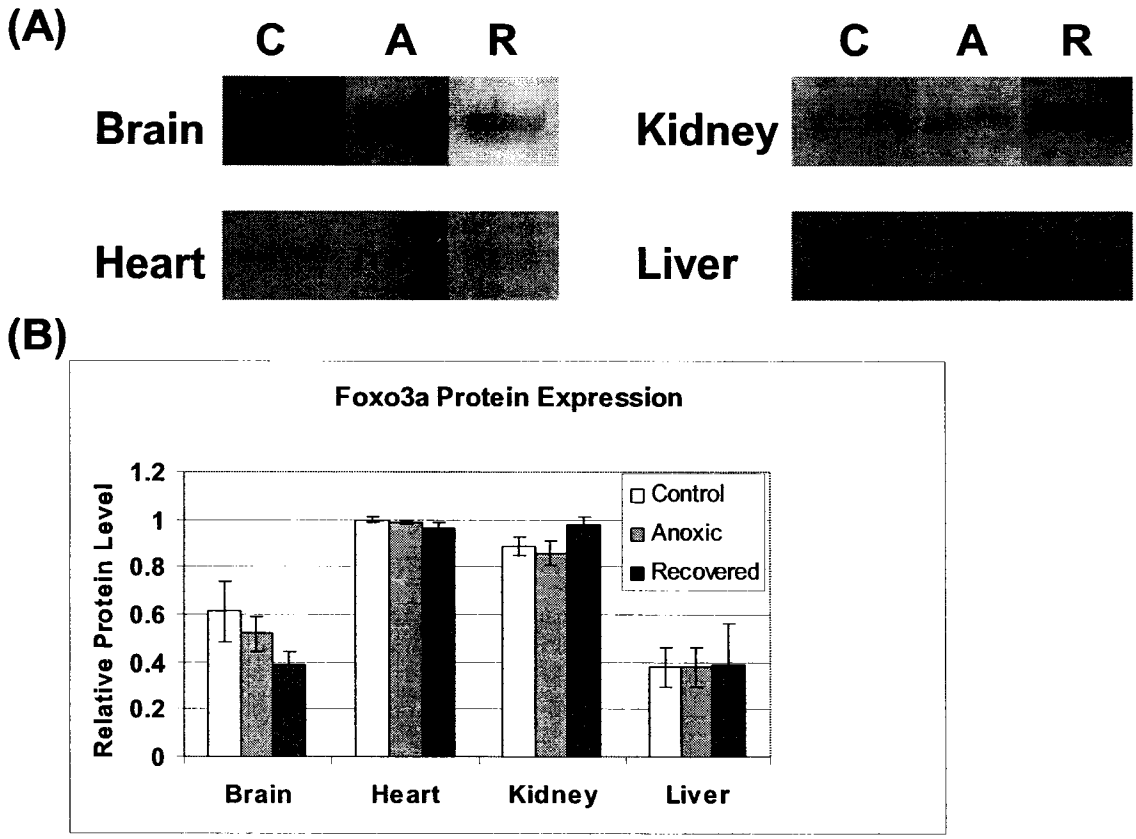


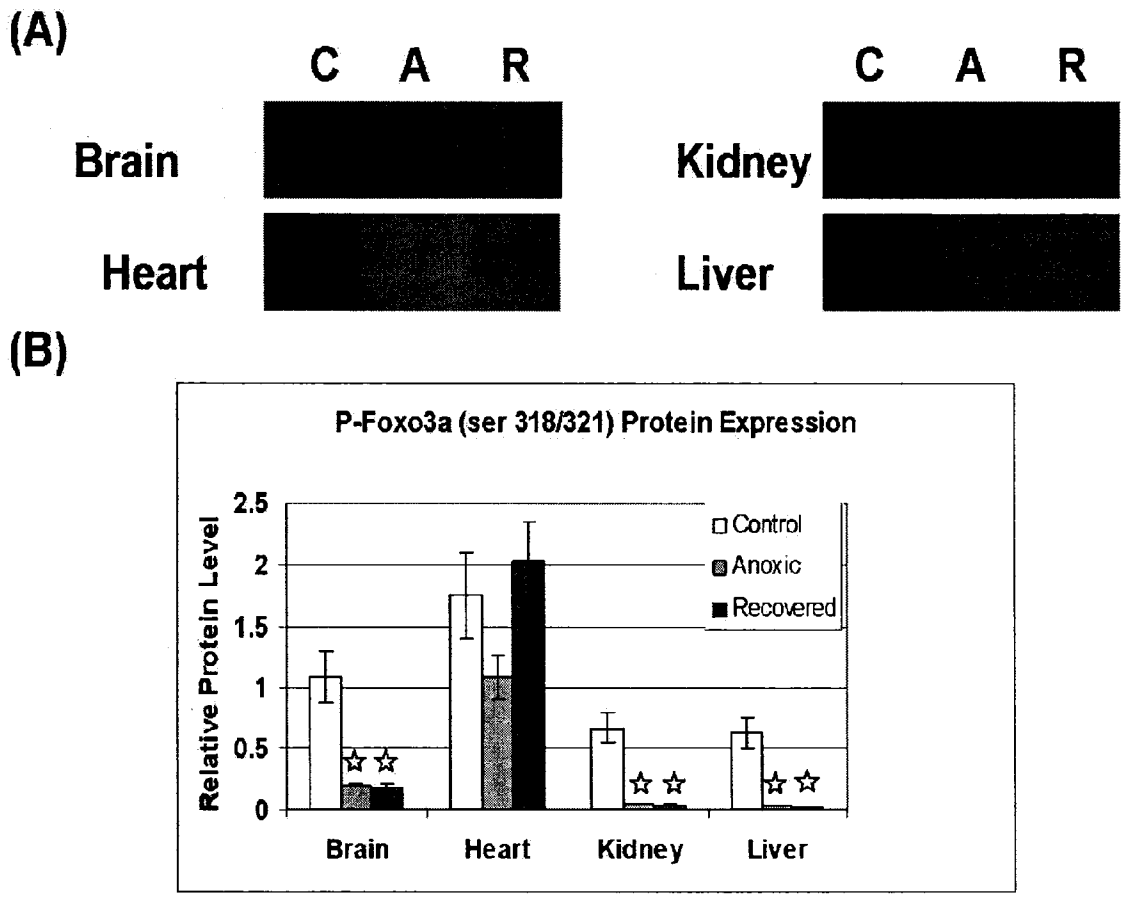
Figure 2.8

Western blot analysis of phospho-FOXO3 (ser318/321) protein content in brain, heart, kidney and liver of wood frogs from three conditions: control (5°C acclimated), anoxic (24 h at 5°C) and aerobic recovery (4 h at 5°C after 24 h anoxic)

(A) Representative Western blots showing phospho-FOXO3 (ser318/321) protein levels in tissues from control (C), anoxic (A) and recovered (R) frogs.
(B) Relative levels of phospho-FOXO3 (ser 318/321) protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 2.8



CHAPTER 3:

Regulation of Glycogen Synthase Kinase 3

Introduction

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase, once thought to be capable only of phosphorylating and inactivating glycogen synthase, the final enzyme in glycogen biosynthesis (Embi *et al.*, 1980). In mammals, there are two closely related isoforms, GSK3α (51 kDa) and GSK3β (47 kDa), which are ubiquitously expressed in tissues (Woodgett, 1991). The difference in molecular weight between the two isoforms is mainly due to a glycine-rich extension at the N-terminus of GSK3α (Woodgett, 1990). When cells are stimulated by insulin, inactivation of GSK3 occurs through a phosphoinositide 3-kinase (PI3K)-dependent mechanism. PI3K-induced activation of protein kinase B (PKB, also known as Akt) results in PKB phosphorylation of both GSK3 isoforms (on serine 9 for GSK3β and serine 21 for GSK3α), which consequently inhibits GSK3 activity (Cross *et al.*, 1995). By inhibiting GSK3, glycogen synthase can then be activated allowing the insulin-stimulated uptake of glucose into cells to be channeled into glycogen synthesis. GSK3 can also be phosphorylated on ser9/ser21 by the most downstream kinase of the classical mitogen-activated protein kinase (MAPK) cascade, called MAPK-activated protein kinase-1 (MAPKAP-K1, also known as RSK) (Saito et al., 1994). Many other stimuli also cause the inactivation of GSK3 by phosphorylation of the serine residues: activators of p70 ribosomal S6 kinase (p70S6K) such as amino acids, activators of cAMP-activated protein kinase (PKA) and protein kinase C (PKC) activators (Armstrong *et al.*, 2001; Fang *et al.*, 2000, 2002).

The specificity of GSK3 for its substrates is exceptional since it requires a priming phosphate at n + 4 (where n is the site of phosphorylation by GSK3). The optimal consensus site for phosphorylation is Ser/Thr-Xaa-Xaa-Xaa-pSer/pThr (where Xaa is any

amino acid and pSer/pThr is either phosphoserine or phosphothreonine) (Fiol *et al.*, 1987).

Substrates phosphorylated by GSK3 have roles in many different cellular processes, which include glycogen metabolism, transcription, translation, cytoskeletal regulation, intracellular vesicular transport, cell cycle progression and apoptosis. Phosphorylation of protein substrates by GSK3 is usually inhibitory. GSK3 phosphorylates and inactivates a variety of transcription factors such as β-catenin (transcription transactivator), c-Jun (component of activator protein-1 which regulates many diverse genes), c-Myc (regulates genes involved in cell growth, differentiation and apoptosis) and cyclin D1 (cell cycle regulation) (Yost *et al.*, 1996; Boyle *et al.*, 1991; Pulverer *et al.*, 1994; Diehl *et al.*, 1998). GSK3 action can also increase the transcriptional activity of some transcription factors such as the cAMP response element binding protein which regulates cAMP-responsive genes (Fiol *et al.*, 1994). GSK3 also inhibits the activity of several enzymes including glycogen synthase and eIF-2B, an enzyme critical for translation initiation by ribosomes (Singh *et al.*, 1996; Dent *et al.*, 1989).

Since GSK3 is involved in processes such as cell cycle arrest and inhibition of protein translation, and since wood frogs need to depress ATP-expensive processes such as cell division and protein synthesis for long term survival in the ischemic, frozen state, I hypothesized that GSK3 would be more active during freezing.

Materials and Methods

Animal experiments, preparation of tissue extracts and soluble protein determination and SDS polyacrylamide gel electrophoresis were as done in Chapter 2.

Sample preparation for protein kinase assays

Frozen tissue samples were homogenized 1:5 w:v in cold (4°C) buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM NaF, 4 mM EDTA, 1mM Na_3VO_4 and 0.1% (v/v) 2-mercaptoethanol. A few crystals of phenylmethylsulfonyl fluoride and 1 μL (per ml of homogenization buffer) of Sigma Protease Inhibitor Cocktail were added immediately prior to homogenization. Homogenates were centrifuged 10,000 x g for 25 minutes. The supernatant was removed and the concentration of protein was determined using the Bio-Rad reagent as described above.

Radioactive glycogen synthase kinase 3 assays

GSK3 activity was determined using a radioactive assay that measured the incorporation of radiolabeled γ-^{32}P from γ-^{32}P-ATP (3000 Ci/mmol; GE Healthcare Biosciences, Piscataway, NJ) onto a specific GSK3 peptide substrate. Assays were initiated by addition of γ-^{32}P-ATP to the appropriate reaction mixture in 500 μL Eppendorf tubes. The reaction volume was 25 μL and the amount of crude extract used to assay GSK3 was optimized to contain 25 μg of total soluble protein per assay. The GSK3 peptide substrate was obtained from Upstate (Millipore Corp. catalogue # 12-241) and had the amino acid sequence: YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE (similar to the sequence around the phosphorylation site in human skeletal muscle glycogen synthase; pS is phosphorylated serine). Optimum assay conditions were 2 mM $MgCl_2$, 200 μM Mg^{2+}-ATP (containing 0.5 μCi γ-^{32}P-ATP), 0-75 μM peptide (20 μM for the metabolic study) and assay buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.15 M NaCl and 0.1% v/v 2-mercaptoethanol). The assay also contained 0.5 mM of specific peptide inhibitors

of protein kinases A and C (PKA fragment 5-24 and PKC fragment 19-31) (added as 1.25 μL from a 10 mM stock). Zero substrate blanks were always run to account for nonspecific background signals. The reaction time was 30 minutes at 22°C.

For a study of metabolite effects on GSK3 activity, the standard amount of peptide substrate used was 20 μM and the metabolites to be tested were prepared in Tris buffer, pH 7.5. Metabolites were tested at the following concentrations: 5 mM glucose-6-phosphate (G6P), 10 mM L-alanine, 10 mM L-aspartate, 5 mM succinate, 1 mM AMP, 5 mM phosphoenolpyruvate (PEP), or 250 mM D-glucose.

Signal detection

GSK3 activity was quantified based on concepts modified from Asensio and Garcia (2003). Reactions were stopped by addition of one-half reaction volume of 0.2 M EDTA containing 0.1% (w:v) bromophenol blue. Aliquots of 2 μL of the stopped reaction mix were then spotted onto penciled circles (5 mm diameter) drawn in a grid on P81 paper. The P81 paper array was allowed to air dry under a fume hood followed by 4 quick washes in 0.8% v:v phosphoric acid, 2 long washes (10 minutes) with 0.8% phosphoric acid, and lastly 1 quick wash with 95% ethanol.

After washing, the paper array was allowed to air dry and was then placed under saran wrap and exposed to a storage phosphor screen in autoradiography cassettes. Prior to use, storage phosphor screens were stripped for at least 10 minutes using a white light box. The exposure time for visualizing ^{32}P-labeled GSK3 peptide was 1 h. After exposure, phosphor screen signals were captured using a BioRad PhosphoImager (Hercules, CA). Quantification, background correction, and data analysis was performed

using Quant One software, and relative signal density was transformed into units of activity using a standard curve of γ-^{32}P-ATP that was prepared using the same conditions as those for GSK3 activity (*e.g.* same spot volume, exposure time, etc.). One unit is defined as the amount of enzyme that phosphorylates 1 μmol of peptide per minute. Kinetic parameters were determined using a nonlinear least squares regression computer program (Kinetics 3.51) (Brooks, 1992). Data are expressed as mean ± S.E.M. with statistical testing performed using analysis of variance followed by the Student-Newman-Keuls test.

Results

GSK3 and phospho-GSK3 (ser9) were detected in all wood frog tissues tested: brain, heart, kidney, liver and skeletal muscle. The activity of GSK3 was also measured, using a radioactive assay, under various conditions: at 22°C, at 4 °C, and at both temperatures in the presence of 250 mM glucose (a level mimicking the natural concentration of this cryoprotectant in vivo). The effects of other metabolites on GSK3 activity were also assessed at both high and low assay temperatures including: 5 mM glucose-6-phosphate (G6P), 10 mM L-alanine, 10 mM L-aspartate, 5 mM succinate, 1 mM AMP, 5 mM phosphoenolpyruvate (PEP), or 250 mM D-glucose.

GSK3 total protein levels

Total GSK3 protein levels was quantified using Western blotting in wood frog tissues under three experimental conditions: control (acclimated at 5°C), 24 h frozen (at -2.5°C) and 8 h thawed recovery (at 5°C) after 24 h frozen (Figure 3.1). The GSK3

antibody crossreacted with a single band at the expected molecular weight of 50 kDa. GSK3 protein was detected in all five tissues but protein levels did not change during freezing and thawing in brain, liver or muscle. In the heart, however, the GSK protein content decreased significantly ($P<0.05$) to 57.5% of control values when frogs were frozen, but returned to control levels when animals were thawed. In the kidney, GSK3 protein content did not change during freezing, but fell significantly ($P<0.05$) to 48.5% of the control value when the frogs were thawed.

Phospho-GSK3 (ser9) levels

GSK3 is inactivated by phosphorylation on serine residue 9 and Western blotting was also used to determine the relative amounts of the phosphorylated form of GSK3 (ser9) in control, frozen and thawed frogs (Figure 3.2). The phospho-GSK3 antibody crossreacted with a single band at the expected molecular weight of 50 kDa. Phospho-GSK3 (ser9) was again detected in all five tissues tested. The levels of phospho-GSK3 (ser9) decreased in all organs during freezing and remained suppressed after 8 h thawing in three tissues. In brain, phospho-GSK3 content decreased significantly ($P<0.05$) to 50.7% of the control value during freezing and remained low at 44.9% of control levels after 8 h thawing. A similar pattern was seen in muscle tissues, with phospho-GSK3 levels that were 27.3% and 38.6% in heart and 15.0 and 26.5 % in skeletal muscle of frozen and thawed frogs, respectively, as compared with controls ($P<0.05$). In the kidney and liver, however, phospho-GSK3 content decreased significantly ($P<0.05$) during freezing (to 50.0% in kidney and 43.1 % in liver of controls) but rebounded again when frogs thawed to ~89% of the control value in each case.

GSK enzyme activities

GSK3 activity was measured in extracts of skeletal muscle of control wood frogs (acclimated at 5°C) and animals frozen for 24 h at -2.5 °C. A specific peptide substrate was used which mimics the phosphorylation site on skeletal muscle glycogen synthase. Velocity versus peptide concentration curves were generated under four different conditions: at high (22°C) and low (4°C) temperatures (Figures 3.3 and 3.4) and in the presence of 250 mM glucose at each temperature (Figures 3.5 and 3.6). V_{max}, K_m, and Hill coefficient (n_H) values were calculated using a kinetics program (Brooks, 1992) with the data from the velocity vs substrate concentration curves. The values for four independent trials using different preparations of enzyme are summarized in Table 3.1.

The velocity versus [peptide] curve at 22°C was near hyperbolic for frog muscle GSK3 (Fig. 3.3) with n_H values that were close to 1.0. Enzyme affinity for peptide did not change between control (5°C acclimated) and frozen (-2.5°C) situations, being 23.5 ± 4.2 μM and 33.1 ± 4.4 μM for GSK3 from control and frozen frogs, respectively. V_{max} values were calculated (from the computer analysis of the velocity vs substrate concentration curves using the kinetics program) to be 1.83 ± 0.30 units/mg for control and 2.27 ± 0.18 units/mg soluble protein for frozen frogs, both assayed at 22°C. When the enzyme was assayed at 4°C, V_{max} was strongly reduced as is expected for the effects of temperature on enzyme reactions. The K_m value for GSK3 from control muscle did not change significantly when assayed at 4°C but the K_m for the enzyme from frozen frogs was reduced by 50% as compared to the 22°C assay ($P<0.05$). The Hill coefficient values were unchanged for the frozen enzyme but increased for the control enzyme assayed at

4°C. Glucose is the natural cryoprotectant in wood frogs and rises to high levels in frog organs during freezing. To determine if glucose affected GSK3 activity, assays at 22 and 4°C were repeated in the presence of 250 mM glucose. The addition of glucose significantly changed both V_{max} and K_m of the enzyme from both 5°C acclimated and -2.5°C frozen frogs in 22°C assays. For the enzyme from control frogs, both K_m and V_{max} increased significantly; for example, K_m increased by 73%. On the other hand, the properties of the enzyme from frozen frogs were opposite with K_m decreasing by 34% and V_{max} by 50% as compared to the values in assays without glucose. When GSK3 was assayed at 4°C in the presence of 250 mM glucose, the sigmoidicity of the reaction increased substantially (Figure 3.6) with Hill coefficients that rose to 2.6-2.8 for the enzyme from both control and frozen frogs (Table 3.1). V_{max} values were also reduced as compared with the enzyme assayed at 4°C without glucose. The K_m value for the enzyme from control frogs was the same as the value for 4°C assays in the absence of glucose but for the enzyme from frozen animals, the K_m was significantly higher than the value in the absence of glucose (about 2-fold, $P<0.05$) and equivalent to the value determined in 22°C assays without glucose.

Metabolic study of GSK3 effectors

The effects of different metabolites on GSK3 activity was assessed at a constant peptide substrate concentration of 20 μM at 22°C (Figure 3.7) and at 4°C (Figure 3.8). Seven metabolites that were predicted to change in vivo in muscle during freezing were tested at levels that are at the high end of the normal range of these metabolites in frog organs: 5 mM G6P, 10 mM alanine, 10 mM aspartate, 5 mM succinate, 1 mM AMP, 5

mM PEP, 250 mM glucose. Activities are reported as the percentage of the activity obtained in the absence of added metabolites.

Analysis at 22°C

When the activity of GSK3 was assessed at 22°C, several metabolites had no significant effect on enzyme activity, either when compared with assays run without added metabolites or in assays comparing activities of GSK3 in extracts from control (5°C acclimated) versus frozen frogs (Figure 3.7). These included 10 mM alanine, 10 mM aspartate, 5 mM succinate and 5 mM PEP. When 5 mM G6P was present, significant increases in activity were seen for the enzyme from both 5°C acclimated and frozen frogs (by 1.32 and 1.84 fold, respectively) ($P<0.05$). Furthermore, the effect was significantly greater for the enzyme from frozen frogs. By contrast both 1 mM AMP and 250 mM glucose significantly reduced the activity of GSK3 from muscle of control 5°C acclimated frogs by 33.6% and 7.1% ($P<0.05$), respectively. However, these metabolites did not affect enzyme activity in muscle from frozen frogs.

Analysis at 4°C

When the same assays were conducted at 4°C, activity of GSK3 was again unaffected by the addition of 10 mM alanine, 10 mM aspartate or 5 mM succinate. G6P again activated the enzyme from frozen muscle (by 1.18- fold, $P<0.05$), an effect that was less than that seen at 22°C, but the control enzyme was not affected by G6P in the cold. By contrast, 1 mM AMP was a stronger effector at 4°C, decreasing activity by 61.1% and 36.2% for the enzyme from control and frozen frogs, respectively. Glucose at 250 mM glucose was also a stronger inhibitor in 4°C assays, reducing activities significantly by 21.6% and 19.2% ($P<0.05$) for the enzyme from control and frozen frogs, respectively. In

addition, 5 mM PEP had a small inhibitory effect (a 13.1% decrease, $P<0.05$) on GSK3 activity from control muscle but did not affect the enzyme from frozen muscle.

Discussion

In 1980, glycogen synthase kinase 3 (GSK3) was identified as one of protein kinases that phosphorylates glycogen synthase, the enzyme that catalyses the last step in glycogen synthesis (Embi *et al.*, 1980). The phosphorylation of glycogen synthase by GSK3 inactivates the enzyme. Protein kinase B (also known as Akt) was later shown to be responsible for controlling the insulin-induced inhibition of GSK3 by catalyzing the phosphorylation of a serine residue in the amino acid terminus of GSK3 (Cross *et al.*, 1995). When GSK3 activity is inhibited, its substrates such as glycogen synthase and the eukaryotic initiation factor 2B (eIF2B) can be dephosphorylated (and therefore activated) by protein phosphatases, assisting the insulin-induced activation of both glycogen synthesis and protein synthesis (Parker *et al.*, 1983; Hughes *et al.*, 1992). GSK3 is unique among protein kinases since it requires that many of its substrates are first phosphorylated by another protein kinase on a serine or threonine residue (termed the priming substrate) situated four residues carboxy-terminal to the site of GSK3 phosphorylation (Fiol *et al.*, 1987). When the serine residue near the amino terminus of GSK3 becomes phosphorylated, it interacts with the same residues that are involved in binding the priming phosphate (in the active site) and so phosphorylation suppresses activity by turning the amino terminus of GSK3 into a simulated substrate. This not only prevents substrates from binding, but also blocks access to the catalytic center, thereby shutting down GSK3 activity (Frame *et al.*, 2001).

The same residue on GSK3 that is targeted by PKB/Akt is also known to be phosphorylated by other protein kinases in response to different signals. For example, growth factors can inhibit GSK3 activity through the classical MAPK cascade, as well as by the PtdIns(3,4,5)P_3-dependent pathway (Stambolic and Woodgett, 1994; Shaw and Cohen, 1999). Amino acids can also reduce GSK3 activity through a pathway that involves the mammalian target of rapamycin (mTOR) (Armstrong *et al.*, 2001). The phosphorylation of GSK3 can additionally be catalyzed by cyclic-AMP-dependent protein kinase (PKA) in response to agonists that elevate the intracellular concentration of cAMP (Fang *et al.*, 2000; Li *et al.*, 2000). GSK3 also has other downstream targets than those mentioned above (glycogen synthase and eIF2B); these include cyclin D1 and the transcription factor c-myc. Phosphorylation of these proteins targets them for ubiquitylation and subsequent proteolytic destruction. Cyclin D1 facilitates entry into the S phase of cell-division while c-myc stimulates cell proliferation. GSK3 also phosphorylates several residues in the transcription factor c-jun, suppressing its binding to DNA and thereby inhibiting the transcription of several c-jun regulated genes, including cyclin D1 (Sabbah *et al.*, 1999). Therefore, GSK3 exerts negative controls a number of key downstream targets that regulate in biosynthesis, cell cycle and growth processes.

GSK3 Protein Levels

In the current study, the levels of GSK3 and a phosphorylated form of GSK3 (at serine position 9) were assessed in control (acclimated at 5°C), 24 h frozen (at -2.5°C) and 8 h thawed (at 5°C) wood frogs in 5 tissues (brain, heart, kidney, liver, muscle). The

protein was detected in all tissues tested. Total GSK3 protein levels did not change significantly in the brain, liver and muscle during freezing and thawing (Figure 3.1). Since this antibody does not distinguish between the unphosphorylated and phosphorylated forms of GSK3, it represents the total amount of GSK3 present in the tissues during control, frozen and thawed conditions. However, total GSK3 protein levels did decrease significantly during freezing in heart, and then increased again to near control levels during thawing. In the kidney, the total amount of GSK3 did not change significantly during freezing, but did decrease significantly when frogs were thawed for 8 hours. Overall, therefore, total GSK3 protein is maintained at fairly constant levels over cycles of freeze/thaw which has two implications: (a) the enzyme is always present to respond when needed, and (b) the regulation of the activity of this kinase probably comes mainly from posttranslational controls.

Indeed, the levels of phosphorylated GSK3 (Ser 9) were significantly reduced in all tissues of frozen frogs, as compared with controls, and remained low after 8 h thawed in brain, heart and skeletal muscle (Figure 3.2). Since GSK3 is inactivated by phosphorylation, the phosphorylated form represents the relative amount of inactive GSK3 present in the tissues. The amount of active GSK3 would have the opposite pattern therefore. Since the total amount of GSK3 did not change significantly in four tissues during freezing, and since the phosphorylated (inactive) form of the enzyme decreased significantly during freezing, this implies that the amount of active GSK3 strongly increases during freezing in liver, kidney, muscle and brain. In heart, the situation is a little harder to evaluate but the decrease in total GSK3 protein was ~42% whereas phospho-GSK3 content was more strongly reduced by 73%. Overall, then, this would be

consistent with a higher relative amount of dephosphorylated active GSK3 in heart of frozen frogs. In the liver and kidney, the relative amount of phosphorylated GSK3 rose again to near control levels after thawing. A rapid reversal is key in liver which has to begin clearing the massive load of glucose that was produced as a cryoprotectant and restore it as liver glycogen. Skeletal muscle showed a small increase in phosphoenzyme content during thawing but did not return to control levels whereas there was no change in phospho-GSK3 amount in brain and heart after 8 h of thawing (although these tissues might just require longer recovery times). In heart, however, the rise in total GSK3 protein during thawing, when coupled with sustained low phospho-GSK3, would indicate an active GSK3 protein in thawed heart.

The freeze/thaw changes in GSK3 in frog organs can be interpreted with respect to what is already known about glycogen metabolism during freezing in wood frogs. Freezing triggers a rapid increase in glycogen phosphorylase (GP) activity, particularly in liver, that initiated glycogen breakdown and a strong increase in glucose within minutes (Storey and Storey, 1988). The production and export of glucose as a cryoprotectant continues over several hours until all organs have high glucose levels; in core organs glucose rise to 150-300 mM compared with about 5 mM in control frogs. GP is activated as a result of adrenergic signals that elevate cyclic AMP and activate PKA (Figure 3.9). When GP is active and breaking down glycogen, it is important that GS is turned off to prevent a wasteful ATP-dependent recycling of glucose into glycogen. Indeed, measurement of GS activity showed that it was reduced to very low levels during freezing following an inverse pattern to that seen for GP activity (Russell and Storey, 1995). Since GSK3 inhibits GS, the key regulatory enzyme in the glycogen synthesis

pathway, then a more active GSK3 during freezing would be consistent with the inactivation of GS that is needed during freezing.

GSK3 is also known to inhibit other key proteins such as c-myc, a transcription factor involved in cell growth, cyclin D1, a protein needed for cell differentiation, and eIF-2B, an enzyme that is critical for translation initiation. Cell growth, cell differentiation and translation are all suppressed during freezing, as part of the metabolic rate depression experienced by the wood frog, and so the increase in active GSK3 during freezing, observed from Western blotting, is consistent with GSK3-mediated inhibition of these processes (Storey and Storey, 2004a). Active GSK3 would turn off all these processes in the frozen frog. During thawing, however, these cell functions should be reactivated and, indeed, after just 8 h back at 5°C (typically it takes up to 4 h for all body ice to melt at this temperature; J. Storey, unpublished results) the amount of phosphorylated inactive GSK3 had risen back to control levels in liver and kidney. The sustained low levels of phospho-GSK3 in the other tissues after 8 h may simply reflect a slower recovery of metabolic processes in these tissues. The rise in inactive phospho-GSK3 content back to control levels within 8 h in liver would allow the reactivation of GS and the reconversion of cryoprotectant back into glycogen stores in liver. Although it takes many days to reduce glucose levels back to normal (Storey and Storey, 2004b), the process gets underway quite quickly. Thus, frogs thawed for 24 h at 5°C showed high GS activity so glycogen resynthesis seems to be well established by that time. Russell and Storey (1995) found that the percentage of GS in the active form in wood frog liver was 81.5% in 24 h thawed frogs compared with a control level of 34% and a level in liver of 24 h frozen frogs of just 11%.

GSK3 enzyme properties

GSK3 activity was determined in *Rana sylvatica* muscle extracts at varying peptide concentrations (from 0-75 μM) for control (acclimated at 5°C) and frozen (8h at -2.5°C) conditions. Assays were conducted at 22°C (Figure 3.3) and 4°C (Figure 3.4) and in the absence versus presence of 250 mM glucose (Figure 3.5, 3.6). Assays in the presence of high glucose were used to determine if the frog cryoprotectant that is accumulated during freezing affected the kinetics of frog GSK3. V_{max}, K_m and Hill coefficients were determined for each condition (Table 3.1). The Hill coefficients were greater than one in all cases, which indicates a positive cooperation reaction; once one ligand molecule is bound to the enzyme, its affinity for other ligand molecules increases. This positive cooperation reaction typically occurs with proteins that have multiple subunits, and so this result makes sense since GSK3 is a dimer. However, the Hill coefficients between control and frozen conditions were not significantly different in any of the conditions assayed. The GSK3 enzyme velocity determined at room temperature for control and frozen frog muscle did not differ and there was no significant difference found between the K_m and V_{max} between control and frozen tissues. For control and frozen muscle, when the assay was performed at 4°C, the V_{max} was not significantly different, but there was a significant difference ($P<0.05$) between the K_m for control (29.7 ± 2.6 μM) and frozen (16.6 ± 0.7 μM). The K_m value for the enzyme from frozen tissue was lower, which indicates that GSK3 from frozen tissue has a higher affinity for its peptide when assayed at 4°C, which could result from a modification to the frozen enzyme. A significant difference was also seen between the K_m of the frozen enzyme at 4°C and at 22°C (a 50%

decrease), which could also be due to the frozen enzyme being modified to have a higher affinity for its substrate. When 250 mM glucose was included in the assay, and the assay was performed at room temperature, a significant difference in K_m was seen between GSK3 from control (40.6 ± 4.0 μM) and frozen (21.8 ± 2.9 μM) muscle and since the K_m value was lower for the enzyme from frozen tissue, this indicates that GSK3 in the frozen state has a higher affinity for its peptide substrate. GSK3 V_{max} in extracts from frozen muscle assayed at 22°C with 250 mM glucose was found to be significantly lower ($P<0.05$) at 1.14 ± 0.35 units/mg compared to the control value of 2.89 ± 0.64 units/mg. The lower V_{max} found in frozen tissue implies that the enzyme is less active at room temperature with 250 mM glucose. When the activity of GSK3 was assayed at 4°C and the assay contained 250 mM glucose, there was no significant difference between the K_m and V_{max} values between control and frozen tissues. Generally, GSK3 is much less active when assayed at 4°C, indicated by the lower V_{max} observed compared to the assays performed at room temperature. This lower activity at low temperatures is the expected temperature effect on any reaction rate. Enzyme rates typically decrease by ~2-fold for a 10°C decrease in temperature, which was seen for the control enzyme without glucose and the frozen enzyme with and without glucose (a 3-4-fold decrease). A much higher decrease in enzyme rate (9.6-fold) was seen for the control enzyme with glucose, which could be due to a glucose-induced enzyme modification in the control state. It also seems as if GSK3 from frozen muscle could have a higher affinity for the peptide compared to the control. An enzyme that binds its substrate more effectively could be advantageous when it is crucial to quickly turn off pathways in order to conserve energy to survive.

When the effect of glucose on the enzyme is analyzed at 22°C, an opposing effect is observed for the K_m and V_{max} values for the control and frozen enzymes. For the control enzyme, the K_m and V_{max} values significantly increase in the presence of glucose, while for the frozen enzyme, the values significantly decrease. This result implies that GSK3 in the control frog has a lower affinity and a higher maximum activity in the presence of glucose, while in the frozen frog, the enzyme has a higher affinity for the substrate and has a lower maximum activity in the presence of glucose. The higher affinity and lower maximum activity implies that the enzyme is more efficient in the frozen state and could be beneficial to the frog since it allows the enzyme to remain active when its metabolic rate is depressed. At 4°C, the K_m of the frozen enzyme with glucose was significantly higher when compared to the enzyme without glucose, but was similar to the frozen enzyme at 22°C without glucose. This result implies that, at 4°C, GSK3 from frozen tissue is less active in the presence of glucose.

Metabolite study

The velocity of GSK3 with 20 μM of peptide was measured in the presence of several metabolites (5 mM G6P, 10 mM alanine, 10 mM aspartate, 5 mM succinate, 1 mM AMP, 5 mM PEP, 250 mM glucose) to determine if any of these could be allosteric effectors of the enzyme. Glucose-6-phosphate (G6P) is at the start of glycolysis and the pentose phosphate pathways and can also be converted to glycogen for storage or to glucose for export. It was found to be present at concentrations of ~0.3 mM in control liver tissue wood frogs and rose to ~0.9 mM in frozen liver during active glucose synthesis for cryoprotection (Storey, 1987b). G6P is also an important metabolite

regulator of GS, causing the allosteric activation of the enzyme (via a conformational rearrangement) that also converts GS into a better substrate for protein phosphatases, which can dephosphorylate GS to activate it (Ferrer *et al.*, 2003). Hence, in most vertebrates, high G6P is typically a signal that sugar availability is high and that glycogen synthesis should be activated. Alanine and aspartate are amino acids, whereas succinate is in intermediate of the tricarboxylic acid cycle (energy-producing); in many anoxia tolerant species, alanine and succinate are additional products of anaerobic carbohydrate catabolism (in addition to or instead of L-lactate) whereas aspartate is an anaerobic substrate. In wood frog control muscle, alanine and aspartate are present at approximately 5 mM and 2 mM while in the frozen tissue, they are present at concentrations of 10-15 mM and 1 mM, respectively (Storey and Storey, 1985). Alanine accumulates along with lactate as a product of glycolysis in the anoxic and ischemic tissues of frozen frogs (Storey and Storey, 1985). Phosphoenolpyruvate (PEP) is a late intermediate in glycolysis and its production by the enzyme PEP carboxykinase is the rate-limiting step of gluconeogenesis, the pathway by which excess carbohydrates and amino acids can be converted back to glucose. Adenosine monophosphate (AMP) is one of the three adenylates (ATP, ADP, AMP) that make up the primary energy currency of cells. The concentration of PEP did not change significantly at ~0.05 mM between control and frozen wood frog liver, whereas AMP in control and frozen muscle was about 0.02 mM and 0.05 mM, respectively (Storey, 1987). AMP accumulates in tissues under energy stress, including during anoxia and freezing. It is an allosteric activator of various enzymes involved in pathways of ATP production, and stimulates the AMP-dependent protein kinase which in turn also regulates a variety of enzymes of energy metabolism.

Glucose is an energy source and in the wood frog, a cryoprotectant. It is found at concentrations of about 5 mM in control muscle and in the 150-300 mM range in the tissues of frozen frogs. GSK3 activity was determined in extracts from control (acclimated at 5 °C) and frozen (24 h at -2.5°C) muscle in the presence versus absence of the various metabolites and their effects are shown in shown in Figure 3.7 (for assays at 22°C) and Figure 3.8 (for assays at 4°C).

Analysis at 22°C

When assayed at room temperature, the velocity of GSK3 at 20 μM peptide did not change significantly between control and frozen tissues and also when assayed with and without selected metabolites: 10 mM alanine, 10 mM aspartate, 5 mM succinate and 5 mM PEP. Glucose had a small effect on the control enzyme but G6P and AMP showed substantial regulatory effects. G6P at 5 mM significantly increased GSK3 velocity by 33% and 84% for the enzyme from control and frozen muscle, respectively ($P<0.05$), whereas 1 mM AMP produced an inhibition of activity by about 34% for the control enzyme. To our knowledge, this may be the first report of metabolite effectors changing the activity of the active form of GSK3. When frogs start to freeze, glycogen phosphorylase (GP) is rapidly activated and G6P levels quickly rise and are converted into glucose which accumulates in high levels inside cells and is also exported by the liver to other organs (Storey and Storey, 1988). However, G6P can also be reconverted to glycogen, a process that that would be futile for producing cryoprotectant. An activation of GSK3 by G6P could enhance its ability to phosphorylate and inhibit GS and prevent glycogen resynthesis while the frog is freezing. This would help to keep all glucose as a

cryoprotectant. The effect of G6P was also stronger with GSK3 from frozen frogs versus controls which might also argue for a stable modification of GSK3 during freezing that makes it more susceptible to G6P allosteric activation. When the frogs thaw, G6P levels fall rapidly (Storey, 1987b) and this would remove the activating effect and might make GSK3 more susceptible to inactivation by phosphorylation. The increase in GSK3 velocity in the control tissue was unexpected since glycogen synthesis would be expected to be occurring in active wood frogs to store G6P when it is present at an increased level, and consequently, GSK3 should be less active when frogs are in a control state in the presence of G6P compared to when G6P is not added. A possible explanation to this unexpected result is that there is less active enzyme in the control muscle, and so such a high concentration of G6P (much higher than physiological levels) should have some sort of effect on the portion of active GSK3. Another explanation is that the addition of sufficient G6P was found to be able to overcome the inactivating effects of phosphorylation and restore nearly full activity to the phosphorylated glycogen synthase enzyme in mammalian muscle tissue (Wilson *et al.*, 2005). And so, in the control state, GSK3 is more active when 5 mM G6P is present, but glycogen synthase enzyme inhibition is overridden by the high G6P concentration, enabling glycogen synthesis to occur.

By contrast when 1 mM AMP was included in the assay, the velocity of GSK3 was significantly reduced ($P<0.05$) for the control enzyme. Activity was reduced by about 34%. When AMP levels rise, cells are running out of energy and often this is due to conditions such as hypoxia/ischemia or high rates of muscle work that compromise the ability to produce ATP by oxidative metabolism in the mitochondria alone. High AMP is

typically a signal that glycogenolysis needs to be activated to increase flux through glycolysis. Furthermore, it is necessary to conserve ATP as much as possible and energy-expensive pathways are shut down. This includes many biosynthetic processes, several of which are regulated by GSK3. Thus, GSK3 inhibits not just glycogen synthesis but also protein synthesis by inhibiting eIF-2B, a critical protein for translation initiation by ribosomes. Also, by inhibiting glycogen synthesis, available glucose can be metabolized by glycolysis when needed to fuel ATP production. AMP is a known activator of glycogen breakdown via GP. Although 1mM AMP appears to significantly inhibit GSK3 in vitro, this concentration is 50-100 fold higher than physiological concentrations, which means that this observed effect probably has no role in regulating the enzyme in vivo. It does imply that adenylates have an effect on the enzyme.

Analysis at 4°C

The velocity of GSK3 did not change significantly for extracts of control of frozen frog muscle when 10 mM alanine, 10 mM aspartate or 5 mM succinate were included in the assay performed at 4°C. GSK3 was slightly inhibited by PEP and glucose at 4°C but the major effectors were again G6P and AMP. GSK3 velocity was significantly higher (by 18%, $P<0.05$) in the presence versus absence of 5 mM G6P for the enzyme from frozen tissue but G6P did not affect the control enzyme when assayed at 4°C. Again, this effect of G6P would help to inhibit glycogen resynthesis under cold or frozen conditions when glycogen breakdown needs to be directed into the synthesis of glucose as the cryoprotectant. AMP also inhibited GSK3 activity at low temperature and here the effects were greater than seen at 22°C and the effect on the control enzyme was substantially

greater with activity reduced by about 60% as compared with the frozen enzyme where AMP inhibited by only about 38%.

Conclusion

In the current study, the levels of GSK3 and a phosphorylated form of GSK3 (ser 9) were assessed in control (acclimated at 5°C), 24 h frozen (at -2.5°C) and 8 h thawed (at 5°C) wood frogs in 5 tissues (brain, heart, kidney, liver, muscle). Total protein levels of GSK3 did not change during freezing and thawing in the brain, liver and muscle. They did decrease significantly during freezing in the wood frog heart, and then increased to near control levels. In the kidney, the total amount of GSK3 protein decreased significantly when thawed for 8 hours.

The levels of phosphorylated GSK3 were significantly lower in all tissues from frozen frogs. This implies that there is more active enzyme present when tissues freeze. Phosphorylated GSK3 levels remained low after 8 h of thawing in brain and heart, but increased somewhat in skeletal muscle and rebounded completely to control levels in liver and kidney. The rapid reversal in liver, in particular, may be closely related to the need to begin to clear the high glucose cryoprotectant levels and restore the carbon as glucose. High levels of phosphorylated inactive GSK3 would allow GS to be dephosphorylated and activated by protein phosphatases.

When looking at the temperature effects on the V_{max} values of the GSK3 enzyme, an expected drop of 3-4-fold is seen in the control enzyme without glucose and the frozen enzyme with and without glucose, when this enzyme is assayed at 4°C compared to 22°C. An unexpected 10-fold decrease in V_{max} for the control GSK3 enzyme with 250

mM glucose could be due to a glucose-induced enzyme modification in the control state which lowers the maximum velocity of the enzyme. When comparing the frozen enzyme assayed at 22°C and 4°C, the K_m dropped by 50% when assayed at 4°C, implying that the frozen enzyme could be modified to have a higher affinity for its substrate. The addition of glucose lowers enzyme activity and increases maximum velocity of control GSK3 enzyme while having the opposite effect on the frozen enzyme. Glucose seems to have somewhat of an inhibitory effect on the control enzyme, but increases the specificity of the frozen enzyme. When the affinity of GSK3 for its peptide is compared in control and frozen tissue at 4°C without glucose, the frozen enzyme has a significantly higher affinity, implying that the enzyme in the frozen tissue is more efficient than that in the control tissue.

Aspartate, alanine and succinate were not allosteric modifiers of GSK3. G6P was found to have an activating effect on the enzyme, whereas AMP suppressed enzyme activity. PEP and glucose also had slight inhibitory effects on GSK3. To our knowledge, this may be the first report of metabolite effectors changing the activity of the active form of GSK3.

Table 3.1

Summary of kinetic data for GSK3 for four different assay conditions: at high (22°C) and low (4°C) temperatures and in the absence versus presence of 250 mM glucose at each temperature. V_{max}, K_m, and Hill coefficient (n_H) values were obtained from the computer analysis of the velocity vs substrate concentration curves, using a Kinetics program. Statistical testing used analysis of variance followed by the Student-Newman-Keuls test. Data are means $\pm$ SEM, n = 4 determinations on separate extracts of control and frozen muscle.

	K_m (μM)	V_{max} (U/mg)	Hill coefficient
Control, 5°C acclimated			
22°C	23.5 ± 4.2	1.83 ± 0.30	1.34 ± 0.13
4°C	29.7 ± 2.6	0.60 ± 0.02 b	1.93 ± 0.55
22°C + 250 mM glucose	40.6 ± 4.0 c	2.89 ± 0.64 c	1.46 ± 0.11
4°C + 250 mM glucose	30.1 ± 2.2	0.30 ± 0.04 b	2.80 ± 0.38
Frozen, -2.5°C (24 hours)			
22°C	33.1± 4.4	2.27 ± 0.18	1.53 ± 0.20
4°C	16.6 ± 0.7 a,b	0.59 ± 0.08 b	1.34 ± 0.18
22°C + 250 mM glucose	21.8 ± 2.9 a,c	1.14 ± 0.35 a,c	2.09 ± 0.26
4°C + 250 mM glucose	33.2 ± 4.5 c	0.41 ± 0.04	2.56 ± 0.80

a = significantly different ($P<0.05$) from the corresponding value from control frogs at the same temperature and glucose concentration

b = significantly different ($P<0.05$) from the corresponding value at 22°C at the same glucose concentration

c = significantly different ($P<0.05$) from the corresponding in the absence of glucose at the same temperature

Figure 3.1

Western blot analysis of GSK3 protein content in brain, heart, kidney, liver and muscle of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen)

(D) Representative Western blots showing GSK3 protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.

(E) Relative levels of GSK3 protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 3.1

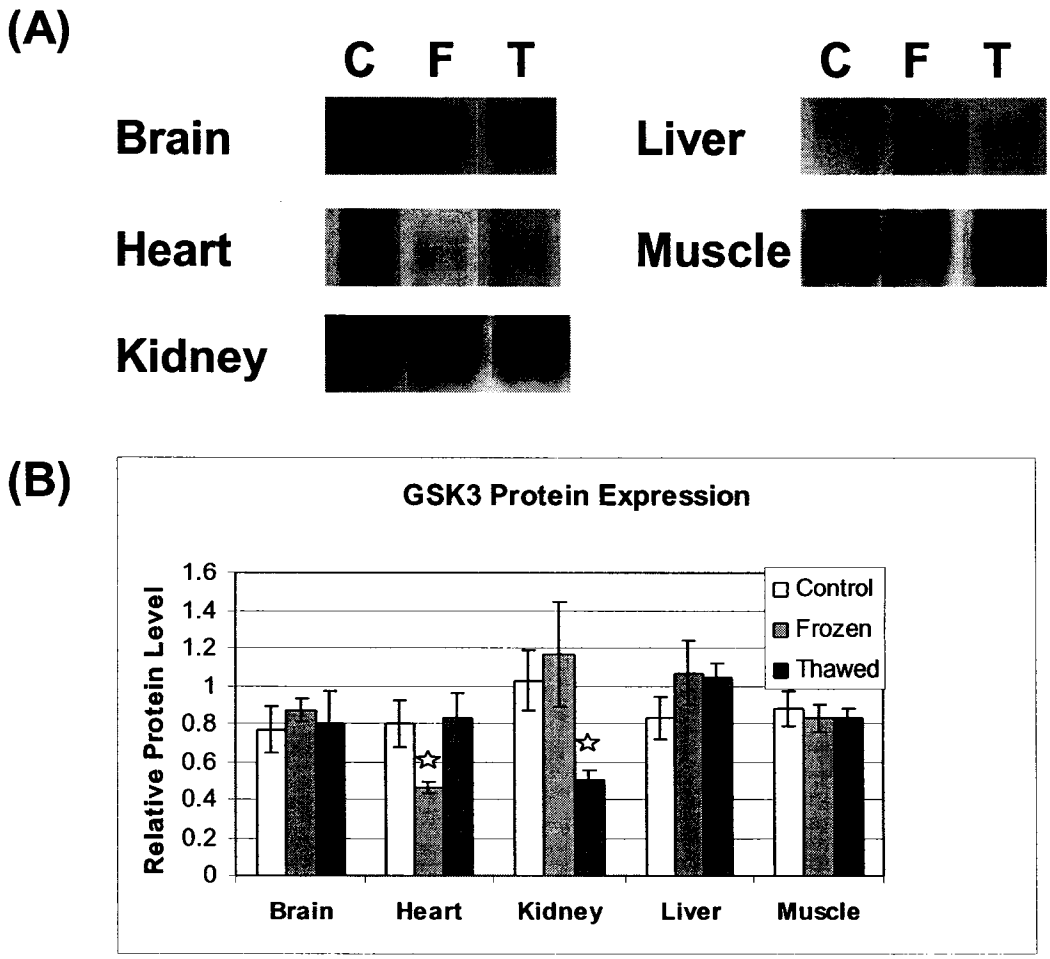


Figure 3.2

Western blot analysis of phospho-GSK3 (ser 9) protein content in brain, heart, kidney, liver and muscle of wood frogs from three conditions: control (5°C acclimated), frozen (24 h at -2.5°C) and thawed (8 h at 5°C after 24 h frozen)

(A) Representative Western blots showing phospho-GSK3 (ser9) protein levels in tissues from control (C), frozen (F) and thawed (T) frogs.
(B) Relative levels of phospho-FOXO1 (ser9) protein in frozen and thawed frogs as compared with controls. Histograms shows mean ± SEM, n=4 independent determinations.

Stars show values that are significantly different from the corresponding control for the same tissue as determined by the Student's t-test, $P < 0.05$.

Figure 3.2

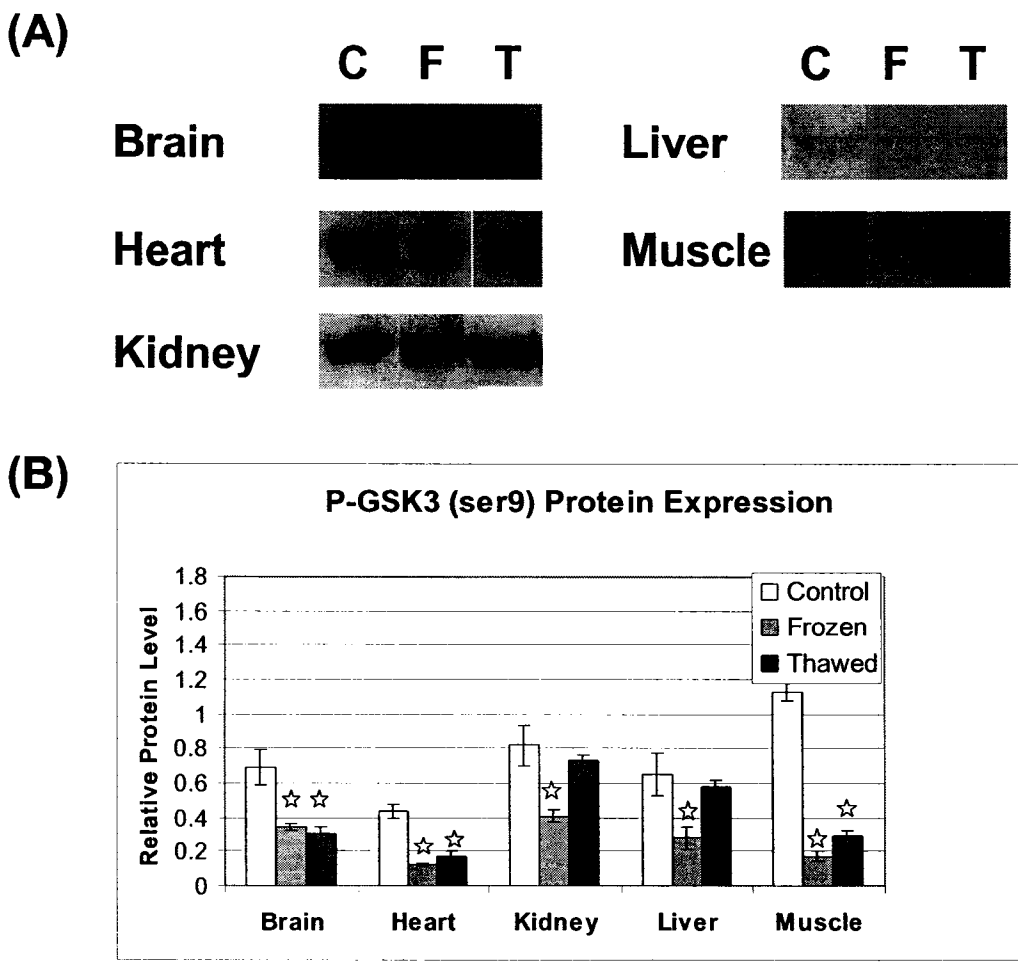


Figure 3.3

Velocity of GSK3 (in units/mg of GSK3) in Rana sylvatica muscle with increasing peptide concentration at 22 °C. Control frog tissue was obtained from frogs acclimated at 5 °C and frozen frog tissue was obtained from frogs frozen at 2.5 °C for 24 hours. One unit is defined as the amount of enzyme that phosphorylates 1 μmol of peptide per minute.

Figure 3.3

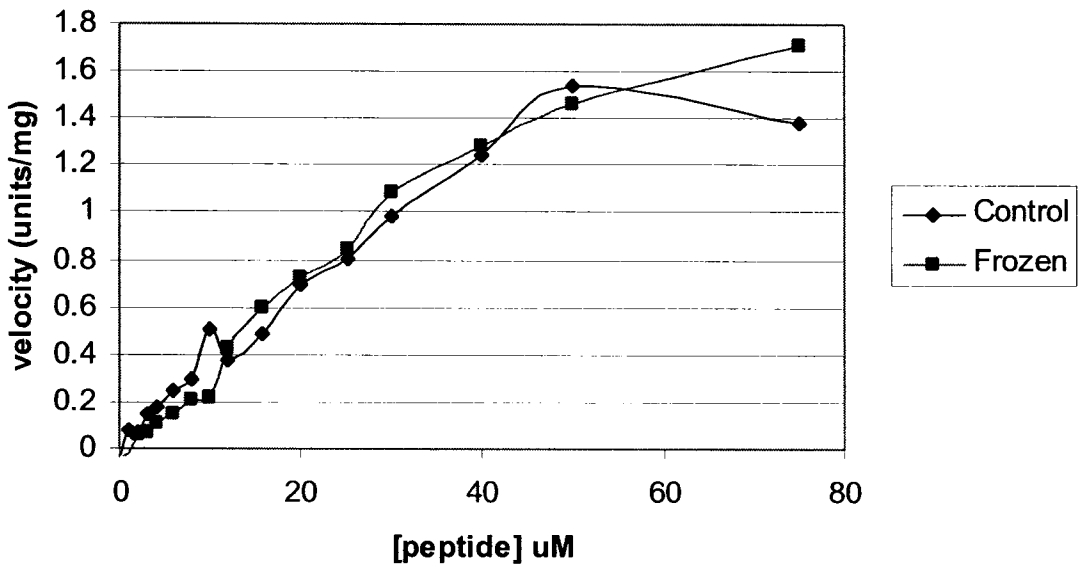


Figure 3.4

Velocity of GSK3 (in units/mg of GSK3) in Rana sylvatica muscle with increasing peptide concentration at 4 °C. Control frog tissue was obtained from frogs acclimated at 5 °C and frozen frog tissue was obtained from frogs frozen at 2.5 °C for 24 hours. One unit is defined as the amount of enzyme that phosphorylates 1 μmol of peptide per minute.

Figure 3.4

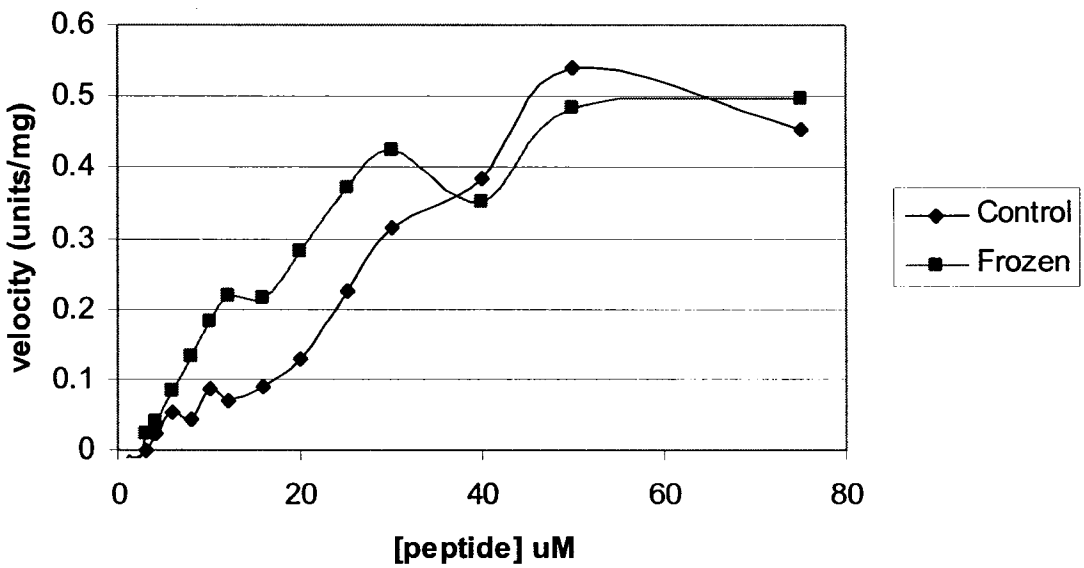


Figure 3.5

Velocity of GSK3 (in units/mg of GSK3) in Rana sylvatica muscle with increasing peptide concentration at 22 °C. Each assay contained 250mM glucose. Control frog tissue was obtained from frogs acclimated at 5 °C and frozen frog tissue was obtained from frogs frozen at 2.5 °C for 24 hours. One unit is defined as the amount of enzyme that phosphorylates 1 μmol of peptide per minute.

Figure 3.5

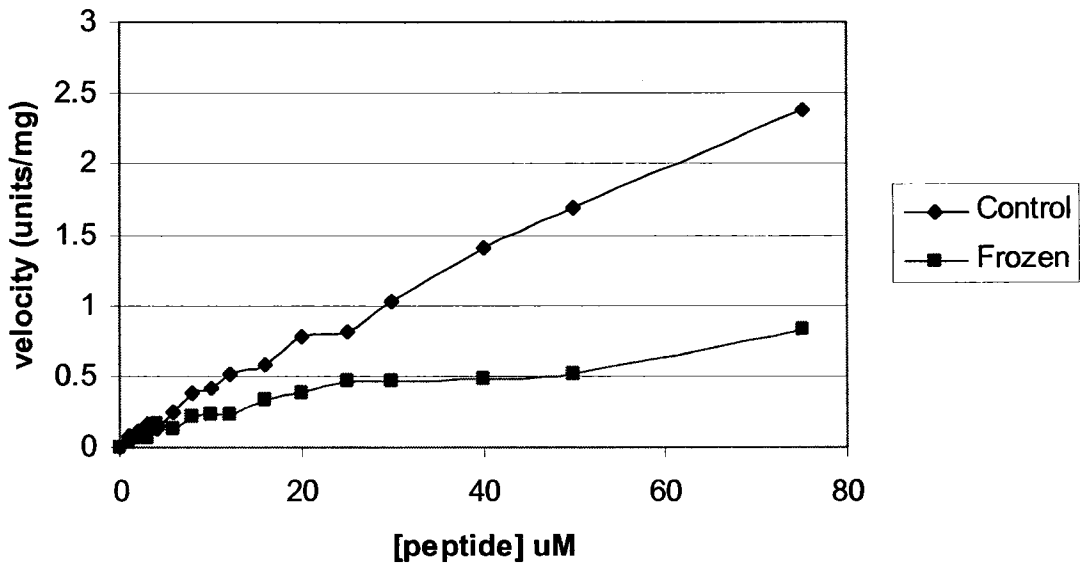


Figure 3.6

Velocity of GSK3 (in units/mg of GSK3) in Rana sylvatica muscle with increasing peptide concentration at 4 °C. Each assay contained 250mM glucose. Control frog tissue was obtained from frogs acclimated at 5 °C and frozen frog tissue was obtained from frogs frozen at 2.5 °C for 24 hours. One unit is defined as the amount of enzyme that phosphorylates 1 μmol of peptide per minute.

Figure 3.6

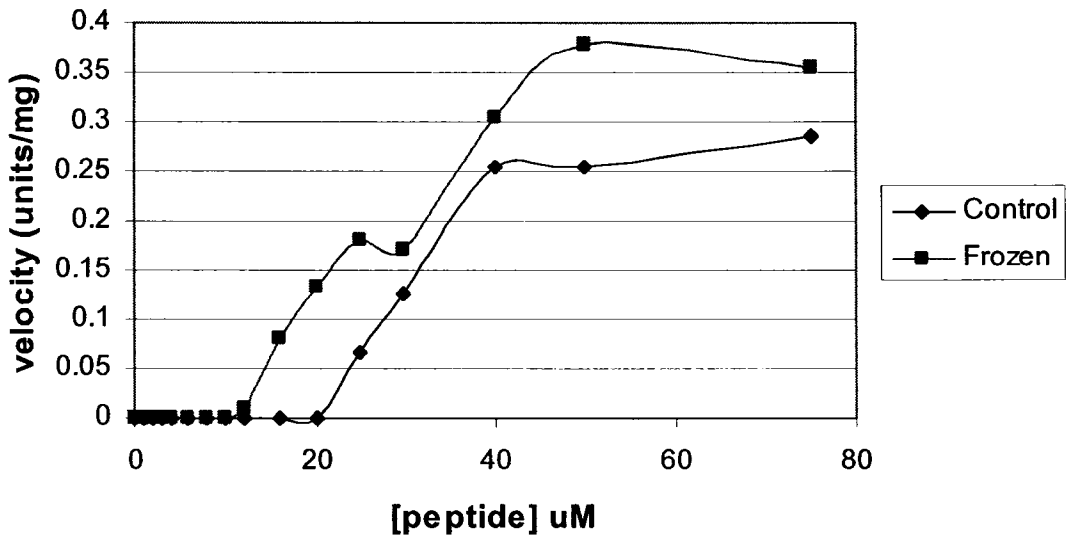


Figure 3.7

Activity of GSK3 with various metabolites (5 mM G6P, 10 mM alanine, 10 mM aspartate, 5 mM succinate, 1 mM AMP, 5 mM PEP, 250 mM glucose) for control (acclimated at 5 °C) and frozen (24 h at 2.5°C) wood frogs. The assays were performed at 22 °C. Histograms shows mean ± SEM, n=4 independent determinations.The bars represent percent of activity compared to GSK3 with no metabolites. Stars show values that are significantly different from the corresponding control with the same metabolite, and diamonds show values that are significantly different from the activity with no metabolite, as determined by the Student's t-test, $P < 0.05$.

Figure 3.7

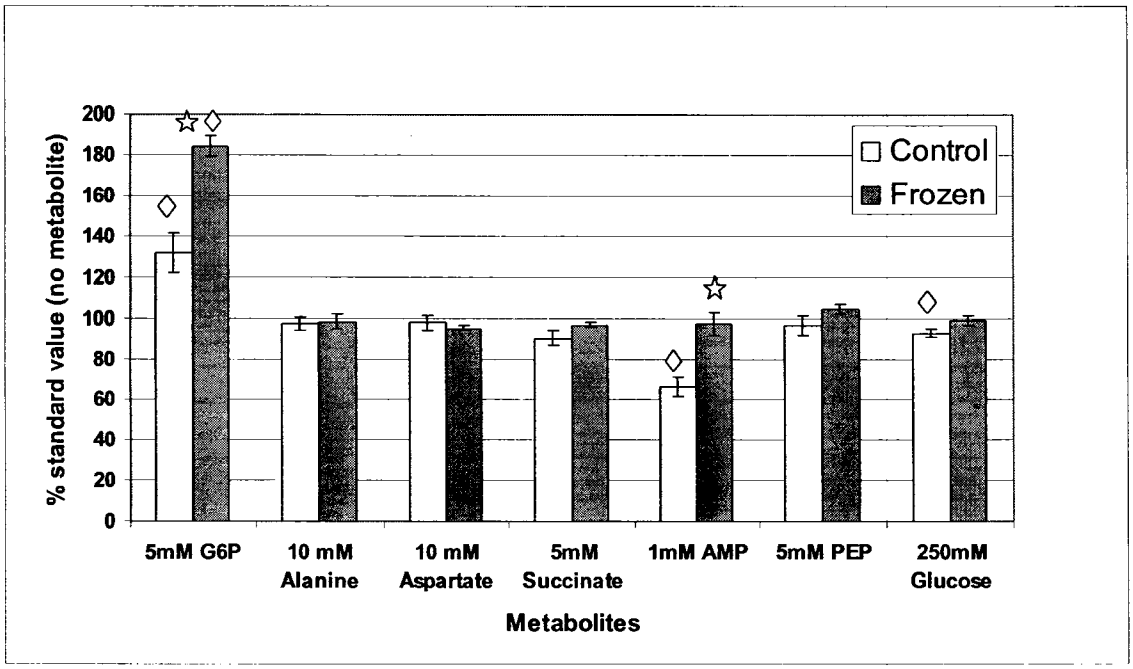


Figure 3.8

Activity of GSK3 with various metabolites (5 mM G6P, 10 mM alanine, 10 mM aspartate, 5 mM succinate, 1 mM AMP, 5 mM PEP, 250 mM glucose) for control (acclimated at 5 °C) and frozen (24 h at 2.5°C) wood frogs. The assays were performed at 4 °C. Histograms shows mean ± SEM, n=4 independent determinations.The bars represent percent of activity compared to GSK3 with no metabolites. Stars show values that are significantly different from the corresponding control with the same metabolite, and diamonds show values that are significantly different from the activity with no metabolite, as determined by the Student's t-test, $P < 0.05$.

Figure 3.8

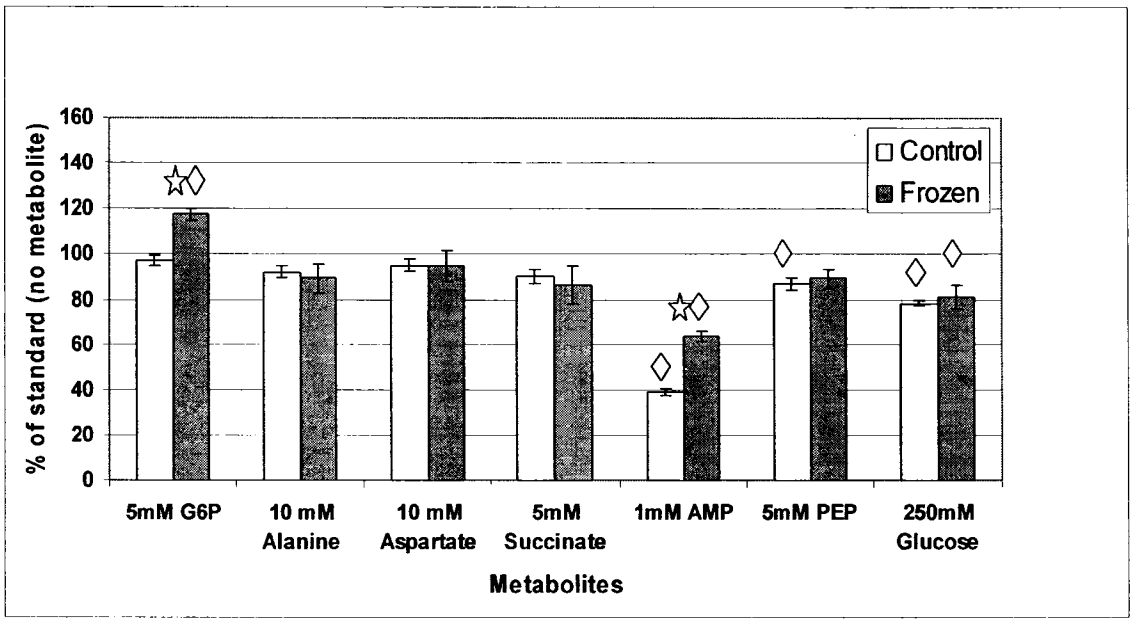
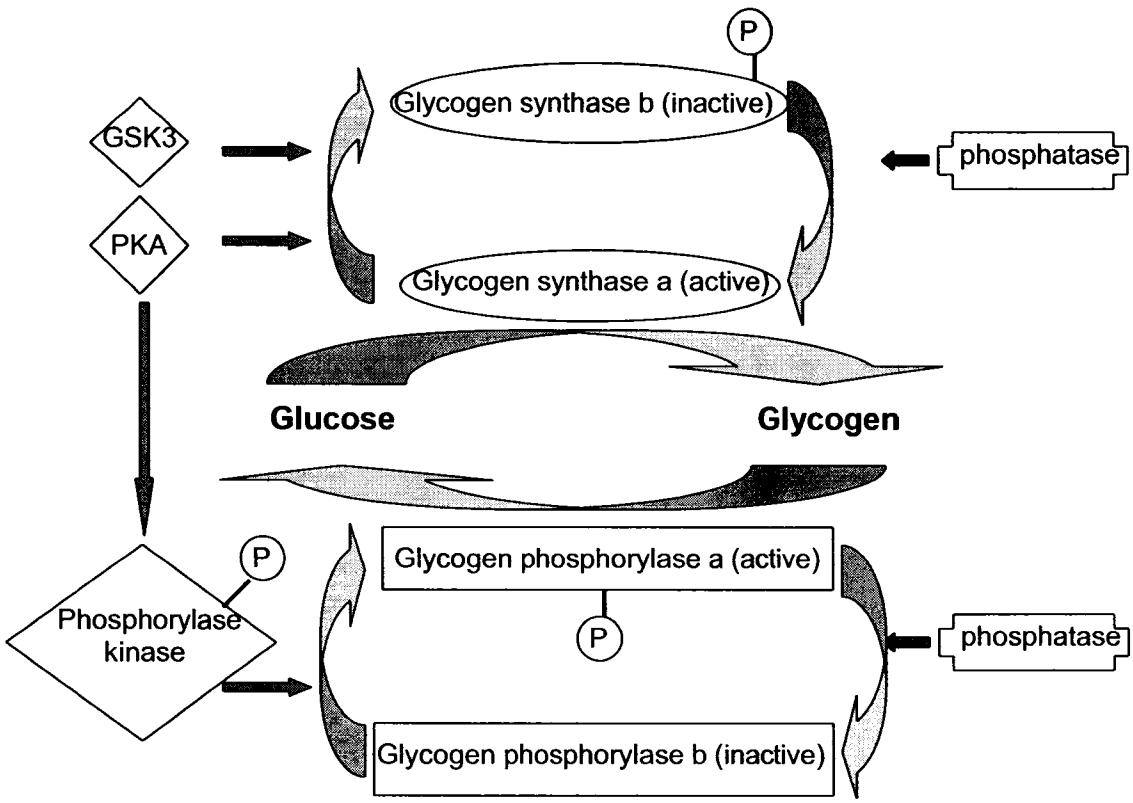


Figure 3.9

General schematic showing the regulation of glycogen synthesis and breakdown.

Figure 3.9



CHAPTER 4:

General Discussion

The Wood Frog

The wood frog, *Rana sylvatica*, is the primary animal used for studies of vertebrate freeze tolerance and is able to survive weeks completely frozen. When the frog is frozen, oxygen-dependent energy production is stopped and intertissue transport of fuels is halted. To survive weeks of freeze-induced ischemia, the wood frog must find ways to survive on fixed supplies of fermentable fuels (mostly carbohydrates and some amino acids) and using just the ATP output from glycolysis which is just one-eighteenth of the amount available from the oxidative catabolism of carbohydrate. To do this, most ATP-consuming metabolic processes are strongly suppressed to 1-5% of normal, only keeping those of which are needed for survival. In this study, the activity of two signaling proteins involved in the insulin signal transduction cascade, Forkhead box other (FOXO) transcription factors and glycogen synthase kinase 3 (GSK3) were analyzed, and their role in the wood frog's ability to survive freezing was determined.

Metabolic Rate Depression

The ability to highly reduce metabolic rate and enter a hypometabolic state is a life-saving mechanism for many organisms. When animals are faced with environmental conditions that would limit their ability to survive, they can enter a state of dormancy until the conditions are acceptable. Some examples of environmental conditions that induce hypometabolism are: high and low temperature, oxygen deprivation, food restriction and water limitation (Storey and Storey, 1990). In most cases of hypometabolism, the organism experiences little or no change to the internal environment of their cells and organs, even though their metabolic rate is lowered to between 5 and

40% of the resting rate of the normal state. This lack of change enables a rapid arousal when favourable environmental conditions return (Guppy and Withers, 1999).

The rate of ATP utilization by ATP-consuming processes must match the rate of ATP production by central pathways of fuel catabolism to maintain homeostasis in any cell. To survive, all cells have ways of increasing ATP production when energy demand is high and of scaling back production when energy demand declines. Also, situations that limit ATP production will quickly affect the rates of ATP-utilizing processes. Studies have shown that the pathways of macromolecular biosynthesis (such as protein synthesis, RNA/DNA synthesis) are more sensitive to ATP availability than various other activities such as transmembrane ion pumping (Buttgereit and Brand, 1995). In stress intolerant organisms, a drop in ATP production due to a limitation such as oxygen depletion or ischemia leads quickly to an energy crisis in cells because the demand by ATP-utilizing reactions cannot be met. The key factor that allows long term survival in organisms that can enter hypometabolic states is, therefore, the ability to coordinate the suppression of ATP-utilizing reactions to match the rate of ATP production; the rate of ATP use by cellular reactions is rebalanced and reprioritized in order to achieve a net overall reduction in ATP turnover that can be sustained over the long term in the hypometabolic state. This typically involves shutting down energy expensive cellular activities such as biosynthesis, the cell cycle, growth/differentiation and minimizing the rates of ATP use by vital processes such as the maintenance of membrane potential difference. This latter is one of the most energy-expensive and energy-sensitive functions of cells. When ATP levels are low, membrane potential difference is quickly dissipated with many negative consequences due to a rapid imbalance in the opposing rates of ion transport across

membranes by ATP-dependent ion pumps versus ATP-independent ion channels (Perez-Pinzon *et al.*, 1992; Hochachka and Lutz, 2001). Stress tolerant organisms quickly suppress and rebalance the activities of ion pumps and ion channels so that membrane potential is maintained but at a much lower rate of ATP turnover.

Protein synthesis is another highly energy-expensive process, requiring about five ATP equivalents per peptide bond formed and consuming a substantial portion of the total ATP budget of all cells (e.g. ~36% in normoxic turtle liver; Hochachka *et al.*, 1996). Protein synthesis is well known to be affected by the availability of energy and amino acids and is suppressed, for example, during starvation or hypoxia (Casey *et al.*, 2002; DeGracia *et al.*, 2002; Mordier *et al.*, 2002). Not surprisingly, suppression of protein synthesis is a key characteristic of hypometabolic states (Joplin and Denlinger, 1989) and, in addition, the proportion of cellular ATP turnover devoted to protein synthesis is greatly lowered in hypometabolism (Land *et al.*, 1993). Primary control over protein synthesis is due to the strong regulation of ribosomal proteins, particularly selected initiation and elongation factors. Inhibition of translation initiation is often due to inhibitory control of the eukaryotic initiation factor 2 (eIF2). The mechanism involved is phosphorylation of the alpha-subunit of eIF2 (eIF2α) (Rhoads, 1993; Mikulits *et al.*, 2000), phospho-eIF2α being a dominant inhibitor of the guanine nucleotide exchange factor eIF2B to prevent the recycling of eIF2α between successive rounds of peptide synthesis (Clemens, 2001; DeGracia *et al.*, 2002).

Reversible protein phosphorylation is a major mechanism responsible for coordinating and rebalancing not just membrane ion transport and cellular protein synthesis but many other cell functions and plays a dominant role in regulating entry into

hypometabolic states (Storey and Storey, 2004b). Not only does covalent modification mechanism allow for rapid changes in activity states of enzymes and functional proteins but it is easily reversible to allow a quick return to normal metabolic functions when environmental conditions permit. Reversible protein phosphorylation regulates the activities, kinetic properties, binding interactions and subcellular locations of a huge number of metabolic enzymes, signal transduction enzymes, functional proteins, and transcription factors. Signal transduction cascades use this technique for the transmission and amplification of signals starting from membrane receptors, spreading through multiple intermediary steps, and ending in the alteration of protein function or gene expression (Cowan and Storey, 2003; MacDonald, 2004).

The activities the FOXO transcription factor family as well as GSK3 are controlled by reversible protein phosphorylation. Both FOXO factors and GSK3 are inactivated by phosphorylation of various protein kinases acting at multiple phosphorylation sites. FOXO transcription factors and GSK3 also catalyze the reversible protein phosphorylation to control their downstream targets, either activating or repressing them. This study shows that when the wood frog is frozen, in general, there is a reduced amount of the phosphorylated forms of FOXO1 and FOXO3, which implies that these transcription factors are more active during freezing (and during anoxia for FOXO3). By being more active, they can upregulate the transcription of important downstream targets such as manganese superoxide dismutase and catalase to help deal with oxidative damage and also repress genes such as cyclin D1 to induce cell cycle arrest to conserve energy in the frozen state. This study also demonstrated that the relative amount of phosphorylated GSK3 was reduced during freezing in frog organs

implying an opposite increase in the amount of dephosphorylated active enzyme in the frozen frog. GSK3 in muscle of frozen frogs also had a higher affinity for its substrate when compared to the enzyme in control tissue. A more active GSK3 could then phosphorylate and regulate key enzymes as the frog enters the hypometabolic frozen state including the eukaryotic initiation factor 2B (an enzyme critical for translation initiation) and glycogen synthase (the final enzyme of glycogen biosynthesis). Phosphorylation of these enzymes by GSK3 renders them inactive, and would consequently inhibiting protein translation, to save energy, and glycogen synthesis, to maintain glucose pools as a cryoprotectant.

Implications of the Insulin Pathway

Insulin controls a wide variety of biological responses including stimulation of glucose uptake, glycogen, lipid and protein synthesis, antilipolysis, activation of transcription of specific genes, and modulation of cellular growth and differentiation. Protein phosphorylation and dephosphorylation is the main mechanism involved in mediating and coordinating the molecular actions of insulin.

The insulin receptor is an α2/β2 tetramer. The α-subunit contains the insulin binding site and is located entirely at the extracellular face of the plasma membrane, whereas the β-subunit is a transmembrane peptide (Van Obberhen *et al.*, 1981). The intracellular portion of the β-subunit contains the insulin-regulated tyrosine protein kinase (Kasuga *et al.*, 1983). When insulin binds to the α-subunit, it activates the tyrosine kinase in the β-subunit which leads to the autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit and also to the phosphorylation of the insulin

receptor substrate (IRS) proteins. Tyrosine-phosphorylated IRS-proteins generate downstream signals by direct binding to the SH2 domains of various signaling proteins. Several enzymes and adaptor proteins have been identified that associate with IRS proteins, including phosphatidylinositol-3-kinase (PI3K) (White, 1998).

One of the earliest steps in the insulin signaling pathway is the activation of PI3K. One of its major actions is to phosphorylate phosphatidylinositol 4,5-diphosphate (PtdIns(4,5)P_2) to produce phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) which is an important second messenger in signal transduction (Stephens *et al.*, 1991). PI3K plays a major role in many insulin-regulated responses including the stimulation of glucose uptake (Clarke *et al.*, 1994), general and growth-specific protein synthesis (Mendez *et al.*, 1997) and cell growth and proliferation (Jhun *et al.*, 1994). PI3K also participates in regulating the expression of key genes such as the phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (Agati *et al.*, 1998 ; Dickens *et al.*, 1998). Induction of hexokinase II (Osawa *et al.*, 1996), glucose-6-phosphate dehydrogenase (Wagle *et al.*, 1998), and GLUT4 gene expression (Valverde *et al.*, 1999) also requires PI3K. In addition, the activation PI3K has been shown to be both necessary and sufficient for the activation of protein kinase B (PKB, also known as Akt). The primary mechanism for the activation of PKB is phosphorylation on two sites (Thr 308 and Ser 473). Phosphorylation at Thr 308 is achieved by the phosphoinositide-dependent protein kinase (PDK1) (Walker *et al.*, 1998). The kinase responsible for Ser473 phosphorylation has not been cloned yet, but is tentatively named PDK2 (Alessi and Cohen, 1998). The mechanism for PKB activation involves interaction with PtdIns(3,4,5)P_3 through its PH domain which recruits PKB to the plasma membrane and

results in conformational changes to PKB so that Thr and Ser become accessible to phosphorylation by PDK1 and PDK2, respectively (Alessi *et al.*, 1996b). Many PKB substrates have been identified and include, among others, BAD, CREB, members of the forkhead family of transcription factors, Iκ-B kinase, procaspase-9, GSK3 and mTOR/FRAP (Rodiguez-Viciana *et al.*, 1994). The large variety of proteins that are phosphorylated by PKB explains why this kinase is a key mediator of cell proliferation, differentiation and survival.

Skeletal muscle is the major site of insulin-stimulated glucose uptake, and most of the glucose that enters human muscle fibers in response to insulin is deposited as glycogen. Insulin causes activation of glycogen synthase by promoting dephosphorylation of multiple sites on the enzyme (Lawrence and Roach, 1997). The enzyme GSK3 has been one of the best-studied kinases for glycogen synthase regulation. An insulin-triggered cascade leads to phosphorylation and inactivation of GSK3 mediated by the actions of PI3K and PKB (Akt) (Welsh and Proud, 1993). Insulin also plays a key role in the overall regulation of protein synthesis. Several initiation and elongation factors are regulated by the hormone, usually as a consequence of changes in their states of phosphorylation. Regulation of eukaryotic initiation factor-2B (eIF-2B) is a key step in translation initiation. eIF-2B is activated by insulin signaling via a PI3K mediated mechanism (Welsh and Proud, 1993) whereas the inactivation of eIF-2B is controlled by GSK3 (Welsh *et al.*, 1997).

The insulin pathway is involved in important, but high ATP-consuming processes, such as the synthesis of glycogen, lipids and proteins, cellular growth and differentiation, and the transcription of specific genes. These processes are important in normal

metabolic states, but under stressed states, they must be suppressed to conserve energy. For example, when the wood frog is frozen, its metabolic rate is depressed to conserve energy in order to extend its survival throughout the winter months. Since the insulin pathway consumes a lot of energy and since the wood frog needs to conserve energy in the frozen state, the insulin pathway needs to be turned off while the frog is frozen. The insulin pathway normally represses the activity of FOXO transcription factors and GSK3, but when the insulin pathway is turned off, these proteins are more active, which was seen in the results obtained in this study. Another reason for the insulin pathway to be less active during freezing is because the insulin pathway is involved in stimulating glucose uptake. When frozen, blood glucose levels of wood frogs rises to 150-300 mM, since it is used as a cryoprotectant. To keep blood glucose levels high, glucose uptake and its catabolism or reconversion to glycogen must be stopped, and since the insulin pathway is involved in stimulating glucose uptake, the insulin pathway needs to be turned off.

Conclusion

To survive whole body freezing for weeks during the winter months, the wood frog uses several energy-saving mechanisms that allow it to survive over the long term using only its internal fuel reserves. Metabolic rate depression, including reversible protein phosphorylation of many key target proteins, is a large part of this energy conservation mechanism. In addition, selected key genes need to be up-regulated to ensure survival. This study determined that two of the forkhead box 'other' transcription factors, FOXO1 and FOXO3, are kept active during freezing. These proteins are kept

active because they are involved in oxidative damage prevention and cell-cycle arrest. In this study, GSK3 was also shown to be more active during freezing, presumably to inhibit protein translation and glycogen synthesis in the frozen state. Both FOXO factors and GSK3 are phosphorylated and inactivated by protein kinase B (also known as Akt), a downstream kinase of the insulin pathway (Figure 4.1). Since FOXO factors and GSK3 are both active during freezing, the insulin pathway must be turned off.

Future Directions

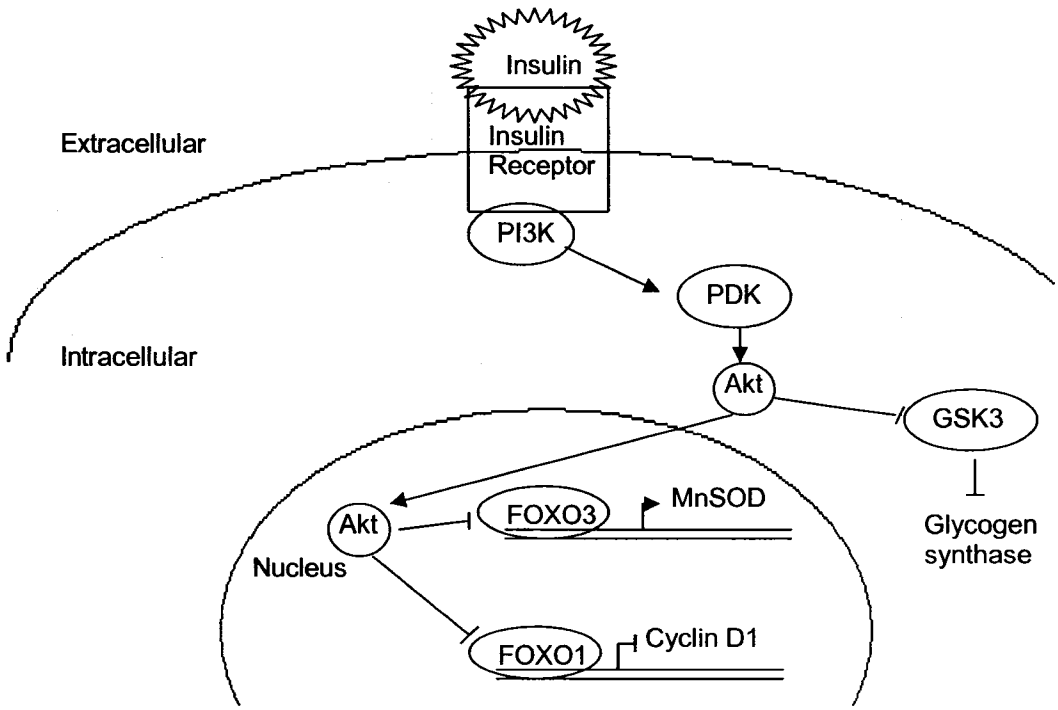
In the present study, I have looked at the implications of FOXO factors and GSK3 on the ability of the wood frog to survive whole body freezing. Both FOXO factors and GSK3 are phosphorylated and controlled by protein kinase B (also known as Akt), and so the properties of this enzyme should be examined. Radioactive kinase assays could be used to look at the activity of Akt in the control and frozen state. The effects of glucose, temperature and different metabolites could also be considered, similar to the GSK3 assays performed in this study. Downstream targets of both FOXO and GSK3 would also be valuable to study. Since FOXO1 and FOXO3 are transcription factors, the polymerase chain reaction could be used to look at changes in the mRNA levels of known downstream targets of FOXO signaling. Examples of important downstream targets of FOXO1 and FOXO3 are cyclin D1 for FOXO1 and MnSOD for FOXO3. Cyclin D1 is also a downstream target for phosphorylation by GSK3, and so the amount of total cyclin D1 protein and also the amount of phosphorylated cyclin D1 protein in the control and frozen wood frog could also be examined using Western blotting and phospho-specific antibodies.

AMP was shown to have an inhibitory effect on the activity of GSK3 when present at a concentration of 1 mM. The physiological concentration of AMP was previously found to be in the 0.02-0.05 mM range, which makes this result inaccurate in vivo. Since a high concentration of AMP inhibited the enzyme, it implies that adenylates have an effect on GSK3 activity. To study the effect of adenylates on the activity of GSK3, the concentration required for 50% inhibition of GSK3 (IC_{50}) could be measured for different adenylates (ATP, ADP, AMP, A, IMP, I).

Figure 4.1

The insulin pathway: phosphorylation of FOXO factors and GSK3 by Akt.

Figure 4.1



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