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ROLE OF DIFFERENTIAL GENE AND PROTEIN REGULATION IN HIBERNATING MAMMALS

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B.Sc. University of New Brunswick, 1998

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

Doctor of Philosophy

Department of Chemistry

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**“ROLE OF DIFFERENTIAL GENE AND PROTEIN REGULATION IN
HIBERNATING MAMMALS”**

submitted by

Sean F. Eddy, B.Sc. (Hons)

in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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January 2003, Carleton University

ABSTRACT

Winter survival for many mammals involves hibernation. By strongly suppressing metabolic rate, animals conserve energy and endure long months of subzero environmental temperatures and lack of food. The little brown bat, *Myotis lucifugus*, and thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, are two such hibernating mammals. Hibernation consists of long periods (1-2 weeks) of cold torpor with body temperature near ambient interspersed with brief periods of arousal to euthermia. Precise control over gene and protein expression is needed to prevent costly overuse of essential fuel reserves while ensuring that selected specific changes are made that aid survival in the cold, torpid state. The present studies evaluated signal transduction pathways and changes in gene expression in skeletal and cardiac muscle during hibernation. The p38 MAP kinase signal transduction pathway in skeletal muscle of both species appears triggered with increased amounts of active phosphorylated p38 found during hibernation along with activation of various downstream targets of p38 including ATF-2, CREB, HSP27 and I κ B. The Akt-mediated insulin-signalling pathway was, by contrast, apparently suppressed in hibernator muscle; this may help restrict the use of carbohydrate fuels. cDNA arrays were used to compare gene expression in euthermic versus hibernating states; selected genes (0.5-2%) were up-regulated during hibernation but most were unaffected. A general suppression of protein synthesis is likely during hibernation and this was supported by elevated levels of phosphorylated initiation (eIF2 α) and elongation (eEF2) ribosomal proteins. Despite the probable suppression of transcription and translation, selected genes and proteins were up-regulated during

hibernation including two involved in fatty acid transport and metabolism: fatty acid-binding protein and carnitine palmitoyl transferase-1. Furthermore, two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry revealed the up-regulation of a thioredoxin peroxidase type enzyme in heart of *M. lucifugus*; both mRNA and protein levels rose during hibernation. This, along with elevated amounts of the active forms of oxidative stress markers, HSP27 and I κ B, demonstrates that oxidative stress has a role in hibernating tissues. The data enhance our knowledge of the molecular mechanisms of hibernation with novel contributions to the understanding of the roles of fatty acid metabolism, oxidative stress, and muscle atrophy.

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List of Abbreviations

AA	amino acid
Akt	protein kinase B
AP-1	activating protein 1
ATF	AP-1 binding transcription factor
BAT	brown adipose tissue
cDNA	complementary DNA
cpm	counts per minute
CPT	carnitine palmitoyl transferase
CREB	cAMP response element binding protein
Cy3/Cy5	Cyanine dye 3 or 5
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
eEF2	eukaryotic elongation factor subunit 2
egr	early growth response
EGTA	ethyleneglycol bis(β -aminoethyl ether) tetra-acetic acid
eIF2 α	eukaryotic initiation factor 2 α subunit
EtBr	ethidium bromide
FABP	fatty acid binding protein
HSP	heat shock protein
I κ B	inhibitor of NF κ B
kb	kilobase
kDa	kilodalton

MAPK	mitogen activated protein kinase
mRNA	messenger RNA
NF κ B	nuclear factor κ -B
ORF	open reading frame
Pag	proliferative associated gene
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PGC	PPAR γ co-activator
PMSF	phenylmethysulfonyl fluoride
PPAR	peroxisome proliferation activated receptor
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcription
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
T _b	body temperature
Tris	tris(hydroxymethyl)aminomethane
UCP	uncoupling protein
WAT	white adipose tissue

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Chapter 1

General Introduction: Hibernation

Mammalian hibernation is a winter survival strategy for conserving energy that would otherwise be needed to maintain a constant high or euthermic body temperature (T_b), normally about 37°C, when air temperatures drop below freezing and food availability is severely restricted or lacking. This energy-saving strategy is characterized by a synchronized suppression of metabolic rate, reduction of heart rate and the rates of all other physiological processes, and a strong reduction in T_b . The T_b of hibernating mammals is routinely lowered to near ambient and values have been recorded as low as -2 or -3°C for Arctic ground squirrels [Wang and Lee, 1996; Boyer and Barnes, 1999; Storey, 2000]. Wang (1979) has estimated that hibernating mammals can conserve up to 88% of the energy that would otherwise be required to maintain a euthermic T_b over the winter. By entering a deep torpor, the hibernator minimizes its consumption of metabolic fuel and the production of waste and can survive for many months fueled only by its lipid reserves.

Prior to hibernation, animals go through a period of hyperphagia, laying down large fat reserves and increasing body weight by as much as 50%. During this prehibernation period, hibernating species change their feeding habits to maximize their intake of fatty acids, in particular increasing their intake of foodstuffs that are high in polyunsaturated fatty acids (PUFAs) [Geiser, 1990; Geiser et al., 1994; Harlow and Frank, 2001]. A high PUFA content in the lipid deposits of white adipose tissue (WAT), brown adipose tissue (BAT), and other tissues is necessary in order to maintain fluidity of the lipids so that they can be metabolized at the low T_b values of the hibernating state [Harlow and Frank, 2001].

Torpor and Hibernation

Torpor occurs in many kinds of mammals as well as some birds. By dropping T_b and metabolic rate animals conserve energy stores or, viewed in another way, animals extend the time that a fixed reserve of body fuel can support life. It has been hypothesized that the ability to enter torpor and hibernation was existent in a common mammalian ancestor, however as new mammals evolved, many did not retain this ability for reasons that are not entirely known [Srere et al., 1992]. The duration of torpor bouts varies from species to species. Torpor may last for a single night in daily heterotherms such as the deer mouse, *Peromyscus maniculatus* [Nestler, 1992] or up to 90 days in the big brown bat, *Eptesicus fuscus* [Yacoe, 1983]. When the duration of torpor increases in long winter months, it is referred to as hibernation. The depth of hibernation is related to the amount of metabolic suppression that occurs. This can be represented by the reduction in T_b , heart rate and O_2 consumption that occurs deep in the hibernation cycles of various animals.

Hibernation consists of a series of torpor bouts, each of which can be divided into entry, hibernation, arousal and a short period of euthermia before the next cycle begins. Changes in photoperiod, ambient temperature, diet composition and food availability during the prehibernation period all influence the development of the capacity to hibernate. These external signals influence internal regulatory mechanisms generating signals in the brain that are sent out to other cells and organs. A neuropeptide known as the hibernation induction trigger (HIT) [Oeltgen et al., 1978] has been proposed to initiate hibernation and may act as a master control for initiating a plethora of cellular

responses that support hibernation. Presently, no conclusive evidence for the HIT exists. Nonetheless, such an internally generated signal, whatever its identity, apparently acts as a master signal for various secondary responses that occur, such as reversible phosphorylation of multiple proteins/enzymes including the eukaryotic initiation factors (eIF) and elongation factors (eEF) of ribosomes [Frerichs et al., 1998; Chen et al., 2001], ion motive ATPases (e.g. NaK-ATPase) [MacDonald and Storey, 1999] and various metabolic enzymes such as pyruvate dehydrogenase (PDH) [Brooks and Storey, 1992; Heldmaier et al., 1999].

Arousal

Arousal from torpor can take as little as 20 minutes or as long as several hours depending on the size of the mammal in question. Non-shivering thermogenesis in BAT provides the initial warming. This occurs by uncoupling the electron transport chain from oxidative phosphorylation via the action of uncoupling proteins (UCP). These proteins create a futile cycle in the proton gradient, which results in proton leakage and the release of energy as heat that would otherwise drive ATP formation. Recent studies have documented tissue specific expression patterns of UCP homologs in hibernating and cold exposed ground squirrels [Boyer et al., 1998, Liu et al., 2001]. After the initial stage of rewarming, shivering thermogenesis by skeletal muscles begins [Wang et al., 1987]. Arousals occur throughout the hibernation season and in mid-winter occur only every 3-4 weeks. In ground squirrels, arousals last up to 20 hours. The process of periodic rewarming is very costly for the animal and accounts for upwards of 90% of the total energy consumption over the hibernation season. Why these arousals occur is not yet

clear. Many hypotheses have been proposed, including the possible need to restore ionic balance, replenish carbohydrate reserves, or eliminate metabolic waste.

Model Animals

Two types of model hibernating animals are used in the studies described in this thesis. The hibernators studied are both small mammals that experience extreme reductions in metabolic rate, heart rate and oxygen consumption. The little brown bat, *Myotis lucifugus*, represents a small group of hibernating bats and the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, a group of ground squirrel species that are obligatory hibernators.

Myotis lucifugus, the little brown bat, is one of the smallest hibernating animals and one of a number of hibernating bats. An insectivorous bat, it populates most of North America from northern Alaska to southern parts of Mexico. Insectivorous bats prepare for hibernation by entering torpor for up to 14 hours a day [Speakman and Rowland, 1999] through selection of colder roosting locations ($\sim 10^{\circ}\text{C}$). By entering torpor prior to hibernation, energy expenditure is significantly reduced allowing bats to control mass balance and build up lipid fuel reserves. Torpor bouts for *M. lucifugus* begin as early as August and hibernation can last for 8 to 9 months of the year.

The thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, is one of several species of hibernating ground squirrels. *S. tridecemlineatus* inhabits most of the North American great plains and feeds primarily on seeds, flowers and insects [Wang and Lee, 1996]. Hibernation cycles routinely begin around late September, when animals go into intermittent states of torpor and arousal before entering deep torpor in late October

[Boyer et al., 1998]. The cycles of torpor followed by interboutal arousals continue throughout the winter months until the animal is fully aroused from hibernation and remains in a euthermic state. Selected studies in this thesis also involve another species, the Richardson's ground squirrel, *Spermophilus richardsonii*. This species mainly inhabits prairie regions and grasslands but has a diet and hibernation cycle similar to *S. tridecemlineatus*.

Hibernating bats and ground squirrels represent a number of unique animals that endure and adapt to harsh climates. The physiological conditions experienced by hibernators would be extremely injurious to other mammals, including humans. For example, humans and most other mammals exhibit severe disruptions in metabolic control when T_b falls below $\sim 25^\circ\text{C}$ [Granberg, 1991], yet during the hibernation season bats and ground squirrels show little, if any, negative effects of prolonged exposure to hypothermia or any of its associated consequences [Hochachka, 1986; Frerichs et al., 1998]. A major consequence of reduced T_b is a large decrease in heart rate, which in turn results in a decrease in oxygen perfusion that would be considered severely ischemic at normal T_b [Frerichs et al., 1994].

Metabolic control of hibernation at the molecular level involves both gene regulation and protein regulation. In the case of protein regulation, this often involves reversible phosphorylation. Genes that are up-regulated in response to hibernation range from a number of stress proteins responding to antioxidant stress to a plethora of genes involved in fatty acid metabolism. Hibernation also includes distinctive organ-specific up-regulation of genes [Wilson et al., 1992; Carey and Martin, 1996; Andrews et al.,

1998; Boyer et al., 1998; Falhman et al., 2000; Bauer et al., 2001; Hittel and Storey, 2001; Buck et al., 2002; Epperson and Martin, 2002].

This thesis focuses on the responses made by muscle tissues (heart and skeletal muscle) in response to hibernation.

Heart

The heart is one of the most profoundly affected organs in a hibernator. Between euthermia and hibernation, heart rates drop from upwards of 300 beats per minute (bpm) to 5 or less [Andrews et al., 1998] in ground squirrels. During hibernation, the heart catabolizes fatty acids almost exclusively and, indeed, the catabolism of carbohydrate fuels is strongly suppressed during torpor. This is achieved through inactivation of pyruvate dehydrogenase (PDH) [Brooks and Storey, 1992] as a result of enhanced expression of PDH kinase isozyme 4 (PDK4) [Andrews et al., 1998], which phosphorylates and inactivates PDH. Lipid metabolism during hibernation is also facilitated by increased expression of pancreatic lipase (PLP) [Andrews et al., 1998]. Another recent study [Belke et al., 1998] has also shown an increase in palmitate oxidation in hibernating hearts, with a concurrent suppression of acetyl-CoA carboxylase. Recent studies in our lab have documented the up-regulation during hibernation of selected genes including NADH ubiquinone oxidoreductase subunit 5 (*Nad5*), the ventricular isoform of myosin light chain 1 (*Mlc1*) [Fahlman et al., 2000] and adipose and heart-type fatty acid binding proteins (*a-* and *h-fabp*) [Hittel and Storey, 2001]. FABPs function in the intracellular transport of fatty acids from the cell membrane or intracellular lipid droplets to the mitochondria, whereupon the fatty acids pass through membrane transporters into the mitochondrial matrix for oxidation. Up-regulation of

Nad2 during hibernation may be due to an increase in the number of mitochondria in the hibernating heart. Up-regulation of *Mlc1* may be a part of myosin restructuring that readjusts the composition of the contractile proteins for optimal functioning at low body temperature.

Skeletal Muscle

Muscle disuse atrophy is a loss of functioning muscle due to extended periods of inactivity. In man, such factors as ageing, lack of exercise or space flight all result in a loss of skeletal muscle strength and function due to atrophy. Hibernating animals provide a natural model for studying disuse atrophy in skeletal muscle. During a typical year, hibernating animals, such as bats and ground squirrels, may spend up to eight months in a state of hibernation, the result of which is very little physical activity or use of their muscles. Hibernating animals experience atrophy to a much lesser extent that might be expected over eight months of disuse. It is believed that in order to retain physical strength required during arousal that hibernating animals must maintain a balance of muscle loss and muscle gain that prevents atrophy from exceeding some critical level. The skeletal muscle of bats must maintain its structure over the hibernation cycle; the leg muscle (gastrocnemius) must maintain itself so bats can roost and flight muscle (pectoralis) must not change fibre composition so that, upon arousal, bats can return to flight. Hibernating rodents on the other hand, do not have to maintain muscle composition to the same extent that is seen in hibernating bats.

One common theme is that both types of hibernators undergo significant muscle atrophy [Egginton et al., 2001; Kim et al., 2000; Wickler et al., 1991]. However, the fact

that muscles atrophy does not necessarily mean that they lose functionality. Quite the contrary, hibernating pectoralis muscle of *M. lucifugus* maintains its fibre composition, while decreasing its oxidative and glycolytic potential [Brigham et al., 1990]. Hibernating muscles increase their ability to exploit fatty acids by increasing lipolytic activity, which is essentially the ability to free fatty acids from liposomes [Wallach, 1970]. This also correlates with increases in amounts of free fatty acids [Brustovetsky et al., 1992]. Comparisons of hibernators and non-hibernators showed that, when exposed to cold temperatures over extended periods, the hibernators showed enhanced atrophy (measured by cross sectional area); yet, the total amount of capillarity to the muscle increased. Coupled with an increase in myoglobin content [Postnikova et al., 1999], this presumably allows hibernators to maintain functional muscle mass during hibernation. At the gene level a number of changes occur in hibernating muscle of ground squirrels including up-regulation of *h-fabp* [Hittel and Storey, 2001], *Mlc1*, *Nad2* [Fahlman et al., 2000], PDK4 [Buck et al., 2002] and *ucp-3* [Boyer et al., 1998]. To date, though, there are no data on gene regulation in skeletal muscle of hibernating bats and only limited data on gene regulation in skeletal muscle of hibernating ground squirrels.

Adipose Tissue

Adipose tissues (brown and white) are central to hibernation survival providing animals with lipid stores required for arousal. Brown adipose tissue (BAT) is the thermogenic workhorse of hibernating mammals. It contains huge lipid reserves that are catabolized by a very high population of mitochondria that, in turn, have uncoupling proteins embedded within their membranes. The tissue serves no other apparent purpose

than to produce heat, which is used to warm the body of hibernators [Foster, 1984; Boyer et al., 1998]. The primary role of BAT is to rapidly raise body temperature during arousal from torpor but, if necessary due to subzero air temperatures in the hibernaculum, the tissue also provides low level thermogenesis to keep core body temperature from falling below 0°C. During thermogenesis in BAT mitochondria, the electron transport system is uncoupled from oxidative phosphorylation so that energy that would normally be captured in ATP synthesis is released as heat instead. Uncoupling of oxidative phosphorylation is achieved through the action of uncoupling proteins, which eliminate the pH gradient across the inner membrane is eliminated.

White adipose tissue (WAT), despite being a major lipid store for hibernators, has received little attention. Wilson et al. (1992) observed seasonal variations in the levels of lipoprotein lipase (LPL) mRNA and hormone sensitive lipase (HSL) mRNA. During feeding, LPL levels were high to allow for the accumulation of fat stores, whereas HSL levels increased during fasting periods (torpor) to allow for the mobilization of fatty acids for export to other organs. The regulation of HSL appears to be in conjunction with pancreatic triacylglycerol lipase (PTL) [Bauer et al., 2001]. Composition of WAT varies with season to allow for the broad range of temperatures typically experienced with hibernators [Cochet et al., 1999a, 1999b]. A rise in levels of PDK4 [Buck et al., 2002], consistent with findings in other tissues, appears to be involved in the suppression of carbohydrate use and the switch to fatty acid utilization in hibernating WAT of ground squirrels as well.

Methodology

A cellular and molecular chicken and egg

In every biological organism or system, there exists a conundrum. Which level of regulation does one study first, nucleic acids (genes) or proteins? With recent advances in microarray availability, a number of researchers are looking at global gene expression under a number of stress states but are the nucleic acids translated into proteins? If they are translated into proteins, these in turn can regulate expression of nucleic acids or genes. So, where does one begin a study on hibernation at the molecular level? I propose to begin with the identification of signaling proteins that are important in regulating expression of a subset of genes and, upon further characterization of these genes, identify the particular proteins that are affecting their expression and what roles these genes and proteins may play in hibernation.

This present study will elucidate changes in gene and protein expression that occur in tissues of two hibernators, *Myotis lucifugus* and *Spermophilus tridecemlineatus*, with an emphasis on muscle tissues. Gene and protein regulation in skeletal muscle is important because of disuse atrophy that exists in hibernating animals during long hibernation periods. Heart becomes important when it is realized that during hibernation, it undergoes changes that allow for continued function at a low T_b and a conversion to lipid metabolism. Unlike other tissues, the heart cannot cease to function during hibernation although it does drop its rate to about 1% of the euthermic rate.

Hypotheses

(a) Stimulation and suppression of selected signal transduction cascades will occur during hibernation. In order to test this hypothesis, differential phosphorylation states of various signal transduction enzymes and transcription factors were tested through the use of protein Western blotting.

The p38 mitogen activated protein kinase cascade responds to a variety of cellular stress states including oxidative stress experienced during ischemia [Ogura and Kitamura, 1998; Luss et al., 2000]. p38 MAPK is of interest because it becomes activated in response to cellular stress and acts to stabilize the actin cytoskeleton through HSP27 [Landry and Huot, 1995]. Also, downstream of p38, there are a number of targets that are potentially activated by p38 including ATF-2, CREB and Elk-1.

(b) Metabolic suppression under the stress of hibernation will lead to a suppression of overall gene expression and protein translation states. Nevertheless, the selective expression of genes is needed to induce and maintain hibernating heart and skeletal muscles. To determine the validity of this, gene expression status was determined by comparing hybridization patterns of probes generated from euthermic and hibernating tissues onto DNA arrays and comparing the expression levels of genes between the two states. Western blotting determined the status of protein translation factors between euthermic and hibernating states.

DNA arrays provide an opportunity for researchers to examine global expression levels of genes. Because hibernation results in such a profound metabolic depression, it is likely that the majority of gene regulation will be maintained or suppressed and that those genes showing up-regulation are important to survival during hibernation. To this end, cDNA arrays of humans and rats, were heterologously probed to determine global mRNA expression levels as a means to determine candidate genes and gene families for future studies.

- (c) Genes and proteins involved in fatty acid transport and consumption are specifically up-regulated during hibernation and, therefore, regulatory proteins that control fatty acid metabolism, such as peroxisome proliferator activated receptors (PPARs), must be differentially regulated during hibernation.

Chapter 4 examines the expression of genes and proteins involved in fatty acid transport and consumption to elucidate further the role and regulation that fatty acids play in hibernation. Northern blot analysis revealed the differential expression of *a-* and *h-fabp* transcript levels in addition to A- and H-FABP protein levels. The PPAR family of transcription factors regulates transcript levels of *a-* and *h-fabp* in a number of systems. The expression levels of PPAR factors were determined via Western blotting. Finally, protein expression levels of carnitine palmitoyl transferase were investigated as this is the mitochondrial enzyme that is the rate-limiting step in fatty acid oxidation and plays a role in fatty acid metabolism during hibernation.

(d) Because arousal from hibernation involves a very rapid increase in oxygen uptake and consumption, hibernator organs, in particular the heart, must be well equipped with strong antioxidant defences to minimize the damage due to reactive oxygen species (ROS) formation during arousal.

Chapter 5 describes the use of 2D-PAGE to discover a protein that whose concentration increased in the heart of hibernating bats. The protein was identified as a thioredoxin peroxidase-like protein called proliferation-associated gene, Pag, which responds to oxidative stress. Relative RT-PCR was used to assess relative gene transcript levels in euthermic versus hibernating tissues that may experience significant oxidative stress upon arousal. Protein levels were confirmed by means of Western blotting between euthermic and hibernating tissues.

Chapter 2
p38 MAPK Signal Transduction and
Akt in euthermic versus hibernating
animals

INTRODUCTION

Protein regulation plays a key role in entry into and maintenance of hibernation. Various studies have identified key changes to a number of metabolic enzymes [Storey, 1997, 2000] and some transcription factors [O'Hara et al., 1999; Carey et al., 2000; Ono et al., 2001] when comparing euthermic versus hibernating states. Many of the changes in activities or properties of proteins/enzymes are mediated via reversible phosphorylation, which suggests important roles for signal transduction mechanisms and protein kinases and/or phosphatases in the regulation of hibernation. Hibernation is a state of profound torpor where both physiological and molecular processes are suppressed to very low levels. At the molecular level, a number of key events must be tightly regulated and maintained in order for cells to make a smooth transition to hypometabolism and re-establish homeostasis at a very much lower rate of ATP turnover. One of the energy-expensive functions that must be strongly reduced during hibernation is protein translation, which is controlled through reversible phosphorylation of selected ribosomal proteins [Frerichs et al., 1998; Chen et al., 2001]. Gene transcription is also strongly reduced [van Breukelen and Martin, 2002]. However, a key element in the control of gene transcription is the activity of transcription factors, most of which are also regulated via reversible phosphorylation. Hence, an investigation of the responses during hibernation of selected transcription factors and the protein kinases that regulate them is justified. For, just as the activities of metabolic enzymes and ion-motive ATPases are strongly suppressed during hibernation via protein phosphorylation mechanisms, it is probable that the same reversible phosphorylation mechanisms (although mediated via

different protein kinases), which do not require a great expenditure of energy, are used to suppress overall gene transcription and protein translation.

Despite the overall state of hypometabolism, selected gene up-regulation and protein synthesis does occur during hibernation. For example, key to long term hibernation is the inhibition of carbohydrate use as a metabolic fuel and a switch to fatty acid oxidation. Suppression of carbohydrate metabolism has been linked to increased expression of pyruvate dehydrogenase kinase isozyme 4 (PDK4), the enzyme that phosphorylates and inactivates pyruvate dehydrogenase in hibernating heart and muscle [Andrews et al., 1998, Buck et al., 2002]. In our lab we have also documented hibernation-induced increases at both the gene and protein level for fatty acid binding proteins (FABP), a factor that would aid lipid metabolism during hibernation [Hittel and Storey, 2001, 2002; Chapter 4]. Signal transduction must play an underlying role in the regulation of these and other genes during hibernation.

One kinase, p38 mitogen activated protein kinase (MAPK), is an important signaling molecule involved in relaying a number of cellular signals/stresses including, but not limited to, inflammation and ischemia [Bogoyevitch et al., 1996], both of which have been implicated previously in hibernation [Frerichs et al., 1994; Yasuma et al., 1997]. In addition to this, recent studies have revealed that p38 activation occurs during hypothermic stress [Aggeli et al., 2002; Roberts et al., 2002]. It is likely that tissues that may experience hypoxia and/or ischemia, as well as hypothermia, may experience a rise in active p38. To assess further whether the p38 MAPK signaling pathway is active, a number of downstream targets can also be investigated (Figure 2.1). This chapter proposes a role for p38, one of the mitogen-activated protein kinases (MAPKs), in the

metabolism of heart and skeletal muscle during hibernation of two different hibernators (bats and ground squirrels). Activated by phosphorylation, p38 can translocate to the nucleus, where it phosphorylates a number of downstream targets and transcription factors. The responses of several potential downstream targets of p38 are also characterized including the AP-1 binding transcription factor 2 (ATF-2) and the cAMP response element binding protein (CREB). Both are members of the CREB family of leucine zipper DNA binding proteins [Abdel-Hafiz et al., 1992]. The transcription factor Elk-1 was also investigated and a downstream target that is indirectly phosphorylated by p38, heat shock protein 27 (HSP27), was also explored. In addition to p38, another kinase molecule, protein kinase B (PKB or Akt) was investigated in euthermic versus hibernating tissues. Exercise can markedly increase the activity of a number of signaling molecules, including Akt [Sakamoto and Goodyear, 2002; Sakamoto et al., 2002] and, in addition, Akt is central to insulin signaling and cellular homeostasis of carbohydrate levels in healthy cells [Summers and Birnbaum, 1997; Smith, 2002]. Hibernation involves a lack of exercise and a change in the state of insulin production and likely insulin signaling [Bauman et al., 1987; Bauman, 1990]. Hence, Akt could be a potential target for regulation in hibernating mammals. Its role in hibernation was also investigated based on the proposal that it could be acting as a suppressor of carbohydrate metabolism in hibernating mammals.

MATERIALS AND METHODS

Animals.

Little brown bats, *Myotis lucifugus* (7-8 g body mass) were collected by Dr. D. Thomas on November 30, 1999 from a disaffected slate mine near Sherbrooke, Quebec

where they had been hibernating since October (cave air temperature was 5°C). Collection aroused the bats and they remained aroused during transport to the Université de Sherbrooke. Upon arrival, ten bats were maintained under euthermic conditions at 23-24°C air temperature; these were kept awake for a total of 48 hours post-collection and were then euthanized by cervical dislocation. Ten others were placed in a cold room at 5°C and allowed to re-enter hibernation; 10-12 hours were required for full torpor to be re-established with a body temperature close to ambient. Hibernating animals remained torpid until sampled 36-38 hours later; measured rectal temperatures at sampling were 5-6°C. Tissues from euthermic and hibernating animals were quickly excised, immediately frozen in liquid nitrogen, and then transported to Ottawa where they were stored at -80°C until analysis.

Thirteen-lined ground squirrels, *Spermophilus tridecemlineatus* (130-180 g) were obtained from a trapper licensed by the U.S. Department of Agriculture (TLS research, Michigan) in September 2000 and transported to the NIH (Bethesda, MD) where they were held at the Animal Hibernation Facility and hibernation experiments carried out by the laboratories of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke) and Dr. R.M. McCarron (Naval Medical Research Center). Animals were kept in a winter day/night light cycle and fed *ad libitum* until they entered and finished the pre-hibernation phase of hyperphagia that maximized body lipid reserves prior to hibernation. When animals had exhibited a rapid increase in body weight to 220-240 g, they were placed in dark chambers at 5-6°C to induce hibernation. Four animals that had been hibernating for 2-5 days (as indicated by a body temperature of ~5°C) were sacrificed by

decapitation and tissues sampled as above. Four other animals kept as controls (euthermic) were sacrificed on the same day as the hibernating animals.

Richardson's ground squirrels, *Spermophilus richardsonii*, were captured near Calgary, Alberta in the summer of 1994. They were held in the animal care facility at the University of Calgary on a natural (fall) photoperiod and fed *ad libitum*. After two months, half of the animals were induced to hibernate by placing their cages in a cold room at 5°C with water but without food. Animals were determined to be truly hibernating if they were torpid for at least five days between periodic arousals. After one month, both euthermic and hibernating animals were sacrificed; in their last cycle, hibernating individuals were sampled after they had been in torpor for at least 2 days. All animals were killed by decapitation and excised tissues were immediately immersed in liquid nitrogen and subsequently stored at -70°C.

Tissue homogenization and protein preparation.

Frozen tissue samples were ground into small pieces in a mortar and pestle under liquid N₂. A 100 mg sample of tissue was placed in 1 ml of homogenization buffer (100 mM MOPS, 25 mM HEPES, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM EGTA, 250 μM NaVO₄, pH 7.4) with 1 mM phenylmethylsulfonyl fluoride added prior to homogenization, and were homogenized by a Polytron PT1000. Samples were centrifuged for 10 minutes at 10000 x g and supernatants were removed. An 250 μl aliquot of supernatant was mixed 1:1 v/v with 2X SDS-PAGE buffer (100 mM Tris-HCl, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.4% w/v bromophenol blue, 10% v/v 2-mercaptoethanol). The samples were then boiled and stored at -20°C. Aliquots of the sample containing 10 or 20 μg protein were removed as necessary. Samples were

electrophoresed on polyacrylamide gels (10 or 15% depending on the molecular mass of the protein under investigation) at 200V for 1 hour. Proteins were transferred onto PVDF membrane for 3 hours at 80V, 350 mA in transfer buffer (25 mM Tris base, 192 mM glycine, 25% v/v methanol).

Akt, ATF-2, CREB, Elk-1, p38 MAP Kinase, phospho-Akt (Ser 473), phospho-ATF-2 (Thr69/71), phospho-CREB (Ser133), phospho-Elk-1 (Ser383), and phospho-p38 MAP kinase (Thr180/Tyr182) antibodies raised in rabbits were purchased from Cell Signaling Technology. These proteins cross react over a range of animal species. HSP 27 antibodies (also raised in rabbits) were purchased from StressGen and phospho-HSP27 (Ser78/82) antibodies were a gift of Colleen Brophy (Arizona State University). Antibodies for HSP25 were also used (StressGen) in an attempt to identify the small HSP family in ground squirrel heart. The correct molecular weights of bands were determined by using a biotinylated protein ladder detection pack according to manufactures protocols (#7727, Cell Signaling).

Western blotting

After transfer of proteins to PVDF, the PVDF membranes were incubated at 4°C overnight with primary antibodies. Each antibody was probed on a separate membrane. Incubations were conducted in Tris buffered saline containing Tween-20 (TBST: 20 mM Tris base, 140 mM NaCl, 0.1% v/v Tween-20) at a 1:1000 v/v dilution of antibodies. The exception to this was HSP27 probing. In this case, the membrane was first blocked in TBST containing 5% w/v non-fat dried milk (NFDM) for 6 hours and then incubated at 4°C in TBST, 5% w/v NFDM overnight at a dilution of 1:10000. After incubations, each blot was washed for 3 x 5 minutes in TBST prior to the addition of HRP-conjugated anti-

rabbit antibody. Secondary incubations were carried out for 2-3 hours at room temperature at a 1:2000 v/v dilution and followed by 3 x 5 minute washes in TBST. Blots were then developed using the Western LightningTM Chemiluminescence *Plus* (NEN, Perkin Elmer) system according to protocols provided with the system. Blots were briefly exposed to Kodak X-OMAT-AR film and developed according to manufacturer's protocols. Multiple exposures of blots were obtained to ensure proper quantification of band density. X-ray images were quantified using densitometric analysis software (Imagequant, Molecular Imaging). Images shown in figures represent longer exposures and were not necessarily used for quantification.

Statistics

All trials are a result of a minimum of 3 independent isolations from pooled tissue (n=10 for bats, n=4 for ground squirrels). Hibernating:euthermic ratios were calculated as follows. Mean band densities $\pm$ SEM for each of euthermic and hibernating samples were calculated by using values obtained from analysis software (Imagequant, Molecular Imaging) and significance was determined using the Student's t-test ($P < 0.05$ unless otherwise stated). The ratio of hibernating:euthermic represented in histograms is a result of the hibernating intensity expressed as a function of the euthermic intensity. Error bars shown on the final histograms are the sum of SEM values for euthermic and hibernating trials.

RESULTS

Western blotting was used to assess the relative amounts of p38 MAPK and related transcription factors in bat and ground squirrel muscles. Figures 2.2 to 2.5 show

both total protein amounts and the amounts of phosphorylated, active protein for five proteins in bat skeletal muscle, bat heart, ground squirrel skeletal muscle and ground squirrel heart, respectively.

1. **p38 mitogen activated protein kinase**

p38 MAP kinase is an important signaling molecule involved in relaying a number of cellular signals including, but not limited to, pro-inflammatory cytokines and stresses on cells that can be in the form of ischemia, osmotic shock or in the case presented here, hibernation. In skeletal muscle, there was a substantial increase in the phosphorylation state of p38 in both species during hibernation; the ratio hibernating:euthermic for phospho-p38 was 2.2 ± 0.4 ($n=3$, $P<0.05$) for bats and 1.6 ± 0.2 ($n=3$, $P<0.05$) for ground squirrels (Fig. 2.2, 2.4). Total p38 content increased only slightly in skeletal muscle of hibernating *M. lucifugus* (1.2 ± 0.1 , $P<0.05$) but rose strongly by 17.9 ± 3.3 fold ($P<0.01$) in ground squirrel muscle during hibernation. Contrary to the results for skeletal muscle, there was no appreciable effect of hibernation on either total or phosphorylated amounts of p38 in hearts of either species (Fig. 2.3, 2.5).

2. **ATF-2 and CREB: members of the CREB family of transcription factors**

ATF-2 and CREB make up a family of transcription factors that bind to DNA at a cyclic AMP response element (CRE). They contain a basic leucine zipper domain that binds to a consensus DNA sequence located upstream of specific genes [Hai and Hartman, 2001]. Binding to the consensus sequence allows these proteins to activate the expression of specific genes. In *M. lucifugus* skeletal muscle, the total amounts of ATF-2 and CREB did not change between euthermic and hibernating states (Fig. 2.2). Whereas, the amount of phospho-protein rose substantially during hibernation by 11.1 ± 3.7 fold

for ATF-2 ($P<0.05$) and 2.2 ± 0.2 fold for CREB ($P<0.001$). Both total and phosphorylated amounts of ATF-2 and CREB were unchanged in *M. lucifugus* heart during hibernation (Fig. 2.3).

Skeletal muscle of *S. tridecemlineatus* behaved much like bat skeletal muscle. Levels of phospho-ATF-2 and phospho-CREB were higher in hibernating animals, by 5.1 ± 1.8 fold ($P<0.05$) and 2.0 ± 0.5 fold ($P<0.05$), respectively, and total CREB levels were lower in hibernating animals (ratio was 0.5 ± 0.2 , $P<0.05$) (Fig. 2.4). In ground squirrel heart, both total and phospho-ATF-2 decreased in hibernation (values were 0.5 ± 0.2 , $P<0.05$ and 0.4 ± 0.3 , $P<0.05$, compared with euthermic) (Fig. 2.5). Total CREB did not change but phospho-CREB content rose significantly by 3.4 ± 0.9 fold ($P<0.05$) in heart of hibernating ground squirrels.

3. Elk-1

Cells respond to a variety of stresses by stimulating members of the ternary complex factor (TCF) family to which Elk-1 belongs. Members the TCF family can be activated by the ERK, JNK and p38 MAPK pathways [Treisman, 1996]. Stimuli including glutamine, glutamic acid and insulin act via these pathways to activate Elk-1. Phosphorylation is required for transcriptional activation particularly at Ser383 [Gille et al., 1995]. The effects of hibernation on Elk-1 in skeletal muscle and heart of bats are shown in Figures 2.2 and 2.3. In skeletal muscle, total Elk-1 did not significantly differ between euthermic and hibernating states but phospho-Elk-1 rose by 4.2 ± 1.7 fold ($P<0.05$) (Fig. 2.2). Total levels of Elk-1 in hearts of hibernating bats were lower than those found in euthermic hearts (0.3 ± 0.1 , $P<0.002$) as were levels of Ser383 phosphorylated Elk-1 (0.3 ± 0.1 , $P<0.005$) (Fig. 2.3).

Total Elk-1 in skeletal muscle of thirteen-lined ground squirrels decreased slightly during hibernation (ratio hibernating:euthermic was 0.7 ± 0.1 , $P < 0.005$) whereas phospho-Elk-1 content rose by 3.3 ± 0.4 fold ($P < 0.001$) during hibernation (Fig. 2.4). Total Elk-1 levels in euthermic and hibernating hearts were approximately equal whereas the amount of Ser383 phosphorylated Elk-1 was strongly reduced in hibernation (0.1 ± 0.2 , $P < 0.01$) (Fig. 2.5).

4. HSP 25/27

The HSP25 and HSP27 are similar proteins that differ only in their post secondary modifications, which are tissue dependent. This family of proteins increases under a number of stresses and they may function as chaperones. An increase in phosphorylation at Ser78/82/86 of HSP27 has been associated with increases in actin and cytoskeleton stability [Benndorf et al., 1994, Preville et al., 1998]. Previous studies have shown phosphorylation of HSP27 arising from p38 acting through MAPKAP-2/3 [Dalle-Donne et al., 2001]. In skeletal muscle of hibernating bats, total levels of HSP27 remained the same, whereas a higher level of phosphorylation was evident (hibernator:euthermic ratio for phospho-HSP27 was 3.2 ± 0.3 , $P < 0.001$) (Fig. 2.2). In bat heart both HSP27 and phospho-HSP27 rose during hibernation by 4.4 ± 1.4 fold ($P < 0.05$) and 2.2 ± 0.5 fold ($P < 0.05$), respectively (Fig. 2.3).

In ground squirrel skeletal muscle total HSP27 was unchanged between the two states whereas phospho-HSP27 was 2.0 ± 0.3 fold higher during hibernation ($P < 0.02$) (Fig. 2.4). Immunoblotting failed to detect either total or phospho-HSP27 in ground squirrel heart. Further attempts to find the protein using antibodies for HSP25 failed.

5. Akt

Analysis of Akt responses during hibernation is shown in Figure 2.6 for bats and Figure 2.7 for ground squirrels. During hibernation in bats neither total nor phospho-Akt content was affected in heart or skeletal muscle (Fig. 2.6) but other organs showed substantial changes. Akt in brown adipose tissue was strongly affected with total Akt 2.5 ± 0.7 fold higher during hibernation ($P < 0.05$) and active phospho-Akt also elevated by 2.9 ± 0.6 fold ($P < 0.05$). The total levels of Akt did not change between euthermic and hibernating states in brain, kidney, or liver and decreased significantly in WAT (0.3 ± 0.2 , $P < 0.05$). Lower levels of phosphorylated Akt were observed in brain (0.3 ± 0.2 , $P < 0.001$), kidney (0.3 ± 0.1 , $P < 0.05$), liver (0.4 ± 0.1 , $P < 0.001$) and WAT (0.4 ± 0.1 , $P < 0.01$) during hibernation.

By contrast with the results for bats, a highly consistent response by Akt was seen in the four tissues of ground squirrels tested (Fig. 2.7). The amount of phosphorylated active Akt was strongly suppressed in all organs, the ratio hibernating:euthermic falling in brain, brown adipose, kidney and skeletal muscle to 0.09 ± 0.10 ($P < 0.0005$), 0.04 ± 0.57 ($P < 0.05$), 0.39 ± 0.08 ($P < 0.0005$) and 0.12 ± 0.24 ($P < 0.005$), respectively. Total Akt was not affected during hibernation in any organ.

DISCUSSION

MAP kinases play an essential role in transmitting extracellular signals to specific nuclear signals and responses. The activation of nuclear signals occurs via a number of interactions that lead to differential phosphorylation of key intermediates in MAP kinase cascades and ultimately fall on various transcription factors. Reversible protein phosphorylation appears to play a key role in regulating metabolism in hibernating

animals [Storey, 1997; Frerichs et al., 1998; Ohtsuki et al., 1998; Chen et al., 2001]. Differential phosphorylation of specific enzymes in hibernating animals is correlated with a suppression of protein synthesis, glycolytic metabolism and an enhancement of lipid metabolism. The present study shows that the mechanism of reversible phosphorylation likely extends to the control of nuclear gene expression by altering phosphorylation status of protein kinases and signal transduction factors that affect gene expression.

1. Skeletal muscle

Some of the hibernation-associated changes reported in the present chapter appear to be common between evolutionarily distinct hibernating mammals. This is the case with the p38 MAPK signaling pathway in hibernator skeletal muscle of *M. lucifugus* and *S. tridecemlineatus*. Higher levels of active phosphorylated p38 are seen in both types of skeletal muscle during hibernation along with elevated amounts of the phosphorylated forms of various downstream transcription factors controlled by p38 including ATF-2, CREB and Elk-1. In addition, the amount of phospho-HSP27, another protein indirectly controlled by p38, is elevated in hibernation. Previous studies have shown that signaling by p38 increases in response to a number of stresses including oxidative stress and ischemia [Kim et al., 1999; Bradbury et al., 2001]. It also increases during differentiation and when organs increase their reliance on fats as fuel [Puigserver et al., 2001]. Ischemia tolerance and utilization of fats are both strategies that are employed during hibernation [Frerichs et al., 1994; Hittel and Storey, 2001].

CREB and ATF-2 belong to the CREB family of transcription factors. Although they are members of the same family, they tend to act on diverse targets. CREB is involved in the activation of genes in conjunction with sterol regulatory element binding

proteins. Recent evidence implicates CREB involvement in regulating genes involved in fat metabolism and non-shivering thermogenesis such as liver carnitine palmitoyl transferase-1 (L-CPT-1), a rate limiting enzyme in fatty acid oxidation [Louet et al., 2002] and uncoupling protein-1 (UCP-1) [Rim and Kozak, 2002]. Since hibernators rely almost exclusively on lipid oxidation to fuel their energy needs over the winter, it is not surprising to observe an increase in the expression of a number of the genes and their protein products that are involved in the consumption and regulation of lipids and their changes may be mediated by CREB. ATF-2 has been shown to be involved in the regulation of tumor necrosis factor alpha (TNF α) gene expression [Ivanov and Ronai, 1999], a key gene in insulin resistance and obesity [Uysal, et al., 1998]. A-FABP also appears to be a central link between fatty acid metabolism and TNF α expression [Hotamisligil et al., 1996] and TNF α may also affect FABP expression levels [Memon et al., 1999]. Hibernator skeletal muscle may indeed deal with an increased fatty acid load as evidenced by increases in FABP in *S. tridecemlineatus* [Hittel and Storey, 2001] and in *M. lucifugus* (Chapter 4).

Elk-1 is a downstream target of signaling pathways and has been described as a point of convergence for several different signals [Zinck et al., 1995; Raingeaud et al., 1996]. However, the actions of p38 in controlling Elk-1 may be minimal as p38 poorly phosphorylates Elk-1 [Janknecht and Hunter, 1997] and this opens the possibility that a number of signal transduction pathways participate in and are closely regulated during torpor bouts. Oxidative stress, which will be discussed in more detail in subsequent chapters, may play a role in Elk-1 activation via the action of extracellular regulated kinase 2 (ERK2) [Muller et al., 1997; Ogura and Kitamura, 1998]. Upon activation via

phosphorylation, Elk-1 binds to the c-fos serum response element [Vanhoutte et al., 1999] activating the transcription of genes such as c-fos and egr-1 [Lim et al., 1998]. Defining a role for Elk-1 in hibernation is difficult since relatively little is known about the precise mechanism of its activation at this time but the 3-4 fold increase in phospho-Elk-1 in skeletal muscles in hibernation suggests that a significant role is played by this transcription factor. Glutamate and insulin appear to be involved in regulating activation of Elk-1 [Vanhoutte et al., 1999] and both may be altered during hibernation; glutamate levels change during periods of inactivity [Jaspers et al., 1986, 1989] and insulin levels increase in hibernating bats [Bauman, 1990].

HSP27 acts in a number of roles including the response to oxidative stress [Dalle-Donne et al., 2001]. Upon, exposure to stress, HSP27 becomes phosphorylated and associates with the actin cytoskeleton to help preserve cytoskeletal integrity. Phosphorylation of HSP27 during hibernation in skeletal muscle likely acts to add structural stability during long periods of cold exposure. The lack of detection of HSP27 in *S. tridecemlineatus* heart is to some extent perplexing and may possibly be due to novel postsecondary modifications occurring in the antigenicity site that prevent the antibody from identifying its inherent target in the ground squirrel heart protein.

2. Heart

The effects of hibernation on p38 and transcription factors in heart were quite different than in skeletal muscle. There were no significant changes in either total or phospho-p38 levels in hearts from euthermic versus hibernating bats or ground squirrels. Total and phosphorylated (active) ATF-2 and CREB did not change in euthermic versus hibernating *M. lucifugus* and phospho-ATF-2 decreased in *S. tridecemlineatus* heart

during hibernation whereas phospho-CREB increased in *S. tridecemlineatus* heart. The different responses of these transcription factors between heart and skeletal muscle could be due to the fact that the heart continues beating, and thus its muscle continues to work while animals are torpid, whereas skeletal muscle remains motionless during deep torpor and during hibernation bouts. Elk-1 similarly responded differently between heart and skeletal muscle. Both total and active Elk-1 decreased during hibernation in hearts of *M. lucifugus* and active levels decreased in hibernating hearts of *S. tridecemlineatus*. Whereas the skeletal muscle appears to remain inactive, the heart must maintain its activity throughout torpor and this may account for the differences seen with the p38 MAPK signal cascade.

One of the requirements of hibernation is strong suppression of the use of carbohydrates and a greater reliance on fatty acids and lipids for energy production. Signal transduction is likely key in this and most likely involves the insulin receptor signal transduction pathway that is mediated through a number of protein kinases and phosphatases. A key signaling molecule that responds to insulin is Akt or protein kinase B. Analysis of Akt in ground squirrel tissues revealed a general, strong suppression of the amount of active phospho-enzyme during hibernation in all tissues tested and in bat brain, kidney, liver and WAT as well. This suggests that a general suppression of Akt-mediated signal transduction systems may be important during torpor. However, the responses of Akt in brown adipose tissue of bats were anomalous; the amount of active phospho-Akt was strongly suppressed in ground squirrel brown adipose but both total and phospho-Akt increased 2.5-3 fold in the same tissue of bats. Skeletal muscle also showed species-specific differences in phospho-Akt, decreasing in ground squirrels but not changing in

bats. These may be related to differing levels of insulin in hibernating bats and ground squirrels. Ground squirrels show distinct decreases in pancreatic insulin during hibernation whereas bats show increases throughout hibernation, then a rapid decrease upon arousal [Bauman et al., 1987, Bauman, 1990]. Many of these changes may be associated with tissue specific function during hibernation as well.

In summary, the phosphorylation state of key transcription factors increases in skeletal muscle of *M. lucifugus* and *S. tridecemlineatus* during hibernation. The enhanced levels of active transcription factors demonstrated by the data in this chapter may result in increased gene transcription followed by translation and synthesis of selected proteins that are key to survival in the hibernating state. Alternatively, increased levels of selected mRNA transcripts may not be immediately translated, but may be kept available for rapid protein synthesis when animals enter an arousal phase. This latter idea will be developed further in later chapters of this thesis. The former idea is supported by changes in known downstream target genes of phospho-CREB, which include hibernation-responsive up-regulation of CPT-1 (Chapter 4) and uncoupling proteins (UCP) [Nizielski et al., 1995]. The data presented in this chapter do not rule out the possibility that other signaling molecules play a role in hibernation, but rather it provides an initial step in the study and characterization of signaling pathways in hibernating heart and skeletal muscle.

Figure 2.1 An overview of the p38 MAP kinase signal transduction pathway. Included are potential downstream targets as well as other signalling molecules that may act as intermediates between p38 and its targets.

Figure 2.2 The effects of hibernation on *M. lucifugus* skeletal muscle p38 and its downstream targets, ATF-2, CREB, Elk-1 and HSP27. Representative Western blots show total protein and phospho-protein levels in samples from euthermic (E) versus hibernating (H) animals. Mean ($\pm$ SEM) values for band intensities were calculated from n=3 trials for both euthermic and hibernating samples, followed by significance testing using the Student's t-test (* - hibernator value is significantly different from euthermic value, $P < 0.05$). The ratios of Hibernating:Euthermic mean values were then calculated and are presented in the histogram. The molecular weight of each antibody was determined by comparison with a biotinylated protein ladder. p38 MAPK was approximately 45 kDa, ATF-2 was approximately 70 kDa, CREB was approximately 40 kDa, Elk-1 was approximately 60 kDa and HSP27 was approximately 30 kDa.

M. lucifugus skeletal muscle

Figure 2.3 The effects of hibernation on *M. lucifugus* heart p38 and its downstream targets, ATF-2, CREB, Elk-1 and HSP27. Other information as in Figure 2.2.

M. lucifugus heart

Figure 2.4 The effects of hibernation on *S. tridecemlineatus* skeletal muscle p38 and its downstream targets, ATF-2, CREB, Elk-1 and HSP27. Other information as in Figure 2.2.

S. tridecemlineatus skeletal muscle

Figure 2.5 The effects of hibernation on *S. tridecemlineatus* heart p38 and its downstream targets, ATF-2, CREB, Elk-1 and HSP27. Other information as in Figure 2.2.

S. tridecemlineatus heart

Figure 2.6 The effects of hibernation on *M. lucifugus* total and phosphorylated levels of protein kinase B (PKB or Akt). The bar graph shows the Hibernating:Euthermic ratio (mean $\pm$ SEM, n=3) for the protein. Abbreviations used are BAT, brown adipose tissue, Sk Mus, skeletal muscle and WAT, white adipose tissue. The molecular weight of Akt was determined to be 60 kDa.

M. lucifugus Akt

Figure 2.7 The effects of hibernation on *S. richardsonii* total and phosphorylated levels of protein kinase B (PKB or Akt). Displayed on the bar graph is the average ratio of Hibernating:Euthermic levels of each protein (n=3 trials) where error bars represent SEM. Other information as in Figure 2.2.

S. richardsonii Akt

Figure 2.8 An overview of the insulin receptor-signalling pathway. Included are potential downstream targets as well as other signalling molecules that may act as intermediates between the insulin receptor and its downstream targets

Chapter 3

Assessment of gene expression and translation state in heart and skeletal muscle during hibernation

INTRODUCTION

Although not an old technique itself, the screening of cDNA libraries is considered to be the "classical" method of gene discovery against which other methods are compared. cDNA library screening has multiple benefits including results that have a low number of false positives (Figure 3.1) and a capacity to detect novel and unknown genes that are unique to the animal/tissue in question. However, the differential screening of cDNA libraries is arduous, time-consuming and costly, has limitations with some tissue sources, and is relatively poor for the detection of gene transcripts that are present in low copy number. More recently, other techniques have been developed. For example, differential display PCR (ddPCR) [Liang and Pardee, 1992] has the advantage of needing very little experimental material, but is often plagued by a high number of false positives [Miele et al., 1998]. The newest method for differential screening of gene expression is cDNA array technology which provides the means to screen the responses of hundreds or thousands of genes simultaneously and so gain a broad picture of gene expression responses, representing multiple categories of cell functions, to an imposed stress or experimental condition.

In the present chapter, experiments are described that use heterologous probing of human and rat cDNA arrays using probe derived from the tissues of euthermic and hibernating animals to identify potential genes that are up-regulated during hibernation. The ability to screen thousands of genes simultaneously using arrays represents a recent advance in biotechnology although the underlying methodology has been prevalent in molecular biology for the past 25 years. The number of applications for which cDNA arrays can be used appears to be almost limitless and is bounded only by the researcher's

imagination. All but a few studies to date have used homologous probing of cDNA arrays using arrays that are available for the major model systems including human, mouse, rat, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Escherichia coli*, and *Saccharomyces cerevisiae*. The costs associated with producing arrays, not to mention the genome sequencing that is first required, has so far limited the production of arrays to these species. In this chapter, I will discuss the viability of using cDNA arrays for heterologous probing and demonstrate their usefulness for hibernation-responsive gene discovery in heart and skeletal muscle of *M. lucifugus* and *S. tridecemlineatus*.

Since the advent of the Southern blot in the 1970's, researchers have found a number of ways to use nucleic acids on solid supports including membranes or glass. DNA microarrays are the newest addition to this field. DNA array technology is still much in its infancy but already numerous reviews and original research papers have been produced on the subject [Schena et al., 1995; Cunningham, 2000; Jain, 2000; Burgess, 2001; Blohm and Guiseppi-Elie, 2001; Dolan et al., 2001; Lobenhofer et al., 2001]. There are two major procedures for producing the commercially available DNA arrays (Figure 3.2), the one that spots PCR amplified cDNAs onto solid supports, glass, nylon or plastic being the most common. The method of spotting cDNAs onto treated glass slides was developed at Stanford University [Schena et al., 1995] and is currently used by a number of commercial ventures. The other procedure for producing cDNA microarrays was developed by Affymetrix [Pease et al., 1994] and involves the synthesis of oligonucleotides directly onto solid supports through the use of chemical protecting groups and photolithographic masks.

Gene up-regulation, as demonstrated by increased numbers of mRNA transcripts, generally leads to increased synthesis of the protein coded by the mRNA. In some cases the two processes are "unhooked" and an increase in mRNA transcripts does not always correlate with an increase in translated protein. Control over the synthesis of most proteins occurs via regulation of the ribosomal translation complexes. Two of these complexes, the eukaryotic initiation factor 2 (eIF2) and the eukaryotic elongation factor (eEF2) are responsible for key stages of protein synthesis. For example, by phosphorylation of serine 51 of the eIF2 α subunit, initiation of protein synthesis is blocked by preventing the exchange of GDP for GTP (Figure 3.3a); phosphorylation of this residue is stimulated in response to a variety of stresses such as heme deficiency, endoplasmic reticulum stress, heat shock and amino acid starvation. This event subsequently blocks the transfer of the Met-tRNA complex (Figure 3.4). Elongation factor 2 can also be phosphorylated in response to stress. Under normal conditions, eEF2 catalyses the transfer of peptidyl-tRNA from the A site to the P site on the ribosome (Figure 3.4). Phosphorylation of eEF2 at threonine 56 blocks this transfer; the protein kinase that catalyzes this event is dependent on Ca^{2+} and calmodulin and can also be activated by PKA and increased cAMP that occurs under cellular stress (Figure 3.3b).

The present chapter uses heterologous probing of cDNA arrays to search for and identify genes that are putatively up-regulated in bat or ground squirrel muscles during hibernation. The data also show that the majority of gene transcripts do not change during hibernation, as would be expected in the hypometabolic state, and additional studies demonstrate that overall protein synthesis rates are likely reduced in these tissues via

regulation of ribosomal translation involving reversible phosphorylation control of eIF2 α or eEF2.

MATERIALS AND METHODS

Animals

Animals were prepared and treated as described in Chapter 2.

RNA preparation

All materials used for RNA preparation were treated with 0.1% v:v diethylpyrocarbonate (DEPC) and autoclaved. Total RNA was isolated from euthermic and hibernating animals using Trizol (Invitrogen Gibco BRL) reagent (1.5 ml per 100 mg of tissue) according to the manufacturer's protocol and then poly (A)+ RNA was isolated from total RNA using Qiaquick poly(A)+ isolation kits (Qiagen) and the manufacturer's protocol. Concentrations of RNA were determined spectrophotometrically at 260 nm. Quality of total RNA was determined by separation on a 1.2% denaturing formaldehyde agarose gel by the presence of sharp and distinct 28S and 18S rRNA bands through visual inspection. RNA was stored at -80°C until required for further use.

First strand cDNA synthesis

³²P-labelled cDNA. First strand synthesis of labelled cDNA was prepared for hybridization to cDNA arrays. Total RNA (2-5 μ g) in a volume of 2 μ l was added to 1 μ l of CDS primer mix (primers specific for genes found on Clontech arrays) in a 0.5 ml Eppendorf tube and heated for 2 minutes at 70°C. Tubes were then placed at 50°C for 2 minutes to allow for the annealing of primers. A master mix was prepared containing (per reaction) 2 μ l 5X reaction buffer (Superscript RT, Invitrogen), 1 μ l of 10mM dNTP stock

(without dATP) and 3.5 μl α - ^{32}P dATP (10 $\mu\text{Ci}/\mu\text{l}$) (AP Biotech). While annealing primers, 1 μl of Superscript RT (Invitrogen) was added to the master mix (per reaction) and mixed. After 2 minutes at 50°C, 7 μl of master mix was added to the tubes, which were then incubated for 25 minutes. Column chromatography was performed to separate unincorporated nucleotides from labelled probe. Briefly, a tube comprised of Sephadex G50 suspended in TE (10 mM Tris base, 1 mM EDTA, pH 8.0) buffer was added to a 1 cc syringe barrel and spun at 500 rpm for 2 minutes. Probe was boiled with salmon sperm and cold-snapped on ice to remove any secondary structure.

Fluorescently labelled cDNA. Cy3 or Cy5 probe was prepared according to protocols obtained from the Ontario Cancer Institute Microarray facility with a few modifications. For 10-20 μg of total RNA, the reaction volume was 40 μl with 8 μl 5X first strand buffer, 1.5 μl Anchored T mRNA primer (5'-T₂₀VN, 100 pmol/ μl), 3 μl dNTP (20 mM total, no dCTP), 1 μl 2 mM dCTP, 1 μl of 1 mM Cy3 or Cy5 labelled dCTP, 4 μl DTT (0.1 M) and ddH₂O to a final volume of 40 μl . The reaction mix was heated at 65°C for 5 minutes and then cooled to 42°C for 5 minutes. Following this, 2 μl Superscript II (Invitrogen) was added and 1 μl of RNAsin. The reaction was incubated for 2-3 hours at 42°C. After the incubation, 4 μl of 50 mM EDTA and 2 μl of 10 M NaOH were added to tubes and incubated at 65°C for 20 minutes (to degrade RNA that could potentially act as an inhibitor of the fluorescently labelled cDNA). Acetic acid (4 μl , 5 M) was added to neutralize the solution (as determined by spotting on pH paper) and 100 μl of isopropanol was added and the tubes were incubated on ice for 30 minutes. Tubes were centrifuged for 10 minutes at 10000 rpm at 4°C to precipitate the cDNA. Precipitated cDNA was

washed with 70% ethanol and allowed to dry before resuspending in 5 μ l of H₂O. From this point, cDNA was either frozen for future hybridization or hybridized immediately.

DNA array hybridization and analysis

Clontech rat arrays were hybridized at 55°C in modified Church's buffer (0.5 M sodium phosphate, 7% SDS) overnight and subsequently washed in 2X SSC, 1X SSC and 0.5X SSC at room temperature (5 minutes each wash) until counts per minute (cpm) were between 800-2000. Multiple exposures of the arrays to X-ray film was done providing a good dynamic range for quantification. Quantification of gene expression data was done by one of two methods, either by using Imagequant and normalizing to genes near the differentially regulated gene or by using the freeware program, EST (www.uni-mainz.de/FB/Medizin/Tumorgenetik/array/download.htm). Comparable results were obtained with both programs.

Human 19K cDNA arrays were purchased from the Ontario Cancer Institute Microarray facility. Fluorescently labelled cDNA was added to prepared hybridization buffer. Hybridization buffer was prepared (per reaction) by taking 100 μ l DIG easy hyb solution (Roche), adding 5 μ l yeast tRNA (Life Technologies, 10 mg/ml), 5 μ l calf thymus DNA (10 mg/ml) and incubating this for 2 minutes at 65°C and then cooling to room temperature. An 80 μ l aliquot of hybridization solution was added to each labelled probe (Cy 3 or Cy 5) and the tubes were then heated to 65°C for an additional 2 minutes and cooled to room temperature before adding to the microarray slides. Slides and fluorescent cDNA were hybridized for 18 hours in a microscope slide holder at 37°C in an air incubator and kept level to ensure even distribution of cDNA. Washing was done the following day beginning with 5X SSC, 0.1% w/v SDS for 10 minutes at room

temperature. Subsequent washes reduced the SSC concentration (2X, 1X and 0.5X). All washes were for 10 minutes. Following washing, slides were dipped in isopropanol and spun dry in 50 ml conical tubes, with kimwipes placed in the bottom of the tubes, for 10 minutes at 500 g in table top centrifuge. Human 19K cDNA arrays were initially analyzed using Scanalyze software and later quantified with ArrayPro.

Western blotting

Western blotting was performed as described in Chapter 2. Antibodies specific for eIF2 α , phospho-eIF2 α (serine 51), eEF2 and phospho-eEF2 (threonine 56) were purchased from Cell Signaling.

RESULTS

Transcriptional profiling of heart and skeletal muscle using DNA array screening

Clontech AtlasTM Rat cDNA arrays were probed with ³²P-labelled probe derived from RNA isolated from tissues of euthermic and hibernating *M. lucifugus* and *S. tridecemlineatus*. AtlasTM Rat cDNA arrays contained 588 PCR amplified cDNA targets (series 1) or 1176 PCR amplified cDNA targets (series 1.2 and series 1.2 II). Target cDNAs are immobilized on nylon membranes in a macroarray format. Fluorescently labelled dyes were used for hybridization of cDNA derived from bats and ground squirrels to human cDNA microarrays containing 19,200 immobilized cDNA targets. Typical hybridization results are shown in Figure 3.5, part A showing a Clontech AtlasTM cDNA array probed with radiolabelled cDNA derived from *M. lucifugus* heart and part B showing a subarray (1 of 32 arrays contained on a microscope slide) from the human 19K cDNA microarray probed with fluorescently labelled cDNA derived from *M. lucifugus*

skeletal muscle. As can be seen from the figure, significant levels of hybridization are possible with heterologous probing mainly because the PCR amplified targets contain conserved, or at minimum, partially conserved regions of the genes in question. The lack of hybridization to negative controls on the DNA arrays (PCR amplified fragments from *Arabidopsis thaliana*) increases confidence in heterologous probing of cDNA microarrays. Through repeated trials, a more accurate representation of gene expression profiling in heart and skeletal muscle could be obtained, however, these experiments were performed to provide insight into gene up-regulation that can provide the basis for future studies. Undoubtedly, the genes presented here must be repeated and confirmed using standard and accepted techniques of gene expression determination such as northern blotting or quantitative RT-PCR.

Analysis of the array data for heart and skeletal muscle of both species showed species- and tissue-specific variation in gene expression during hibernation and identified a number of genes in each case as putatively up-regulated during hibernation. Putatively up-regulated genes are listed in Appendix 3.1 for *S. tridecemlineatus* and Appendix 3.2 for *M. lucifugus*. Analysis of gene expression in heart of *S. tridecemlineatus* showed no apparent up-regulation of genes during hibernation when a minimum 2-fold cut-off was applied to cross-hybridization with human 19K cDNA arrays whereas 7 genes were identified as putatively up-regulated by 3-fold or greater when Clontech Atlas cDNA arrays (rat series 1.2) were used (Appendix 3.1). These 7 potentially up-regulated mRNA transcripts represented 0.6% of the total cDNA targets present on the array. On average, approximately 75-90% of the cDNAs on the Atlas array displayed a signal with significant intensity to be considered quantifiable and, by contrast with the very low

percentage of up-regulated genes, approximately 10% of the cross-reacting genes showed strong down-regulation during hibernation.

Studies with *M. lucifugus* heart, in contrast to *S. tridecemlineatus* heart, revealed approximately 20 genes that were up-regulated by at least 3-fold during hibernation as assessed on Rat Atlas cDNA arrays (Rat series 1.2 and 1.2 II for a total of 2.4K genes); this represented up-regulation of close to 0.9% of the total genes on the array (Appendix 3.2). There was not a sufficient fluorescent signal derived from labelled cDNA to generate hybridization patterns with human 19K cDNA arrays.

Skeletal muscle is often thought to be dormant during hibernation but heterologous array screening revealed a substantial number of genes that were hibernation responsive. Screening with probe generated from *S. tridecemlineatus* skeletal muscle revealed 5 genes (0.9 % of the 588 on the array) that showed a minimum 3-fold up-regulation on the Rat cDNA arrays (Series 1) (Appendix 3.1). The same 5 genes were identified as up-regulated during screening with Series 1.2 cDNA arrays, although at less than the 3-fold threshold, whereas an additional 15 targets were identified as up-regulated (3-fold minimum) using rat Series 1.2 arrays (representing 1.3% of the genes on the array). Heterologous probing of the human 19K cDNA arrays, paradoxically, indicated only 2 genes that were up-regulated by 2-fold or greater. One would assume that with increased immobilized targets, an increase in the number of genes identified as putatively up-regulated would occur (as was seen when Clontech series 1 and series 1.2 rat cDNA arrays were compared), yet this was not the case.

Use of the human 19K gene arrays to assess gene expression during hibernating in *M. lucifugus* skeletal muscle revealed approximately 50 clones that were up-regulated on

reciprocally labelled microarrays with a minimum of 2-fold up-regulation. The genes, their accession numbers and fold up-regulation are listed in Appendix 3.2. The genes that were up-regulated during hibernation constituted 0.28% of the total number of genes on the array, again suggesting that very few genes need to be up-regulated to make the transition from euthermic to hibernating states. Similarly, heterologous probing with Clontech cDNA rat arrays (Series 1.2) revealed 5 potentially up-regulated genes or approximately 0.4% of the total genes found on the array. The percentage of down-regulated genes on the same array was closer to 10% suggesting that differential regulation during hibernation is skewed towards reduction in the overall transcript pool.

Translational status of heart and skeletal muscle

The phosphorylation status of eIF2 α has previously been linked to the rate of translation in brain and kidney of hibernating ground squirrels [Frerichs et al., 1998; Hittel and Storey, 2002] however; the effects in heart and skeletal muscle have not yet been investigated. Western blotting was used to assess both the total levels of eIF2 α in euthermic and hibernating muscles and, using antibodies raised against the serine 51 phospho-peptide, the relative amount of eIF2 α that is phosphorylated. As Figure 3.6 shows, total levels of eIF2 α did not change between euthermic and hibernating states in either heart or skeletal muscle of bats or ground squirrels. The amount of eIF2 α present in the phosphorylated (Ser51) form increased during hibernation in all cases. In hibernating *M. lucifugus*, the amount of phospho-eIF2 α was 5.2 ± 1.4 fold (mean $\pm$ SEM, n=3) higher in hearts and 2.1 ± 0.5 fold higher in skeletal muscle (Figure 3.6b). In *S. tridecemlineatus*

higher levels of phospho-eIF2 α were also found in hibernation, 3.0 ± 0.3 and 9.0 ± 1.7 -fold higher for heart and skeletal muscle, respectively.

Figure 3.7 shows a similar analysis of eEF2. Again, the total amount of eEF2 did not change between euthermia and hibernation in any case (Fig. 3.7). Phospho-eEF2 (Thr 56) content rose in skeletal muscle of both species during hibernation by 14.7 ± 3.4 and 2.3 ± 0.4 fold in *M. lucifugus* and *S. tridecemlineatus*, respectively (Figure 3.8b). By contrast, phospho-eEF2 did not change in hibernating heart of either species.

DISCUSSION

Differential gene expression and gene screening, once an arduous task, has become rather quick and unproblematic over the last few years with the advent of cDNA arrays. Gone are the days of blindly screening a cDNA library, which tends to present a bias towards genes with high abundance transcript levels. Arrays allow for the identification of genes expressed at low levels that may be significant in regulating cellular homeostasis during prolonged exposure to stress. Screening using immobilized cDNAs on an array offers a number of advantages. The most obvious is the time required to screen an array, which is typically about one week from the isolation of RNA to the completion of computer-assisted analysis of differential gene expression for the thousands of genes on the array. By contrast, many months and years can be required to construct a cDNA library, differentially screen it, isolate putatively up-regulated clones, sequence them and search for their identity. cDNA arrays also offer the advantage of allowing researchers to look at specific subsets of genes that are may be differentially

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greater fold up-regulated of genes than do microarrays. For example, a comprehensive study by Taniguchi et al. (2001) quantitatively comparing DNA microarrays and Northern blots found that for 4 of 46 genes compared, DNA arrays did not detect expression changes that were evident on Northern blots. This suggests that the sensitivity of microarrays is inferior to that of Northern blotting. However, for the majority of the genes that were induced or suppressed, DNA microarray results appeared to be quantitatively similar to those found on Northern blots with the exception of one gene where DNA microarrays detected a 4-fold induction but Northern blotting revealed a 60-fold increase [Taniguchi et al., 2001].

Initial experiments on the 19K cDNA arrays compared gene expression in heart and skeletal muscle of euthermic versus hibernating *M. lucifugus* and *S. tridecemlineatus*. A two-fold up-regulation was identified as the minimum that should be used for cDNAs that will be actively pursued in future research, as this is the level that is considered to be the cut-off by most researchers [Hedge et al., 2000]. Changes of lesser magnitude are typically within the range of variation and noise on DNA microarrays.

The use of heterologous screening on human cDNA arrays to assess gene expression in hibernating mammals opens the possibility for global profiling of gene expression in all tissues of hibernating mammals, however, the up-regulation of specific targets must still be confirmed via northern blotting or PCR techniques. Good fluorescence signal intensity on the spotted cDNAs was achieved and, after optimizing washing temperature, excellent cross-reactivity of up to 85% was attained. This bodes well for the future use of heterologous screening as a mechanism of gene discovery. The advent of DNA arrays has allowed researchers to bypass the long and arduous tasks

involved in differential screening of cDNA libraries. Although library screening is a trusted method of gene discovery, it consumes time and resources that can be saved by moving to a DNA array screening protocol. Our lab has encountered few problems with using commercially available DNA arrays to investigate gene expression in other mammalian species. For example, the up-regulation of H-FABP and A-FABP during hibernation in *S. tridecemlineatus* was identified using the use of Clontech ATLASTM arrays [Hittel and Storey, 2001], demonstrating the value of cDNA arrays for gene discovery via heterologous probing. The roles of each, A- and H-FABP, in hibernating bats are investigated in Chapter 4. Recent studies on cardiovascular disease models using pigs have also demonstrated the benefits of cross-species hybridization using cDNA arrays [Medhora et al., 2002].

Of the putatively up-regulated genes on the arrays, several generalizations can be made. Routinely, genes were identified that could fit one of five broad categories for heart and skeletal muscle. These were: genes involved in the switch from carbohydrate to fat metabolism through insulin-mediated signaling, oxygen-responsive genes, signaling molecules, solute carriers and active transporters, and structural genes involved in preserving the integrity of heart and skeletal muscle.

Of the putatively up-regulated genes, eIF2 α appeared to be more highly expressed in hibernating hearts of *M. lucifugus*, implicating it as playing a role in hibernation. However, whether or not transcript levels actually increased, there was no increase in total eIF2 α protein in hibernating bat hearts although as discussed below the phosphorylation state of eIF2 α certainly changed during hibernation. Genes involved in oxidative stress response that were up-regulated in hibernating bat heart included:

interleukin 9 receptor, trefoil factor, and acid phosphatase (also important in signal transduction). Genes involved in energy metabolism and regulation identified included: 5'AMP kinase, neurogenin, and malate dehydrogenase. Structural integrity of the heart must also be maintained and this may occur through a number of genes. The basic helix-loop-helix transcription factor *Mist1* appeared to be up-regulated, which is an inhibitor of *MyoD* (an important gene in muscle growth). Kinesin also appeared up-regulated. Active transport is responsible for maintaining cellular integrity under a number of different stresses. Regucalcin expression increased in the heart, as did aquaporin expression, which may be related to evaporative water loss occurring in hibernating animals [Speakman and Thomas, 2002].

Skeletal muscle of *M. lucifugus* showed some interesting genes that were apparently up-regulated during hibernation including soluble superoxide dismutase 1, which may play a role in the oxidative stress response. Also of interest were the observed changes in insulin responsive genes such as: receptor docking protein (*p60^{dok}*), insulin-like growth factor binding protein 6 (*IGFBP6*), insulin-like growth factor II, hydroxy steroid dehydrogenase, cytochrome P450, protein tyrosine phosphatase 1B, CCAAT-box binding transcription factor and interferon gamma (also involved in immune response). Genes involved in signal transduction that are up-regulated consisted of: tetraspan, CDC-like 2 protein and EGF receptor. Translation rates are likely suppressed in skeletal muscle as evidenced by the phosphorylation status of eEF2 and eIF2 α and, in addition, a number of genes involved in transcription and translational suppression were putatively up-regulated in hibernating bat skeletal muscle. These included: translation termination factor, CTD phosphatase and CCCTC binding zinc finger transcription factor. Structural

integrity of the muscle may be preserved by putative up-regulation of Ca^{2+} binding atrophy-related autoantigen 1, fidgetin – a chaperone type protein, syntrophin, collagen, dynamin and fibrinogen, which is a clotting factor whose expression in mammals is typically only in the liver.

In skeletal muscle of *S. tridecemlineatus*, the genes for heat shock protein 70 (*hsp70*) and heat shock protein 90 (*hsp90*) also appeared to be up-regulated in hibernation. Although named heat shock proteins, these proteins are general stress response proteins that act as cellular chaperones to protect other proteins from damage encountered during an external stress. Heart of *S. tridecemlineatus* displayed increases in insulin-like growth factor binding protein 5 (*IGFBP5*), a potential downstream target of retinoic acid receptors and peroxisome proliferator activated receptors (PPARs, which will be discussed in more detail in Chapter 4) [Dailly et al., 2001; Lewitt et al., 2001], and acyl coenzyme dehydrogenase, reiterating the role of fatty acid metabolism during hibernation.

Translation states in heart and skeletal muscle were investigated in both species to determine the likelihood that increases in gene expression levels observed on cDNA array screening would actually result in increased protein synthesis. As with previous studies in the field of hibernation that have looked at translational status of tissues and organs [Frerichs et al., 1998; Chen et al., 2001; van Breukelen and Martin, 2001; Hittel and Storey, 2002], the phosphorylation state of eIF2 α was investigated and found to be higher in both the heart and skeletal muscles of *M. lucifugus* and *S. tridecemlineatus* during hibernation. Higher levels of phosphorylation have been associated with a decrease in protein translation rate during hibernation [Frerichs et al., 1998; Hittel and Storey, 2002].

The phosphorylation state of eEF2 has recently been implicated as playing a key role in suppression of protein synthesis in hibernator brain [Chen et al., 2001], where higher levels of eEF2 were linked to overall suppression of translation rates and suppression of protein synthesis. The current study on skeletal muscle expands the possibility that the same mechanism of metabolic suppression may be occurring in other organs and tissues. Increased phosphorylation of eEF2 did not occur in heart of either species, suggesting that the hibernation-related suppression of protein synthesis may not be as great in the heart, which may be due to the continuing function of heart during torpor.

The overall increase in phosphorylation of eIF2 α and eEF2 in skeletal muscle represents two key stages of suppression of protein synthesis in *M. lucifugus* and *S. tridecemlineatus* during torpor. The probable reduction of protein synthesis rate in hibernating heart and skeletal muscles represents a key component to the overall metabolic suppression that is needed for long term torpor.

This chapter has introduced the notion that cDNA arrays can be used for heterologous probing to identify the global levels of gene expression between euthermic and hibernating tissue samples. With many of the transcripts not changing expression or appearing to be down-regulated during hibernation, it is probable that this is another key strategy for overall metabolic rate reduction. Screening of cDNA microarrays provides potential targets for further gene studies on gene expression in hibernating animals. With phosphorylation of eIF2 α and eEF2 (in skeletal muscle), whether or not an increase in gene expression leads to an increase in protein is still questionable and warrants further study.

Figure 3.1. Differential screening of control versus experimental clones in a cDNA library. Typically, clones are bound to duplicate membranes (nylon), which are then screened with probe (often radioactive) derived from control and experimental samples. Clones that are differentially expressed between the two states can be isolated and identified and used to confirm differential expression.

Control

Experimental

Clone and confirm

Figure 3.2. Two main methods of cDNA array production. (A) PCR amplified cDNA is spotted onto a solid support such as glass or nylon and then fixed onto the support. (B) The method developed by Affymetrix in which short oligonucleotides are synthesized directly onto a support using chemical protecting groups and photolithography.

Repeat steps 1-3

M: photolithographic mask

Figure 3.3. Phosphorylation of initiation complexes and elongation complexes in response to stress. Various stress stimuli can have an effect on the phosphorylation states of eIF2 α (A) and eEF2 (B). Some of these stimuli are listed. In each case, phosphorylation results in a suppression of translation.

- A. Amino acid starvation
Heat shock
Hypoxia

- B. Ca^{2+}
cAMP

Figure 3.4. Overview of the initiation and elongation phases of protein synthesis. (A) Initiation of protein synthesis involves the transfer of Met-tRNA to the 43S preinitiation complex. This process is GTP dependent but upon phosphorylation of eIF2 α the exchange of GDP for GTP is blocked, thus suppressing transfer of any further Met-tRNA to the preinitiation complex. (B) Phosphorylation of eEF2 effectively blocks the transfer of peptidyl-tRNA from the A to P sites on the ribosome thus blocking elongation of the growing peptide chain.

Figure 3.5. Representative examples of cDNA arrays hybridized with cDNA derived from *M. lucifugus*. (A) Clontech Rat 1.2 II nylon macroarray containing 1188 individual cDNAs hybridized with ³²P-labeled heart cDNA. (B) Human 19K cDNA microarrays from the University of Toronto Clinical Genomics Centre heterologously probed with fluorescently labelled cDNA derived from *M. lucifugus* skeletal muscle. Euthermic samples are labelled with Cy3 (green) and hibernating samples are labelled with Cy5 (red). The relative intensity of green or red determines differential expression. A signal of yellow indicates nearly identical levels of expression between euthermic and hibernating derived probes. The subarray shown is one of 64 in the 19K human gene set. This particular subarray shows 300 cDNAs spotted in duplicate for a total of 600 spots.

A.

B.

Figure 3.6. The state of eIF2 α in tissues of euthermic (E) and hibernating (H) animals. Shown are representative western blots of total and phosphorylated eIF2 α in (a) little brown bat heart, (b) little brown bat skeletal muscle, (c) ground squirrel heart, (d) ground squirrel skeletal muscle. Mean ($\pm$ SEM) values for band intensities were calculated from three trials for both euthermic and hibernating samples, followed by significance testing using the Student's t-test (* - hibernator value is significantly different from euthermic value, $P < 0.05$). The ratio of Hibernating:Euthermic mean values was then calculated and these are presented in the histogram. eIF2 α antibodies were detected at 40 kDa.

Figure 3.7. The state of eEF2 in tissues of euthermic (E) and hibernating (H) animals. Shown are representative western blots of total and phosphorylated eEF2 in (a) little brown bat heart, (b) little brown bat skeletal muscle, (c) ground squirrel heart, (d) ground squirrel skeletal muscle. Other information as in Figure 3.6. eEF2 antibodies were detected at 100 kDa.

A.3.1 Putative up-regulation of genes during hibernation in ground squirrels determined through cDNA array screening.

S. tridecemlineatus heart assessed via Clontech Rat Array series 1.2

Gene	Genbank Accession Number	Fold Increase
Ribosomal protein S29	X59051	7.1
Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	M32247	5.5
Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight-chain	J02791	4.5
Serine/threonine kinase PCTAIRE2 (PCTK2)	AB005540	4.1
Insulin-like growth factor-binding protein 5	M62781	3.1
Syntaxin 2	M95735	3.1
Cytochrome P450, subfamily I (aromatic compound-inducible), member A1 (C6, form c)	X00469	3.0

S. tridecemlineatus skeletal muscle assessed via Clontech Rat Array series 1

Gene	Genbank Accession Number	Fold Increase
OCT-1A	U76379 (E5m)	>10
SH-PTP2 protein tyrosine phosphatase, non-receptor type 11	U09307 (B2f)	5.5
Murine thymoma viral (v-akt) oncogene homolog 1	D30040 (C2c)	4.6
Cadherin 22	D83349 (D7a)	3.1
Heat shock protein 70-1	Z27118 (A7g)	3.0

S. tridecemlineatus skeletal muscle assessed via Clontech Rat Array series 1.2

Gene Name	Genbank accession number	Fold Increase
GTPase Rab14	M83680 (E14k)	5.1
Neuronal pentraxin receptor	AF005099 (D11m)	4.6
Arginine vasopressin receptor 1B	D45400 (D04c)	4.4
v-crk-associated tyrosine kinase substrate	D29766 (F05a)	4.3
DNA-damage-inducible transcript 1	L32591 (A08c)	4.1
Dopamine receptor D4	M84009 (D03d)	3.9
Aquaporin 2	D13906 (B12b)	3.8
Cyclin-dependent kinase inhibitor 1B, (p27, Kip1)	D83792 (A07j)	3.5
solute carrier family 22 (organic anion transporter), member 6	AB004559 (B01j)	3.2
Adenylyl cyclase 2	M80550 (F03f)	3.1
Synuclein, alpha	S73007 (C10l)	3.1
Heat shock 90-kDa protein beta (HSP90-beta); HSP84; HSPCB	S45392 (A13k)	3.0
Protein tyrosine phosphatase, non-receptor type 1	M33962 (E12f)	3.0
Retinoblastoma-related gene	D55627 (A13h)	3.0
Small inducible cytokine B subfamily (Cys-X-Cys), member 10	U17035 (F02c)	3.0
Protein tyrosine phosphatase, non-receptor type 1	M33962 (E12f)	3.0

S. tridecemlineatus skeletal muscle assessed via human 19K cDNA arrays

Gene	Genbank Accession Number	Fold Increase
Potassium inwardly rectifying channel, subfamily J, member 4	NM004981	6.5
Receptor tyrosine kinase-like orphan receptor 1	NM005012	2.3

A.3.2 Putative up-regulation of genes during hibernation in bats determined through cDNA array screening.

M. lucifugus heart assessed via Clontech Rat array series 1.2 II

Gene	Genbank Accession Number	Fold Increase
Interleukin 9 receptor	NM 017021	9.4
Regucalcin	D38467	7.1
Trefoil factor 1	D83231	6.9
5'-AMP activated protein kinase, β subunit	X95577	5.5
eIF2 α	U05821	5.3
Neurogenin	U67777	5.0
Acid phosphatase 2	NM 016988	4.1
Basic helix-loop-helix transcription factor	NM 012863	4.1
R calcium activated potassium channel (rSK3)	U69884	3.8
Thyroid hormone responsive protein	U94904	3.8
Kinesin light chain A	M75146	3.6
Thyroid hormone responsive protein 2	K01934	3.5
Cytosolic malate dehydrogenase	AF093773	3.4
Vasopressin	NM 012996	3.4
Chondroitin sulfate proteoglycan 3	M97161	3.2

M. lucifugus heart assessed via Clontech Rat array series 1.2

Gene	Genbank Accession Number	Fold Increase
Interleukin 1 receptor, type 1	M99578	5.8
Tumor associated protein 1	U00995	5.2
Ras related GTP binding protein 4b	X78605	3.2
Protein tyrosine phosphatase, non receptor type 1	M33962	3.0
Regulator of G-protein signaling 4	U27767	3.0

M. lucifugus skeletal muscle assessed via Clontech Rat Array series 1.2

Gene	Genbank Accession Number	Fold Increase
Hydroxysteroid dehydrogenase, 11 beta type 2	U22424	>10
Insulin-like growth factor II (somatomedin A)	M13969	6.0
Cytochrome P450 1b1	U09540	5.7
Tumor-associated protein 1	U00995	5.3
Superoxide dismutase 1, soluble	Y00404	3.2

M. lucifugus skeletal muscle assessed via human 19K cDNA array

Gene	Genbank Accession Number	Fold Increase
Docking protein 1, 62kD (downstream of tyrosine kinase 1)	NM001381	3.7
Phospholamban	NM002667	3.2
potassium voltage-gated channel, shaker-related subfamily, beta member 2	NM003636	3.1
Protein tyrosine phosphatase, non-receptor type 12	NM002835	3.0
tetraspan 2	NM_005725	2.9
DAZ associated protein 2	NM014764	2.8
interferon, gamma-inducible protein 16		2.8
cell recognition molecule Caspr2	AF193613	2.7
EBNA-2 co-activator (100kD)	XM011618	2.7
Fanconi anemia, complementational group G	BC000032	2.7
Granulin	BC010577	2.7
HT017 protein	NM020678	2.7
membrane component, chromosome 11, surface marker 1	NM005898	2.6
signal-induced proliferation-associated gene 1	NM006747	2.6
Uncharacterized bone marrow protein, BM040	AF217516	2.6
zinc finger protein 162		2.6
Apical protein, Xenopus laevis-like	NM001649	2.5
CCAAT-box-binding transcription factor	NM005760	2.5
microphthalmia-associated transcription factor	NM000248	2.5
Mitogen-activated protein kinase kinase kinase 4	NM005922 (variant 1) NM006724 (variant 2)	2.5
pregnancy specific beta-1-glycoprotein 1	NM006905	2.5
tetraspan transmembrane 4 super family	AF027204	2.5

M. lucifugus skeletal muscle assessed via human 19K cDNA array - continued

transcription termination factor, RNA polymerase II	AF073771	2.5
Calcium binding atrophy-related autoantigen 1	NM006077	2.4
cytokine receptor-like molecule 9	AF120151	2.4
Fidgetin	XM171005	2.4
Fusion, derived from t (12;16) malignant liposarcoma	NM004960	2.4
Galectin 9	NM002308 (short variant) NM009587 (long variant)	2.4
MARK		2.4
MUF1	XM170939	2.4
Serine protease, umbilical endothelium		2.4
Solute carrier family 12 (potassium/ chloride transporters), member 6	NM005135	2.4
Syntrophin, beta 1 (dystrophin-associated protein A1, 59kD, basic component 1)	NM_021021	2.4
Angiogenin, ribonuclease, RNase A family, 5		2.3
Collagen, type IV, alpha 1	NM001845	2.3
Cyclin-dependent kinase (CDC2-like) 10		2.3
Dynamin 1		2.3
Insulin-like growth factor binding protein 6	NM002178	2.3
Nucleoporin 50kD	NM007172	2.3
Putative protein O-mannosyltransferase	NM013382	2.3
REV1 protein	AF206019	2.3
Ribosomal protein L24	NM000986	2.3
SFRS protein kinase 1	NM003137	2.3
Small nuclear RNA activating complex, polypeptide 3, 50kD	NM003084	2.3
Bone morphogenetic protein 1	M22488	2.2
CD151 antigen	BC013302	2.2
Epidermal growth factor receptor (avian erythroblastic leukemia viral (v-erb-b) oncogene homolog)	NM005228	2.2
Lymphocyte-specific protein 1	NM002339	2.2
CATX-8 protein	BC009831	2.1
CCCTC-binding factor (zinc finger protein)	NM006565	2.1
CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1		2.1
Epithelial membrane protein 1	NM001423	2.1
Fibrinogen, B beta polypeptide	NM005141	2.1
Myosin regulatory light chain 2, smooth muscle isoform	NM_004715	2.1
Sex hormone-binding globulin	NM001040	2.1
RaP2 interacting protein 8	U93871	2.0

Chapter 4
**Molecular response of fatty acid
transport and metabolism; a possible
role for PGC-1 and PPARs in
hibernation**

INTRODUCTION

The key to initiating and maintaining the hibernating state is a coordinated suppression of the rates of multiple cellular systems allowing homeostasis to be re-established and sustained at a new low body temperature. For example, protein synthesis during hibernation is strongly suppressed [Frerichs et al., 1998] by means of multiple mechanisms including polysome disaggregation [Knight et al., 2000; Hittel and Storey, 2002], suppression of polypeptide initiation and elongation functions via phosphorylation of eukaryotic initiation (eIF2 α) and elongation (eEF2) factors [Frerichs et al., 1998; Chen et al., 2001], and a reduction in mRNA transcript availability [van Breukelen and Martin, 2002]. Carbohydrate catabolism is also suppressed through reversible phosphorylation control of key enzymes, including phosphofructokinase and pyruvate dehydrogenase, and the activities of energy-expensive membrane ion pumps (e.g. NaK-ATPase) are strongly reduced during hibernation [Storey, 1997].

Differential gene expression plays an important role in hibernation. Initial studies ten years ago [Srere et al., 1992] postulated that gene expression is essential for the establishment and maintenance of a hibernating state, and to date, significant increases in steady state mRNA transcript levels of a variety of genes have been shown to occur in hibernators. For example, levels of the transcription factors c-fos, junB and c-Jun increase in brain during hibernation [O'Hara et al., 1999]. Various recent publications have also identified specific changes in the expression of genes involved in fatty acid metabolism and mitochondrial-encoded genes. Genes encoding pyruvate dehydrogenase kinase isozyme 4 (PDK4) and pancreatic lipase (PLP) were up-regulated in hearts of

hibernating ground squirrels [Andrews et al., 1998]. Phosphorylation and inhibition of pyruvate dehydrogenase by way of PDK4 is key to suppressing carbohydrate usage whereas PLP is involved in the liberation of fat stores. Heart and skeletal muscle of *Spermophilus lateralis* showed up-regulation of myosin light chain 1 (MLC1) and NADH-ubiquinone oxidoreductase subunit 2 (ND2) [Fahlman et al., 2000] and *S. tridecemlineatus* showed increased transcripts of ATPase 6/8 and cytochrome c oxidase subunit 1 (Cox1) in BAT, heart and kidney [Hittel and Storey, 2002]. Elevated levels of the three mitochondrial-encoded transcripts (ND2, ATPase 6/8, Cox 1) may be due to a proliferation of mitochondria during hibernation or may function to increase the oxidative capacity of mitochondria to permit a heavy reliance on fatty acid oxidation as the primary fuel of metabolism during both hibernation and interboutal arousals. Adipose and heart type fatty acid binding proteins (A-, H-FABP), thought to play a central role in the intracellular transport of fatty acids, are also up-regulated in multiple tissues, including brown adipose tissue (BAT), heart and skeletal muscle of ground squirrels [Hittel and Storey, 2001]. These two genes are also markedly up-regulated in BAT of cold-exposed rats, suggesting that these proteins have a central role in the intracellular transport of fatty acids to be used for thermogenesis [Daikoku et al., 1997]. Uncoupling proteins (UCP) are also up-regulated during hibernation [Liu et al., 1997]. UCP-1 is critical for nonshivering thermogenesis (NST) by BAT; by creating a futile cycling in the proton gradient spanning the inner mitochondrial membrane of BAT they allow the release of energy as heat that would normally used for ATP synthesis [Boyer et al., 1998]. UCP-2 and UCP-3 also increase in white adipose tissue (WAT) and skeletal muscle, respectively, in response to hibernation [Boyer et al., 1998]. Up-regulation of UCP

transcripts also occurs in cold-exposed rats suggesting that UCP proteins play a role in cold tolerance [Yamashita et al., 1999].

Fatty acid oxidation is the principle source of energy for hibernating mammals during torpid bouts [Burlington et al., 1972]. Levels of enzymes and proteins involved in fatty acid oxidation appear to be controlled at the transcriptional level through a family of retinoic acid receptor related proteins called peroxisome proliferator activated receptors (PPAR) [Green and Wahli, 1994; Reddy and Mannaerts, 1994]. PPARs are nuclear receptors, that upon stimulation via a signaling cascade, translocate to the nucleus and act as transcription factors to increase the expression of a number of genes involved in fatty acid oxidation and regulation and maintenance of energy metabolism [Armstrong and Towle, 2001]. These include carnitine palmitoyl transferase 1 (CPT-1), fatty acyl-CoA oxidase, FABPs, HMG-CoA synthase and UCPs [Berger and Moller, 2002].

The present study further addresses the role and regulation of FABPs in hibernating mammals. Previously, our lab has identified up-regulation of *a-fabp* and *h-fabp* transcripts during hibernation in a ground squirrel, *S. tridecemlineatus*, and proposed that this up-regulation supports the expanded reliance of hibernator metabolism on fatty acid oxidation as its metabolic fuel source. The present study examines *fabp* gene expression in organs of the little brown bat, *M. lucifugus*, in order to determine if up-regulation of this gene/protein is a conserved mechanism of hibernation in an evolutionarily distinct species. In addition, the first analysis of the mechanism and co-factors involved in FABP regulation in a hibernator is conducted by analyzing the role of PPARs (PPAR α and PPAR γ) and the PPAR gamma coactivator-1 (PGC-1) in the control of *fabp* gene expression during hibernation. To assess the role that fatty acid oxidation

plays in hibernation further, total protein levels of CPT-1 were assessed in organs and tissues revealing expression of A- and H-FABP.

MATERIALS AND METHODS

Animals

Little brown bats (*M. lucifugus*) were captured near Sherbrooke, QC and treated as previously described in this thesis (Chapter 2).

RNA preparation

All materials used for RNA preparation were treated with 0.1% v:v diethylpyrocarbonate (DEPC) and autoclaved. Total RNA was isolated from BAT, heart, skeletal muscle and WAT of euthermic and hibernating bats using Trizol (Invitrogen Gibco BRL) reagent (1.5 ml per 100 mg of tissue) according to the manufacturer's protocol. Poly (A)+ RNA was isolated from total RNA using Qiaquick poly(A)+ isolation kits (Qiagen) according to the manufacturer's protocol. Concentrations of RNA were determined spectrophotometrically at 260 nm. RNA was stored at -80°C until required for further use.

cDNA synthesis and PCR amplification of H-FABP

First strand cDNA synthesis was performed on 1 µg poly (A)+RNA from *M. lucifugus* using Superscript II reverse transcriptase according to the manufacturer's protocol. An aliquot of first strand cDNA (0.5 µl) was used for PCR amplification of H-FABP. Primers for amplification of *h-fabp* were designed using Primer Designer version 3.0 (Scientific and Educational Software) and are based on the consensus mammalian sequences. Primers were: forward, 5'-GGTACCTGGAAGCTAGTGG-3' and reverse, 5'-

CGCCTCCTTCACGTAAGT-3'. PCR amplification was performed in a mixture of 5.0 μ l 10X PCR buffer (Invitrogen), 2.5 μ l $MgCl_2$, 1 μ M primer (0.5 μ M forward and 0.5 μ M reverse), 1 μ l dNTPs (10mM), 0.25 μ l Taq polymerase (5U/ μ l) (Invitrogen) with 0.5 μ l of the reverse transcribed cDNA, which was then diluted to 50 μ l with sterile H_2O . Cycles for amplification were 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute repeated a total of 40 times followed by a final step at 72°C for 7 minutes on a PTC-100 (MJ Research). A single product with a size of approximately 400 bp corresponding to the bat homolog of *h-fabp* was found and confirmed as *h-fabp* by DNA sequencing.

A modified 3' RACE protocol was used to amplify the 3' region of the *h-fabp* mRNA. The cDNA was amplified using the forward primer 5'-GGTACCTGGAAGCTAGTGG-3' with an oligo dT₂₅ reverse primer. The protocols described above were used with a few modifications. Annealing temperature was reduced to 50°C and the oligo dT primer concentration was 2.5 μ M. A single product corresponding to approximately 600 bp was obtained.

The 5' end of *h-fabp* was amplified using a SMART RACE amplification kit (Clontech). Poly(A)⁺ RNA was reverse transcribed into cDNA according to the manufacturer's protocol. Amplification of the 5' end of the gene was performed as described above but with a Universal Primer Mix (Clontech) and the reverse primer 5'-CGCCTCCTTCACGTAAGT-3', both at a final concentration of 1 μ M. PCR was performed as before with a modified cycle for amplification: denature at 94°C for 45 seconds, anneal at 55°C for 50 seconds and extension at 72°C for 1 minute. The above cycle was repeated 35 times and a final extension step at 72°C for 5 minutes was

performed. Electrophoresis on a 1% TAE gel revealed a product approximately 500 bp in size corresponding to the amplified 5' end of *M. lucifugus h-fabp*.

Sequencing

Sequencing was performed at Canadian Molecular Research Services (Ottawa, ON). The sequences for 5' RACE and 3' RACE were confirmed twice through sequencing revealing the same sequence each time and the sequence 383 bp amplified partial sequence of *h-fabp* was confirmed three times. Each sequence was verified as *h-fabp* by submitting sequences to BLAST. Sequences were then assembled and the complete transcript of *h-fabp* was obtained. The *M. lucifugus* sequence was submitted to Genbank (accession number AF530469).

Northern Blotting

A 15 µg aliquot of total RNA was electrophoresed on a 1% denaturing formaldehyde agarose gel. The quality of RNA was determined by the relative amounts of 28S and 18S ribosomal RNA bands. Blots were transferred overnight onto Hybond N nylon membrane in 10X SSC. The following day, blots were crosslinked in a UV crosslinker and baked at 80°C prior to probing. Blots were prehybridized in Church's buffer (0.25 M Na₂HPO₄, 0.25 M NaH₂PO₄, pH 7.5, 7% w:v SDS) at 55°C for at least 1 hour prior to hybridization. A clone for rat *α-fabp* was purchased from Research Genetics (IMAGE ID 1771436); which was previously used to retrieve *α-fabp* from a *S. tridecemlineatus* cDNA library [Hittel and Storey, 2001]; and excised from the plasmid. Purified rat *α-fabp* and partial *M. lucifugus h-fabp* were ³²P labelled by the random primer method as previously described [Hittel and Storey, 2001]. Blots were incubated overnight at 48°C with radiolabeled probes, washed the following day in 2X SSC:1%

SDS, 1X SSC:1% SDS and 0.1X SSC, 1% SDS at room temperature (5 minutes each wash), and exposed to X-ray film (X-OMAT AR, Kodak) and/or phosphor screens (BioRad). Quantification of *fabp* mRNA bands (size of ~700 bp) was performed using Imagequant (Molecular Dynamics) or software included with the BioRad Personal FX phosphorimager. The molecular weight of *fabp* mRNA was estimated from molecular weight markers run concurrently on the 1% formaldehyde gels.

Western Blotting

Tissues were homogenized in sample buffer (250 mM sucrose, 50 mM HEPES, 5 mM EDTA, 2mM EGTA, pH 7.4) and placed on ice. The quantity of protein was determined using the BioRad protein assay. Equal amounts of protein (10 or 20 µg) were electrophoresed on 15% SDS polyacrylamide gels using the MiniPROTEAN II system (BioRad) at 200 V for 1 hour followed by protein transfer to PVDF membrane at 400 mA for 2 hours at 4°C according to BioRad protocols. Blots were then blocked in 5% non-fat milk in TBST for 3 hours. Polyclonal antibodies were then added to the solution followed by overnight incubation at 4°C (antibody dilution of 1:1000). Human A-FABP and H-FABP antibodies were a gift of Dr. F. Spener (University of Munster, Germany). CPT-1 antibodies were a gift of Victor Zammit (Hannah Research Institute). The PGC-1 antibody was a kind gift of Dan Kelly (Washington University, St. Louis). Rabbit polyclonal human PPAR α antibody was purchased from Geneka (Montreal, QC) and mouse PPAR γ was purchased from Cederlane (Hornby, ON). After incubation with primary antibody, blots were washed three times successively for 5 minutes in 1X TBST and incubated with HRP linked Anti-Rabbit IgG (1:2000) in 1X TBST for 1 hour at room temperature. Blots were reacted with Renaissance Western Blot Chemiluminescence

Reagent Plus (Perkin Elmer) for 1 minute and exposed to X-ray film (X-OMAT AR, Kodak). Quantification of bands was performed using Imagequant software (Molecular Dynamics).

PPAR gamma DNA binding activity

Commercially available kits for the study of transcription factor binding were purchased from Active Motif. A 20-50 mg sample of tissue was homogenized in lysis buffer (20 mM HEPES, pH 7.5, 350 mM NaCl, 20% v/v glycerol, 1% Igepal-CA630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA) and centrifuged. Supernatant containing soluble protein was removed and placed protein concentrations were determined by using the Bio-Rad protein assay and concentrations of extract were adjusted to 0.5-1 $\mu g/\mu l$ with lysis buffer. The assay mixture in each well of the microplate contained 22.2 μl of cellular protein extract in lysis buffer, 0.23 μl of protease inhibitor cocktail and 0.11 μl of 1M DTT. Added to this was 30 μl of complete binding buffer solution, which consisted of 0.06 μl 1M DTT, 0.3 μl herring sperm DNA and 29.64 μl binding buffer (4 mM HEPES, pH 7.5, 120 mM KCl, 8% v/v glycerol, 1% w/v bovine serum albumin). Wells were incubated for 1 hour at room temperature with slight agitation after which wells were washed to remove excess protein with 1X washing buffer (10 mM phosphate buffer, pH 7.5, 50 mM NaCl, 0.1% Tween 20). A 100 μl aliquot (1:1000 dilution) of PPAR γ antibody was added and incubated for 1 hour at room temperature. After incubation, wells were washed three times with 200 μl with 1X washing buffer followed by addition of 100 μl of HRP conjugated secondary antibody and incubation for 1 hour at room temperature. Wells were then washed with four times with 200 μl 1X washing buffer. Developing solution (100 μl) was added to each well (TMB substrate in 1% DMSO) and

incubated for 2-10 minutes until the appearance of a dark blue colour. Finally, 100 μ l of 0.5 M H_2SO_4 were added to stop the reaction. Absorbance was measured at 460 nm with a microplate reader. Results were done in triplicate from pooled tissues of euthermic (n=10) and hibernating (n=10) bats.

RESULTS

Cloning of *M. lucifugus h-fabp*

Cloning of the complete bat *h-fabp* transcript was done using a variety of PCR techniques. A large portion of the open reading frame was amplified using primers developed from consensus mammalian sequences. The 3' portion of the gene was then amplified using a modified 3' RACE protocol using a gene specific primer and a dT_{25} primer with a reduced annealing temperature. Finally, a 5' RACE procedure was used to obtain the remaining portion of the sequence (Figure 4.1). BLAST searching of each of the sequenced PCR products confirmed that each represented a portion of *h-fabp*. Figure 4.2a shows the assembled sequence containing the full open reading frame (ORF) that runs from nucleotide position 120 to 518 (Genbank accession number AF530469). The start and stop codons are underlined in Figure 4.2 along with the deduced amino acid sequence. A comparison of the nucleotide sequence of *h-fabp* with the ORFs from other mammals revealed that the bat ORF was 88%, 87%, 86%, 85%, 82% and 80% identical with the sequences of the human, pig, cow, 13-lined ground squirrel, rat and mouse ORFs, respectively.

Translation of the open reading into the amino acid sequence (Fig. 4.2) revealed a protein of 133 amino acids with a molecular weight of 14.8 kDa and basic isoelectric

point (8.48). Figure 4.3 compares the amino acid sequence of *M. lucifugus* H-FABP with the sequences of the protein from another hibernator (the ground squirrel, *S. tridecemlineatus*) and several nonhibernating mammals. The bat protein is 90% identical to *S. tridecemlineatus* H-FABP, 87% identical to the human and rat proteins, and shows 89%, 85% and 86% identity with pig, mouse and cow H-FABP, respectively (Figure 4.4). Several amino acid substitutions are evident in the bat sequence, as compared with the others. Deduced from the mature protein after cleavage of the N-terminal methionine, these included an F→L substitution at position 70 in the bat sequence that is not observed in other mammalian species (residue underlined in Fig. 4.3). The significance of this substitution lies in the fact that phenylalanine is a fairly restricted residue whereas leucine is fairly flexible. At position 72, an E→K substitution replaces an acidic amino acid with a basic amino acid (lysine). This substitution occurs in the same structural area of the protein as a previously reported lysine substitution in the *S. tridecemlineatus* sequence (position 68) [Hittel and Storey, 2001]. Three more substitutions involving a switch to basic amino acids occur in the bat H-FABP, lysine at position 103 and asparagines at 120 and 121. Finally, at position 83, hydrophobic residues (isoleucine or valine) in nonhibernating mammals are substituted by threonine, a charged polar residue, in the two hibernators (the ground squirrel substitution was previously reported; [Hittel and Storey, 2001]). The substitutions mentioned may assist in the function of H-FABP at low temperatures. Figure 4.5 is a ribbon diagram with the approximate positions of F→L (70), E→K (72) and I/V→T (83) represented.

***h-fabp* and *a-fabp* transcript levels**

A partial cDNA for bat *h-fabp* was ^{32}P -labeled. A cDNA clone for rat *a-fabp* was purchased from Research Genetics and the gene insert was isolated from the plasmid and ^{32}P labelled. Both radiolabeled cDNAs were used to probe northern blots. A single transcript was detected for each of *h-fabp* and *a-fabp*, of a size of ~700bp. It is likely that the rat *a-fabp* cDNA hybridized only with bat *a-fabp*, and not with *h-fabp*. This is supported by studies with the ground squirrel genes that showed no cross-hybridization between *h-fabp* and *a-fabp* and only 47% nucleotide sequence identity between the two [Hittel and Storey, 2001], compared with the very high nucleotide sequence identity (>85%) between species when either *h-fabp* or *a-fabp* are considered individually. Furthermore, northern blots hybridized with *a-fabp* probe from *S. tridecemlineatus* produced identical patterns of *a-fabp* expression in bat tissues as were generated with the rat probe (data not shown).

Figure 4.6 shows expression levels of *a-fabp* and *h-fabp* mRNA transcripts in tissues of *M. lucifugus*. Neither transcript was detected in brain kidney or liver but both were found in BAT, heart, skeletal muscle and WAT. Transcript levels of *a-fabp* increased significantly during hibernation only in BAT (the hibernating:euthermic transcript ratio was 3.9 ± 1.4 , mean $\pm$ SEM, $n=3$, $P<0.03$) (Fig. 4.6a,c). *H-fabp* transcript levels rose significantly during hibernation in both BAT and skeletal muscle; the ratios hibernating:euthermic were 1.7 ± 0.1 and 1.8 ± 0.3 , respectively ($P<0.01$) (Fig. 4.6b,d).

A-FABP and H-FABP protein expression

Western blotting was used to determine whether increased transcript levels correlated to a rise in FABP protein during hibernation. Figure 4.7 shows representative western blots and displays the hibernating:euthermic ratio for A- and H-FABP levels in four tissues. Both proteins were detected in BAT, heart, skeletal muscle and WAT but their expression patterns in euthermia versus hibernation differed from those of the mRNA transcript. Levels of A-FABP protein did not increase significantly in any of the tissues analyzed, and was only slightly detectable in heart and skeletal muscle. H-FABP expression increased in three tissues during hibernation. The hibernating:euthermic ratio for protein levels was 1.7 ± 0.1 ($P < 0.005$) in BAT, 2.7 ± 1.0 ($P < 0.05$) in heart and very high at 7.1 ± 1.8 ($P < 0.05$) in skeletal muscle. The increase in protein levels in hibernating heart and skeletal muscle greatly exceeds mRNA expression levels in the same tissues.

CPT-1 protein expression

Given that in elevated levels of FABP, which transports fatty acids through the cytoplasm, were found in hibernator tissues, it seemed logical to predict that levels of the carrier protein that catalyzes the transfer of the fatty acid chain from CoA to carnitine allowing for the transport of fatty acids into the mitochondria may also be raised during hibernation to further assist in fatty acid oxidation. Transport of fatty acids into the mitochondria via the mitochondrial transporter, CPT-1, is the rate-limiting step in fatty acid oxidation [Foxworthy et al., 1990]. Western blotting was used to assess levels of two isoforms of CPT-1, the liver isoform CPT-1 α and the skeletal muscle isoform CPT-1 β , in BAT, heart, and skeletal muscle of euthermic versus hibernating bats. Neither heart nor skeletal muscle showed increased levels of either isoform of CPT-1 during

hibernation but both rose significantly in BAT. CPT-1 α levels were 3.0 ± 0.6 fold higher ($P < 0.001$) in BAT from hibernating animals whereas CPT-1 β levels were 2.1 ± 0.3 ($P < 0.05$) fold higher (Figure 4.8).

PPAR and PGC-1 protein levels: regulators of FABP transcripts?

FABP gene expression in other mammals is regulated by PPARs (PPAR α and PPAR γ) and the PPAR gamma coactivator-1 (PGC-1). Both PPAR α and PPAR γ can differentially activate different FABPs [Mochizuki et al., 2001]. Much has been hypothesized about a possible role for PPARs in hibernation but there has been little study to date. Western blotting was used to assess levels of PPAR α , PPAR γ and PGC-1 in *M. lucifugus* BAT, heart, skeletal muscle and WAT. Levels of PPAR α protein increased significantly during hibernation only in WAT (hibernating:euthermic ratio 3.5 ± 1.2 , $P < 0.01$) (Figure 4.9a,c), whereas PPAR γ levels increased significantly in all four tissues with hibernating:euthermic protein ratios of 3.0 ± 0.3 in BAT, 1.4 ± 0.2 in heart, 2.6 ± 0.5 in skeletal muscle and 1.7 ± 0.1 in WAT ($P < 0.05$ for all) (Figure 4.9b,d). Levels of PGC-1, a potent co-activator of PPAR α and PPAR γ , also rose significantly during hibernation in BAT (3.0 ± 0.5), skeletal muscle (2.2 ± 0.6) and WAT (1.8 ± 0.3) ($P < 0.05$).

Transcription factors act by binding to DNA so increased activity of transcription factors depends both on their protein concentration and the amount of binding to DNA. To assess PPAR γ binding to DNA, a commercially available DNA binding kit was used (TransAm transcription factor assay from Active Motif). Relative DNA binding activities by PPAR γ were determined for extracts from euthermic versus hibernating samples of each of the four tissues. DNA binding activity increased slightly in BAT

(hibernating:euthermic ratio was 1.2 ± 0.1 , $P < 0.05$) and skeletal muscle (1.7 ± 0.3 , $P < 0.05$) during hibernation but no significant differences were seen heart and WAT.

DISCUSSION

The heart isoform of fatty acid binding protein from *M. lucifugus* showed strong sequence identity at both the nucleotide and amino acid level with the gene/protein from other mammals. Over 80% nucleotide identity and >85% amino acid identity indicates that the protein is highly conserved among mammals. Several significant substitutions were noted. Both hibernator species, *M. lucifugus* and *S. tridecemlineatus* showed a threonine substitution at position 83 of the mature protein (replacing valine or isoleucine)(Figure 4.3, 4.5). This amino acid residue is found at the bottom of the fatty acid binding pocket, however, its function at this point is not entirely clear and may represent a possible phosphorylation site specific to hibernators [Hittel and Storey, 2001]. *M. lucifugus* H-FABP also showed a unique lysine substitution at position 72 (replacing glutamate) which is in the same structural region of the protein as the lysine substitution at position 68 (replacing valine) that is found in ground squirrel H-FABP. Glutamate at position 72 is a conserved region through all of the FABP members [Zimmerman et al., 1999], nonetheless, in hibernating bats it is substituted for a lysine. Site directed mutagenesis of this residue to serine resulted in a reduction in stability of H-FABP [Zimmerman et al., 1999], nevertheless, it cannot be assumed that the same would result would be observed in a hibernating animal where fatty acids are transported at low temperatures. In fact, the affinity of various fatty acids for isolated L-FABP from ground squirrels has been shown to equal or greater at low temperatures, to mimic the

hibernating state, as compared to normal assay conditions, to mimic euthermia [Stewart et al., 1998]. It is quite possible that these substitutions aid the function of H-FABP at the low body temperatures encountered during hibernation.

Three other unique substitutions are present in the bat sequence at position 70 (leucine), 103 (lysine) and 120-121 (asparagine). H-FABP also has an unusually high pI (8.48) relative to other mammalian H-FABPs (6.2). The phenylalanine to leucine substitution may allow for increased flexibility of the H-FABP binding pocket as phenylalanine is fairly rigid structure that has the capability to for π -bonding interactions through its aromatic ring, whereas leucine is fairly flexible with no such π -bonding capabilities. The presence of lysine a couple of lysine and asparagine substitutions likely account for this and may improve binding of H-FABP to the phospholipid membranes [Herr et al., 1996] and thus improve transfer of fatty acids through the cytosol during hibernation.

Identification of *a-fabp* transcripts and A-FABP protein in the skeletal muscle is a novel finding and goes along with the novel identification of *a-fabp* in ground squirrel heart [Hittel and Storey, 2001], a finding that also true of bat heart. The presence of the adipose form of FABP in skeletal muscle and hearts of hibernators may aid specific needs for fatty acid transport in hibernating species. Up-regulation of *a-fabp* transcript levels occurred in BAT of *M. lucifugus* during hibernation, and *h-fabp* transcripts also increased in BAT and skeletal muscle. The same pattern of *h-fabp* up-regulation was seen in ground squirrels [Hittel and Storey, 2001]. Elevated transcript levels do not always mean a comparable increase in the content of the protein that they encode. Indeed, recent studies in our lab on *S. tridecemlineatus* [Hittel and Storey, 2002a] and the marine snail

Littorina littorea [Larade and Storey, 2002] have shown that transcription and translation can be "uncoupled" under situations of environmental stress. Thus, these studies have identified several instances where transcript levels of selected genes are elevated in response to stress but the transcripts remain in the translationally silent monosome fraction throughout the stress; protein levels remain constant or decline. Gene up-regulation in these cases may put the transcripts in place so that they can be rapidly translated when the stress is lifted, such as during arousal in hibernators. Hence, it is crucial for a full understanding of the adaptive significance of gene up-regulation that not only mRNA transcript levels but also protein levels be quantified under control and stress conditions.

Elevated *α-fabp* transcript levels in BAT of *M. lucifugus* correlated with an increase in A-FABP protein content. Elevated *h-fabp* transcripts in BAT and skeletal muscle were consistent with increase H-FABP protein content in both tissues (although heart H-FABP also increased without a concurrent increase in transcripts). Both transcript levels and protein content of H-FABP also increased in BAT of *S. tridecemlineatus* during hibernation [Hittel and Storey, 2002]. The presence of A-FABP in hibernation has been proposed to transport fatty acids from intracellular lipid droplets by way of its association with hormone sensitive lipase that reside on the interface between the lipid droplet and the cytoplasm [Shen et al., 1999], thus enhancing transport and availability of fatty acids during hibernation [Hittel and Storey, 2001]. The similar up-regulation in both species, *M. lucifugus* and *S. tridecemlineatus*, of H-FABP, suggests an important role for elevated H-FABP content in BAT and skeletal muscle of hibernators, and with its high pI in *M. lucifugus*, a better association with the phospholipid membrane is possible [Fiona et al., 1999]. It has been proposed to be primarily involved in transport of blood borne fatty

acids [Hittel and Storey, 2001]. Having two forms of FABP present probably enhance the ability for BAT, heart, skeletal muscle and WAT to utilize fatty acids rapidly during hibernation and arousal.

The transcript in WAT displays a reduced size by approximately 50 nucleotides in both euthermic and hibernating isolations. Studies on poly A tail lengths in hibernating animals have shown that they are preserved in liver [Knight et al., 1998] through association with a poly A binding protein. Since WAT is mainly used for lipid storage there may not be a requirement for the same protective mechanisms displayed in liver during hibernation.

Levels of CPT-1, both α and β isoforms, increased during hibernation in BAT. CPT-1 is the rate limiting step in fatty acid oxidation and the protein responsible for transferring CoA for carnitine on long chain fatty acids and thus allowing them to enter into the mitochondria. Elevated CPT-1 content is consistent with the demand for high rates of lipid oxidation in BAT to supply the heat needed to rewarm the hibernator's body during arousal. A well-developed capacity for nonshivering thermogenesis by BAT must also be present during torpor because BAT is called upon to provide low level heating in torpor at any time when ambient temperature drops below 0-2°C. This prevents the hibernator from freezing. Elevated H-FABP and CPT-1 levels in BAT help to provide the necessary capacity for thermogenesis during both torpor and arousal. The increase in CPT-1 content may be related to higher levels of the active form of Akt in hibernating BAT, which has been linked to lipid metabolism [Sul et al., 2000]. Higher levels of CPT-1 in torpor may cause an increased utilization of fats, but this is not the case, rather it increases the capacity for fatty acid oxidation under optimal conditions. Because

metabolic rate is highly suppressed during hibernation [Storey, 1997] and because of the Q10 effect, which over a 30°C temperature range should suppress transport rates 8-10-fold, it is not likely that substantial fat loss is occurring in torpor and that the need for the protein is more likely for oxidation of fats during interboutal arousals.

Genes involved in regulating fatty acid metabolism in all tissues and organs have been shown to work through the PPAR response element (PPRE) with binding of PPAR α or PPAR γ and then activation by a number of factors, one of which is PGC-1. All genes involved in fat metabolism are thought to contain a PPRE, to which PPARs bind as a heterodimer complex with activated retinoic acid receptors (RXR) [Green and Wahli, 1994]. PPAR α is a known co-activator of nuclear-encoded mitochondrial genes involved in fatty acid oxidation [Vega et al., 2000]. Recent studies have shown that phosphorylation of PPAR α by p38 increases its ability to activate transcription [Barger et al., 2001] whereas p38 effects on PPAR γ remain uncertain [Hu et al., 1996, Oberkofler et al., 2002]. This indicates that the activation of PPAR isoforms is regulated differently. PGC-1 appears to be regulated by p38 as well [Puigserver et al., 2001], and p38 activation has already been described in skeletal muscle of hibernating little brown bats (Chapter 2). PPAR α levels did not change during hibernation in three of the bat tissues studied but increased in WAT together with an increase in PGC-1. It is not known whether this increase conferred an increase in DNA binding activity and, thus, increased transcription of a suite of genes downstream of the PPAR α binding element but it would seem likely as PGC-1 acts to increase DNA binding affinity of PPAR proteins [Knutti and Kralli, 2001]. PPAR proteins control a number of genes involved in fatty acid metabolism, including *a-fabp* and *h-fabp* in other species [Mohizuki et al., 2001, Guan et

al., 2001]. However, neither *a-fabp* or *h-fabp* transcript levels were elevated during hibernation in WAT so the effects of elevated PPAR α in WAT during hibernation may be regulating other aspects of fatty acid metabolism.

By contrast, a strong correlation can be drawn between the increased PGC-1 protein levels, increased PPAR γ protein levels, increased PPAR γ DNA binding activity, and increased *h-fabp* transcripts in BAT and skeletal muscle. The correlation also extends, in skeletal muscle, to the increased levels of H-FABP protein of hibernators. The parallel patterns of PGC-1 and PPAR γ changes suggest coordinate regulation and, in skeletal muscle at least, these may be linked with elevated p38 MAPK activity in hibernation. A coordinated expression of multiple gene/proteins involved in lipid catabolism would aid the shift to a dependence on lipid fuels during hibernation and contrasts with the suppression of the use of carbohydrate fuels (through inhibitory control of pyruvate dehydrogenase and other enzymes) during torpor.

PGC-1 likely interacts with PPAR α and/or PPAR γ to stimulate the gene expression of *a-fabp* and *h-fabp*, which may lead to the subsequent translation of the gene into functional protein in BAT, skeletal muscle and WAT (as these tissues showed increases in *a-fabp* and/or *h-fabp*). Having increased fatty acid binding protein allows for the binding and solubilization of fatty acids at low temperatures routinely experienced in hibernation and torpid bouts. These results lead us to believe that there is indeed an increase in PPAR γ DNA binding in hibernation. Since an overall increase in protein does not necessarily reflect an increase in transcription factor activity and an increase in a co-activator does not confer an increase in activity, I sought to determine whether or not PPAR γ had an increased affinity for the PPARE region during hibernation. Binding

activity of PPAR γ increases in BAT and in skeletal muscle, two tissues displaying the greatest increase of *h-fabp* transcript levels.

A number of recent studies on nutrition and hormonal regulation have focussed on PPAR mechanisms of activation. The result of these studies show striking similarity to the responses seen in hibernating animals. Hibernators show altered insulin levels [Bauman, 1990], increased DNA binding activity of PPAR γ (current study) and increases in fat transporting and metabolizing genes [Andrews et al., 1998, Bauer et al., 2001, Buck et al., 2002, Hittel and Storey, 2001]. All of these are also characteristic responses to starvation in nonhibernating mammals. Indeed, this is not surprising as hibernation is a starved state and animals must make adjustments to switch their metabolism to a dependence on body fuel reserves (primarily lipids) as the sole means of sustaining life throughout the winter. Hence, it is not surprising that the signal transduction pathways involved in mammalian responses to nutrition and starvation are utilized in hibernators.

Figure 4.1. cDNA cloning of H-FABP from *M. lucifugus*. Consensus primers were developed from available mammalian sequences and used to amplify *h-fabp*. Lane 1: DNA ladder, Lane 2-3: negative controls, Lane 4: Amplification of partial cDNA using consensus primers. Lane 5: 5' RACE amplified cDNA from Clontech Smart RACE kit using the 3' consensus primer and 5' consensus primer.

Lane 1: DNA ladder

Lane 4: PCR amplified partial H-FABP

Lane 5: 5' RACE amplified H-FABP

Figure 4.2. Complete cDNA sequence of *h-fabp* and deduced amino acid sequence of H-FABP from *M. lucifugus*. Start and stop codons are underlined along with the polyadenylation signal. The complete cDNA sequence was assembled from sequences of partially amplified cDNA and 5' and 3' RACE amplified cDNAs.

1 TCAGCCTAGCCCAGCCTCACCATGGCGGACGCCTTCGCAGGCACCTGGAAGCTGGTGGAC
 1 M A D A F A G T W K L V D

 61 AGCAAGAACTTCGATGACTACATGAAGTCCATCGGTGTGGGTTTTGCTACCAGGCAGGTG
 21 S K N F D D Y M K S I G V G F A T R Q V

 121 GCCAGTATGACCAAGCCTACCACAATCATCGAAATAAATGGGGACACGATCATCCTAAAA
 41 A S M T K P T T I I E I N G D T I I L K

 181 ACACAAAGCACCTTCAAGAACACAGAGATCAGCTTCAAGCTGGGGGTGGAGCTTGACAAG
 61 T Q S T F K N T E I S F K L G V E L D K

 241 ACAACGGCAGATGACAGGAAGGTCAAGTCCACCGTGACGCTGGATGGAGGCAAACCTTGTC
 81 T T A D D R K V K S T V T L D G G K L V

 301 CATGTGCAGAAATGGGACGGGCAAGAAACAAAACCTTGTGCGGGAGCTCGTGGATGGCAAA
 101 H V Q K W D G Q E T K L V R E L V D G K

 361 CTCATCCTGACACTCACCCACAATAATGTAGTGTGTACGCGGACTTACGAGAAGGAGGCA
 121 L I L T L T H N N V V C T R T Y E K E A

 421 TGACCAGCCCACTGCTTCGCTGACTGCTCCGCTGCCAACTGGCTACCCCTGGACTCAGCA
 141 *

 481 CCAGATTGCCTCGTCTTTTCCTCTGGCGTTTTTGTATAAATCCACCTTGGGGGGGAAATT

 541 CTTCTGGGGTCAGGTGCACCATCCTGGATACAGTTCCAGTTCCCATTGTGTTTGTCTGTC

 601 TTAAGTGCATCCAAAGAGTGCTCTGAGGTCAATAAAGCAGAGCCAAGGCC

Figure 4.3. Multiple alignment of *M. lucifugus* H-FABP protein sequence with other known mammalian sequences. Dashed lines represent identical amino acids; significant substitutions are underlined in the *M. lucifugus* sequence. Sequence substitutions at positions 70, 72 and 83 are underlined; these may have a marked effect on protein function. Accession numbers are as follows: AAD32219, NP_004093, NP_034304, NP_077076, O02772, P10790 and Q99P61 for horse, human, mouse, rat, pig, cow and squirrel H-FABPs, respectively.

ORIGIN		
bat	MADAFAGTWKLVDSKNFDDYMKSIGVGFATRQVASMTKPTTIIIEINGDTI	50
squirrel	-v---v-----l-----k-----	50
human	-v---l-----l-----k---il	50
mouse	----v-----l-----k-----	50
rat	----v-----l-----k-----	50
pig	-v-----n-----v-----	50
cow	-v---v-----l-----gn-----v---v	50
bat	ILKTQSTFKNTEISFKLGVELDKTTADDRKVKSTVTLDGGKLVHVQKWDG	100
squirrel	-----q--k-f-e-----	100
human	t---h-----f-e-----i-----l----	100
mouse	ti-----n-q--i-f-ev-----l-----i---n-	100
rat	ti--h-----q---f-ev-----v-----	100
pig	-i-----s-----f-e-----i-----l---n-	100
cow	-i-----f-e-----i-----n-	100
bat	QETKLVRELVDGKLILTLTHNNVVCTRTRYEKEA	133
squirrel	---t---n-----gs-----	133
human	---t---i-----gta-----	133
mouse	---t-t-----gs-s-----	133
rat	---t-t---s-----g--s-----	133
pig	---t-----gsa-----	133
cow	---s---m-----gta-----q-	133

Figure 4.4. Homology tree of heart type mammalian FABP deduced amino acid sequences. *M. lucifugus* and *S. tridecemlineatus* have very similar amino acid sequences that may be related to the ability of these two animals to hibernate. The similarity of protein sequences between these two hibernating mammals suggests convergent evolution.

H-FABP identities across species

Figure 4.5. Ribbon diagram of mammalian H-FABP. Areas containing key substitutions in *M. lucifugus* H-FABP are circled and listed below the diagram. Ribbon diagram taken from Vogel-Hertzel and Bernlohr, 2000.

Key substitutions:

V $\rightarrow$ T 83

F $\rightarrow$ L 70

E $\rightarrow$ K 72

Figure 4.6. mRNA expression levels of *a-fabp* and *h-fabp* in euthermic and hibernating tissues of *M. lucifugus*. (A) Representative Northern blot of *M. lucifugus* tissues heterologously probed with rat *a-fabp*; 10µg of total RNA was loaded per lane. B – brown adipose tissue, Br – brain, H – heart, K – kidney, L – liver, M – skeletal muscle, W – white adipose tissue. (B) Representative Northern blot of *M. lucifugus* probed with *h-fabp*. Histograms show the ratio of *a-fabp* (C) and *h-fabp* (D) transcript levels in hibernating versus euthermic bats; data are means $\pm$ SEM, n=3 or more trials. *a-fabp* and *h-fabp* probes bound to transcripts at approximately 750 nucleotides except in the case of WAT where probe bound to transcripts approximately 700 nucleotides long.

Figure 4.7. Representative western blots of A-FABP (A) and H-FABP (B) protein levels in *M. lucifugus* tissues. Aliquots (10 µg) of total protein extracts were electrophoresed on 15% SDS-polyacrylamide gels and incubated with human anti-A-FABP and human anti-H-FABP. Histograms show the ratio of A-FABP (C) and H-FABP (D) protein levels in hibernating versus euthermic bats; data are means $\pm$ SEM, n=3 trials. Abbreviations are: E - Euthermic; H - Hibernating; BAT - brown adipose tissue; WAT - white adipose tissue. Determination of protein molecular weights was determined by comparison with a protein ladder standard. Molecular weights were found to be 14-15 kDa for each protein (methods found in Chapter 2).

Figure 4.8. Western blots showing levels of carnitine palmitoyl transferase 1 liver isoform (α) and muscle isoform (β) in BAT, heart and skeletal muscle of euthermic versus hibernating bats. (A) Representative Western blots showing CPT-1 α protein levels in tissues from euthermic (E) and hibernating (H) bats. (B) Comparable Western blots probed for CPT-1 β . Histograms show the ratios of CPT-1 α and CPT-1 β in euthermic and hibernating tissues, data are means $\pm$ SEM, n=3 trials. Both proteins were found to be approximately 90 kDa.

Figure 4.9. Representative Western blots showing levels of PPAR α (A) and PPAR γ (B) protein in four tissues of euthermic (E) and hibernating (H) *M. lucifugus*. Histograms show the ratios of PPAR α (C) and PPAR γ (D) protein levels in hibernating versus euthermic bats; data are means $\pm$ SEM, n=3 trials. PPAR α and PPAR γ were found to be approximately 55 kDa.

Figure 4.10. (A) Representative Western blots showing levels of PGC-1 in four tissues of euthermic (E) and hibernating (H) *M. lucifugus*. (B) Histograms show the ratio of PGC-1 protein levels in hibernating versus euthermic bats; data are means $\pm$ SEM, n=3 trials. The molecular weight of PGC-1 was 90 kDa.

Chapter 5

Up-regulation of proliferation-associated gene, pag; a member of the thioredoxin peroxidase family of proteins; in hearts of hibernating mammals

INTRODUCTION

Hibernating mammals experience large changes in metabolic rate. Oxygen consumption can drop to just 1-5% of the resting euthermic rate when the animal is in torpor and then rises again within minutes during arousal. Such major changes in oxygen consumption mean that the potential for oxidative damage due to the formation of reactive oxygen species (ROS) also varies widely over the hibernation-arousal cycle. In particular, effective antioxidant defenses must be in place to deal with a rapid rise in ROS production during arousal when oxygen consumption increases nearly 100-fold. In addition, hibernators must have good antioxidant defenses in place throughout torpor to limit the accumulation of oxidative damage products during the long weeks of torpor [Carey et al., 2000].

Thioredoxin and its associated redox enzymes have recently garnered much attention. It was first proposed that the system played a role in cellular defenses against oxidative stress [Butterfield et al., 1999], but more recently it has been suggested that the main function of the thioredoxin peroxidase system may be in signal transduction in response to oxidative stress [Hoffman et al., 2002]. HSP27 phosphorylation is associated with oxidative stress and Chapter 2 of this thesis reported elevated phospho-HSP27 content during hibernation, implying that oxidative stress may occur during hibernation in heart and skeletal muscle. Indeed, cold-acclimation and hibernation dependent increases in the antioxidant defenses of brown adipose tissue of ground squirrels have been previously documented and linked with the need for protection against ROS generation during the periods of intense uncoupled respiration that generate the heat to re-warm the arousing animal [Buzadzic et al., 1990; Barja de Quiroga et al., 1991]. Improvement of antioxidant defenses during hibernation may

be key for other organs as well, and hence, a role for the thioredoxin system in hibernation might also be predicted.

Thioredoxin peroxidases are a family of enzymes that are thiol specific, meaning that they reduce ROS in cells using thiol groups of conserved cysteine residues (positions 52 and 173) through coupled reactions with thioredoxin proteins (Figure 5.1). Members of the thioredoxin peroxidase family also appear to be directly involved with the activation of nuclear factor kappa B (NF κ B) [Jin et al., 1997; Haridas et al., 1998]. NF κ B is an important transcription factor that regulates gene expression in cells. It exists as a homo- or heterodimer of p65 and p50 subunits and, under normal cellular conditions; an inhibitor, I κ B, which blocks its function, caps it. In response to one of a number of stresses, including oxidative stress, I κ B is phosphorylated and ubiquitinated, causing its dissociation from NF κ B and allowing NF κ B to translocate to the nucleus and activate the transcription of various target genes. Thioredoxin may be involved in oxidative stress by acting as a cellular scavenger of reactive species and/or free radicals as is shown in figure 5.1.

In this chapter, I present evidence that a potent antioxidant protein of the thioredoxin peroxidase family known as proliferation-associated gene (*pag*), responds to hibernation showing increased expression in heart of hibernating little brown bats. In addition, markers for oxidative stress appear to be up-regulated in both the heart and skeletal muscle of little brown bats and in the skeletal muscle of ground squirrels.

MATERIALS AND METHODS

Animals.

All animals were treated as previously described in earlier chapters.

2-Dimensional Polyacrylamide Gel Electrophoresis.

2-D PAGE was performed using the Mini-PROTEAN II system as outlined by the manufacturer (BioRad). For the first dimension, 4% acrylamide gels containing 1.6% (v/v) ampholines (pH 4-6) and 0.4% (v:v) ampholines (pH 3.5-10) (Sigma) were prepared in 0.75 mm x 150 mm capillary tubes. Samples of bat hearts (100 mg) were homogenized with a Polytron homogenizer in 1.5 ml of buffer (25 mM HEPES, 25 mM KCl, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, pH 7.4). A small amount of the protease inhibitor, phenylmethylsulphonyl fluoride, was added immediately after homogenization and samples were then centrifuged at 3000 rpm for 5 minutes in a Biofuge 15 (Baxter Canlab). Supernatant was removed, protein content was measured, and an aliquot of 10 µg of soluble protein was combined with an equal volume of sample buffer (9.5 M urea, 2% v/v Triton X-100, 5% v/v β-mercaptoethanol, 1.6% ampholines pH 4-6 and 0.4% ampholines pH 3.5-10) and loaded onto the surface of the first dimension gel. Samples were covered with sample overlay buffer (9 M urea, 0.8% ampholines pH 4-6 and 0.2% ampholines pH 3.5-10, 0.0025 % w/v bromophenol blue) and then isoelectric focusing was carried out at 500 V for 8 hours. First dimension gels were removed from capillary tubes and loaded onto an SDS polyacrylamide gel (12%) for second dimension separation by molecular mass. Second dimension gels were run at 100 V for 2 hours then fixed in 25% methanol (v/v) and 10% acetic acid (v/v) for 1 hour. After this, gels were stained in 25% methanol (v/v), 10% acetic acid (v/v) and 0.2 % (w/v) colloidal Coomassie blue for 2 hours and then destained overnight in 25% methanol (v/v) and 10% acetic acid (v/v). Spots showing differential expression were excised from the gels, placed in Eppendorf tubes and destained. Colloidal Coomassie blue stained gels were destained with 100 mM ammonium bicarbonate (BDH, Mississauga, ON)

in 30% acetonitrile (Anachemica, Mississauga, ON). Gel spots were then rinsed 3 times with distilled water.

Tryptic digestion.

Excised gel spots were shrunk by incubation in acetonitrile and were then reswollen in digestion buffer: 20 μ l of 50 mM ammonium bicarbonate containing 200 ng of modified sequencing grade trypsin (Promega, Madison, WI, USA). Sufficient 50 mM ammonium bicarbonate was added to each tube to cover the rehydrated gel pieces (50 μ l). Eppendorf tubes were then sealed and digestion was carried out overnight at 37°C. After digestion, the tryptic peptides were recovered as follows. The buffer solution surrounding a gel piece was transferred to a fresh sterile Eppendorf tube. The gel piece was then extracted first with 50 μ l of 5% acetic acid and then with 50 μ l of 5% acetic acid, 50% methanol. First and second extracts were pooled, evaporated to dryness on a centrifugal evaporator (Savant) and stored at -20°C until required for analysis.

Analysis of tryptic digests by rapid capillary-LC-MS/MS.

Capillary LC-MS/MS was carried out in the laboratory of Dr. Thibault, National Research Council (Sussex Dr., Ottawa). The instrumentation used was a Waters CapLC liquid chromatograph (Waters, Milford, MA, USA) coupled to a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) using an electrospray ionization interface. Digest extracts were redissolved in 15 μ l of 5% (v/v) acetonitrile, 0.5% acetic acid and a 10 μ l aliquot was loaded. Tryptic peptides were concentrated on a 0.3 mm i.d. x 5 mm μ -precolumn cartridge (LC-Packings, San Francisco, CA, USA). Rapid peptide elution was achieved in 6 minutes (flow rate of 1 μ l/min) using a linear gradient of 5% to 60% acetonitrile in 0.2% formic acid (Anachemica, Mississauga, ON).

The mass spectrometer was operated in data dependent acquisition mode. Briefly, a fast survey scan was carried out to profile the ions entering the instrument. The instrument was programmed to switch to MS/MS sequencing mode upon detection of doubly and triply protonated ions (a background of singly charged ions is constantly observed and arises from the HPLC solvent). Three multiply-charged ions could be selected per survey scan and MS/MS scans were acquired for 2 seconds on each ion selected. The collision gas was nitrogen and the collision offset (voltage gradient across the collision cell) was automatically selected. The instrument returned to survey scan mode after the MS/MS spectra were acquired.

Protein Identity and Database Searching.

The peptide MS/MS spectra acquired for each protein sample were used to search an in-house non-redundant protein sequence database (NCBI + Swiss-Prot) using the instrument manufacturer's proprietary software (ProteinLynx). In some cases, the spectra could not be matched with known sequences in the database. In these cases, the sequence was assigned manually and submitted to the NCBI nr protein sequence database using MS-Seq (Protein Prospector™, Mass Spectrometry Facility, UCSF, San Francisco, CA, USA) or the NCBI BLAST searching algorithms. One of the sequences obtained was homologous to a member of human thioredoxin peroxidases, proliferation associated gene (*pag*) (accession number X67951).

RNA Isolation.

All protocols were as previously described in Chapter 3.

cDNA synthesis and PCR amplification of pag.

Synthesis of cDNA and PCR amplification of *pag* cDNA were performed as previously described (Chapter 4) using primers designed from human *pag* (accession number: X67951). Primers were synthesized and purchased from Bio S&T (Montreal, QC). The forward primer spanned positions 46-64 of human *pag*, 5'-ACTGCTGATAGGAAGATGTC-3' (45% GC). The reverse primer extended from positions 664-649, with the sequence 5'-AGCGCTCACTTCTGCTT-3' (53% GC). An amplified product corresponding to approximately 620 bp was obtained and confirmed through sequencing (CMRS, Ottawa, ON). The identity of the gene was determined through homology searching of the NCBI database.

Gene expression of pag

Reverse transcription was performed as described (Chapter 4). First strand cDNA that was generated from the reaction had to be normalized to confirm that equal amounts of starting material between euthermic and hibernating heart extracts. To determine this, serial dilutions were made of first strand cDNA synthesis and PCR was performed as described (Chapter 4) on these dilution series using a control gene, α -tubulin. Agarose gel electrophoresis on serial dilutions of the PCR amplified α -tubulin was done to confirm expression remained constant between euthermic and hibernating extracts. Once cDNA levels were normalized, PCR amplification was performed on serial dilutions with *pag* primers to identify its relative expression levels between euthermic and hibernating hearts.

Tissue homogenization and protein preparation

Soluble protein extracts were prepared from tissues as described in Chapter 2.

Western Blotting.

Antibodies to I κ B α and phospho-I κ B α were purchased from Cell Signaling Technologies. The anti-human pag antibody (SC-7381) raised in goats was purchased from Santa Cruz Biochemicals. All primary antibody incubations for western blotting were carried out at 4°C overnight in 1X TBST at a 1:1000 v/v dilution, except for pag, which was incubated in 1% nonfat dried milk in 1X TBST overnight at 4°C. Each blot was washed for 3 x 5 minutes in 1X TBST prior to the addition of secondary antibody. Secondary antibody incubations were carried out for 2-3 hours at room temperature at a 1:2000 v/v dilution and followed by 3 x 5 minute washes in 1X TBST. Blots were developed using the Western Lightning™ Chemiluminescence *Plus* (NEN, Perkin Elmer) system and briefly exposed to Kodak X-OMAT AR film.

NF κ B DNA binding assay

Commercially available kits for the study of transcription factor binding were purchased from Active Motif. A 20-50 mg sample of tissue was homogenized in lysis buffer (20 mM HEPES, pH 7.5, 350 mM NaCl, 20% v:v glycerol, 1% Igepal-CA630, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA). Protein concentrations in cell extracts were determined via the Bio-Rad protein assay and extract concentrations were then adjusted to 0.1-1 μ g/ μ l in lysis buffer. For each well of the assay, 22.2 μ l of cellular protein extract in lysis buffer, 0.23 μ l protease inhibitor cocktail and 0.11 μ l of 1M DTT were used. Added to this was 30 μ l of complete binding buffer solution, which consisted of: 0.06 μ l 1M DTT, 0.3 μ l herring sperm DNA and 29.64 μ l binding buffer (4 mM HEPES, pH 7.5, 120 mM KCl, 8% glycerol, 1% bovine serum albumin). Wells were incubated for 1 hour at room temperature with slight agitation after which wells were washed to remove excess protein with 1X washing buffer

(10 mM phosphate buffer, pH 7.5, 50 mM NaCl, 0.1% Tween 20). A 100 μ l aliquot of NF κ B p65 subunit antibody (dilution 1:1000) was then added and incubated for 1 hour at room temperature. After incubation, wells were washed three times with 200 μ l with 1X washing buffer and then 100 μ l of HRP conjugated secondary antibody was added to the wells and incubated for 1 hour at room temperature. Wells were then washed with four times with 200 μ l 1X washing buffer. A 100 μ l aliquot of developing solution was then added to each well (TMB substrate in 1% DMSO) and incubated for 2-10 minutes until the appearance of a dark blue colour. Finally, 100 μ l 0.5 M H₂SO₄ was added to stop the reaction. Absorbance was measured at 460 nm on a microplate reader. Experiments were performed in triplicate, each trial from pooled tissues of euthermic (n=10) and hibernating (n=10) bats.

RESULTS

2D Gel Electrophoresis

Figure 5.2 shows the separation of soluble proteins in *M. lucifugus* heart via 2D PAGE with Coomassie blue staining. One protein stood out as very strongly expressed in hearts from hibernating bats but was barely detectable in euthermic hearts (Fig. 5.2). This protein had an approximate mass of 20 kDa and an isoelectric point (pI) of ~ 7.0 . The content of this protein was determined from euthermic and hibernator gels using densitometry and Imagequant; levels were 6.20 ± 0.88 fold higher in heart samples from hibernating bats, compared with euthermic (n=3 euthermic and n=3 hibernating samples; $P < 0.005$). Levels of the protein were normalized against values for other nearby proteins whose densities did not appear to change between the two states. The gel area surrounding the protein of interest was excised using aseptic technique, destained and the protein was

subjected to tryptic digestion followed by mass spectrometry analysis. The total mass of the protein was found to be 21.8 kDa. Two tryptic peptides retrieved from the in gel digestion of the protein provided sequence information, GLFIIDGK and QITVNDLPVGR. With the above data, searches were undertaken to determine the identity of the protein in question. The peptide sequences showed a high degree of similarity to human *pag* (accession number X67951) and this putative identity was assigned to the protein in question. Other proteins whose expression appears lower during hibernation (Figure 5.2), yet these proteins did not display a consistent expression pattern on repeated trials.

Western Blotting

Pag is a member of the thioredoxin peroxidase family of proteins. Commercial antibodies were purchased for the human protein to confirm the identity and up-regulation of the unknown protein found from 2D PAGE experiments on euthermic and hibernating bat hearts. Immunoblotting using the pag antibody was used to assess soluble protein extracts from heart of euthermic and hibernating bats (Fig. 5.3). The antibody crossreacted with a single protein band of approximately 23 kDa in both states and densitometry analysis showed that protein content was 2.30 ± 0.13 fold ($P < 0.05$) higher in heart of hibernating, compared with euthermic, bats. Western blotting of extracts from BAT, skeletal muscle and WAT also showed the presence of a protein crossreacting with the pag antibody but there were no appreciable differences between hibernating and euthermic states (data not shown).

Gene expression and amplification of ORF

Sequences of *pag* genes from other mammalian sources were used to identify conserved regions in the mRNA sequence. Once conserved regions were found, primers were

designed from the human *pag* sequence (X67951) to amplify *pag* from *M. lucifugus*. Thirty-seven cycles of PCR were required to visualize a product of the appropriate molecular weight, 620 bp. Sequencing was used to confirm its identity and its coverage of the open reading frame of the protein (Figure 5.4). The *M. lucifugus pag* cDNA sequence was 95% identical to human *pag* and the complete deduced amino acid sequence was 99% identical to human Pag and displayed no significant substitutions. The bat sequence contained the conserved cysteine residues at positions 52 and 173, which are underlined in Figure 5.4. The substitutions present were a leucine (human) to isoleucine (bat) change at position 68 and a valine (human) to isoleucine (bat) change at position 87. Neither of these substitutions would be likely to have any significant effect on the function of the Pag protein product. The gene and protein appear to be highly conserved among mammalian species (Figure 5.5); for example, there was much greater sequence identity between *pag* sequences from a number of mammalian species compared with the H-FABP sequence (Chapter 4).

To determine whether the increase in Pag protein correlated with gene expression levels, I sought to determine whether or not there was an increase in mRNA levels of *pag*, the technique of relative RT-PCR was used with heart RNA extracts of euthermic versus hibernating bats. Transcript levels of α -tubulin do not change during hibernation in bat hearts (noted on cDNA arrays) and thus, α -tubulin was chosen as a control gene. Initially, first strand cDNA was prepared from 0.5 μ g of poly (A)⁺ RNA. Since the PCR amplification is done on first strand cDNA, it was then necessary to normalize these cDNA levels. PCR reactions were carried out with α -tubulin at different dilution sets (e.g. 10, 100-, and 1000-fold dilution) to confirm that starting cDNA concentrations were equal in euthermic versus hibernating heart samples prior to amplification of bat *pag*. Figure 5.6 shows a representative

amplification of *pag* from euthermic versus hibernating heart cDNA. Lanes 1-3 are serial dilutions from euthermic first strand cDNA and lanes 4-6 represent normalized dilutions from hibernating first strand cDNA. Hibernating levels of normalized cDNA were determined to be 4.6 ± 1.6 fold higher ($P < 0.05$) than euthermic levels of normalized cDNA, representing initial levels of mRNA expression. RT-PCR amplification of *pag* transcripts in skeletal muscle did not show hibernation-related changes in expression level (data not shown).

Oxidative stress markers in the heart and skeletal muscle

The *pag* protein is part of a large family of thioredoxin peroxidases, which are among the most potent of cellular antioxidants. This up-regulation of *pag* and its protein product suggests that tissues may be experiencing oxidative stress during hibernation or may be preparing themselves for oxidative stress upon arousal. To investigate these possibilities the effect of hibernation on a well-known marker of oxidative stress was analyzed. One of the prominent markers of oxidative stress is the phosphorylation of I κ B. Phosphorylation of the inhibitor protein, I κ B, at serine 32 leads to its ubiquitination and dissociation from the transcription factor, NF κ B, which in turn activates NF κ B. Immunoblotting with an antibody specific for the phospho-peptide of I κ B was used to assess the levels of phosphorylated I κ B in hibernator tissues. Phospho-I κ B levels were higher during hibernation, compared with euthermia, in all three tissues tested; levels were 2.0 ± 0.4 fold (mean $\pm$ SEM, $P < 0.05$) higher in hibernating bat heart, and 5.9 ± 1.4 fold ($P < 0.05$) and 5.6 ± 1.5 ($P < 0.05$) fold higher in hibernator skeletal muscle of *M. lucifugus* and *S. tridecemlineatus*, respectively (Fig. 5.7). Comparable assessment of total I κ B protein levels showed no change between

hibernating and euthermic states in either heart or skeletal muscle of bats or in skeletal muscle of ground squirrels (data not shown).

To determine directly if hibernation affected NF κ B, the *in vitro* DNA binding activity of the p65 subunit of NF κ B was assessed to determine whether increased phosphorylation of I κ B correlated with an increased ability of NF κ B to activate transcription of target genes. The amount of NF κ B p65 subunit binding to DNA was measured in extracts of both euthermic and hibernating tissues and the hibernating:euthermic ratio of binding activity was calculated. Values for this ratio were 0.88 ± 0.44 , 1.18 ± 0.05 ($P < 0.05$) and 1.44 ± 0.22 ($P < 0.05$) for *M. lucifugus* heart and skeletal muscle and *S. tridecemlineatus* skeletal muscle, respectively. The increases in DNA binding ability seen in skeletal muscle of hibernators were small but suggest that significant changes in NF κ B-mediated gene expression may occur in hibernation. This is because the assay probably under-represents the change in DNA binding capacity by the fraction of NF κ B that is localized in the nucleus since the method assesses the total cellular content of NF κ B (not just the nuclear content).

DISCUSSION

Hibernation appears specifically to increase the mRNA and protein levels of a bat homolog of Pag, a thioredoxin peroxidase type protein, in the heart of little brown bats, *M. lucifugus*. Numerous increases in protein and gene expression have been reported in a variety of tissues during hibernation, both in this thesis and in the literature [Wilson et al., 1992; Andrews et al., 1998; Fahlman et al., 2000; Hittel and Storey, 2002b; Yu et al., 2002]. Nevertheless, some researchers subscribe to the notion that cells are in a dormant state during hibernation with no appreciable transcription or translation occurring [Van Breukelen and

Martin, 2002] and that the observed increases in gene transcript or protein levels are the result of decreased rates of degradation during hibernation. This conclusion was drawn from studies with liver and brain [Frerichs et al., 1998; Chen et al., 2001] but evidence now indicates that this is not true of all tissues. In fact, recent evidence from our lab has shown that BAT is quite active in hibernating ground squirrels and shows no appreciable loss of ability to translate new proteins during torpor as evidenced by the status of eIF2 α , polysome profiles and *in vitro* translation rates [Hittel and Storey, 2002a]. Results presented in this chapter also indicate that levels of selected proteins, in this case Pag, can increase in vital organs during hibernation (higher levels of selected stress proteins have also been observed in liver from hibernating bats; personal observation). One could argue that the accumulation of Pag protein could be because the rate of protein degradation is retarded during torpor. However, there was no appreciable amount of the protein detected by 2D-PAGE in the euthermic state and this suggests that the protein was newly synthesized within the time frame of hibernation and tissue sampling in little brown bats.

The role of thioredoxin peroxidase proteins, of which Pag is a member, is to prevent the accumulation of reactive oxygen species, in particular peroxides, within cells. The proteins do this by first complexing to peroxides (ROOH) and reducing them to alcohols (ROH) by utilizing the thiol groups on cysteine residues at the active site. Bat pag was 99% identical with human pag making it likely that the bat protein functions similarly to its human counterpart. There were no significant amino acid substitutions in the bat sequence that might allow the enzyme to function at the lower T_b values encountered during hibernation. In fact, only two minor differences were noted between the human and bat proteins, an isoleucine substitution for leucine and a valine substitution for isoleucine in the bat heart protein.

Neither of these amino acid substitutions would greatly alter enzyme function, which is dependent on conserved thiol groups on cysteine residues of the protein. In previous studies on H-FABP [Hittel and Storey, 2001; Chapter 4], amino acid substitutions that appeared only in hibernating mammals were observed and are thought to aid low temperature function of H-FABP.

Two possible reasons can be suggested for the up-regulation of Pag in hibernation. One is that the increase in Pag protein aids the heart in dealing with a burst of ROS generation that would accompany the rapid increase in oxygen consumption of the heart during arousal. The other is that protein levels increase because protein degradation mechanisms are suppressed in hibernation. However, the first possibility seems most likely because both *pag* mRNA transcripts and Pag protein are higher during hibernation and other markers of oxidative stress are also seen during hibernation. The reduction in oxygen intake by the heart during hibernation initially give the indication that the heart hypoxic, although, there is also a strong reduction in metabolic rate and a reduction in the requirement and usage of oxygen, which may help reduce a hypoxic response. There are striking similarities to the hibernating heart and hypoxic hearts. Some changes found during hypoxia or ischemia (reduced blood flow) in nonhibernating mammals are expression of HSPs and antioxidant genes [Das et al., 1995] during ischemia and this is certainly correlated with results from hibernating bat hearts. Both I κ B (this chapter) and HSP27 (Chapter 2) show increased phosphorylation during hibernation. HSP27 is a known marker of oxidative stress and I κ B phosphorylation results in its dissociation from NF κ B and allows NF κ B transcription factor to activate its target genes, many of which are known to be involved in antioxidant defense. Thus, there is evidence to suggest that the up-regulation of Pag during hibernation is in

response to oxidative stress incurred by hibernating bat hearts. Although the DNA binding assay gave inconclusive evidence of NF κ B activation in hibernator tissues, a recent study has documented NF κ B activation in the intestine (smooth muscle) of ground squirrels during hibernation [Carey et al., 2000]. The work presented in this chapter shows that further investigation of the role of NF κ B in hibernator hearts is warranted.

Recent reports have shown that p38 and PKC signaling play a role in *pag* regulation [Li et al., 2002], however, there does not appear to be an increase in the active form of p38 in hibernating hearts. Luss et al. (2000) have also shown a role for p38 in ischemic porcine hearts. The phosphorylation of p38 in hibernating skeletal muscle may indeed have a role in oxidative stress signal transduction and p38 has recently been shown to be a potential upstream effector of NF κ B [Bradbury et al., 2002]. Increased phosphorylation of both of these markers was also seen in skeletal muscle during hibernation but without increases in *pag* protein levels, possibly because skeletal muscle is not the vital organ that the heart is.

Previous work in this thesis showing enhanced eIF2 α phosphorylation during hibernation (Chapter 3) suggested that overall protein initiation was suppressed in hibernating heart and skeletal muscle and, at first glance, an increase in *pag* protein contradicts this notion. Overall rates of protein synthesis are undoubtedly reduced in virtually all tissues of bats during hibernation, as has been reported previously for ground squirrels [Frerichs et al., 1998; Hittel and Storey, 2002] but, nevertheless, the possibility of a selective increase in the synthesis of some specific proteins exists. For example, this is clearly the case for FABP in ground squirrel brown adipose [Hittel and Storey, 2002a] and in H-FABP in brown adipose of bats (Chapter 4). This indicates that selected proteins required for the fitness of a species will be actively translated under stress [Larade, 2002].

Figure 5.1. A typical reaction of a pag dimer with a peroxide (ROOH). Thioredoxin (TXN) reduces the disulfide bond between the two proteins and is itself reduced via mechanisms not shown. Two pag proteins are in close proximity and negative charges are stabilized through hydrogen bonding (step 1). They then form a transition state (TS) complex with a reactive peroxide (step 2) resulting in the reduction of the peroxide to an alcohol (ROH). A hydroxide ion forms and is released oxidizing the sulfur atoms and forming a disulfide bridge between two Pag proteins (step 3 and 4). Reduction of the double bond occurs through concerted reactions with the thiol group of thioredoxin (steps 5-7). An oxidized thioredoxin is released and the cycle begins anew.

From Hoffman et al., 2002

TXN: thioredoxin
TS: Transition state

Figure 5.2. Representative 2D-PAGE gels stained with Coomassie Blue showing the separation of soluble proteins in extracts from *M. lucifugus* hearts. A. Euthermic and B. Hibernating. One protein that is strongly expressed in hearts of hibernators but not apparent in euthermia is circled in B. Trials were repeated n=3 times from pooled tissue (n=10 euthermic hearts and n=10 hibernating hearts).

Euthermic

Hibernating

Figure 5.3. The unknown protein seen in Figure 5.2 was identified as the bat equivalent of human proliferation-associated gene (pag) and confirmation of up-regulation during hibernation (as determined by 2D-PAGE) was performed by immunoblotting. Equal amounts of euthermic (E) and hibernation (H) protein were electrophoresed on a 15% PAGE gel. (A) Shown is a representative Western blot, which was repeated in triplicate (pooled tissue n=10 euthermic hearts and n=10 hibernating hearts). (B) Bar graphs depicting mean levels of pag in euthermic and hibernating hearts (mean + SEM).

A.

Euthermic

Hibernating

B.

Figure 5.4. Partially amplified cDNA sequence of bat pag. The sequence was amplified using RT-PCR and primers designed from consensus mammalian sequences. Start and stop codons are underlined in the DNA sequence. The deduced amino acid sequence is also shown with conserved cysteines at positions 52 and 173 underlined.

ACTGCTGATAGGAAGATGTCTTCAGGAAATGCTAAAATCGGCCACCCTGCCCCCAACTTC

M S S G N A K I G H P A P N F

AAAGCCACAGCTGTTATGCCTGATGGTCAGTTCAAAGATATCAGCCTATCTGACTACAAA

K A T A V M P D G Q F K D I S L S D Y K

GGAAAATATGTGGTGTTCCTCTTTTACCCTCTTGACTTTACCTTTGTGTGCCCCACGGAG

G K Y V V F F F Y P L D F T F V C P T E

ATCATTGCTTTCAGTGATAGGGCAGAAGAATTTAAGAAAATCAACTGCCAAGTGATTGGT

I I A F S D R A E E F K K I N C Q V I G

GCTTCTGTGGATTCTCACTTCTGTACCTGGCATGGATCAACACACCCAAGAAACAAGGA

A S V D S H F C H L A W I N T P K K Q G

GGACTGGGTCCCATGAACATTCCTTTGGTGTGACACCCCAAGCGCACCATTGCTCAGGAC

G L G P M N I P L V S D P K R T I A Q D

TACGGAGTCTTAAAGGCTGATGAAGGCATCTCATTGAGGGCCTCTTTATCATTGATGAT

Y G V L K A D E G I S F R G L F I I D D

AAAGGTATCCTTCGCCAGATCACTGTGAATGACCTTCCTGTTGGCCGCTCTGTGGATGAG

K G I L R Q I T V N D L P V G R S V D E

ACTCTGAGACTAGTTCAGGCTTTCCAGTTCAGTACAAACATGGGGAAGTGTGCCCAGCT

T L R L V Q A F Q F T D K H G E V C P A

GGCTGGAAACCTGGCAGTGATACCATCAAGCCTGATGTCCAGAAGAGCAAAGAATATTC

G W K P G S D T I K P D V Q K S K E Y F

TCTAAGCAGAAGTGAGCGCT

S K Q K

Figure 5.5 Cladograms depicting nucleic acid (A) and amino acid (B) identity of *M. lucifugus* Pag with other mammalian sources. For nucleic acid identities, comparisons between the bat ORF and ORFs of other mammalian species. CHO = Chinese hamster ovary cells.

Gene

Protein

Figure 5.6 Representative relative RT-PCR. cDNA was normalized with alpha tubulin. Dilution sets of cDNA were made to allow for standardization of α -tubulin levels providing better quantification of the degree of up-regulation of *pag*. (A) Lanes 1-3 show euthermic heart cDNA (10^{-1} , 10^{-2} and 10^{-3} dilutions, respectively) whereas lanes 4-6 show hibernating heart cDNA at the same dilutions. (B) Bar graphs representing mRNA levels derived from three independent trials of RT-PCR. Data in the histogram are derived from the 10^{-2} dilution sets.

Figure 5.7. Western blotting showing total protein levels of phospho-I_KB in euthermic and hibernating states. (A) Representative blots for *M. lucifugus* heart, skeletal muscle (Sk Mus) and *S. tridecemlineatus* muscle (Sk Mus). (B) Bar graphs showing the relative amounts of I_KB(P) in tissues from hibernating versus euthermic animals; data are means $\pm$ SEM, n=3.

Chapter 6

General Discussion

This thesis has examined the underlying molecular responses to the physiological state of hibernation at the gene and protein levels. Hibernation is a state of extreme metabolic suppression and precise control and coordination must be utilized at the molecular level in order to make smooth transitions to and from the hypometabolic state and re-establish long term homeostasis in the torpid state. Within the cells and organs of animals, two of the major mechanisms of molecular response to external signals are changes in gene expression and post-translational modifications to proteins. Gene activity is regulated through transcriptional control, via multiple mechanisms that act to increase or decrease the steady-state levels of mRNA as well as mechanisms that process the RNA including the splicing of pre-mRNA transcripts, 5' capping of the transcripts and export of the processed mRNA from the nucleus to the cytosol. Gene activity is also controlled at the translational level, where proteins are produced from the processed mRNA transcripts. Regulation is applied at both the initiation and elongation phases of protein synthesis. Multiple forms of post-translational modifications to proteins can alter their function or subcellular location in response to stimuli. The most common form of post-translational modification that is involved in the reversible control of cellular metabolism is protein phosphorylation. The addition or removal of covalently bound phosphate via the actions of protein kinases or protein phosphatases causes significant changes to the conformation of proteins that can have major effects on protein/enzyme activity, in many cases acting as virtual on/off switches.

Hibernating animals endure the long winter months by entering a deep torpor and dropping their T_b from 37°C to near ambient (often as low as 0-5°C). Accompanying this are multiple physiological changes including a major reduction in heart rate and a virtual

lack of skeletal muscle activity. Although its rate is suppressed, the heart continues to play a vital role in hibernation to pump the blood that delivers oxygen and metabolic fuels to all tissues. Skeletal muscle is inactive during torpor but during the arousal process, shivering thermogenesis by muscle plays a key role by supplementing nonshivering thermogenesis by BAT to rewarm the animal rapidly. In this thesis, I studied heart and skeletal muscle at the molecular level in a comparative manner, to analyze the responses to hibernation by both tissues in two distinct hibernator species.

The regulation of gene activity was first examined by evaluating steady state mRNA levels on a global scale via the novel use of cDNA array screening. Further investigation of gene expression then focused on a selected subset of genes and analyzed stress-specific and organ-specific gene expression by northern blotting and other techniques. After confirming a gene as up-regulated, it is necessary to determine whether the corresponding protein also shows a marked increase, as it is the protein that provides the basis for function. The levels of multiple selected proteins were then analyzed with western blotting and other techniques. However, protein function is often regulated by post-translational modification, and so control of the activity state of various proteins was also examined by analyzing hibernation-induced changes to protein phosphorylation state. Together these multiple techniques and approaches provided an integrated view of the different levels of molecular control that assist mammals in their ability to survive through the long winter months by hibernating.

Gene and Protein regulation during hibernation

This thesis has examined the molecular basis of metabolic suppression, specifically the up-regulation of selected genes and proteins during hibernation. The genes and proteins that were identified are involved in the adjustment of metabolism for long term function during cold torpor and contribute to several important issues in hibernation including muscle atrophy, a change to lipid fuels, and oxidative stress.

In Chapter 3, I utilized cDNA array screening for a novel analysis of gene responses to hibernation. The screening revealed that that levels of the majority of mRNA transcripts did not change in skeletal muscle and heart between euthermic and hibernating states. Only a selected few (<2%) were up-regulated, including genes associated with muscle regeneration and muscle atrophy. Heterologous probing of cDNA arrays revealed a number of potential targets, some that were further analysed in this thesis and some that will provide the basis for future studies. Of the up-regulated genes, a number were involved in fat metabolism, signal transduction, oxidative stress response, and the structural integrity of cells. Studies of all of these will enhance the understanding of hibernation at the molecular level.

Hibernators switch to a fat-based metabolism during torpor and studies in Chapter 4 addressed the responses of two of the key factors that promote use of fatty acids as a source of fuel, PGC-1 and PPAR γ . My research showed increased levels of both in BAT and skeletal muscle of *M. lucifugus* during hibernation (Figure 4.9, 4.10) and PGC-1 was also elevated in *S. tridecemlineatus* skeletal muscle (Figure 6.1). PPARs bind to promoter regions of a number of genes involved in fatty acid metabolism, including α - and *h-fabp*. The binding of these to promoter regions enhances gene expression of downstream genes.

The up-regulation of *h-fabp* (Figure 4.6) correlated with protein expression levels of PPAR γ and PGC-1, and although the evidence presented does not investigate and prove the functional binding and activation of PPAR γ , there certainly exists a strong correlation between expression patterns of PGC-1, PPAR γ and *h-fabp*. The roles and regulation of A- and H-FABP were investigated and correlate with previous studies that have analyzed fatty acid binding protein involvement in hibernation. Of particular interest is the occurrence of the A-FABP transcripts and protein within skeletal muscle of little brown bats and this warrants further study in other hibernating animals to determine whether or not this is specific for bats or occurs in hibernators in general. Remarkably, the H-FABP from little brown bats is 93% identical to H-FABP from thirteen-lined ground squirrels with some significant substitutions in common between the two animals that are not seen in nonhibernating mammals. Further studies on H-FABP and A-FABP in other hibernating mammals (and other mammals as well) are warranted to determine whether or not these substitutions are hibernation-specific. Elevated levels of both fatty acid binding proteins in selected hibernator organs enhance the capacity for intracellular fatty acid transport to contribute to sustained function during torpor (heart) and the high rates of lipid oxidation that are needed for thermogenesis during arousal (BAT, skeletal muscle).

PGC-1 and PPAR γ may also be involved in a proliferation of mitochondria in red skeletal muscle during hibernation. This correlates with the increased expression of mitochondrially-encoded gene transcripts that was observed in muscle of hibernating *Spermophilus lateralis* [Fahlman et al., 2000]. Indeed, an increase in mitochondria resulting from PGC-1 stimulation could be linked with a promotion of the growth of red

muscle fibres [Lin et al., 2002], which might be important for increasing the capacity for shivering thermogenesis by muscle during arousal. It has been reported that CaMK is required for mitochondrial biogenesis acting through PGC-1 [Wu et al., 2002] and p38 MAPK signalling pathways have been implicated in promoting muscle maintenance, as well [Xu et al., 2002]. Expression of PGC-1 also appears to drive skeletal muscle towards the development of slow twitch, or red muscle fibres, from white muscle [Lin et al., 2002] and this may be important in limiting atrophy of red muscles during hibernation.

My data also show an increase in the total cellular protein levels of early growth response 1 (Egr-1) in hibernating skeletal muscle (Figure 6.2). Furthermore, screening of cDNA arrays, putatively identified Id-1 as an up-regulated gene in hibernating skeletal muscle (Appendix 3.1). Id-1 is a potential downstream target of Egr-1 [Tournay and Benezra, 1996], and both have been proposed to be involved in muscle atrophy [Gundersen and Merlie, 1994; Tournay and Benezra, 1996; Alway et al., 2002]. The debate over whether or not significant atrophy occurs during hibernation remains a hot topic among hibernation researchers. Atrophy during hibernation has been reported in several cases [Brigham et al., 1990; Steffen et al., 1991; Kim et al., 2000], but it appears that it is limited mainly to white muscle and that red muscle is, in fact, protected [Egginton et al., 2001]. The gene for Egr-1 is induced rapidly and may be responding to Elk-1 activation [Lim et al., 1998; Ayadi et al., 2001], which displays higher levels of its active phosphorylated form in hibernating skeletal muscle of *M. lucifugus* and *S. tridecemlineatus* in Chapter 2 (Figure 2.2, 2.4).

Clearly, there are genes that are up-regulated during hibernation that are associated with both myogenesis and atrophy. This up-regulation of factors that appear to

work on opposing pathways in skeletal muscle may, in fact, result from the selective up-regulation of atrophy-related factors in white muscle fibres and myogenic factors in red muscle fibres. Future research on gene and protein expression in hibernator skeletal muscle will require the independent investigation of responses in each fibre type. From the research presented in this thesis it is clear that skeletal muscle is dynamic in its adaptation to disuse and cold tolerance during the winter as evidenced by the observed differential gene and protein regulation.

Studies in Chapter 5 took a different approach to identifying gene and protein changes during hibernation. Two-dimensional gel electrophoresis coupled to mass spectrometry was used to compare protein expression between euthermic and hibernating states in hearts of little brown bats. One protein displayed a marked increase in steady state levels and was identified as a thioredoxin peroxidase type protein, Pag (Figure 5.2). Confirmation of its up-regulation in hibernator heart was achieved via Western blotting (Figure 5.3) and relative RT-PCR was used to assess transcript levels (Figure 5.5) and document an up-regulation of gene expression during hibernation. This protein is known to play a role in oxidative stress tolerance in conjunction with a host of other factors including, HSP27, I κ B and p38 MAPK, that are discussed in greater detail in the following section.

Post-translational modifications

The most common form of post-translational modification is protein phosphorylation, which can significantly alter the activity of proteins. I hypothesized that signalling pathways must be activated and/or inactivated in order to regulate hibernation

at the cellular level and to explore this hypothesis, I analyzed the phosphorylation status of p38 MAPK and a host of its downstream targets along with the status of Akt (protein kinase B). Skeletal muscle of hibernating *M. lucifugus* and *S. tridecemlineatus* showed elevated levels of the active form of p38 MAPK and a number its downstream targets including ATF-2, CREB, Elk-1, HSP27 and I κ B (Figures 2.2, 2.4) implying that p38 MAPK plays an important role in hibernation and may possibly play a role in metabolic stress response [Barger et al., 2001]. p38 MAPK appears to be one of the key master switches in controlling stress response in skeletal muscle. A key to investigate hibernation further will be the identification of ATF-2, CREB and NF κ B target genes in hibernating animals. Recent evidence suggests that p38 is involved in PPAR activation [Barger et al., 2001; Oberkofler et al., 2002] and thus may be affecting up-regulation of *h-fabp* in skeletal muscle. By contrast, Akt was completely suppressed in *S. richardsonii* (Figures 2.7), which correlates with a suppression of carbohydrate use and the promotion of a metabolism based primarily on lipid catabolism during hibernation. However, the status of Akt in hibernating bats is less clear (Figure 2.6).

Oxidative stress may be encountered by hibernating animals, as evidenced by both the increased levels of Pag (noted above) in *M. lucifugus* heart and the elevated levels of the active, phosphorylated forms of two oxidative stress markers, HSP27 and I κ B, in *M. lucifugus* and *S. tridecemlineatus* skeletal muscle and *M. lucifugus* heart during hibernation. Oxidative stress can result from a number of sources including links to hypoxia (reduced oxygen availability), ischemia (reduced blood flow) and reperfusion (re-oxygenation). Phosphorylation of I κ B leads to its dissociation from the transcription factor, NF κ B, and allows NF κ B to activate the expression of multiple genes involved in

antioxidant defense. Notably, NF κ B has previously been shown activated in smooth muscle of hibernating ground squirrels [Carey et al., 2000]. Das et al. (1995) have shown that oxidative stress in heart cells leads to increases in antioxidant gene and HSP expression. Since the heart must continue to function at low temperatures, it is likely that the degree of oxidative stress is lessened through one or more internal protective mechanisms, potentially via NF κ B mediation. It appears that Pag may be serving in this role in hibernating hearts.

As mentioned previously, HSP27 appears to play a significant role in hibernating heart and skeletal muscle as evidenced by higher levels of phospho-HSP27 in each. It appears that HSP27 serves a dual role in cells, and that the post-translational modification of HSP27 through phosphorylation is not the only significant means of altering the protein. The protein can exist in at least two completely distinct forms within cells. In one form, it exists as an oligomer complex displaying chaperone activity and in the other form, it is a monomer that serves as a stabilizer of the cytoskeleton [Dalle-Donne et al., 2001]. Until recently it was believed that under normal cellular conditions, HSP27 existed as a non-phosphorylated oligomeric complex acting as a chaperone protein and that when stress was applied, HSP27 was phosphorylated and the complex dissociated. The newly phosphorylated HSP27 monomers would then associate with actin to promote stabilization of the cytoskeleton. Recent evidence now suggests that phosphorylation is not required or even necessary for the dissociation of oligomeric complexes of HSP27. Instead, it appears that a change in the redox state of the cell affects disulfide bonds between HSP27 monomers and that it is this that is responsible for the dissociation of oligomeric HSP27 [Eaton et al., 2002]. Oxidative stress can cause a rise in intracellular

Ca^{2+} levels [Bellomo and Mirabelli, 1987-88], causing actin dissociation from α -actinin. The result is a loss of stability of the cytoskeletal structure that can be stabilized by phosphorylated HSP27 monomers. It appears that Ca^{2+} levels rise in skeletal muscle hibernating animals [Agostini et al., 1991] and from the data presented here HSP27 phosphorylation may assist in protecting the cytoskeleton. In addition, the cold temperatures experienced by hibernators could also lead to disruption of cytoskeletal integrity.

To assess the role that HSP27 plays in hibernating heart and skeletal muscle further, I investigated the oligomeric status of HSP27. Figure 6.3 shows that HSP 27 exists mainly as an oligomer in skeletal muscle of both euthermic and hibernating *M. lucifugus*, indicating that the major role played by HSP27 is that of a chaperone protein. The data presented in Chapter 2 showed that HSP27 displayed higher levels of phosphorylation in hibernation (Figure 2.2, 2.3, 2.4), which, under previous thought, would have suggested an increase in the dissociation of the oligomer complex. However, it now appears that the redox state of the cell regulates oligomer versus monomer with phosphorylation occurring after dissociation of the oligomer [Eaton et al., 2002]. From the data presented, a dual model of HSP27 activity can be proposed for hibernating heart and skeletal muscle. Figure 6.2 shows that the majority of HSP27 exists as an oligomer chaperone protein to stabilize intracellular proteins during cold torpor and that a small subset of HSP27 proteins may indeed become phosphorylated to promote stabilization of the cytoskeleton during hibernation. Further investigation is required to elucidate this model including Western blotting using phospho-HSP27 antibodies on non-reduced total

protein. It would be interesting to investigate the various roles that HSP27, and the small HSP family in general, plays during hibernation in various organs and tissues.

Although specific genes are up-regulated in hibernation, I hypothesized that an overall suppression of gene transcription and protein translation would occur in hibernation. The results from cDNA array screening supported this by showing very few up-regulated genes in hibernator muscles whereas suppression of protein translation was supported by the observed hibernation-induced changes to the phosphorylation state of ribosomal initiation and elongation factors. Two key proteins controlling protein translation were investigated, eIF2 α and eEF2, which are major regulators of the initiation and elongation phases of protein synthesis. Both were phosphorylated in skeletal muscle during hibernation and eIF2 α was also phosphorylated in heart. Regulation at both of these key sites will strongly suppress protein synthesis in hibernator tissues. These data for skeletal muscle and heart agree with studies of hibernator brain and kidney that have similarly demonstrated the phosphorylation of these ribosomal proteins during hibernation and also documented strong inhibition of that rates of radiolabeled amino acid incorporation into proteins during hibernation [Hittel and Storey, 2002a].

In addition to the phosphorylation of ribosomal factors, other mechanisms also contribute to the overall suppression of transcription and translation during hibernation. Rates of transcript elongation are strongly suppressed by low temperature so that a virtual arrest of transcription is implied at the low T_b values in hibernation [van Bruekelen and Martin, 2002]. Polysome dissociation into monosomes has also been documented in several cases [Frerichs et al., 1998; Hittel and Storey, 2002a; Knight et al., 2000] so that

the translation machinery is largely dismantled during torpor. Since there are relatively few genes that are up-regulated in torpor, and since translation rates are strongly reduced in torpor, it is possible that the products of many of the hibernation-induced genes are actually required during the arousal from hibernation. Transcripts may be produced during entry into torpor, or even during torpor, and then transcripts are protected and subsequently translated rapidly during arousal. Evidence for this exists in the liver [Knight et al., 2000], where the lengths of polyA tails of mRNA transcripts were measured and found to be protected during hibernation, probably through association with a polyA binding protein [Hittel and Storey, 2002a].

Outlook

A number of molecular and biochemical techniques have been used in this thesis to study the role of signal transduction and gene regulation during hibernation in skeletal muscle and heart of little brown bats and thirteen-lined ground squirrels. These techniques have revealed both similarities and differences in the molecular responses to hibernation between the two mammals. However, in both species, the data emphasize a role for the selected up-regulation of specific genes and their products in order to sustain tissues and organs during extended periods of torpor.

Future studies investigating gene and protein regulation in animal models of environmental stress will likely make further use of DNA microarray technology, newly developed protein microarrays, and 2-D PAGE coupled with mass spectroscopy to fully elucidate the gene and protein expression of various tissues and organs under stress. DNA microarrays are becoming more commonplace in many molecular biology labs, particularly due to lowered costs as the production of microarrays improves. Indeed, there

appears to be no end in sight to the plethora of uses for DNA arrays. Recent advances in 2-D PAGE coupled mass spectroscopy are now allowing the identification of minute amounts of proteins within cells along with the identification of proteins within complex mixtures. Progress in new technologies will eventually allow for the complete identification of a cell's gene and protein components and their changes in response to stress, age, disease and numerous other external factors.

With cross-species hybridization (or heterologous probing), two types of arrays were employed and the most consistent data was obtained from repeated trials using nylon arrays. Results from heterologously probed microarrays are beginning to be accepted [Hittel and Storey, 2001; Medhora et al., 2002] and this technique is providing valuable insights into the gene responses of organisms for which species-specific DNA arrays are unavailable. When dealing with tissues such as skeletal muscle, which tends to show very few alterations in its gene expression profile between euthermic and hibernating states, one must be wary of data that may be due to experimental variability. The easiest and most obvious solution to this is to perform a number of repeated trials; however, when tissue samples are limited, this poses a problem, as is the case with hibernating animals. Species-specific DNA arrays will undoubtedly provide a more accurate portrayal of relative gene expression and this is already beginning as arrays become available for more and more species; for example, arrays have recently become available for the goby fish, *Gillichthys mirabilis* [Gracey et al., 2001]. The production of species-specific DNA arrays is, of course, time intensive but the ease with which one can confirm gene up-regulation from an in-house produced array is much greater than that with heterologously probed arrays, not to mention the reduced likelihood of producing

false positives and false negatives. Species-specific hybridized DNA arrays also allow for better estimation of gene expression relative to other genes on the array that may be in the same family or pathway than do cross-species hybridized DNA arrays.

This thesis addressed four hypotheses about the molecular mechanisms of mammalian hibernation. The role played by differential signal transduction in hibernation was addressed with a primary focus on the p38 MAPK pathway that is activated during hibernation. Transcription factors that may be regulated by p38 showed higher levels of their active, phosphorylated forms when animals were torpid and these may be involved in regulating critical hibernation-specific gene expression and/or may also aid in the general suppression of metabolic functions during torpor. Second, the hypothesis of an overall suppression of transcription and translation during hibernation was supported by the low numbers of hibernation-responsive genes identified during cDNA array screening and by the inactivation of two key ribosomal factors, eIF2 and eEF2, during hibernation. However, the up-regulation of selected gene/proteins occurred in skeletal muscle and heart. Some of the up-regulated genes produce proteins that appear to be involved in muscle atrophy during hibernation. Additional studies on skeletal muscle at both the protein and gene level will determine whether there is a molecular basis for the controlled atrophy of white muscle fibres. The third hypothesis dealt with the probability that factors involved in fatty acid transport and metabolism were up-regulated in hibernation. One of the significant changes observed was the up-regulation of fatty acid binding proteins and this was analyzed along with the changes in the transcription factors PGC-1, PPAR α and PPAR γ that are likely involved in FABP control. Up-regulation of the transcript for *h-fabp* in BAT and skeletal muscle correlated with increased levels of the transcription

factors PPAR γ and PGC-1. Last, the need for increased antioxidant defences to counteract oxidative stress during hibernation or arousal was hypothesized. A common belief among hibernation researchers is that oxidative stress does not occur during torpor itself, but research reported in this thesis has identified the increased expression of some significant markers of oxidative stress during hibernation, including HSP27 and I κ B, as well as a marker of antioxidant defense, pag. This suggests the occurrence of oxidative stress during hibernation and correlates with other recent research has shown that lipid peroxidation occurs in torpid animals [Carey et al., 2000].

In summary, this thesis has examined the molecular basis for the metabolic regulation of hibernation by investigating transcriptional, translational and post-translational responses. The research has identified new signal transduction and gene expression responses in hibernation. The activation of the p38 MAPK signalling pathway has been identified in skeletal muscle during hibernation. Transcription factor activation in hibernators has only recently begun to be investigated and this thesis identifies new targets that play a role in regulating gene expression. The application of new technologies to the hibernator model, such as cDNA array screening and 2D-PAGE has provided the foundation for numerous future gene expression studies and has opened up new areas of hibernation research. These studies have also provided the first in depth examination of the molecular mechanisms of hibernation in bats.

Figure 6.1. Total protein levels of PGC-1 levels in euthermic versus hibernating skeletal muscle of *S. tridecemlineatus*. Results are representative blots with three trials.

S. tridecemlineatus PGC-1

Euthermic Hibernating

Figure 6.2. Egr-1 protein levels in euthermic versus hibernating tissues of *M. lucifugus* and *S. tridecemlineatus*. Representative blots are shown from three independent trials. Bar graphs are means $\pm$ SEM.

Early growth response: Egr-1

Figure 6.3. Total HSP27 protein investigating oligomeric status in euthermic and hibernating *M. lucifugus* skeletal muscle. Since HSP27 oligomers use disulfide bonding to maintain their structure, it is required to investigate the reduced and non-reduced forms of HSP27 total protein isolates. Samples were isolated in sample buffer (100 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA, 1mM PMSF, pH 7.4) and homogenized. Samples were then centrifuged and supernatants were removed and reconstituted in SDS sample buffer (with no reducing agent). To confirm that oligomers comprised primarily of disulfide bonds, an aliquot of the total protein (in SDS sample buffer) was first boiled for 2 minutes (Lane 2, 5) revealing no size difference non-boiled total protein (Lane 1, 4). Lanes 3 and 6 were reduced with β -mercaptoethanol and revealed that HSP27 oligomers primarily existed through disulfide bond formation. SDS-PAGE was carried out on a 12% polyacrylamide gel for 2 hours at 200 V and transferred overnight to PVDF membranes. Antibody incubations with HSP27 were as described in Chapter 2.

M. lucifugus skeletal muscle
HSP27

PUBLICATION LIST

Chapters and Reviews

Eddy, Sean F. and Kenneth B. Storey. (2002) Chapter 22: *Dynamic Use of cDNA Arrays: Heterologous probing for gene discovery and exploration of organismal adaptation to environmental stress* In Cell and Molecular Responses to Stress Vol. 3 (Storey, K.B. and Storey, J.M. Eds.). Elsevier Press, Netherlands.

Research Articles in Preparation

Eddy, S. F. and Kenneth B. Storey. The regulation of fatty acid binding proteins in hibernation by PGC-1, PPAR α and PPAR γ .

Eddy, S. F. and Kenneth B. Storey. Antioxidants in the hibernation heart: Up-regulation of a bat thioredoxin peroxidase.

Eddy, S. F. and Kenneth B. Storey. Activation of p38 MAPK signal transduction pathway in hibernating heart and skeletal muscle of little brown bats.

Eddy, S.F. and Kenneth B. Storey. Up-regulation of CPT-1 α and CPT-1 β in hibernating BAT

Eddy, S.F. and Kenneth B. Storey. Signal transduction in *Spermophilus richardsonii* during hibernation.

Eddy, S.F. and Kenneth B. Storey. Phosphorylation of eIF2 α and eEF2 in hibernating heart and skeletal muscle.

Genbank submissions

Myotis lucifugus heart type fatty acid binding protein (H-FABP) mRNA, complete cds
AF530469

Communications at Scientific Meetings

S. F. Eddy and K. B. Storey. Gene Expression in Hibernation: Testing Skeletal Muscle of Little Brown bats, *Myotis Lucifugus*, using commercially available cDNA Microarrays. *Proc. Virt. Conf. Genom. and Bioinf.* October 15-16, 2001. (<http://midas-10.cs.ndsu.nodak.edu/bio/papers/hibernation.pdf>)

Eddy, Sean F. and Kenneth B. Storey. *Increased mRNA of Heart Type Fatty Acid Binding Protein in Skeletal Muscle of the Little Brown Bat, Myotis lucifugus.* Ottawa Life Sciences National Conference, Ottawa, Canada, December 2000.

Eddy, Sean F. and Kenneth B. Storey. *Down Regulation of Ribosomal Protein Genes during Hibernation in Skeletal Muscle of Thirteen-Lined Ground Squirrels.* Life in the Cold, 11th International Hibernation Symposium, Jungholz, Austria, August 13-18, 2000.

Eddy, Sean F. and Kenneth B. Storey. *Gene Expression of Ribosomal Proteins During Hibernation.* Society for Experimental Biology, Cambridge, UK, July 31-August 2, 2000.

Eddy, Sean F. and Kenneth B. Storey. *cDNA Arrays as Tools for Profiling Gene Expression in Hibernating Mammals.* European Society for Comparative Physiology and Biochemistry, Liege, Belgium, July 23-28, 2000.

Eddy, Sean F. and Kenneth B. Storey. *Differential Gene Expression in Skeletal Muscle of the Thirteen-Lined Ground Squirrel Detected by cDNA Arrays.* Canadian Federation of Biological Sciences Conference, Ottawa, Canada, June 22-25, 2000.

Eddy, Sean F. and Kenneth B. Storey. *Analysis of Gene Regulation during Hibernation in the 13-Lined Ground, *Spermophilus tridecemlineatus*, Through the use of cDNA Arrays*. Ottawa Life Sciences National Conference, Ottawa, Canada, November 1-3, 1999.

Dissertation

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REFERENCES

- Abdel-Hafiz, H.A., Heasley, L.E., Kyriakis, J.M., Avruch, J., Kroll, D.J., Johnson, G.L., Hoeffler, J.P.** (2002) Activating transcription factor-2 DNA-binding activity is stimulated by phosphorylation catalyzed by p42 and p54 microtubule-associated protein kinases. *Mol. Endo.* 6, 2079-2089.
- Aggeli, I.K., Gaitanaki, C., Lazou, A., Beis, I.** (2002) Hyperosmotic and thermal stresses activate p38-MAPK in the perfused amphibian heart. *J. Exp. Biol.* 205, 443-54.
- Agostini, B., De Martino, L., Soltau, B., Hasselbach, W.** (1991) The modulation of the calcium transport by skeletal muscle sarcoplasmic reticulum in the hibernating European hamster. *Z. Naturforsch.* 46, 1109-26.
- Alway, S.E., Degens, H., Lowe, D.A., Krishnamurthy, G.** (2002) Increased myogenic repressor Id mRNA and protein levels in hindlimb muscles of aged rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 282, R411-22.
- Andrews, M.T., Squire, T.L., Bowen, C.M., Rollins, M.B.** (1998) Low-temperature carbon utilization is regulated by novel gene activity in the heart of a hibernating mammal. *Proc. Natl. Acad. Sci. USA* 95, 8392-7.
- Armstrong, M.B., Towle, H.C.** (2001) Polyunsaturated fatty acids stimulate hepatic UCP-2 expression via a PPAR alpha-mediated pathway. *Am. J. Physiol. Endocrinol. Metab.* 281, E1197-204.
- Ayadi, A., Zheng, H., Sobieszczuk, P., Buchwalter, G., Moerman, P., Alitalo, K., Wasylyk, B.** (2001) Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. *EMBO J.*, 20, 5139-52.

- Barger, P.M., Browning, A.C., Garner, A.N., Kelly, D.P.** (2001) p38 mitogen-activated protein kinase activates peroxisome proliferator-activated receptor alpha: a potential role in the cardiac metabolic stress response. *J. Biol. Chem.* 276, 44495-501.
- Bartosiewicz, M., Trounstein, M., Barker, D., Johnston, R. and Buckpitt, A.** (2000). Development of a toxicological gene array and quantitative assessment of this technology. *Arch. Biochem. Biophys.* 376, 66-73.
- Bauer, V.W., Squire, T.L., Lowe, M.E., Andrews, M.T.** (2001) Expression of a chimeric retroviral-lipase mRNA confers enhanced lipolysis in a hibernating mammal. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281, R1186-92.
- Bauman, W.A.** (1990) Seasonal changes in pancreatic insulin and glucagon in the little brown bat (*Myotis lucifugus*). 5, 342-6.
- Bauman, W.A., Meryn, S., Florant, G.L.** (1987) Pancreatic hormones in the nonhibernating and hibernating golden mantled ground squirrel. *Comp. Biochem. Physiol. A* 86, 241-4.
- Belke, D.D., Wang, L.C., Lopaschuk, G.D.** (1998) Acetyl-CoA carboxylase control of fatty acid oxidation in hearts from hibernating Richardson's ground squirrels. *Biochim. Biophys. Acta* 1391, 25-36.
- Bellomo, G., Mirabelli, F.** (1987-88) Oxidative stress injury studied in isolated intact cells. *Mol. Toxicol.*, 1, 281-93.
- Berger, J., Moller, D.E.** (2002) The mechanisms of action of PPARs. *Ann. Rev. Med.* 53, 409-35.

- Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J., Lutsch, G. B** (1994) Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *J. Biol. Chem.* 269, 20780-4.
- Blohm, D.H. and Guiseppi-Elie, A.** (2001) New developments in microarray technology. *Curr. Opin. Biotechnol.* 12, 41-47.
- Bogoyevitch, M.A., Gillespie-Brown, J., Kettermann, A.J., Fuller, S.J., Ben-Levy, R., Ashworth, A., Marshall, C.J., Sugden, P.H.** (1996) Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ. Res.* 79, 162-73.
- Borchers, T., Hohoff, C., Buhlmann, C., Spener, F.** (1997) Heart-type fatty acid binding protein - involvement in growth inhibition and differentiation. *Prostaglandins Leukot. Essent. Fatty Acids* 57, 77-84.
- Boyer, B.B., Barnes, B.M., Lowell, B.B., Grujic, D.** (1998) Differential regulation of uncoupling protein gene homologues in multiple tissues of hibernating ground squirrels. *Am. J. Physiol.* 275, R1232-8.
- Bradbury, C.M., Markovina, S., Wei, S.J., Rene, L.M., Zoberi, I., Horikoshi, N., Gius, D.** (2001) Indomethacin-induced radiosensitization and inhibition of ionizing radiation-induced NF-kappaB activation in HeLa cells occur via a mechanism involving p38 MAP kinase. *Cancer Res.* 61, 7689-96.

- Brigham, R.M., Ianuzzo, C.D., Hamilton, N., Fenton, M.B.** (1990) Histochemical and biochemical plasticity of muscle fibers in the little brown bat (*Myotis lucifugus*). J. Comp. Physiol. B 160, 183-6.
- Brooks, S.P.J., Storey, K.B.** (1992) Mechanisms of glycolytic control during hibernation in the ground squirrel *Spermophilus lateralis*. J. Comp. Physiol. B 162, 23-28.
- Brustovetsky, N.N., Egorova, M.V., Gnutov, D.Yu, Gogvadze, V.G., Mokhova, E.N., Skulachev, V.P.** (1992) Thermoregulatory, carboxyatractylate-sensitive uncoupling in heart and skeletal muscle mitochondria of the ground squirrel correlates with the level of free fatty acids. FEBS Lett. 305, 15-17.
- Buck, M.J., Squire, T.L., Andrews, M.T.** (2002) Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. Physiol. Genomics 8, 5-13.
- Burgess, J.K.** (2001). Gene expression studies using microarrays. Clin. Exp. Pharmacol. Physiol. 28, 321-328.
- Burlington, R.F., Bowers, W.D. Jr., Daum, R.C., Ashbaugh, P.** (1972) Ultrastructural changes in heart tissue during hibernation. Cryobiology 9, 224-8.
- Butterfield, L.H., Merino, A., Golub, S.H., Shau, H.** (1999) From cytoprotection to tumor suppression: the multifactorial role of peroxiredoxins. Antioxid. Redox Signal. 1, 385-402.
- Carey, H.V., Martin, S.L.** (1996) Preservation of intestinal gene expression during hibernation. Am. J. Physiol. 271, G804-13.

- Carey, H.V., Frank C.L., Seifert, J.P.** (2000) Hibernation induces oxidative stress and activation of NK-kappaB in ground squirrel intestine. *J. Comp. Physiol. B* 170, 551-9.
- Chen, Y., Matsushita, M., Nairn, A.C., Damuni, Z., Cai, D., Frerichs, K.U., Hallenbeck, J.M.** (2001) Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. *Biochemistry* 40, 11565-70.
- Cochet, N., Georges, B., Meister, R., Florant, G.L., Barre, H.** (1999a) White adipose tissue fatty acids of Alpine marmots during their yearly cycle. *Lipids* 34, 275-81.
- Cochet, N., Meister, R., Florant, G.L., Barre, H.** (1999b) Regional variation of white adipocyte lipolysis during the annual cycle of the alpine marmot. *Comp. Biochem. Physiol. C* 123, 225-32.
- Cunningham, M.J.** (2000). Genomics and proteomics: the new millennium of drug discovery and development. *J. Pharmacol. Toxicol. Meth.* 44, 291-300.
- Daikoku, T., Shinohara, Y., Shima, A., Yamazaki, N., Terada, H.** (1997) Dramatic enhancement of the specific expression of the heart-type fatty acid binding protein in rat brown adipose tissue by cold exposure. *FEBS Lett.* 410, 383-6.
- Dailly, Y.P., Zhou, Y., Linkhart, T.A., Baylink, D.J., Strong, D.D.** (2001) Structure and characterization of the human insulin-like growth factor binding protein (IGFBP)-6 promoter: identification of a functional retinoid response element. *Biochim. Biophys. Acta.* 1518, 145-51.

- Dalle-Donne, I., Rossi, R., Milzani, A., Di Simplicio, P., Colombo, R.** (2001) The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic. Biol. Med.* 31, 1624-32.
- Das, D.K., Maulik, N. Moraru, I.I.** (1995) Gene expression in acute myocardial stress. Induction by hypoxia, ischemia, reperfusion, hyperthermia and oxidative stress. *J. Mol. Cell. Cardiol.* 27, 181-93.
- Dolan, P.L.; Wu, Y., Ista, L.K., Metzenberg, R.L., Nelson, M.A., Lopez, G.P.** (2001). Robust and efficient synthetic method for forming DNA microarrays. *Nucleic Acids Res.* 29, e107.
- Dunn, S.E., Chin, E.R., Michel, R.N.** (2001) Matching of calcineurin activity to upstream effectors is critical for skeletal muscle fiber growth. *J. Cell. Biol.* 151, 663-72.
- Eaton, P., Fuller, W., Shattock, M.J.** (2002) S-thiolation of HSP27 regulates its multimeric aggregate size independently of phosphorylation. *J. Biol. Chem.*, 277, 21189-96.
- Egginton, S., Fairney, J., Bratcher, J.** (2001) Differential effects of cold exposure on muscle fibre composition and capillary supply in hibernator and non-hibernator rodents. *Exp. Physiol.* 86, 629-39.
- Epperson, L.E., Martin, S.L.** (2002) Quantitative assessment of ground squirrel mRNA levels in multiple stages of hibernation. *Physiol. Genomics* 10, 93-102.
- Fahlman, A., Storey, J.M., Storey, K.B.** (2000) Gene up-regulation in heart during mammalian hibernation. *Cryobiology* 40, 332-42.

- Foster, D.O.** (1984) Quantitative contribution of brown adipose tissue thermogenesis to overall metabolism. *Can. J. Biochem. Cell. Biol.* 62, 618-22.
- Foxworthy, P.S., Perry, D.N., Hoover, D.M., Eacho, P.I.** (1990) Changes in hepatic lipid metabolism associated with lipid accumulation and its reversal in rats given the peroxisome proliferator LY171883. *Toxicol. Appl. Pharmacol.* 106, 375-83.
- Frerichs, K.U., Kennedy, C., Sokoloff, L., Hallenbeck, J.M.** (1994) Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia." *J. Cereb. Flow. Metab.*, 14, 193-205.
- Frerichs, K.U., Smith, C.B., Brenner, M., DeGracia, D.J., Krause, G.S., Marrone, L., Dever, T.E., Hallenbeck, J.M.** (1998) Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc. Natl. Acad. Sci. USA* 95, 14511-6.
- Friday, B.B., Horsley, V., Pavlath, G.K.** (2000) Calcineurin activity is required for the initiation of skeletal muscle differentiation. *J. Cell. Biol.* 149, 657-66.
- Geiser, F.** (1990) Influence of polyunsaturated and saturated dietary lipids on adipose tissue, brain and mitochondrial membrane fatty acid composition of a mammalian hibernator. *Biochim. Biophys. Acta.* 1046, 159-66.
- Geiser, F., McAllan, B.M., Kenagy, G.J.** (1994) The degree of dietary fatty acid unsaturation affects torpor patterns and lipid composition of a hibernator. *J. Comp. Physiol. B* 164, 299-305.
- Gille, H., Strahl, T., Shaw, P.E.** (1995) Activation of ternary complex factor Elk-1 by stress-activated protein kinases. *Curr. Biol.* 5, 1191-200.

- Gracey, A.Y., Troll, J.V., Somero, G.N.** (2001) Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc. Natl. Acad. Sci. USA* 98, 1993-8.
- Granberg, P.O.** (1991) Human physiology under cold exposure. *Arctic Med. Res.*, 50, supplement 23-27.
- Green, S., Wahli, W.** (1994) Peroxisome proliferator-activated receptors: finding the orphan a home. *Mol. Cell Endocrinol.* 100, 149-53.
- Guan, Y., Zhang, Y., Schneider, A., Davis, L., Breyer, R.M., Breyer, M.D.** (2001) Peroxisome proliferator-activated receptor-gamma activity is associated with renal microvasculature. *Am. J. Physiol. Renal Physiol.* 281, F1036-46.
- Gundersen, K., Merlie, J.P.** (1994) Id-1 as a possible transcriptional mediator of muscle disuse atrophy. *Proc. Natl. Acad. Sci. USA* 91, 3647-51.
- Hai, T., Hartman, M.G.** (2001) The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 273, 1-11.
- Haridas, V., Ni, J., Meager, A., Su, J., Yu, G.L., Zhai, Y., Kyaw, H., Akama, K.T., Hu, J., Van Eldik, L.J., Aggarwal, B.B.** (1998) TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase. *J. Immunol.* 161, 1-6.
- Harlow, H.J., Frank, C.L.** (2001) The role of dietary fatty acids in the evolution of spontaneous and facultative hibernation patterns in prairie dogs. *J. Comp. Physiol. B* 171, 77-84.

- Hegde, P., Qi, R., Abernathy, K., Gay, C., Dharap, R., Gaspard, R., Hughes, J.E., Snesrud, E., Lee, N., Quakenbush, J.** (2000). A concise guide to cDNA microarray analysis. *Biotechniques* 29, 548-562.
- Heldmaier, G., Klingenspor, M., Werneyer, M., Lampi, B.J., Brooks, S.P., Storey, K.B.** (1999) Metabolic adjustments during daily torpor in the Djungarian hamster. *Am. J. Physiol.*, 276, E896-E906.
- Herr, F.M., Aronson, J., Storch, J.** (1996) Role of Portal Region Lysine Residues in Electrostatic Interactions between Heart Fatty Acid Binding Protein and Phospholipid Membranes. *Biochemistry*, 35, 1296-1303.
- Hittel, D., Storey, K.B.** (2001) Differential expression of adipose- and heart-type fatty acid binding proteins in hibernating ground squirrels. *Biochim. Biophys. Acta* 1522, 238-43.
- Hittel, D., Storey, K.B.** (2002a) The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*). *Arch. Biochem. Biophys.* 401, 244-54.
- Hittel, D.S., Storey, K.B.** (2002b) Differential expression of mitochondria-encoded genes in a hibernating mammal. *J. Exp. Biol.* 205, 1625-31.
- Hochachka, P.W.** (1986) Defense strategies against hypoxia and hypothermia. *Science*, 231, 234-41.
- Hoffmann, T., Schutz, A., Brosius, M., Volker, A., Volker, U., Bremer, E.** (2002) High-salinity-induced iron limitation in *Bacillus subtilis*. *J. Bacteriol.* 184, 718-27.

- Holden, C.P., Storey, K.B.** (1998) Protein kinase A from bat skeletal muscle: a kinetic study of the enzyme from a hibernating mammal. *Arch. Biochem. Biophys.* 358, 243-50.
- Hotamisligil, G.S., Johnson, R.S., Distel, R.J., Ellis, R., Papaioannou, V.E., Spiegelman, B.M.** (1996) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 274, 1377-9.
- Hu, E., Kim, J.B., Sarraf, P., Spiegelman, B.M.** (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR γ . *Science* 274, 2100-3.
- Humphries, M.M., Thomas, D.W., Speakman, J.R.** (2002) Climate-mediated energetic constraints on the distribution of hibernating mammals. *Nature* 418, 313-6.
- Ivanov VN, Ronai Z.** (1999) Down-regulation of tumor necrosis factor alpha expression by activating transcription factor 2 increases UVC-induced apoptosis of late-stage melanoma cells. *J. Biol. Chem.* 274, 14079-89.
- Jain, K.K.** (2000). Applications of biochip and microarray systems in pharmacogenomics. *Pharmacogenomics* 1, 289-307.
- Janknecht, R., Hunter, T.** (1997) Convergence of MAP kinase pathways on the ternary complex factor Sap-1a. *EMBO J.* 16, 1620-7.
- Jaspers, S.R., Jacob, S., Tischler, M.E.** (1986) Metabolism of amino acids by the atrophied soleus of tail-casted, suspended rats. *Metabolism* 35, 216-23.
- Jaspers, S.R., Henriksen, E.J., Satarug, S., Tischler, M.E.** (1989) Effects of stretching and disuse on amino acids in muscles of rat hind limbs. *Metabolism* 38, 303-10.

- Jin, D.Y., Chae, H.Z., Rhee, S.G., Jeang, K.T.** (1997) Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. *J. Biol. Chem.* 272, 30952-61.
- Kim, M.H., Park, K., Gwag, B.J., Jung, N.P., Oh, Y.K., Shin, H.C., Choi, I.H.** (2000) Seasonal biochemical plasticity of a flight muscle in a bat, *Murina leucogaster*. *Comp. Biochem. Physiol. A* 126, 245-50.
- Kim, S.O., Baines, C.P., Critz, S.D., Pelech, S.L., Katz, S., Downey, J.M., Cohen, M.V.** (1999) Ischemia induced activation of heat shock protein 27 kinases and casein kinase 2 in the preconditioned rabbit heart. *Biochem. Cell Biol.* 77, 559-67.
- Knebel, B., Kotzka, J., Avci, H., Schiller, M., Bruning, J.C., Hafner, M., Krone, W., Muller-Wieland, D.** (2000) Characterization of a postreceptor signaling defect that impairs cfos expression in cultured fibroblasts of a patient with insulin resistance. *Biochem. Biophys. Res. Commun.* 268, 577-82.
- Knight, J.E., Narus, E.N., Martin, S.L., Jacobson, A., Barnes, B.M., Boyer, B.B.** (2000) mRNA stability and polysome loss in hibernating Arctic ground squirrels (*Spermophilus parryii*). *Mol. Cell. Biol.* 20, 6374-9.
- Knutti, D., Krali, A.** (2001) PGC-1, a versatile co-activator. *Trends. Endocrinol. Metabol.*, 12, 360-365.
- Landry, J., Huot, J.** (1995) Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. *Biochem. Cell Biol.* 73, 703-7.

- Larade, K., Storey, K.B.** (2002) Reversible suppression of protein synthesis in concert with polysome disaggregation during anoxia exposure in *Littorina littorea*. *Mol. Cell. Biochem.* 232, 121-7.
- Lewitt, M.S., Brismar, K., Wang, J., Wivall-Helleryd, I.L., Sindelar, P., Gonzalez, F.J., Bergman, T., Bobek, G.A.** (2001) Responses of insulin-like growth factor (IGF)-I and IGF-binding proteins to nutritional status in peroxisome proliferator-activated receptor-alpha knockout mice. *Growth Horm. IGF Res.* 11, 303-13.
- Li, B., Ishii, T., Tan, C.P., Soh, J.W., Goff, S.P.** (2002) Pathways of induction of peroxiredoxin I expression in osteoblasts: roles of p38 mitogen-activated protein kinase and protein kinase C. *J. Biol. Chem.* 277, 12418-22.
- Liang, P., Pardee, A.B.** (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257, 967-71.
- Lim, C.P., Jain, N., Cao, X.** (1998) Stress-induced immediate-early gene, *egr-1*, involves activation of p38/JNK1. *Oncogene* 16, 2915-26.
- Lin, J., Wu, H., Tarr, P.T., Zhang, C.Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., Lowell, B.B., Bassel-Duby, R., Spiegelman, B.M.** (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418, 797-801.
- Liu, X., Li, Q., Lin, Q., Sun, R.** (2001) Uncoupling protein1 mRNA, mitochondrial GTP-binding, and T4 5'-deiodinase of brown adipose tissue in euthermic Daurian ground squirrel during cold exposure. *Comp. Biochem. Physiol. A* 128, 827-35.
- Lobenhofer, E.K., Bushel, P.R., Afshari, C.A., Hamadeh, H.K.** (2001) Progress in the application of DNA microarrays. *Environ. Health Perspect.* 109, 881-91.

- Louet, J.F., Hayhurst, G., Gonzalez, F.J., Girard, J., Decaux, J.F.** (2002) The coactivator PGC-1 is involved in the regulation of the liver carnitine palmitoyltransferase I gene expression by cAMP in combination with HNF4a and CREB. *J. Biol. Chem.* 277, 37991-38000.
- Luss, H., Neumann, J., Schmitz, W., Schulz, R., Heusch, G.** (2000) The stress-responsive MAP kinase p38 is activated by low-flow ischemia in the in situ porcine heart. *J. Mol. Cell. Cardiol.* 32, 1787-94.
- MacDonald, J.A., Storey, K.B.** (1999) Regulation of ground squirrel Na^+K^+ -ATPase activity by reversible phosphorylation during hibernation. *Biochem. Biophys. Res. Comm.* 254, 424-9.
- MacDonald, J.A., Storey, K.B.** (2001) Reassessment of the cold-labile nature of phosphofructokinase from a hibernating ground squirrel. *Mol. Cell Biochem.* 225, 51-7.
- Malysheva, A.N., Storey, K.B., Ziganshin, R.K., Lopina, O.D., Rubtsov, A.M.** (2001) Characteristics of sarcoplasmic reticulum membrane preparations isolated from skeletal muscles of active and hibernating ground squirrel *Spermophilus undulatus*. *Biochemistry (Mosc)* 66, 918-25.
- Martin, S.L., Maniero, G.D., Carey, C., Hand, S.C.** (1999) Reversible depression of oxygen consumption in isolated liver mitochondria during hibernation. *Physiol. Biochem. Zool.*, 72, 255-64.
- McLeod, L.E., Wang, L., Proud, C.G.** (2001) beta-Adrenergic agonists increase phosphorylation of elongation factor 2 in cardiomyocytes without eliciting calcium-independent eEF2 kinase activity. *FEBS Lett.* 489, 225-8.

- Medhora, M., Bousamra, M., Zhu, D., Somberg, L., Jacobs, E.R.** (2002) Upregulation of collagens detected by gene array in a model of flow-induced pulmonary vascular remodeling. *Am. J. Physiol. Heart Circ. Physiol.*, 282, H414-422.
- Miele, G., MacRae, L., McBride, D., Manson, J., Clinton, M.** (1998) Elimination of false positives generated through PCR re-amplification of differential display cDNA. *Biotechniques* 25, 138-44.
- Mochizuki, K., Suruga, K., Kitagawa, M., Takase, S., Goda, T.** (2001) Modulation of the expression of peroxisome proliferator-activated receptor-dependent genes through disproportional expression of two subtypes in the small intestine. *Arch. Biochem. Biophys.* 389, 41-8.
- Memon, R.A., Bass, N.M., Moser, A.H., Fuller, J., Appel, R., Grunfeld, C., Feingold, K.R.** (1999) Down-regulation of liver and heart specific fatty acid binding proteins by endotoxin and cytokines in vivo. *Biochim. Biophys. Acta* 1440, 118-26.
- Miele, G., MacRae, L., McBride, D., Manson, J., Clinton, M.** (1998) Elimination of false positives generated through PCR re-amplification of differential display cDNA. *Biotechniques* 25,138-44.
- Muller, J.M., Cahill, M.A., Rupec, R.A., Baeuerle, P.A., Nordheim, A.** (1997) Antioxidants as well as oxidants activate c-fos via Ras-dependent activation of extracellular-signal-regulated kinase 2 and Elk-1. *Eur. J. Biochem.* 15, 45-52.
- Nestler, J.R.** (1992) Tissue-specific metabolism during normothermy and daily torpor in deer mice (*Peromyscus maniculatus*). *J. Exp. Zool.* 261, 406-13.

- Nizielski, S.E., Billington, C.J., Levine, A.S. (1995)** Cold-induced alterations in uncoupling protein and its mRNA are seasonally dependent in ground squirrels. *Am. J. Physiol.* 269, R357-64.
- Oberkofler, H., Esterbauer, H., Linnemayr, V., Strosberg, A.D., Krempler, F., Patsch, W. (2002)** Peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1 recruitment regulates PPAR subtype specificity. *J. Biol. Chem.* 277, 16750-7.
- Oeltgen, P.R., Bergmann, L.C., Spurrier, W.A., Jones, S.B. (1978)** Isolation of a hibernation inducing trigger(s) from the plasma of hibernating woodchucks. *Prep. Biochem.* 8, 171-88.
- Ogura, M., Kitamura, M. (1998)** Oxidant stress incites spreading of macrophages via extracellular signal-regulated kinases and p38 mitogen-activated protein kinase. *J. Immunol.* 161, 3569-74.
- O'Hara, B.F., Watson, F.L., Srere, H.K., Kumar, H., Wiler, S.W., Welch, S.K., Bitting, L., Heller, H.C., Kilduff, T.S. (1999)** Gene expression in the brain across the hibernation cycle. *J. Neurosci.* 19, 3781-90.
- Ohtsuki, T., Jaffe, H., Brenner, M., Azzam, N., Azzam, R., Frerichs, K.U., Hallenbeck, J.M. (1998)** Stimulation of tyrosine phosphorylation of a brain protein by hibernation. *J. Cereb. Blood Flow Metab.* 18, 1040-5.
- Oliner, J.D., Andresen, J.M., Hansen, S.K., Zhou, S., Tjian, R. (1996)** SREBP transcriptional activity is mediated through an interaction with the CREB-binding protein. *Genes Dev.* 10, 2903-11.

- Ono, M., Hosoe, Y., Azuma, S., Shoji, M., Nara, K., Kondo, N., Shiba, T., Takamatsu, N.** (2001) HNF-1 regulates the liver-specific transcription of the chipmunk HP-20 gene. *Gene* 277, 121-7.
- Pease, A.C., Solas, D., Sullivan, E.J., Cronin, M.T., Holmes, C.P., Fodor, S.P.** (1994). Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. USA* 91, 5022-5026.
- Postnikova, G.B., Tselikova, S.V., Kolaeva, S.G., Solomonov, N.G.** (1999) Myoglobin content in skeletal muscles of hibernating ground squirrels rises in autumn and winter. *Comp. Biochem. Physiol. A* 124, 35-7.
- Preville, X., Schultz, H., Knauf, U., Gaestel, M., Arrigo, A.P.** (1998) Analysis of the role of Hsp25 phosphorylation reveals the importance of the oligomerization state of this small heat shock protein in its protective function against TNF α - and hydrogen peroxide-induced cell death. *J. Cell Biochem.* 69, 436-52.
- Puigserver, P., Rhee, J., Lin, J., Wu, Z., Yoon, J.C., Zhang, C.Y., Krauss, S., Mootha, V.K., Lowell, B.B., Spiegelman, B.M.** (2001) Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPAR γ coactivator-1. *Mol. Cell* 8, 971-82.
- Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B., Davis, R.J.** (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* 16, 1247-55.
- Reddy, J.K., Mannaerts, G.P.** (1994) Peroxisomal lipid metabolism. *Ann. Rev. Nutr.* 14, 343-70.

- Rim, J.S., Kozak, L.P.** (2002) Regulatory motifs for CREB-binding protein and Nfe2l2 transcription factors in the upstream enhancer of the mitochondrial uncoupling protein 1 gene. *J. Biol. Chem.* 277, 34589-600.
- Roberts, J.R., Rowe, P.A., Demaine, A.G.** (2002) Activation of NF-kappaB and MAP kinase cascades by hypothermic stress in endothelial cells. *Cryobiology* 44, 161-9.
- Sakamoto, K., Goodyear, L.J.** (2002) Invited Review: Intracellular signaling in contracting skeletal muscle. *J. Appl. Physiol.* 93, 369-83.
- Sakamoto, K., Hirshman, M.F., Aschenbach, W.G., Goodyear, L.J.** (2002) Contraction regulation of Akt in rat skeletal muscle. *J. Biol. Chem.* 277, 11910-7.
- Schena, M., Shalon, D., Davis, R.W. Brown, P.O.** (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467-470.
- Shen, W.J., Sridhar, K., Bernlohr, D.A., Kraemer, F.B.** (1999) Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. *Proc. Natl. Acad. Sci. USA*, 96, 5528-5532.
- Smith, U.** (2002) Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance--is insulin resistance initiated in the adipose tissue? *Int. J. Obes. Relat. Metab. Disord.* 26, 897-904.
- Speakman, J.R., Rowland, A.** (1999) Preparing for inactivity: how insectivorous bats deposit a fat store for hibernation. *Proc. Nutr. Soc.* 58, 123-31.

- Speakman, J.R., Thomas, D.W.** (2002) Physiological ecology and energetics of bats. Phys. Ecol. In T.H. Kunz & M. B. Fenton. Ecology of bats II. Academic Press. (in press)
- Srere, H.K., Belke, D., Wang, L.C., Martin, S.L.** (1995) alpha 2-Macroglobulin gene expression during hibernation in ground squirrels is independent of acute phase response. Am. J. Physiol. 268, R1507-12.
- Steffen, J.M., Koebel, D.A., Musacchia, X.J., Milsom, W.K.** (1991) Morphometric and metabolic indices of disuse in muscles of hibernating ground squirrels. Comp. Biochem. Physiol. B 99, 815-9.
- Stewart, J.M., English, T.E., Storey, K.B.** (1998) Comparisons of the effects of temperature on the liver fatty acid binding proteins from hibernator and nonhibernator mammals. Biochem. Cell Biol. 76, 593-9.
- Storey, K.B.** (1997) Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. Comp. Biochem. Physiol. A 118, 1115-1124.
- Storey, K.B.** (2001) Turning down the fires of life: metabolic regulation of hibernation and estivation. In: Molecular Mechanisms of Metabolic Arrest (Storey, K.B., ed.) BIOS Scientific Publishers, Oxford, pp. 1-21.
- Sul, H.S., Latasa, M.J., Moon, Y., Kim, K.H.** (2000) Regulation of the fatty acid synthase promoter by insulin. J. Nutr. 130, 315S-320S.
- Summers, S.A., Birnbaum, M.J.** (1997) A role for the serine/threonine kinase, Akt, in insulin-stimulated glucose uptake. Biochem. Soc. Trans., 25, 981-8.
- Taniguchi, M., Miura, K., Iwao, H., Yamanaka, S.** (2001). Quantitative assessment of DNA microarrays - comparison with Northern blot analyses. Genomics 71, 34-39.

- Thatcher, B.J., Storey, K.B.** (2001) Glutamate dehydrogenase from liver of euthermic and hibernating Richardson's ground squirrels: evidence for two distinct enzyme forms. *Biochem. Cell Biol.* 79, 11-9.
- Tournay, O., Benezra, R.** (1996) Transcription of the dominant-negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene Egr-1. *Mol. Cell Biol.* 16, 2418-30.
- Treisman, R.** (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Biol.* 8, 205-215.
- Uysal, K.T., Wiesbrock, S.M., Hotamisligil, G.S.** (1998) Functional analysis of tumor necrosis factor (TNF) receptors in TNF-alpha-mediated insulin resistance in genetic obesity. *Endocrinology* 139, 4832-8.
- van Breukelen, F., Martin, S.L.** (2001) Translational initiation is uncoupled from elongation at 18°C during mammalian hibernation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281, R1374-9.
- van Breukelen, F., Martin, S.L.** (2002) Reversible depression of transcription during hibernation. *J. Comp. Physiol. B* 172, 355-61.
- Vanhoutte, P., Barnier, J.V., Guibert, B., Pages, C., Besson, M.J., Hipkind, R.A., Caboche, J.** (2001) Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. *Mol. Cell. Biol.* 19, 136-46.
- Vega, R.B., Huss, J.M., Kelly, D.P.** (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of

nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol. Cell. Biol.* 20, 1868-76.

Vogel-Hertzel, A., Bernlohr, D.A. (2002) The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends Endocrinol. Metab.* 11, 175-180.

Wallach, D.P. (1970) Enhancement of the lipolytic activity of skeletal muscle in certain physiological states. *Can. J. Biochem.* 48, 547-52.

Wang, L.C.H., Lee, T.F. (1996) Torpor and hibernation in mammals: metabolic, physiological, and biochemical adaptations. In *Handbook of Physiology, Environmental Physiology*. (ed. M.J. Fregely and C.M. Blatteis) pp. 507-532. New York, Oxford University Press.

Wang, L.C., Lee, T.F., Jourdan, M.L. (1987) Seasonal difference in thermoregulatory responses to opiates in a mammalian hibernator. *Pharmacol. Biochem. Behav.* 26, 565-71.

Wentworth, B.M., Donoghue, M., Engert, J.C., Berglund, E.B., Rosenthal, N. (1991) Paired MyoD-binding sites regulate myosin light chain gene expression. *Proc. Natl. Sci. USA* 88, 1242-1246.

Wickler, S.J., Hoyt, D.F., van Breukelen, F. (1991) Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis*. *Am. J. Physiol.* 261, R1214-7.

Wilson, B.E., Deeb, S., Florant, G.L. (1992) Seasonal changes in hormone-sensitive and lipoprotein lipase mRNA concentrations in marmot white adipose tissue. *Am. J. Physiol.* 262, R177-81.

- Wu, H., Kanatous, S.B., Thurmond, F.A., Gallardo, T., Isotani, E., Bassel-Duby, R., Williams, R.S.** (2002) Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* 296, 349-52.
- Xu, Q., Yu, L., Liu, L., Cheung, C.F., Li, X., Yee, S.P., Yang, X.J., Wu, Z.** (2002) p38 Mitogen-activated protein kinase-, calcium-calmodulin-dependent protein kinase-, and calcineurin-mediated signaling pathways transcriptionally regulate myogenin expression. *Mol. Biol. Cell* 13, 1940-52.
- Yamashita, H., Sato, Y., Mori, N.** (1999) Difference in induction of uncoupling protein genes in adipose tissues between young and old rats during cold exposure. *FEBS Lett.* 458, 157-61.
- Yasuma, Y., McCarron, R.M., Spatz, M., Hallenbeck, J.M.** (1997) Effects of plasma from hibernating ground squirrels on monocyte-endothelial cell adhesive interactions. *Am. J. Physiol.* 273, R1861-9.
- Yu, E., Hallenbeck, J., Cai, D., McCarron, R.** (2002) Elevated arylalkylamine-N-acetyltransferase (AA-NAT) gene expression in medial habenular and suprachiasmatic nuclei of hibernating ground squirrels. *Brain Res. Mol. Brain Res.* 102, 9-17.
- Zimmerman, A.W., Rademacher, M., Ruterjans, H., Lucke, C., Veerkamp, J.H.** (1999) Functional and conformational characterization of new mutants of heart fatty acid-binding protein. *Biochem. J.*, 344, 495-501.
- Zinck, R., Cahill, M.A., Kracht, M., Sachsenmaier, C., Hippskind, R.A., Nordheim, A.** (1995) Protein synthesis inhibitors reveal differential regulation of mitogen-

activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1. *Mol. Cell. Biol.* 15, 4930-8.