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**ANTIOXIDANT DEFENSE IN THE HIBERNATING THIRTEEN-LINED
GROUND SQUIRREL SPERMOPHILUS TRIDECEMPLINEATUS**

By

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B.Sc. East China Normal University, 2002

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

Master of Science
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Abstract

Dramatic changes in blood flow and oxygen consumption during torpor-arousal cycles could increase the risk of oxidative stress to tissues of mammalian hibernators. Changes to antioxidant defense systems during hibernation were assessed in thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*, focusing on heme oxygenase 1 (HO1), superoxide dismutase (SOD) and the thioredoxin (Trx) system. Compared with euthermic control squirrels, inducible HO1 was elevated at both the transcriptional and translational level in multiple tissues of hibernating squirrels. The Nrf2/ARE signal transduction pathway commonly regulates HO1 and protein levels of both the transcription factor Nrf2 and its cofactor MafG were elevated in concert with HO1 implicating this pathway in HO1 expression in hibernation. Both cytosolic Cu/Zn SOD and mitochondrial Mn SOD increased significantly in squirrel muscle during hibernation, probably contributing to antioxidant defense during arousal from torpor when oxygen consumption by muscle increases rapidly to support shivering thermogenesis. Proteins of the thioredoxin system (Trx1, Trx2, TrxR2) also rose in multiple tissues during hibernation, perhaps to remedy a glutathione redox imbalance during hibernation. Amino acid sequencing of HO1 and SOD showed strong conservation of sequence compared with nonhibernating mammals but selected substitutions were found that could aid enzyme function at low body temperatures. The data support the idea that hibernators experience oxidative stress over torpor-arousal cycles and in response activate antioxidant defenses to minimize damage. A better understanding of antioxidant defense systems in hibernation may guide the use of antioxidants in medical treatments for ischemia/reperfusion damage and technology for cold preservation of organs.

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List of Abbreviations

2D-PAGE	Two-Dimensional Poly-Acrylamide Gel Electrophoresis
AP-1	activator protein 1
ARC	arcuate nucleus
ARE	antioxidant response element
B.A.T.	brown adipose tissue
BLAST	Basic Local Alignment Search Tool
cDNA	complementary DNA
Ct	cycle threshold
DEPC	diethylpyrocarbonate
EDTA	ethylenediamine tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
FABP	fatty acid binding protein
GPx	glutathione peroxidase
GRP75	glucose regulated protein 75
GSH	glutathione
GST	glutathione S-transferase
HIF-1	hypoxia-inducible factor-1
HO	heme oxygenase
HSP	heat shock protein
kb	kilobase

kDa	kilo Dalton
Keap1	kelch-like ECH-associated protein 1
MafG	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G
MAPK	mitogen-activated protein kinase
MARE	Maf-recognition element
MLC1v	ventricular isoform of myosin light chain 1
MOPS	2-[N-morpholine] proanesulfonic acid
ND2	NADH–ubiquinone oxidoreductase
mRNA	Messenger RNA
NPY	neuropeptide Y
NF- κ B	nuclear factor-kappa B
Nrf2	nuclear factor-erythroid 2 related factor -2
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene fluoride
RACE	rapid amplification of cDNA ends
PCR	polymerase chain reaction
PDK	pyruvate dehydrogenase kinase
ROS	reactive oxygen species
RT	room temperature $\sim 21^{\circ}\text{C}$
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate

SOD	superoxide dismutase
StRE	stress-response element
Ta	ambient temperature
Tb	body temperature
TEMED	N,N,N',N'-tetramethylethylenediamine
Trx	thioredoxin
TrxR	thioredoxin reductase
UCP	uncoupling protein
W.A.T.	white adipose tissue

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Chapter 1

General Introduction

1.1 General Introduction of Hibernation

If an unprotected human is exposed to low environmental temperatures, his body temperature (T_b) will decrease and soon hypothermia will set in. The shivering response helps to generate heat but ultimately as T_b falls, the heart fibrillates and ventilation fails at a T_b of 23-30°C, and death from hypothermia soon occurs (Hirvonen, 1979; Ivanov, 2000). However, some small mammals can survive low environmental temperatures and avoid the injuries normally associated with hypothermia by utilizing hibernation.

Hibernation is a common winter survival strategy of many small mammals that allows them to survive both the cold weather of winter and a limited or absent food supply in this season. Hibernation consists of multiple bouts of torpor that range in duration from a few days to up to 5 weeks in some species (Figure 1.1). During torpor, the metabolic rate falls to very low values (typically to <5% of euthermic resting rate) and T_b is maintained within just a few degrees above ambient temperature (T_a) (Wang and Lee, 1996). Physiological functions are strongly suppressed during torpor. For example, heart rate and respiration are greatly reduced, some muscle atrophy occurs, and renal function is significantly reduced or halted. Torpor bouts are interspersed with periodic arousals that return T_b to euthermic values (~37°C). 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 and the animals typically remain aroused for up to 1 day before sinking back into torpor. Why these arousals occur is not yet clear. Many hypotheses have been proposed, including the possible need to restore ionic balance, replenish carbohydrate reserves, eliminate metabolic waste, and revitalize neurological or immunological functions (Larkin and Heller, 1999; Prendergast et al., 2002).

By hibernating, the total energy expenditure during torpor can be anywhere from 60% to 98% less than normal, depending on the core temperature and the size of the animal (smaller animals generally save more energy) (Wang et al., 1989). Furthermore, Wang et al. (1989) calculated that energy savings were as much as 88% for ground squirrels over the full winter season counting both torpor bouts and periodic arousals, compared with the cost of remaining euthermic over the winter. Animals spend days or weeks with a core T_b that is often near to 0°C , yet they show none of the metabolic injuries that rapidly accrue in the organs of humans and most other mammals when core T_b falls below approximately 25°C . Heart beat of hibernators is also reduced to just a fraction of normal (Frerichs et al., 1994), and the blood supply to body tissues decreases dramatically, creating conditions that would constitute severe ischemia in most mammals, including man, but are noninjurious to hibernators. How do hibernators prevent or endure these potential injuries? And why can't we? Or can we? Hibernators offer very good natural models of mammalian organ systems that can withstand long-term hypothermia and ischemia.

Hibernation is a state in which all physiological and metabolic activities are profoundly depressed in a highly controlled and coordinated manner. This phenomenon has inspired several generations of research into the physiological mechanisms that govern this remarkable adaptation. Recent studies have begun to probe hibernation-specific gene expression and other forms of animal dormancy in response to environmental stress (Srere et al., 1992). Because the hibernating mammal arouses periodically from torpor throughout the hibernation season, the ability to function at euthermic temperatures must also be maintained continuously. At the molecular level,

this predicts that the steady-state levels of mRNA and protein expressed from the majority of genes will remain relatively constant between the euthermic and hibernating states (Ohara et al., 1999; Epperson and Martin, 2002) and that a relatively small number of genes that perform crucial functions for hibernation will be up-regulated during the winter (Carey et al., 2000; Fahlman et al., 2000; Storey and Storey, 2004). These could include genes that regulate metabolic suppression, genes that improve ischemia resistance, antioxidant defense, and hypothermia tolerance, and genes that address organ-specific needs for the maintenance of long term viability while torpid. Other gene products that are not essential during hibernation may be down-regulated. By understanding the patterns of gene expression in mammalian hibernation we can gain knowledge of both the key metabolic problems faced by different organs and the natural solutions that have been developed. All of the answers gained have obvious applications for hypothermic organ preservation.

1.2 Model Animal: Thirteen-lined ground squirrels

The thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, was chosen as the model organism for our laboratory studies to explore the biochemical and genetic basis of mammalian hibernation. This species is one of several species of hibernating ground squirrels. In nature, 13-lined ground squirrels inhabit most of the North American great plains (Figure 1.2 A and B). They feed primarily on seeds, flowers and insects. Adults weigh 130-180 g but they gain considerable weight (up to 4g fat per day) in late summer to raise their body mass to 220-240 g prior to hibernation. Hibernation begins in September or early October and finishes between late March and early May. The cycles

of torpor followed by short arousals continue throughout the winter months until the animal is fully aroused from hibernation and remains in a euthermic state. During hibernation, core Tb of the animals can drop to near 5°C, the heart rate slows dramatically, and organ perfusion rate falls by ~90%, creating conditions that would constitute severe ischemia in human and most mammals but have no ill effects on the hibernating squirrels (Hochachka, 1986; Frerichs et al., 1998). In addition, apnoic breathing patterns are common in torpor so tissues experience long periods of hypoxia.

Hibernating thirteen-lined ground squirrels are, therefore, excellent research models to use to investigate a number of medically important problems including hypothermia resistance, cold or freezing preservation of transplantable organs, and the prevention of damage to the heart, brain and other ischemia-sensitive tissues during heart attack or stroke. Elucidating the biochemical and genetic mechanisms of hibernating ground squirrels will lead to some potential applications of hibernation biology to biomedicine.

This thesis focuses on the responses made by brown adipose tissue, white adipose tissue, brain, heart, skeletal muscle, kidney, liver and lung of ground squirrels in response to hibernation.

Adipose Tissue (Brown and White)

Adipose tissue must remain responsive and retain function at the low Tb values of torpor. White adipose tissue (W.A.T.) is the dominant source of the major metabolic fuel, triglycerides, for the hibernation season; the quantity and quality (e.g. percentage of polyunsaturated fatty acids) of the lipids in these fat stores has consequences of the timing and patterns of torpor (Frank, 1992; Frank and Storey, 1995). Brown adipose tissue (B.A.T.) is found only in placental hibernators (Hayward and Lisson, 1992). The

primary function of B.A.T. is nonshivering thermogenesis. Heat production by B.A.T. is very important for the survival of many small mammals in cold environments and also for arousal from torpor in hibernating species. The tissue has a high density of mitochondria and a high level of vascularization. High rates of lipid oxidation in B.A.T. mitochondria are primarily directed toward heat generation by uncoupling respiration from oxidative phosphorylation. Hence, energy that would otherwise be trapped as ATP is released as heat. A protein unique to brown adipocytes, the uncoupling protein (UCP1), is central to the uncoupling of brown fat mitochondrial respiratory chain (Kuroshima, 1993).

Brain

Hibernation is a natural model of tolerance to cerebral ischemia. It represents a state of pronounced fluctuation in cerebral blood flow where no brain damage occurs. Numerous neuroprotective aspects may contribute in concert to such tolerance (Zhou et al., 2001). Several immediate-early genes, including transcription factors, c-fos, c-jun, and junB, are differentially expressed in the brain across the hibernation cycle (O'Hara et al., 1999), likely reflecting a link between neuronal activation and immediate-early gene expression. It will be interesting to identify the downstream target genes that are regulated by the increase in these transcription factors early in arousal. Other studies have shown that levels of the mRNA encoding neuropeptide Y (NPY) exhibited a significant increase in expression in the arcuate nucleus (ARC) of jerboas during hibernation (El Ouezani et al., 2001). Since NPY is known to be transmitter in the nervous system which is involved in the regulation of feeding behavior including food intake and carbohydrate preference as well as metabolic and lipogenic rates, the stimulation of NPY

gene within neurons of the ARC may play an integrative role in the control of energy metabolism in hibernating jerboas.

Heart

One of the hallmarks of hibernation is the continued and regulated contractile function of cardiac muscle at the low Tb values of deep torpor (Milsom et al., 1999). In contrast, hearts of nonhibernators are much more sensitive to cold. Studies show that changes in metabolic fuel use in heart during hibernation may play a role in continued function at low Tb values (Belke et al., 1998). A switch from carbohydrate to fatty acid metabolism is regulated in part by differential gene expression in the heart. Elevated levels of pyruvate dehydrogenase kinase (PDK) isozyme 4 mRNA in the heart of hibernating ground squirrels, leading to increased PDK-4 activity, is undoubtedly the basis of the strong suppression of pyruvate dehydrogenase activity during hibernation and a key factor in overall glycolytic rate suppression (Brooks and Storey, 1992; Andrews et al., 1998). Recent studies in our lab have shown that transcript levels of the ventricular isoform of myosin light chain 1 (MLC1v) and the mitochondrially-encoded subunit 2 of NADH-ubiquinone oxidoreductase (ND2) are also up-regulated in heart of hibernating ground squirrel (Fahlman et al., 2000) and heart type fatty acid binding protein (H-FABP) was also up-regulated in hearts of both ground squirrels and little brown bats during hibernation (Hittel and Storey, 2001; Eddy and Storey, 2004). Hibernation-induced up-regulation of MLC1v suggests that a restructuring of myosin subunit composition could contribute to changes in muscle contractility needed for hypothermic function, whereas changes in ND subunit composition may affect the function of the electron transport chain during hibernation. Fatty acid binding proteins are a multigene family of small

proteins that reversibly bind fatty acids and other hydrophobic ligands for transport through the cytoplasm. Up-regulation of H-FABP in heart and skeletal muscle likely reflects the increased reliance of these muscles on lipid fuels to support the specialized needs of hibernation.

Kidney

During hibernation the kidney is in a hypometabolic condition where renal blood flow is minimal and urine production is much reduced (Zatzman, 1984). These processes resume during periodic arousals, concomitant with the rise in Tb and cardiac output. Thus, there are only moderate effects of hibernation on plasma osmolality and electrolyte concentration, and the renin-angiotensin system appears to be functional during periodic arousals (Zatzman, 1984; Anderson et al., 1990). Electron microscopy studies of dormouse kidney have shown that the fine structure of the kidney cortex is well preserved during hibernation, whereas most apparent ultrastructural changes during hibernation take place in glomerular endothelial cells and podocytes (Zancanaro et al., 1999). A reduction of the endothelial pore number and epithelial filtration slits during hibernation has been consistently reported both qualitatively and quantitatively in different species (Zimny et al., 1996; Anderson et al., 1990). This would imply a reduced permeability of the ultrafilter. However, hibernators must be able to protect their kidneys from damage because urine production begins immediately upon arousal. As observed in other hibernating tissues, the underlying mechanisms of this physiological adaptation must be regulated, in part, by the differential expression of genes.

Liver

Liver is very important to hibernators because of its key role in metabolic aspects of

physiological homeostasis. To date, the known genes that are hibernation-responsive in ground squirrel liver include α_2 -macroglobulin, apolipoprotein A1, thyroxine-binding globulin, and cathepsin H (Srere et al., 1992; Epperson and Martin, 2002). α_2 -Macroglobulin is a broad-spectrum protease inhibitor that is synthesized by liver and excreted into the blood. It inhibits blood factor X and is partly responsible for the decrease in clotting capacity that occurs during hibernation and prevents spontaneous clot formation in delicate capillary beds at low temperatures and blood pressures (Srere et al., 1995). Apolipoprotein A1 is a component of high-density lipoprotein (HDL) that transports long-chain fatty acids through the plasma between storage and target tissues. Given that hibernators switch from a reliance on carbohydrates to lipids for fuel during torpor, proteins of lipid metabolism and transport should be up-regulated during hibernation. Hibernation also requires strict control of metabolic rate, logically involving thyroid hormone. Thyroxine-binding globulin binds the T3 and T4 forms of thyroid hormone, which upon delivery to target tissues activate receptors and transcription of many metabolic regulators (Mutvei et al., 1989). Cathepsin H is a lysosomal (early endosomal) cysteine protease that is endogenous to the liver and could play a role in protein degradation and removal (Claus et al., 1998) that must occur rapidly during interbout arousal.

Lung

The lung has received remarkably little attention from hibernation researchers. During torpor, blood flow to the lungs is significantly decreased and ventilation rate is markedly reduced. Lung experiences long periods of ischemia and hypoxia in torpor, but, upon arousal, breathing frequency rapidly resumes its normal, active pattern (McArthur

and Milsom, 1991; Mellen et al., 2002). The cellular and genetic basis for change in lung metabolism during torpor-arousal is not yet unknown. However, as in other tissues, the underlying mechanisms of hibernation-responsive adaptations must be regulated, at least in part, by the differential expression of genes.

Skeletal Muscle

Prolonged muscle disuse in most vertebrates, including man, typically leads to a pathological change resulting in a loss of muscle mass and a decrease in oxidative capacity in skeletal muscle. Hibernating animals, such as ground squirrels, may spend up to eight months in a state of hibernation, during which they experience very little physical activity or use of their muscles. However, hibernators show very little atrophy over this long period of muscle disuse (Wickler et al., 1991). In previous studies by our lab and others, a number of hibernation-responsive changes in gene expression have been documented in ground squirrel skeletal muscle including up-regulation of H-FABP (Hittel and Storey, 2001), MLC1v and ND2 (Fahlman et al., 2000), PDK4 (Buck et al., 2002) and uncoupling protein 3 (UCP3) (Boyer et al., 1998). The first three genes are also up-regulated in hibernator heart. UCP3 is a homologue of UCP1 and is expressed at high levels in human skeletal muscle and in rat and mouse BAT and skeletal muscle. The up-regulation of UCP3 suggest that this proton translocators may participate in muscle thermogenesis and energy balance.

1.3 Antioxidant Defense in Hibernation

Hibernators may experience ischemic events during hibernation because of the highly variable rates of tissue perfusion during torpor-arousal cycles. Because metabolic rate, Tb

and tissue perfusion all decrease during torpor, it is likely that the greatly reduced delivery of oxygen to cells during torpor is matched by a reduced energetic demand during hibernation, and thus the stresses induced by ischemia, such as production of free radicals and oxidative attack on cellular macromolecules, are probably also lower in torpid compared with active animals. However, the conditions of ischemia could potentially favor reactive oxygen species (ROS) generation despite the low levels of O₂, since the highly reduced mitochondrial redox state would promote electron donation to residual O₂ to form superoxide (Hess and Manson, 1984; Vanden Hoek et al., 1997). So hibernators may still be at risk for oxidative attack during hibernation. Furthermore, the rapid increase in Tb during periodic arousals to euthermia may increase the risk of regional ischemia-reperfusion injury in areas where blood flow is not fully restored before elevations in Tb.

In prior work on antioxidant defense in hibernators (Buzadzic et al., 1990; Carey et al., 2000; Hermes-Lima and Zenteno-Savin, 2000), evidence has shown that activities of several antioxidant enzymes and amounts of other antioxidants increased during hibernation. This indicates that antioxidant defense systems are enhanced during hibernation. Results from our lab using screening of cDNA nylon macroarrays to search for hibernation-responsive genes also revealed up-regulation of several genes associated with antioxidant defense during hibernation (Storey, 2003). For example, compared with the euthermic state, differential screening indicated significant up-regulation (by >2-fold) of glutathione-S-transferase (GST), glutathione peroxidase (GPX) and superoxide dismutase (SOD) in ground squirrel kidney during hibernation. These same enzymes as well as peroxiredoxin (PRX) and metallothionein (MT) also showed significant up-

regulation in liver. It has become increasingly apparent that differential gene regulation play an important role in the antioxidant defense required for hibernation.

1.4 Hypothesis

The thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*), is one of the most thoroughly investigated hibernating mammals. This species of ground squirrel has been used frequently as a “model hibernator” when describing the physiological and molecular changes associated with this state. During hibernation, organs of thirteen-lined ground squirrels can experience ischemia which could trigger antioxidant difference. Based on previous studies in our lab, I hypothesize that the differential expression of selected antioxidant related genes and their protein products occurs during hibernation and plays an important role in the antioxidant defense. To test this hypothesis I used relative RT-PCR, semi-quantitative PCR and Western blotting to identify genes which are differentially expressed at both the mRNA and protein level in organs of hibernating thirteen-lined ground squirrels.

In Chapter 2, data are presented from relative RT-PCR and Western blotting studies of heme oxygenase 1 (HO1). HO1 is a stress protein induced in response to a variety of oxidative challenges. The up-regulation of HO1 was detected in brain, kidney, liver and several other tissues during hibernation at both the mRNA and protein level. HO1 is regulated by Nrf2 and MafG transcription factors through the antioxidant response element (ARE). Changes in the protein contents of Nrf2 and MafG in hibernator organs are also presented.

In Chapter 3, data are presented from relative RT-PCR, semi-quantitative PCR and

Western blotting of Cu/Zn SOD and Mn SOD. As described before, previous studies in our lab using nylon microarray screening have provided evidence that SOD increased in kidney at the transcriptional level during hibernation. Western blotting documented the consequences of gene up-regulation on protein levels. SOD was found increase only in skeletal muscle. The Relative RT-PCR and semi-quantative PCR studies quantified the expression of these genes in muscle and found no changes.

In Chapter 4, data are presented from Western blot analysis of proteins in the thioredoxin system. The thioredoxin system is formed by thioredoxin reductase (TrxR) and its characteristic substrate thioredoxin (Trx), and together they form an important constituent of the intracellular redox milieu. The elevation of Trx1, Trx2 and TrxR2 was detected during hibernation in liver and several other tissues.

Figure 1.1

Typical pattern of changes in body temperature (T_b) for a hibernator over 1 year, starting in June. Shown on the x-axis is time, from the beginning of June through to the end of May, and on the y-axis, body temperature ($0\text{--}40^\circ\text{C}$); SA, summer active (Epperson and Martin, 2002).

Figure 1.2

Thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, and distribution map.

A) Distribution map for thirteen-lined ground squirrels. They currently occur in all or part of 22 U.S. states and 3 Canadian prairie provinces (Cleary and Craven, 1994).

B) Thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*.

A)

B)

Chapter 2

Heme oxygenase 1 and antioxidant defense in hibernation

2.1 Introduction

Accumulating evidence suggests that differential expression of selected antioxidant related genes plays a central role in antioxidant defense during hibernation. Using microarray screening much progress has been made in our understanding of some "classic" antioxidant enzymes (e.g., glutathione-S-transferase, superoxide dismutase, glutathione peroxidase) in mediating the resistance against oxidants in hibernation. However, it is becoming clear that other oxidant-induced gene products may also play vital roles in the ischemia protective response to oxidative stress. One such stress-response protein is heme oxygenase 1, HO1.

Heme oxygenase (HO) catalyzes the first and rate-limiting step of heme degradation producing CO, biliverdin, and ferrous iron (Figure 2.1A) (Tenhunen et al., 1968). Biliverdin, and its subsequent metabolite bilirubin, have antioxidant properties. Furthermore, iron released from HO activity stimulates the synthesis of ferritin (an iron storage protein), which ultimately provides an iron detoxification mechanism that may account for long-term cytoprotection observed after HO induction. HO1 is the inducible isoform of HO and is identical to heat shock protein 32 (HSP32), whereas HO2 is constitutive isoform (Maines, 1998). HO1 is not only induced in response to accumulation of its substrate heme (Maines, 1998), but also by various stress stimuli such as UV light, heat shock (Keyse and Tyrrell, 1989), sulfhydryl reactive reagents (Applegate et al., 1991), heavy metals, anoxia, hypoxia, hyperoxia (Lee et al., 1997; Lee et al., 2000) and ischemia (Semenza, 2000). A common feature among these inducers, including heme, is that they generate reactive oxygen species (ROS) and/or diminish glutathione levels.

Induction of HO1, regulated at the level of gene transcription, is essential for manifestation of the enzyme's cytoprotective function. It is known that hypoxia-inducible factor-1 mediates transcriptional activation of the HO1 gene in response to hypoxia (Lee et al., 1997). Current studies by our lab verified that in brown adipose tissue (B.A.T.) of thirteen-lined ground squirrels, HIF-1 is up-regulated at the protein level and showed increased in DNA-binding activity in tissues of hibernators in comparison with euthermic controls. As a downstream gene under HIF-1 control, HO1 may be transcriptionally activated in B.A.T. of hibernating ground squirrels. Furthermore, extensive analysis has identified a common mechanism of HO1 gene regulation: the gene is regulated via the antioxidant response element (ARE) in response to nuclear factor-erythroid 2 related factor -2 (Nrf2) transcription factor pathway (Alam et al., 1999; Owuor et al., 2002). This is a major pathway by which various environmental and industrial toxins increase the expression of antioxidant and detoxification enzymes (e.g. GSTs and HO1) in mammalian cells (Owuor and Kong, 2002). One model has been proposed to explain how Nrf2 senses oxidative stress (Figure 2.1 B). Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, a multidomain, cysteine-rich protein that is bound to the actin cytoskeleton. Upon activation by oxidative stress, the Keap1-Nrf2 complex uncouples due to the phosphorylation of Nrf2, and Nrf2 translocates into the nucleus. In the nucleus, Nrf2, a member of the bZIP transcription factor family, heterodimerizes with Maf family members, binds to the ARE and transcriptionally activates ARE. Recent studies by Kang et al. (2002), revealed that Nrf2 binds with actin and that the rearrangement of actin microfilaments in response to oxidative stress and depolymerization of actin causes a complex of Nrf2 bound with actin to translocate in to nucleus for ARE activation.

Given the evidence that ground squirrels experience oxidative stress and generate ROS during hibernation, I hypothesized that the transcription and translation of HO1 may be activated in hibernating ground squirrels to minimize stress-induced damage at the extremes of their normal physiology. Furthermore, I hypothesized that the ARE/Nrf2 pathway may be involved in this activation.

The current experiments were designed to investigate the regulation of gene and protein expression of HO1 in different tissues of hibernating ground squirrels, *Spermophilus tridecemlineatus*. In order to assess whether HO1 is regulated via the ARE/Nrf2 pathway, the protein expression and translocation of Nrf2 and MafG were also examined. Actin was detected by western blot to test whether there is actin in a putative complex with Nrf2.

2.2 Materials and Methods

2.2.1 Animal Treatments

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were captured in the autumn in Illinois and shipped to an animal housing facility on the National Institutes of Health campus (Bethesda, MD). All animal studies were performed under a protocol approved by Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke. Hibernation experiments were conducted by the laboratories of Dr. J. Hallenbeck (National Institute of Neurological Disorders and Stroke) and Dr. D. McCarron (Naval Medical Research Centre). I am thankful to them for the supply of hibernator tissue samples. Prior to hibernation, animals (130-180 g body mass) were housed individually in cages in a facility with an ambient room temperature

of 21°C and a 12/12-h light/dark cycle. They were fed standard rodent chow and water *ad libitum* and were weighed weekly to monitor their seasonal weight gain. When animals had exhibited a rapid increase in body weight to 220-240 g body mass, they reached the end of the pre-hibernation phase of hyperphagia that maximizes body lipid reserves. They were then placed in a cold chamber at 5-6 °C, at 60% humidity, and in constant darkness to facilitate hibernation. Noise within the chamber was kept to a minimum. These environmental conditions approximate those to which the animals are normally exposed in the winter season. After hibernation for 2-6 days (as indicated by a constant low body temperature of ~5°C), animals were sacrificed by decapitation. Animals that were kept as euthermic controls at 21°C were sacrificed at the same time as hibernating animals. Brain, liver, kidney, heart, lung, brown adipose tissue (B.A.T.), white adipose tissue (W.A.T.) and muscle were removed quickly, frozen immediately in liquid nitrogen, and then stored at -80°C. The tissues were packed in dry ice and air-freighted to Carleton University where they were again stored at -80 °C until use.

2.2.2 Semi-Quantitative PCR of HO1 and HO2 mRNA

RNA preparation: To avoid RNAase contamination, all materials and solutions used for RNA isolation were treated with 0.1% v/v diethylpyrocarbonate (DEPC) at room temperature (RT, ~21°C) for 12-24 h and subsequently autoclaved. Frozen tissue samples of approximately 50-100 mg were weighed and placed in 2 ml Eppendorf tubes, 1 ml of Trizol™ reagent (Invitrogen) was added, and then samples were homogenized by a Polytron PT1000 and allowed to sit for 2-3 min at RT. Chloroform was then added (0.2 ml/ml of Trizol reagent), and after mixing for 15 s, samples were incubated for 2-10 min

at RT. Samples were then centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous layer was removed and transferred to an autoclaved 1.5ml Eppendorf tube containing 800 µl isopropanol. After gentle shaking, the RNA was precipitated at RT for 10 min. The samples were centrifuged at 12,000 x g for 15 min to pellet the RNA, the supernatant was discarded and 70% ethanol was used to remove lingering chemicals from the RNA pellet. After gentle shaking and then centrifugation at 12,000 x g for 5 min, the RNA was dissolved in 50 µl DEPC-treated water. RNA was separated on a 1.2% denaturing formaldehyde agarose gel and high quality was assessed by the presence of sharp and distinct 28S and 18S rRNA bands through visual inspection using the ChemiGenius Bio Imaging System (SynGene). The concentration of RNA in the prepared samples was determined spectrophotometrically by absorbance at 260 nm. RNA was stored at -80°C until required for further use.

First Strand cDNA synthesis: A volume that contained 10-15 µg total RNA was removed from each RNA isolation, diluted with DEPC water to 10 µl final volume and then 1 µl of 200 ng/µl oligo-dT (5'-TTTTTTTTTTTTTTTTTTTTTTTTTV-3'; V=A or G or C)(Sigma Genosys) was added to each sample. The sample was placed in a 68°C water bath for 5 min to denature secondary structures. The mixture was then chilled rapidly on ice to 4°C and then 4 µl 5X first strand buffer (Invitrogen), 2 µl 10 mM DTT (Invitrogen), 2 µl dNTPs (Invitrogen) and 1 µl M-MLV Reverse Transcriptase (Superscript) was added for a total volume of 19 µl. The mix was incubated at 42°C for 45 min and then the cDNA stock was stored at -20°C. Dilutions of the cDNA stock were

prepared (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}) before performing semi-quantitative PCR.

Degenerate PCR: For Semi-Quantitative-PCR analysis, degenerate primers for alpha-tubulin, HO1 and HO2 were designed from the conserved sequences of human, mouse, rat, rabbit, and pig (Figure 2.2). Primer design was carried out using DNAMAN (v 4.11) and Primer Designer (v 3.0). DNAMAN makes alignments of multiple sequences and shows regions of homology in aligned sequences. These conserved regions are then assessed in Primer Designer and possible primers are selected based on a number of characteristics, including melting temperature and GC base pair content. The program typically eliminates primers with runs of a single base greater than 3 and those capable of forming dimers or hairpins. Chosen primers were then synthesized by Sigma Genosys. Primer sequences, optimal PCR annealing temperatures and their product size are listed in Table 2.1.

Polymerase chain reactions were performed on a Mastercycler gradient PCR machine (Eppendorf) or a iCycler PCR machine (BioRad). Each PCR reaction was carried out in a final volume of 25 μ l, using 5 μ l 10^{-2} cDNA dilution, 15 μ l H_2O , 2.5 μ l 10 $\times$ PCR Rxn buffer (- $MgCl_2$) (Invitrogen), 1.25 μ l 50 mM $MgCl_2$ (Invitrogen), 0.5 μ l 10 mM dNTPs (Invitrogen), 0.125 μ l *Taq* DNA Polymerase (Invitrogen) and 2.5 μ l 30 nmol/ml forward and reverse primer mixture (1:1). Temperature cycles for PCR were as follows: denaturation at 95°C for 30 sec, primer annealing at their optimal PCR annealing temperature (Table 2.1) for 30 sec, and primer extension at 72°C for 30 sec. These cycles were carried out 35 times. The PCR products were run on a 0.8% agarose/ethidium bromide gel, and the intensity of the single specific cDNA bands of interest were

analyzed using the ChemiGenius Bio Imaging System (SynGene) to determine whether amplification occurred.

Sequencing: PCR products were sequenced by either Canadian Molecular Research Services (Ottawa, ON) or by CORTEC (Kingston, ON). Product sequences were verified as the sequence expected using the program BLASTX at the NIH. The deduced amino acid sequences were also verified to be the protein of interest by using the program BLASTP at the same site.

Relative RT-PCR: Gene specific primers for HO1 and HO2 were designed from squirrel HO1 and HO2 sequences (Table 2.2). Two different PCR reactions were performed with each set of cDNA sample dilutions, one to evaluate the quantity of the cDNA of interest (HO1 or HO2) and the other to evaluate the quantity of the housekeeping gene, alpha-tubulin. A tube with reaction mix and alpha-tubulin primers but with no cDNA was also used as a negative control. A tube with reaction mix and alpha-tubulin primers but with total RNA was used as control to avoid genomic contamination. Reaction mixtures and cycling conditions were as described for degenerate PCR above. The PCR products were separated in a 0.8% agarose/ethidium bromide gel. The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene) and densitometry values were measured with GeneTools software (SynGene). By comparing the intensity of PCR products at each dilution, the lowest dilutions for alpha-tubulin and HO1/HO2 which have visible bands were chosen for quantification, the relative amounts of HO1/HO2 mRNA transcripts, relative to alpha-

tubulin, were determined for hibernating versus euthermic situations. For example, in Figure 2.11, the lowest dilutions for alpha-tubulin and HO1 which have visible bands were chosen for quantification (10^{-5} for tubulin and 10^{-4} for HO1). The formula below was used to calculate the mRNA regulation of HO1 relative to alpha-tubulin.

$$\text{Relative mRNA level} = (\text{hibernating HO1} / \text{hibernating tubulin}) / (\text{euthermic HO1} / \text{euthermic tubulin})$$

2.2.3 Western Blotting of HO1, Nrf2, MafG and actin

Tissue homogenization and protein preparation: Total soluble protein was extracted from frozen samples of squirrel tissues by homogenizing (1:10 w/v) in buffer containing 250 mM sucrose, 25 mM HEPES, 25 mM KCl, 1 mM EGTA, and 1 mM EDTA. The protease inhibitor PMSF was added immediately prior to homogenization. Samples were centrifuged at 5,000 rpm for 5 min at 4°C in a Biofuge 15 (Baxter Canlab), supernatant was collected, and the protein concentration was determined using the BioRad protein assay with bovine serum albumin as the standard (Bradford, 1976).

SDS–Polyacrylamide Gel Electrophoresis: Stock solutions for electrophoresis were as follows. The 2× sample buffer contained 100 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v) and 0.2% bromophenol blue (w/v). The 10x stock of running buffer contained 30.3 g Tris base, 144 g glycine, and 10 g SDS per liter, pH ~8.3. Protein samples were mixed with 2x SDS-PAGE sample buffer (1:1, v/v) and boiled 3-5 min. Aliquots containing 10 µg of protein were loaded into each

well. Depending on subunit molecular mass of the protein of interest, proteins were separated on 7.5-15% gels (acrylamide:bis-acrylamide 29.2:0.8; w/w) using the discontinuous buffer system of Laemmli (1970). Separation generally required ~1 h at 200 V for the BioRad Mini-PROTEAN 3 System. Proteins were blotted to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore Corp. Bedford, MA) by wet transfer with pre-chilled solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 20% v/v methanol at 4°C overnight at 30V or 2 h at 70V.

Immunoblotting and protein visualization: The general immunoblotting procedure was as follows. The PVDF membrane was first rinsed with TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) and then incubated in blocking buffer (TBST with 5% non-fat dry milk) for 30 min at RT. The blot was then added to 5 ml TBST buffer containing polyclonal rabbit antibody against the protein of interest and incubated at RT for 2 h or at 4°C overnight. In the case of HO1, the antibody was rabbit anti-HO1 at 1:2000 v:v (SPA-896, Stressgen). A duplicate blot was incubated with 5 ml TBST buffer containing HO1 antibody (1:2000 v:v) and 10 µg HO1 peptide (SPT-896E, Stressgen) as a negative control. In case of Nrf2 and MafG, the antibody was rabbit anti-Nrf2 at 1:2000 v:v and rabbit anti-MafG at 1:1000 v:v (gifts from Dr. John Hayes, Biomedical Research Centre, University of Dundee). In the case of actin, the antibody was mouse anti-actin (JLA20, University of Iowa) at 1:2000 v:v. After incubation, blots were washed 3-5 times in TBST (5 min, RT) and then incubated in 5 ml TBST solution with secondary antibody overnight at 4°C. For HO1, Nrf2 and MafG studies, HRP-linked goat anti-rabbit IgG (H&L) (1:2000 v:v) (Cell Signaling) was used as the secondary

antibody,. For actin studies HRP-linked horse anti-mouse IgG(H&L)(1:2000v:v)(Cell Signaling) was used as the secondary antibody. The target protein was visualized by the enhanced chemiluminescence assay using SuperSignal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene). Equal loading of proteins in the samples was confirmed by Coomassie blue staining of the blots. Densitometry values for Western blots and Coomassie stained bands were quantified using GeneTools software (SynGene).

Statistics: Data result from a minimum of 3-4 independent isolations from pooled tissue. Hibernating:euthermic ratios were calculated as follows. Mean band intensities $\pm$ SEM were quantified for euthermic and hibernating samples using GeneTools and then the Student's t-test ($P < 0.05$ unless otherwise stated) was used to test for significant differences. The ratio of hibernating:euthermic was then calculated and plotted in histograms. Error bars shown on the final histograms are the sum of SEM values for euthermic and hibernating trials.

2.2.4 Two-Dimensional Polyacrylamide Gel electrophoresis to detect Nrf2

Preparation of Protein Samples: 2D-PAGE was performed using the Modular Mini-Protean II System (Bio-Rad). Total soluble protein was extracted from frozen samples of squirrel tissues as described in section 2.2.3 above.

Isoelectric Focusing of Proteins: Separation of total soluble protein based on isoelectric point (pI) was performed in a capillary tube containing first dimension gel

monomer solution [9.2 M urea, 4% acrylamide, 1.6% pH 5-8 ampholytes (Sigma), 0.4% pH 3.5-10 ampholytes (Sigma), 0.01% ammonium persulfate, 0.1% TEMED]. The upper chamber running buffer was 100 mM NaOH, and lower chamber running buffer was 10 mM H₃PO₄. Following polymerization, the capillary gels were pre-electrophoresed for 10 min at 200 V, 15 min at 300 V and 15 min at 400 V. First dimension sample buffer contained 9.5 M urea, 5% β -mercaptoethanol, 1.6 % ampholytes (pH 5-8 range), and 0.4 % ampholytes (pH 3.5-10 range). Soluble protein extracts were mixed with the first dimension sample buffer (1:1 v/v) and allowed to incubate at RT for 15 min. Aliquots of 20-30 μ g protein were loaded into capillary sample reservoirs and overlaid with first dimension sample overlay buffer (9 M urea, 0.8% pH 5-8 ampholytes, 0.2% pH 3.5-10 ampholytes, 0.005% w/v bromophenol blue). Separation generally occurred over 10 min at 500 V and then 3 h at 750V. Following isoelectric focusing the gels were removed from the capillary tubes and incubated for 10 min in SDS equilibration buffer containing 62.5 mM Tris-HCl, pH 6.8, 2.3% w/v SDS, 5.0% β -mercaptoethanol, 10% w/v glycerol and 0.00125% w/v bromophenol blue.

SDS-Polyacrylamide Gel Electrophoresis: The proteins in the first dimension gel were further separated based on molecular weight using 12 % acrylamide gels (acrylamide:bis-acrylamide 29.2:0.8; w/w) and the discontinuous buffer system of Laemmli (1970) with a Bio-Rad mini-gel apparatus. Prior to second dimension electrophoresis, the first dimension gels were sandwiched between a 12% acrylamide separating gel, and a sealing gel (1% w/v agarose in SDS equilibration buffer). Running buffer contained 3.03 g Tris base, 14.4 g glycine, and 1.0 g SDS per liter, pH ~8.3.

Separation generally required ~1 h when run at 200 V with the Mini-PROTEAN 3 System.

Immunoblotting and protein visualization: Immunoblotting and protein visualization were performed as described in section 2.2.3. Rabbit polyclonal antibody Nrf2 (a gift from Prof. John Hayes, Biomedical Research Centre, University of Dundee) was used at a dilution of 1:5000. The second antibody was HRP-linked goat anti-rabbit IgG (H&L) (Cell Signaling) used at a dilution of 1:2500.

2.2.5 Analysis of Nrf2 and MafG Protein Expression in Nuclear and Cytoplasmic Fractions

Isolation of Nuclear and Cytoplasmic Extracts from Frozen Tissue: Samples (~0.5 g) of frozen tissue were crushed under liquid nitrogen and then homogenized using a Dounce homogenizer in 1 ml hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM DTT and 10 µl of a protease inhibitor mixture (1 mM PMSF, 1 µg/ml aprotinin, 0.01 mg/ml of pepstatin A, and 1 µg/ml antipain.) The lysates were centrifuged at 10,000 g for 10 min at 4°C. Supernatants were removed as the cytoplasmic fraction for the assay of Nrf2 and stored at -80°C. Pellets containing crude nuclei were resuspended in 200 µl extraction buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1.5 µl protease inhibitor, and then incubated for 1 h on ice with gentle rocking. The samples were then centrifuged at 10,000g for 10 min at 4°C and the supernatants representing the nuclear fraction was collected. Nuclear fractions were stored at -80°C to use.

Western Blot Analysis: SDS-PAGE and immunoblot analyses were performed as described in section 2.2.3. The samples were fractionated on 12% gels, transferred to PVDF membrane. For the Nrf2 study, the membrane was incubated with polyclonal rabbit anti-Nrf2 antibody (1:5000 v:v), followed by incubation with HRP-linked goat anti-rabbit IgG (H&L) antibody (1:2000 v:v). For the MafG study, the membrane was incubated with polyclonal rabbit anti-MafG antibody (1:2000 v:v), followed by incubation with HRP-linked goat anti-rabbit IgG(H&L) antibody (1:2000). Development used the enhanced chemiluminescence assay using SuperSignal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene).

2.3 Results

2.3.1 cDNA Analysis of Squirrel HO1 and HO2

Degenerate PCR of HO1 and HO2 cDNA: Degenerate primers were designed for use in amplifying cDNAs that encoded the ground squirrel specific forms of inducible HO1 and constitutive HO2. Forward and reverse primers were designed for each based on the consensus sequences identified from a comparison of the cDNA sequences for HO1 and HO2 in other mammals (human, rat, mouse, pig, rabbit) (Figure 2.2). Sequences of the primers used are shown in Table 2.1A. The HO1 primers were used to amplify a PCR product of 575 bp using a 68°C annealing temperature (Table 2.1A; Figure 2.3A). A PCR product of 692 bp was amplified using the HO2 primers at an annealing temperature of 65°C (Table 2.1A; Figure 2.3B).

HO1 sequence analysis: Nucleotide and deduced amino acid sequences of the 581 bp cDNA fragment amplified with the HO1 primers are shown in Figure 2.4. Analysis using the NIH BLAST database indicated that the sequence encoded a mammalian HO1 and, therefore, validated the specificity of the degenerate primers that were designed. The nucleotide sequence and the three possible open reading frames were loaded into a Blast program at NCBI for a similarity search in GenBank. Analysis of the putative amino acid sequence of ground squirrel cDNA showed that it encoded 193 of the 288-289 amino acids of the full mammalian HO1 sequence (Figure 2.5). The segment lacked both the N- and C-termini. Figure 2.6 shows that the ground squirrel sequence is very similar to the sequence from other mammals. The homology tree and homology matrix shown in Figure 2.6 show that the ground squirrel HO1 amino acid sequence shared 75.1%, 71.5%, 71.0% and 76.7% amino acid identity with human, mouse, rat and pig HO1, respectively.

Comparison of HO1 deduced amino acid sequences of squirrel with those from other mammals revealed that HO1 protein may have distinct amino acid substitutions which could significantly alter protein functionality. Using the X-ray protein crystal structure of human HO1 as a model (Protein Data Bank, ID# 1N45), we found that the Gly143 residue is substituted by Arg143. This substitution occurs in a region that is normally very highly conserved Gly139-Asp140-Leu141-Ser142-Gly143-Gly144. The other distinct amino acid substitutions in ground squirrel are His136 and Asn140 (Figure 2.5). These two residues are among a cluster of polar residues that appear near pyrrole B of heme (Schuller et al., 1999).

HO2 sequence analysis: Figures 2.7, 2.8 and 2.9 show a comparable sequence analysis of ground squirrel HO2. The nucleotide and deduced amino acid sequences are shown in Figure 2.7. The amplified cDNA encoded 230 of the 312-316 amino acid residues in the full sequence of HO2 from other mammals (Figure 2.8). Very high sequence similarity was again evident among the five mammals and Figure 2.9 revealed 90.3%, 89.1%, 91.3% and 90.9% amino acid identity between ground squirrel HO2 and human, mouse, rat and rabbit HO2, respectively. Compared to HO1, HO2 protein structure has received remarkably little attention from researchers. No X-ray protein crystal structure of HO2 has been reported to date.

2.3.2 Relative RT-PCR of HO1 and HO2 mRNAs in Tissues

Gene specific PCR of HO1 and HO2: Because degenerate primers for squirrel HO1 and HO2 amplified only weak bands using high concentrations of cDNA, which were not easy to quantify, squirrel-specific forward and reverse primers for HO1 and HO2 were designed from the ground squirrel HO1 and HO2 cDNA sequences (Table 2.1B). When the specific primers were used, a 394 bp PCR product was amplified using the HO1-specific forward/reverse primers at a 68°C annealing temperature with 10^{-2} diluted squirrel liver cDNA (Table 2.1B; Figure 2.10A,B). Similarly, a 493 bp PCR product was amplified using HO2-specific forward/reverse primers at a 65°C annealing temperature using 10^{-2} diluted squirrel liver cDNA. (Table 2.1 B; Figure 2.11A,B). Sequencing showed that these PCR products were identical to the sequences shown in Figures 2.4 and 2.7.

Relative RT-PCR of HO1 and HO2 mRNA levels: Relative RT-PCR was used to examine transcriptional regulation of HO1 and HO2 in ground squirrel tissues to evaluate the effects of hibernation on mRNA levels. Relative RT-PCR involves amplifying an internal control simultaneously with the gene of interest. The internal control is used to normalize the samples. Once cDNA is normalized, direct comparisons of relative abundance of a specific mRNA can be made across the samples. Figure 2.12 shows an example of the results obtained; PCR was conducted using 5 dilutions of cDNA prepared from kidney mRNA of euthermic versus hibernating squirrels and using either squirrel-specific HO1 primers or alpha-tubulin primers (Table 2.2). PCR products were separated on agarose/ethidium bromide gels and band intensities were quantified. Visual inspection of these results for kidney indicates higher levels of HO1 mRNA in kidney of hibernating animals than in euthermic squirrels; thus, PCR product can be seen in the 10^{-4} dilution sample of hibernator cDNA but not in the same dilution of euthermic cDNA and at all other lesser dilutions the bands for hibernator PCR products are more intense than the corresponding euthermic bands. However, there is little, if any, difference in band intensities between the euthermic versus hibernating pairs at any dilution when alpha-tubulin was being amplified. To quantify the relative levels of PCR products (representing relative mRNA levels) in the hibernating versus euthermic samples, the lowest visible dilution (10^{-4} for HO1 and 10^{-5} for alpha-tubulin) was chosen to avoid the measurements of the plateau phase of PCR. The intensity of HO1 bands are all normalized to the intensity of their corresponding tubulin bands and then the ratio of hibernating vs. euthermic HO1 levels was calculated. The formula in Figure 2.12 was

used.

Relative RT-PCR analysis of HO1 transcript levels in hibernation versus euthermia was conducted for eight tissues of ground squirrels and the ratio of transcript levels, hibernation:euthermia is shown in Figure 2.13. HO1 mRNA was detected in B.A.T., brain, heart, kidney, liver, lung and muscle. However, HO1 could not be amplified from RNA extracts of W.A.T. suggesting that the gene is not expressed or is in extremely low levels in this tissue. During hibernation, HO1 mRNA levels rose significantly in four organs: by 3.8-fold in brain, 1.4-fold in kidney, 2.4-fold in liver, 2.8-fold in lung ($P < 0.05$) (Figure 2.13A).

Relative RT-PCR analysis was also applied to analyze levels of HO2 mRNA in ground squirrel tissues (Figure 2.13B). Transcripts were detected in brain, lung, heart, liver, kidney, B.A.T., and W.A.T. but were not detected in skeletal muscle. This suggests that HO2 is not expressed in muscle or is in extremely low levels. The data in Figure 2.13B show results from $n=1$ trials only but are consistent in suggesting that no significant changes occurred in the mRNA levels of the constitutive HO2 during hibernation.

2.3.3 Western Blotting analysis of HO1 expression

Western blotting was performed to study HO1 translational regulation in ground squirrels. For HO1 a polyclonal antibody raised against human HO1 (residue 1-30) was used. Western blot analysis of squirrel muscle using this antibody showed crossreaction with two bands (Figure 2.14A, lanes 1 and 2), a 32 kDa band that corresponded to the known molecular mass of HO1 and a 40 kDa band. However, in paired runs after

preabsorption of the HO1 antibody with its immunizing peptide (Human HO1 synthetic peptide aa. 1-30) immunoreactivity with the 32 kDa band was abolished but the 40 kDa band remained unchanged (Figure 2.14A, Lane 3, 4). This shows that the 40 kDa band is a non-specific reaction and confirmed that the 32 kDa band is specific to squirrel HO1. The 32 kDa band was quantified in all other experiments.

Figure 2.14 B and C shows the results of Western blot analysis of HO1 protein levels in seven tissues of ground squirrels, plotting the ratio hibernating:euthermic protein levels. HO1 levels were significantly elevated ($P < 0.05$) in four organs during hibernation, by 1.5-fold in brain and kidney and by 1.9-fold in liver and heart. The results for brain, kidney and liver were consistent with the transcriptional up-regulation shown in Figure 2.13A. No HO1 protein was detected in lung, suggesting that the translation level of HO1 in lung is very low.

2.3.4 Analysis of transcription factor Nrf2 protein expression in tissues

2-Dimentional Gel analysis of Nrf2 antibody specificity: Induction of HO1, regulated at the level of gene transcription, is essential for its cytoprotective function. A common mechanism of HO1 gene regulation is activation of transcription via the antioxidant response element (ARE) in response to the Nrf2 transcription factor pathway (Alam et al., 1999, Owuor et al., 2002). Previous studies showed that Nrf2 occurred in both 57 kDa and 100 kDa forms but immunoblotting and immunoprecipitation assays were able to show that the 100 kDa protein was comprised of both Nrf2 and actin (Kang et al., 2002). Figure 2.15A shows the results from two dimensional gel electrophoresis

that was used to examine this situation in squirrel liver and confirm the specificity of the Nrf2 antibody. Proteins were resolved in the first dimension by isoelectric point (pI), and in the second dimension by molecular mass. Treatment with Nrf2 antibody revealed crossreaction with several spots. One spot was detected at 57 kDa with a pI of ~4.5, consistent with the known molecular mass of Nrf2 and the theoretical pI value of human Nrf2 (Accession number Q16235, PI=4.67) calculated by SWISS-2D. Another spot was detected at 100 kDa and pI of ~5.4, which may composed of a Nrf2 and actin pair. Because the proteins were fractionated in SDS-PAGE under the denatured condition, we raised the hypothesis that Nrf2 might covalently bind with actin

Western blot analysis of Nrf2 protein expression in tissues: Western blot analysis Nrf2 in ground squirrel tissues revealed that the content of the 57 kDa Nrf2 protein increased significantly in B.A.T., heart, and liver during hibernation (by 3.2-fold, 2.2-fold, and 1.6-fold respectively, $P>0.05$) (Figure 2.15 B and C). Levels of the 100 kDa Nrf2 also increased in these three tissues by 3.2-fold, 2.2-fold, and 1.4-fold respectively ($P<0.05$). Levels of both the 57 and 100 kDa proteins were unaffected in lung, muscle and W.A.T. during hibernation but both decreased significantly ($P<0.05$) in brain to just 70% of the euthermic value. In kidney, levels of the 57 kDa protein decreased to 60% ($P<0.05$), but the 100 kDa protein was unaffected.

2.3.5 Nuclear Translocation of Nrf2 in liver and muscle

To elicit gene expression, a transcription factor must be present in the nucleus

and translocation to the nucleus is one of the mechanisms that is involved in controlling various transcription factors. To determine whether Nrf2 moved into the nucleus during hibernation, nuclear and cytoplasmic fractions were isolated from liver and skeletal muscle and the distribution of Nrf2 in these fractions was studied by Western blotting. Our results showed that the expression pattern of Nrf2 in liver and muscle subcellular fractions was different (Figure 2.16).

In liver, the amount of 57 kDa Nrf2 in the nuclear fraction increased by 1.4-fold ($P<0.05$) during hibernation and, oppositely, decreased by 50% ($P<0.05$) in the cytoplasmic fraction (Figure 2.16 A and B). These results indicate that the 57 kDa Nrf2 may translocate from the cytoplasm into nucleus during hibernation. By contrast, the 100 kDa Nrf2, which is proposed to bind to actin was stable in both the nucleus and cytoplasm during hibernation (Figure 2.16 A and B).

In muscle, a very different situation was seen. Levels of the 57 kDa Nrf2 in the nucleus were significantly reduced during hibernation to just 30% of the euthermic value ($P<0.05$) but the amount of cytoplasmic 57 kDa Nrf2 remained stable (Figure 2.16 A and C). Levels of the 100 kDa Nrf2 decrease to 80% ($P<0.05$) in the nucleus during hibernation compared with, and were also reduced in the cytoplasm to 40% ($P<0.05$) of the euthermic value (Figure 2.16 A and C).

2.3.6 Analysis of transcription factor MafG protein expression in tissues

The small Maf proteins function as obligatory heterodimeric partner molecules with Nrf2. Western blot analysis MafG in eight tissues of ground squirrels revealed that

the content of MafG protein was elevated in B.A.T., heart, liver and lung during hibernation (by 1.6-fold, 1.5-fold, 1.8-fold and 3.8-fold, respectively, $P < 0.05$) (Figure 2.17 A). Levels of MafG were unaffected in muscle and W.A.T. and decreased in brain and kidney. The results of MafG for B.A.T, heart and liver were consistent with the up-regulation of Nrf2 shown in Figure 2.15C.

2.3.7 Nuclear Translocation of MafG in liver and muscle

To determine whether the MafG moved into the nucleus during hibernation, nuclear and cytoplasmic fractions were isolated from liver and skeletal muscle and the distribution of MafG in these fractions was studied by Western blotting. In liver, the amount of MafG in the nuclear fraction increased by 1.4-fold ($P < 0.05$) during hibernation and, oppositely, decreased to 75% ($P < 0.05$) of the control level in the cytoplasmic fraction (Figure 2.17 B and D). These results indicate that MafG may translocate from the cytoplasm into the nucleus in liver during hibernation. By contrast, in muscle, relative levels of MafG in the nucleus and cytoplasm were constant during hibernation (Figure 2.17 B and D).

2.3.8 Analysis of actin protein expression in tissues

Given the possible interaction between Nrf2 and actin, Western blot were used to detect the protein expression profile of actin and whether the protein expression of actin in a putative complex followed the same pattern as for Nrf2. Western blot analysis of eight tissues of ground squirrels revealed that levels of the 43 kDa actin protein are

unaffected during hibernating (Figure 2.18 A and B). The 100k Da protein (putative complex with Nrf2) was detected only in brain and heart by anti-actin antibody (Figure 2.18 C). In heart, the relative amount of the 100 kDa protein increased by 1.5-fold during hibernation ($P<0.05$) (Figure 2.18 D). Conversely, in brain, the relative amount of the 100 kDa protein decreased to 75% ($P<0.05$) of the euthermic level (Figure 2.18 D).

2.4 Discussion

Hibernation is accompanied by extreme changes in physiology. Body temperature, metabolic rate, heart rate, respiration and other parameters fall during torpor to levels that would be lethal for mammals that do not hibernate (Hirvonen, 1979; Ivanov, 2000). With such low rates of heart rate and respiration, hibernators may experience ischemia and hypoxia stress and be exposed to high rates of free radical production and oxidative attack on cellular macromolecules, particularly during the arousal process when the rate of oxygen consumption rises many-fold within minutes (Vanden Hoek et al., 1997). Oxidative stress conditions provoke cellular responses, principally involving transcriptional activation of genes encoding proteins that participate in the defense against oxidative injuries to tissues. An excess of free heme, which is released from heme proteins under these conditions, may constitute a major threat because it catalyzes the formation of ROS.

Exposure of mammalian cells to oxidative stimuli induces HO1, the rate-limiting enzyme in heme degradation, as well as the 32 kDa heat shock protein. In various tissue injury systems, HO1 induction has been shown to confer protection, while its abrogation

has been shown to accelerate cellular injuries. The present study demonstrates that hibernation induced expression of HO1, a critical enzyme in heme metabolism that exhibits antioxidant and cytoprotective effects in hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*.

2.4.1 Analysis of the structures of ground squirrel HO1

Hibernators lower their core Tb to close to 0°C in winter, a value that is lethal to human and most other animals. Temperature change can have deleterious effects on proteins affecting, for example, their conformation, their kinetic/regulatory properties, their binding interactions with subcellular structures, and their integrated functioning within metabolic pathways. Proteins in mammals are often optimized for function over a fairly narrow temperature range around 37°C whereas proteins in ectotherm species may have to function over very wide temperatures ranges. Hibernating mammals presumably also need proteins that are functional over a wide temperature range. Adaptations in proteins, such as to conserve ligand binding and conformational flexibility, can often be achieved by only minor differences in sequence (Somero, 2003). Selected structural differences in hibernator proteins, arising from distinct amino acid substitutions, may allow them to function over a broad temperature range.

The amino acid sequence alignments of ground squirrel HO2 with their homologues from human, mouse, rat and rabbit revealed a high degree of sequence similarity between species, about 90% in all cases (Figure 2.9). Significantly, however, HO1 showed three distinct amino acid substitutions as compared with the protein from

the other species. The side chains of all 3 of these amino acids project outwards from the plane of the secondary structure and may serve to stabilize the structure of the protein, allowing it to function effectively at low temperatures.

The reported crystal structure of human and rat HO1 reveals a helical fold with the heme sandwiched between two helices forming a heme pocket (Schuller et al., 1999, Sugishima et al., 2000). The proximal helix provides a heme iron ligand, His 25. Conserved glycines in the distal helix near the oxygen binding site allow close contact between the helix backbone and heme in addition to providing flexibility for substrate binding and product release. Regioselective oxygenation of the *α-meso* heme carbon is due primarily to steric influence of the distal helix.

Comparison of the partial amino acid sequence of squirrel HO1 and HO1 from other mammalian species revealed that the overall structures are similar except for a segment of distal helix from Gly139 to Gly144 (Figure 2.5). This region is known to be highly conserved in all other HO1 amino acid sequences in non-hibernators and provides the required flexibility and also allows the helix to approach closer to the heme than in true hemoproteins such as the globins and peroxidases (Schuller et al., 1999). As a result, backbone atoms of Gly139 and Gly143 directly contact the heme. The Gly143 residues are present in all of the previously known sequences of HO1. Gly143N forms a hydrogen bond to the distal ligand of heme. However, in ground squirrels, we found the Gly143 residue was substituted by Arg143. In the case of globins and peroxidases, a polar side chain of distal amino acids such as histidine is considered to stabilize an oxygen ligand. In the case of human and rat HO1, Gly143 and Gly139 have no polar side chain. Schuller et al. (1999) suggested that the carbonyl oxygen of Gly139 and nitrogen of

Gly143 are candidates for the distal polar groups. However, in ground squirrel, the Arg143 substitution in ground squirrel places a polar amino acid where the nonpolar Gly143 was previously. The side chain of Arg143 may provide favorable electrostatic interactions, possibly including specific hydrogen bonds, for an oxygen ligand. It may provide alterations to protein binding of ligands that counteract the effects of low temperature on hydrogen bonds. Hence, substitution could be needed to allow the protein to function effectively both at the low body temperatures (near 0°C) during hibernating and over the wide range of body temperatures (0-37°).

The other distinct amino acid substitutions in ground squirrel are His136 and Asn 140. None of these residues is close enough to the iron or α -meso edge of heme to take part directly in the reaction, but these residues do contribute to the polar nature of the heme binding pocket, which may have importance for both ligand discrimination and regioselectivity. They might be important in helping to maintain an active conformation of the enzyme over the wide range of body temperatures under which the hibernator enzyme has to function.

2.4.2 Transcription and translation induction of HO1

Changes in the expression of specific genes and proteins in specific tissues is clearly important and has a defined role in supporting hibernation. Our results for relative RT-PCR and western blots show that mRNA expression levels and protein levels of inducible HO1 are differently regulated in different tissues of hibernating ground squirrels. By contrast, HO2 is known to be a constitutive isoform of HO and the relative

RT-PCR result demonstrated that HO2 mRNA expression was not affected in tissues of hibernating ground squirrels.

In brain, kidney and liver, HO1 expression was increased at both the transcription level (by 1.5-3.5 fold) and translation level (1.5- to 2-fold) during hibernation, compared with euthermic controls. By contrast, in B.A.T. and skeletal muscle, both the mRNA and protein contents remained constant during hibernation. Hence, the expression patterns of mRNA and protein correlated well in each of these five organs and this suggests that HO1 mRNA substrate availability is a primary determinant of the protein level in these most tissues. However, mRNA substrate availability is not the only factor that contributes to the regulation of protein levels during hibernation. For example, in heart, the mRNA level of HO1 decreased to 50% of the control level but the protein content of HO1 increased by about two-fold in heart. Ribosome aggregation state and suppression of protein degradation may also affect the regulation of HO1 protein level. During hibernation, most mRNA types move into the translationally silent monosome fraction but selected mRNAs for a few protein (e.g. FABP) are preferentially found in the polysome fraction where they are translationally active, so they are preferentially translated during hibernation (Hittel and Storey, 2002). So, even with overall lower mRNA levels, there might be a need to keep translating HO1 in heart. Furthermore, net protein content is affected by the rates of both protein synthesis and protein degradation. Because protein synthesis is strongly suppressed to save energy during hibernation, but animals show no significant deficit of cellular proteins, it is obvious that the rate of protein degradation must also be strongly suppressed during hibernating (Storey and Storey, 2004). This suggests that a suppressed rate of HO1 degradation may also

contribute to the up-regulation of HO1 in hibernating squirrel heart. In lung a significant up-regulation of HO1 mRNA transcripts was detected, but no HO1 protein was detected in either control or hibernating animals. In W.A.T., there was no HO1 mRNA detected in either control or hibernating animals, but a constant protein level was detected in hibernating versus control animals. In these last two cases it is probable that there are extremely low levels of either HO1 mRNA in W.A.T. or HO1 protein in lung, levels that were below the limits of detection of our experiment techniques.

The specific adaptive response of HO1 protein expression in brain, heart, kidney and liver during hibernation suggests an important role of HO1 in the protection against oxidative stress conditions. Free heme, largely liberated from hemeproteins under oxidative conditions, is highly lipophilic and will likely intercalate into the lipid bilayers of cells. HO1 catalyses the metabolism of heme to CO, biliverdin, and free iron (Figure 2.1A). CO produced from heme by HO can suppress apoptosis of endothelial cells via the activation of p38 MAPK (Brouard et al., 2000). Biliverdin is rapidly converted by biliverdin reductase to bilirubin, which is an anti-oxidant (Stocker et al., 1987). Iron, which is an oxidant, is directly sequestered and inactivated by co-induced ferritin (Vogt et al., 1995). Thus, the CO and bilirubin metabolites that result from the HO reaction contribute directly to the protective response against oxidative stress. It would be interesting to determine whether ferritin is also co-induced with HO1 during hibernation to provide for the sequestering of iron released by the HO reaction.

2.4.3 ARE/Nrf2 pathway and gene regulation of HO1

Recent evidence points to the fact that a group of oxidative stress inducible genes is under the immediate transcriptional control of Nrf2-small Maf heterodimer-regulatory protein (Figure. 2.1B). Nrf2 forms a heterodimer with a small Maf protein, interacts with an antioxidant responsive element (ARE), and induces transcription of a set of genes that encode antioxidant functions. Thus, Nrf2 regulates stress inducible protein genes via the ARE. Indeed, Nrf2-deficient cells have been shown to be hypersensitive to oxidative stresses (Itoh et al., 1997). In this manner, Nrf2 regulates a wide range of metabolic responses to oxidative stress, which include, among others, HO1 (Ishii et al., 2000). It was reported that HO1 gene induction by cobalt in Chinese hamster ovary cells involves the formation of Nrf2·MafG heterodimers (Gong et al., 2001).

Our results indicate that induction of HO1 in hibernating ground squirrel liver is probably mediated, at least in part, by the Nrf2·MafG / ARE pathway. Western blot analysis of Nrf2 and MafG revealed that levels of both proteins increased in B.A.T., heart and liver during hibernation. However, of these three tissues, HO1 mRNA levels were elevated only in liver during hibernation; they remained constant in B.A.T. and decreased in heart. In addition, the protein content of HO1 increased in liver. Taken together, this provides strong evidence that Nrf2 and MafG transcriptionally activate HO1 gene expression in liver of hibernating ground squirrels, leading to elevated levels of HO1 protein. A correlation between elevated Nrf2 and MafG and increased HO1 protein was also seen in heart although, as mentioned earlier, heart mRNA levels actually declined during hibernation. Skeletal muscle and W.A.T. showed no changes in any component of the HO1/ Nrf2 / MafG system during hibernation whereas in other organs, although Nrf2 and MafG levels were generally correlated, the HO1 response did not correlate with the

changes in the transcription factors. In brain and kidney, in particular, the elevated HO1 mRNA and protein contents could not be accounted for by the changes in Nrf2 and MafG which decreased by 30-50%. Hence, another mechanism of transcriptional up-regulation may be involved in the hibernation-responsive expression of HO1 in these organs.

The protein level of Nrf2 and MafG were not compromise in lung. Protein level of Nrf2 had no change in this tissue, but protein level of MafG significantly increased. In lung, a very strong increase (nearly 4-fold) in MafG was seen during hibernation but without a corresponding change in Nrf2. MafG can serve not only as a cofactor of Nrf2 in the Nrf2/ARE pathway, but it also participates in other forms of transcription regulation. The ARE cognate sequence shares a high degree of homology to the consensus Maf recognition element (MARE) sequence, permitting AREs to be competitively bound by a number of bZip transcription factors in the Maf, Jun, Fos and Cap'n'Collar families (Ishii et al., 2000). Because of their responsiveness to a wide variety of stress agents including oxidative stresses, the MAREs were also named stress-responsive elements (StREs) (Alam et al., 1995). The small Maf proteins form homodimers or heterodimers with other b-Zip proteins present in the cell and bind to Maf recognition elements (MARE) in DNA and exert finely articulated transcriptional control over gene expression during development, differentiation and oncogenesis.

Western blot analysis detected Nrf2 in both 57 kDa and 100 kDa bands in all the eight tissues. The expression patterns of the 57kDa and 100kDa proteins correlated in all organs except in kidney where the 57 kDa protein decreased to 60% of control values while the 100kDa protein remained stable. It is known that Nrf2 has a 57 kDa molecular mass whereas previous studies identified the 100 kDa protein as a complex of Nrf2 and

actin (Kang et al., 2002). The denaturing conditions of the SDS-PAGE typically disrupt protein-protein interactions (e.g. polymers are separated into their constituent monomers). Since this was not the case for the putative Nrf2-actin pair, this suggests that the binding of Nrf2 with actin might be covalent. Indeed, studies by Kang et al. (2002) showed that treatment of the 100 kDa Nrf2 with a phosphatase released a 57 kDa Nrf2. Hence, it is possible that the 100 kDa protein was formed as a consequence of phosphorylation of Nrf2 and/or actin. Actin is a building block of microfilaments. It may participate in the process of nuclear localization of Nrf2. Actin as a complex bound with Nrf2 may link the ARE-bound transcription factor to microfilaments in the nucleus and thus contribute to the stability of the DNA protein complex.

To test this hypothesis, western blots were performed to detect actin in ground squirrel organs. The actin antibody crossreacted with 43 kDa and 100 kDa bands in brain and heart but in other organs, only one 43 kDa band was seen. Actin is a housekeeping gene which is constantly expressed in all the tissues and the current results confirm this by showing that levels of the 43 kDa actin were unchanged during hibernation in all eight tissues. However, during hibernation, levels of the 100 kDa putative actin complex in brain decreased to 75% of the control value whereas a 1.5-fold increase occurred in heart. Furthermore, the expression patterns of 100 kDa putative actin complex in brain and heart were identical with the expression pattern of the 100 kDa protein detected by Nrf2 antibody in these two tissues. This suggests that the actin may complex with Nrf2 in brain and heart and participate in the process of Nrf2 translation. In the other six tissues, a 100 kDa actin complex was not detected. This differed from what we found in heart and brain and may have resulted from a noncovalent interaction of Nrf2 with actin in these six

tissues or perhaps the strong reducing conditions of SDS-PAGE cleaved the complex. Another reason could be that the relative level of the 100 kDa Nrf2-actin complex is too low compared to the highly expressed 43 kDa actin. Although the 43 kDa actin band could be detected by the antibody; the 100 kDa band might not be detected by the same antibody at such a low level of Nrf2-actin complex.

Oxidative stress causes the Keap1 protein to disassociate from Nrf2 and Nrf2 then translocates into the nucleus to induce transcription of antioxidant genes (Itoh et al., 1999). Thus, activation of Nrf2 should change the distribution of Nrf2 between cytoplasmic and nuclear fractions. Subcellular fractionation of liver and muscle followed by western blot analysis of Nrf2 abundance demonstrated that a higher proportion of the liver 57 kDa Nrf2 translocated into the nucleus during hibernation whereas the distribution of the 100 kDa Nrf2 content was unchanged. The 100 kDa Nrf2 and actin complex is suggested to aid nuclear localization of Nrf2. This indicates that the translocation process of Nrf2 is stable and the binding activity of Nrf2 is increased in liver. This further confirms that Nrf2 may participate in the transcriptional activation of HO1 in liver of hibernating ground squirrels. In muscle, less 57 kDa Nrf2 was found in the nucleus of hibernating animals, which indicates that Nrf2 did not translocate into the nucleus during hibernation in this tissue. The 100 kDa Nrf2 decreased both in nucleus and cytoplasm fractions in muscle, which demonstrates a decreased translocation process in hibernating muscle. This is consistent with the lack of HO1 activation in hibernator muscle.

To further confirm that HO1 gene expression was regulated by the Nrf2/ARE pathway in liver, the translocation of MafG induced by hibernation was also studied.

Subcellular fractionation and western blots revealed that MafG showed increased translocation into the nucleus in liver during hibernation, but not in muscle. As a cofactor of Nrf2, the movement of MafG into liver nuclei during hibernation could increase the level of Nrf2•MafG complex present and increase ARE binding activity.

In summary, my data demonstrate that HO1 increased at both transcriptional and translational levels in multiple tissues in hibernating ground squirrels. The Nrf2/ARE pathway, a common pathway regulating HO1 gene expression was investigated. The data presented are consistent with a specific requirement of the Nrf2/ARE pathway in the activation of HO1 by oxidative stress, particularly in liver. Both Nrf2 and its cofactor MafG increased and translocated into the nucleus in liver during hibernation. A putative Nrf2-actin complex was detected in heart and brain. These findings suggest that tissue-specific HO1 expression in hibernating ground squirrels may be regulated via the Nrf2/ARE pathway and offer a new mode of antioxidant defense of hibernating animals. In the case of Nrf2, further work remains to be done, in particular to evaluate the ARE binding activity of Nrf2 and the role of microfilaments in Nrf2 nuclear translocation.

Table 2.1

Primers used to amplify squirrel HO1 and HO2 cDNA and relative quantitative-PCR

A) Degenerate primers used to amplify squirrel HO1 and HO2 cDNA

Transcript detected	Oligonucleotides (5'-3')	Annealing Temperature	Product size
HO1 Degenerate	Forward: TGAAGGAGGCCACCAAGGAG Reverse: GTTGAGCAGGAASGCRGTCTT	68°C	575bp
HO2 Degenerate	Forward: ATGGABCGCAAYAAGGAC Reverse: TAGTACCAGGCCAAGAGTCC	65°C	692bp

B) Squirrel gene specific primers used for relative-quantitative-PCR

Transcript detected	Oligonucleotides (5'→3')	Annealing Temperature	Product size
Alpha-tubulin	Forward: AAGGAAGATGCTGCCAATAA Reverse: GGTCACATTTACCATCTG	57°C	635bp
HO1 Gene Specific	Forward: TCCAAGCTGGTGATGGTGTC Reverse: GCTTGAAGCTGGTGGAAGTCTG	68°C	394bp
HO2 Gene Specific	Forward: CTGACCAAGGACATGGAGTA Reverse: CTTGCTGAGCAGCATAGAAG	68°C	493bp

Figure 2.1

Mechanism of heme oxygenase enzyme reaction and its transcription regulation via the ARE/Nrf2 pathway.

- A) The heme oxygenase enzyme reaction. Heme oxygenase catalyzes the first and rate-limiting step of heme degradation producing CO, ferrous iron and biliverdin.

- B) Mechanism of Nrf2 response to oxidative stress. This model suggests that the cytosolic protein Keap1, which is bound to the actin cytoskeleton, suppresses Nrf2 transcriptional activity by coupling and retaining Nrf2 in the cytosol. Upon activation by oxidative stress, the Keap1-Nrf2 complex is disassociated and Nrf2 translocates into nucleus, heterodimerizes with a Maf family member, binds to the antioxidant response element (ARE) and thereby leads to the expression of genes encoding antioxidant stress proteins.

Figure 2.2

Example of degenerate primer design for heme oxygenase 1.

A) HO1 forward primer designed from *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* HO1, and *Sus scrofa* HO mRNA consensus sequences.

B) HO1 reverse primer was designed from the same species with two wobbles.

R=A or G; S=C or G.

A)

Human	ggcc tgaaggaggccaccaaggaggt cac	157
Mouse	ggcc tgaaggaggccaccaaggaggt cac	205
Rat	ggcc tgaaggaggccaccaaggaggt cac	77
Pig	ggcc tgaaggaggccaccaaggaggt cac	151
Consensus	ggcc tgaaggaggccaccaaggaggt cac	

5' -TGAAGGAGGCCACCAAGGAG-3'

B)

Human	gc aagac gc ttcttgctcaacat agct	718
Mouse	gc aagac gc ttcttgctcaacat agct	766
Rat	gc aagac gc ttcttgctcaacat agct	638
Pig	gc aagac gc ttcttgctcaacat agct	712
Consensus	gc aagac gc ttcttgctcaacat agct	

5' -GTTGAGCAGGAASGCRGTCTT-3'

Figure 2.3

Degenerate PCR amplification of squirrel kidney cDNA.

A) Left: Degenerate PCR product of HO1 Forward/Reverse primers at 68°C annealing temperature. Right: Molecular weight marker (1 Kb ladder).

B) Left: Degenerate PCR product of HO2 Forward/Reverse primers at 65°C annealing temperature. Right: Molecular weight marker (1 Kb ladder).

A)

B)

Figure 2.4

Partial nucleotide and deduced partial amino acid sequence of the ground squirrel (*Spermophilus tridecemlineatus*) HO1 gene product that was amplified from squirrel kidney cDNA. Nucleotides and amino acids are numbered on the left. A single open reading frame was predicted from the nucleotide sequence and coded for a polypeptide with 191 residues. ? represents an unknown nucleotide. X represents an unknown amino acid.

1 TGAAGGAGGCCACCAAGGAGGTGCACACCCAGGCAGAGAATGCCGAGTTCATGAGGAACT
 1 K E A T K E V H T Q A E N A E F M R N
 61 TCCGGAAGGGCCAGGTGACCAGAAAGGGCTCCAAGCTGGTGATGGTGTCCCTGTACCACG
 20 F R K G Q V T R K G S K L V M V S L Y H
 121 TCTACTTGGCCCTGGAGGAGCAGATC?AGCACAAACACGGACAACCCCGTCTTCGCCCCCG
 40 V Y L A L E E Q I X H N T D N P V F A P
 181 TCTACTTCCCCGAGGAACTGCACCACAGGGCCACACTGGAGCAGGACATGGCCTTCTGGT
 60 L Y F P E E L H H R A T L E Q D M A F W
 241 ACAGGCCATGCTGGCAGCAGGCCGTCCCCTACACAGCAGCTACGTGG?GTCAGGTA????
 80 Y R P C W Q Q A V P Y T A A T W X Q V X
 301 GGCTCCAGCAGGTAGAGCGCAGGGAGCCGGAGATGCTGGTGGCCCACGCCTACACCCACT
 100 X L Q Q V E R R E P E M L V A H A Y T H
 361 ATCTGGGCAACTTGTCCAGGGGCCAGGTCTCTCAAGAAGATCACACATAAGGCCCTGGACC
 120 Y L G N L S R G Q V L K K I T H K A L D
 421 TGCCCAGCTCCAGAGAGGGCCTGGCCTTCTTCACCTTCCCCAAGGTCGCCAGTGCCACCA
 140 L P S S R E G L A F F T F P K V A S A T
 481 AGTTCAAGCAGCTCTACCACTCACTCATGAACACTCTGGAGATGACCCCTGAGTTCAGGC
 160 K F K Q L Y H S L M N T L E M T P E F R
 541 AGAGGGTTATTAAAGAGGCCAAGACCGCCTTCCTGCTCAAC
 180 Q R V I K E A K T A F L L N

Figure 2.5

Ground squirrel (*Spermophilus tridecemlineatus*) HO1 partial amino acid sequence aligned with human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*) HO1 and pig (*Sus scrofa*) HO amino acid sequences (Genbank accession numbers NP_002124, NP_034572, NP_036712, and P32394, respectively).

For convenience only those amino acids differing from the squirrel sequence are indicated; distinct amino acid substitutions in the squirrel sequence are indicated in bold underline. Dashes (-) represent amino acids in the human, mouse, rat or pig sequences that are identical with the squirrel sequence. Periods are present in the alignment to indicate when an amino acid is not present in the coding region of one of the species.

squirrel	KEATKEVHTQAENAEFMRNFRKG	23
human	merpqpdsmppqdlseal-----q--	40
mouse	merpqpdsmppqdlseal-----i-----k--q--	40
rat	merpqldsmssqdlseal-----ir---s-----q--	40
pig	mehsqpnsmppqdlseal-----v-----k--q--	40
squirrel	QVTRKGSKLVMVSLYHVYLALEEQIXHNTDNPVFAPLYFP	63
human	----d-f----a----i-v----e-er-kes-----v---	80
mouse	--s-e-f----a----i-t----e-er-kq---y-----	80
rat	--s-e-f----a----i-t----e-er-kq---y-----	80
pig	e---e-f----a----i-d----e-e--ke---yt-----	80
squirrel	EELHHRATLEQDMAFWYRPCWQQAVPYTAATWXQVXXLQQ	103
human	----rk-a----l----g-r--evi---p-mqry-kr-he	120
mouse	----r--a-----g-h--eii-c-p--qhy-kr-he	120
rat	----r--a-----g-h--e-i---p--qhy-kr-he	120
pig	----r--a-----g-r--e-i---q--kry-rr---	120
squirrel	VERREPEMLVAHAYTHYLGNLSRGQVLKKITHKALDLPSS	143
human	-g-t---l-----r---d--g-----aq-----	160
mouse	-g-th--l-----r---d--g-----aq--ma----	160
rat	-ggth--l-----r---d--g-----aq--ma----	160
pig	-g-f---l-----r-m-d--g-----aq-----	160
squirrel	REGLAFFTFPKVASATKFKQLYHSLMNTLEMTPEFRQRVI	183
human	g-----ni-----r-r--s-----av-----	200
mouse	g-----nid-p-----rar-----vkh--t	200
rat	g-----sidnp-----rar-----vkh--t	200
pig	g-----n--n-----r-r-----vkh--l	200
squirrel	KEAKTAFLLN.....	193
human	e-----iqlfeelqellthdtkdqspsrappglrqlra	240
mouse	e-----ielfeelqvmhteekdqspsqmaslrqrp	240
rat	e-----ielfeelqallteekdqspsqteflrqrp	240
pig	e-----iqlfeevqelltqdtkdqrpsqasdirkra	240
squirrel		193
human	snkvqdsapvetprgkplntr.sqapllrwvltlsflva	279
mouse	aslvqdtapaetprgkpqistsssqtpllqgwltlsflila	280
rat	aslvqdttsaetprgkqistsssqtpllrwvltlsflila	280
pig	gsrvqdstpvtprgkpqlsvl.sqvplirwvltlsflva	279
squirrel		193
human	tvavglyam	288
mouse	tvavgiam	289
rat	tvavgiam	289
pig	tvamglyam	288

Figure 2.6

Putative squirrel HO1 amino acid sequence homology analysis.

(A) Homology tree of partial squirrel, human, mouse, rat and pig HO1 amino acid sequences.

(B) Homology matrix of the five amino acid sequences.

A.

B.

Homology matrix of 5 sequences

squirrel	100%				
human	75.1%	100%			
mouse	71.5%	86.0%	100%		
rat	71.0%	84.5%	95.9%	100%	
pig	76.7%	86.0%	85.5%	84.5%	100%

Figure 2.7

Partial nucleotide and deduced amino acid sequence of the ground squirrel (*Spermophilus tridecemlineatus*) HO2 gene product that was amplified from squirrel kidney cDNA. Nucleotides and amino acids are numbered on the left. A single open reading frame was predicted from the nucleotide sequence to code for a polypeptide with 230 residues.

1 ATGGAGCGCAACAAGGACCACCCAGCCTTTGCCCCCTTGTACTTCCCCACGGAGCTGCAC
1 M E R N K D H P A F A P L Y F P T E L H

61 CGGAAGGAGGCACTGACCAAGGACATGGAGTACTTCTTTGGTGAAAACCTGGGAGGAGCTG
21 R K E A L T K D M E Y F F G E N W E E L

121 GTGAAGTGCTCTGAGGCTGCCCAGAAGTATGTAGAGCGGATCCACTATATAGGGCAGAAT
41 V K C S E A A Q K Y V E R I H Y I G Q N

181 GAGCCAGAGCTGCTGGTGGCCCATGCATACACCCGCTACATGGGGGACCTCTCGGGAGGC
61 E P E L L V A H A Y T R Y M G D L S G G

241 CAGGTGCTGAAGAAGGTGGCCCAGCGGGCACTAAACCTCCCCAGCACAGGGGAAGGGACC
81 Q V L K K V A Q R A L K L P S T G E G T

301 CAGTTCTACCTGTTTGAGCATGTGGACAATGCCCAGCAGTTCAAACAGTTCTACCGGTCC
101 Q F Y L F E H V D N A Q Q F K Q F Y R S

361 AGGATGAATGCCCTGGACCTGAATTTGAAGACCAAAGAGAGGATTGTGGAGGAGGCCAAC
121 R M N A L D L N L K T K E R I V E E A N

421 AAGGCCTTTGAGTACAACATGCAGATATTCAATGAACTGGACCAGGCTGGCTCTGCTTTG
141 K A F E Y N M Q I F N E L D Q A G S A L

481 GCCAGAGAAACCCTGGAGGATGGGCTCCCAGGGCACGACGGGAAGGGAGATGTACGTAAA
161 A R E T L E D G L P G H D G K G D V R K

541 TGCCCCCTTCTATGCTGCTCAGCAAGACAAAGGTGCCCTGGAGGGCAGCAGCTGCCCCCTTC
181 C P F Y A A Q Q D K G A L E G S S C P F

601 CGGACAGCCATGGCTGTGCTGAGGAAGCCCAGTCTCCAGTTTATTCTGGCTGCTGGTGTG
201 R T A M A V L R K P S L Q F I L A A G V

661 GCCCTGGCTGCTGGACTCTTGGCCTGGTACTA
221 A L A A G L L A W Y

Figure 2.8

Translation of ground squirrel HO2 partial amino acid sequence and its alignment with corresponding human, mouse, rat and rabbit (*Oryctolagus cuniculus*) HO2 partial amino acid sequences (Genbank accession numbers S34389, NM_010443, J05405 and S61699, respectively).

Squirrel		0
Human	msaevetsegvdesekknscalekenqmrmdl sellkeg	40
Mouse	mssevetsegvdeseknsmap.ekenhtkmdl sellkeg	39
Rat	mssevetsegvdesennstap.ekenhtkmdl sellkeg	39
Rabbit	msaevetsegvdepeeknfgnhi....rmdl sellkeg	36
Squirrel		0
Human	tkeahdraentqfvkdfkgnikkelfklattalyftysa	80
Mouse	tkeahdraentqfvkdfkgnikkelfklattalyftysa	79
Rat	tkeahdraentqfvkdfkgnikkelfklattalyftysa	79
Rabbit	tkeahdraentkfvkdfkgnikkeifklattalyftysa	76
Squirrel	MERNKDHPAFAPLYFPTELHRKEALTKDMEYFFGEN	36
Human	leee-----m-----	120
Mouse	leee-d-----a--i---k-----	119
Rat	leee-d-----i-----	119
Rabbit	leee-d-----m-----	116
Squirrel	WEELVKCSEAAQKYVERIHYIGQNEPELLVAHAYTRYMGD	76
Human	---q-q-pk-----	160
Mouse	---q-----d---v-----	159
Rat	---q-----d---v-----	159
Rabbit	---q-q-----	156
Squirrel	LSGGQVLKKVAQRALKLPSTGEGTQFYLFHV DNAQQFKQ	116
Human	-----n-----	200
Mouse	-----	199
Rat	-----	199
Rabbit	-----n-----	196
Squirrel	FYRSRMNALDLNLKTKERIVEEANKAFEYNMQIFNELDQA	156
Human	l--a-----m-----	240
Mouse	---a-----s-----	239
Rat	---a-----sm-----s-----	239
Rabbit	---a-----s-----	236
Squirrel	GSALARETLEDGLPGHDGKGDVRKCPFYAAQQDKGALEGS	196
Human	--t-----f-v-----m-----e-----	280
Mouse	--m-----v-----i-----p---t-g--	279
Rat	--m-tk-----v-----p---t-g--	279
Rabbit	--p-s--v--ri-v-----y--g-vn-----	276
Squirrel	SCPFRTAMAVLRKPSLQFILAAGVALAAGLLAWY..	230
Human	-----ym	316
Mouse	n---q-tv-----l---s---v-----ym	315
Rat	n-----l---s---v-----ym	315
Rabbit	----a-----lv---a-----ym	312

Figure 2.9

Putative squirrel HO2 amino acid sequence homology analysis.

(A) Homology tree of partial squirrel, human, mouse, rat and pig HO2 amino acid sequences.

(B) Homology matrix of the five amino acid sequences.

A.

B.

Homology matrix of 5 HO2 sequences

Squirrel HO2	100%				
Human_HO2	93.9%	100%			
Mouse_HO2	90.4%	87.9%	100%		
Rat_HO2	91.3%	89.2%	95.7%	100%	
Rabbit_HO2	90.9%	90.9%	87.1%	88.4%	100%

Figure 2.10

Amplification of HO1 from ground squirrel liver using species-specific HO1 gene primers.

(A) Left: species-specific PCR product from amplification with HO1 Forward/Reverse gene specific primers at 68°C annealing temperature. Right: Molecular weight markers (1 kb ladder).

(B) Nucleotide sequence of the PCR product from squirrel liver.

A)

B)

```

1      TCCAAGCTGG TGATGGTGTC CCTGTACCAC GTCTACTTGG CCCTGGAGGA GCAGATCAGC
61     ACAACACGGA CAACCCCGTC TTCGCCCCC TCTACTTCCC CGAGGAACTG CACCACAGGG
121    CCACACTGGA GCAGGACATG GCCTTCTGGT ACAGGCCATG CTGGCAGCAG GCCGTCCCCT
181    ACACAGCAGC TACGTGGGTC AGGTAGGCTC CAGCAGGTAG AGCGCAGGGA GCCGGAGATG
241    CTGGTGGCCC ACGCCTACAC CCACTATCTG GGCAACTTGT CCAGGGGCCA GGTCCCTCAAG
301    AAGATCACAC ATAAGGCCCT GGACCTGCCC AGCTCCAGAG AGGGCCTGGC CTTCTTCACC
361    TTCCCCAAGG TCGCCAGTGC CACCAAGTTC AAGC

```

Figure2.11

Amplification of HO2 from ground squirrel liver using species-specific HO2 gene primers

(A) Left: species-specific PCR product from amplification with HO2 Forward/Reverse gene specific primers at 68°C annealing temperature. Right: Molecular weight markers (1 kb ladder).

(B) Nucleotide sequence of the squirrel liver PCR product.

A)

B)

1	CTGACCAAGG	ACATGGAGTA	CTTCTTTGGT	GAAAACTGGG	AGGAGCTGGT	GAAGTGCTCT
61	GAGGCTGCCC	AGAAGTATGT	AGAGCGGATC	CACTATATAG	GGCAGAATGA	GCCAGAGCTG
121	CTGGTGGCCC	ATGCATACAC	CCGCTACATG	GGGGACCTCT	CGGGAGGCCA	GGTGCTGAAG
181	AAGGTGGCCC	AGCGGGCACT	AAAACTCCCC	AGCACAGGGG	AAGGGACCCA	GTTCTACCTG
241	TTTGAGCATG	TGGACAATGC	CCAGCAGTTC	AAACAGTTCT	ACCGGTCCAG	GATGAATGCC
301	CTGGACCTGA	ATTTGAAGAC	CAAAGAGAGG	ATTGTGGAGG	AGGCCAACAA	GGCCTTTGAG
361	TACAACATGC	AGATATTCAA	TGAACTGGAC	CAGGCTGGCT	CTGCTTTGGC	CAGAGAAACC
421	CTGGAGGATG	GGCTCCAGG	GCACGACGGG	AAGGGAGATG	TACGTAAATG	CCCCTTCTAT
481	GCTGCTCAGC	AAG				

Figure 2.12

Example of relative RT-PCR analysis on kidney cDNA showing amplification of alpha-tubulin (left) and HO1 (right) at each dilution.

Band intensities of PCR products at each dilution were compared: each HO1 band was first normalized relative to its corresponding alpha-tubulin band, and then differences between hibernating versus euthermic situations were assessed. The lowest dilutions for alpha-tubulin and HO1 which have visible bands were marked in squares and chosen for quantification (10^{-5} for tubulin and 10^{-4} for HO1). The formula below the picture was used to calculate the mRNA regulation of HO1 relative to alpha-tubulin.

$$\text{Relative mRNA level} = \frac{(\text{hibernating HO1 } 10^{-4} / \text{hibernating tubulin } 10^{-5})}{(\text{euthermic HO1 } 10^{-4} / \text{euthermic tubulin } 10^{-5})}$$

Figure 2.13

Analysis of HO1 and HO2 transcript levels in ground squirrel organs during hibernating.

- A) Relative HO1 transcript levels in brain, lung, heart, liver, kidney, skeletal muscle and brown adipose tissue (B.A.T.) of hibernating versus euthermic squirrels, as determined from semi-quantitative PCR. The lowest dilutions for alpha-tubulin and HO1 which have visible PCR products (normally at dilution 10^{-3} ~ 10^{-5}) were chosen for quantification. Data are means $\pm$ S.E.M., n= 3 independent trials with both hibernating and euthermic samples. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.
- B) Relative HO2 transcript levels in brain, lung, heart, liver, kidney, B.A.T., and white adipose tissue (W.A.T.) of hibernating versus euthermic squirrels. Data are for n= 1 trial with both hibernating and euthermic samples.

A)

Relative change in HO1 mRNA transcript levels in hibernating vs.euthermic squirrels

B)

Relative change in HO2 mRNA transcript levels in hibernating vs euthermic squirrels

Figure 2.14

Relative levels of HO1 protein in tissues from euthermic versus hibernating ground squirrels as determined from Western blotting.

A) Peptide competition experiments to confirm the specificity of polyclonal rabbit anti-HO1 antibody. Lane 1 and 2 show results from Western blotting with extracts of squirrel muscle using polyclonal rabbit anti-HO1 antibody. Lane 3 and 4 show comparable Western blot results using polyclonal rabbit anti-HO1 in the presence of its immuno peptide. Pre-incubation with the antigenic peptide abolished immunoreactivity to HO1 and the band at 32 kDa disappeared.

C) Representative Western blots showing HO1 protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.

A)

B)

C)

Relative HO1 protein levels hibernating vs.euthermic squirrels

Figure 2.15

Western blots showing Nrf2 protein content in tissues of hibernating versus euthermic ground squirrels.

- A) Left: Representative 2D-PAGE western blot showing Nrf2 from ground squirrel liver. Right: Representative western blots showing Nrf2 antibody crossreacting with several bands, one at 57 kDa and one at 100 kDa.
- B) Representative Western blots show Nrf2 protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.
- C) Relative levels of Nrf2 (57 kDa and 100 kDa) proteins in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean $\pm$ SEM, n=4 independent determinations.
- * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

Figure 2.16

Nrf2 (57 kDa and 100 kDa) protein distribution between nuclear and cytoplasmic fractions.

A) Representative Western blots showing Nrf2 protein distribution between nuclear and cytoplasmic fractions in liver and muscle from euthermic (E) and hibernating (H) ground squirrels.

B) Nrf2 (57 kDa and 100 kDa) protein distribution between nuclear and cytoplasmic fractions in liver: relative levels in hibernating versus euthermic squirrels. Histogram shows mean $\pm$ SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

C) Nrf2 (57 kDa and 100 kDa) protein distribution between nuclear and cytoplasmic fractions in muscle: relative levels in hibernating versus euthermic squirrels. Histogram shows mean $\pm$ SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)

B)

Nrf2 (57 KDa and 100 KDa) Protein Expression in Liver Nuclear and Cytoplasmic Fractions

C)

Nrf2 (57 KDa and 100 KDa) Protein Expression in Muscle Nuclear and Cytoplasmic Fractions

Figure 2.17

Western blots showing MafG protein content in tissues of hibernating versus euthermic ground squirrels.

- A) Representative Western blots showing MafG protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.
- B) Representative Western blots showing MafG distribution between nuclear and cytoplasmic fractions in liver and muscle from euthermic (E) and hibernating (H) ground squirrels.
- C) Relative levels of MafG proteins in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean + SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.
- D) MafG protein distribution between nuclear and cytoplasmic fractions in liver and muscle: relative levels in hibernating versus euthermic squirrels. Histogram shows mean + SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

Figure 2.18

Western blots showing actin protein content in tissues of hibernating versus euthermic ground squirrels.

- A) Representative Western blots showing actin protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.
- B) Relative levels of actin proteins in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean + SEM, n=4 independent determinations.
- C) Representative Western blots showing actin and its putative complex distribution in brain and heart from euthermic (E) and hibernating (H) ground squirrels.
- D) Actin putative complex protein distribution in brain and heart: relative levels in hibernating versus euthermic squirrels. Histogram shows mean + SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

D) Protein expression of actin in a putative complex in ground squirrel (hibernating vs. euthermic)

Chapter 3

Superoxide dismutase and antioxidant defense in hibernation

3.1 Introduction

The conditions of ischemia/reperfusion in hibernation could potentially favor reactive oxygen species (ROS) generation. The idea that hibernators may be at risk for oxidative stress is supported by previous studies, for example, in ground squirrels levels of some antioxidant enzymes and ROS scavengers are increased during hibernation (Buzadzic et al., 1990; Storey 2003). Superoxide dismutase (SOD) is the first and most important line of antioxidant enzyme defense against ROS. Results from our lab revealed up-regulation of the SOD gene in kidney of hibernating ground squirrels. It has become increasingly apparent that SOD (see reaction below) plays an important role in antioxidant defense during hibernation.

SOD catalyzes the dismutation reaction of superoxide radical anions (O_2^-) to form hydrogen peroxide and oxygen with the H_2O_2 then catabolized to O_2 and H_2O by either glutathione peroxidase or catalase (McCord and Fridovich, 1969). Two genetically distinct mammalian isoforms of SOD are known. Cu/Zn SOD is a well-characterized cytosolic scavenger of oxygen free radicals that requires copper and zinc binding to potentiate its enzymatic activity (Bewley, 1988). Cu/Zn SOD is ubiquitously expressed in somatic cells and functions as a homodimer (Chang et al., 1988). Supplying nerve cells with Cu/Zn SOD can protect them against damage such as that occurring in stroke, where the oxygen and glucose supply to the brain is cut off. Defects in the gene encoding Cu/Zn SOD have been implicated in the progression of neurological diseases, including amyotrophic lateral sclerosis (a neurodegenerative disease characterized by the loss of

spinal motor neurons), Down's syndrome, and Alzheimer's Disease (Shaw et al., 1998). Mn SOD, exists as a tetramer and is located in the mitochondrial matrix (Weisiger and Fridovich et al., 1973). Mn SOD has been shown to play a major role in promoting cellular differentiation and tumorigenesis (St.Clair et al., 1994) protecting against hypoxia-induced pulmonary toxicity (Wise et al., 1992), and providing cellular resistance to cytotoxicity by tumor necrosis factor (TNF)(Wong and Goeddel, 1988; Wong et al., 1989).

Transcriptional regulation of SODs is highly controlled in response to both extra- and intracellular conditions. Despite the fact that Cu/Zn SOD and Mn SOD are constitutively expressed in many cell types and tissues, their mRNA levels can be dramatically altered in response to a wide array of mechanical, chemical, and biological messengers such as heat shock, shear stress, UVB- and X-irradiation, heavy metals, hydrogen peroxide, and xenochemicals (Zelko et al., 2002).

Evidence from cDNA array screening indicated that SOD mRNA was up-regulated in ground squirrel kidney during hibernation (S. Eddy and K. Storey, unpublished). Based on this, I hypothesized that SOD protein levels would broadly increase in ground squirrel organs during hibernation and the present chapter analyzes the responses of Cu/Zn SOD and Mn SOD in different tissues of hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. Western blot analysis demonstrated that both Cu/Zn SOD and Mn SOD proteins increased only in muscle but, by contrast, the mRNA transcript levels of Cu/Zn SOD and Mn SOD was unchanged in muscle as assessed by both relative RT-PCR and semi-quantitative PCR.

3.2 Materials and Methods

3.2.1 Animal Treatments

Animals were treated as previously described in Chapter 2.

3.2.2 2-Dimensional Polyacrylamide Gel electrophoresis of SOD

2D PAGE was performed using the Modular Mini-Protean II System (Bio-Rad) as described in Chapter 2. Aliquots (30 µg protein) of squirrel liver extracts were loaded onto the surface of the first dimension gel (pH 3.5 to 10), and then isoelectric focusing was carried out. After this, first dimension gels were removed from capillary tubes and loaded onto an SDS polyacrylamide gel (12%) for second dimension separation by molecular mass, followed by immunoblotting and protein visualization. Primary antibodies to rabbit Cu/Zn SOD (#SOD 100) and rabbit Mn SOD (#SOD 110) were purchased from Stressgen Biotechnologies. Secondary HRP-linked goat anti-rabbit IgG (H&L) (#7047) was purchased from Cell Signaling Technology. Primary antibody was used at a dilution of 1:5000 v/v in 1X TBST. The secondary antibody was used at a dilution of 1:2500 v/v in 1X TBST. Blots were developed using the enhanced chemiluminescence assay with SuperSignal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned by ChemiGenius Bio Imaging System (SynGene).

3.2.3 Western Blotting of Cu/Zn SOD and Mn SOD

Western blotting using one dimensional SDS-PAGE was performed as described in Chapter 2. Total protein was isolated from samples of B.A.T., brain, heart, kidney,

liver, lung, muscle and W.A.T. of euthermic and hibernating ground squirrels. Aliquots containing 10 µg of protein were loaded into each well of 12% SDS gels, followed by electrophoresis, and then transfer to PVDF membrane. The same primary and secondary antibodies were used as in section 3.2.2. All primary antibody incubations for western blotting were carried out at 4°C overnight in 1X TBST at a 1:5000 v/v dilution. Each blot was washed for 3 x 5 minutes in 1X TBST prior to the addition of secondary antibody. Secondary antibody (1:2500 v/v dilution) incubations were carried out for 2-3 hours at room temperature and followed by 3 x 5 minute washes in 1X TBST. Blots were developed using the enhanced chemiluminescence assay using SuperSignal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene).

3.2.4 Relative RT-PCR of Cu/Zn SOD and Mn SOD mRNA

Relative RT-PCR was performed as described in Chapter 2. Total RNA was isolated from squirrel muscle and assessed on a formaldehyde agarose gel. First strand cDNA was synthesised from total RNA, and then five dilutions were prepared (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}) before performing semi-quantitative PCR.

Degenerate primers for Cu/Zn SOD and Mn SOD were designed from the conserved sequences of human, mouse, and rat and then synthesized by Sigma Genosys. Primer sequences, optimal PCR annealing temperatures and their product sizes are listed in Table 3.1. PCR products were sequenced by Cortec DNA Service Laboratories (Kingston, ON), and then verified as the sequence expected using the program BLASTX

at the NIH. The deduced amino acid sequences were also verified to be the protein of interest by using the program BLASTP at the same site.

Two different PCR reactions were performed with each set of cDNA sample dilutions, one to evaluate the quantity of the cDNA of interest (Cu/Zn SOD or Mn SOD) and the other to evaluate the quantity of the housekeeping gene, alpha-tubulin. The PCR products were separated in a 0.8% agarose/ethidium bromide gel. The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene) and densitometry values were measured with GeneTools software (SynGene). By comparing the intensity of PCR products at each dilution, the lowest dilutions for alpha-tubulin and Cu/Zn SOD (or Mn SOD) which had visible bands were chosen for quantification and the relative amounts of Cu/Zn SOD (or MnSOD) mRNA transcripts, relative to alpha-tubulin, were determined. The formula below was used to calculate the relative mRNA levels for hibernating versus euthermic situations:

$$\text{Relative mRNA content} = (\text{hibernating SOD/hibernating tubulin}) / (\text{euthermic SOD/euthermic tubulin}).$$

3.2.5 Semi-Quantitative PCR of Cu/Zn SOD and Mn SOD mRNA

Semi-quantitative PCR was done in addition to relative RT-PCR to quantify PCR template concentration. The same annealing temperature and primer concentration used in relative RT-PCR was also used in this new technique and the same amount of RNA from euthermic and hibernating muscle was used for reverse transcription into cDNA by first strand cDNA synthesis as described in Chapter 2. The cDNA content in euthermic and hibernating samples was also normalized by comparing the intensity of serial

dilutions as described in Chapter 2. The lowest dilution pair (euthermic control and hibernation sample) which had visible Cu/Zn SOD or Mn SOD PCR products was chosen for use with the semi-quantitative PCR reaction. Two different PCR reactions were performed, one to evaluate the quantity of the cDNA of euthermic controls and the other to evaluate the quantity of hibernation sample. Each PCR reactions master mix contained 10 μ l normalized first strand cDNA, 60 μ l ddH₂O, 10 μ l PCR Running buffer (-MgCl₂) (Invitrogen), 5 μ l 50 mM MgCl₂ (Invitrogen), 2 μ l 10 mM dNTPs (Invitrogen), 0.5 μ l *Taq* DNA Polymerase (Invitrogen) and 2.5 μ l 30 nmol/ml forward and reverse primer mixture (1:1). The master mix was aliquoted into 10 tubes each containing 10 μ l of the PCR reaction. Polymerase chain reactions were performed on a iCycler PCR machine (BioRad). Temperature cycles for PCR were as follows: denaturation at 94°C for 30 seconds, primer annealing at their optimal PCR annealing temperature (Table 3.1) for 1 minute, and primer extension at 72°C for 1 minute. These cycles were carried out 42 times. At the end of cycle 24, 1 tube of euthermic control and 1 tube of hibernating sample were removed and then subsequently tubes were removed every 2 cycles until cycle 42. The PCR products and 4 μ l of low mass DNA ladder (Invitrogen) were run on a 0.8% agarose/ethidium bromide gel. The bands were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene) and densitometry values were measured with GeneTools software (SynGene) (Figure 3.10 B).

This technique is based on a parameter called the cycle threshold (Ct). The Ct is the number of cycles required for the PCR product to be detected by your method. The Ct values of a standard dilution series plotted against the log of their known initial concentrations yields a straight line. The calibration curve used in semi-quantitative PCR

was constructed by Dave McMullen (Carlton University) (Figure 3.10 A). When analyzing the gel on GeneTools, the background correction was set as automatic, and the 400bp band in the low mass DNA ladder was set as 100. This normalizes the gel to the same intensity values as the gels used to create the standard curve. The intensities of the bands was used to generate a plot of relative intensity (y-axis) versus number of cycles. The slope of the graph was calculated in Excel (Microsoft Office). The bands whose intensities appearing to be saturated was not used in calculation. The rate of growth of PCR products is related to the number of template molecules in the reaction mixture. For example, in the case of eutermic control Cu/Zn SOD in muscle (Figure 3.10 B), a plot of relative intensity (y-axis) versus number of cycles was generated as in Figure 3.10 C. The cycle threshold for the intensity of 100, [Ct(100)] was calculated as follows: $[Ct(100)] = 100 - (-694.8)/29.201 = 27.218$. Then the Ct calibration curve was used to calculate the original template concentration. The equation of the line for the Ct calibration curve is: $\log(\text{copy \#}) = [Ct(100) - 45.641]/-3.3888 = 5.436$, so $\text{copy \#} = 275427$.

3.3 Results

3.3.1 *Western Blotting analysis of Cu/Zn SOD protein expression in tissues*

Figure 3.1A shows the two dimensional gel electrophoresis and western blotting that were used to examine the specificity of the Cu/Zn antibody. Squirrel liver soluble proteins were resolved in the first dimension by isoelectric point (pI), and in the second dimension by molecular mass. Treatment with Cu/Zn SOD antibody revealed a reaction

with a single spot at ~17 kDa with a pI of ~5.5, consistent with values in the literature for SOD (Hochstrasser et al., 1992).

Western blotting was then used to assess total Cu/Zn SOD content in tissues from euthermic versus hibernating ground squirrels. As Figure 3.1 B and C shows, the level of Cu/Zn SOD did not change between euthermic and hibernating states in B.A.T., brain, heart, kidney, liver, lung or W.A.T.. Only the amount of Cu/Zn SOD in muscle of hibernators was significantly higher (2.3 ± 0.2 fold) than in the euthermic situation (Figure 3.1 C).

3.3.2 Western Blotting analysis of Mn SOD protein expression in tissues

Figure 3.2 shows a similar analysis of Mn SOD. Two dimensional gel electrophoresis and western blotting were again used to examine the specificity of the Mn SOD antibody. Treatment with Mn SOD antibody revealed reaction with three spots at ~25 kDa with pI values of approximately 6, 6.5, 7, respectively (Figure 3.2 A), which are consist with the pI values in the literature is 6.7~7 (Hochstrasser et al., 1992). Because Mn SOD has no isoforms, we raised the hypothesis that Mn SOD might be modified by posttranscriptionally (such as by protein phosphorylation), which could change the pI of the protein.

As occurred for Cu/Zn SOD, the total amount of Mn SOD did not change between euthermia and hibernation in any of the tissues except muscle (Fig. 3.2 B and C). Mn SOD content rose in skeletal muscle by 1.6 ± 0.12 fold during hibernation (Figure 3.2 C).

3.3.3 cDNA Analysis of Squirrel Cu/Zn SOD and Mn SOD

Degenerate PCR of Cu/Zn SOD and Mn SOD: Degenerate primers were designed for use in amplifying cDNAs that encoded the ground squirrel specific forms of Cu/Zn SOD and Mn SOD. Forward and reverse primers were designed for each based on the consensus sequences identified from a comparison of the cDNA sequences for Cu/Zn SOD and Mn SOD in other mammals (human, rat, and mouse). Sequences of the primers used are shown in Table 3.1. The Cu/Zn primers were used to amplify a PCR product of 381 bp using a 60°C annealing temperature (Table 3.1; Figure 3.3A). A PCR product of 542bp was amplified using the Mn SOD primers at an annealing temperature of 56°C (Table 3.1; Figure 3.3B).

Cu/Zn SOD sequence analysis: Figures 3.4 and 3.5 show the sequence analysis of ground squirrel Cu/Zn SOD. The putative amino acid sequence of the ground squirrel cDNA encoded 117 of the 154 amino acids of the full mammalian Cu/Zn SOD sequence (Figure 3.4 A). The segment lacked both the N- and C-termini. Figure 3.4 B shows that the ground squirrel sequence is very similar to the sequence from other mammals. The homology tree and homology matrix shown in Figure 3.5 show that the ground squirrel Cu/Zn SOD amino acid sequence shared 80.3%, 88.0%, and 86.3% amino acid identity with human, mouse and rat Cu/Zn, respectively.

Mn SOD sequence analysis: Figure 3.6 shows that the amplified cDNA encoded 180 of the 222 amino acid residues in the full length sequence of Mn SOD from other mammals. Very high sequence identity was again evident among the four mammals (Figure 3.7). The homology tree and homology matrix shown in Figure 3.8 revealed

93.9%, 94.4%, and 93.9% amino acid identity between ground squirrel Mn SOD and human, mouse, and rat Mn SOD, respectively.

3.3.4 Analysis of Cu/Zn SOD and Mn SOD transcript levels in muscle

Because both Cu/Zn SOD and Mn SOD protein levels were elevated in skeletal muscle during hibernation, it is important to know whether they also increased at the transcriptional (mRNA) level. Relative RT-PCR analysis of Cu/Zn SOD and Mn SOD transcript levels in hibernating versus euthermic states was conducted for muscle of ground squirrels (Figure 3.9 A). The ratio of transcript levels, hibernating:euthermic is shown in Figure 3.9 B. Neither Cu/Zn SOD or Mn SOD mRNA levels changed significantly during hibernation (Figure 3.9 B).

To confirm the results of relative RT-PCR, semi-quantitative PCR was conducted to quantify HO1 cDNA template levels in equal concentrations of euthermic and hibernating cDNA samples. This technique is based on a parameter called the cycle threshold (Ct). The Ct is the number of cycles required for the PCR product to be detected by the PCR method. The Ct values of a standard dilution series plotted against the log of their known initial concentrations yields a straight line, which is used as standard curve (Figure 3.10 A). Figure 3.10 B shows a semi-quantitative PCR gel sample and the intensity of each band after a different number of cycles. A plot of relative intensity (y-axis) versus number of cycles was generated as in Figure 3.10 C. Since the intensity of PCR products is related to the number of template molecules in the reaction mixture, we are able to calculate the Ct of the sample, and then calculate the initial concentration of the template for the sample using the Ct standard curve.

The hibernating versus euthermic cDNA samples were normalized before use to ensure that they had equal cDNA concentration. After running semi-quantitative PCR, the number of Cu/Zn SOD or MnSOD cDNA templates in each sample was calculated by using the Ct calibration curve. Hibernation samples contained 271089 ± 34436 copies of Cu/Zn cDNA, and 109506 ± 342 copies of Mn cDNA in it. Euthermic control samples contained 237746 ± 20428 copies of Cu/Zn cDNA and 115510 ± 3218 copies of Mn cDNA. These results show no change in SOD copy numbers between the two states, the same outcome as determined from the relative RT-PCR. Thus, neither of Cu/Zn SOD or Mn SOD changed significantly at the gene transcription level during hibernation (Figure 3.11).

3.4 Discussion

Adaptations that aid hibernation include well-developed antioxidant defenses that allow cells to mount an effective defense against oxidative challenges. SOD, one of the most important antioxidant enzymes, serves as a front-line molecular defense against superoxide, a reactive free radical oxygen metabolite that is thought to be responsible for the oxidative damage to living cells underlying aging, cancer, and neurodegenerative disease (Stadtman, 1992). The activities of Cu/Zn SOD and Mn SOD are highly regulated by the redox state of cells. Under oxidative stress conditions, it is common to detect increased cellular activities of both enzymes. In the present work, I studied the transcription and translation regulation of Cu/Zn SOD and Mn SOD in tissues of hibernating ground squirrels.

3.4.1 Analysis of the structures of ground squirrel Cu/Zn SOD and Mn SOD

The cDNA attained from RT-PCR encoded 75~80% of the amino acid sequences of Cu/Zn SOD and Mn SOD. Both of the sequences from ground squirrels shared a high degree of structural homology with the sequences from other mammals.

Cu/Zn SOD occurs as a dimer of identical 16 kDa subunits, with a total protein mass of 32 kDa. Each 151 amino acid monomer folds as an eight-stranded Greek-key beta-barrel connected by three external loops. The active site of Cu/Zn SOD lies at the base of a deep cavity formed by two loops. These loops contain charged residues which facilitate the electrostatic guidance of superoxide to the active site (Hough and Hasnain, 2003). Cu/Zn SOD has both a Cu and a Zn ion embedded in its structure. The Zn is bound to three His and one Asp residues; these are His72, His81, Asp84 and His 63 in the human sequence. The Cu is bound to four His residues – His44, His46, His63 and His 121 in the human sequence. The two metal ions are connected via a histidine bridge. Comparison of the partial amino acid sequence of squirrel Cu/Zn SOD with those of the other mammals revealed that their overall structures are similar and that they are identical at these key metal sites.

Mn SOD is a tetramer of identical subunits that is found in mitochondria (Borgstah et al., 1992). Each newly synthesized human MnSOD subunit has 222 amino acids. After transport into the mitochondria and cleavage of a presequence that is a mitochondrial localization signal, each mature MnSOD subunit has 198 amino acids (Wispe et al., 1989). The monomers fold into two domains. The N-terminal domain consists of two long antiparallel alpha-helices. The C-terminal domain contains a central beta-sheet formed by three antiparallel beta strands with 4-6 surrounding alpha-helices.

The manganese atom is liganded by two residues from each N-terminal helix and two residues from the loops in the C-terminal domain. In all Mn SOD structures that have been characterized, the active site is highly conserved, being composed of three histidines and one aspartic acid residue; these are His 26, His74, Asp159 and His163 in the mature human Mn SOD sequence. Comparison of the partial amino acid sequence of squirrel Mn SOD and the sequences from other mammals revealed that their overall structures are similar. Residues that are expected to represent groups essential for catalytic function are strictly conserved in ground squirrel Mn SOD.

3.4.2 Transcriptional and translational control of Cu/Zn SOD and Mn SOD

Up-regulation of selected genes, with enhanced production of their protein products, contributes to adaptation during hibernation (Storey and Storey, 2004). The nylon microarray screening studies by our lab indicated that the SOD mRNA level was elevated in ground squirrel kidney during hibernation and this suggested that SOD protein might be elevated in hibernation. However, my results from western blotting of eight tissues of ground squirrels failed to find increased levels of either Cu/Zn and Mn SOD protein in kidney during hibernation. Cu/Zn SOD and Mn SOD protein levels were similarly unchanged in B.A.T, brain, heart, kidney, liver, lung and W.A.T. Interestingly, only skeletal muscle, showed significantly elevated levels the enzymes during hibernation (2.3-fold for Cu/Zn SOD and 1.6-fold for Mn SOD higher than euthermic levels).

Transcript levels of squirrel SOD in kidney were putatively elevated in the hibernation state but no increase in the level of the corresponding protein product

occurred. One reason for this may be SOD protein synthesis was arrested during hibernation. Protein synthesis is an energy-expensive process. Recent studies with ground squirrels confirmed that strong reductions in the rate of protein synthesis occur as part of hibernation. In squirrel kidney, the rate of protein synthesis in tissue samples from hibernating animals was just 15% of its euthermic level (both rates measured in vitro at a constant temperature) (Hittel and Storey, 2002). Two mechanisms may contribute to the general arrest of protein synthesis (including SOD synthesis). The first mechanism involves phosphorylation and inhibition of several important ribosomal initiation factors and their binding proteins (Frerichs et al., 1998; Storey and Storey, 2004) whereas the second mechanism is the state of ribosomal assembly (Hittel and Storey, 2002; Storey and Storey, 2004). Active translation occurs on polysomes whereas monosomes are translationally silent. It is possible that most of the transcripts of SOD were stored in the translationally silent monosome fraction during hibernation so that translation would be delayed until later, such as during the arousal process. During arousal, body temperature is rapidly rewarmed to 37°C supported by a massive increase in oxygen-based fuel consumption that is also accompanied by a large increase in oxygen free radical production. A rapid increasing of SOD protein during arousal could be achieved using stored mRNA transcripts that were previously elevated during hibernation. The same anticipatory up-regulation of mRNA during hibernation but delay of translation until arousal was also seen for the organic cation transporter gene in ground squirrel kidney (Hittel and Storey, 2002). Hence, this may be a common way to achieve rapid synthesis of selected proteins during arousal. This would allow the fastest resumption of kidney functions during the brief hours of the interbout.

Since both Cu/Zn SOD and Mn SOD were significantly elevated at protein level in skeletal muscle of hibernating ground squirrels, the transcription of both genes was also investigated. Both relative RT-PCR and semi-quantitative PCR showed that the increased Cu/Zn SOD and Mn SOD protein seen in hibernating ground squirrels was not associated with higher levels of gene transcription. So, how did SOD protein levels increase when there was no change in mRNA levels? Two possible methods could be suggested. One is a selective increase in the translation rate of SOD mRNA transcripts during hibernation, probably by maintaining SOD transcripts in the polysome fraction. The mRNA for several other genes is also selectively sequestered into the polysome fraction during hibernation to enhance the synthesis of a few specific proteins; the mRNA for fatty acid binding protein is one example (Hittel and Storey, 2002). The other possibility is a selective reduction in the rate of SOD degradation by proteolytic systems during hibernation. The overall rates of both protein synthesis and protein degradation are suppressed during hibernation as part of the coordinated metabolic rate depression; hence, the half-life of all proteins is extended (Storey and Storey, 2004). However, it is possible that mechanisms may be present that differentially affect the degradation of selected proteins.

Hibernating ground squirrels may spend up to eight months in a state of hibernation. The skeletal muscles of the animals endure low temperature and low blood flow while in torpor and physical activity of their muscles is very low. However, hibernating squirrels experience oxidative damage and atrophy to a much lesser extent that would be expected over eight months of cold ischemia and disuse in other species. Previous studies show that muscle 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). Furthermore, 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. Potential damage due to ischemia and atrophy during hibernation in skeletal muscle may be counteracted by the up-regulation of specific cytoprotective proteins. Previous studies has revealed several proteins, for example, H-FABP (Hittel and Storey, 2001), MLC1v and ND2 (Fahlman et al., 2000), PDK4 (Buck et al., 2002), and uncoupling protein 3 (UCP3) (Boyer et al., 1998), that are up-regulated and play protective roles in muscle during hibernation. The results of the current study add to this list both Cu/Zn SOD and Mn SOD, the enzymes that catalyze the removal of superoxide radicals. SOD protein content increased during hibernation, undoubtedly also enhancing net SOD enzymatic activity. SODs are thought to be the first line of cellular defense against oxidative damage by superoxide anion radicals. Cu/Zn SOD appears to destroy radicals which are normally produced within the cytoplasm. Mn SOD performs a similar role in the mitochondrial matrix, in particular dealing with superoxide radicals that are produced by the electron transport chain.

In conclusion, this study identified Cu/Zn SOD and Mn SOD protein as increased in skeletal muscle of hibernating thirteen-lined ground squirrels. However, transcripts of both genes were unchanged in skeletal muscle during hibernation versus euthermia. Hence, elevated SOD protein levels are probably due to altered translation of SOD mRNA during hibernation or reduced SOD protein degradation. Elevated Cu/Zn SOD and Mn SOD protein levels would result in increased activities of these enzymes and

enhanced antioxidant defense in skeletal muscle during hibernation. This could contribute to ischemia tolerance and/or defend against oxidative stress damage during periods of arousal when oxygen consumption by muscle increases rapidly to support shivering thermogenesis. These data proved targets for further research on cold ischemia survival and muscle disuse atrophy.

Table3.1

Degenerate primers used to amplify squirrel Cu/Zn SOD and Mn SOD cDNA.

Transcript detected	Oligonucleotides (5'→3')	Annealing Temperature	Product size
Cu/Zn SOD Degenerate	Forward: AARGCSGTGTGCGTGHTGAA Reverse: CACCTTTGCCCAAGTCATCT	60°C	381bp
Mn SOD Degenerate	Forward: GACCTGCCBTACGACTAYGG Reverse: CTCCCAGTTGATYACATTCC	56°C	542bp

Figure 3.1

Western blots showing Cu/Zn SOD protein content in tissues of hibernating versus euthermic ground squirrels.

A) Representative 2D-PAGE western blot showing Cu/Zn SOD from ground squirrel liver.

B) Representative Western blots showing Cu/Zn SOD protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.

C) Relative levels of Cu/Zn SOD protein in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean $\pm$ SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly greater than the corresponding euthermic value, $P < 0.05$.

B)

C)

Figure 3.2

Western blots showing Mn SOD protein contents in tissues of hibernating versus euthermic ground squirrels.

A) Representative 2D-PAGE western blot showing Mn SOD from ground squirrel liver.

B) Representative Western blots showing Mn SOD protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.

C) Relative levels of Mn SOD protein in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean $\pm$ SEM, n=4 independent determinations. * - Mean value for hibernating sample was significantly greater than the corresponding euthermic value, $P < 0.05$.

Figure 3.3

Degenerate PCR amplification of squirrel muscle Cu/Zn SOD and Mn SOD cDNA.

A) Left lane: Molecular weight markers (1 Kb ladder). Right lane: PCR product from amplification of Cu/Zn SOD cDNA using degenerate forward/reverse primers at 60°C annealing temperature.

B) Left lane: Molecular weight marker (1 Kb ladder). Right lane: PCR product from amplification of Mn SOD cDNA using degenerate forward/reverse primers at 56°C annealing temperature.

Figure 3.4

Partial nucleotide and deduced partial amino acid sequence of the ground squirrel (*Spermophilus tridecemlineatus*) Cu/Zn SOD and ground squirrel Cu/Zn SOD partial amino acid sequence aligned with human, mouse, and rat Cu/Zn SOD amino acid sequences

- A) Gene product that was amplified from squirrel muscle cDNA. Nucleotides and amino acids are numbered on the left. A single open reading frame was predicted from the nucleotide sequence to code for a polypeptide with 117 residues.
- B) Ground squirrel (*Spermophilus tridecemlineatus*) Cu/Zn SOD partial amino acid sequence aligned with human (*Homo sapiens*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*) Cu/Zn SOD amino acid sequences (Genbank accession numbers NP_000445, NP_035564, NP_058746, respectively). For convenience only those amino acids differing from the squirrel sequence are indicated; dashes (-) represent amino acids in the human, mouse, or rat sequences that are identical with the squirrel sequence. Periods are present in the alignment to indicate when an amino acid is not present in the coding region of one of the species. Bold and underlined amino acids are active sites.

A)

```

1      AAGCCGGTGTGCGTGCTGAAAGGCGACGGCCCGGTTAGGGCACCATCCACTTCGAGCAG
1      K P V C V L K G D G P V Q G T I H F E Q
61     AAGGCAAATAAAGTTGTGGTATCGGGACGCATTACAGGATTGGCTGAAGGCCAGCACGGA
21     K A N K V V V S G R I T G L A E G Q H G
121    TTCCACGTCCATCAGTTTGGCGATAACACACAAGGCTGTACAAGTGCAGGTCCTCACTTT
41     F H V H Q F G D N T Q G C T S A G P H F
181    AATCCTCAATCCAAAAACATGGTGGGCCAACAGATGAAGAAAGGCACGTTGGAGACCTG
61     N P Q S K K H G G P T D E E R H V G D L
241    GGCAATGTGATTGCTGACAAAGATGGTGTGGCCAATGTGTCCATTGAAGATTCTCTGATC
81     G N V I A D K D G V A N V S I E D S L I
301    TCACTCTCAGGACAGCATTCCATCATTGGCCGCACAATGGTGGTCCATGAAA
101    S L S G Q H S I I G R T M V V H E

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B)

Squirrel	...KPVCVLKGDGPVQGTIHFQKANK..VVVSGRITGLA	35
Human	mat-a-----i-n---esngp-k-w-s-k--t	40
Mouse	mam-a-----sgep--l--q---t	40
Rat	mam-a-----v-----sgep----q---t	40
Squirrel	EGQHGFHVHQFGDNTQGCTSAAGPHFNPQSKKHGGPTDEER	75
Human	--l-----e-----a-----l-r----k----	80
Mouse	-----y-----h-----a----	80
Rat	--e-----y-----t-----h-----a----	80
Squirrel	HVGD DL LGNV IA DKDGVANVSIEDSLISLSGQHSIIIGRTMVV	115
Human	-----t-----d-----v-----d-c-----l--	120
Mouse	-----t-g-----rv-----e-----	120
Rat	-----a-g-----rv-----e-----	120
Squirrel	HE.....	117
Human	--kaddlgkkgneestktgnagsrlacgvigiaq	154
Mouse	--kqddlgkkgneestktgnagsrlacgvigiaq	154
Rat	--kqddlgkkgneestktgnagsrlacgvigiaq	154

Figure 3.5

Putative squirrel Cu/Zn SOD amino acid sequence homology analysis.

A) Homology tree of partial squirrel, human, mouse, and rat Cu/Zn SOD amino acid sequences.

B) Homology matrix of the four amino acid sequences.

A)

B)

Homology matrix of Cu/Zn SOD sequences

Squirrel	100%			
Human	80.3%	100%		
Mouse	88.0%	80.7%	100%	
Rat	86.3%	79.8%	95.8%	100%

Figure 3.6

Partial nucleotide and deduced amino acid sequence of the ground squirrel (*Spermophilus tridecemlineatus*) Mn SOD gene product that was amplified from squirrel muscle cDNA. Nucleotides and amino acids are numbered on the left. A single open reading frame was predicted from the nucleotide sequence to code for a polypeptide with 180 residues.

1 TGCCGTACGACTACGGCGCCCTGGAGCCGCACATCAACGCCCAGATCATGGAGCTGCACC
 1 P Y D Y G A L E P H I N A Q I M E L H

 61 ACAGCAAGCACCACGCGGCCTACGTGAACAACCTGAACGTCACGGAGGACAAGTACCGCG
 20 H S K H H A A Y V N N L N V T E D K Y R

 121 AGGCGCTGGCCAAGGGAGATGTTACCGCTCAGGTAGCCCTTCAACCTGCACTGAAGTTCA
 40 E A L A K G D V T A Q V A L Q P A L K F

 181 ATGGCGGAGGTCATATCAATCACACCATTTTCTGGACAAATTTGAGTCCTAATGGTGGTG
 60 N G G G H I N H T I F W T N L S P N G G

 241 GAGAACCCAAAGGAGAGTTGCTGGAAGCTATCAAACGTGACTTTGGTTCTTTTGACAAGT
 80 G E P K G E L L E A I K R D F G S F D K

 301 TTAAGGAGAAGCTGACAACCTGTCTCTGCTGGTGTCCAGGGCTCAGGTTGGGGTTGGCTCG
 100 F K E K L T T V S A G V Q G S G W G W L

 361 GTTTCATAAAGGAGCAGGGACGCTTGCAGATTGCTGCCTGTGCTAATCAGGATCCATTGC
 120 G F N K E Q G R L Q I A A C A N Q D P L

 421 AAGGAACAACAGGTCTTATCCCACTGCTGGGGATTGATGTATGGGAGCATGCTTATTACC
 140 Q G T T G L I P L L G I D V W E H A Y Y
 481 TTCAGTATAAAAATGTCAGACCCGATTATCTAAAAGCTATTTGGAATGTCATCAACTGGG
 160 L Q Y K N V R P D Y L K A I W N V I N W

 541 AG
 180 E

Figure 3.7

Ground squirrel (*Spermophilus tridecemlineatus*) Mn SOD partial amino acid sequence aligned with the amino acid sequences of the precursor form of human (*Homo sapiens*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*) Mn SOD (Genbank accession numbers NP_000627, NP_038699, NP_058747, respectively) .

Only those amino acids differing from the squirrel sequence are indicated; dashes (-) represent amino acids in the human, mouse, or rat sequences that are identical with the squirrel sequence. Periods are present in the alignment to indicate when an amino acid is not present in the coding region of one of the species. Bold and Italic amino acids are only present in the precursor form Mn SOD, but not in the mature form of the enzyme. Bold and underlined amino acids are active sites.

Squirrel	PYDYGALEP	9
Human	mlsravcgtsrqlapvlgylgsrq khslpdl-----	40
Mouse	mlcraacstgrrlgpvavaagsrh khslpdl-----	40
Rat	mlcraacsagrrlgpaastagsrh khslpdl-----	40
Squirrel	HINAQIMEL <u>H</u> HSKHHAAYVNNLNVTEDKYREALAKGDVTA	49
Human	-----q-----e-q-----	80
Mouse	-----q-----a-e-h-----t	80
Rat	-----q-----t-----e-h-----t	80
Squirrel	QVALQPALKFNGGGHIN <u>H</u> TIFWTNLSPNGGGEPKGELLEA	89
Human	-i-----s-----	120
Mouse	-----k-----	120
Rat	-----s-----k-----	120
Squirrel	IKRDFGSFDKFKEKLTTVSAGVQSGWGWLGFNKEQGRLQ	129
Human	-----aa-v-----r-h--	160
Mouse	-----e-----am-v-----	160
Rat	-----e-----a-v-----	160
Squirrel	IAACANQDPLQGTGLIPLLGI <u>D</u> VWE <u>H</u> AYYLQYKNVRPDY	169
Human	---p-----	200
Mouse	---s-----	200
Rat	---s-----	200
Squirrel	LKAIWNVINWE.....	180
Human	-----nvterymackk	222
Mouse	-----nvterytackk	222
Rat	-----nvsqryivckk	222

Figure 3.8

Putative squirrel Mn SOD amino acid sequence homology analysis.

A) Homology tree of partial squirrel, human, mouse and rat Mn SOD amino acid sequences.

B) Homology matrix of the four amino acid sequences.

A)

B)

Homology matrix of 4 sequences

Squirrel	100%			
Human	93.9%	100%		
Mouse	94.4%	93.9%	100%	
Rat	93.9%	94.4%	98.3%	100%

Figure 3.9

Transcript expression analysis of Cu/Zn SOD and Mn SOD mRNA in ground squirrel muscle during hibernation by relative RT-PCR.

- A) Representative relative RT-PCR of Cu/Zn SOD and Mn SOD relative to alpha-tubulin in euthermic (C) and hibernating (H) ground squirrel muscle. Dilutions are 10^{-1} , 10^{-2} and 10^{-3} .
- B) Histogram showing relative Cu/Zn SOD and Mn SOD transcript levels in muscle of hibernating versus euthermic squirrels, as determined from semi-quantitative PCR. Data are means $\pm$ S.E.M., n= 4 independent trials with both hibernating and euthermic samples.

A)

B)

Figure 3.10

Example of Semi-quantitative PCR Calculation.

A) Ct Calibration curve used in semi-quantitative PCR (Dave McMullen, Carleton University).

B) Gel example for Semi-quantitative PCR in euthermic control muscle.

C) Calculation of slope of the PCR amplification. The band at cycle 24 was not used in the calculation because it was not visible. The bands at cycles 40 and 42 were not used because they were too saturated.

A)

B)

C)

Figure 3.11

Transcript expression analysis of Cu/Zn SOD and Mn SOD mRNA in ground squirrel muscle during hibernation by semi-quantitative PCR.

Histogram showing quantity of Cu/Zn SOD and Mn SOD templates in muscle of hibernating and euthermic squirrels, as determined from semi-quantitative PCR. Data are means $\pm$ S.E.M., n= 3 independent trials with both hibernating and euthermic samples.

Chapter 4

Thioredoxin system in hibernation

4.1 Introduction

Hibernating mammals undergo reductions in body temperature to near ambient and a strong reduction in heart rate and organ perfusion that can last for three weeks or more during a single torpor bout, a situation that would be ischemic under normal conditions. Every few days or weeks, hibernating animals periodically arouse, re-warming themselves to 37°C fueled by shivering and nonshivering thermogenesis that involves a huge increase in oxygen uptake and oxidative fuel catabolism. The torpor and arousal events resemble the phenomenon of ischemia-reperfusion which is well known to induce oxidative damage. Protective mechanisms must be in place in hibernators to counteract the effects of oxidative damage. The regulation of cellular redox balance is critically determined by the activity of several antioxidant systems. The cellular redox milieu with its metabolic, antioxidative, and regulatory aspects is largely maintained and regulated by two enzyme-based systems: the glutathione (GSH) and the thioredoxin (Trx) systems.

The classical thioredoxin system is formed by thioredoxin reductase (TrxR) and its characteristic substrate, the redox active protein thioredoxin (Trx), whose reduction at the expense of NADPH occurs by the following reaction.

In mammals, there are two main forms of Trx: Trx1 is the cytosolic and nuclear form and Trx2 is the mitochondrial form. Trx in the oxidized form (Trx-SS) is recycled to the reduced form, Trx-(SH)₂, by TrxR (Andoh et al., 2002). Reduced Trx is oxidized whilst providing reducing equivalents to target molecules (Arner and Holmgren, 2000).

Specific protein disulfide targets for reduction by Trx include protein disulfide isomerase (PDI) (Lundstrom and Holmgren, 1990) and a number of transcription factors such as p53 (Nordberg and Arner, 2001), NF- κ B and AP-1 (Matthews et al., 1992). Trx is also capable of removing H_2O_2 , particularly when it is coupled with either methionine sulfoxide reductase or several isoforms of peroxiredoxins (Wei et al., 2000; Chae et al., 1999). The members of the TrxR family are composed of three isoforms including cytosolic TrxR1, mitochondrial TrxR2, and TrxR3, also known as Trx and GSSG reductase (TGR). TrxR plays a key role in the protection of cells against oxidative stress and the redox-regulatory mechanisms of transcription factors and various biological phenomena (Mustacich and Powis, 2000).

Hibernation is a state of profound metabolic depression in which animals experience a number of natural stresses, including oxidative stress. The regulation of cellular redox balance is critically determined by activity of several antioxidant systems. The thioredoxin system-formed by TrxR and its substrate Trx, is an important constituent of the intracellular redox milieu. I hypothesized that changes in the cellular Trx system (Trx and TrxR) would aid the organs of hibernators in dealing with oxidative stress during hibernation and arousal. Using western blot analysis, the data in this chapter show that Trx1, Trx2 and TrxR2 proteins increased in multiple tissues in hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. These data strongly support the notion that the thioredoxin system is of importance in hibernation.

4.2 Materials and Methods

4.2.1 Animal Treatments

Animals were treated as previously described in Chapter 2.

4.2.2 2-Dimensional Polyacrylamide Gel electrophoresis

2-D PAGE was performed using the Modular Mini-Protean II System (Bio-Rad) as described in Chapter 2. Aliquots (30 µg protein) of squirrel liver extracts were loaded onto the surface of the first dimension gel (pH 3.5 to 10), and then isoelectric focusing was carried out. After this, first dimension gels were removed from capillary tubes and loaded onto an SDS polyacrylamide gel (12%) for second dimension separation by molecular mass, followed by immunoblotting and protein visualization. Primary antibodies to rabbit Trx1(LF-PA0002), Trx2 (LF-PA0012), TrxR1 (LF-PA0023), and TrxR2 (LF-PA0024) were purchased from Lab Frontier (Seoul, Korea). Secondary HRP-linked goat anti-rabbit IgG (H&L) (#7047) was purchased from Cell Signaling Technology. Primary antibody was used at a dilution of 1:2000 v/v in 1X TBST. The secondary antibody was used at a dilution of 1:2000 v/v in 1X TBST. Blots were developed using the enhanced chemiluminescence assay with SuperSignal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned by ChemiGenius Bio Imaging System (SynGene).

4.2.3 Western Blotting

Western blotting using one dimensional SDS-PAGE was performed as described in Chapter 2. Total protein was isolated from samples of B.A.T., brain, heart, kidney, liver, lung, muscle and W.A.T. of euthermic and hibernating ground squirrels. Aliquots containing 10 µg of protein were loaded into each well of 12% SDS gels, followed by electrophoresis, and then transfer to PVDF membrane. Each blot was incubated in 2.5%

milk for 30 minutes. The same primary and secondary antibodies were used as in section 4.2.2. All primary antibody incubations for western blotting were carried out at 4°C overnight in 1X TBST at a 1:2000 v/v dilution. Each blot was washed for 3 x 5 minutes in 1X TBST prior to the addition of secondary antibody. Secondary antibody (1:2000 v/v dilution) incubations were carried out for 2-3 hours at room temperature and followed by 3 x 5 minute washes in 1X TBST. Blots were developed using the enhanced chemiluminescence assay using SuperSignal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene).

4.3 Results

4.3.1 Western Blotting analysis of Trx1 protein expression in tissues

Two dimensional gel electrophoresis and western blotting were used to examine the specificity of the Trx1 antibody (Figure 4.1 A). Squirrel liver soluble proteins were resolved in the first dimension by isoelectric point (pI), and in the second dimension by molecular mass. Treatment with Trx1 antibody revealed a reaction with a single spot at ~12 kDa with a pI of about 4.5, consistent with the molecular mass (12kDa) and pI value (4.67) in the literature for Trx1 (Pasquali et al., 1996).

Western blotting was performed to study Trx1 translational regulation in squirrels. Figure 4.1 B and C show the results of western blot analysis of Trx1 protein levels (quantifying a 12 kDa band) in six tissues of ground squirrels and plotting the ratio hibernating:euthermic protein levels. Trx1 levels were significantly elevated ($P < 0.05$) in three organs during hibernation, by 1.4-fold in B.A.T. and lung and by 1.9-fold in liver.

However, Trx1 levels fell in hibernating brain, kidney and muscle to 35%, 42% and 73% of the corresponding euthermic values, respectively. No Trx1 protein was detected in heart and W.A.T. using this Trx1 antibody. This may be because the concentration of Trx1 in these tissues is very low.

4.3.2 Western Blotting analysis of Trx2 protein expression in tissues

Figure 4.2 shows a similar analysis of Trx2. The 2-D electrophoresis showed crossreaction of the Trx2 antibody with one spot at ~12 kDa with a pI of ~7, consistent with the theoretical pI value of Rat Trx2 (Accession number P97615, pI = 7.74) as calculated by SWISS-2D search (<http://us.expasy.org/cgi-bin/ch2d-search-ac>).

Figure 4.2 B and C show the results of Western blot analysis of Trx2 protein levels in five tissues of ground squirrels and plotting the ratio hibernating:euthermic protein levels. Trx2 levels were elevated ($P < 0.05$) by 1.4-fold in B.A.T. and by 1.2-fold in liver during hibernation. However, the amount of Trx2 decreased in hibernating heart, kidney and muscle to 86%, 52% and 70% of the euthermic levels, respectively. No Trx2 protein was detected in brain, lung and W.A.T. using this Trx2 antibody. It may be because the concentration of Trx2 in these tissues is very low, the technique we use were not able to detect it.

4.3.3 Western Blotting analysis of TrxR2 protein expression in tissues

Figure 4.3 shows an analysis of TrxR2 protein levels. The 2 dimensional gel electrophoresis followed by treatment with TrxR2 antibody revealed a crossreaction with

one spot at ~60 kDa with a pI of ~6, consistent with the known molecular mass of TrxR2 and the theoretical pI value of human TrxR2 (Accession number Q9NNW7, PI=7.23) calculated by SWISS-2D search

Figure 4.3 B and C show the results of Western blot analysis of TrxR2 protein levels in six tissues of ground squirrels and plot the ratio hibernating:euthermic protein levels. TrxR2 levels were significantly elevated ($P < 0.05$) in three organs during hibernation, by 1.9-fold in brain, by 2.1-fold in liver and by 2.2-fold in lung. The amount of TrxR2 did not change in the other tissues during hibernation. No TrxR2 protein was detected in W.A.T. using this TrxR2 antibody.

4.4 Discussion

Abrupt changes in metabolic rate in ground squirrels arousing from hibernation may set up a condition of increased ROS formation. The most commonly observed response to oxidative stress is an enhancement of antioxidant defenses by increasing the baseline activities of key antioxidant enzymes and donors of reducing equivalents (e.g. thioredoxin, glutathione). The thioredoxin (Trx) system formed by Trx and TrxR plays an important role in the intercellular redox milieu (Mitsui et al., 1992; Nakamura et al., 1997). Trx is a small protein having strong antioxidant quenching capabilities and other multiple functions depending on the cellular redox state. Reduced Trx catalyzes the reduction of disulfide bonds in many proteins, and oxidized Trx is reduced by the action of Trx reductase (TrxR) and NADPH. As Trx is a strong antioxidant protein that has significant reducing capability against induced oxidative stress, it may serve to reduce

cellular oxidative stress in hibernating animals. Hibernation-induced changes to the Trx system have not previously received attention except for a study from our lab showing increased thioredoxin peroxidase activity in heart of hibernating bats (S. Eddy, 2003. Ph.D. thesis).

The present study shows that levels of both the cytosolic/nuclear Trx1 and mitochondrial Trx2 increased in B.A.T. and liver during hibernation and Trx1 also rose in lung. However, kidney and muscle showed a consistent reduction in both Trx1 and Trx2 during hibernation. Other tissues were harder to evaluate because of low Trx levels and/or lack of antibody crossreaction. Trx1 acts as a hydrogen donor for ribonucleotide reductase, which is critical for DNA synthesis, and modulates the DNA-binding activity of several transcription factors, including NF- κ B, AP-1 and p53, (Junn et al., 2000; Arner and Holmgren, 2000). Trx1 also stimulates cell growth, is an inhibitor of apoptosis and plays a role in the protection against oxidative stress (Matthews et al., 1992; Tanaka et al., 2000). Trx2 is uniquely expressed in the mitochondria and plays a crucial role in scavenging ROS in mitochondria and regulating the mitochondrial apoptosis signaling pathway, where it regulates the mitochondrial redox state and plays an important role in cell proliferation (Damdimopoulos et al., 2002; Tanaka et al., 2002). Since both Trx1 and Trx2 are known as regulators of the manifestation of apoptosis under redox-sensitive signaling, potential coordinated actions between Trx1 and Trx2 may be possible. The simultaneous up-regulation of Trx1 and Trx2 in B.A.T. and liver (and elevated Trx1 in lung) during hibernation suggests that overexpression of Trx may protect these tissues from oxidative stress and suppress an apoptosis response to oxidative stress during hibernation or arousal. The response in B.A.T. is particularly interesting because other

antioxidant defenses are known to be up-regulated in hibernator brown adipose in order to deal with high rates of oxyradical production associated with the huge increase in oxygen consumption that supports thermogenesis during arousal. The reduced levels Trx1 and Trx2 protein in brain, heart, kidney and muscle might suggest that these organs are less susceptible to oxidative stress during hibernation/arousal or that some other mechanism of antioxidant defense (e.g. the GSH system) may be of greater importance in these organs.

Mitochondrial TrxR2, the enzyme responsible for reducing oxidized Trx2, may be a key player in the regulation of mitochondria-dependent apoptosis and antioxidant defense (Paternaude et al., 2004). However, little is known on the functions of the mitochondrial thioredoxin system and its potential role in the regulation of cell survival. Recent studies suggest that the mitochondrial Trx system is essential for mammalian development since disruption of the Trx2 gene in the mouse confers a lethal embryonic phenotype associated with massive apoptosis during early embryogenesis (Nonn et al., 2003). The present data show that TrxR2 increased in brain, liver and lung during hibernation and was unchanged in B.A.T, heart and muscle. Thus, liver showed a coordinated increase during hibernation in all three components of the thioredoxin system that were measured. Enhanced TrxR2 protein content indicates a need for elevated activity of this enzyme and, therefore, suggests that there is a need for higher rates of Trx reduction during hibernation and/or arousal, probably arising due to higher rates of consumption of reduced Trx under oxidative stress conditions.

In conclusion, hibernation and/or arousal can induce oxidative damage to the organs of hibernating animals. Protective mechanisms must be in place to counteract the

effects of oxidative damage. Trx is a strong redox-regulating antioxidant protein that has significant quenching capacity against induced oxidative stress. The results of the present study demonstrate that cytosolic/nuclear Trx1, mitochondrial Trx2 and mitochondrial TrxR2 increased in multiple tissues of hibernating ground squirrels and this demonstrates that the Trx system contributes significantly to antioxidant defense during hibernation.

Figure 4.1

Western blots showing Trx1 protein content in tissues of hibernating versus euthermic ground squirrels.

- A) Representative 2D-PAGE western blot showing Trx1 from ground squirrel liver.
- B) Representative Western blots showing Trx1 protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.
- C) Relative levels of Trx1 protein in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean $\pm$ SEM, n=3 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

B)

C)

Figure 4.2

Western blots showing Trx2 protein content in tissues of hibernating versus euthermic ground squirrels.

A) Representative 2D-PAGE western blot showing Trx2 from ground squirrel liver.

B) Representative Western blots showing Trx2 protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.

C) Relative levels of Trx2 protein in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean $\pm$ SEM, n=3 independent determinations. * - Mean value for hibernating sample was significantly different from the corresponding euthermic value, $P < 0.05$.

A)

B)

C)

Squirrel Trx2 protein level (hibernating vs control)

Figure 4.3

Western blots showing TrxR2 protein content in tissues of hibernating versus euthermic ground squirrels.

- A) Representative 2D-PAGE western blot showing TrxR2 from ground squirrel liver.
- B) Representative Western blots showing TrxR2 protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels.
- C) Relative levels of TrxR2 protein in tissues from hibernating versus euthermic ground squirrels as determined from Western blotting. Histogram shows mean $\pm$ SEM, n=3 independent determinations. * - Mean value for hibernating sample was significantly greater than the corresponding euthermic value, $P < 0.05$.

A)

B)

C)

Chapter 5

General Discussion

Hibernation is a state of profound torpor where metabolic rate is typically reduced to less than 5% of the euthermic resting rate and core body temperature (T_b) often drops to near 0°C. During torpor, heart beat is also reduced to just a fraction of normal so that organ perfusion rates fall by approximately 90%, creating conditions that would constitute severe ischemia in most mammals but are noninjurious to hibernators (Frerichs et al, 1994). Previous studies have demonstrated the potentially destructive role of ROS during prolonged ischemia/reperfusion, which cause oxidative stress (Ambrosio et al., 1993; Nohl et al., 1993). During ischemia/reperfusion, electrons were found to leak from the mitochondrial respiratory chain to O_2 and generate active O_2^- (Nohl et al., 1993). At the same time, production of oxygen free radicals increases in cells and toxic oxygen metabolite production is exacerbated by re-admission of oxygen during reperfusion (Ferrari et al., 2004). However, hibernating animals appear to utilize a variety of adaptive processes that could act in concert to protect vital organs during the pronounced fluctuations in blood flow and oxidative metabolism characteristic of hibernation and periodic arousal.

Overall, the work presented in this thesis demonstrates that antioxidant defense may play an important role in the survival of oxidative stress during mammalian hibernation. The goal of the research in this thesis was to analyze the expression of several proteins with key roles in antioxidant defense using PCR and western blotting techniques to examine both gene and protein expression. Heme oxygenase 1 (HO1) was shown to increase at both the mRNA and protein level in liver and other tissues of hibernating ground squirrels. It is believed that increased expression of HO1 increases the capacity to degrade the free heme which promotes the conversion of low-density

lipoprotein to cytotoxic oxidized products, and produces biliverdin and CO which contribute to the protective response against oxidative stress. In addition, this study presented data that suggests that small amino acid differences in HO1 of hibernating animals may aid the continued optimal function of the enzyme at the low body temperatures characteristic of the hibernating state. The Nrf2/ARE pathway is a common pathway regulating HO1 gene expression. My data on the transcription factor Nrf2 and its cofactor MafG are consistent with the specific requirement for the Nrf2/ARE pathway in the activation of HO1 in hibernators, particularly in liver. These findings suggest that tissue-specific HO1 expression in hibernating ground squirrels may be regulated via the Nrf2/ARE pathway and offer a new mode of antioxidant defense for hibernating animals.

The increased protein expression of both cytosolic and mitochondrial superoxide dismutase (Cu/Zn SOD and Mn SOD, respectively) in skeletal muscle would probably increase the catalytic capacity for the dismutation of superoxide radicals that can leak from the electron transport chain under ischemia stress. As a consequence this would enhance antioxidant defense in skeletal muscle during hibernation. The increased levels of thioredoxin (Trx) and the mitochondrial thioredoxin reductase (TrxR2) in multiple tissues of ground squirrels would also contribute to the balance of the intracellular redox milieu during hibernation. Overall, these changes in the gene and protein expression of antioxidant genes/proteins in hibernation contribute substantially to our understanding of the antioxidant defenses adaptations needed for hibernation.

This investigation also revealed that the transcript levels of some genes are not always consistent with their translation level in hibernators. Furthermore, the transcription and translation regulation of genes is highly tissue dependent. For example,

in liver, HO1 increased at both the mRNA and protein level; in heart, HO1 decreased at the mRNA level in heart, but increased at the protein level. In most cases, the availability of mRNA substrate is a primary determinant of protein level. However, changes in mRNA transcript levels do not always imply changes of equal magnitude in protein levels or in the impact on metabolism. Protein levels are also influenced by several additional layers of control including post-transcriptional processing of mRNA, differential mRNA stability, selective translation of transcripts by ribosomes, suppression of protein degradation, etc (Storey and Storey, 2004). Moreover, each tissue of hibernators contains different cell types and plays a different role in hibernation. Many genes that are known to respond to hibernation are expressed in a tissue-specific pattern and provide a particular protective mechanism to the specific tissue. Examples of organ-specific up-regulation of genes include alpha-macroglobulin in liver, fatty acid binding protein in B.A.T. and heart, and the ventricular isoform of myosin light chain 1(MLC1v) in heart and skeletal muscle (Srere et al., 1995; Hittel and Storey, 2001; Fahlman et al., 2000). In our study, the differential expression patterns of antioxidant genes and proteins also suggest that the needs for antioxidant defense and/or mechanisms providing antioxidant defense are also highly tissue dependent.

Hibernation has been regarded as a natural model for studying ischemia tolerance by a variety of researchers (Carey et al., 2001; Zhou et al., 2001). A growing literature exists showing the importance of antioxidant defenses against ischemia-reperfusion injury during torpor. Indications of the need for adequate antioxidant defenses in hibernators include demonstrations of increased concentrations of plasma ascorbate (Drew et al., 1999), an increased amount of the stress protein GRP75 and activation of

nuclear factor- κ B in gut (Carey et al., 2000), a shift in glutathione redox balance to the more oxidized state (Carey et al., 2003b), and higher activities of SOD and glutathione peroxidase during hibernation (Buzadzic et al., 1990; Buzadzic et al., 1992; Buzadzic et al., 1997). My study supplied additional data about antioxidant defenses and a new model for the regulation of antioxidant defense in hibernation. The demonstration of increased activities of the oxidative stress indicator, HO1 in multiple tissues of hibernating ground squirrels could be interpreted as evidence that hibernation causes increased oxidative stress and, therefore, elicits enhanced antioxidant defense. The data also indicate that HO1 (and perhaps other antioxidant adaptations) is regulated during hibernation by a common antioxidant regulation pathway, the Nrf2/ARE pathway. The up-regulation of Cu/Zn SOD and Mn SOD in muscle is consistent with the previous finding of increased SOD enzyme activity during hibernation (Buzadzic et al., 1997). The increased Trx1, Trx2 and TrxR2 in multiple tissues of ground squirrels indicated a shift in cellular redox balance to a more reduced state and may remedy the glutathione redox imbalance in hibernating ground squirrels (Carey et al., 2003b). In conclusion, previous and current studies both suggest enhanced antioxidant defense during hibernation constitutes an important protective component of hibernation physiology.

Perspectives

In this thesis I applied PCR and western blot techniques to describe the regulation of several important antioxidants at gene and protein levels. The protein products encoded by these genes likely alter the physiological parameters of the tissues and organs of hibernating animals so that they survive under conditions of reduced metabolic rate,

low blood flow and low body temperatures during torpor. The partial protein sequence of ground squirrel HO1 presented some distinct amino acid substitutions compared to other mammals which might be important in helping to maintain an active conformation of the enzyme over the wide range of body temperatures under which the hibernator enzyme has to function. Further work could determine the full length cDNA and protein sequence of ground squirrel HO1 using 5' and 3' RACE PCR in order to determine whether other key regulatory elements of the protein are similar or differ from the protein structure in other mammals.

The data on HO1, Nrf2 and MafG raised the possibility that HO1 was regulated via Nrf2/ARE pathway in response to oxidative stress during hibernation. Further analyses of the binding ability of Nrf2•MafG to the ARE in hibernating versus euthermic squirrels using EMSA technology will be necessary in order to determine if binding of the Nrf2•MafG heterodimer with the ARE increases during hibernation and transcriptionally activates the ARE. Additionally, the antioxidant response element (ARE) is a cis-acting DNA regulatory element located in the regulatory regions of multiple genes including antioxidant proteins as well as phase II detoxification enzymes including glutathione-S-transferases, NAD(P)H:quinineoxidoreductase-1, gamma-glutamylcysteine synthase, ferritin and HO1 (Chen and Kunsch, 2004). If Nrf2/ARE pathway activation can be established in hibernating ground squirrels, then the numerous downstream antioxidant gene and phase II detoxification enzymes, especially ferritin, potentially provide new targets for studies of antioxidant defense in hibernator. HO degrades heme and produces biliverdin, CO and iron. Both biliverdin and CO have antioxidant characteristics, thus the CO and bilirubin metabolites that result from the HO

reaction contribute directly to the protective response against oxidative stress. However, iron, which is an oxidant, is directly sequestered and inactivated by co-induced ferritin (Vogt et al., 1995). It would be interesting to determine whether ferritin is also co-induced with HO1 during hibernation via the Nrf2/ARE pathway to provide for the sequestering of iron released by the HO reaction.

Currently existing life forms are equipped with a very complex system for the control of free radical damage to cell constituents. The key antioxidant network includes antioxidant enzymes such as catalase, SOD, GPx, GST, HO1; cellular redox balancing systems such as the glutathione and thioredoxin systems; and, low molecular weight radical scavengers such as melatonin, urate and ascorbate. In the present study, HO1, Cu/ZnSOD, Mn SOD, Trx1, Trx2 and TrxR2 were shown to increase in multiple tissues of hibernating ground squirrels and, therefore, probably play important protective roles against oxidative stress during hibernation. It would be meaningful to test some other related members of the antioxidant defense to determine if the full antioxidant response is stimulated in different tissues of hibernating ground squirrels.

In summary, this thesis examined the differential expression of antioxidants and antioxidant-related genes and proteins in hibernating thirteen-lined ground squirrels. In some cases, amino acid substitutions in the encoded proteins (e.g. HO1) may support/enhance their function at low temperatures. I believe that up-regulation of antioxidant and antioxidant defense related genes at protein and mRNA levels essential for hibernating ground squirrels to tolerate the large potential for oxidative stress during hibernation. HO1 and some other antioxidant genes may be regulated by the Nrf2/ARE

pathway and the combinatorial nature of their in vivo expression patterns may lead to a unique antioxidant protein expression. These data also support the idea that antioxidant defense may be impaired during ischemia/reperfusion in non-hibernating species and suggest targets for directed studies to improve oxidative stress survival and cold preservation of organs in medical applications.

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