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The Caspase Cascade during Hibernation in the Golden-Mantled Ground Squirrel, Spermophilus lateralis

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THE CASPASE CASCADE DURING HIBERNATION IN THE GOLDEN-MANTLED GROUND SQUIRREL, *SPERMOPHILUS LATERALIS*

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ABSTRACT

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Spermophilus Lateralis

by

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In several human pathologies like heart attack, stroke, neurodegenerative diseases, and autoimmune disorders, widespread cell death, or apoptosis, is a major cause of organ dysfunction and death. Hibernating golden-mantled ground squirrels, *Spermophilus lateralis*, experience numerous conditions during the winter that are known to be pro-apoptotic in other mammal systems (e.g. extreme hypothermia, ischemia and reperfusion, acidosis, increased reactive oxygen species, bone and muscle disuse). However, studies suggest that hibernators may invoke a protective phenotype to limit widespread cell damage and loss during the hibernation season. Could regulating apoptosis provide protection against the harmful conditions experienced during the hibernation season? Could the lessons learned from studying the mechanisms of hibernation provide insights into new therapies for human pathologies? To address potential apoptotic regulation, I systematically examined a class of crucial apoptotic regulators, the caspase cascade (caspases 1-12), for evidence of apoptotic signaling and regulation during hibernation. Caspases comprise a family of cysteine-aspartate proteases that, upon proteolytic processing and activation, participate in a complex signaling cascade involved in apoptosis and inflammation.

Using ground squirrel liver, I determined the availability and activation status of caspases with western blots, performed caspase-specific enzymatic activity assays, and analyzed multiple caspase-mediated cellular events for indications of downstream caspase signaling during hibernation. Surprisingly, I found the canonical apoptotic executioner caspases 3 and 6, as well as inflammatory caspases 11 and 12, appeared activated during hibernation. Caspase activation typically has dramatic effects on enzymatic activity. For instance, in other systems, when caspase 3, the key executioner of apoptosis, is processed into the active 17 kDa (p17) fragment, caspase 3 enzymatic activity can increase >10,000X compared to the procaspase form. Therefore, caspase 3 activation is thought to commit a cell to apoptosis. I found caspase 3 p17 increased ~2X during hibernation which may indicate significant apoptotic commitment. Did these seemingly winter-activated caspases display increased activity? Using *in vitro* enzymatic assays, I found no indications of dramatically increased caspase activity. To better understand the implications of seeming caspase activation during hibernation, I used a systems-level approach to analyze several events downstream of caspase activation. I looked for indications of caspase 3 activity via degradation of the inhibitor of caspase-activated DNAse (ICAD), inactivation of DNA repair enzyme poly (ADP-ribose) polymerase (PARP), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) activity. Caspase 6 activity was determined via nuclear lamin A cleavage and inflammatory caspase activity was analyzed through IL-1β and IL-18 cleavage as well as serum transaminase levels. Despite the pro-apoptotic conditions of hibernation and the seeming caspase activations, I found no evidence of increased downstream caspase activity or evidence of widespread apoptosis and inflammation during hibernation. My data demonstrate that regulation of apoptosis during hibernation does not involve the *prevention* of caspase activation. Instead, partial activation of the caspase cascade does not result in predictable

downstream processing, thus demonstrating regulation of apoptosis during hibernation occurs at an unexpected locus. These data demonstrate the importance and utility of the systems-level approach in studying complex cellular signaling pathways like apoptosis during hibernation.

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CHAPTER 1

INTRODUCTION

Hibernation Overview

Metabolic depression in response to unfavorable environmental conditions is a common biological strategy employed in diverse species. Presumably in response to the limited resources associated with winter, many temperate mammals invoke a seasonal metabolic depression and hibernate. Hibernation is characterized by repeated oscillations between extended bouts (~1-3 weeks) of metabolic depression or torpor, wherein body temperature (T_b) and oxygen consumption are depressed below basal levels, and brief periods (~12-24 h) of euthermy called interbout arousals (IBA), wherein T_b and oxygen consumption are elevated to euthermic levels (Figure 1; for reviews, see van Breukelen and Martin 2002; Carey et al. 2003; Ruf and Geiser 2015).

Ground squirrels are exemplary hibernators and tolerate extreme physiological conditions while hibernating. While torpid, ground squirrels experience core T_bs as low as -2.9 °C, oxygen consumption rates as low as $1/100^{th}$ of active rates, extreme bradycardia (2-3 erratic heart beats per minute (bpm)), and low ventilatory rates (Barnes 1989; Frank 1992; Carey et al., 2003). Along with the severe depression in T_b and aerobic metabolism, numerous homeostatic processes (e.g. transcription, translation, protein degradation, mitosis, immune activity, and renal function) are known to be fundamentally depressed during torpor (Carey et al, 2003; van Breukelen 2016). The dramatic depression in physiology and metabolism seen in ground squirrel hibernation results in ~90% seasonal energetic savings. During interbout arousals, the physiological

depression is reversed. In the typical 6- to 9-month hibernation season, ground squirrels may experience ~15 to 20 torpor cycles.

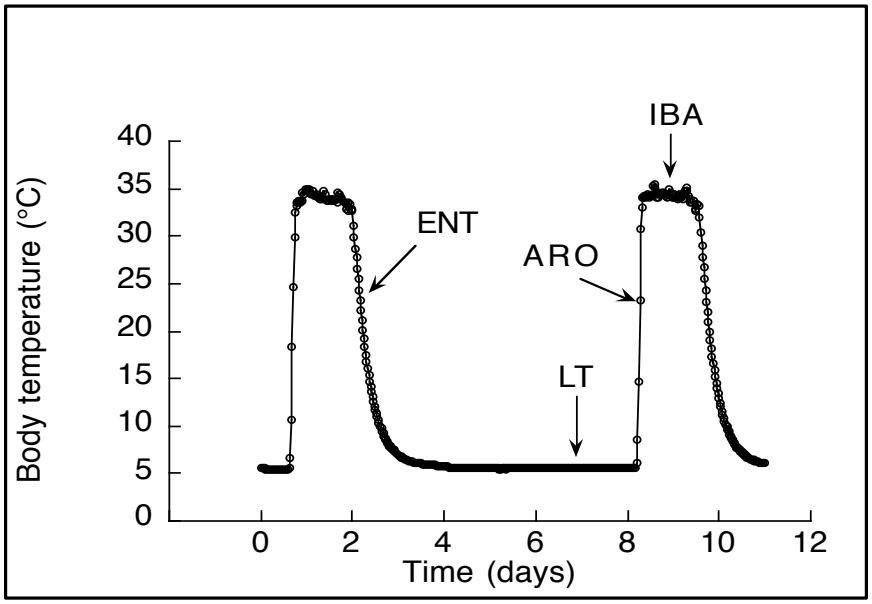

Figure 1. Body temperature during a typical hibernation cycle in the laboratory for golden-mantled ground squirrels, *Spermophilus lateralis.* Animals were implanted with temperature-sensitive radiotelemeters that allow for precise determination of torpor state. Major stages of the torpor cycle are indicated: ENT = entrance into torpor; LT = late torpor; ARO = arousal from torpor; IBA = interbout aroused. These torpor cycles are repeated approximately 15 times per season. The environmental chamber was maintained at 4 °C

During hibernation, ground squirrels experience conditions such as hypoxia, intracellular acidification, imbalances in ion homeostasis, mitochondrial dysfunction, increased oxidative damage, and the various effects associated with hypothermia, starvation, muscle and bone disuse, and ischemia/ reperfusion injury (van Breukelen et al., 2010). These conditions are known to be potent activators of programmed cell death, or apoptosis, in non-hibernating mammals like mice, rats, and humans. For instance, the transitions between torpor and IBA states likely represent periods of physiological mismatches.

When ground squirrels enter torpor, they experience dramatic and sudden changes in heart rate that precedes appreciable changes in T_b (Milsom et al., 1999). Increased

parasympathetic vagal tone during entrance results in a ~50% decrease in heart rate with only a 1 °C drop in T_b. Typically, ischemia/reperfusion injury occurs when blood flow to metabolically-active tissue is suddenly stopped for a period of time (ischemia) and then restored (reperfusion). Thus, entrance into torpor may represent a natural ischemia/reperfusion injury. Hibernating ground squirrels appear more tolerant to ischemia/reperfusion injury compared to non-hibernators (Kurtz et al. 2006; Otis et al., 2017; Bhowmick et al., 2017). Hippocampal slices from ground squirrels experienced a ~2 to 3-fold increase in cell survival during oxygen and glucose deprivation compared to non-hibernating rats (Frerichs and Hallenbeck 1998; Bhowmick et al., 2017). One explanation may be that ground squirrels are simply more tolerant to physiological insult than rats. However even within ground squirrels, seasonal differences in stress tolerance are evident. In a cold-storage paradigm (as is utilized in organ transplants), livers from hibernating squirrels had fewer signs of damage and increased survivorship compared to livers from non-hibernating ground squirrels sampled in the summer (Lindell et al. 2005; Otis et al. 2017). These data suggest a seasonal protective phenotype. Typically, apoptosis is widespread during the ischemia/reperfusion injury associated with heart attack and stroke in other model systems (Serracino-Inglott et al., 2001; de Oliveira et al., 2018). Could ground squirrels simply be adept at mitigating apoptosis in the face of physiological insult or are they invoking a protective phenotype?

Widespread apoptosis during hibernation is at odds with both conserving energy during a severely energy-limited state and overall animal survival, and, therefore, I hypothesize apoptosis is depressed in hibernating ground squirrels. The question of how hibernators specifically limit apoptotic induction in the face of numerous pro-apoptotic stressors remains.

Experimental System

Hibernation is widespread amongst the various mammalian taxa. However, most experimental research has been conducted in sciurid rodents (van Breukelen and Martin, 2015). Ground squirrels have been of particular interest due to their wide population distribution and deep hibernation with T_bs below -2 °C and oxygen consumption rates around 1% of active rates (Barnes 1989; Carey et al., 2003). Additionally, ground squirrels will readily hibernate and experience highly predictable torpor bouts when housed in environmental chambers in the laboratory.

All experiments herein were conducted with golden-mantled ground squirrels (*Spermophilus lateralis*) at the University of Nevada, Las Vegas. Animals were live trapped from local populations in Southern Nevada, Southern California, and Southern Utah. Prior to the onset of the hibernation season, ground squirrels had temperature-sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR) and/or iButton temperature loggers (model DS1922L; Maxim Inc., San Jose, CA) surgically implanted into the abdominal cavity. The radiotelemeters/ loggers allow for precise measurement of T_b across the hibernation season and determination of physiological state. For the entire duration of the hibernation season, animals were individually housed in constant dark conditions in environmental chambers set at T_a=4 °C and allowed to hibernate naturally. The University of Nevada, Las Vegas Institutional Animal Care and Use Committee approved all procedures related to the experiments detailed in this dissertation.

Historical Perspectives and Rationale for the Work

Despite over a century of interest in trying to understand hibernation in mammals, we are only recently gaining more mechanistic insights into this phenotype through comprehensive, systems-level studies. Part of the problem was the belief that hibernators were highly adapted to function in a steady-state fashion (e.g. maintain transcription, translation, protein metabolism) during torpor. Unfortunately, despite the years of research, very few *bona fide* hibernation-specific adaptations have been found that specifically enhance hibernator function while torpid and cold (van Breukelen and Martin, 2002). More recent work has demonstrated that numerous essential homeostatic processes like transcription, translation, and protein metabolism are essentially arrested at the low T_bs experienced during torpor. Hibernation, therefore, is not a steady-state condition (van Breukelen et al., 2008). Interestingly, while most cellular processes are coordinated during euthermy (e.g. protein synthesis and protein degradation rates are approximately matched) not all cellular processes are completely coordinated during torpor resulting in imprecise coordination of processes and the accumulation of various physiological mismatches that must be rectified in subsequent IBAs (van Breukelen et al., 2008; van Breukelen 2016). This lack of coordination has been termed 'homeostasis on hold' (van Breukelen et al., 2008). For instance, protein ubiquitylation is only moderately temperature sensitive and continues at ~30% even at 0 °C. Protein degradation at the 26S proteasome, however, is more temperature sensitive and is essentially arrested at the cold T_bs experienced during torpor. Decoupling the ubiquitylation-degradation pathway results in the non-specific accumulation of ubiquitylated proteins during torpor that must be rectified during IBA (Velickovska and van Breukelen, 2007). Clearly, steady-state assumptions are inappropriate in the non-steady-state condition of hibernation. Mechanistic investigations of hibernation, therefore, require a more

thorough and systems-level approach (van Breukelen, 2016). In the context of this study, a systems-level approach involves examination of upstream and/or downstream components of a pathway for indications of regulation.

The benefit of employing a systems-level approach for hibernation studies was also evident when our laboratory investigated the transcription factor p53 in hibernating ground squirrels (Pan et al. 2014). Previously, transcription was found to be virtually arrested during hibernation due to extremely limited transcriptional elongation rates at low T_bs (van Breukelen and Martin 2002). Other investigators found movement of transcription factors into or out of the nucleus during torpor and presumed functional significance to these movements (Fleck and Carey, 2005; Allan and Storey, 2012). Pan et al. (2014) found the expression of known activators or repressors of p53 function to be consistent with an activation of p53 in winter (both torpid and IBA) squirrels. Furthermore, p53 localized to the nucleus, associated with DNA, and partially recruited RNA polymerase II. However, and most importantly, the severe elongational depression of transcription did not result in predicted changes in p53 target gene expression. These data clearly indicated that while a seeming activation of p53 occurs during the hibernation season, the systems-level approach demonstrated ineffective p53 function consistent with the known depression of transcription. Without the systems-level approach used in this study, these data could have been readily misinterpreted.

Application of the systems-level approach towards the study of apoptosis during hibernation seems particularly warranted. Apoptotic regulation is complex with numerous factors acting in opposition to one another. There are numerous pro-apoptotic effectors that operate at various levels including extrinsic or intrinsic signaling pathways, the initiation of the apoptotic machinery, integration and spread of apoptotic signals, and modulation of the apoptotic cascade.

Similarly, anti-apoptotic effectors operate at all of these levels of regulation. Given this complexity, one requires a synthetic and exhaustive understanding. Simply measuring a particular regulator or two of apoptosis in the non-steady state condition of hibernation is unlikely to elucidate apoptotic control. Rather, a systematic examination of various components of a pathway, and reconciliation of those data, is required.

Mechanisms of apoptosis

Apoptosis is a form of programmed cell death that is important in development, homeostasis (e.g. maintaining tissue cell populations), immunity, and in response to cell stress or damage (Elmore, 2007; Ramirez and Salvesen, 2018). Different cell types respond differently to the various apoptosis-inducing stimuli, however, most cell types typically undergo similar morphological and biochemical changes once apoptosis has been initiated. Morphologically, apoptotic cells shrink and detach from neighboring cells, chromatin condenses (pyknosis), the cytoskeleton is modified and the plasma membrane starts blebbing, the nuclear envelope is disassembled (karyorrhexis), and finally apoptotic bodies are formed and released (Ramirez and Salvesen, 2018). These apoptotic bodies contain components of the disassembled cell and are phagocytosed by macrophages or neighboring cells and degraded in phagolysosomes. Apoptosis is a unique form of cell death considered to be immunologically silent in that it does not elicit secondary inflammatory responses and lytic cell death in surrounding cells.

The caspases: death by a thousand cuts

At the cellular level, apoptotic induction is highly complex and is dependent on the balance of the activities of cellular regulators that can induce apoptosis signaling (e.g. pro-

apoptotic BCL-2 family members like BAD, BAX, and BAK, the caspases, apoptosis-inducing factor (AIF)), or inhibit apoptosis signaling (e.g. anti-apoptotic BCL-2 family members like BCL-2 and BCL-XL, inhibitor of apoptosis proteins (IAPs), Akt; Czabotar et al., 2014; Philchenkov, 2004). My focus here will be on the components and mechanisms of apoptotic induction and execution. Apoptosis is typically induced via one or both of two major pathways (Logue and Martin, 2006; Elmore, 2007). The extrinsic pathway is mediated through plasma membrane death receptors (e.g. TNFR and FasR) that are responsive to extracellular death-inducing signals (e.g. tumor necrosis factor (TNF) and Fas ligand (FasL)). The intrinsic pathway is responsive to intracellular death-inducing signals (e.g. growth factor removal, pathogen infection, ROS) and is mediated through various apoptosis-inducing elements released from the mitochondria (e.g. cytochrome c, AIF; Fulda et al., 2010). In mammals, both of these pathways converge on a family of at least 12 cysteine-aspartate proteases essential to the execution of apoptosis known as the caspases (Ramirez and Salvesen, 2018). All caspases are produced as inactive zymogens (procaspases) which contain three domains: a pro-domain which typically has a recruitment motif, a large catalytic subunit, and a small catalytic subunit. Caspase activation is dependent on both intradomain proteolytic cleavage (often performed by other caspases) and large and small catalytic unit heterodimerization (Fuentes-Prior and Salvesen, 2004; Ramirez and Salvesen, 2018). The active site of caspases is a three-dimensional, four amino acid substrate binding pocket that is formed during dimerization in a region that typically spans the large and small catalytic units (Earnshaw et al., 1999; Fuentes-Prior and Salvesen, 2004). Due to the dramatic structural changes between procaspase and active caspase forms, activation of caspases has been shown to result in dramatic increases in caspase activity compared to the procaspase forms. For instance, upon activation, caspase 9 enzymatic activity increased ~2000-fold while

caspase 3 activity increased >10,000-fold (Boatright and Salvesen, 2003). Canonical apoptotic caspase signaling is a two-phase proteolytic signaling cascade where initiator caspases are activated and then activate executioner effector caspases. In addition to apoptosis, a subset of caspases were originally found to be involved in inflammation (McIlwain et al., 2013). Therefore, based on their canonical functions, the caspases were historically classified into three categories: initiator caspases (caspase 2, 8, 9, and 10), executioner caspases (caspase 3, 6, and 7), and inflammatory caspases (caspase 1, 4, 5, 11, 12). Most caspases are now known to participate in non-canonical pathways (Figure 2; Creagh, 2014). For instance, inflammatory caspase 1 is capable of activating executioner caspases 6 and 7 (Guo et al., 2006; Lamkanfi et al., 2008), while apoptotic initiator caspase 8 is known to be able to directly process inflammatory cytokine IL-1β (Man and Kanneganti, 2016).

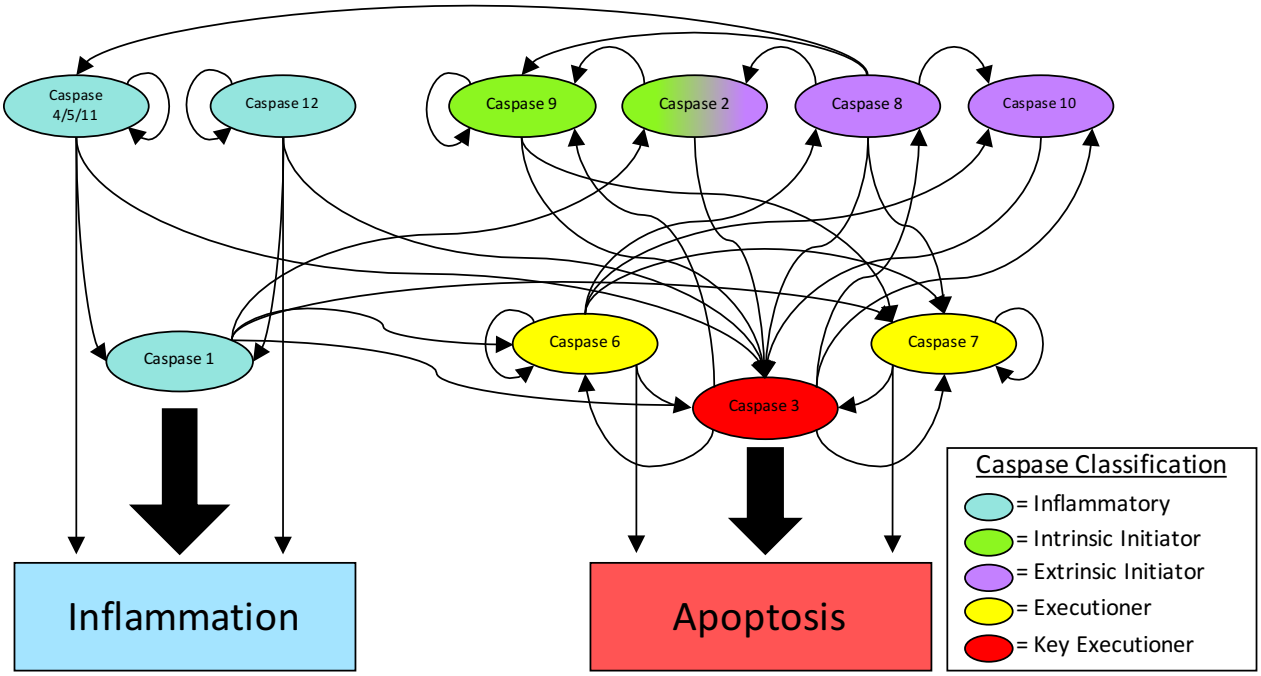

Figure 2. Caspase cascade activation network in mammals. Arrows represent known caspase-caspase activation interactions. Please note the complexity and interwoven nature of these caspase interactions with crosstalk between inflammatory and apoptotic caspases. For instance, classical inflammatory caspases, like caspase 1, are capable of apoptotic signaling through direct activation of caspases 3, 6, and 7 (Denes et al., 2012; Guo et al., 2006; Lamkanfi et al., 2008).

The initiation phase of apoptosis involves the integration of extrinsic and/or intrinsic death signals through formation of large cytosolic multiprotein structures that recruit and facilitate the activation of initiator caspases. After death receptors bind ligand, adapter proteins containing death effector domain (DED), such as Fas-associated death domain (FADD), are recruited to the cytosolic domains of the stimulated death receptors. A DED in the pro-domain of procaspase 8 facilitates recruitment to this death-inducing signaling complex, or DISC. Once associated with the DISC, procaspase 8 can be activated. Significant cellular stress or damage (e.g. DNA damage, unfolded protein response, hypoxia) results in intracellular signaling that act to stimulate mitochondrial outer membrane permeabilization (MOMP; McIlwain et al., 2013). Upon MOMP, a myriad of pro-apoptotic factors are released into the cytosol. In particular, cytochrome c release and cytosolic protein apoptotic protease activating factor-1 (Apaf-1) are integral in caspase 9 activation (Elmore, 2007; Parrish et al., 2013). Apaf-1 contains two regions critical for apoptotic signaling: a cytochrome c binding domain and a caspase-activating recruitment domain, or CARD. Cytochrome c binds to Apaf-1 causing a conformational change exposing Apaf-1's CARD and facilitates ~7 cytochrome c-bound Apaf-1 monomers to spontaneously assemble into a ring-like structure (Jiang and Wang, 2004). Similar to DED function in the extrinsic initiator pathway, the pro-domain of procaspase 9 also contains a CARD which is recruited to the CARD of Apaf-1, resulting in the formation of a structure known as the 'apoptosome' wherein caspase 9 is activated (Logue and Martin, 2006).

Presumably, crosstalk between extrinsic and intrinsic pathways exists to promote robust apoptotic signaling. Active caspase 8 cleaves pro-apoptotic BCL-2 family member BID (called tBID) that localizes to the mitochondria, stimulates MOMP, and results in intrinsic caspase pathway activation via caspase 9 (Gross et al., 1999; Kruidering and Evan, 2000).

Active initiator caspases specifically target and activate the executioner caspases 3, 6, and 7 in the execution phase of apoptosis (Earnshaw et al., 1999; Elmore, 2007; McIlwain et al., 2013). Executioner caspases are known to cleave ~300 cellular targets which results in the characteristic changes seen during apoptosis (Fischer et al., 2003). Caspase 3 has a central position in the caspase cascade and can process a majority of the cellular targets essential for proper apoptotic execution (Elmore, 2007). Significant caspase 3 activation in most cases is believed to be a point of no return in apoptosis due to caspase 3's central role in the caspase cascade and dramatic increases in caspase 3 enzymatic activity (>10,000-fold activity increase upon activation; Boatright and Salvesen, 2003; Rehm et al., 2002).

Most of the data currently available on apoptosis during hibernation focus on attempts to find increased activation of pro-survival, cellular stress response pathways like Akt and NF-κB (Carey et al., 2000; Allen and Storey, 2012) or anti-apoptotic regulators like anti-apoptotic BCL-2 members or inhibitors of apoptosis proteins (IAPs; Jain et al., 2016; Logan et al., 2016). Few efforts have been made towards examination of activation of the caspase cascade. Perhaps not surprisingly then, no definitive regulatory motif has been identified (Carey et al., 2003; Fleck and Carey, 2005; Zhang et al., 2011). Akt is a serine/threonine protein kinase that has anti-apoptotic activity (Zhang et al., 2011). Akt can affect apoptosis in two ways: 1) Akt phosphorylates and inhibits pro-apoptotic BCL-2 member, BAD; 2) Akt phosphorylates caspase 9 at Ser196 and directly inhibits activation of caspase 9 (Cai et al., 2004; Datta et al., 1997; Parrish et al., 2013). One study found a 3-fold increase in phosphorylated Akt (p-Akt) in bat brains during arousals from hibernation (Lee et al., 2002). Another study on hibernating bats showed p-Akt levels decreased in brain, kidney, and liver; showed total Akt and p-Akt levels decreased in white adipose tissue (WAT); and yet in brown adipose tissue (BAT), found that

both total Akt and p-Akt levels went up significantly during hibernation (Eddy and Storey, 2003). For activation of Akt, phosphorylation at S473 and T308 are required. In hibernating ground squirrels, phosphorylation at Ser473 decreased during hibernation while phosphorylation at T308 was maintained (Cai et al., 2004). Another study on various anti-apoptotic regulators like anti-apoptotic BCL-2 members: BCL-2, BCL-XL, BI-1, and MCL-1, and inhibitor of apoptosis proteins (IAP): cIAP1/2 and XIAP in hibernating 13-lined ground squirrels found coordinated increases in these regulators only in brain and heart tissue, and more variable levels were found in liver, kidney, skeletal muscle, and BAT (Rouble et al., 2013). It should be noted that the authors concluded that an outcome of these various phosphorylation motifs was always an adaptive mitigation of apoptosis.

The most comprehensive study of apoptosis in hibernators came from the Carey lab group. In a study by Fleck and Carey (2005), various anti-apoptotic (e.g. BCL-2, p-BCL-2, BCL-XL, Akt, and p-Akt) and pro-apoptotic (e.g. BAX, caspase 8 and 9 activation, caspase 3- activity, DNA laddering, and TUNEL) effectors and indicators were analyzed in intestinal mucosa from hibernating and non-hibernating 13-lined ground squirrels. This study found significant increases in TUNEL-positive cells during hibernation. However, no DNA laddering was evident. All BCL-2 family members in this study (both pro- and anti-apoptotic) increased during hibernation. Caspases 3 and 8 were not activated during hibernation. Caspase 9 was activated in early-season hibernators but not in late-season hibernators. The authors conclude there was no activation of apoptosis during hibernation even though they acknowledge increased DNA damage. Careful analysis of their images reveals cytosolic TUNEL staining suggesting high background may not have allowed for effective conclusion-making (van Breukelen et al., 2010).

In addition to apoptosis, caspases 1, 4, 5, 11, and 12, function as regulators of inflammation through activating and releasing pro-inflammatory cytokines like IL-1β and IL-18 in response to pathogens or cellular damage (McIlwain et al., 2013). Caspase 1 is the canonical inflammatory caspase as it directly cleaves and actives numerous pro-inflammatory cytokines. Caspase-mediated inflammatory signaling is similar to initiator caspase 8 and 9 activation as it relies on the formation of multimeric protein platforms, known as inflammasomes, that recruit and activate inflammatory caspases (Man and Kanneganti, 2016). An essential component of inflammasomes are NOD-like receptors (NLRs) that sense various pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs; Szabo and Petrasek, 2015). Similar to Apaf-1 function, NLRs are held in an inactive conformation until they bind PAMP- or DAMP-associated ligands which induces inflammasome assembly (McIlwain et al., 2013). NLRs also have a NACHT domain that, after inflammatory ligand binding, recruits adapter protein apoptosis-associated speck-like protein containing a CARD (ASC/PYCARD), known simply as ASC. In canonical inflammatory signaling, ASC association with NLRs permits recruitment and activation of caspase 1. Inflammatory caspases are also known to induce a distinct form of cell death known as pyroptosis that results in lytic cell death which is presumed to exacerbate the inflammatory, and hence, immune response to a specific area in an organism.

Although complex, apoptotic signaling may be studied in mammalian hibernation. The physiological consequences of torpor use would be pro-apoptotic in other systems. However, widespread apoptosis is at odds with the energy-saving strategy of hibernation. I chose to focus on caspase function since these proteases are responsible for the execution of apoptosis. The complex nature of caspase activation and regulation requires a systems-level approach that will

allow dissection of the pathways at multiple levels. By identifying the locus/loci of regulation, I hope to resolve an otherwise complex apoptotic signaling in the context of a non-steady state condition like mammalian hibernation.

CHAPTER 2

CASPASE 3 REGULATION DURING HIBERNATION

Abstract

Hibernating mammals naturally experience conditions that in other systems result in apoptosis. A family of zymogenic cysteine-aspartate proteases known as the caspases regulates apoptosis through a proteolytic signaling cascade. Active caspase 3, known as the key executioner of apoptosis, cleaves numerous cellular targets and results in processes like rapid membrane breakdown and DNA fragmentation. I used a systems-level approach to investigate caspase 3 and the status of apoptosis during mammalian hibernation. I found caspase 3 to be activated ~2-fold in winter squirrels. Caspase 3 activation typically results in >10,000-fold increased enzymatic activity. Activity assay data demonstrate no increases in caspase 3 activity as would be expected with *bona fide* caspase 3 activation. Analysis of downstream caspase 3 targets, ICAD and PARP, as well as TUNEL assays revealed no indications of increased *in-vivo* caspase 3 activity. These data demonstrate that despite the *seeming* caspase 3 activation in winter squirrels, there is no downstream commitment to undergo apoptosis during hibernation. These results are consistent with the idea that hibernation is a non-steady state condition where homeostatic processes, including important signaling pathways, are suspended.

Introduction

Presumably in response to low environmental temperatures and limited resources, many mammals enter into an extended state of torpor known as hibernation (for review see, Carey et al., 2003, van Breukelen and Martin, 2002). Ground squirrels are particularly adept at

hibernating. During hibernation, ground squirrels enter torpor where oxygen consumption may be as low as 1/100th of active rates and T_b may approach ambient temperature (T_a) to as low as –2.9 °C (Wang and Lee, 2011; Barnes, 1989). The physiological changes are also reflected on the cellular level where hibernators experience a virtual block in homeostatic cellular processes like transcription and translation (van Breukelen and Martin, 2002; van Breukelen et al., 2002). However, hibernation is not a static state. Instead, extended bouts of torpor (up to 3 weeks depending on time of season, species, and T_a) are interrupted by brief, 12-20 h bouts of euthermy, known as interbout arousals (IBAs). Hibernation is a dynamic state wherein animals experience repeated shifts in T_b, metabolism, and physiology as they transition between torpor and euthermy.

Despite a common misperception that ground squirrels are highly adapted to hibernate, the hibernation season is associated with high mortality. As many as 40% of adult ground squirrels and up to 70% of juvenile ground squirrels may not survive the hibernation season (Sherman and Morton, 1984). During hibernation, ground squirrels experience 1) extreme bradycardia as heart rate is reduced from ~250 beats per minute (bpm) to 2-3 erratic bpm, 2) extreme hypothermia with T_b to as low as –2.9 °C in some species (Barnes 1989), 3) acidosis (Clausen and Ersland, 1968; Bock et al., 2002), 4) oxidative damage during transitions into and out of torpor (Carey et al., 2000), and 5) anorexia for the 6- to 9-month hibernation season. These five conditions are well-characterized stressors known to result in apoptosis in other systems (van Breukelen et al., 2010). Could widespread apoptosis during hibernation be partially responsible for the high mortality seen in hibernating ground squirrels?

When mammalian cells are exposed to significant levels of stress or damage, they typically undergo apoptosis. Apoptosis is regulated by cysteine-aspartate proteases called caspases (see Elmore, 2007; Ramirez and Salvesen, 2018 for review). Caspases are produced as

inactive zymogenic procaspases that require internal processing from other proteases (typically other caspases) in order to become fully active. Traditionally, caspases are thought to signal in a cascade fashion with most upstream signaling converging on and activating caspase 3. Due to caspase 3's central role in integrating upstream apoptotic signals, processing numerous apoptotic substrates, and directly executing the apoptotic program, it is known as the key executioner of apoptosis (Hirata et al., 1998). Activation of procaspase 3 to the p17 fragment results in a >10,000-fold increase in enzymatic activity (Boatright and Salvesen, 2003; Stennicke and Salvesen, 1998). Caspase 3 activation typically commits cells to apoptosis (Porter and Janicke 1999).

Despite the pro-apoptotic conditions of hibernation, widespread apoptosis would be detrimental to survivorship. Are hibernators more tolerant to the stresses of hibernation? Hibernators do appear more tolerant to reduced blood flow or ischemia than non-hibernators (Frerichs and Hallenbeck, 1998; Frerichs et al., 1994, 1995; Kurtz et al., 2006). For example, *in vitro* studies on hibernator brain slices demonstrated that ground squirrel brain slices survived oxygen and glucose deprivation 2-3 fold longer than rat counterparts (Frerichs and Hallenbeck, 1998; Bhowmick 2017). Although it is plausible to attribute these results to inherent differences between rats and squirrels (i.e. squirrels are better able to withstand harsher conditions), additional data indicate that hibernators may exploit a seasonal shift in their physiology. Using a cold ischemia and reperfusion paradigm, increased survivorship has been noted in livers derived from ground squirrels in the winter compared to both rat and summer active (SA) squirrels (Lindell et al. 2005). A recent study also confirms increased resistance to cellular damage in hibernating ground squirrels when livers were subjected to warm ischemia and reperfusion (Otis et al., 2017). These data suggest that ground squirrels may invoke seasonal protective

mechanisms during hibernation, although the precise nature of this seasonal difference remains to be elucidated. One possibility is that increased tolerance may be achieved through increased regulation of caspases and mitigation of apoptotic processes.

Caspase 3 function during hibernation has been studies previously. Activated caspase 3 during torpor and IBA has been suggested in kidney (Jani et al., 2011; Jain et al., 2016). Activate caspase 3 was also evident in white adipose tissue during late torpor and early arousal (Logan et al., 2016). In these studies, activation of caspase 3 was presumed based on cleavage of the procaspase. An appropriate question is if cleavage of procaspase 3 translates into *bona fide* activity? Fleck and Carey (2005) further elaborated on caspase activation by showing that interbout aroused squirrels assayed at 37 °C had measureable caspase 3 activity, although reduced from that of summer squirrels. Confusingly though, Fleck and Carey found evidence for increase TUNEL activity but no DNA laddering. Was apoptosis really occurring? Importantly, the TUNEL staining was not restricted to the nucleus but instead was evident in the cytosol suggesting their interpretations may have been misguided. I will clarify these inconsistencies by applying a systems-level approach to better define the locus/loci of regulation.

Materials and Methods

Animal care and tissue collection

Adult golden–mantled ground squirrels (*Spermophilus lateralis*) were captured during the summer from southern Nevada, southern Utah, and California. Some animals were trapped and killed immediately as a seasonal control (summer active, SA). The remaining squirrels were implanted with temperature sensitive radiotelemeters as described previously in order to allow for precise determination of torpor status. (Martin et al., 1999). Implanted squirrels were housed

in an environmental chamber at 4° C to facilitate hibernation. The body temperature of torpid squirrels was ~ 5° C. In some cases, torpor status was tracked through surface temperatures using an infrared thermometer. All animals were killed by CO_2 asphyxiation except for the torpid and early arousal animals which were killed by decapitation because of their low respiratory rates. Tissues were collected and snap frozen in liquid nitrogen and stored at -80° C until use. Tissues were collected from animals in the summer (Summer Active, SA), while torpid (T), or while hibernating animals were euthermic between torpor bouts (Interbout Aroused; IBA). As indicated later, TUNEL assays were performed on additional tissues obtained from squirrels that were naturally arousing from torpor and whose body temperatures were approximately 10, 20, and 30 °C at the moment of sampling.

Sample preparation and western blot analyses

Livers were pulverized in liquid N_2 and homogenized in three volumes of 50 mM tris•HCl, pH 8.3, 20% glycerol, 2% SDS, and 0.4 M β-mercaptoethanol using glass/glass homogenization. The homogenate was centrifuged at 20,000 X g @ 4 °C to remove cellular debris. Protein concentration of all supernatants was determined using a modified Lowry assay. Seventy µg of total protein from 3 SA, 3 T, and 3 IBA squirrels was subjected to standard SDS-PAGE 4-20% gradient gels. Following electrophoresis, blots were electrotransferred to PVDF (400 mA for 3 h). Blots were allowed to completely dry before blocking. Non-specific protein binding was blocked by incubation of the membrane in 3% milk in 10 mM tris•HCl, pH 8, 150 mM NaCl (tris buffered saline; TBS) with 0.5% tween-20 (TTBS) for 1 h. Incubation with a polyclonal primary antibody for caspases 3 (Santa Cruz Biotechnology; rabbit polyclonal used at 1:300) was done overnight at 4 °C. Following washing, blots were exposed to HRP-conjugated

secondary antibody (Amersham) for 1 h in TTBS with 1% BSA to block non-specific binding. Washing between incubation steps consisted of one 5 min wash in TBS, followed by two 5 min washes in TTBS, and a final 5 min wash in TBS. All visualizations (except ICAD and PARP) were performed using ECL+ on a Typhoon imager (GE Health Sciences). For ICAD and PARP analyses, 45 µg total protein from three squirrels in each state (SA, T, and IBA) were run on 17% (ICAD) or 14% (PARP) SDS-PAGE gel. The ICAD primary antibody was used at 1:1000 (rabbit polyclonal; ProSci Inc.) while the PARP primary antibody was used at 1:200 (rabbit polyclonal; Roche Diagnostics). Following washing, blots were exposed to IRDye 680LT-conjugated secondary antibodies (LiCor, Inc.). ICAD and PARP blots were visualized on a LiCor Odyssey (Model # 9120; LiCor Inc.).

Caspase 3 activity assays

Livers were pulverized in liquid N_2 and homogenized in one volume of 25 mM HEPES, pH 7.8, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, 10 mM DTT, 5 mM $MgCl_2$, and 10 mM KCl using glass/glass homogenization. The homogenate was centrifuged at 20,000 X g @ 4 °C to remove cellular debris. Protein concentration of all supernatants was determined using a modified Lowry assay. Caspase 3 reactions consisted of 20 mM HEPES, pH 7.6, 2 mM EDTA, 5 mM DTT, and either 500 µg protein (rat) or 1000 µg protein (SA, T, IBA) in a total volume of 100 µl. Assay mixtures were allowed to pre-incubate on ice for 30 min to reduce background. Reactions were initiated with addition of 40 µM caspase 3 tetrapeptide substrate DEVD conjugated with aminomethylcoumarin fluorophore (AMC; Sigma Chemical Corp.). Following incubation at indicated temperatures and times, reactions were stopped with addition of $1/10^{th}$ volume concentrated HCl. Following centrifugation at 10,000 g for 12 m, supernatants were

diluted with water and fluorescence was measured with excitation/emission spectra of 360/460 nm. Addition of 100 μM of the specific competitive inhibitor of caspases 3/7, Ac-DEVD-CHO, reduced activity to 26.6 ± 4.6% (n = 3, ANOVA $p < 0.05$) of the non-inhibited rate. However, we found that assaying parallel reactions wherein HCl was added prior to substrate provided a more consistent basis for comparison and all data are presented as such. Reactions were repeated for three different animals from each state with similar results. Linearity of the caspase 3 activity assay was measured in both rats and ground squirrels (data not shown; r^2 of linear fit was > 0.99 and 0.96, respectively). Reaction incubation times are as indicated in figure legends and are within the linear range for both species. In these and our previous experiences with utilizing AMC-linked peptides as substrates, we have found significant day-to-day variation in the extent of AMC cleavage (Velickovska and van Breukelen, 2005). For that reason, I expressed all data as a percentage of maximum to allow for direct comparison of different states.

TUNEL assay

Caspase 3 activity results in the cleavage of the inhibitor of caspase-activated deoxyribonuclease (ICAD; Sakahira et al., 1998). The caspase-activated deoxyribonuclease (CAD) moves into the nucleus and nicks DNA. As an indicator of downstream effects of caspase 3 activity, I performed terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays to detect DNA nicking. Glass slides were dipped in 0.01% poly(l)-lysine for 5 min and allowed to dry overnight before use. Tissues were embedded in O.C.T. (Tissue-Tek) solution and frozen. Livers, kidneys, and hearts of ground squirrels from each state were used (n = 3 of each for T, IBA, SA, and naturally arousing animals whose core body temperatures were 9.97° ± 0.07 °C, 20.37° ± 0.07 °C, and 31.13° ± 1.07 °C at

the moment of sampling). For comparison and to ensure the reaction was working, I performed TUNEL assays on liver slices from rats, n = 3. Tissues were sectioned on a cryostat at 7 μm thickness. Following application of the tissue slice to the slide, the slide was fixed in 4% paraformaldehyde for 20 min. Slides were washed three times for 5 min each in phosphate buffered saline (PBS). Tissues were permeablized for 5 min with 0.1% Triton X-100 before 3 washes 5 min each in PBS. TUNEL reagent was added per manufacturer's instructions (Roche Diagnostics *in situ* Cell Death Detection kit). Reactions were visualized on a confocal microscope. To ensure efficacy of the assay, some sections from each tissue were subjected to DNase treatment as per the manufacturer's instructions (Roche Diagnostics). All TUNEL-positive cells were counted in each tissue slice. For an estimate of total cell numbers for each tissue slice, cells were counted in 5 separate fields and the average cell count was multiplied by the number of fields per slice. The validity of this approach was confirmed by counting all cells on some representative tissue slices.

Statistical analysis

Data are presented as mean ± standard error (SE). Where appropriate, statistical analyses were performed using ANOVA with Fisher's LSD post-hoc analysis for specific comparisons. Statistical significance was assumed when $p<0.05$.

Results

Western blot analyses of caspase 3

Caspase 3 activation occurs when procaspase 3 is cleaved to form the active caspase p17 fragment (Elmore, 2007; Ramirez and Salvesen, 2018). Our western blots resolved three bands,

p32 procaspase, p20, and the most active p17 fragment (Han et al., 1997). Caspase 3 is seemingly activated in both T and IBA winter animals as evidenced by an approximately 2-fold increase in p17 concentrations as compared to SA squirrels (Figure 3; ANOVA, $p < 0.05$;). For simplicity, only the most active caspase fragment is shown and data are normalized to the SA state. See Table 4 in Appendix for total fragment expression data.

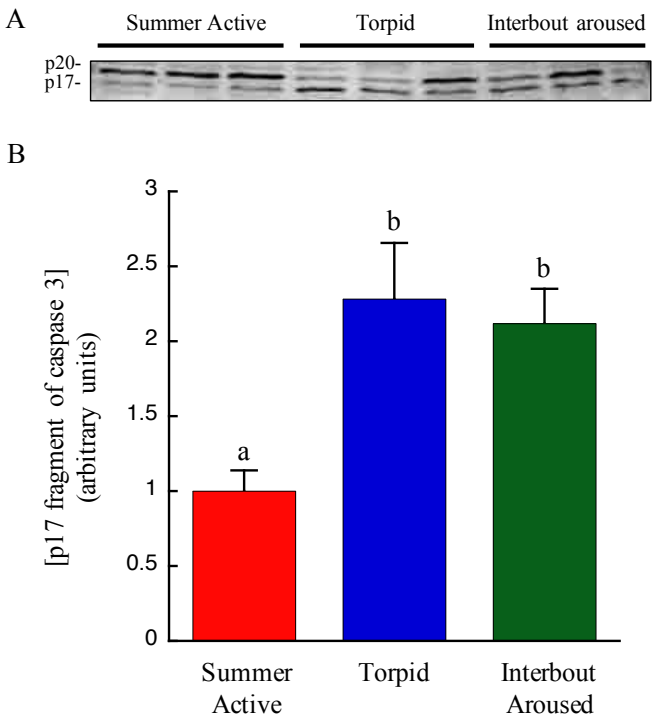

Figure 3. p17 fragment of caspase 3 increases during hibernation. Caspase 3 is produced as an inactive 32 kDa (p32) proenzyme and, upon activation, gets processed into the active 17 kDa (p17) fragment. Ground squirrel livers (n=3 per state) were obtained in the summer (Summer Active) and during hibernation when animals were torpid or between bouts of torpor (Interbout Aroused). For clarity, only the p17 fragment (the most active detectable caspase fragment) is displayed. Caspase 3 p17 is increased ~2-fold in winter squirrels (both LT and IBA; ANOVA, $p<0.05$) potentially representing significant apoptotic signaling in hibernating animals. Data for graphs represent means ± standard error (SE).

Caspase 3 activity assays

Liberation of p17 typically results in caspase 3 activation. Western blot data do not address actual enzymatic activity *per se*. Therefore, I directly examined the activity of caspase 3

by using an artificial substrate that is linked to the fluorescent molecule, AMC (see Materials and Methods). Upon cleavage of the substrate by caspase 3, the AMC is released and then fluoresces. Although the low temperatures typical of a torpor bout (e.g. 0 to 10 °C) resulted in a predictable depression in caspase 3 activity, there was still measurable activity even at 0°C in all ground squirrel states (SA, T, and IBA) and even rats (Figure 4). Interestingly, caspase 3 activity was markedly and specifically depressed at 37 °C in winter squirrels (T and IBA; ANOVA $p < 0.05$) but not in SA or rats. Activity at 37 °C was depressed 34.85 ± 8.39% of the rate at 30 °C for IBA animals and 32.33 ± 14.76% for T animals (n = 3 different animals). In viewing Figure 4, background fluorescence represents a greater proportion of maximum rate in torpid hibernating squirrels than in other groups, suggesting less total activity. Although results for only one animal are shown in the figure, two additional animals per state demonstrated similar results. Simple passive temperature effects appear to severely depress the enzymatic activity of caspase 3 at low temperatures. These data do not support expected activation of caspase 3 based on the western blot data.

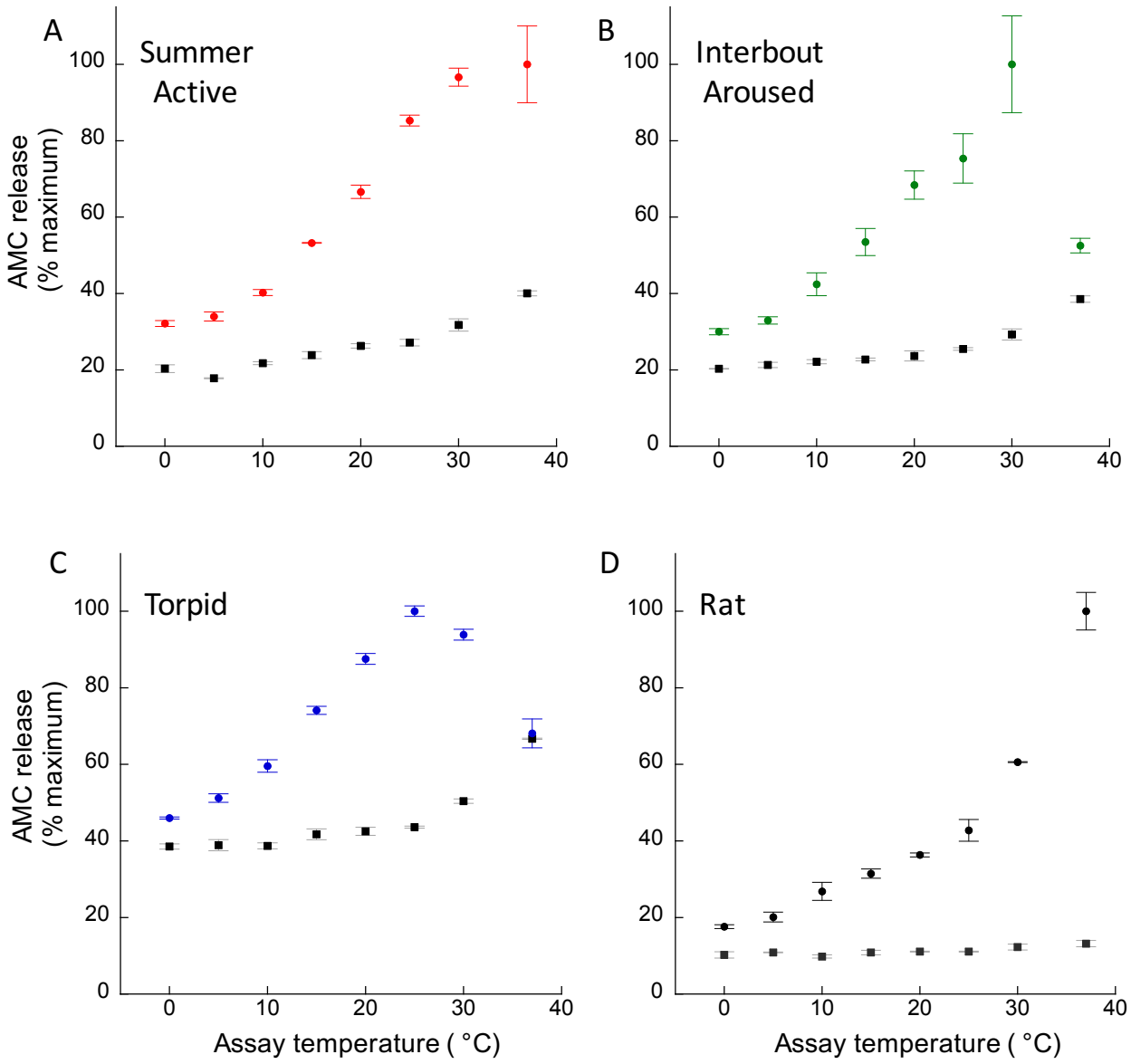

Figure 4. Caspase 3 enzymatic activity as a function of assay temperature and state with comparison to non-hibernating rat. Representative results for the effects of assay temperature on caspase 3 activity. Hepatic lysates from A) Summer Active B) Interbout Aroused, C) Torpid squirrels, and D) rat were incubated in the presence of DEVD-AMC, a substrate used for the estimation of caspase 3/7 protease activity. Assays were incubated for either 1 h (rats) or 4 h (squirrels). Circles represents uninhibited reactions while squares represent lysates that were quenched with HCl prior to the addition of substrate. Values represent means ± SE, (n =3 replicates). Similar results were obtained using lysates derived from two additional animals from each state.

ICAD and PARP cleavage

Activated caspase 3 cleaves cellular targets like the inhibitor of caspase-activated DNase (ICAD) and DNA repair enzyme poly (ADP-ribose) polymerase (PARP; Fischer et al., 2003; Rehm et al., 2002). I examined ICAD and PARP to determine if the seemingly winter-activated caspase 3 was functioning *in vivo*. I found no evidence of increased ICAD (Figure 5A) or PARP (Figure 5C) cleavage during winter compared to SA (ANOVA, $p > 0.05$ for both analyses). These data do not support activation of caspase 3 in winter squirrels.

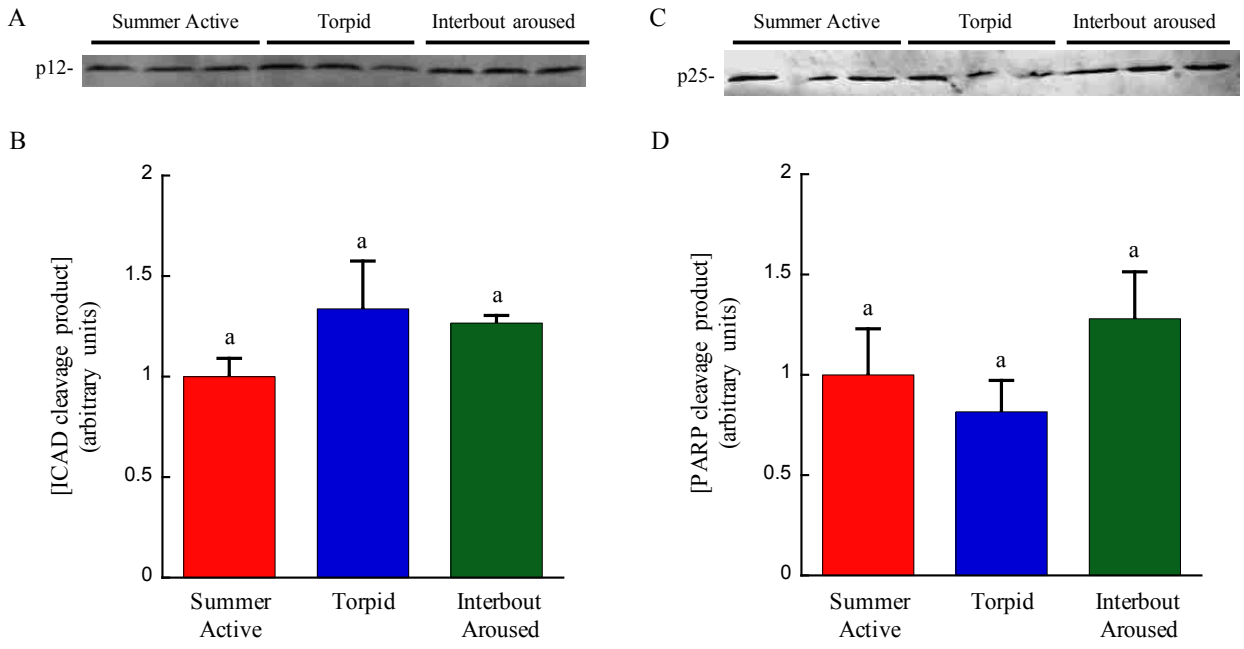

Figure 5. Downstream caspase 3 targets ICAD and PARP are not cleaved during hibernation. Western blots were performed on ground squirrel hepatic lysates from all three states (SA, T, and IBA; n=3 per state). Data represent mean ± SE. (A and B) The 12 kDa ICAD cleavage product. (C and D) The 25 kDa PARP cleavage product. No statistical differences were found in cleavage products as a function of state (ANOVA, $p>0.05$ for all comparisons).

TUNEL assays

I examined three different tissues (heart, liver, and kidney) for DNA nicking for evidence of caspase 3 activity. The level of TUNEL activity was remarkably low in all states for all tissues (Figure 6; Table 1). We verified the assay by treating some slides with DNase as a positive control. Further verification of the TUNEL assay using rat livers demonstrated much higher levels of TUNEL positive cells (data not shown). To address if there was an increase in DNA nicking activity as animals arouse from torpor, but that the putative activity would have ceased by the time animals completed arousing (i.e. in IBA squirrels), I sampled livers from animals that were naturally arousing at 10, 20 and 30 °C (Table 2). I found no evidence for widespread DNA nicking.

Hibernators experience tremendous bradycardia (heart rate may be reduced from 200-250 bpm to 2-3 erratic bpm; Milsom et al., 1999), extremely low body temperatures (core body temperatures may be below –2 °C; Barnes, 1989), evidence of acidosis (despite an expected alkalosis from simple temperature effects, pH_i remains surprisingly constant thus reflecting an input of H^+ into the system; Clausen and Ersland, 1968; Bock et al., 2002), and oxidative stress (Carey et al., 2000). All of these conditions are known to be pro-apoptotic in other systems (for review, see van Breukelen et al., 2010). I employed a systems level approach towards understanding the regulation of a critical stage in the apoptotic signaling cascade in hibernators. Although key aspects of apoptosis signaling appear to be activated, caspase 3 activity seems limited which results is very little apoptosis in the seemingly pro-apoptotic conditions of hibernation.

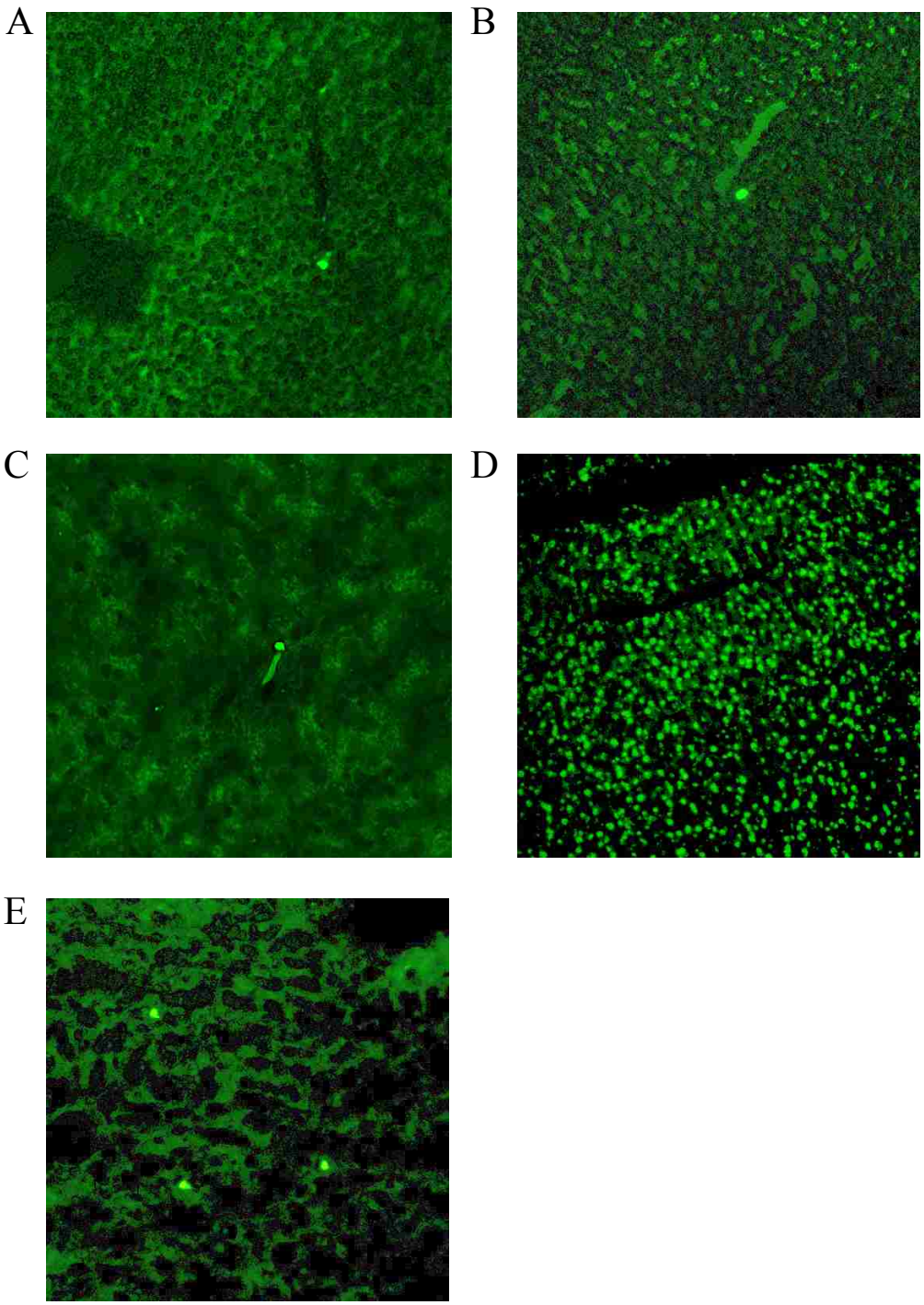

Figure 6. Apoptosis is extremely limited in ground squirrel hepatocytes regardless of state. Representative results of TUNEL assays for the detection of nicked DNA. Representative TUNEL activity for the detection of nicked DNA in hepatocytes from ground squirrels that were sampled as they were (A) arousing, (B) torpid, (C) interbout aroused. (D) DNase-treated and (E) non-hibernating rat were included as assay controls. Brightness and contrast were adjusted in order to visualize surrounding cells.

Table 1. Results of TUNEL assay for DNA damage in ground squirrel tissues

Tissue	State	TUNEL positive nuclei/slide	Estimated total nuclei counted	% TUNEL positive
Liver	summer active	2.00 ± 0.58	74636 ± 10389	$3.01 \times 10^{-3} \pm 1.03 \times 10^{-3}$
	torpid	0.67 ± 0.33	45462 ± 7405	$1.73 \times 10^{-3} \pm 9.45 \times 10^{-4}$
	interbout aroused	0.33 ± 0.33	35051 ± 7334	$1.41 \times 10^{-3} \pm 1.41 \times 10^{-3}$
Heart	summer active	0.67 ± 0.33	18183 ± 1286	$3.70 \times 10^{-3} \pm 1.89 \times 10^{-3}$
	torpid	0.67 ± 0.33	27864 ± 9257	$2.27 \times 10^{-3} \pm 1.34 \times 10^{-3}$
	interbout aroused	0.67 ± 0.67	12890 ± 2192	$7.76 \times 10^{-3} \pm 7.76 \times 10^{-3}$
Kidney	summer active	0.33 ± 0.33	138053 ± 29343	$2.76 \times 10^{-4} \pm 2.76 \times 10^{-4}$
	torpid	2.00 ± 1.00	120526 ± 25337	$2.13 \times 10^{-3} \pm 1.12 \times 10^{-3}$
	interbout aroused	2.33 ± 1.45	121527 ± 155572	$1.72 \times 10^{-3} \pm 1.08 \times 10^{-3}$

Data represent means ± SE for n=3 animals for each state and tissue. There are no statistical differences (ANOVA, $p>0.05$).

Table 2. Results of TUNEL assay for DNA damage in tissues of ground squirrels arousing from torpor

Tissue	T_b at time of sampling	TUNEL positive nuclei/slide	Estimated total nuclei counted	% TUNEL positive
Liver	10°C	1.00 ± 1.00	52379 ± 13330	$3.24 \times 10^{-3} \pm 3.24 \times 10^{-3}$
	20°C	2.67 ± 2.19	53805 ± 12624	$8.56 \times 10^{-3} \pm 7.76 \times 10^{-3}$
	30°C	1.33 ± 1.33	788998 ± 14491	$1.40 \times 10^{-3} \pm 1.40 \times 10^{-3}$
Heart	10°C	0.33 ± 0.33	18694 ± 3114	$2.52 \times 10^{-3} \pm 2.52 \times 10^{-3}$
	20°C	0.67 ± 0.67	19754 ± 4055	$2.43 \times 10^{-3} \pm 2.43 \times 10^{-3}$
	30°C	1.00 ± 0.57	20097 ± 3024	$5.35 \times 10^{-3} \pm 2.73 \times 10^{-3}$
Kidney	10°C	4.00 ± 2.08	101399 ± 23188	$4.17 \times 10^{-3} \pm 2.43 \times 10^{-3}$
	20°C	4.00 ± 2.31	103877 ± 15314	$4.67 \times 10^{-3} \pm 2.89 \times 10^{-3}$
	30°C	3.67 ± 1.20	57428 ± 12719	$6.11 \times 10^{-3} \pm 6.57 \times 10^{-4}$

Data represent means ± SE for n=3 animals for each temperature and tissue. There are no statistical differences (ANOVA, $p>0.05$).

Discussion

Caspase 3 is widely regarded as the key executioner of apoptosis due to its direct role in processing numerous apoptotic substrates that lead to apoptosis (Fischer et al., 2003; Budihardjo et al., 1999). Liberation of caspase 3 p17 results in a >10,000-fold enzymatic increase (Boatright and Salvesen, 2003; Stennicke and Salvesen, 1998). My western blot data demonstrate what appears to be a 2-fold increase in the active caspase 3 p17 during winter (Figure 3). In light of the considerable increase in caspase 3 enzymatic processing upon activation, this seemingly modest 2-fold increase in p17 during the winter potentially represents a dramatic increase in caspase 3 activation, activity, and commitment to apoptosis during hibernation.

True activation of caspase 3 should result in dramatically increased enzymatic activity. While western blots can readily resolve protein expression and zymogen activation status, they cannot address enzyme *activity* per se. Using caspase-specific fluorescent peptides, I directly examined caspase 3 activity as a function of temperature with the rationale that perhaps the cold temperatures of torpor would passively control the function of this enzyme. A predictable temperature effect on enzymatic activity was observed; caspase 3 activity is reduced (but still measurable) even at 0 °C and increased predictably with increasing assay temperature (Figure 4). However, activity was markedly and specifically depressed by as much as ~2/3 at 37 °C in both torpid and interbout aroused animals but not in summer squirrels or rats. Initially, I interpreted this finding as potential evidence for a *bona fide* hibernation-specific adaptation to depress global apoptosis during IBA. However, activity data collected on additional caspases (presented in subsequent chapters), showed similar depression in activity at 37 °C. Further, I utilized a heterologous assay wherein torpid squirrel lysate was added to summer active squirrel lysate and there was no depression of enzymatic activity (data not shown). Importantly, procaspase 3

processing into the active fragment was expected to increase activity 20,000-fold. The data in Figure 4 do not support this expectation suggesting caspase 3 activity is depressed in winter animals.

In light of a seeming activation of caspase 3 by being processed into smaller fragments, but a lack of associated increased activity in the proteolytic assay, I examined downstream targets of caspase 3 for indications of activation. Expected downstream effects of activated caspase 3 include the cleavage of the inhibitor of the caspase-activated deoxyribonuclease (ICAD; Fischer et al., 2003) and the cleavage of DNA repair enzyme poly-ADP-ribose-polymerase (PARP; Rehm et al., 2002). Under non-apoptotic conditions, the 40 kDa caspase-activated deoxyribonuclease (CAD) is bound to the 45 kDa inhibitory unit (ICAD). When caspase 3 is activated, ICAD gets cleaved and liberates a non-functional 12 kDa fragment. This ICAD cleavage activates CAD. Caspase 3 also cleaves the 113 kDa PARP into non-functional 89 kDa and 25 kDa fragments (Nicholson et al., 1995). The cleavage of PARP ensures robust and non-reversible DNA degradation during apoptosis. As evidenced by ICAD or PARP cleavage (Figure 5), there is no indication of caspase 3 activation. TUNEL assays also confirmed no effective activation of caspase 3 in winter animals. Apoptosis can be completed in 1-3 h (Tyas et al., 2000). Since it only takes 3 to 4 h to arouse from hibernation, apoptosis might have occurred *during* the arousal process. TUNEL activity for arousing animals was also very low suggesting that I did not simply miss the apoptosis process (Table 2).

Several previous studies have examined aspects of apoptotic control in regards to hibernation (Fleck and Carey, 2005; Kurtz et al., 2006; Cai et al., 2004; Tamura et al., 2006). Fleck and Carey (2005) report TUNEL-positive nuclei in ~1/2 of all enterocytes. I found TUNEL levels in the liver, heart and kidney to be so low that I confirmed the validity of the

assay using DNase treatment. Fleck and Carey (2005) found no evidence for DNA laddering. Importantly, their TUNEL staining was not restricted to the nucleus but was found in the cytosol (van Breukelen et al., 2010).

I found caspase 3 seemingly activated during hibernation in liver of both torpid and interbout aroused ground squirrels with no apparent downstream caspase activity or widespread apoptosis. How might this be possible? Caspase 3 is known to be regulated in various ways even after activation. Heat shock protein 70 (HSP70) overexpression is known to block caspase 3 activity after activation (Xanthoudakis and Nicholson 2000). Carey et al. (1999 and 2003) found that HSP70 expression was not increased during hibernation in 13-lined ground squirrel intestinal mucosa compared to summer squirrels, however, they did not address HSP70 activity. The X-linked inhibitor of apoptosis protein (XIAP) can bind to the active site of active caspase 3 and block caspase activity (Jaattela et al 1998; Callus and Vaux, 2007). IAPs are also ubiquitin ligases and cIAP2 is known to interact with caspase 3 and ubiquitylate it, however, functional outcomes are unknown (Huang et al., 2000). My lab previously looked at XIAP protein expression and found XIAP expression was incredibly consistent regardless of state (data not shown). Obviously, in addition to protein expression levels, a better understanding of HSP70 and XIAP activity is required in order to distinguish what these expression levels may mean in the context of hibernation.

Through employing a systems-level approach, I was able to identify of a novel locus for caspase regulation in hibernating ground squirrels. Despite caspase 3 being *seemingly* activated during hibernation, apoptotic signaling is mitigated through inhibition of caspase activity after caspase activation.

CHAPTER 3

INCOMPLETE APOPTOTIC CASPASE SIGNALING DURING HIBERNATION

Abstract

Typically, when mammalian cells experience extreme levels of stress or damage, the caspase cascade is activated and executes programmed cell death, or apoptosis. Despite the numerous cellular insults inherent to hibernation, no indications of significant cellular damage or tissue depletion have been found in hibernating mammals, suggesting a level of apoptotic mitigation in hibernators. In chapter 2, I focused on regulation of the key executioner caspase 3. In this chapter, I focus on the other caspases associated with apoptosis. Examination of caspases 2, 6, 7, 8, 9, and 10 revealed caspases 6 and 9 may be seemingly activated during hibernation. Liberation of executioner caspase 6 active fragment increased ~4- to 8-fold in winter squirrels, while caspase 9 active fragment increased ~2-fold during interbout arousals only. Caspases 6 and 9 enzymatic activity data does not reflect expected increases in enzymatic activity consistent with an activation during hibernation. Mitochondrial permeabilization, a critical event that results in caspase 9 activation, was not detected during hibernation A specific downstream target for caspase 6, lamin A, was not cleaved during hibernation. I conclude that incomplete apoptotic signaling through the caspase cascade does not result in apoptosis during hibernation.

Introduction

As discussed in chapters 1 and 2, many of the conditions that ground squirrels experience during hibernation result in widespread apoptosis in other mammals (for review, see van Breukelen et al. 2010). The caspases are a family of cysteine-aspartate proteases essential to

executing apoptosis and inducing inflammation (for review see Fuchs and Steller, 2015; Man and Kanneganti, 2016). Caspases 2, 3, 6, 7, 8, 9, and 10 are all considered apoptotic caspases (Elmore, 2007). In this chapter, I address the role of these caspases in promoting apoptosis during mammalian hibernation. The systems-level approach used in chapter 2 will be employed here to determine if seeming activation events translate into *bona fide* apoptotic signaling.

Materials and Methods

Animal care and tissue collection

Adult golden–mantled ground squirrels (*Spermophilus lateralis*) were captured and tissues were collected from summer active (SA), torpid (T), and interbout aroused (IBA) as described in Chapter 2.

Sample preparation and western blot analyses

Western blots were performed essentially as described in Chapter 2. Conditions for SDS-PAGE were: caspase 7 (50 µg total protein samples on a 12% acrylamide gel), caspase 8 (70 µg total protein samples on a 12% acrylamide gel), caspase 9 (50 µg total protein samples on a 12% acrylamide gel), lamin A (50 µg total protein samples on a 12% acrylamide gel), and AIF (50 µg total protein samples on a 12% acrylamide gel)) and 15% (caspase 2 (30 µg total protein samples on a 15% acrylamide gel), caspase 6 (50 µg total protein samples on a 15% acrylamide gel), and caspase 10 (50 µg total protein samples on a 15% acrylamide gel)). The following antibodies were used: anti-caspase 2 (Santa Cruz Biotechnology; rabbit polyclonal used at 1:1000), anti-caspase 6 (Cell Signaling; rabbit polyclonal used at 1:1000), anti-caspase 7 (Cell Signaling; goat polyclonal used at 1:200), anti-caspase 8 (BD Biosciences; rabbit polyclonal used at 1:800), anti-

caspase 9 (ProSci Inc.; rabbit polyclonal used at 1:1000), anti-caspase 10 (Santa Cruz Biotechnology; goat polyclonal used at 1:200), anti-lamin A (Morris, G.E. via DSHB; mouse monoclonal used at 1:1000), and anti-Apoptosis Inducing Factor (AIF; Novus Biologicals, rabbit polyclonal used at 1:2000).

Caspase activity assays

Sample preparation and assay protocol for caspase activity assays are described in Chapter 2. Reactions were initiated with addition of 40 μM VEID-AMC (caspase 6) or LEHD-AMC (caspase 9) of specific caspase substrate (Enzo Life Sciences). In order to test the specificity of the caspase reaction and understand background fluorescence, 100 μM of the specific competitive inhibitor of caspase 6 (Ac-VEID-CHO; Enzo Life Sciences) and caspase 9 (Ac-LEHD-CHO; Enzo Life Sciences) was added and reduced caspase 6 and 9 activity 82.4± 12.9% and 62.8 ± 6.9%, respectively (ANOVA $p < 0.05$), of the non-inhibited rate. However, inhibiting parallel reactions with concentrated HCl prior to substrate addition provided a more complete inhibition.

Apoptosis Inducing Factor (AIF) immunohistochemistry/ immunofluorescence

Frozen livers were embedded in Tissue-Tek OCT compound (VWR Scientific) and equilibrated to −20 °C for 20 min. Frozen sections were cut at a thickness of 7 μm with a Vibratome cryostat and subsequently mounted on glass slides coated with freshly prepared 0.01% poly-L-lysine (Sigma-Aldrich). Sections were fixed with 1:1 methanol/acetone (v/v) solution for 10 min at −20 °C and then rehydrated in PBS with 0.3% Triton-X (PBST) for 10 min at room temperature. The fixed slides were immersed in blocking solution (5% milk in PBST) overnight at 4 °C. After

three 5 min washes in PBST, the sections were incubated with rabbit anti-AIF polyclonal IgG antibody (Novus Biologics) diluted 1:100 in blocking solution overnight at 4 °C. After three 5 min washes in PBST, the sections were reacted with goat α-rabbit IgG antibody conjugated to BODIPY FL (Invitrogen B2766) diluted 1:1000 in blocking solution for 1 h at room temperature. After another three 5 min washes in PBST, the sections were counterstained with DAPI (Vectashield mounting medium with DAPI; Vector Laboratories, Inc. #H-1200) to label nuclei. Finally, tissue sections were visualized with laser scanning confocal fluorescent microscopy (Carl Zeiss, Jena, Germany). One hundred clearly identified nuclei were used for quantification for each animal.

Results

Availability and activation status of the caspase cascade during hibernation

Seemingly activated apoptotic caspases: Caspases 6 and 9

Caspase 6 is an executioner caspase that, through its specific cleavage of nuclear lamins, plays an essential role in dismantling the nuclear envelope during the terminal stages of apoptosis (Ruchaud et al., 2002). Liberation of the caspase 6 p15 fragment is indicative of fully active caspase 6 (Wang et al., 2010). I found that caspase 6 appears significantly activated during the winter in both torpid or interbout aroused. I found a significant increase in the caspase 6 p15 fragment during hibernation. Specifically, when compared to SA squirrels, caspase 6 p15 increased 3.67 ± 1.29-fold during torpor (T) and up to 8.26 ± 0.70-fold in interbout arousals (IBA; Figure 7; ANOVA $p<0.05$ for IBA vs. SA and T). For simplicity, only the most active

caspase fragment is shown and data are normalized to the SA state. For total fragment expression, see Table 4 in the Appendix.

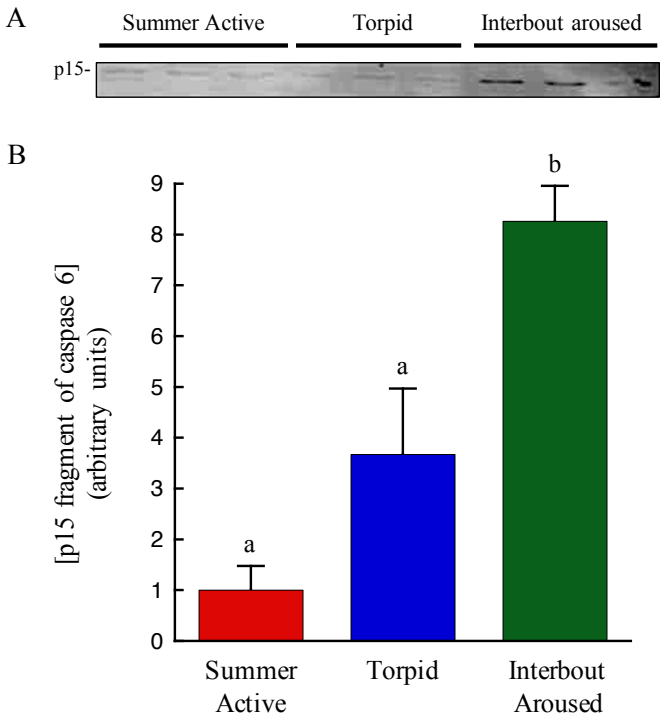

Figure 7. p15 fragment of caspase 6 increases during hibernation. Executioner caspase 6 gets processed into a 15 kDa (p15) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p15 fragment (most active caspase fragment detectable) is displayed. Caspase 6 p15 increased ~4-fold in torpid and ~8-fold in interbout aroused squirrels (ANOVA, $p<0.05$) Data for graphs represent means ± standard error (SE)

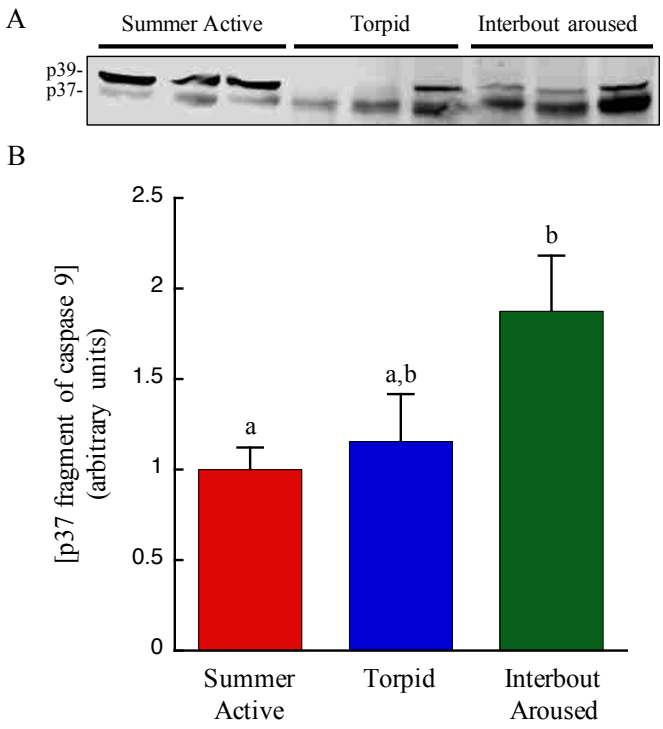

Figure 8. p37 fragment of caspase 9 increases during hibernation. Intrinsic initiator procaspase 9 is processed into a 37 kDa intermediate and a 25 kDa (p18) fully active fragment. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p37 fragment (most active fragment detected) is displayed. Caspase 9 p37 was increased ~2-fold only in interbout aroused winter squirrels when compared to summer animals (ANOVA, $p<0.05$). Data for graphs represent means ± standard error (SE)

Caspase 9

Caspase 9 is considered the primary initiator caspase of the intrinsic apoptotic pathway (Logue and Martin, 2006). Upon apoptotic stimuli and permeabilization of the outer mitochondrial membrane, cytochrome c is released, binds to Apaf-1, and stimulates formation of the apoptosome with subsequent caspase 9 activation (Logue and Martin, 2006). Interestingly, while associated with the apoptosome, caspase 9 may be enzymatically active without needing procaspase 9 internal cleavage (Boatright and Salvesen, 2003). In the interbout aroused state during hibernation, increased liberation of caspase 9 p37 fragment was found compared to SA animals (Figure 8; ANOVA $p<0.05$). For simplicity, only the most active caspase fragment

detectable is shown and data are normalized to the SA state. See Table 4 in Appendix for total fragment expression.

The non-activated apoptotic caspases

Caspases 8 and 10

Caspases 8 and 10 are both extrinsic apoptotic initiator caspases and function primarily by propagating death signals received extracellularly at cell surface receptors (i.e. TNF-a, Fas ligand (FasL); Elmore, 2007; Logue and Martin, 2006; Ramirez and Salvesen, 2018). After death ligands bind, a transmembrane protein complex is assembled known as the death inducing signaling complex, or DISC. The DISC recruits caspases 8 and 10 which results in their activation. Active caspase 8 and 10 then process downstream targets like caspase 2, BID, or executioner caspases. During hibernation, no indications of increased caspase 8 activation or caspase 10 availability were found (Figure 9 and Figure 10; ANOVA $p>0.05$). For simplicity, only the most active caspase fragment detected is shown and data are normalized to the SA state. See Table 4 in Appendix for total fragment expression.

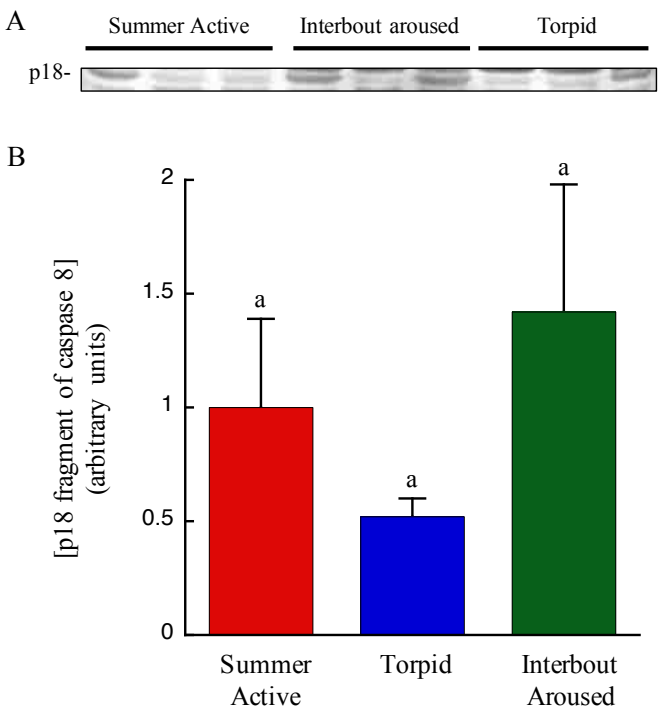

Figure 9. p18 fragment of caspase 8 does not increase during hibernation. Extrinsic initiator caspase 8 is processed into an 18 kDa (p18) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p18 fragment (most active caspase fragment detectable) is displayed. Active caspase 8 fragment was not increased in winter squirrels (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE)

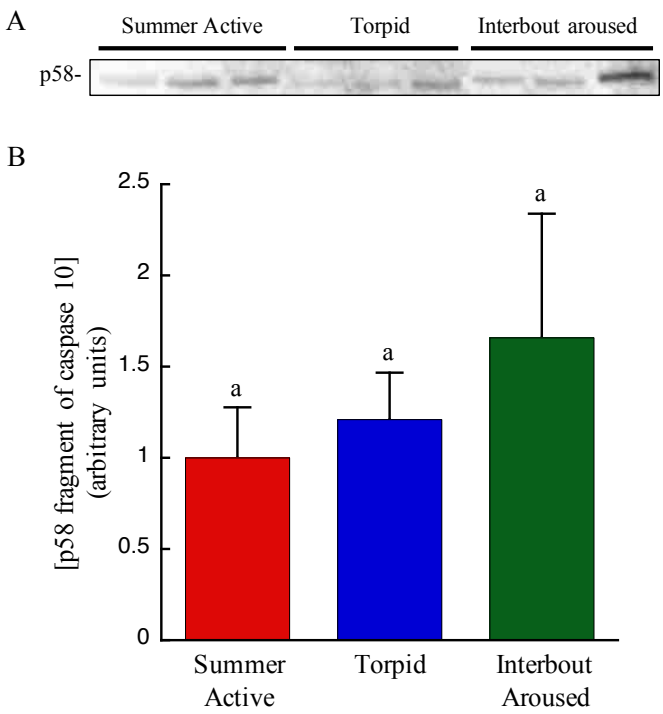

Figure 10. p58 fragment of caspase 10 does not increase during hibernation. Extrinsic initiator caspase 10 is produced as a 58 kDa procaspase. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. Caspase 10 antibody only identified the procaspase p58 fragment and was not increased in winter squirrels (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE)

Caspase 2

Caspase 2, considered an intrinsic pathway caspase, has been shown to function upstream of mitochondrial permeabilization, is activated in response to DNA damage, and may also act as a link between extrinsic and intrinsic apoptotic pathways (Lassus et al., 2002; Aksenova et al., 2016). Caspase 2 does not appear to activate other caspases directly but rather acts to produce a more robust caspase response by processing pro-apoptotic BCL-2 member BID to tBID which then targets the mitochondria to stimulate cytochrome c release (Logue and Martin, 2006). Caspase 2 does not appear to be activated in hibernating ground squirrels (Figure 11; ANOVA $p>0.05$). For simplicity, only the most active caspase fragment is shown and data are normalized to the SA state. See Table 4 in Appendix for total fragment expression.

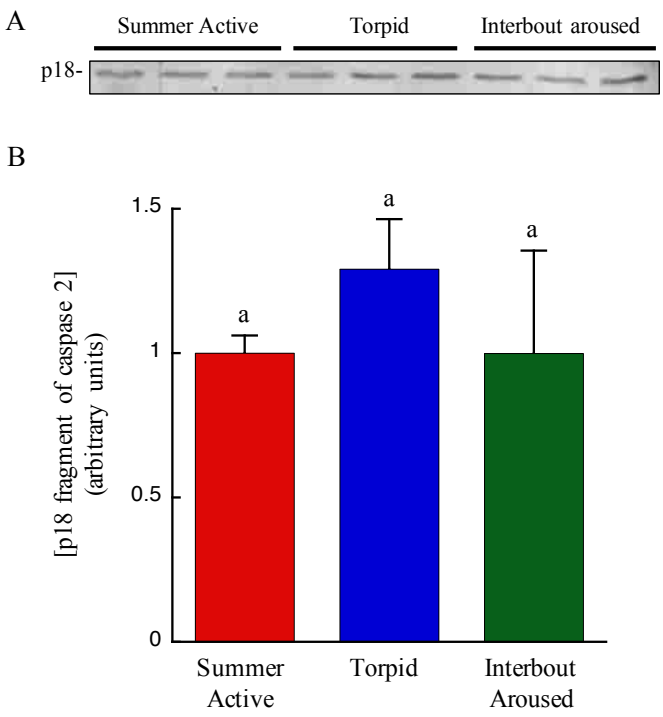

Figure 11. p18 fragment of caspase 2 does not increase during hibernation. Caspase 2 is processed into an 18 kDa (p18) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p18 fragment (most active caspase fragment detectable) is displayed and is normalized to summer active. Active caspase 2 fragment was not increased in winter squirrels (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE)

Caspase 7

Caspase 7 is an executioner caspase with high substrate overlap with caspase 3. Caspase 7 is not activated in hibernating ground squirrels (Figure 12; ANOVA $p>0.05$). For simplicity, only the most active caspase fragment is shown and data are normalized to the SA state. See Table 4 in Appendix for total fragment expression.

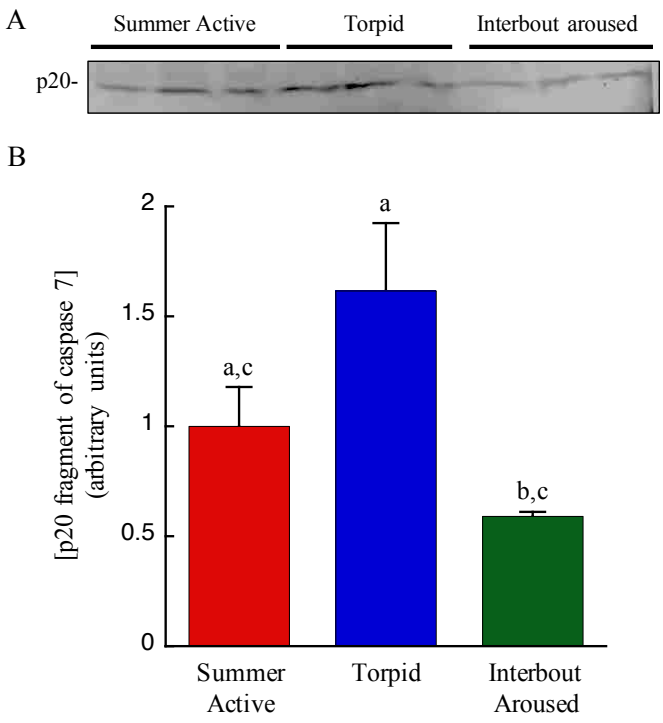

Figure 12. p20 fragment of caspase 7 does not increase during hibernation. Executioner caspase 7 is processed into a 20 kDa (p20) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p20 fragment (most active caspase fragment detectable) is displayed. Active caspase 7 fragment was not increased in winter squirrels (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE).

Caspase activity assays

In the previous section, caspases 6 and 9 appeared to be activated during hibernation. *In vitro* enzymatic activity of caspase 6 (Figure 13) and caspase 9 (Figure 14) was predictable and activity was low at low temperatures and then increased with assay temperature. Similar to what was found in chapter 2, caspase 6 and caspase 9 activity typically peaked in the 30 °C assay samples and was depressed at the 37 °C in hibernating animals (T and IBA). Surprisingly unlike caspase 3, however, the depressions found at 37 °C were also occurring in SA animals in both caspase 6 and 9 (Figure 13 and Figure 14, respectively). For caspase 6, the average activity depression at 37 °C vs. 30 °C was 17.95± 8.32% in SA animals, 9.02± 7.99% in IBA animals, and 18.25± 1.16% in T animals (Figure 13; n=3 different animals per state). For caspase 9, the

average activity depression at 37 °C vs. 30 °C was 14.65 ± 3.64% in SA animals, 23.28 ± 3.42% in IBA animals, and 14.97 ± 2.37% in T animals (Figure 14; n = 3 different animals per state).

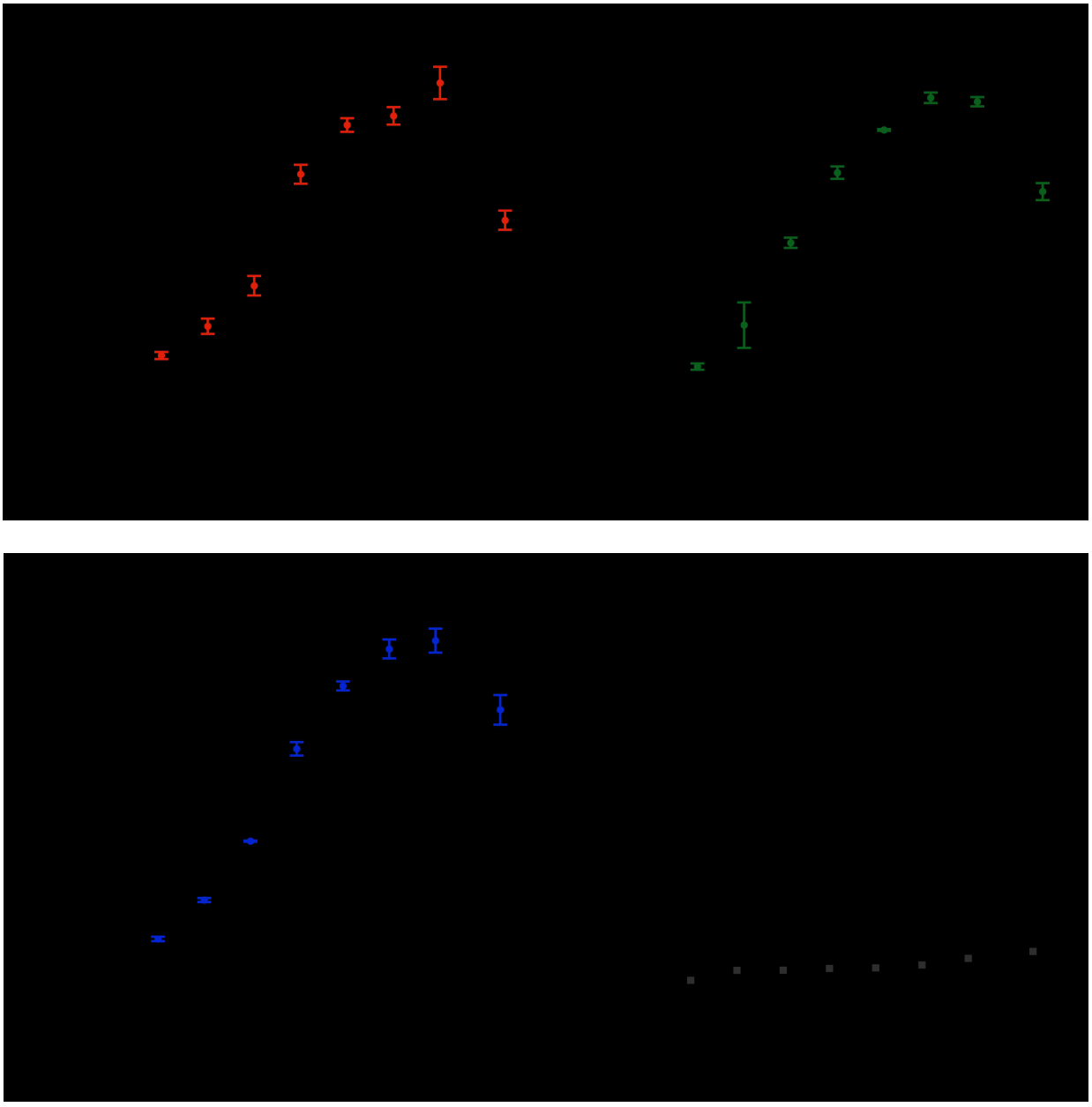

Figure 13. Caspase 6 enzymatic activity as a function of assay temperature and state with comparison to non-hibernating rat. Representative results for the effects of assay temperature on caspase 6 activity. Hepatic lysates from A) Summer Active B) Interbout Aroused, C) Torpid squirrels, and D) rat were incubated in the presence of VEID-AMC, a substrate used for the estimation of caspase 6 peptidase activity. Assays were incubated for either 1 h (rats) or 4 h (squirrels). Circles represents uninhibited reactions while squares represent lysates that were quenched with HCl prior to the addition of substrate. Values represent means ± SE, (n =3 replicates). Similar results were obtained using lysates derived from two additional animals from each state.

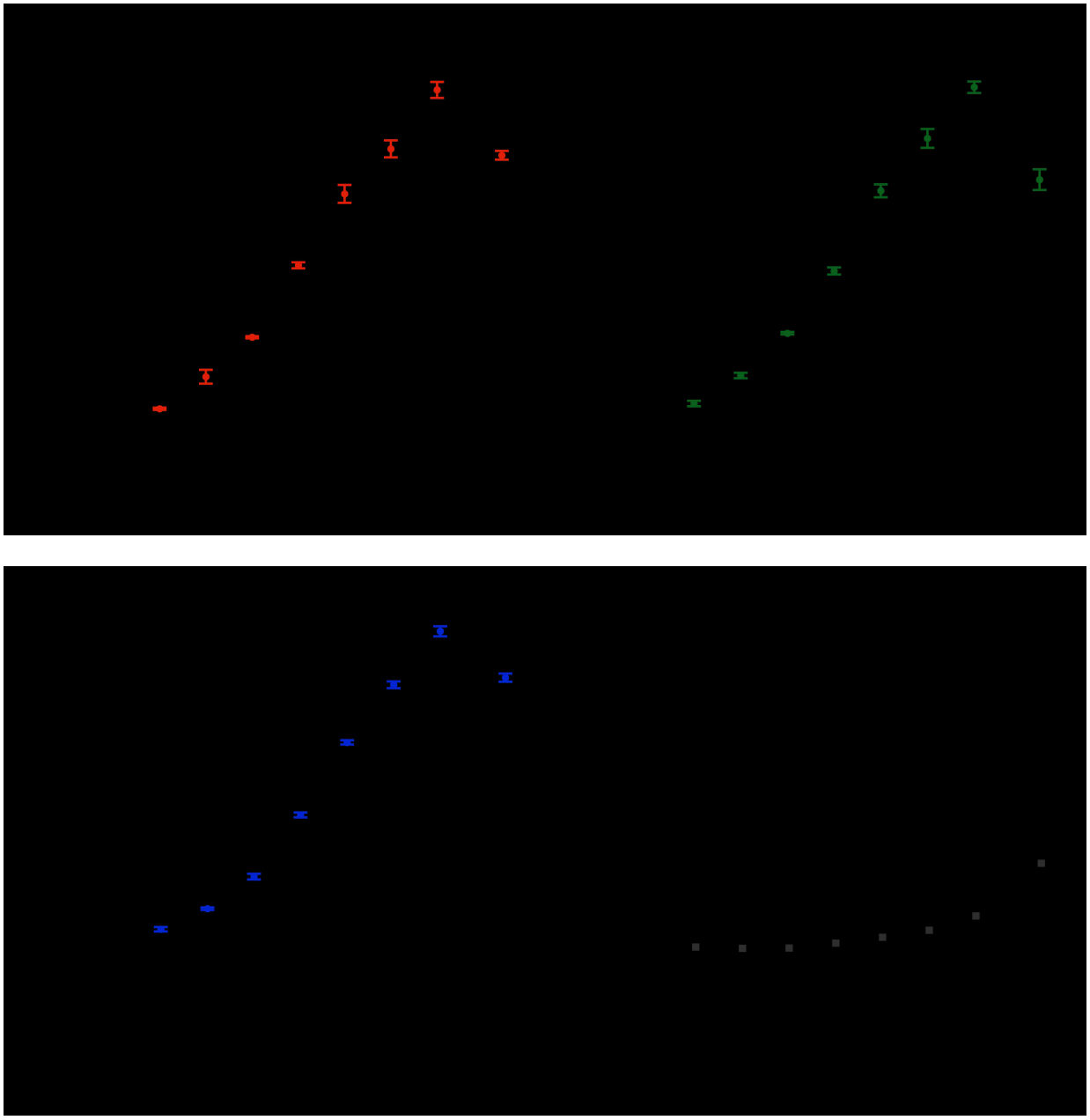

Figure 14. Caspase 9 enzymatic activity as a function of assay temperature and state with comparison to non-hibernating rat. Representative results for the effects of assay temperature on caspase 6 activity. Hepatic lysates from A) Summer Active B) Interbout Aroused, C) Torpid squirrels, and D) rat were incubated in the presence of LEHD-AMC, a substrate used for the estimation of caspase 9 peptidase activity. Assays were incubated for either 1 h (rats) or 4 h (squirrels). Circles represents uninhibited reactions while squares represent lysates that were quenched with HCl prior to the addition of substrate. Values represent means ± SE, (n =3 replicates). Similar results were obtained using lysates derived from two additional animals from each state.

Downstream indicators of apoptotic caspase activation

Lamin A cleavage-

Nuclear lamins are essential structural elements of the nuclear envelope and specific targets for active caspase 6 (Ruchaud et al., 2002; Fischer et al., 2003). There is no cleavage of lamin A during winter (Figure 15; ANOVA $p>0.05$).

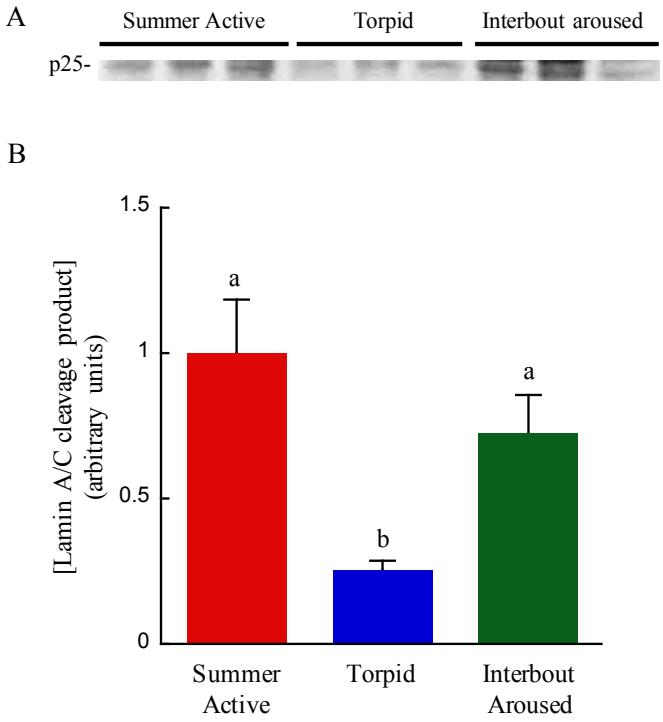

Figure 15. Downstream caspase 6 target nuclear lamin A/C is not cleaved during hibernation. During apoptosis, caspase 6 cleaves lamin A/C into large (41-50 kDa) and small (24-28 kDa) fragments. For clarity, only the small fragment is displayed. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. Lamin A/C cleavage decreased significantly in torpid squirrels compared to both summer active and interbout aroused squirrels (ANOVA, $p<0.05$). Data for graphs represent means ± standard error (SE)

Apoptosis Inducing Factor (AIF) localization-

During apoptosis, the outer mitochondrial membrane becomes permeabilized and numerous factors are released from the intermembrane space. Cytochrome c release is integral to

the caspase 9-mediated intrinsic apoptotic pathway, however, is difficult to reliably visualize due to its small size and propensity to diffuse.

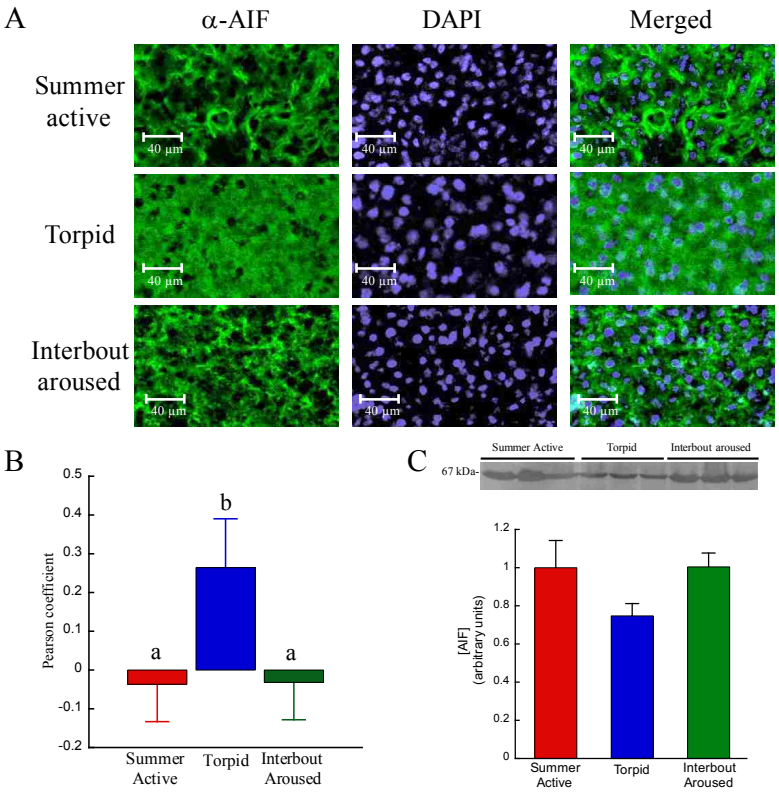

Figure 16. Apoptosis inducing factor (AIF) nuclear localization increases during torpor. During apoptosis, the outer mitochondrial membrane becomes permeabilized leading to cytosolic release of several pro-apoptotic molecules such as cytochrome c and AIF. Upon mitochondrial permeabilization, AIF is released into the cytosol and rapidly moves into the nucleus and assists in nuclear disassembly. Immunohistochemistry (IHC) using an α-AIF antibody was performed on 7µm liver sections from summer active, torpid, and interbout aroused ground squirrels (N=1 animal from each state; 3 different sections were analyzed per animal). (A) Typical AIF immunohistochemistry results showing AIF signal (left column), nuclear DAPI stain (middle column), and merged AIF and DAPI (right column). (B) Pearson's correlation analysis of α-AIF and DAPI in liver sections from summer active (r= -0.037), torpid (r=0.264), and interbout aroused (r=-0.032) ground squirrels. (C) Western blot analysis of AIF demonstrates consistent AIF concentrations regardless of state (n=3 animals per state; ANOVA, $p<0.05$).

AIF is also released from the mitochondria during apoptosis and is rapidly translocated to the nucleus where it functions in nuclear disassembly (Daugas et al., 2000; Cande et al., 2002). In non-apoptotic cells, AIF should be sequestered to the mitochondria and visualized only in the cytosolic fraction (Daugas et al., 2000). AIF in the nucleus increases during torpor (Figure

16A,B). Pearson's correlation coefficient during torpor are modestly positive (r=0.264) compared to both summer active and interbout aroused squirrels (r=-0.037 and r=-0.032, respectively; Figure 16B).

Discussion

In chapter 2, I demonstrated that caspase 3 was seemingly activated based on western blotting. However, there was no expected increases in *in vitro* enzymatic activity or indications of downstream target processing. Those data suggested an incomplete initiation of apoptosis consistent with the requirements of apoptosis-mitigation and appropriate for the non-steady state conditions of hibernation. The locus for regulation of caspase 3 during hibernation appears to be at the level of activity. In other words, caspase 3 does not cleave the intended targets. Does this mode of regulation hold true for all apoptotic caspases? Or, might the non-steady state conditions of hibernation allow some caspases to be regulated in other manners? Could cleavage of another caspase result in some downstream processing? In this chapter, I present data for the other caspases known to be involved in apoptosis, caspases 2, 6, 7, 8, 9, and 10.

Western blotting revealed that caspases 6 and 9 showed indication of seeming activation during hibernation. The p15 fragment of caspase 6 increased ~4- to 8-fold during winter (Figure 7). Based on other caspases, such as caspases 9 and 3, liberation of this fragment would be expected to result in a large (potentially thousands of fold) activation of caspase 6 (Boatright and Salvesen, 2003). *In vitro* enzymatic activity does not support an increased activity of caspase 6 in winter animals (Figure 13). Furthermore, downstream analysis of lamin A processing further supports a lack of caspase 6 activation during winter. Caspase 9 processing into the p37 fragment increased during interbout arousals (Figure 8). However, caspase 9 may also be enzymatically

active while associated with the apoptosome without requiring procaspase 9 internal cleavage (Boatright and Salvesen, 2003). Due to its importance in initiating intrinsic apoptotic signaling and this peculiar activation strategy, I utilized a systems-level approach to investigate caspase 9 function in hibernation. No indications of increased caspase 9 activity were evident based on the *in vitro* proteolysis assay. However, the modest release of AIF from the mitochondrial intermembrane space during torpor may indicate some level of *bona fide* upstream apoptotic signaling during hibernation (Figure 16).

These data are consistent with the mitigation of apoptosis during hibernation. Perhaps this result is not surprising in the context of what was previously learned about the regulation of caspase 3. However, there are additional implications to investigating the other apoptotic caspases. We may gain further insight into the mechanism of apoptotic mitigation. Although caspase 6 undergoes a seeming activation during hibernation, there is no evidence for downstream activity. Both caspases 3 and 6 share this mode of regulation. One might have expected an almost haphazard aspect to the homeostasis on hold notion of mammalian hibernation. For instance, two non-regulated pathways that are simply depressed by passive temperature effects may be depressed at different locations. Shared regulatory loci may be indicative of a sensitive regulatory point. If a disconnect in processes were accidental, one would not expect both caspases 3 and 6 to share similar regulation. My data here suggest a common locus for regulation is the prevention of caspase activity not at the level of procaspase activation but at the level of translating that activation into enzymatic activity, *per se*.

When examining the activity of caspase 3, I noted a ~2/3 depression of activity at 37 °C (Figure 4). This depression was not evident at any other temperature or in summer squirrels. In other words, there appeared to be an active suppression of caspase 3 activity specifically in

winter squirrels. A similar depression of enzymatic activity was evident at 37 °C for both caspases 6 and 9 (Figures 13 and 14, respectively). It is important to note that this depression was not restricted to winter squirrels. Instead, summer active animals experienced this depression of caspase activity. These results argue against a specific suppressive mechanism to mitigate the activity of caspases at 37 °C in winter animals. Potential modes for the regulation of caspase activity are discussed in chapter 5.

CHAPTER 4

INCOMPLETE INFLAMMATORY CASPASE SIGNALING DURING HIBERNATION

Abstract

In addition to apoptosis, some caspases have been demonstrated to be important in regulating aspects of the innate immune response and inflammation in mammals. In this chapter, the canonical inflammatory caspases 1, 4, 5, 11, and 12 were investigated using a systems-level approach. Based on western blotting, caspases 5, 11, and 12 were seemingly activated during hibernation. There was no evidence for increased enzymatic activity as would be expected with *bona fide* activation in the in vitro proteolysis assays. Analysis of downstream targets, such as inflammatory cytokines IL-1β and IL-18 were not cleaved during hibernation. Finally, comparisons of plasma aspartate and alanine transaminases showed no indications of global liver inflammation or damage occurring during hibernation. I conclude an incomplete inflammatory caspase activation occurred that did not translate into caspase activity, *per se*.

Introduction

Hibernation allows numerous species to survive unfavorable environmental conditions by depressing most physiological processes and surviving on stored resources. Ground squirrels are exceptional hibernators and experience extreme changes in their physiology during the hibernation season. For instance, ground squirrels depress metabolism during torpor for 1 to 3 weeks where T_b approximates T_a (to as low as -2.9 °C) and rates of oxygen consumption are $1/100^{th}$ of active rates (Barnes, 1989; Carey et al., 2003). Repeatedly throughout the hibernation season, these animals will spontaneously arouse from torpor wherein T_b, metabolism, and

physiology return to non-torpid, euthermic levels. These interbout arousals last between 12-24h before the animal goes torpid again. Transitions between torpor and interbout arousals occur ~15 to 20 times during the hibernation season.

During hibernation, ground squirrels may experience repeated exposure to profound hypothermia, bradycardia, hypoxia, ischemia, and increased ROS production (van Breukelen et al., 2010). These conditions are pro-inflammatory and pro-apoptotic in other systems. In mammals, inflammation is a function of the innate immune system and occurs in response to diverse stimuli such as cell damage, pathogen infection, environmental irritants, metabolite accumulation (McIlwain et al., 2013). Inflammation can be initiated by numerous immune cell types (i.e. macrophages, dendritic cells, mast cells, neutrophils, and lymphocytes) and a few non-immune cell types (i.e. epithelial cells, endothelial cells, and fibroblasts; Ahmed, 2011). Similar to initiator caspase activation, inflammation relies on the formation of large multi-protein complexes, known as inflammasomes, that serve to sense pro-inflammatory signals and recruit and activate inflammatory procaspases (Ramirez and Salvesen, 2018).

Caspases are a family of cysteine-aspartate proteases with described functions in executing apoptosis and inducing inflammation (for review see Fuchs and Steller, 2015; Man and Kanneganti, 2016). Caspases 1, 4, 5, 11, and 12 are considered inflammatory caspases (Elmore, 2007). Inflammatory caspases are produced as single-chain inactive zymogens with three functional domains: a non-catalytic CARD-containing prodomain, a large catalytic subunit, and a small catalytic subunit. Inflammatory procaspase activation, like in apoptotic pathways, requires proteolytic cleavage, conformational changes and dimerization of large and small subunits in order to establish a fully-functional catalytic domain (Earnshaw et al., 1999).

During ischemia and reperfusion injury, the reduction of blood flow results in resident immune cell activation and endothelial cell swelling resulting in a vasoconstriction (Abu-Amara et al., 2010; Szabo and Petrasek, 2015). During reperfusion, activated resident immune cells release pro-inflammatory cytokines. These cytokines further exacerbate inflammation (Ahmed, 2011). Swelling endothelial cells increase expression of adhesion molecules which results in aggregation of platelets and immune cells (Abu-Amara et al., 2010). In other words, there is a positive feedback system resulting in impaired microcirculation following reperfusion. In some cases, this uncontrolled primary inflammatory response may lead to widespread apoptosis (Abu-Amara et al., 2010).

In chapters 2 and 3, I found that canonical apoptotic caspases, including key executioner caspase 3, were *seemingly* activated during hibernation. However, this seeming activation was not translated into caspase enzymatic activity, *per se*. Is this seeming activation restricted to the apoptotic caspases only? Or, are inflammatory caspases subject to similar seeming activation? Elucidation of this phenomenon will provide insight into apoptosis-specific regulation during hibernation.

Here, I employed a systems-level approach to determine inflammatory caspase availability and activation status, *in vitro* enzymatic activity, and evidence for downstream activity for a more complete understanding on caspase signaling during hibernation.

Materials and Methods

Animal care and tissue collection

Adult golden–mantled ground squirrels (*Spermophilus lateralis*) were captured and tissues were collected from summer active (SA), torpid (T), and interbout aroused (IBA) as described previously in Chapter 2.

Sample preparation and western blot analyses

Western blots were performed essentially as described in Chapter 2. Conditions for SDS-PAGE were: caspase 1 (30 µg total protein samples on a 15% acrylamide gel), caspase 4 (30 µg total protein samples on a 17% acrylamide gel), caspase 5 (50 µg total protein samples on a 15% acrylamide gel), caspase 11 (50 µg total protein samples on a 15% acrylamide gel), caspase 12 (50 µg total protein samples on a 17% acrylamide gel), IL-1ß (50 µg total protein samples on a 15% acrylamide gel), and IL-18 (50 µg total protein samples on a 15% acrylamide gel).The following antibodies were used: anti-caspase 1 (Cell Signaling; rabbit polyclonal used at 1:1000), anti-caspase 4 (ProSci Inc.; rabbit polyclonal used at 1:800), anti-caspase 5 (Santa Cruz Biotechnology; goat polyclonal used at 1:200), anti-caspase 11 (Santa Cruz Biotechnology; goat polyclonal used at 1:200), anti-caspase 12 (ProSci Inc.; rabbit polyclonal used at 1:800), anti-IL-1ß (Novus Biologics; rabbit polyclonal used at 1:2000), anti-IL-18 (Clinical Proteomics Technologies via DSHB; mouse monoclonal used at 1:1000). Visualizations for all blots, except caspase 12, were performed using ECL+ on a Typhoon imager (GE Health Sciences). The caspase 12 blot was exposed to IRDye 680LT-conjugated secondary antibodies (LiCor, Inc.) and was visualized on a LiCor Odyssey (Model # 9120; LiCor Inc.).

Caspase activity assays

Sample preparation and assay protocol for caspase activity assays are described in Chapter 2. Reactions were initiated with addition of 40 μM WEHD-AMC substrate (shared substrate by inflammatory caspases 1, 4, 5, and 11; Enzo Life Sciences).

Serum alanine aminotransferase (ALT) assay

General hepatocytoxicity (the combined effects of both inflammation and cell death in liver) with reduced liver function can be assessed by measuring serum aminotransferase alanine aminotransferase (ALT) levels. ALT levels have been used clinically to diagnose various forms of liver damage (Giannini et al., 2005). Typically, ALT values ≥500 IU/l denotes significant liver damage (Gowda et al., 2009). Serum ALT levels were measured spectrophotometrically using an ALT detection kit (Pointe Scientific) in summer active (SA), early torpor (ET: ≤ 20% of previous bout length), late torpor (LT: ~80% of previous bout length), and interbout aroused (IBA) animals (n=3 animals per state). Units are expressed in IU•liter^{-1}. An IU is defined as the amount of enzyme that catalyzes the transformation of one μmole of substrate • min^{-1}.

Results

Availability and activation status of the caspase cascade during hibernation

Caspases 11 and 12

Caspase 11 participates in non-canonical inflammatory signaling and may activate caspase 3 (Kang et al., 2000; Kumar et al., 2007; Ramirez and Salvesen, 2018). When compared to SA squirrels, a 3.07 ± 0.40-fold and 4.45 ± 0.41-fold increased liberation of the p20 fragment was found in torpid and interbout aroused (IBA) squirrels, respectively (Figure 17; ANOVA $p<0.05$).

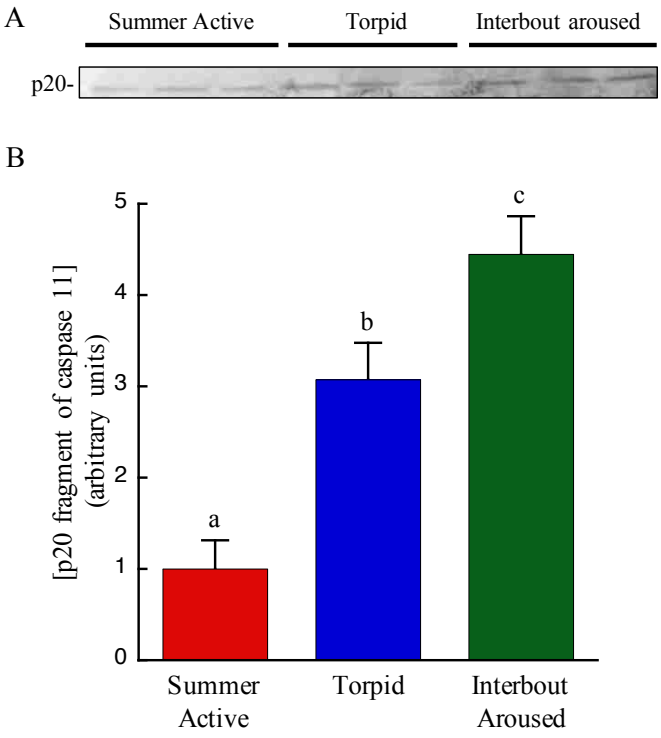

Figure 17. p20 fragment of caspase 11 increases during hibernation. Caspase 11 is processed into a ~20-25 kDa (p20) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p20 fragment (most active caspase fragment detectable) is displayed. Caspase 11 p20 is increased ~3-fold in torpid and ~4.5-fold in interbout aroused squirrels (ANOVA, $p<0.05$) Data for graphs represent means ± standard error (SE).

Caspase 12 is classified as an inflammatory caspase based on amino acid sequence similarities to caspases 1, 4, 5, and 11 (Kalai et al., 2003; Lamkanfi et al., 2004; Ramirez and Salvesen, 2018). When compared to SA squirrels, a 7.11 ± 0.29-fold and a 7.03 ± 2.82-fold liberation of the p20 fragment was found in torpid (T) and interbout aroused (IBA) animals, respectively (Figure 18; ANOVA $p<0.05$).

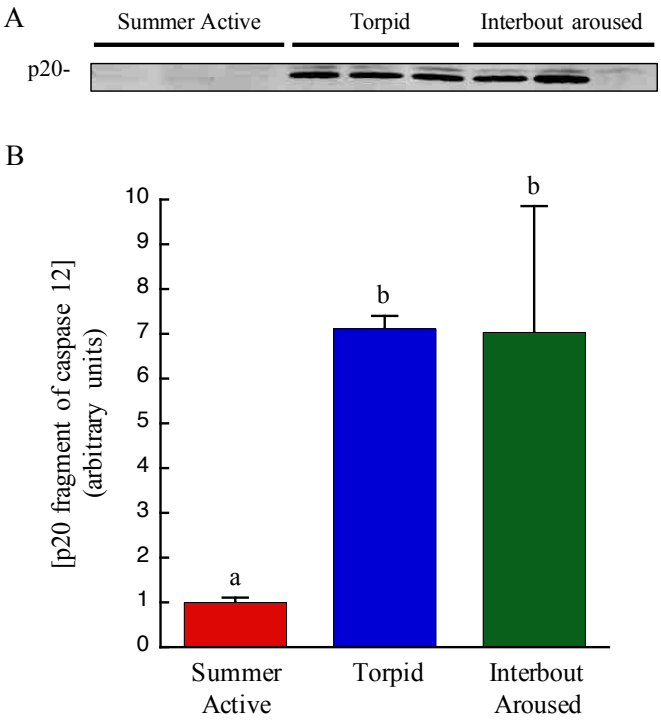

Figure 18. p20 fragment of caspase 12 increases during hibernation. Caspase 12 is processed into a 20 kDa (p20) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p20 fragment (most active caspase fragment detectable) is displayed. Caspase 12 p20 is increased ~7-fold during hibernation in both torpid and interbout aroused ground squirrels (ANOVA, $p<0.05$). Data for graphs represent means ± standard error (SE).

Caspases 1, 4, and 5

Caspase 1, originally named interleukin-1 converting enzyme (ICE), is the primary inflammatory caspase responsible for processing numerous pro-inflammatory cytokines such as IL-1β, IL-18, and IL-33 (Fernandez and Lamkanfi, 2015). No differences in caspase 1 p20 availability were

found during hibernation (Figure 19, ANOVA $p>0.05$). Caspase 4 and 5 are considered homologs of murine caspase 11 (McIlwain et al., 2013). While there was an increase in caspase 5 p10 availability during IBA (~0.5X higher compared to SA, ANOVA $p<0.05$), no other indications of caspase 4 or 5 activation were found during the hibernation season (Figure 20 and Figure 21; ANOVA $p>0.05$).

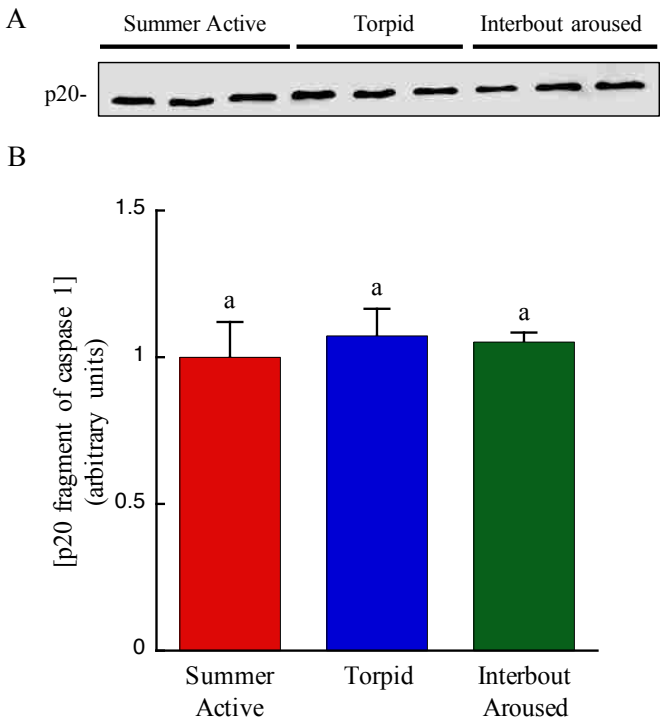

Figure 19. p20 fragment of Caspase 1 is not increased during hibernation. Caspase 1 is processed into a 20 kDa (p20) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p20 fragment (most active caspase fragment detectable) is displayed. No significant differences were found (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE).

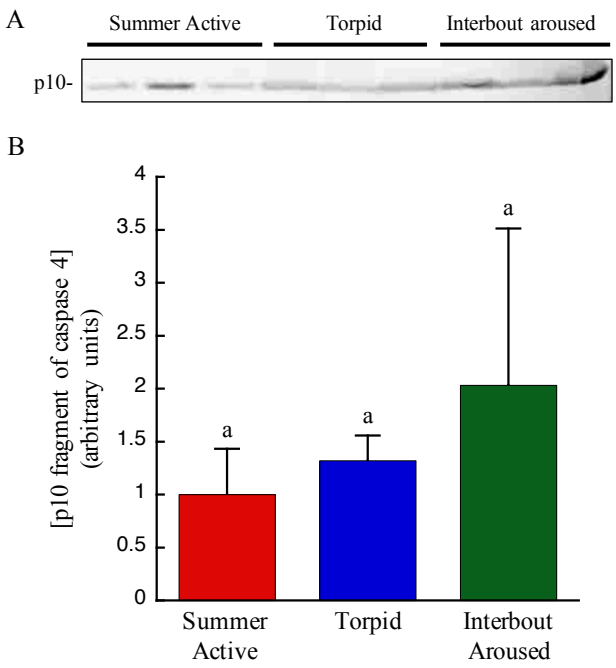

Figure 20. p10 fragment of caspase 4 does not increase during hibernation. Caspase 4 is processed into a 20 kDa (p20) and 10 kDa (p10) fragment upon activation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the p10 fragment (most active caspase fragment detectable) is displayed. Caspase 4 was not activated during hibernation (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE).

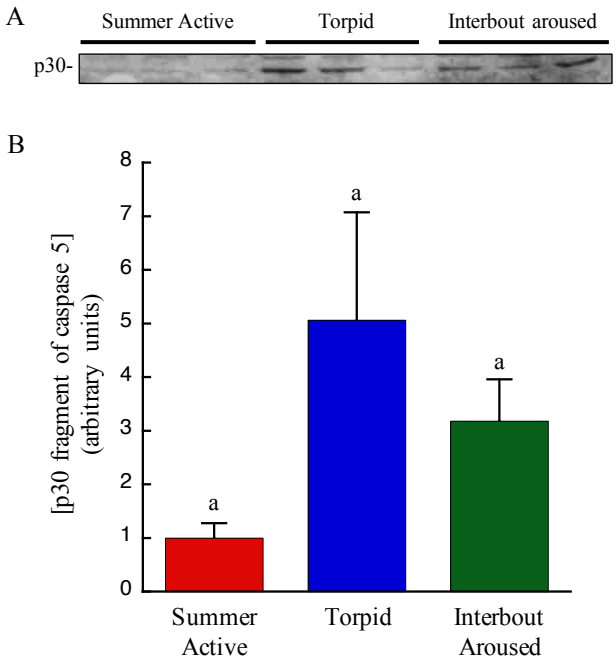

Figure 21. p30 fragment of caspase 5 does not increase during hibernation. Ground squirrel livers (n=3 per state) were obtained from summer active, torpid, and interbout aroused squirrels. For clarity, only the most active detectable caspase fragment (p30 fragment) is displayed. Caspase 5 was not activated during hibernation (ANOVA, $p>0.05$). Data for graphs represent means ± standard error (SE).

Caspase activity assays

To verify if changes in procaspase processing translated into bona fide enzymatic activity, I performed assays wherein the caspase cleaved specific substrates into a fluorescent form. Specific substrates are not available for caspase 11 and 12. Therefore, a shared substrate that is processed by caspases 1, 4, 5, and 11 was utilized. Enzymatic activity of inflammatory caspases was depressed at the low temperatures typical of torpor (e.g. 0-10 °C; Figure 22). Similar to what was found in caspase 6 and 9, activity depression at 37 °C was found in all states (SA, T, and IBA; Figure 22). The average activity depression at 37 °C, when compared to the 30 °C assay, in inflammatory caspases was 20.72 ± 14.55% in SA animals, 23.04 ± 1.44% in T animals, and 36.94 ± 4.43% in IBA animals (Figure 22; n=3 different animals per state). If caspase 11 were truly activated, we would have expected increased enzymatic activity, however, no such increase was noted (Figure 22).

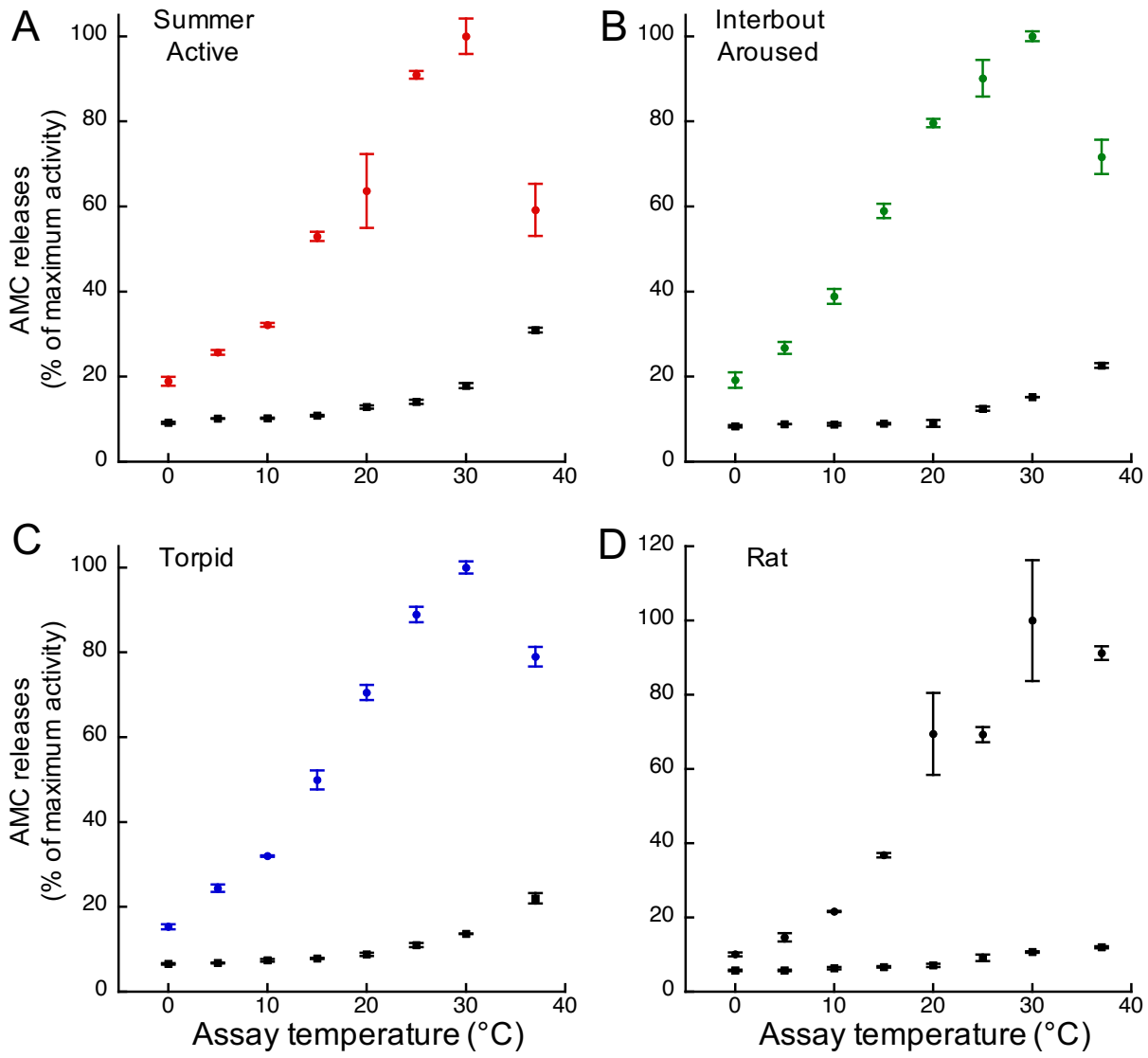

Figure 22. Inflammatory caspase enzymatic activity as a function of assay temperature and state with comparison to non-hibernating rat. Representative results for the effects of assay temperature on general inflammatory caspase activity. Hepatic lysates from A) Summer Active B) Interbout Aroused, C) Torpid squirrels, and D) rat were incubated in the presence of WEHD-AMC, an inflammatory substrate shared by caspases 1, 4, 5, and 11. Assays were incubated for either 1 h (rats) or 4 h (squirrels). Circles represents uninhibited reactions while squares represent lysates that were quenched with HCl prior to the addition of substrate. Values represent means ± SE, (n =3 replicates). Similar results were obtained using lysates derived from two additional animals from each state.

Downstream targets of inflammatory caspases

IL-1β and IL-18 processing

IL-1β is a major pro-inflammatory cytokine that is produced as an inactive 31 kDa form that requires processing into a 17 kDa (p17) active form by caspase 1 (McIlwain et al., 2013). Caspases 4, 5, and 11 stimulate this activation. Unfortunately, there is no appropriate direct downstream target for caspases 11 and 12. As such, I used IL-1β as a proxy for caspases 11 and 12 downstream activity. There is no increase in IL-1β p17 during hibernation (Figure 23, ANOVA, $p>0.05$). Similarly, IL-18 is cleaved by caspase 1 from a 24 kDa protein to the active 18 kDa form. There is no increase in IL-18 p18 during hibernation (Figure 24, ANOVA, $p<0.05$).

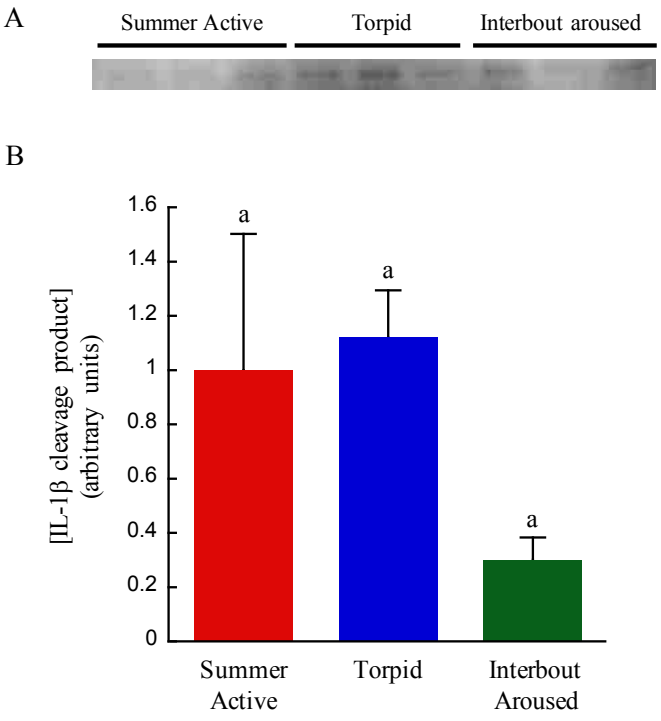

Figure 23. p17 fragment of IL-1β availability during hibernation. Active inflammatory caspase 1 cleaves pro-IL-1β (31 kDa) into active IL-1β (17 kDa). For clarity, only the active 17 kDa fragment is displayed and normalized to summer active levels. IL-1β cleavage did not increase during hibernation and was reduced significantly in interbout aroused squirrels compared to both summer active and torpid squirrels (ANOVA, $p<0.05$). Data for graphs represent means ± standard error (SE).

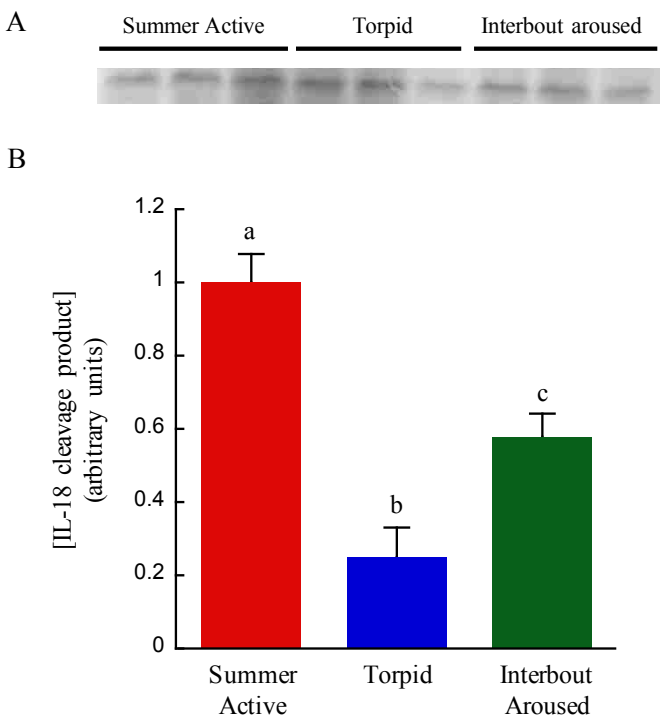

Figure 24. p18 fragment of IL-18 availability during hibernation. Active inflammatory caspase 1 cleaves pro-IL-18 (24 kDa) into active IL-18 (18 kDa). For clarity, only the active 18 kDa fragment is displayed and normalized to summer active levels. IL-18 cleavage did not increase during hibernation and was reduced significantly in winter squirrels compared to both summer active (ANOVA, $p<0.05$). Data for graphs represent means ± standard error (SE).

Alanine aminotransferase levels in serum

Alanine aminotransferase (ALT) is an enzyme with high concentrations in liver with low concentrations found in other tissues such as skeletal muscle and kidney (Giannini et al, 2005). ALT release into the blood is indicative of hepatocellular damage (Gowda et al., 2009). In humans, non-pathological serum ALT levels are ~7-56 IU/l (Gowda et al., 2005). Marked elevations of serum ALT (>500 IU/l) suggests significant hepatocellular damage from acute damage from events such as hepatic ischemia and reperfusion injury. We sampled the entire torpor cycle and found that, while ALT values appear elevated compared to baseline human levels, ALT levels during torpor were not different than summer squirrels (Figure 25).

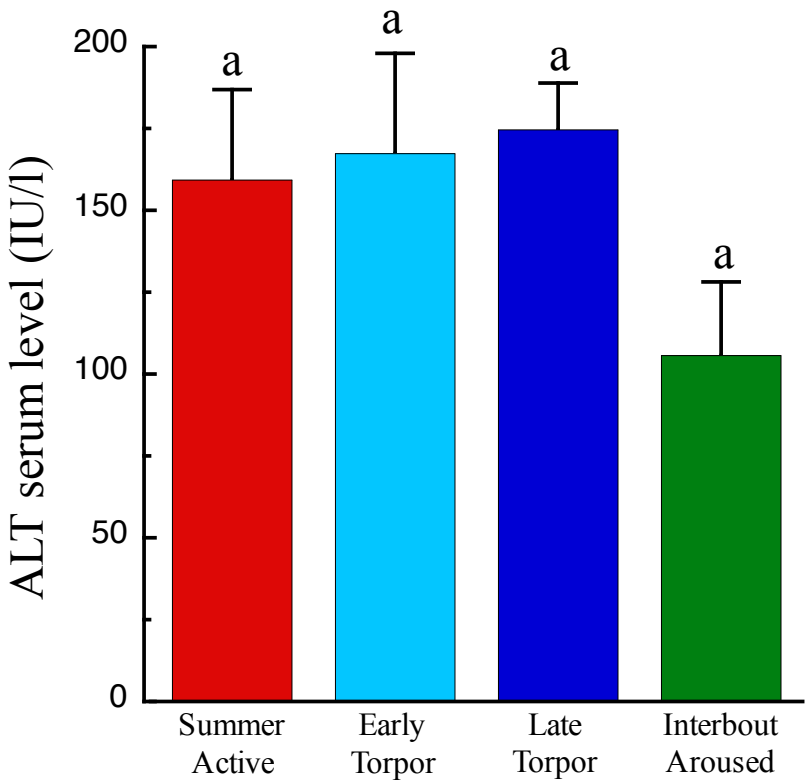

Figure 25. Alanine aminotransferase (ALT) levels do not increase during the hibernation season. Ground squirrels (n=3 animals per state) were analyzed from summer as well as from various points during hibernation (early torpor: <25% of torpor bout; late torpor: >80% of torpor bout; interbout aroused). While the hibernation values appear modestly elevated compared to reference values in humans, no significant increases were found compared to summer squirrels. Furthermore, values ≥500 IU/l are indicative of liver inflammation (ANOVA; $p>0.05$).

Discussion

The caspase cascade is complex with diverse functions in development, homeostasis, and disease (Elmore, 2007). In addition to regulating apoptotic execution, the caspases are well-characterized regulators of inflammation through cleaving and activating various pro-inflammatory cytokines (Ramirez and Salvesen, 2018). Caspase 1 is responsible for directly processing cytokines while caspases 4, 5, 11, and 12 are involved in supporting caspase 1 function (McIlwain et al., 2013). Pathological states like ischemia and reperfusion injury and acidosis begin as inflammatory signaling that, if unmitigated, eventually results to widespread apoptosis, tissue damage, and even death (van Breukelen et al., 2010; Abu-Amara et al., 2010).

Here, I utilized a systems-level approach to determine the activation status and function of inflammatory caspases during mammalian hibernation.

Based on western blotting, the active fragment (p20) of caspase 11 increased ~3- to 4-fold in winter ground squirrels (Figure 17). Caspase 11 is considered a non-canonical inflammatory caspase because it does not process inflammatory cytokines directly (Kayagaki et al., 2011). In addition to participating in its own inflammasome structure, caspase 11 also interacts in the caspase 1 inflammasome. Current though is that caspase 11 exacerbates caspase 1 function (Kayagaki et al., 2011). Much like what occurs for caspase 1, lipopolysaccharide (LPS) administration results in increase caspase 11 expression and activity. Interestingly, caspase 11 participates in lytic cell death of immune cells that, if unchecked, may result in a cytokine storm (Kayagaki et al., 2011; Crowley et al., 2017). Caspase 11 also participates in apoptotic signaling (Ramirez and Salvesen, 2018; Kang et al., 2000). Caspase 11 activates caspase 3 in ischemia and reperfusion injury (Ramirez and Salvesen, 2018; Kang et al., 2000).

As was evident in chapters 2 and 3, liberation of the active form of a caspase does not necessarily result in increased enzymatic activity. The enzymatic activity assay (shared substrate for caspases 1, 4, 5, and 11) demonstrated no increased activity as what would be expected given the western blot data (Figures 17, 18, and 22). To date, there are no identified unique downstream targets for caspase 11. Given its role in supporting caspase 1 activity, we indirectly assessed caspase 11 activity by examining IL-1β and IL-18 processing (Figures 23 and 24). There are no indications of downstream processing suggesting a limited role for caspase 11 activation in hibernation.

A robust liberation (~7-fold increase in p20) of the active fragment of caspase 12 was found in winter squirrels based on western blotting. Caspase 12, unlike other inflammatory

caspases, is not found in immune cells like macrophages and neutrophils, but rather is found in liver, skeletal muscle, heart, brain, testis, and eye tissue (Kalai et al., 2003). Although classically defined as a pro-inflammatory caspase, caspase 12 has a very well defined role in promoting apoptosis (Nakagawa et al., 2000). Endoplasmic reticulum (ER) stress specifically activates caspase 12 through calcium release. Caspase 12 activates procaspase 3 resulting in execution of apoptosis (Hitomi et al., 2003). I am unable to find a specific downstream target for caspase 12. However, and as evidenced in chapter 2, there is no indication for increased enzymatic activity of caspase 3. Thus, I conclude caspase 12 is not having downstream effects during hibernation.

A global perspective of inflammatory processes may be obtained through analysis of serum alanine aminotransferase (ALT; Giannini et al., 2005). Release of this enzyme can indicate inflammatory damage to the liver. I found no increases in ALT serum levels in winter animals (Figure 25). Furthermore, ALT levels above 500 IU/l suggest hepatic inflammation and ground squirrel values were never above 175 IU/l, which does not support significant hepatocellular damage due to inflammation.

Could regulation of inflammatory caspases inform on the regulation of apoptotic caspases? In chapters 2 and 3, I identified liberation of the active fragments of caspases 3 and 6 during hibernation. Similar liberation of these active fragments was found here for caspases 11 and 12. Importantly, the systems-level approach applied here, and in chapters 2 and 3, revealed downstream targets were not processed consistent with a *bona fide* activation of these caspases. The *in vitro* enzymatic assays for caspases 3, 6, 9, and 11 shared the characteristic of depressed activity specifically at 37 °C (Figures 4, 13, 14, and 22). In the caspase 3 study, the depression at 37 °C was limited to the winter animals. These data suggested a potential hibernation-specific regulation. That this depression at 37 °C is found in summer active animals for caspases 6, 9, and

11 argues against a hibernation-specific mechanism. Further, caspase 11 is pro-inflammatory. The seeming activation of caspases 11 and 12 without downstream effect further supports the notion of caspase regulation *after* seeming activation. In other words, more than just the apoptotic caspases may experience processing leading to liberation of the active fragment without associated increases in enzymatic activity. These data argue against an apoptosis-specific regulation and suggest a common mechanism for all caspases.

As indicated earlier, caspases 11 and 12 have well defined roles in processing procaspase 3 into its active form (Kang et al., 2000; Hitomi et al., 2003). It is interesting to speculate that perhaps the activities of caspases 11 and 12 may have been responsible for the observed liberation of the active p17 fragment from caspase 3. However, there was no processing of the inflammatory caspase targets, IL-1β or IL-18 (Figures 23 and 24). It seems unlikely that procaspase 3 would be a more sensitive substrate for caspases 11 or 12 than procaspase 1 or the downstream inflammatory targets.

Taken together, the data from chapters 2, 3, and 4 argue for inhibition of caspases after seeming procaspase activation. I suspect the depression of activity specifically at 37 °C may be an artifact of the assay more than an indication of specific regulation. Potential modes for the regulation of caspase activity are discussed in chapter 5.

CHAPTER 5

POTENTIAL MODES OF CASPASE REGULATION AND ALTERNATIVE ROLES FOR CASPASES

In chapters 2, 3, and 4, I demonstrated that procaspases were seemingly activated into active caspases based on western blotting. Tempering that conclusion were both enzymatic activity assays for the caspases and downstream target analyses. These latter data do not support the expected increased activity suggested by the western blotting. The obvious question is how might caspase activity be depressed after seeming activation.

Ockham's razor is a heuristic guide which suggests following the simplest explanation for a problem. In the previous chapters, I obtained a large amount of information of what may or may not be regulating caspases.

1. Are caspases allowed to be activated during hibernation? Caspases 3, 6, 9, 11, and 12 all show signs of seeming activation, wherein the most active fragment was released during hibernation. These data suggest caspases are allowed to be activated during hibernation. This finding eliminates regulatory processes that prevent the activation of caspases having a major role in regulating apoptosis during hibernation.
2. Is there significant apoptosis during hibernation? Despite the seeming activation of multiple caspases, there does not appear to be significant apoptosis during hibernation. TUNEL assays and analyses of downstream caspase targets (ICAD, PARP, lamin A/C, IL-1β, IL-18) all support that an activation of the procaspase is not simply translated into enzymatic activity.

3. Is the depression of caspase enzymatic activity at the low temperature typical of torpor sufficient to explain a lack of apoptosis? While the cold temperatures of torpor would depress caspase enzymatic activity, these data do not explain how hibernators may avoid apoptosis. Seeming activation was evident in both torpor and/or interbout arousals for caspases 3, 6, 9, 11, and 12. There was high caspase enzymatic activity at temperatures typical of a euthermic squirrel. While temperature may explain depression for torpid squirrels, apoptosis would be expected for interbout aroused squirrels. This observation is not supported by the TUNEL assays.
4. Are caspases specifically inhibited at 37 °C during hibernation? The data for the enzymatic activity assays for caspases 3, 6, 9, and 11 demonstrated a specific depression in enzymatic activity at 37 °C. It was tempting to speculate that these data represented a hibernation-specific mechanism to depress apoptosis. However, this depression is observed in summer active animals for caspases 6, 9, and 11. Further, the depression was observed for both apoptotic and inflammatory caspases suggesting this phenomenon is not apoptosis-specific. I suggest this phenomenon may have simply been an artifact of the assay as opposed to a meaningful regulatory mechanism.
5. Are the western blot data reliable? Could perceived increases in protein availability be inaccurate? One might argue that western blotting could be considered semi-quantitative. I employed an amplification process in the detection step e.g. western blots were visualized using horseradish peroxidase. Are these observed changes real? I used three samples for each state. The standard error within state is relatively small suggesting consistency as a function of state. Caspase activation typically resulted in

the liberation of 1-4 fragments. When possible, I measured all of these fragments (Appendix). Typically, there was good consistency between fragments e.g. when the active fragment increased, corresponding changes were observed in other fragments or the procaspase form. Were the appropriate states examined? I included animals sampled in the summer in order to establish a baseline expectation for apoptosis. Within the hibernation season, I included animals that were torpid and interbout aroused as these states are on opposite sides of the metabolism spectrum. Furthermore, where concerns that the processes of apoptosis may have been concluded by the time sampling occurred, I included arousing animals e.g. TUNEL assays. One might argue that a 2-fold change in active fragment availability is not much of a change. However, it should be noted caspase 9 increases in activity by ~2,000-fold and caspase 3 increases in activity by >10,000-fold following processing of the procaspase form (Boatright and Salvesen, 2003). In addition, some caspases e.g. caspases 11 and 12, experienced an increase in the active fragment as much as 8-fold. For caspase 5, there was only a ~0.5-fold increase in the active fragment of interbout aroused squirrels. In this particular case, parallel changes in other caspase 5 fragments was not observed. One might conclude that the observed changes in caspase 5 were erroneous but given the more substantive changes to the other caspases, such as conclusion is probably isolated to just this particular caspase. It seems unlikely that the western blot data are unreliable.

In the following section, I will address putative modes of apoptotic regulation. Numerous anti-apoptotic mechanisms exist that act to depress caspase signaling after caspase activation which could be exploited during hibernation.

Alternative splicing modulates caspase function

Alternative splicing of caspases generates multiple caspase isoforms from a single caspase gene often resulting in distinct pro- or anti-apoptotic forms. Caspases 2, 3, 6, 7, 8, and 9 have all been shown to undergo differential mRNA splicing (Jiang et al., 1999). Caspase mRNA alternative splicing typically results in "long" enzymatically competent (pro-apoptotic) or "short" enzymatically incompetent (anti-apoptotic) forms. In general, the short isoforms are created by addition of gene segments containing premature stop codons or through exclusion of exons containing essential catalytic domain elements (Wang et al., 1994; Parrish et al., 2013; Paronetto et al., 2016). These non-functional isoforms are considered to be anti-apoptotic because they retain their activation recruitment domain and are still recruited to activation complexes, however, they have no proteolytic function. Caspase splice variants, therefore, function as dominant negatives by reducing functional caspase access to the DISC, apoptosome, or inflammasome (Paronetto et al., 2016). These isoforms will oftentimes be expressed simultaneously in cells, therefore the ratio of the various isoforms may be important in determining the function of the caspases (Jiang et al., 1999). Although the procaspase form of these anti-apoptotic splice variants may be processed (Jiang et al., 1999), active fragment size would be different since the anti-apoptotic splice variants lack exons. The identified fragments were of expected size for the pro-apoptotic splice forms in the western blots for caspases 3, 6, 11,

and 12 (Figures 3, 7, 17, and 18). Splice variant availability is unlikely to explain the mitigation of apoptosis during hibernation.

Inhibitors of apoptosis proteins inhibit caspases

The inhibitors of apoptosis proteins (IAPs) are a family of proteins that function in inhibiting caspases (Vasilikos et al., 2017). IAPs contain small, highly-conserved, 70-80 residue zinc-binding motifs originally discovered in insect p35 baculovirus known as baculoviral IAP repeat (BIR) domains that are essential to IAP's ability to inhibit caspase function (Fuentes-Prior and Salvesen, 2004). Of the numerous mammalian IAPs, only the X-linked IAP (XIAP) has been shown to be able to directly inhibit caspases (Vasilikos et al., 2017). XIAP contains three BIR domains which allow it to directly bind to and inhibit the active site of caspases 3, 7, and 9. XIAP can only inhibit active caspases, and hence, represents an important regulatory mechanism for controlling spurious or subthreshold caspase activation (Fuentes-Prior and Salvesen, 2004). XIAP inhibits caspase 3 and 7 via a linker region between the BIR1 and BIR2, while caspase 9 inhibition occurs at a region linking BIR3 and the RING domain (Fuentes-Prior and Salvesen, 2004). Interestingly, the regional difference in caspase 3/7 and 9 inhibition sites in XIAP allows for simultaneous binding and inhibition of caspase 3/7 and apoptosome-associated caspase 9 (Fuentes-Prior and Salvesen, 2004).

In the related thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, XIAP expression varies by tissue and state (Rouble et al., 2013). Further, 13-lined ground squirrel kidneys subjected to experimental ischemia/reperfusion injury showed consistent XIAP expression associated with low levels of apoptosis (Jain et al., 2016). When XIAP expression was knocked down with siRNA, caspase 3 activation and TUNEL activity increased (Jain et al.,

2016). Our laboratory examined XIAP availability in liver as a function of state and found no changes. To date, XIAP has not been shown to function with caspases 6, 11 and 12. As such, and while there is the possibility that XIAP may be actively suppressing the active caspase 3, this mechanism would not be effective for caspases 6, 11, and 12.

Post-translational modifications of caspases regulate caspase activity

The activities of most proteins can be regulated directly through a staggering number of small-molecule modifications known as post-translational modifications (PTM). Caspases are no different, and are currently known to be ubiquitylated, phosphorylated, nitrosylated, SUMOylated, acetylated, and glutathionylated (Parrish et al., 2013; Zamaraev et al., 2017). Caspases 2, 3, 6, 7, 8, and 9 are all known to be subject to various PTMs (Zamaraev et al., 2017).

Phosphorylation

Phosphorylation of caspases typically results in inhibition, however, in some cases, phosphorylation may foster increased caspase activation and apoptotic signaling (Parrish et al., 2013; Raina et al., 2005; Zamaraev et al., 2017). Caspase 9 is the most well-characterized caspase regarding phosphorylation. Phosphorylation of caspase 9, and most likely other caspases, can result in either pro- and anti-apoptotic effects depending on which residues were phosphorylated (Parrish et al., 2013). For instance, caspase 9 gets phosphorylated at T125, which lies between the CARD pro-domain (apoptosome recruitment motif) and the large catalytic subunit, by various kinases (e.g. ERK, cdk1, DYRK1A, and p38α; Parrish et al., 2013). Presumably, phosphorylation at this residue represents one mechanism to prevent significant caspase 9 activation upon spurious cytochrome c release from the mitochondria. Another

inhibitory phosphorylation is carried out by PKCζ at caspase 9 Ser144 which results in suppression of caspase 9 activity and has been proposed as a cellular survival mechanism in response to hyperosmotic stress (Brady et al., 2005). In newborn piglets, caspase 9 was shown to be phosphorylated at Ser196 when exposed to hypoxia (Levenbrown et al., 2008). Caspase 9 is phosphorylated by p-Akt at Ser196 which downregulates caspase 9 activity and ability to effect apoptosis (Sangawa et al., 2014). In contrast, phosphorylation of caspase 9 by tyrosine kinase c-Ab1 at Tyr153 after DNA damage facilitates increased caspase 9 activation and exacerbates apoptotic signaling (Raina et al., 2005). Caspase 6 is also known to be phosphorylated by ARK5 at Ser257 resulting in complete inactivation through modification of the substrate binding groove (Velazquez-Delgado and Hardy, 2012). This phosphorylation results is a misaligned substrate-binding groove and inhibits proper substrate binding. Interestingly, this phosphorylation affects the activity of fully-activated caspase 6. Therefore, we can see how phosphorylation networks may serve to fine-tune the cellular activities of caspases under various contexts.

In the non-steady state condition of hibernation, phosphorylation may or may not be important. Numerous examples of phosphorylation and presumed regulatory effect have been identified (Eddy and Storey, 2003; van Breukelen et al., 2004; Frerichs et al., 1998). Complicating the understanding of phosphorylation effects are that phosphorylation may simply be spurious in that unused proteins (as the result of other mechanisms) may be more prone to phosphorylation (van Breukelen et al., 2004). Hindle and colleagues (2014) demonstrated proteins that were phosphorylated during entrance into hibernation did not experience expected dephosphorylation events during arousal. Such data emphasize the spurious nature of phosphorylation during the non-steady state condition of hibernation.

I did not identify any changes in phosphorylation status in my western blots for caspases

1 through 12. It remains unknown if phosphorylation may be playing a role in the depression of caspases 3, 6, 11, and 12.

Ubiquitylation

Another common PTM known to affect caspase activity is ubiquitylation. Ubiquitin is a small ubiquitously expressed protein that is conjugated to lysine residues in thousands of target proteins by ubiquitin ligases (Rape, 2018). Interestingly, many of the identified IAPs may be moonlighting E3 ubiquitin ligases (Huang et al., 2000; Bertrand et al., 2008; Zamaraev et al., 2017). While one typically associates ubiquitylation with protein degradation, there are numerous examples of regulation using ubiquitin (Rape, 2018). The X-linked IAP (XIAP) is a potent inhibitor of caspases 3, 7, and 9. This inhibition occurs when the BIR domain of XIAP associates with the active site of these caspases. However, XIAP may also foster ubiquitylation of caspases 3, 7, and 9. For at least caspase 3, this ubiquitylation event is not associated with degradation, but rather inhibits the activity of the caspase enzyme (Schile et al., 2008; Bader and Steller; 2008). Similar to canonical XIAP caspase inhibition, XIAP can only ubiquitylate the activated forms of caspase 3, 7, and 9 but has no effect on the procaspase forms (Zamaraev et al., 2017). During torpor in ground squirrels, protein ubiquitin conjugates increase 2- to 3-fold depending on tissue type (Velickovska and van Breukelen, 2007). This increase in ubiquitin-conjugates during torpor appears to be the result of a lack of coordination between the ubiquitin conjugation system and protein degradation by the 26S proteasome (van Breukelen and Carey, 2002; Velickovska and van Breukelen, 2005 and 2007). Specifically, the ubiquitylation machinery is only moderately temperature sensitive and continues at ~30% even at 0 °C (Velickovska and van Breukelen, 2007). Protein degradation at the 26S proteasome, however, is

more temperature sensitive and is essentially depressed at the cold T_bs experienced during torpor (Velickovska and van Breukelen, 2007). Decoupling the ubiquitylation-degradation pathway results in the non-specific accumulation of ubiquitylated proteins during torpor that must be rectified during IBA. Given that caspase ubiquitylation may depress caspase activity *after* activation and ubiquitin conjugates are known to increase during hibernation this mechanism seems plausible to explain the observed depression of caspase activity (Figure 26).

Other caspase PTMs

Characterization of other PTMs of caspases is limited. Currently, caspases are known to be subject to nitrosylation, SUMOylation, acetylation, and glutathionylation (Zamaraev et al., 2017). SUMO-conjugates increase 10- to 30-fold in the brain during hibernation (Lee et al., 2018). However, it is unknown if SUMOylation could be responsible for caspase regulation. SUMOylation of caspases 2, 7, and 8 seem likely, but not caspase 3 and 9 (Hayashi et al., 2006). Furthermore, caspase 8 SUMOylation appears to help regulate nuclear localization but not activity (Besnault-Mascard et al., 2005). At this point, too little is known to implicate these other PTMs in the regulation of caspase activity during hibernation.

Other anti-apoptotic pathways

There are numerous other anti-apoptotic pathways. Akt activity can directly inhibit the pro-apoptotic activities of BCL-2-associated death promoter (BAD) and caspase 9 (Zhang et al., 2011). These activities would prevent the activation of procaspases. Such a mechanism would not explain the seeming activation of procaspases that I observed.

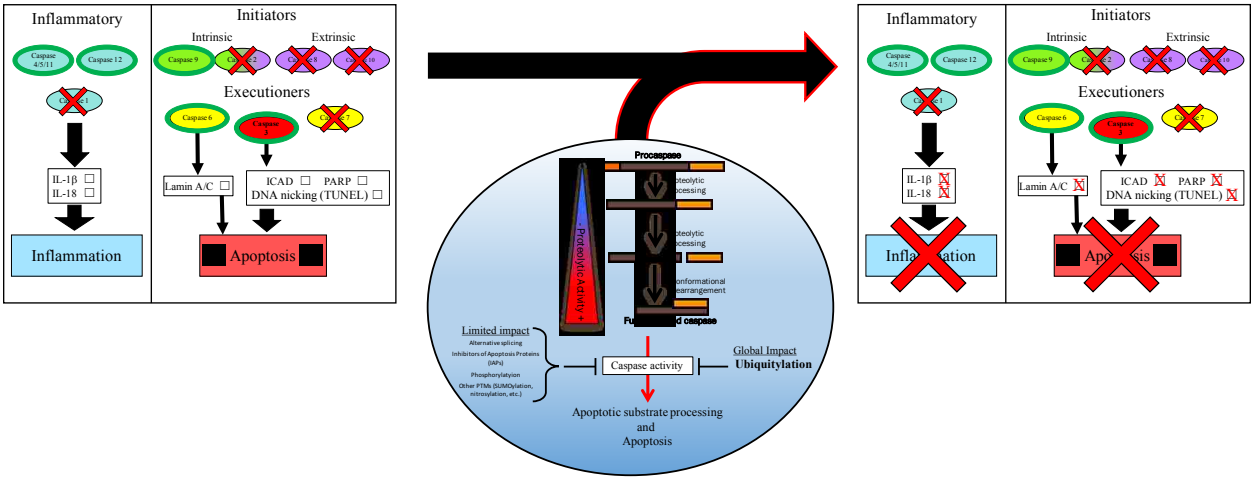

Figure 26. Proposed model for controlling caspase activity after activation during hibernation in ground squirrels. Using western blotting, caspases 3, 6, 9, 11, and 12 were found seemingly activated during hibernation. However, caspase activity assays and analysis of downstream caspase targets did not indicate the increased caspase activity expected with liberation of active caspase fragments. While several mechanisms are known to regulate caspase activity *after* activation, ubiquitylation seems most parsimonious with the caspase cascade data from hibernating ground squirrels.

Non-apoptotic caspase functions

Through manipulation of *Drosophila* genetics, more nuanced, non-apoptotic caspase functions have been elucidated (Mukherjee and Williams, 2017). Caspases, when not executing apoptosis, are implicated in important processes like development, cellular plasticity, and immune function (Mukherjee and Williams, 2017; Nakajima and Kuranaga, 2017; Baena-Lopez et al., 2017). *Drosophila* Apaf-1, caspase 9, and caspase 2 (known as Dark, Dronc, and Dcp-1, respectively) were found to be involved in spermatid individualization and development (Mukherjee and Williams, 2017). Dronc (caspase 9) was then found to be required in numerous developmental processes in *Drosophila* such as compensatory cell division in wing discs, regulating Wingless signaling, and was crucial for proper border cell migration in oogenesis (Mukherjee and Williams, 2017; Nakajima and Kuranaga, 2017).

The non-apoptotic functions of the caspases are also important in various forms of cellular plasticity and have been well-documented in neurons and stem cells from various tissue

types (Mukherjee and Williams, 2017; Baena-Lopez et al., 2017). Non-apoptotic caspase activity is involved in a process referred to as neuronal pruning wherein neurons can selectively eliminate peripheral elements (e.g. synapses and dendrites) without death of the primary neuron (Mukherjee and Williams, 2017). Through use of gene knockouts, caspases 3, 6, and 9 as well as BAX and XIAP have been confirmed to play roles in pruning. In stem cells, the caspases demonstrate interesting non-apoptotic functions. In mouse embryonic stem cells (mESCs), sub-lethal caspase 3 and 9 activity was shown to promote proliferation and differentiation in various cell types. Another example of non-lethal caspase activity in stem cells was demonstrated in the development of induced pluripotent stem cells (iPSCs; Baena-Lopez et al., 2017). Caspase 3 and 8 activation is required for the dedifferentiation of human fibroblasts into iPSCs as this process requires the caspase-mediated degradation of retinoblastoma protein (Rb). To confirm this role, use of a mutant, non-cleavable Rb protein effectively prevented iPSC formation (Baena-Lopez et al., 2017).

Caspases also demonstrate important, non-apoptotic roles in the immune system. For instance, caspase 8 is essential for proper T-cell proliferation upon cytokine stimulation, as well as being important for the development of resting macrophages (Baena-Lopez et al., 2017; Solier at al., 2017). Caspase 3 has also been demonstrated in dendritic cell formation (Baena-Lopez et al., 2017). Caspases are implied in macrophage polarization as well (Baena-Lopez et al., 2017).

Obviously, our understanding of the full biological significance of a broad protein family like the caspases is far from complete. Once associated exclusively with “all-or-nothing” inflammatory and apoptotic signaling pathways, recent research on non-canonical caspase functions have revealed a nuanced class of proteases with more and more realized connections to life sustaining and pro-survival processes.

CHAPTER 6

SUMMARY, SIGNIFICANCE, AND FUTURE DIRECTIONS

Controlling widespread inflammation and apoptosis is critically important during hibernation. Unmitigated apoptosis is at odds with an energy-sparing strategy of hibernation. Furthermore, unmitigated apoptosis might lead to organ dysfunction and even death. Perhaps not surprisingly, mortality during hibernation is high. In ground squirrels, ~40% of adult squirrels and up to ~70% of juveniles may die each winter (Sherman and Morton, 1984). I speculate that mismatches of homeostasis may deplete energetic stores and lead to death. Hibernators repeatedly experience conditions that are pro-inflammatory and pro-apoptotic in non-hibernating mammals. Despite these conditions, no evidence of widespread cellular damage, tissue depletion, or apoptosis has been found in hibernating mammals to date. Indeed, that hibernators survive at all suggests mitigation of apoptosis. Simplistically, controlling apoptosis can be achieved through two general mechanisms: 1) bolster cell survival, or 2) inhibit apoptosis. Most animals undoubtedly employ a combination of both mechanisms.

Common approaches to the study of apoptosis typically includes a limited analysis of pro-apoptotic or anti-apoptotic regulators. Conclusions are based on a limited snapshot of one or a few apoptotic regulators. This approach is unlikely to yield satisfactory results. The tremendously complex and interwoven nature of apoptotic regulation warrants a more comprehensive and systematic analysis. Investigators may approach the study of apoptosis by focusing on either the caspase cascade or the myriad of identified regulators that influence caspase activity. These regulators include the BCL family members, inhibitor or apoptosis

proteins (IAPs), the anti-IAPs, the intrinsic and extrinsic signaling pathways. I chose to focus on the caspase cascade as it is required for the execution of apoptosis, *per se*.

Of the 15 known mammalian caspases, I studied caspases 1-12. The other caspases are not available in ground squirrel liver. This comprehensive analysis enhanced the likelihood of finding the regulatory nodes in this interwoven pathway. Hibernation represents a non-steady state condition. The use of systems-level approaches in previous investigations revealed incomplete pathway commitment during hibernation. For instance, p53 function involves recruitment to DNA before the cold temperatures of torpor result in no elongation of pre-initiated transcripts (Pan et al., 2014). Such a result might be considered surprising since one might assume that p53 function would have been regulated much more upstream in the pathway. I felt compelled to use a systems level approach toward the study of apoptosis in hibernation. For my analyses, I wanted to include downstream targets of caspase function.

In my studies, I found a seeming activation of caspases during hibernation. This seeming activation was evidenced by increased liberation of the most active fragment in winter animals. For instance, the p17 fragment of caspase 3 was increased ~2-fold in winter squirrels (Figure 3). Such an increase would be predicted to translate into a ~20,000-fold increase in enzymatic activity of the caspase (Boatright and Salvesen, 2003). Importantly, caspase 3 activation is typically thought to commit a cell to apoptosis (Elmore 2007; Ramirez and Salvesen, 2018).

Using a systems-level approach, I showed that in the non-steady state condition of hibernation, the seeming caspase 3 activation does not commit cells to apoptosis. Analysis of enzymatic activity and downstream targets demonstrated a lack of execution of apoptosis during mammalian hibernation. Specifically, I was able to identify the locus of regulation of apoptosis during hibernation is not via prevention of caspase activation, but instead, is associated with

depression of enzymatic activity. This result may be unexpected. After all, wouldn't a more precise regulatory mechanism involve the *prevention* of caspase activation?

Early hibernation research predicted that the hibernation phenotype was a result and dependent on unique adaptations the allowed animals to function well in the cold. van Breukelen and Martin (2002) challenged these assumptions and demonstrated a lack of specific adaptations for hibernation. When one combines this lack of adaptation with the previous results involving systems-level approaches of hibernation, the partial commitment toward apoptosis is no longer surprising. One of the lessons learned from hibernation research is that regulation does not have to be precise, it simply must be effective. It is unclear if there are consequences to this partial commitment towards apoptosis. Could some activity of these caspases result in homeostatic mismatches?

Irrespective, my study demonstrates the value of a systems-level approach. If I had only analyzed caspase 3 via western blot analysis, I would have concluded significant apoptosis was occurring during hibernation consistent with the pro-apoptotic conditions. The use of the systems-level approach allowed me to clarify the regulation of a complex pathway in a non-steady state condition. More specifically, my data suggest hibernators cannot prevent the activation of caspases but effectively mitigate apoptosis. My data further suggest that the regulatory mechanism involves a depression of caspase enzymatic activity. Furthermore, this mechanism is not exclusive to the apoptotic caspases as inflammatory caspases were similarly affected.

Future directions might include the characterization of additional pro- and anti-apoptotic elements during hibernation. This includes but is not limited to: the roles for Inhibitor of Apoptosis proteins (IAP), anti-IAPs, anti-inflammatory cytokines, Bcl-2 members, caspase

superstructure assembly e.g. apoptosome, DISC function, inflammasomes, post-translational modifications of caspases including potential ubiquitylation, and more.

APPENDIX

List of abbreviations

1. T_b- body temperature
2. h- hours
3. IBA- interbout arousals
4. ENT- animal entering torpor
5. LT- animal at a period late in a torpor bout
6. ARO- animal arousing from torpor
7. bpm- beats per minute
8. T_a- ambient temperature
9. IAPs- inhibitor of apoptosis proteins
10. TNFR- tumor necrosis factor receptor
11. FasR- Fas receptor
12. ROS- reactive oxygen species
13. AIF- apoptosis inducing factor
14. DED- death effector domain
15. FADD- Fas-associated death domain
16. DISC- death-inducing signaling complex
17. MOMP- mitochondrial outer membrane permeabilization
18. Apaf-1- apoptotic protease activating factor 1
19. CARD- caspase-activating recruitment domain
20. WAT- white adipose tissue
21. BAT- brown adipose tissue
22. NLR- NOD-like receptor
23. PAMP- pathogen-associated molecular pattern
24. DAMP- damage-associated molecular pattern
25. ASC-apoptosis-associated speck-like protein containing a CARD
26. ICAD- inhibitor of caspase-activated DNase
27. CAD- caspase-activated DNase
28. PARP- poly-ADP ribose polymerase
29. TUNEL- terminal UTPase nicked-end label
30. SA- summer active
31. PVDF- poly-vinyl difluoride
32. HRP- horse radish peroxidase
33. AMC- aminomethylcoumarin
34. PBS- phosphate buffered saline
35. TBS- tris buffered saline
36. ANOVA- analysis of variance
37. IU- international unit
38. ICE- interleukin-1 converting enzyme
39. ALT-Alanine aminotransferase
40. LPS- lipopolysaccharide
41. iPSC- induced pluripotent stem cells
42. mESC- mouse embryonic stem cells

Table 3. Western blot analysis of caspase fragments

		State		
Caspase	**Fragment**	**Summer Active**	**Torpid**	**Interbout Aroused**
Caspase 1	p48	1.00 ± 0.32	0.94 ± 0.27	0.40 ± 0.05
	p40	0.51 ± 0.03	0.37 ± 0.02	0.44 ± 0.06
	p20	3.61 ± 0.43	3.87 ± 0.33	3.80 ± 0.12
Caspase 2	p51	1.00 ± 0.01	0.78 ± 0.10	0.91 ± 0.19
	p30	0.11 ± 0.03	0.09 ± 0.01	0.11 ± 0.02
	p18	0.13 ± 0.01	0.17 ± 0.02	0.13 ± 0.05
Caspase 3	p32	1.00 ± 0.10 ^{A}	2.18 ± 0.10 ^{AB}	1.43 ± 0.19 ^{B}
	p20	0.74 ± 0.08	0.52 ± 0.19	0.33 ± 0.19
	p17	0.17 ± 0.02 ^{AB}	0.38 ± 0.06 ^{A}	0.35 ± 0.04 ^{B}
Caspase 4	p45	1.00 ± 0.14	1.14 ± 0.05	1.07 ± 0.11
	p19	0.47 ± 0.02	0.59 ± 0.16	0.54 ± 0.14
	p10	0.16 ± 0.07	0.21 ± 0.04	0.33 ± 0.24
Caspase 5	p48	1.00 ± 0.03 ^{A}	1.37 ± 0.17	1.59 ± 0.07 ^{A}
	p30	1.35 ± 0.41	1.39 ± 0.30	1.37 ± 0.10
Caspase 6	p35	1.00 ± 0.18	0.85 ± 0.03	1.56 ± 0.27
	p15	0.06 ± 0.03 ^{A}	0.22 ± 0.08 ^{B}	0.49 ± 0.04 ^{AB}
Caspase 7	p35	1.00 ± 0.25 ^{A}	0.68 ± 0.09	0.34 ± 0.09 ^{A}
	p20	0.87 ± 0.15	1.40 ± 0.27 ^{A}	0.51 ± 0.02 ^{A}
Caspase 8	p40	1.00 ± 0.15	1.32 ± 0.22	1.20 ± 0.15
	p18	0.33 ± 0.13	0.17 ± 0.03	0.47 ± 0.19
Caspase 9	p51	1.00 ± 0.13 *	2.26 ± 0.39 *	3.30 ± 0.12 *
	p39	15.98 ± 3.03 ^{AB}	4.36 ± 1.51 ^{A}	5.80 ± 1.73 ^{B}
	p37	6.89 ± 0.85 ^{A}	7.95 ± 1.81	12.91 ± 2.12 ^{A}
Caspase 10	p58	1.00 ± 0.28	1.21 ± 0.26	1.66 ± 0.68
Caspase 11	p48	1.00 ± 0.06 ^{A}	1.36 ± 0.29 ^{B}	2.48 ± 0.43 ^{AB}
	p20	0.13 ± 0.04 *	0.39 ± 0.05 *	0.56 ± 0.05 *
Caspase 12	p55	1.00 ± 0.07	1.10 ± 0.14	1.01 ± 0.11
	p20	0.67 ± 0.07 ^{AB}	4.76 ± 0.19 ^{A}	4.71 ± 1.89 ^{B}

All caspase fragments were normalized to the summer active, procaspase fragment. Data represent mean ± SE. ANOVA with subsequent Fischer's LSD were performed for each detected fragment ($p<0.05$; n=3 animals for each state). * denotes all three states are statistically different from each other. In cases where all three comparisons were not statistically different, letters denote where differences were found.

Table 4. Western blot analysis of caspase target fragments

Downstream Targets				
		State		
Target	**Fragment**	**Summer Active**	**Torpid**	**Interbout Aroused**
ICAD	p45	1.00 ± 0.13	1.45 ± 0.32	1.07 ± 0.08
	p30	0.31 ± 0.15	0.48 ± 0.14	0.07 ± 0.03
	p20	2.22 ± 0.17	1.87 ± 0.28	1.81 ± 0.54
	p12	.94 ± 0.09	1.26 ± 0.22	1.19 ± 0.04
PARP	p116	1.00 ± 0.07	1.45 ± 0.32	1.07 ± 0.08
	p89	0.83 ± 0.22 ^{A}	0.48 ± 0.14 ^{B}	0.07 ± 0.03 ^{AB}
	p25	10.45 ± 2.40	8.52 ± 1.63	13.37 ± 2.44
Lamin A/C	p28	1.00 ± 0.18 ^{A}	0.25 ± 0.03 ^{AB}	0.72 ± 0.13 ^{B}
IL-1β	p40	1.00 ± 0.16 ^{A}	0.42 ± 0.07 ^{AB}	0.82 ± 0.06 ^{B}
	p17	0.29 ± 0.15	0.33 ± 0.05	0.09 ± 0.02
IL-18	p18	1.00 ± 0.08 *	0.25 ± 0.08 *	0.58 ± 0.06 *

All caspase fragments were normalized to the summer active, procaspase fragment. Data represent mean ± SE. ANOVA with subsequent Fischer's LSD were performed for each detected fragment ($p<0.05$; n=3 animals for each state). * denotes all three states are statistically different from each other. In cases where all three comparisons were not statistically different, letters denote where differences were found.

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PUBLICATIONS:

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Treat M.D. and van Breukelen, F. (2014) Apoptotic regulation during mammalian hibernation. *American Physiological Society: Intersociety Meeting,* San Diego, California.

Treat M.D. and van Breukelen, F. (2016) Elucidating the caspase cascade during hibernation. *15th International Hibernation Symposium,* Las Vegas, Nevada.

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