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Mitigating Apoptotic and Inflammatory Signaling via Global Caspase Inhibition in Hibernating Ground Squirrels, *Spermophilus lateralis*⋆

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Accepted 7/29/2022; Electronically Published 11/4/2022

Online enhancement: appendix.

ABSTRACT

In most systems, the caspase cascade is activated during cellular stress and results in inflammation and apoptosis. Hibernators experience stressors such as extremely low body temperatures, bradycardia, possible ischemia and reperfusion, and acidosis. However, widespread inflammation and apoptosis would represent an energetic expense that is incompatible with hibernation. To better understand global caspase regulation during hibernation, we employed a systems-level approach and analyzed 11 caspases in ground squirrel liver that are involved in inflammatory (caspases 1, 4, 5, 11, and 12) and apoptotic (caspases 2, 6, 7, 8, 9, and 10) pathways. Western blots revealed liberation of active forms for two inflammatory (caspases 11 and 12) and two apoptotic (caspases 6 and 9) caspases during hibernation (e.g., p15, the most active fragment of caspase 6, increased 8.26 ± 0.70-fold in interbout-aroused animals). We used specific peptide substrates to interrogate the four seemingly activated caspases and demonstrated no expected increases in proteolytic activity. Specific targets of these four caspases were similarly not cleaved, demonstrating that initiation of caspase activation may occur without concomitant downstream effects. Similarly, we found no evidence for upstream activation for caspase 9 signaling based on permeabilization of the outer mitochondrial membrane. We contend that these caspases are suppressed after seeming activation during hibernation. Incomplete caspase signaling is effectively mitigating the induction of widespread inflammation and apoptosis during hibernation.

Keywords: inflammatory caspases, IL-1β, IL-18, alanine aminotransferase (ALT), apoptosis-inducing factor (AIF), lamin.

Introduction

Hibernation allows animals to survive unfavorable conditions of winter because they are able to depress metabolism and live on stored resources (Carey et al. 2003; van Breukelen and Martin 2015; Andrews 2019). Hibernating ground squirrels experience body temperatures (T_b's) that may be below 0°C and oxygen consumption rates as low as 1/100th of active rates. During the hibernation season, these squirrels will periodically arouse from torpor every 1–3 wk , and T_b's are restored to ~36°C during these interbout arousals. The dynamic and cyclical nature of the torpor cycle means that ground squirrels experience repeated exposure to extremely low T_b's, bradycardia, possible ischemia and reperfusion, and acidosis during the 15–20 torpor bouts of a typical hibernation season (van Breukelen et al. 2010). These conditions are known to be strongly proinflammatory and proapoptotic in other systems (for review, see van Breukelen et al. 2010; Huang et al. 2015).

Both inflammation and apoptosis involve cysteine aspartate protease (caspase) activation (Ramirez and Salvesen 2018). Relatively inactive procaspases require internal proteolytic processing and conformational rearrangements to attain full enzymatic activity. Caspases 2, 3, 6, 7, 8, 9, and 10 have been traditionally assigned an apoptotic role, whereas caspases 1, 4, 5, 11, and 12 are typically associated with inflammatory processes. However, significant overlap in pathways is known. For instance, caspases 1 and 11 can directly activate apoptotic executioner caspases 3, 6, and 7 (Van de Craen et al. 1999).

In our forthcoming related article, we found seeming caspase 3 activation during hibernation (Treat et al. 2023). Importantly, this seeming activation does not result in execution of apoptosis. It is tempting to speculate that our caspase 3 data represent an adaptation that effectively mitigates widespread apoptosis induction

⋆This paper included in the Focused Collection "Time-Out for Survival: Hibernation and Daily Torpor in Field and Lab Studies" is based on research presented at the 2021 16th International Hibernation Symposium (IHS) organized by Guest Handling Editors Rob Henning, Roelof Hut, and Hjalmar Bouma.
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  https://doi.org/10.1086/722133

during the proapoptotic hibernation season. Using a systems-level approach to investigate an additional 11 caspases, we found evidence for incomplete activation of both apoptotic and inflammatory caspases. Despite a seeming activation of four caspases, downstream target analyses suggest no bona fide activation resulting in increased catalysis. These data underscore the value of examining both upstream and downstream effectors during a non-steady-state condition like mammalian hibernation.

Material and Methods

All tissue collection protocols were approved by the University of Nevada, Las Vegas, Institutional Animal Care and Use Committee. A more detailed description of our sampling can be found in our related article (caspase 3; Treat et al. 2023). Briefly, adult golden-mantled ground squirrels (*Spermophilus [Callospermophilus] lateralis*) were captured in August from Kennedy Meadows, California, or Duck Creek, Utah, and transported to the University of Nevada, Las Vegas. Some animals were immediately euthanized as a seasonal control group (summer active [SA]). The remaining squirrels were implanted with temperature-sensitive radiotelemeters to allow for precise determination of torpor status during the hibernation season (previously described in Pan et al. 2014). To facilitate hibernation, telemeter-implanted squirrels were housed in a 4°C environmental chamber starting in early November. Interbout-aroused (IBA) and torpid squirrels were sampled toward the end of the hibernation season from late January to February. Torpid squirrels were torpid for a minimum of 3 d, and their T_b was ~5°C when euthanized. Torpid squirrels were euthanized by decapitation because the low respiratory and heart rates during torpor preclude the use of injectable or inhaled anesthetics. SA and IBA squirrels were euthanized by CO_2 asphyxiation. Squirrels were placed on ice, and tissues (e.g., liver, heart, kidney, brain, gonads, and others) were rapidly collected and snap-frozen in liquid nitrogen before being stored at −80°C until use. Some assays included livers from rats as a nonhibernator comparison.

Liver samples from SA, torpid, and euthermic IBA squirrels were subjected to SDS-PAGE Western blotting. Conditions for SDS-PAGE were as follows: for caspases 1 and 2, 30 μg of total protein samples on a 15% acrylamide gel; for caspase 4, 30 μg of total protein samples on a 17% acrylamide gel; for caspases 5, 6, 9, 10, 11, IL-1β, and IL-18, 50 μg of total protein samples on a 15% acrylamide gel; for caspase 7, lamin A, and apoptosis-inducing factor (AIF), 50 μg of total protein samples on a 12% acrylamide gel; for caspase 8, 70 μg of total protein samples on a 12% acrylamide gel; for caspase 12, 50 μg of total protein samples on a 17% acrylamide gel; and for X chromosome–linked inhibitor of apoptosis protein (XIAP), 10 μg of total protein samples on a 12% acrylamide gel. The following antibodies were used: anti–caspase 1 (Cell Signaling Technology, Danvers, MA; rabbit polyclonal used at 1:1,000), anti–caspase 2 (Santa Cruz Biotechnology, Santa Cruz, CA; rabbit polyclonal used at 1:1,000), anti–caspase 4 (ProSci, Poway, CA; rabbit polyclonal used at 1:800), anti–caspase 5 (Santa Cruz Biotechnology; goat polyclonal used at 1:200), anti–caspase 6 (Cell Signaling Technology; rabbit polyclonal used at 1:1,000), anti–caspase 7 (Cell Signaling Technology; goat polyclonal used at 1:200), anti–caspase 8 (BD Biosciences, San Jose, CA; rabbit polyclonal used at 1:800), anti–caspase 9 (ProSci; rabbit polyclonal used at 1:1,000), anti–caspase 10 (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; rabbit polyclonal used at 1:800), anti–lamin A (G. E. Morris via Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA; mouse monoclonal used at 1:1,000), anti-AIF (Novus Biologicals, Centennial, CO; rabbit polyclonal used at 1:2,000), anti–IL-1β (Novus Biologicals; rabbit polyclonal used at 1:2,000), anti–IL-18 (Clinical Proteomics Technologies via DSHB; mouse monoclonal used at 1:1,000), and anti-XIAP (Abcam, Cambridge, UK; rabbit polyclonal used at 1:1,000). Visualizations for all blots, except caspase 12, were performed using ECL Plus on a Typhoon imager. The caspase 12 blot was exposed to IRDye 680LT dye-conjugated secondary antibodies (LI-COR, Lincoln, NE) and was visualized on a LI-COR Odyssey. For simplicity of data presentation, only the most active caspase fragments are shown, and all caspase data are normalized to the SA state. For total Western blot fragment expression levels included in these analyses, see supplementary table 1.

Caspase activity assays were performed essentially as described in our related article (Treat et al. 2023). Briefly, reactions were initiated with the addition of 40 μM of VEID-AMC (caspase 6), LEHD-AMC (caspase 9), or WEHD-AMC substrate (shared inflammatory substrate for caspases 1, 4, 5, and 11; Enzo Life Sciences, East Farmingdale, NY) to the liver lysates. There is no identified specific substrate for caspase 12. We added 100 μM of the specific competitive inhibitor of caspase 6 (Ac-VEID-CHO) or caspase 9 (Ac-LEHD-CHO; Enzo Life Sciences) to assess reaction specificity. Activity of caspases 6 and 9 was reduced 82.4% ± 12.9% and 62.8% ± 6.9%, respectively (ANOVA, $P < 0.05$), of the noninhibited rate. However, inhibiting parallel reactions with concentrated HCl before substrate addition provided a more complete inhibition, and data shown represent HCl-inhibited reactions.

Frozen liver sections were cut at a thickness of 7 μm and mounted on glass slides coated with 0.01% poly-L-lysine. Sections were fixed in 4% paraformaldehyde solution at room temperature for 15 min. Slides were then washed for 5 min in phosphate-buffered saline (PBS), pH 7.4, 5 min in PBS with 0.3% Triton X-100 (PBST), and finally, 5 min in PBS. The fixed slides were blocked overnight at 4°C in 5% milk in PBST. Following three 5-min washes in PBS, the sections were incubated with anti-AIF polyclonal IgG antibody (Novus Biologicals; rabbit polyclonal antibody) diluted 1:100 in PBST with 5% dry milk overnight at 4°C. After three 5-min washes in PBST, the sections were incubated with goat antirabbit IgG antibody conjugated to BODIPY FL (Invitrogen) diluted 1:500 in PBST with 5% dry milk in a light-safe container for 1 h at room temperature. After another three 5-min washes in PBS, sections were visualized with confocal microscopy (×100 magnification). Background fluorescence was normalized to control sections with secondary antibody-only treatments. Under nonapoptotic conditions, proapoptotic factors such as cytochrome c and AIF are sequestered to the intermembrane spaces of the mitochondria (Arnoult et al. 2002; Muñoz-Pinedo et al. 2006). During apoptosis,

the mitochondrial outer membrane is permeabilized, resulting in a cytosolic release of cytochrome c and AIF (Candé et al. 2002). Cytochrome c localization is difficult to perform because of its small size and propensity to diffuse during fixation. AIF is much larger and therefore easier to localize. AIF contains nuclear localization signals and, when released from the mitochondria, is specifically transported into the nucleus (Susin et al. 1999; Daugas et al. 2000). In other words, if cytochrome c and AIF are released from the mitochondria during apoptosis, we expect an enrichment of AIF in the nucleus. Nuclear boundaries were defined by examination of the DAPI signal. To maximize likelihood of detecting a partial or bona fide release of AIF, cells were deemed positive for AIF when the signal for nuclear AIF was 3 SDs above the cytosolic AIF signal value. $N = 3$ animals per state, with 30 cells scored per animal.

Serum alanine aminotransferase (ALT) concentrations were measured spectrophotometrically using an ALT detection kit (Pointe Scientific, Canton, MI) in SA, early-torpor (≤20% of predicted torpor bout length or ~2 d), late-torpor (~80% of predicted torpor bout length or ~5 d), and IBA animals ($N = 3$ animals per state). We included early- and late-torpor animals in this analysis to better understand whether ALT release, a proxy for liver damage, was influenced by torpor duration. Units are expressed in international units per liter.

Data are presented as mean ± standard error. When normality assumptions were met, ANOVAs were performed using KaleidaGraph version 4.5 for Mac (Synergy Software, Reading, PA). For specific comparisons, Fisher's least significant difference post hoc analysis was utilized. Statistical significance was assumed when $P < 0.05$.

Results

Western Blot Analyses Reveal That Some Apoptotic and Inflammatory Caspases Are Seemingly Activated

We performed Western blot analyses on 11 caspases involved in the inflammatory and apoptotic caspase cascades. We found four caspases (caspases 6, 9, 11, and 12) seemingly activated. Caspase 6 activation leads to the liberation of the p15 fragment (Wang et al. 2010). Caspase 6 p15 increased 8.26 ± 0.70-fold in IBA squirrels compared with SA squirrels (fig. 1*A*; ANOVA, $P < 0.05$). Caspase 9 activation results in the liberation of a p37 fragment (Jiang and Wang 2000). Caspase 9 p37 liberation increased 1.87 ± 0.31-fold in IBA animals compared with SA animals (fig. 1*B*; ANOVA, $P < 0.05$). Caspase 11 activation and caspase 12 activation each results in p20 fragments (Schotte et al. 1998; Morishima et al. 2002). Caspase 11 p20 liberation increased 3.07 ± 0.40-fold and 4.45 ± 0.41-fold in torpid and IBA squirrels, respectively, compared with SA squirrels (fig. 2*A*; ANOVA, $P < 0.05$). Caspase 12 p20 liberation increased 7.11 ± 0.29-fold and 7.03 ± 2.82-fold in torpid and IBA animals, respectively, compared with SA animals (fig. 2*B*; ANOVA, $P < 0.05$). The other caspases analyzed (caspases 1, 2, 4, 5, 7, 8, and 10) showed no signs of activation during hibernation (supplementary table 1; supplementary figure 1).

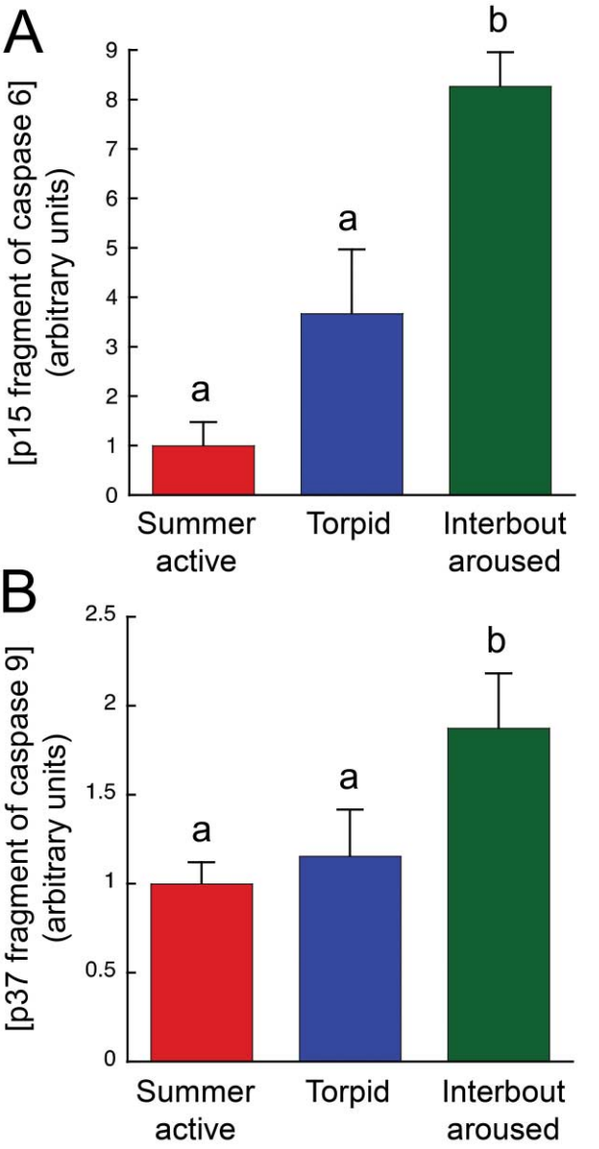

Figure 1. Western blot analyses of apoptotic caspases suggest that caspases 6 and 9 are seemingly activated during hibernation. Only data for the most active fragment are displayed and represent mean ± SE. *A*, Caspase 6 p15 increased 8.26 ± 0.70-fold during interbout arousals. *B*, Caspase 9 p37 increased 1.87 ± 0.31-fold during interbout arousals. Different lowercase letters above the error bars denote statistical difference (ANOVA, $P < 0.05$).

There Is No Increased Cleavage of Artificial Substrates by the Seemingly Activated Caspases

The in vitro enzymatic activity of caspases 6, 9, and 11 increased predictably with assay temperature, except we found that caspase activity was depressed at specifically 37°C in all squirrels (fig. 3). The percentage difference between 30°C and 37°C values were as follows: for caspase 6 SA, 17.95% ± 8.32%; for caspase 6 IBA, 9.02% ± 7.99%; for caspase 6 torpid, 18.25% ± 1.16%; for caspase 9 SA, 14.65% ± 3.64%; for caspase 9 IBA,

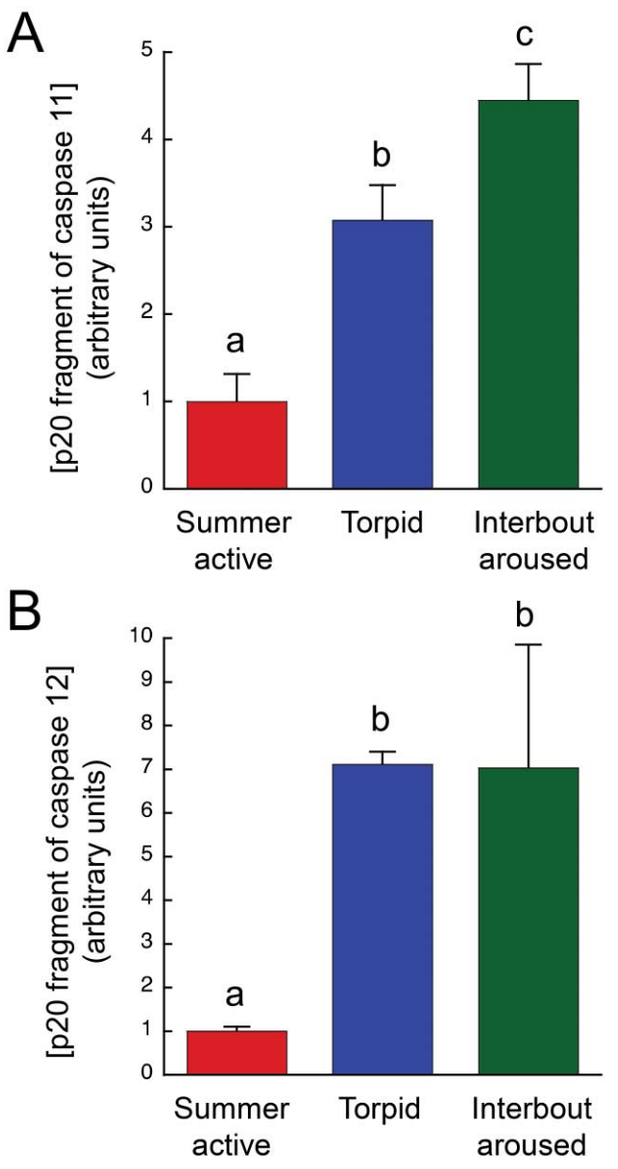

Figure 2. Western blot analyses of inflammatory caspases suggest that caspases 11 and 12 are seemingly activated during hibernation. Only data for the most active fragment are displayed and represent mean ± SE. *A*, Caspase 11 p20 increased 3.07 ± 0.40-fold during torpor and 4.45 ± 0.41-fold during interbout arousals. *B*, Caspase 12 p20 increased 7.11 ± 0.29-fold during torpor and 7.03 ± 2.82-fold during interbout arousals. Different lowercase letters above the error bars denote statistical difference (ANOVA, $P < 0.05$).

23.28% ± 3.42%; for caspase 9 torpid, 14.97% ± 2.37%; for caspase 11 SA, 20.72% ± 14.55%; for caspase 11 IBA, 36.94% ± 4.43%; and for caspase 11 torpid, 23.04% ± 1.44%.

Downstream Indicators of Caspase Activity Do Not Indicate Caspase Activation

Nuclear lamins are specific targets for active caspase 6 (Ruchaud et al. 2002; Fischer et al. 2003). We found no increased cleavage of lamin A/C during winter consistent with a bona fide caspase 6 activation (fig. 4*A*; ANOVA, $P > 0.05$). We found no evidence for increased nuclear localization of AIF for any of the 270 cells that were analyzed ($N = 3$ animals from each of the 3 metabolic states and 30 cells analyzed per animal; fig. 4*C*–4*F*). IL-1β and IL-18 are proinflammatory cytokines. Caspase 1 cleaves IL-1β (31 kDa) into an active 17-kDa form and cleaves IL-18 (24 kDa) into an active 18-kDa form (McIlwain et al. 2013). No increases in IL-1β p17 or IL-18 p18 were found during hibernation (fig. 5*A*, 5*B*; ANOVA, $P > 0.05$).

Aminotransferase Concentration in Serum Does Not Suggest Liver Damage

Normal ALT values in human serum samples are between 7 and 56 IU L^{-1}, and values ≥500 IU L^{-1} denote significant liver damage (Gowda et al. 2009). Ground squirrel serum ALT values are seemingly high (100–180 IU L^{-1}), but concentrations up to 300 IU L^{-1} are considered nonspecific to liver disease in humans. Importantly, ALT concentration does not increase during torpor (fig. 4*C*; ANOVA, $P > 0.05$).

Availability of a Key Caspase Inhibitor Does Not Change in Liver as a Function of Metabolic State

XIAP is a well-characterized inhibitor of activity of caspases 3, 7, and 9 (Takahashi et al. 1998; Shi 2004). We found no changes in XIAP availability in liver regardless of metabolic state (fig. 6; ANOVA, $P > 0.05$)

Discussion

In our related article, we demonstrated that caspase 3 was seemingly activated according to Western blot analyses (Treat et al. 2023). Liberation of the most active proteolytic fragment of caspase 3 (p17) is increased ~2-fold in livers of winter squirrels. Such activation typically results in >10,000-fold increased enzymatic activity (Boatright and Salvesen 2003). Using an in vitro enzyme assay, we found no evidence of increased activation of caspase 3 during winter. Analyses of downstream caspase 3 targets, such as ICAD and PARP cleavage, and TUNEL assays for nicked DNA confirm that there was no downstream commitment to apoptosis. In other words, despite the seeming activation, caspase 3 does not cleave the intended targets. We extend this work by examining other caspases associated with both inflammation and apoptosis signaling to demonstrate that the regulation of caspase 3 was not unique or limited to apoptosis. Instead, the depression of caspase activity after seeming activation occurs for at least four other caspases. However, much like what is seen with caspase 3, our data suggest no commitment of these caspases to downstream processing of substrates.

Western blot analyses revealed that four of the 11 caspases (caspases 6, 9, 11, and 12; figs. 1, 2) had increased liberation of the most active fragment during hibernation. Caspases 6 and 9 typically play important roles in executing and initiating apoptosis, while caspases 11 and 12 are implicated in inflammatory function

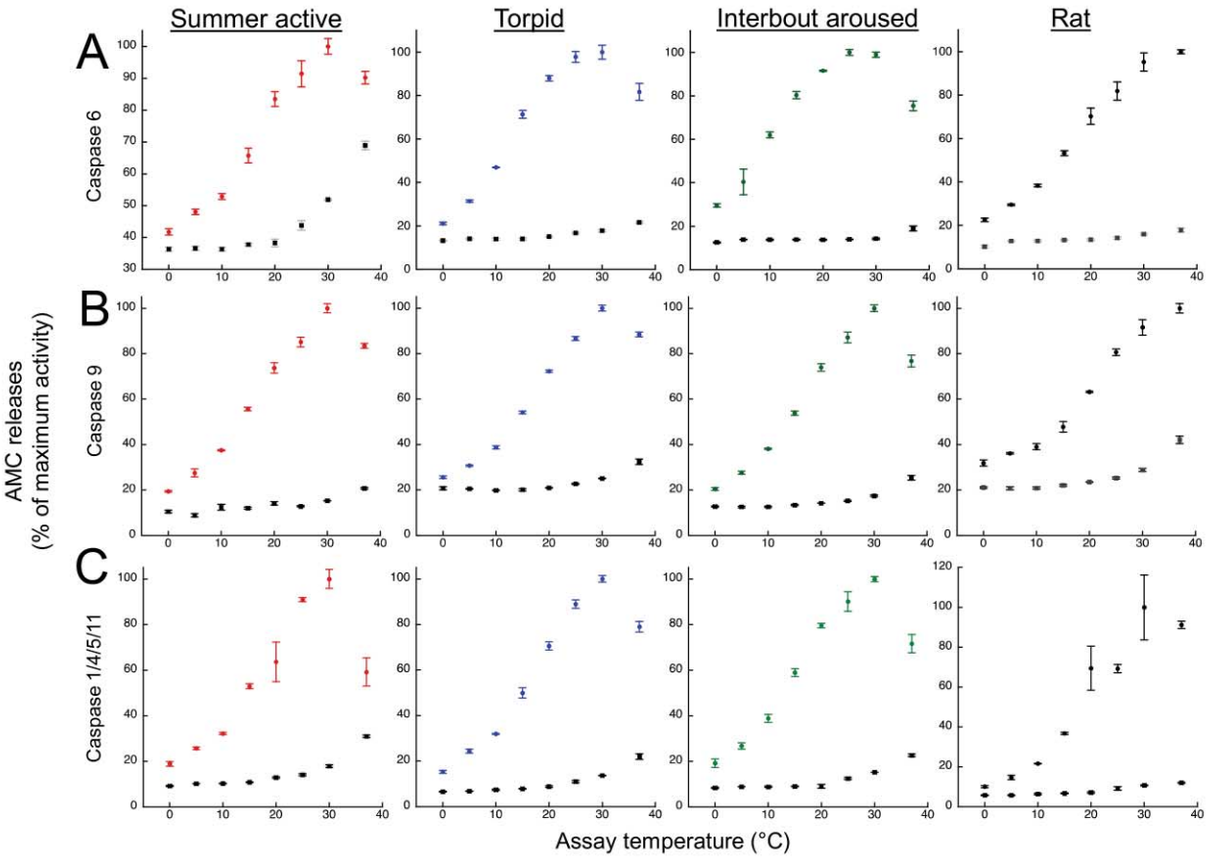

Figure 3. Caspase enzymatic activity as a function of assay temperature and metabolic state with comparison to nonhibernating rat. Representative results for the effects of assay temperature on activity of caspase 6 (*A*), caspase 9 (*B*), and inflammatory caspases 1, 4, 5, and 11 (*C*) in summer active, torpid, and interbout-aroused squirrels and in rats. Samples were incubated in the presence of specific substrates VEID-AMC (caspase 6), LEHD-AMC (caspase 9), and WEHD-AMC (caspases 1, 4, 5, and 11). Assays were incubated for either 1 h (rats) or 4 h (squirrels), which was within the enzymatic linear range for each species. Circles represent uninhibited reactions, while squares represent lysates that were quenched with HCl before the addition of substrate. Values represent mean ± SE ($N = 3$ replicates). Similar results were obtained using lysates derived from two additional animals from each state.

(Ramirez and Salvesen 2018). Therefore, the seeming activation of caspases is not apoptosis specific but instead affects both apoptotic and inflammatory caspases. Furthermore, both initiator and executioner apoptotic caspases are involved.

The executioner caspase 6 cleaves nuclear lamins and helps dismantle the nuclear envelope during apoptosis (Ruchaud et al. 2002). We found that liberation of the active p15 fragment of caspase 6 increased 8.26 ± 0.70-fold in IBA squirrels (fig. 1*A*). Similarly, the liberation of the active p20 fragment of caspase 12 increased >7-fold in both torpid and IBA squirrels (fig. 2*B*). While the twofold activation of caspase 3 may be deemed relatively modest, the profound increase of active caspases 6 and 12 seems incompatible with a spurious activation. These data suggest then that activation of at least a couple of caspases during hibernation may not be accidental. However, we contend that some activations may indeed be accidental. For instance, caspase 3 is typically activated through the initiator caspases 8 and 9. While we did find caspase 9 to be seemingly activated ~2-fold during interbout arousal and such activation would be expected to increase caspase 9 activity by ~2,000-fold, no such increase in p37 was evidenced in torpor (figs. 1*B*, 3*B*; Boatright and Salvesen 2003). Importantly though, upstream analyses for activation of caspase 9 do not suggest a coordinated activation process. Caspase 9 is activated by permeabilization of the outer mitochondrial membrane and release of cytochrome c (Ramirez and Salvesen 2018). This cytochrome c participates in formation of the apoptosome and subsequent activation of caspase 9. We assessed the release of AIF as a proxy for release of cytochrome c and found no evidence for mitochondrial membrane permeabilization (fig. 4*B*–4*F*). Thus, the inconsistency of seeming activation between winter states and the lack of evidence for upstream activation of caspase 9 would suggest that the liberation of caspase 9 p37 during interbout arousal was accidental.

The data presented here also help to clarify the activity assays. In our related article, the activity of caspase 3 was depressed ~2/3 at specifically 37°C in winter squirrels but not in summer squirrels (Treat et al. 2023). It was tempting to speculate that this depression of activity represented a specific adaptation for

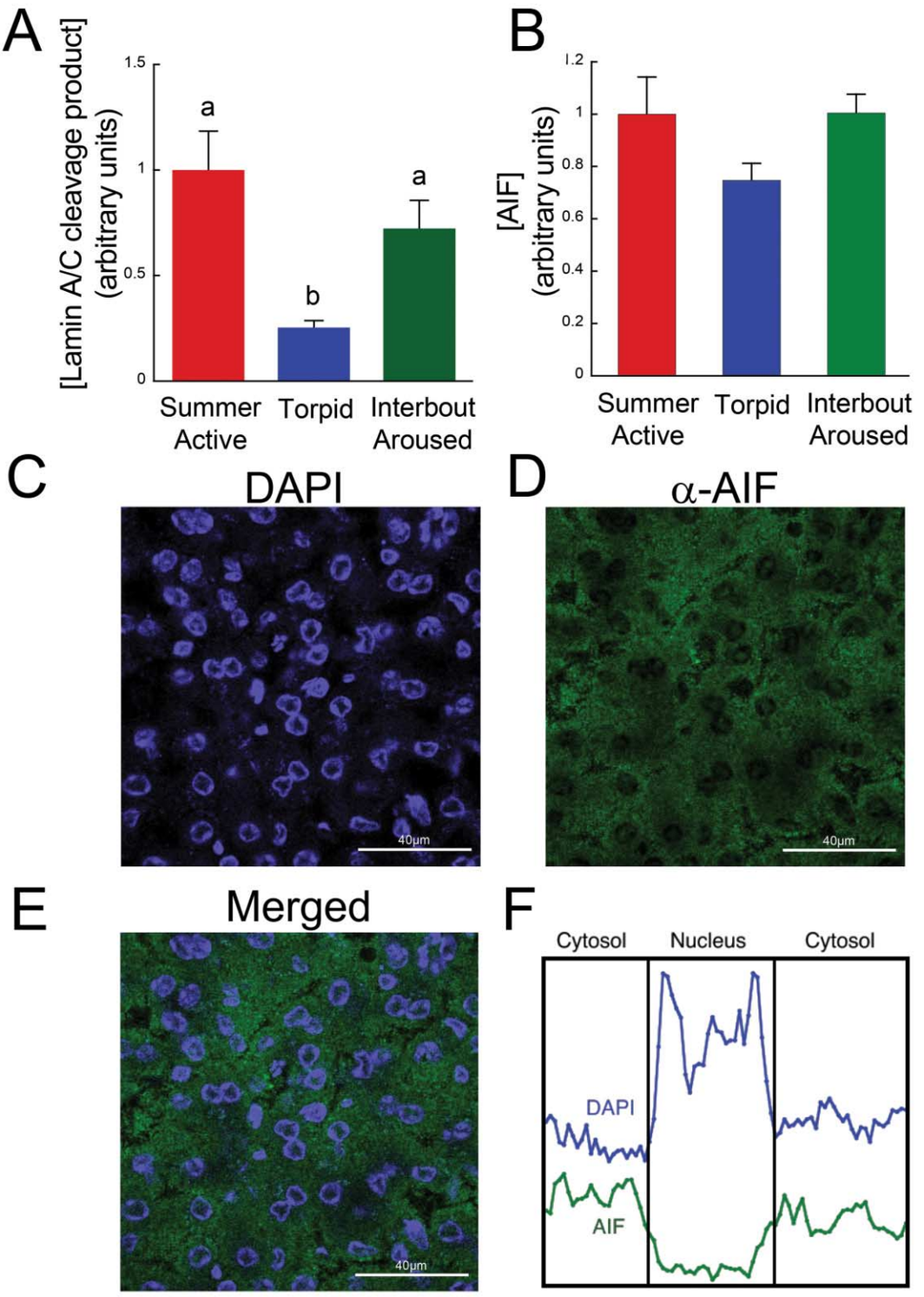

Figure 4. Upstream and downstream analyses of seemingly activated apoptotic caspases 6 and 9 are not consistent with activation during hibernation. *A*, Concentration of the 24–28-kDa nuclear lamin A/C cleavage product is reduced in torpid squirrels compared with both summer active and interbout-aroused squirrels, as revealed by Western blotting (ANOVA, $P < 0.05$). *B*, Concentration of apoptosis-inducing factor (AIF) is unchanged in hibernation, as revealed by Western blotting (ANOVA, $P < 0.05$). *C–E*, Representative confocal images for AIF localization to the nucleus for a single animal. *F*, Representative analysis for a single cell demonstrating exclusion of AIF from the nucleus.

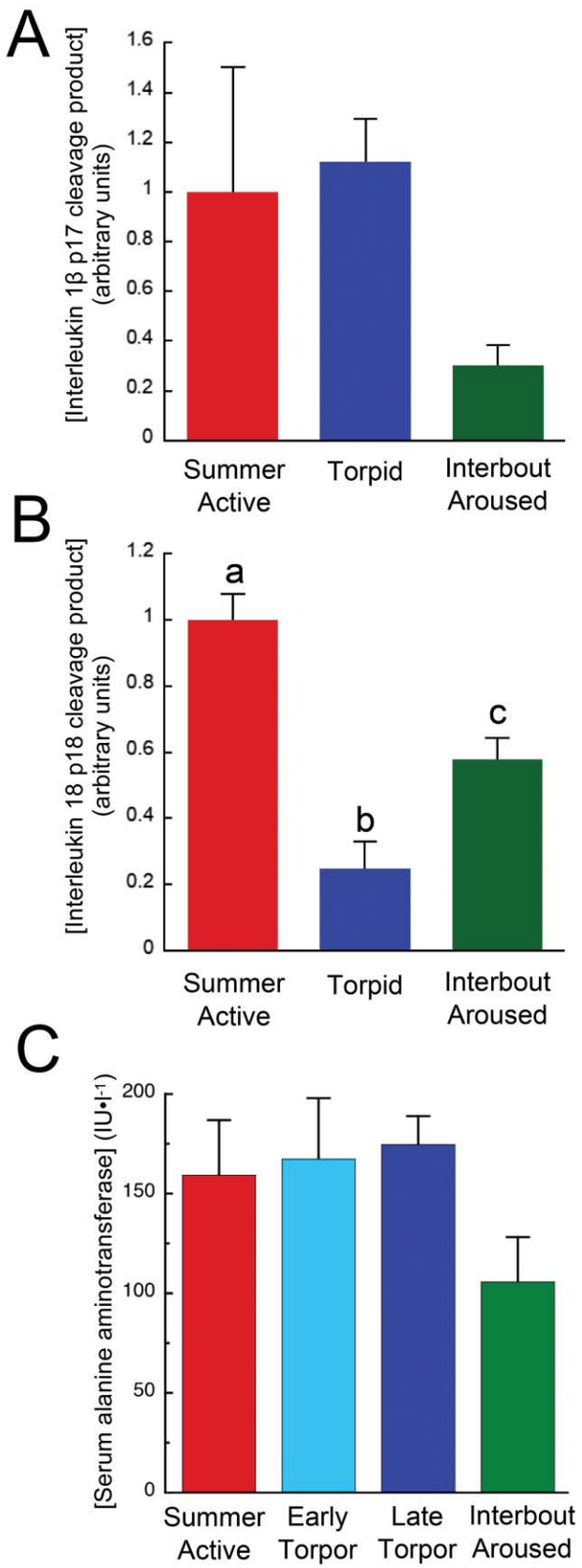

Figure 5. Downstream analyses of targets of the seemingly activated inflammatory caspases 11 and 12 are not consistent with activation during hibernation. *A*, *B*, There was no evidence for cleavage of IL-1β (*A*) or IL-18 (*B*) during hibernation according to Western blotting. IL-1β cleavage decreased significantly during hibernation (ANOVA, $P < 0.05$). *C*, Serum alanine aminotransferase concentrations did not increase during the hibernation season (ANOVA, $P > 0.05$).

the mitigation of apoptosis in hibernators. However, our enzymatic activity data for caspases 6, 9, 11, and 12 showed a moderate depression of activity at 37°C, but the depression was also present for summer animals (fig. 3). This finding suggests that the depression at 37°C may be relatively common in caspases and does not likely reflect a specific adaptation to depress apoptotic signaling during hibernation. We speculate that the effect may simply be an experimental artifact.

A unifying theme for all of these caspases is the lack of activation of downstream targets. Caspase 6 normally cleaves nuclear lamins, but we found no evidence for lamin cleavage (fig. 4*A*). Caspases 11 and 12 regulate caspase 1 in inflammatory responses (Kang et al. 2000; McIlwain et al. 2013). Caspase 1 cleaves IL-1β and IL-18. We found no evidence for interleukin processing, suggesting that the seemingly activated caspases 11 and 12 were not translated into effective downstream inflammatory responses. Finally, ALT is a liver enzyme that is released into the bloodstream upon liver inflammation and damage (Sherman 1991). Serum [ALT] is not increased during torpor, which is also consistent with minimal or no inflammatory response. That all seemingly activated caspases did not result in expected changes of downstream cleavage of cellular substrates suggests a global mechanism to suppress caspase activity. We find it unlikely that there will be specific mechanisms to suppress individual caspases that are all equally effective. Rather, we suspect that there will be a global repressor of caspases that can accommodate relatively large seeming activations of caspases like what we observed in liver for caspase 6 as well as more modest activations.

As indicated in our related article, we were unable to specifically identify such a regulator. We speculated that global regulation of caspases during hibernation may be effected by ubiquitylation. During hibernation, there is imprecise coordination of ubiquitylation versus proteolysis. While proteolytic activity of the 26S proteasome would be markedly affected by the low temperatures of torpor (Velickovska et al. 2005), ubiquitylation is much less temperature sensitive (Velickovska and van Breukelen 2007). The result is a global ubiquitylation of proteins and two- to threefold accumulation of ubiquitin conjugates during torpor depending on tissue type (van Breukelen and Carey 2002). Proteolytic activity of caspase 3 is inhibited by ubiquitylation without actual caspase 3 degradation (Ditzel et al. 2008; Schile et al. 2008; Bader and Steller 2009). Here, it is thought that polyubiquitylation sterically interferes with substrate entry to the caspase. A similar mechanism may be employed even by the more traditional mechanisms used to depress caspase activity. XIAP is an E3 ubiquitin ligase that has been shown to be a very potent modulator of at least caspases 3, 7, and 9 (Takahashi et al. 1998). Interestingly, the RING domain of XIAP is associated with ubiquitylation and degradation of target proteins. However, the BIR domains may be sufficient to depress caspase activity. It is thought that the BIR2

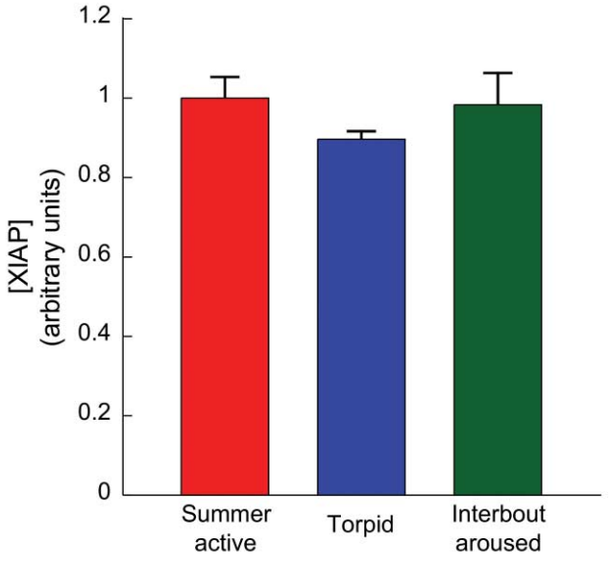

Figure 6. Western blotting reveals that availability of X chromosome–linked inhibitor of apoptosis protein (XIAP) does not change as a function of metabolic state (ANOVA, $P > 0.05$).

domain inhibits caspase function by binding to the active site substrate groove. In other words, steric inhibition at the site where a substrate would enter the caspase seems like a common mechanism for the suppression of caspase activity. Proteins known to possess BIR domains include XIAP, CIAP1 and CIAP2, NAIP, survivin, and BIRC6 (Cao et al. 2008). In our studies, we attempted to address this mechanism by examining the availability of XIAP. As seen in figure 6, there is no evidence for state-specific changes in availability or modification of XIAP. We are unable to make inferences from these data.

Taken together, our data here and in our related article demonstrate that there is no coordinated apoptotic signaling leading to widespread apoptosis despite the seemingly proapoptotic conditions of torpor. Accidental and perhaps more coordinated activation in as many as five different caspases (caspases 3, 6, 9, 11, and 12) may result in as much as an eightfold increase in the liberation of active caspase fragments depending on metabolic state. However, this seeming activation of caspases fails to lead to downstream target activation in all cases. This observation suggests a global mechanism for suppressing caspase function following both spurious and coordinated activation of caspases in the non-steady state of torpor. Our data highlight the need for examining both upstream and downstream indicators of activity when making inferences of pathway function under the non-steady-state conditions inherent with mammalian hibernation.

Acknowledgments

The laboratory is supported by the National Science Foundation (IOS-1655091). We thank members of the laboratory for assistance with experiments and animal care. We thank Dr. Katie Lantz posthumously for assistance with confocal microscopy. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures in studies involving animals were performed in accordance with the ethical standards of the institution or practice where the studies were conducted.

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