Cryobiology 71 (2015) 334–343

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Characterization of the SIRT family of NAD$^+$-dependent protein deacetylases in the context of a mammalian model of hibernation, the thirteen-lined ground squirrel

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ARTICLE INFO

Article history:
Received 11 June 2015
Revised 6 August 2015
Accepted 11 August 2015
Available online 13 August 2015

Keywords:
Hibernation
Sirtuins
Acetylation
SOD2
Skeletal muscle
Liver
Brown adipose tissue
White adipose tissue

ABSTRACT

Hibernating mammals employ strong metabolic rate depression to survive the winter, thereby avoiding the high energy costs of maintaining a euthermic lifestyle in the face of low seasonal temperatures and limited food resources. Characteristics of this natural torpor include a significant reduction in body temperature, a shift to a lipid-based metabolism, global suppression of ATP-expensive activities, and the upregulation of selected genes that mediate biochemical reorganization and cytoprotection. Sirtuin (SIRT) proteins, an evolutionarily conserved family of NAD$^+$-dependent protein deacetylases, have been shown to play important roles in the post-translational regulation of many metabolic and cytoprotective processes, suggesting a potential function for these enzymes in the control of hibernation. To assess this possibility, protein levels of the seven mammalian SIRTs (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7), total SIRT activity, and the acetylation status of two downstream SIRT targets (SOD2K68 and NF-κB p65K310) were measured in skeletal muscle, liver, brown adipose and white adipose tissues of the hibernating thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) over the course of the torpor–arousal cycle. The analysis revealed tissue-specific responses of different SIRTs at various points throughout hibernation, including a potentially interesting correlation between increased levels of SIRT3 protein, heightened total SIRT activity, and decreased acetylation of SIRT3 downstream target SOD2K68 in skeletal muscle during late torpor. These results provide evidence to suggest a possible role for the SIRT family of protein deacetylases in the regulation of the metabolic and cellular protective pathways that mediate the process of mammalian hibernation.

1. Introduction

Mammalian hibernation is a seasonal phenomenon that allows certain species to avoid the severe environmental stresses associated with winter climates (i.e. low temperatures and limited food resources), and is characterized by prolonged bouts (days to weeks) of metabolic rate depression interspersed with brief (hours to a day) periods of arousal when metabolism temporarily returns to normal levels [6]. During deep torpor, metabolic rate often plummets to <5% of euthermic values, a remarkable shift that is accompanied by major changes in vital signs; for example, in ground squirrels this includes reductions in heart rate (from 350–400 beats/min to as low as 5–10 beats/min), breathing rate (from >40 breaths/min to <1 breath/min), organ perfusion rates (<10% of euthermic values), and core body temperature (from euthermic values of 35–38 °C to near-ambient levels during torpor, often <5 °C) that ultimately reduce the animal's energy expenditure [6]. Accompanying these changes is the major remodeling of the hibernator's energy economy, which shifts from carbohydrate pathways of energy production to a reliance on the oxidation of lipids from white adipose tissue (WAT) depots accumulated during the summer months in preparation for hibernation [6,10]. This sustained emphasis on lipid catabolism is also important during the brief periods of arousal, when the rapid reversal of torpor is facilitated by an enormous increase in non-shivering thermogenesis that is fueled by high rates of fatty acid oxidation and oxygen consumption in the specialized mitochondria of brown adipose tissue (BAT). Remarkably, despite exposure to significant oxygen stress during arousal, and the endurance of the harsh conditions of torpor (e.g. cellular inactivity, low temperatures), hibernators avoid damage to their tissues by actively employing protective strategies to mitigate the harm that would otherwise occur under

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http://dx.doi.org/10.1016/j.cryobiol.2015.08.009
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such circumstances in non-hibernating species [6,40]. Because of this capacity to circumvent the potentially harmful repercussions of the torpid state, and due to their remarkable ability to rapidly and effectively modulate their metabolic processes and energy requirements, hibernators have become a very interesting model for studies of metabolic disorders and stress-related cellular damage and are proving to be useful models for conditions such as diabetes, obesity, and aging [47].

The drastic changes in biological function that occur over the course of hibernation are controlled at the molecular level by intricate mechanisms that promote a very strong suppression of energy-intensive cellular processes (e.g. global gene transcription and mRNA translation), while also enabling the specific upregulation of a small number of genes that are essential to the control of the transitions between euthermia and torpor, and which allow the hibernator to maintain the integrity of its cells while in the metabolically depressed state. The control of this simultaneous wide-scale gene suppression and concurrent upregulation of selected biological pathways is thought to be governed in many instances by the post-translational modification of downstream protein effectors that regulate many cell functions [40]. For instance, post-translational modifications (e.g. via phosphorylation, methylation, or ubiquitylation, etc.) of transcription factors (causing increases/decreases in their activity or DNA-binding capabilities), histones (leading to enhanced/lowered accessibility of transcriptional machinery to chromatin), and metabolic enzymes (resulting in an increase/decrease in their cellular activity) may contribute to either downstream reductions in global gene expression or an enhancement of the activities of specific pathways [26,30,40].

One type of modification that is the focus of much recent research in non-hibernating mammals is reversible protein acetylation. Over 2000 acetylated proteins have now been identified in mammals, making this mechanism comparable to other forms of post-translational modification such as phosphorylation and ubiquitylation [9]. The enzymes responsible for reversible acetylation – protein lysine acetyltransferases and deacetylases – have thus become important topics for research on metabolic regulation, with demonstrated roles in chromosomal remodeling, transcriptional regulation, and the control of many different metabolic pathways [16]. One group of protein deacetylases that is of interest with respect to hibernation is the sirtuin (SIRT) family of NAD^+-dependent protein deacetylases (consisting of SIRT1–7) because these have roles in the regulation of glucose metabolism, lipid metabolism, and oxidative stress responses in mammals [17,38]. For example, the most widely studied member of the family, SIRT1, can deacetylate and regulate major transcription factors involved in glucose and lipid metabolism, such as PPARγ, FOXO1 and the transcriptional co-activator PGC-1α, to promote fatty acid oxidation under fasting conditions when glucose availability is low [11,14,15,33,35]. SIRT1 also serves protective and anti-apoptotic roles in response to cellular stress via numerous pathways, including its direct modification of transcription factor p53 and specific histone targets [1,8,25]. Likewise, SIRT2 and SIRT3 play significant roles in promoting lipid metabolism and in controlling responses to oxidative stress [19,34,39,42,45,46] and similar metabolic and cellular protective functions have also been described for other members of the mammalian SIRT family (reviewed in [5,23,38]). Therefore, these deacetylases are intriguing subjects for study as potential targets for novel therapies to treat metabolic disorders and to mitigate cellular damage caused during natural processes such as aging [17]. For similar reasons they are also of interest as potential regulators of the processes that enable hibernating mammals to control metabolism and tissue damage during transitions to and from torpor [18,28].

Numerous studies have identified diverse functions for protein deacetylases in the control of eukaryotic gene expression and cellular metabolism, including potential roles in transcriptional suppression previously implicated for Class I and II deacetylases (also known as histone deacetylases or HDACs) in the context of mammalian hibernation [30,41] and turtle anoxia tolerance [22,41]. Previous studies also hypothesized a possible role for SIRT deacetylases in the regulation of the major metabolic changes that occur over the hibernation torpor–arousal cycle, with pathways of lipid catabolism, oxidative stress resistance, and transcriptional suppression being likely candidates for regulation by SIRTs [28,32]. A recent investigation also found correlative evidence to suggest a possible role for SIRT3 in the regulation of protein acetylation in the liver of thirteen-lined ground squirrels during the hibernation season [18]. In the current study, responses of the SIRT family of deacetylases are characterized in the context of mammalian hibernation. Expression levels of proteins were measured for the seven mammalian SIRTs (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7) over the torpor–arousal cycle in liver, skeletal muscle, brown adipose, and white adipose tissues of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*). In addition, total SIRT activities were evaluated in these tissues comparing euthermic controls with the late torpor phase. Finally, the acetylation status of two proteins known to be targeted by SIRT activity at specific lysine residues was determined in the four tissues throughout the torpor–arousal cycle. These are the antioxidant enzyme superoxide dismutase-2 (SOD2), the mitochondrial Mn-dependent form that is acetylated on lysine 68 [7], and the transcription factor nuclear factor kappa-B (NF-κB) that is acetylated on its p65 subunit at lysine 310 [36,48]. The results suggest a possible role for SIRTs in the regulation of hibernation.

2. Materials and methods

2.1. Animal experiments

Animal experiments were performed as described previously [37], and were carried out at the NIH facility in the laboratory of Dr. J.M. Hallenbeck. Animals were sacrificed at various points throughout the torpor–arousal cycle, and tissues were rapidly excised and immediately frozen in liquid nitrogen. Squirrels were sampled from the following conditions: (1) euthermic in the cold room (EC) – these euthermic control animals had not entered torpor for at least 72 h and maintained a stable body temperature (T_b) of 36–37 °C during this time. (2) Entrance to torpor (EN) – these animals had begun to enter torpor, as shown by a declining T_b of 18–31 °C. (3) Early torpor (ET) – animals were in constant torpor for 24 h, as indicated by a stable T_b of 5–8 °C. (4) Late torpor (LT) – animals had remained in deep torpor for at least 120 h (5 days), as indicated by a stable T_b of 5–8 °C over this time. (5) Early arousal (EA) – animals showed a rising T_b and were sampled when T_b was 9–12 °C. (6) Interbout arousal (IA) – these animals aroused naturally from torpor with T_b returned to ~37 °C for ~18 h. Tissues were transported to Carleton University on dry ice and stored at −80 °C.

2.2. Total protein extraction and immunoblotting

Total protein extraction and the subsequent immunoblotting of the samples were performed as described previously [37]. Membranes were blocked using either skim milk (2.5–5%, 20–30 min) or polyvinyl alcohol (PVA) (1 mg/mL, 30–70 kDa PVA, 45–60 s) in tris-buffered saline with Tween 20 (TBST), and were probed with specific primary antibodies for SIRT1 (Active Motif, Cat# 39354), SIRT2 (Santa Cruz, Cat# sc20966), SIRT3 (Genetex, GTX115701), SIRT4 (Santa Cruz, Cat# sc135053), SIRT5 (Genetex, GTX117825), SIRT6 (Active Motif, Cat# 39912), SIRT7 (Genetex, Cat# GTX105732), SOD2 acetylated at lysine 68

(Abcam, Cat# ab137037), and NF-κB p65 acetylated at lysine 310 (Genetex, Cat# GTX86963) (1:1000–2000 v/v dilution in TBST) at 4 °C overnight. Membranes were then probed with secondary anti-rabbit or anti-mouse IgG Horse Radish Peroxidase (HRP)-linked antibody (1:2000–8000 v/v dilution in TBST) for 30–90 min at room temperature, and developed using enhanced chemiluminescence.

2.3. Cytoplasmic/nuclear protein extraction

Cytoplasmic and nuclear fractions of two tissues (liver and muscle) were prepared for two of the time points of the torpor–arousal cycle (EC and LT, $n = 4$ samples separately extracted from different individuals at each time point). Tissue samples were homogenized using a Dounce homogenizer with 4 (liver) or 10 (muscle) piston strokes and 1 mL of homogenization buffer (10 mM 4-(2-hydroxye thyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.9, 10 mM KCl, 10 mM EDTA, 20 mM β-glycerophosphate) with 10 μL of 100 mM dithiothreitol (DTT) and 10 μL protease inhibitor cocktail (Bioshop) added immediately before homogenization. Samples were then centrifuged at 10,000 rpm for 10 min at 4 °C, and the cytoplasmic fractions (supernatants) removed. The pellets were resuspended in 75 μL extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 10% v/v glycerol, 20 mM β-glycerophosphate) with 0.75 μL of 100 mM DTT and 0.75 μL protease inhibitor cocktail (Bioshop) added immediately before use. The samples were then incubated horizontally on ice while rocking for 1 h, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatants containing nuclear extracts were removed, and their individual protein concentrations were quantified by the Coomassie blue dye-binding assay using the BioRad reagent (BioRad Laboratories, Hercules, CA) with absorbance read at 595 nm on a MR5000 microplate reader. Nuclear extract purity was confirmed via SDS–PAGE followed by immunoblotting with an anti-histone H3 antibody (Cell Signalling, Cat# 9715). Extracts were stored at −80 °C until use.

2.4. SIRT deacetylase activity assay

Total SIRT activity was assayed at 37 °C for nuclear (liver and muscle) and total (liver, muscle, brown adipose tissue, and white adipose tissue) protein fractions using the Epigenase Universal SIRT Activity/Inhibition Assay Kit from Epigentek (Farmingdale, NY), according to the manufacturer's instructions. To measure total SIRT activity, total protein extracts were prepared from tissues sampled from two of the six time points of the torpor–arousal cycle (EC and LT, $n = 4$ independent samples from different individuals at each time point) such that the concentration of each sample was adjusted to a constant 3.25 μg/μL (liver, muscle and brown adipose tissue) or 2 μg/μL (white adipose tissue) by addition of small aliquots of SIRT assay buffer (included with the assay kit). For the preparation of samples to measure nuclear SIRT activity, nuclear extracts were prepared using liver and muscle tissue as described under *Cytoplasmic/Nuclear Protein Extraction* above, with protein concentrations adjusted to 3.25 μg/μL by addition of small aliquots of SIRT assay buffer.

In separate wells of the assay microplate supplied by the manufacturer, 4 μL of each 3.25 or 2 μg/μL sample of total or nuclear protein extracts was added to the following reagents (all provided by the manufacturer): 46 μL of SIRT assay buffer, 1 μL of SIRT substrate, 1 μL of HDAC inhibitor trichostatin A (TSA), and 1 μL NAD^+ co-factor. The microplate was then incubated for 90 min at 37 °C on a shaker while covered with Parafilm, followed by washing each reaction well three times with 150 μL of 1× wash buffer (pH 7.39, 10× stock solution provided by the manufacturer). A 50 μL aliquot of 1:1000 capture antibody (provided by the manufacturer) was then added to each reaction well, and allowed to incubate at room temperature for 60 min on a shaker while covered with Parafilm. The reaction wells were then washed three times with 150 μL of 1× wash buffer, followed by the addition of 50 μL of 1:2000 detection antibody (provided by the manufacturer) and incubation at room temperature for 30 min on a shaker while covered with Parafilm. Each reaction well was then washed four times with 150 μL of 1× wash buffer, followed by the addition of 100 μL of developer solution (provided by the manufacturer) and incubation at room temperature for 10 min on a shaker away from light. A 100 μL aliquot of stop solution (provided by the manufacturer) was then added to each well and allowed to incubate at room temperature for 2 min. The absorbance of each sample was then immediately measured at 450 nm (reference wavelength = 655 nm) using a Powerwave HT spectrophotometer (BioTek). For each assay, three control wells containing all reaction components except the NAD^+ co-substrate were also run concurrently for each tissue. The average absorbance of these three wells was used as the negative control absorbance for their respective tissue, as per the manufacturer's instructions.

2.5. Quantification and statistics

Band densities on chemiluminescent immunoblots were quantified using the ChemiGenius Bio Imaging System GeneTools Software (Syngene, Frederick, MD). Immunoblot band density was standardized against the summed intensity of a group of Coomassie stained protein bands from the same sample lane, chosen based on their lack of variation between experimental conditions, and because they were not located close to the protein band of interest. Data for each time point are expressed as a mean ± SEM, with $n = 3$–4 samples, each from a different animal. Statistical analysis of the data used a one-way ANOVA and a post hoc test (Tukey).

Absorbance values obtained for the SIRT activity assay were corrected using their respective negative control absorbance, and expressed as activity/g wet weight starting tissue. Data for each time point are expressed as a mean ± SEM, with $n = 3$–4 samples, each from a different animal. Statistical analysis compared the activity for a given tissue between time points using the Student's *t*-test.

3. Results

3.1. Analysis of SIRT protein levels over the torpor–arousal cycle

Relative protein levels of the seven SIRTs were assessed in ground squirrel liver (Fig. 1), skeletal muscle (Fig. 2), BAT (Fig. 3) and WAT (Fig. 4) over the six sampling-points of the torpor–arousal cycle using primary antibodies specific for each protein. In liver, SIRT1 protein levels increased significantly during early arousal (EA) by 3.3 ± 0.2-fold as compared with EC and remained elevated during interbout (IA) at 2.0 ± 0.2-fold over controls. Protein levels of SIRT2 responded similarly with levels increased by 1.6 ± 0.04 and 1.6 ± 0.2-fold over EC values in EA and IA, respectively but, in addition, SIRT2 was also elevated during entrance into torpor (EN) by 1.9 ± 0.2-fold. By contrast, SIRT3 protein levels increased significantly only during early torpor (ET), rising by 1.5 ± 0.08-fold. SIRT4, SIRT5, SIRT6, and SIRT7 protein levels did not change significantly as compared to EC at any point over the torpor–arousal course in liver tissue. In muscle, protein levels of SIRT3 were significantly elevated by 1.9 ± 0.2 and 2.6 ± 0.09-fold over controls during ET and late torpor (LT), respectively, while levels of SIRT6 were also elevated by 1.7 ± 0.2-fold over EC during interbout arousal. In contrast, protein levels of SIRT2 were significantly decreased during EN by 0.7 ± 0.05-fold as compared with EC.

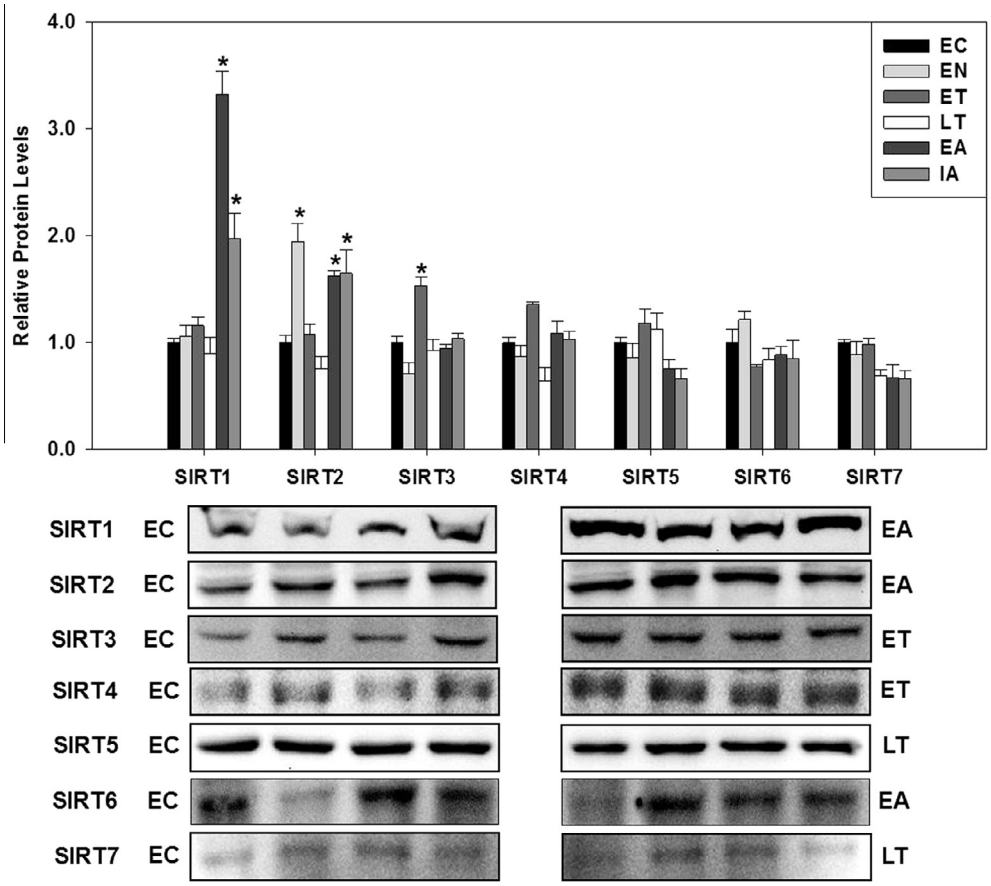

Fig. 1. Relative protein expression of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 in liver of *I. tridecemlineatus* over the torpor–arousal cycle. Representative protein bands are shown for selected sampling points (labeled to the left and right of the blots). Histogram shows mean standardized band densities (± SEM, $n = 3–4$ independent trials on tissue from different animals). Protein bands were standardized against the summed intensity of a group of Coomassie-stained protein bands (loading control) from the same sample lane. Data were analyzed using a one-way ANOVA with a post hoc Tukey test. * = significantly different from the respective EC control, $p < 0.05$.

No significant fluctuations were observed for protein levels of SIRT1, SIRT4, SIRT5, or SIRT7 during any of the sampled time points. In BAT, a significant 1.8 ± 0.1-fold increase in SIRT1 protein levels was observed in EA when compared to EC. However, no other changes were identified for SIRTs 2–7 in this tissue. In WAT, protein levels of SIRT3, SIRT4 and SIRT5 were observed to decrease significantly during ET and LT when compared to controls (SIRT3 0.5 ± 0.06 and 0.6 ± 0.04-fold; SIRT4 0.6 ± 0.03 and 0.6 ± 0.02-fold; SIRT5 0.5 ± 0.1 and 0.4 ± 0.07-fold for ET and LT, respectively). Additionally, SIRT3 levels remained depressed by 0.4 ± 0.02-fold over EC during EA, while SIRT4 levels were also decreased during EA and IA by 0.6 ± 0.1 and 0.6 ± 0.08-fold, respectively, as compared to EC. In contrast, protein levels of SIRT2 increased significantly during LT by 1.7 ± 0.1-fold over EC, as did those of SIRT7 during IA by 1.9 ± 0.3-fold.

3.2. Analysis of total SIRT activity

Total SIRT activity was measured in whole tissue soluble protein extracts of liver (Fig. 5), skeletal muscle (Fig. 6), BAT (Fig. 7) and WAT (Fig. 7), and in the nuclear protein fractions of liver (Fig. 5) and muscle (Fig. 6), comparing EC and LT stages at an assay temperature of 37 °C. In muscle, mean total SIRT activity in whole tissue extracts was significantly increased during LT as compared to EC by 3.6 ± 0.1-fold, while no activity changes were observed between EC and LT in the nuclear protein fraction. In liver, BAT and WAT, mean total SIRT activity measured in either total or nuclear protein fractions did not change significantly during LT as compared to the EC control.

3.3. Analysis of the acetylation status of SIRT targets SOD2 (K68) and NF-κB p65 (K310) during hibernation

Relative protein levels of acetylated SOD2 (K68) (Fig. 8) and NF-κB p65 (K310) (Fig. 9) were assessed in ground squirrel liver, skeletal muscle, BAT and WAT over the six sampling-points of the torpor–arousal cycle using primary antibodies specific for each protein only when acetylated at the indicated lysine residue. The content of acetylated SOD2 (K68) decreased significantly in liver, muscle, and WAT during LT to 0.4 ± 0.05, 0.5 ± 0.1 and 0.4 ± 0.07, respectively, of EC control values. SOD2 (K68) levels also decreased in muscle during IA to 0.5 ± 0.06 of EC. In contrast, levels of SOD2 (K68) increased significantly in BAT by 1.7 ± 0.1 and 1.5 ± 0.1-fold during ET and EA, respectively, over the EC value. Acetylated NF-κB p65 (K310) protein content decreased significantly in liver during LT to just 0.3 ± 0.04 as compared to EC, but increased strongly again during IA to 1.8 ± 0.3-fold above EC. Levels of NF-κB (K310) also increased significantly in BAT during EA by 1.6 ± 0.2-fold as compared to control, while no changes were observed in skeletal muscle or WAT over the torpor–arousal cycle.

4. Discussion

With the goal of providing a preliminary characterization of SIRT involvement in hibernation, this study currently offers the most detailed experimental evidence yet to support a role for these enzymes in the regulation of this form of hypometabolism, with observed changes in SIRT protein expression, total SIRT activity, and acetylation status of SIRT downstream targets potentially

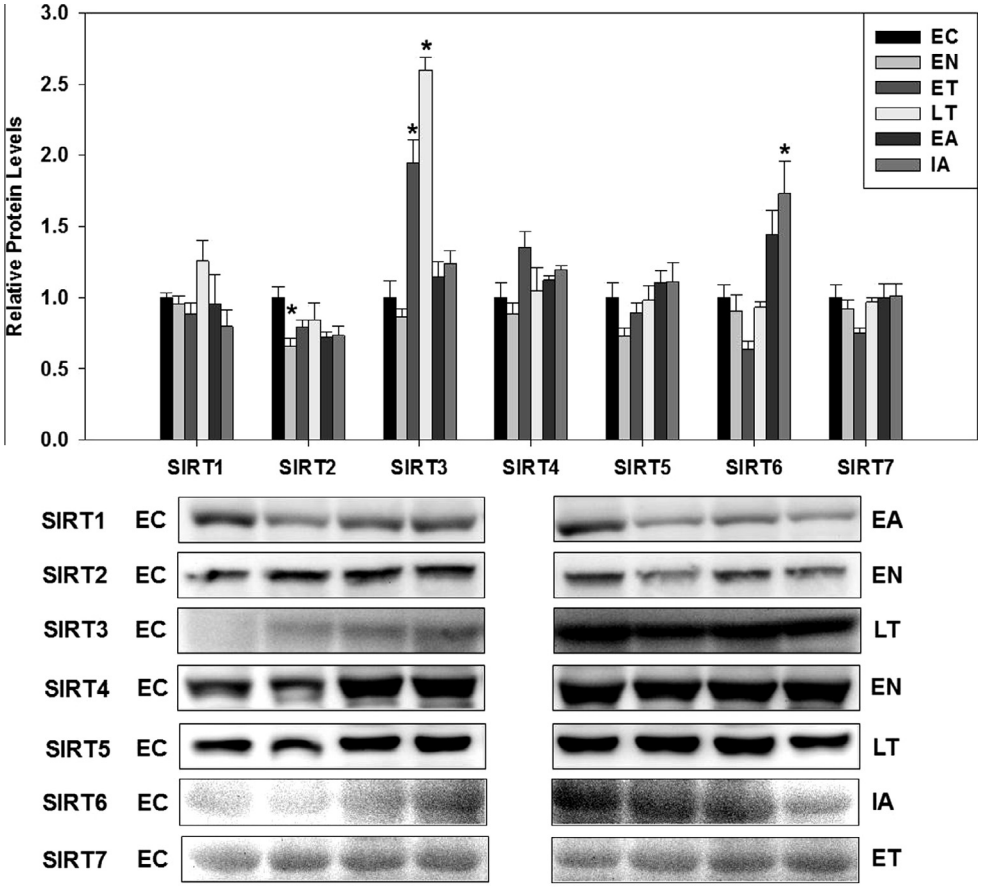

Fig. 2. Relative protein expression of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 in skeletal muscle of *I. tridecemlineatus* over the torpor–arousal cycle. All other information as in Fig. 1.

reflecting a novel regulatory mechanism operating in a tissue-specific manner over the course of the torpor–arousal bouts of hibernation.

The analysis of SIRT protein levels in four ground squirrel tissues revealed changes in the relative abundance of specific SIRTs at various points over the torpor–arousal cycle (Figs. 1–4). In liver, levels of SIRT1 increased significantly during early arousal, with sustained elevation also observed during interbout arousal (Fig. 1). A similar pattern was observed for levels of SIRT2 (significantly elevated throughout arousal as well as during entrance to torpor), while SIRT3 protein increased only during early torpor (Fig. 1). In muscle, protein levels of SIRT2, SIRT3 and SIRT6 fluctuated significantly during hibernation, with SIRT3 showing a particularly interesting increase in expression during early and late torpor (Fig. 2). In BAT, levels of SIRT1 were significantly elevated during early arousal (Fig. 3), similar to the pattern observed in liver. Protein levels of SIRT2, SIRT3, SIRT4, SIRT5 and SIRT7 also demonstrated patterns of differential regulation in WAT, notably characterized by significant reductions in the three mitochondrial SIRTs (SIRT3–5) during early and late torpor, and an increase in SIRT2 and SIRT7 levels during late torpor and interbout arousal, respectively (Fig. 4). Taken together, these results outline what appear to be distinct tissue-specific patterns of differential SIRT expression during hibernation, suggesting that these factors may serve tissue-specific roles in the context of this form of metabolic rate depression.

When considering the current literature, these results are perhaps not surprising; SIRT proteins are involved in the control of an incredibly diverse range of metabolic and protective processes, including those that are highly relevant to the tissues of mammalian hibernators. SIRT3, for example, is known to mitigate oxidative stress in multiple tissues [20,34,42] and regulates fatty acid oxidation and alternative energy pathways via the deacetylation of mitochondrial enzymes [4,19]. In fact, SIRT3 is responsible for the majority of the SIRT activity present in the mitochondria (SIRT4 and SIRT5 have little influence on the acetylation status of most proteins within the mitochondrial compartment), whereby loss of SIRT3 results in global hyperacetylation of mitochondrial proteins [24]. Thus, the apparent elevation of SIRT3 protein levels in hibernator skeletal muscle during deep torpor may suggest that the regulation of mitochondrial protein acetylation is implicated at this time. By extension, this would likely indicate that some of the metabolic and protective functions of SIRT3 are also relevant in this tissue during torpor. A SIRT3-mediated regulation of processes that reduce oxidative stress, for example, would serve a useful role in skeletal muscle during this time, when antioxidant responses are also known to be active in torpid tissues to prevent irreversible cellular damage at low temperatures, and to prepare for the drastic increases in oxygen consumption/ROS production that occur during arousal [3,12,31,44]. Similarly, the apparent upregulation of SIRT1 during arousal in liver and BAT may serve similar roles in promoting protective responses in the face of the increased oxygen stress that characterizes this transitory period, given that SIRT1 expression is known to induce resistance to oxidative stress [2]. Likewise, the observed increase in SIRT6 protein levels in skeletal muscle during interbout arousal may also function with a similar purpose, as SIRT6 protein levels are known to be elevated in response to oxidative stress in other systems, promoting DNA double-strand-break repair to mitigate genomic damage as a result of the increased prevalence of ROS [27]. Since skeletal muscle

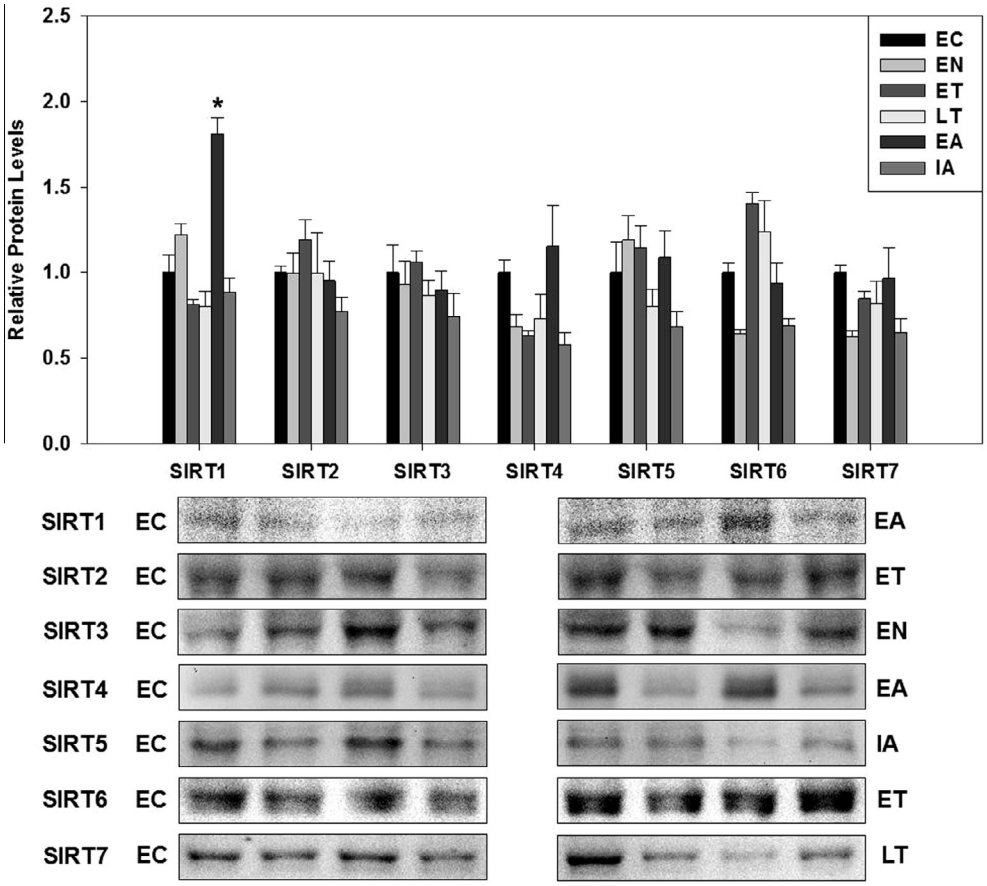

Fig. 3. Relative protein expression of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 in BAT of *I. tridecemlineatus* over the torpor–arousal cycle. All other information as in Fig. 1.

experiences drastic increases in ROS production as a result of shivering thermogenesis during arousal from torpor, this function of elevated SIRT6 in muscle of arousing (an upward trend) and aroused (significantly higher) hibernators may help protect the tissue during this exposure to oxidative stress. In WAT, the increase in SIRT2 levels during late torpor (together with rising trends before LT and decreases after LT) may reflect this tissue's integral function in sustaining the systemic fuel supply requirements of the animal throughout hibernation. Food deprivation has been shown to increase the expression of SIRT2 in white adipocytes of mice, directly leading to the promotion of fatty acid mobilization via lipolysis and the inhibition of adipocyte differentiation [45]. The increased expression of SIRT2 in hibernator WAT could therefore serve a similar purpose, and may contribute to the mobilization of lipid stores that are absolutely necessary to maintain the hibernation state [10]. The decreased expression of mitochondrial SIRT3, SIRT4 and SIRT5 during torpor (and arousal for SIRT3 and SIRT4) in WAT are perhaps surprising considering the known functions of SIRT3 in promoting lipid mobilization in other systems. However, they provide insight into the possible role of mitochondrial protein acetylation in this tissue, suggesting a possible emphasis on lower levels of deacetylation during this time. While the exact functions of the observed changes in SIRT protein expression remain to be determined, these results suggest a role for the differential regulation of SIRT proteins in multiple tissues of the mammalian hibernator.

To gain further insight into the possible significance of the observed fluctuations in SIRT protein levels throughout hibernation, total SIRT activity was assayed and compared between tissues from euthermic (EC time point) and torpid (LT time point) animals (Figs. 5–7). Interestingly, the only significant fluctuation in activity between the two tested time points was observed in whole tissue soluble protein extracts of muscle tissue (Fig. 6), where total SIRT activity was 3.6-fold higher during torpor as compared to euthermia. Given that the activity of muscle nuclear protein extracts did not change significantly under the same conditions (Fig. 6), these results suggest that the increase in total SIRT activity was localized outside of the nucleus, encompassing those SIRTs located in the cytoplasm and mitochondria. This possibility is compelling given the strong increase in SIRT3 protein levels observed in muscle tissue in ET and LT (Fig. 2). Since SIRT3 is mainly a mitochondrial enzyme [24], any increase in its activity would be expected to be detected in whole tissue extracts as opposed to nuclear extracts. However, given that the current assay does not allow the measurement of the activities of specific SIRT isozymes, but instead only total SIRT activity, no conclusions can be drawn regarding the responses of individual SIRTs. This is further emphasized by the observed lack of change in total SIRT activity in WAT between EC and LT (Fig. 7), while protein levels of four different SIRTs (SIRT2, SIRT3, SIRT4 and SIRT5) fluctuated significantly during this time (Fig. 4). As noted, because of the inherent sensitivity limitations associated with a total activity assay, changes in the protein levels of these individual SIRTs may not necessarily be reflected by measureable changes in total SIRT activity. Nonetheless, the correlated increase in SIRT3 protein levels and total SIRT activity in muscle whole tissue soluble protein extracts provides further support for a possible role for SIRTs in the torpid state.

An incredibly diverse range of proteins are influenced by SIRT activity. Indeed, SIRT deacetylation of downstream targets serves

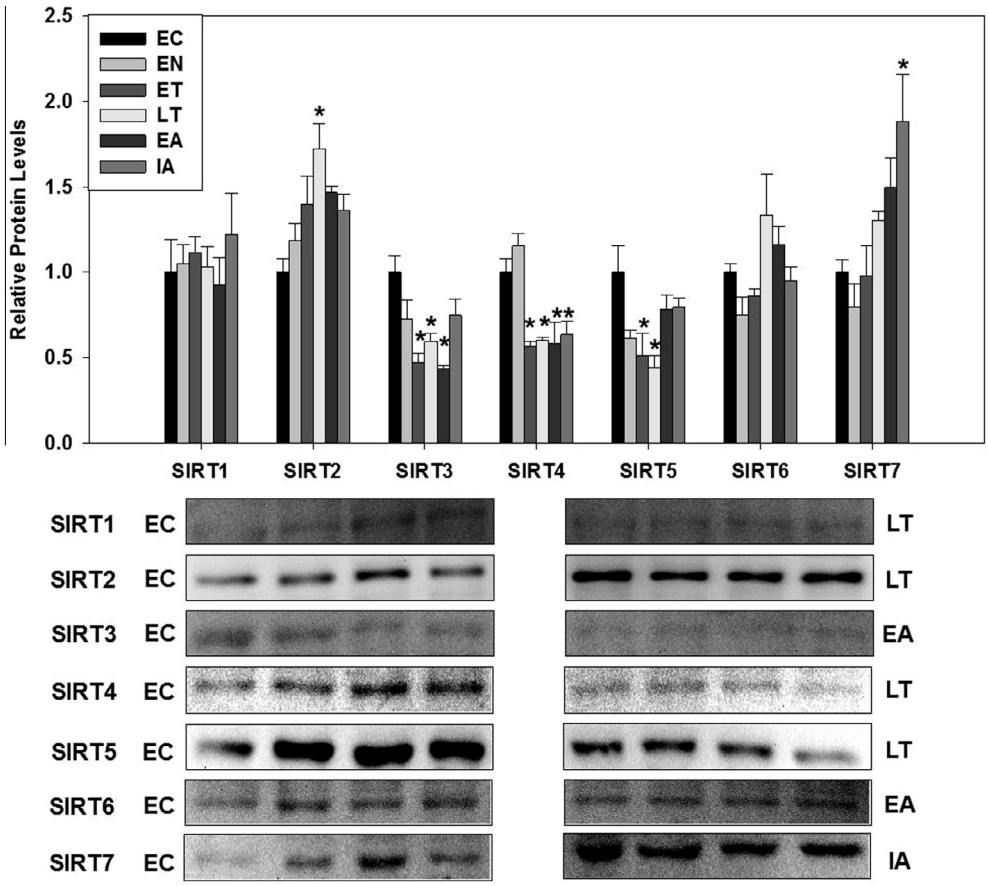

Fig. 4. Relative protein expression of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 in WAT of *I. tridecemlineatus* over the torpor–arousal cycle. All other information as in Fig. 1.

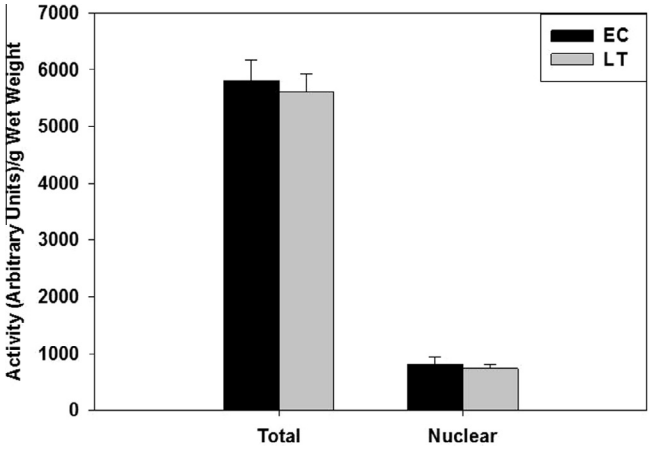

Fig. 5. Total SIRT activity in liver of *I. tridecemlineatus* comparing euthermic control (EC) and late torpor (LT) points of the torpor–arousal cycle at an assay temperature of 37 °C. Enzyme activities in total soluble extracts and nuclear extracts are expressed as activities (arbitrary units) per gram wet weight of starting tissue. Histograms show means ± SEM, $n = 3–4$ independent assays on tissue from different animals. Data were analyzed using the Student's *t*-test. * = significantly different from the respective EC control, $p < 0.05$.

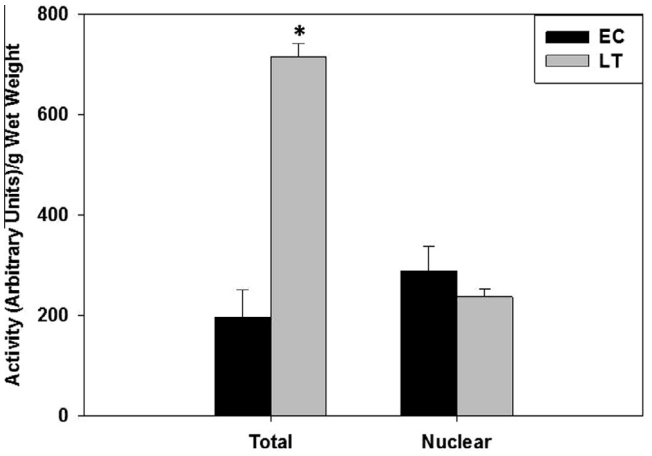

Fig. 6. Total SIRT activity in skeletal muscle of *I. tridecemlineatus* comparing euthermic control (EC) and late torpor (LT) points of the torpor–arousal cycle at an assay temperature of 37 °C. All other information as in Fig. 5.

a wide range of purposes, including the induction of conformational changes, the modulation of protein binding affinities, and the regulation of enzyme activities [17,23]. Two examples of SIRT-targeted proteins are the antioxidant enzyme SOD2 [7], and the p65 subunit of the transcription factor NF-κB [36]. SOD2 is a mitochondrial ROS-scavenging enzyme that prevents the accumulation of the reactive superoxide anion that is generated as a result of normal aerobic metabolism [13]. In response to caloric restriction and oxidative stress, SIRT3 – the major mitochondrial sirtuin – deacetylates SOD2 at lysine residue 68 (K68), causing an increase in its antioxidant activity [7]. Interestingly, levels of SOD2 acetylated at K68 were found to decrease significantly in hibernator liver, muscle, and white adipose during late torpor, as well as in

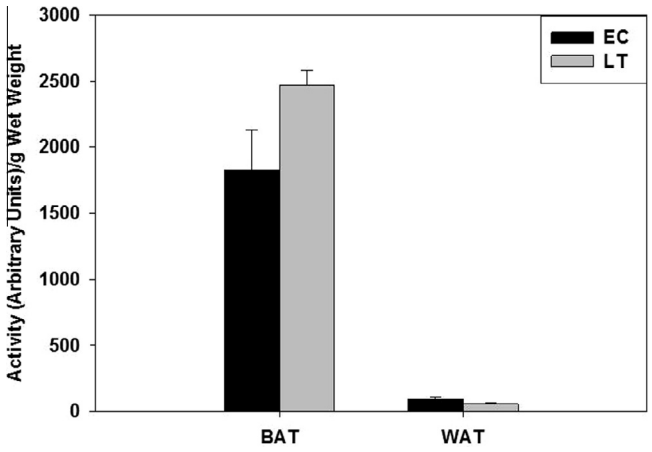

Fig. 7. Total SIRT activity in BAT and WAT of *I. tridecemlineatus* comparing euthermic control (EC) and late torpor (LT) points of the torpor–arousal cycle at an assay temperature of 37 °C. Enzyme activities in total soluble extracts are expressed as activities (arbitrary units) per gram wet weight of starting tissue. All other information as in Fig. 5.

muscle during interbout arousal (Fig. 8), suggesting that SOD2 activity is increased in these tissues during those times. Intriguing still, SOD2 acetylation at K68 appears to decrease in muscle in correlation with increased SIRT3 protein levels and total SIRT activity during late torpor. These correlated observations might reflect a SIRT-mediated control of an oxidative stress response in hibernator muscle during torpor, possibly representing a novel protective strategy employed by these animals. While similar correlations were not observed during interbout arousal, or in liver or white adipose tissue during late torpor, the observed decrease in levels of SOD2 K68 acetylation still supports the general notion of increased antioxidant activity in these tissues as a result of differential acetylation, be it mediated by SIRTs or otherwise. Previous studies have also provided evidence of a role for increased SOD2 activity in the tissues of mammalian hibernators [3,44], but the current results represent the first support for differential acetylation acting as a possible mechanism for promoting SOD2 activity in this context.

Similar conclusions were also drawn for the NF-κB subunit p65. NF-κB itself is an inducible transcription factor that is a heterodimer of p50 and p65 subunits, and is typically present in a latent inactive state in the cytoplasm via its binding to an inhibitor protein (IκB). IκB-binding masks nuclear localization sites on NF-κB and prevents it from entering the nucleus, but various stimuli induce phosphorylation of IκB to target it for ubiquitination and degradation, thereby freeing NF-κB and allowing it to translocate to the nucleus. There, NF-κB mediates the expression of downstream genes that control oxidative stress responses, inflammation, apoptosis, and other important cellular processes [21,29,43]. The p65 subunit is also regulated by SIRT deacetylation at lysine residue 310 (K310), whereby decreased levels of K310 acetylation result in the decreased expression of certain NF-κB target genes [36,48]. In the hibernator, K310 acetylation of p65 decreased significantly in liver during late torpor, but increased during interbout in liver and early arousal in brown adipose tissue (Fig. 9). In the case of liver, these results appear to suggest that the transcriptional activity of NF-κB may be suppressed during late torpor, but stimulated once the animal has returned to euthermia. This differential regulation could serve the hibernator well, depending on the gene targets controlled by such a process. For example, if a reduction in NF-κB activity during late torpor reduced its

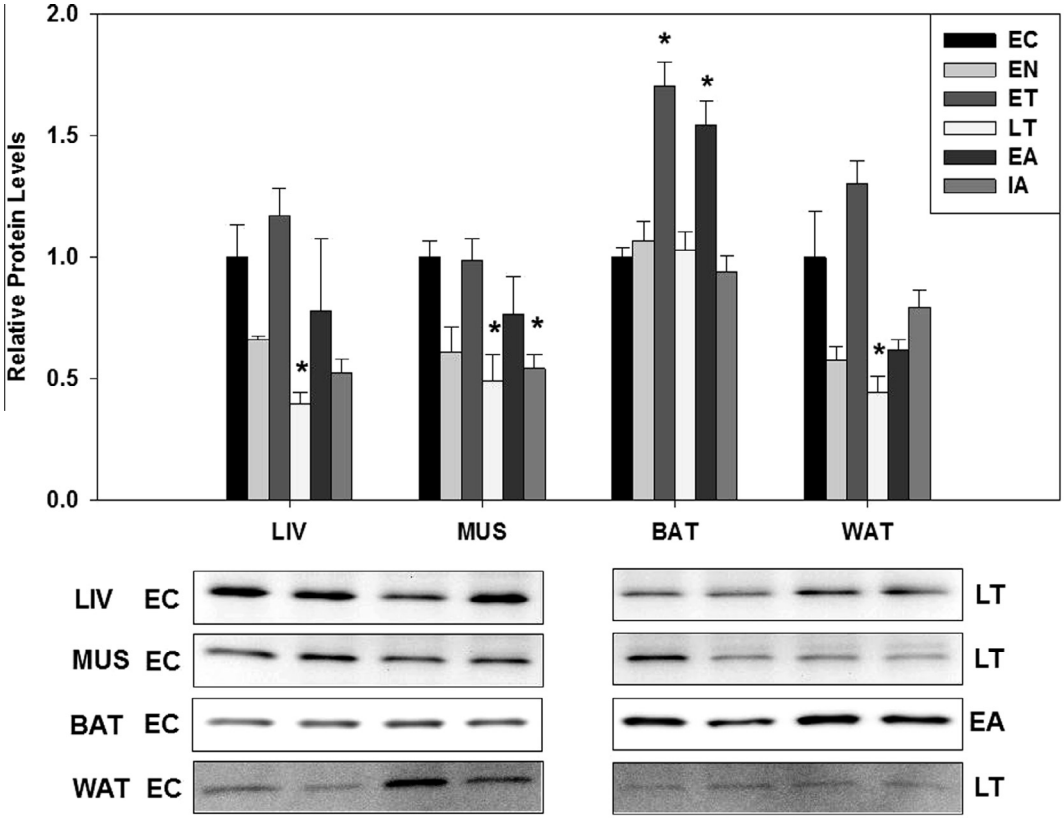

Fig. 8. Relative protein expression of SOD2 acetylated at lysine 68 in liver, skeletal muscle, BAT and WAT of *I. tridecemlineatus* over the torpor–arousal cycle. All other information as in Fig. 1.

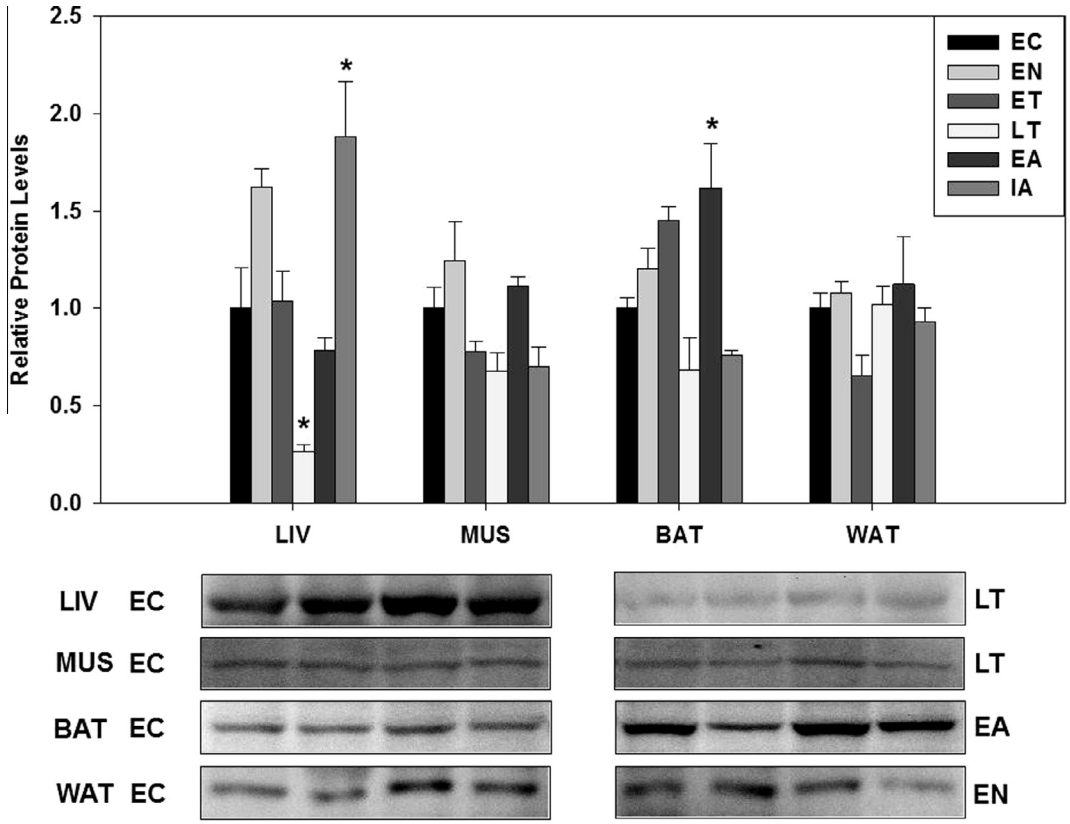

Fig. 9. Relative protein expression of NF-κB p65 acetylated at lysine 310 in liver, skeletal muscle, BAT and WAT of *I. tridecemlineatus* over the torpor–arousal cycle. All other information as in Fig. 1.

pro-apoptotic effects [21], this could serve as a protective mechanism for maintaining cellular integrity during this period of inactivity. Indeed, evidence exists for a role of anti-apoptotic pathways as a protective mechanism in hibernator tissues [37]. Similarly, if the stimulation of NF-κB downstream targets during arousal in liver and BAT lead to enhanced antioxidant responses [29], this would be adaptive for the hibernator. Alternatively, since NF-κB is a diverse transcriptional regulator, a decrease/increase in NF-κB activity could simply reflect the global decrease in transcriptional activity that occurs during torpor, and the subsequent reactivation that proceeds during arousal.

Whatever the ultimate purpose for the observed fluctuations in the acetylation status of these SIRT targets (NF-κB p65 and SOD2), the current results provide further evidence to support a role for SIRT activity during hibernation. While it is possible that other uncharacterized deacetylases may be responsible for the observed changes, the simple occurrence of such fluctuations is also interesting evidence to support the notion that differential protein acetylation likely plays a role in the regulation of the torpor–arousal cycle.

The evolutionarily conserved SIRT family of protein deacetylases regulates a diverse array of metabolic processes. The current study appears to expand upon these previously established functions by providing evidence to support a role for SIRTs in the regulation of the metabolic and protective pathways that mediate the process of mammalian hibernation. With the combined observation of tissue-specific differential SIRT protein expression, enhanced total SIRT activity, and fluctuating acetylation of downstream SIRT targets, the data suggest that these enzymes are likely involved in the control of this form of reversible hypometabolism. Interestingly, Morin and Storey [30] reported a similar conclusion for the expression of class I and II HDACs, whereby specific HDAC protein levels and total HDAC activity increased with a concurrent decrease in downstream acetylation of relevant histone targets in hibernator muscle, suggesting now that all classes of histone deacetylases may be involved in the regulation of hibernation. Although some intriguing hibernator tissues – such as heart and brain – were not studied in this investigation due to their limited availability, the future characterization of SIRT expression and activity in such tissues could potentially provide valuable insight into medically-relevant topics. For instance, given the increasingly understood importance of SIRT function in cardiovascular stress resistance [1,2,8], and since hibernator tissues are remarkable for their ability to avoid reperfusion injury upon arousal from torpor [6,40], the further characterization of SIRT function in hibernator cardiac tissue could conceivably provide unique insight into novel strategies for SIRT-related reperfusion injury-resistance in cardiovascular disease states. However, while much work remains to be done to fully elucidate the possible roles that these enzymes play in regulating the metabolic and protective pathways that are central to hibernation, it appears that the expression of SIRTs may indeed be characteristic of this natural process, and is likely another example of the conserved function of sirtuins in the universal regulation of metabolic homeostasis and cellular protective responses.

5. Statement of funding

This research was supported by a discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to KBS and the Canada Research Chairs program. ANR held a NSERC CGSM Scholarship.

Conflict of interest

None.

Acknowledgments

We would like to thank Dr. J.M. Hallenbeck and Dr. D.C. McMullen (NINDS, NIH, Bethesda) for providing the tissue samples for this study. Thanks also to J.M. Storey for editorial review of the manuscript, and S.N. Tessier for advice and guidance.

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