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Global DNA modifications suppress transcription in brown adipose tissue during hibernation ☆

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ABSTRACT

Hibernation is crucial to winter survival for many small mammals and is characterized by prolonged periods of torpor during which strong global controls are applied to suppress energy-expensive cellular processes. We hypothesized that one strategy of energy conservation is a global reduction in gene transcription imparted by reversible modifications to DNA and to proteins involved in chromatin packing. Transcriptional regulation during hibernation was examined over euthermic control groups and five stages of the torpor/arousal cycle in brown adipose tissue of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*). Brown adipose is crucial to hibernation success because it is responsible for the non-shivering thermogenesis that rewarms animals during arousal. A direct modification of DNA during torpor was revealed by a 1.7-fold increase in global DNA methylation during long term torpor as compared with euthermic controls. Acetylation of histone H3 (on Lys23) was reduced by about 50% when squirrels entered torpor, which would result in increased chromatin packing (and transcriptional repression). This was accompanied by strong increases in histone deacetylase protein levels during torpor; e.g. HDAC1 and HDAC4 levels rose by 1.5- and 6-fold, respectively. Protein levels of two co-repressors of transcription, MBD1 and HP1, also increased by 1.9- and 1.5-fold, respectively, in long-term torpor and remained high during early arousal. MBD1, HP1 and HDACs all returned to near control values during interbout indicating a reversal of their inhibitory actions. Overall, the data presents strong evidence for a global suppression of transcription during torpor via the action of epigenetic regulatory mechanisms in brown adipose tissue of hibernating thirteen-lined ground squirrels.

Introduction

To survive harsh winter conditions, some small mammals enter hibernation, a state characterized by prolonged periods of torpor at low body temperatures that are interspersed with brief arousals back to euthermia. During torpor bouts, metabolic rate may be suppressed to as low as 1–5% of normal resting rates in euthermia, core body temperature can fall as low as −2.9 °C, and physiological processes such as heartbeat and breathing may drop to ~2% of euthermic levels [1,25,17]. These physiological changes are underscored by global suppression of the rates of multiple energy-expensive biochemical processes in cells including the activities of ion motive ATPases, protein synthesis, the cell cycle, etc. [20,27,28]. For example, rates of ^{14}C-leucine incorporation into protein in vivo fell to <0.1% in brain, heart and liver of ground squirrels during torpor as compared to euthermic animals [7]. Strong metabolic controls are applied to suppress and reprioritize energy-expensive cellular processes and selective gene expression is used to reorganize some aspects of metabolism and enhance cytoprotection in the torpid state [20]. Reorganization of cellular processes requires strong reversible controls including post-transcriptional (e.g. via non-coding RNA) regulation of mRNA and post-translational (e.g. protein phosphorylation) regulation of proteins that rapidly and efficiently restructure the cellular landscape of organs [2].

Gene transcription is an energy-expensive cellular activity and, although selective gene up-regulation clearly occurs during hibernation [20], controls that provide a coordinated suppression of global transcriptional activity during torpor (and a reversal during arousal) are clearly required. In eukaryotic cells, several mechanisms can contribute to a global suppression of transcription. One is DNA hypermethylation, which inhibits transcription factor binding and thereby reduces mRNA synthesis. Another is post-translational regulation of chromatin proteins. Eukaryotic DNA is

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organized into a tightly packaged protein–DNA complex called chromatin. The fundamental subunit of chromatin is the nucleosome, primarily composed of an octamer of a four histone protein core. Post-translational modifications to the histone core allow the chromatin to unravel and facilitate the binding of transcription factors and RNA polymerase [6]. Particularly important to regulatory control is the reversible modification of histone H3 such as by acetylation at Lys23 or phosphorylation at Ser10. Both acetylation and phosphorylation of histones are associated with active transcription [4] and, hence, these posttranslational modifications would predictably be suppressed during torpor by the action of appropriate phosphatases and deacetylases. Acetylation is reversed by histone deacetylases (HDACs) that remove acetyl groups from the histone tails, thereby encouraging the closer packing of chromatin that inhibits translational activity.

The present study examines multiple factors that can contribute to global transcriptional suppression over the torpor–arousal cycle in thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*: phosphorylation and acetylation of histone H3, changes in HDAC activity, and DNA methylation. In addition, the recruitment of two co-repressors, methyl-CpG-binding domain protein 1 (MBD1) and heterochromatin protein 1 (HP1) are evaluated. The tissue of interest here is brown adipose tissue (BAT), a unique tissue that produces heat via non-shivering thermogenesis and plays a main role in rewarming hibernating animals as they arouse from torpor [3,16]. BAT also plays a role during torpor in stabilizing body temperature at about 0–5 °C if environmental temperature in the burrow drops to below the hypothalamic body temperature set-point. Hence, BAT has a vital and regulated role in the torpor cycle of hibernating mammals. The present study examines how transcriptional regulation by various epigenetic mechanisms is applied in BAT to control global gene expression in this thermogenic tissue over the torpor–arousal cycle.

Methods

Animal treatment

Thirteen-lined ground squirrels were captured by a United States Department of Agriculture-licensed trapper (TLS Research, Bloomingdale, IL). Animals were transferred to the Animal Hibernation Facility, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD, where the hibernation experiments were conducted as described by McMullen and Hallenbeck (2010). Briefly, animals weighing 150–300 g were housed in individual shoebox cages kept in a room with an ambient temperature of 21 °C. Animals were fitted with a sensor chip (IPTT-300; Bio Medic Data Systems, Seaford, DE, USA) injected subcutaneously while the squirrels were anesthetized with 5% isoflurane. Animals were given standard rodent diet and water *ad libitum* until they gained sufficient lipid stores to enter hibernation. To initiate hibernation experiments, animals were transferred to a cold, dark room at ~5 °C. Body temperature (T_b), time, and respiration rate were monitored to determine the stage of hibernation. All animals had been through several torpor–arousal bouts prior to sampling at a set stage. Animals were sacrificed at different times as determined by T_b and duration of torpor: (1) euthermic in the cold room (EC) (euthermic squirrels had a stable T_b of ~37 °C for 3–5 days, and had previously entered torpor but had not done so in the past 72 h); (2) entrance into torpor (EN) (T_b falling with sampling between 31 and 18 °C); (3) early torpor (ET) (T_b of ~5–8 °C for 24 h); (4) late torpor (LT) (T_b of ~5–8 °C for >5 days); (5) early arousal (EA) (T_b rising to ~12 °C) and (6) interbout arousal (IA) (aroused for ~18 h after a torpor bout with a core body temperature of ~37 °C). Animals were sacrificed by decapitation and tissue samples were quickly excised and immediately frozen in liquid nitrogen. Samples were delivered to Carleton University on dry ice and stored at −80 °C until use.

Protein isolation

Samples of frozen BAT (~0.5 g) were crushed under liquid nitrogen and then homogenized 1:2.5 w:v in homogenizing buffer (20 mM Hepes, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate) with a few crystals of phenylmethylsulfonyl fluoride and 1 μL protease inhibitor cocktail (Cat# PIC001; BioShop) added immediately before homogenization. After centrifugation at 4 °C for 15 min at 10,000×*g*, supernatants were collected and soluble protein concentrations were measured using the BioRad protein assay with bovine serum albumin as the standard. All samples were adjusted to a final concentration of 8 μg/μl by addition of calculated small volumes of homogenizing buffer and then mixed 1:1 v:v with 2× SDS loading buffer (100 mM Tris base, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercaptoethanol). Proteins were denatured by placing the tubes in boiling water for 5 min. Samples were stored at −80 °C until use.

Gel electrophoresis and immunoblotting

Aliquots containing 20 μg protein were loaded onto 10% polyacrylamide gels together with prestained molecular weight standards and separated by electrophoresis for 40–60 min at 180 V in 1× Tris–glycine running buffer. Proteins on the gel were then electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) in transfer buffer for 90 min at 160 mA. The membranes were washed in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) and blocked with 1% polyvinyl alcohol (70–100 kDa) for 45 s. After blocking, the membranes were probed with primary antibody (diluted 1:1000 v:v) for 24 h at 4 °C (Table 1). Membranes were then incubated with HRP-linked anti-rabbit or anti-mouse IgG secondary antibody (diluted 1:4000 v:v in TBST; Cell Signaling Cat# 7074, 7076) for 1 h at room temperature and were then developed using enhanced chemiluminescence. Detection of bands on blots used the ChemiGenius Bio-Imaging System (Syngene, Frederick, MD) and densitometric analysis was performed with the associated Gene Tools software.

Global DNA methylation

Total genomic DNA was isolated using the ZD Genomic DNA-Tissue MiniPrep kit (Cat# D3050; Zymo Research). Briefly, 25 mg samples of frozen tissue were each suspended in a mixture of ddH_2O (95 μl), 2X digestive buffer (95 μl) and proteinase K (10 μl) and left to incubate for 3 h. Subsequently, 700 μl of genomic lysis buffer was added to each sample, followed by centrifugation at 10,000×*g* for 1 min. Supernatant was removed, spun through a Zymo-Spin ICC column, and then 200 μl of DNA prewash buffer was added to elute the DNA into the collection tube. The concentrated product was diluted with 50 μl of ddH_2O and quantified using a GeneQuant Pro spectrophotometer (Pharmacia).

Relative levels of global DNA methylation were assessed using the MethylFlash™ Methylated DNA Quantification Kit (Cat # P-1034; Epigentek), according to manufacturer's instructions. Briefly, 10 ng samples of total DNA isolated from euthermic and hibernating (late torpor) squirrels were bound to an assay plate. The wells were then incubated for 30 min at room temperature with a capture antibody (1 ng/ml) which binds methylated DNA. A detection antibody (0.2 μg/ml) was then added followed by a developing solution to produce a colourmetric chemical reaction. Absorbance was read at 450 nm using a Multiscan Spectrum (Thermo Labsystems).

Table 1
List of primary antibodies used with the expected molecular mass (kDa) of the target protein and the commercial source.

Antibody	Molecular weight (kDa)	Company (catalogue number)
Histone H3	~17	Cell signaling (#9715)
Acetyl-Histone H3 (Lys23)	~17	Cell signaling (#9674s)
Phospho-Histone H3 (Ser10)	~17	Cell signaling (#9701s)
HDAC1	~62	GenScript (#A01279)
HDAC4	~140	GenScript (#A00429)
HP1	~86	NCI-CPTC (#C1A9-s)
MBD1	~22	NCI-CPTC (#CPCT-MBD1-s)

Statistical analysis

Immunoblot band intensity in each lane was standardized against the summed intensity of a group of Coomassie-stained (0.25% w/v Coomassie brilliant blue, 7.5% v/v acetic acid, 50% v/v methanol) protein bands in the same lane that did not show any variation between experimental states and were not near the molecular weight of the protein of interest. All samples were also previously shown to have constant expression of α-tubulin as determined both from immunoreactive band densities alone and also when standardized against the Coomassie stained bands. Statistical analysis used one-way ANOVA with a *post hoc* Holm–Sidak test to compare five stages of torpor–arousal cycle to euthermic control groups (SigmaPlot 11, Systat Software Inc., San Jose, CA).

Results

Histone expression

Relative amounts of total histone H3 protein along with relative levels of acetylation (Lys23) and phosphorylation (Ser10) of the protein were analyzed in BAT of thirteen-lined ground squirrels via immunoblotting. Data were gathered from five stages of the torpor–arousal cycle and compared to EC groups. All three antibodies cross-reacted with a protein band of ~17 kDa, the expected size of histone H3 in other mammals. Total histone (H3) levels did not change significantly over the course of the torpor–arousal cycle or to euthermic controls (Fig. 1A). However, relative acetylation (Lys23) of the protein decreased significantly from euthermic controls (EC) and remained constant throughout the torpor–arousal cycle ($P < 0.05$). Levels of H3(Lys23) decreased to 63 ± 4%, 54 ± 6%, and 49 ± 12% compared to euthermic squirrels in the cold room (EC), during entrance into torpor (EN) and after short and long (ET and LT) durations of torpor ($P < 0.05$; Fig. 1B). Acetylation levels also remained low at 48 ± 4% and 53 ± 6% of control values during both early arousal and interbout arousal (EA and IA) stages, respectively ($P < 0.05$; Fig. 1B). Significant changes in the phosphorylation status of histone H3(Ser10) were found only during EA, a decrease to 63 ± 6% of EC levels, and significantly lower than all other torpor–arousal points ($P < 0.05$; Fig. 1C).

HDAC1 and HDAC4 protein expression

Relative protein levels of HDAC1 and HDAC4 enzymes in BAT were also compared between EC groups and the five stages of the torpor–arousal cycle. Western blotting showed immunoreactive bands for ground squirrel HDAC1 and HDAC4 at approximate molecular weights of 62 and 140 kDa, respectively, as expected for the mammalian proteins from other sources. HDAC1 levels were elevated by 1.43 ± 0.14-fold in EN, 1.42 ± 0.15-fold in LT and 1.41 ± 0.14-fold in EA, as compared with EC ($P < 0.05$; Fig. 2A). However, a temporary drop in HDAC1 content to 66 ± 4% of EC levels ($P < 0.05$), and significantly depressed from all other torpor–arousal points, occurred in ET. HDAC4 levels rose steadily from EC starting at EN with an increase of 2.54 ± 0.23-fold over the EC values and increasing further to 3.83 ± 0.1-fold in ET and to 6.13 ± 0.09-fold in LT higher than control values ($P < 0.05$) (Fig. 2B). Expression of HDAC4 then decreased sharply during arousal from torpor, falling to the level seen during EN but still elevated in EA and IA by 2.61 ± 0.1-fold and 2.44 ± 0.23-fold, respectively, as compared with EC groups ($P < 0.05$).

DNA methylation and expression of MBD1 and HP1 proteins

To evaluate global DNA methylation, the Epigentek *Methyl-Flash*™ kit was used to measure total 5-methylcytosine (5-mC) content in BAT, comparing EC and LT states. Compared with a relative mean level of 1.0 ± 0.15 for EC, DNA methylation levels increased significantly by 1.72 ± 0.22-fold ($P < 0.05$) in late torpor (LT). Relative levels of MBD1 and HP1 proteins were assessed by immunoblotting. Immunoreactive bands were detected at 86 kDa for MBD1 and 22 kDa for HP1, comparable to values for other mammals. MBD1 protein levels were unchanged from EC between the EN and ET stages of torpor, but increased significantly when torpor was prolonged (Fig. 3A). In this regard, MBD1 levels increased by 1.91 ± 0.13-fold in LT and remained high at 1.64 ± 0.09-fold in EA, as compared with EC ($P < 0.05$). Levels returned to values similar to EC and early torpor points (EN and ET), during IA. Relative levels of HP1 followed a similar pattern. HP1 content was also unchanged during EN and ET stages of the torpor–arousal cycle when compared to EC, but increased significantly in LT to 1.46 ± 0.13-fold ($P < 0.05$; Fig. 3B). HP1 remained significantly elevated during EA at 1.25 ± 0.09-fold as compared to EC values ($P < 0.05$). Protein expression of HP1 returned to EC levels during IA. Protein expression of HP1 was not significantly different between EN and LT or EA time points.

Discussion

Entrance into torpor requires the global suppression of energetically expensive processes such as transcription and translation (although selective expression or upregulation of some specific genes remains) [21]. Previous studies have presented evidence for the arrest of both the synthesis of mRNA products and the translation of mRNA into proteins by the ribosomal machinery during mammalian hibernation. For example, van Breukelen and Martin [23] used nuclear run-on assays to show that the rate of transcriptional initiation in liver was reduced by 50% in early and late hibernation in golden-mantled ground squirrels (*Spermophilus lateralis*) as compared to control animals. Frerichs et al. [7] showed elevated phosphorylation of the eukaryotic initiation factor 2α and increased ribosomal disaggregation, both characteristics of arrested initiation of protein synthesis. Hittel and Storey [11] showed that the rate of [^{3}H] leucine incorporation into protein in kidney extracts from

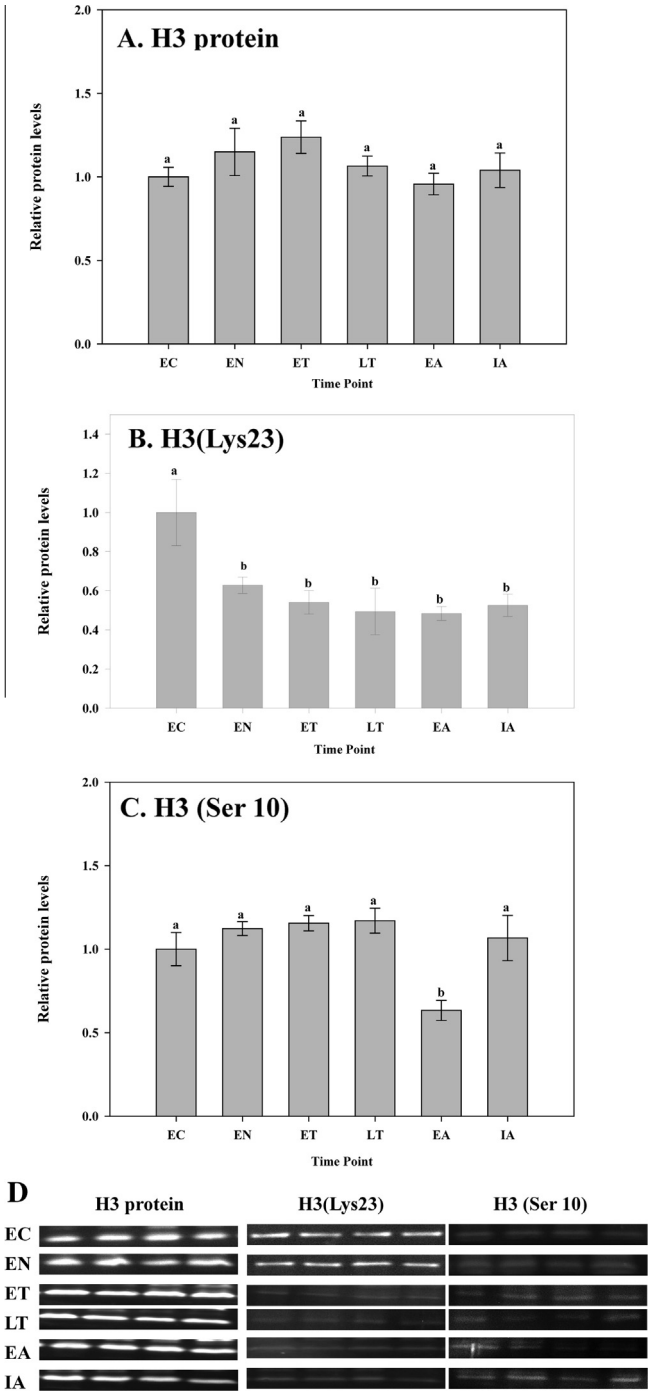

Fig. 1. Changes in histone H3 protein levels and post-translational modifications in brown adipose tissue of thirteen-lined ground squirrels, *I. tridecemlineatus*, over the torpor–arousal cycle. (A) Relative levels of total histone H3 protein in euthermic controls (EC) and over five phases of the torpor–arousal cycle. (B) Relative levels of acetylated histone H3 (Lys9). (C) Relative levels of phosphorylated histone H3 (Ser10). Sampling points are: EC, euthermic in the cold room (T_b 37 °C); EN, entrance into torpor (T_b falling to between 31 and 18 °C); ET, early torpor (T_b ~5–8 °C for 24 h); LT, late torpor (T_b ~5–8 °C for >5 days); EA, early arousal (T_b rising to ~12 °C); IA, interbout arousal (T_b 37 °C for ~18 h). (D) Immunoblot images for total histone H3 protein, acetylated H3 (Lys23) and phosphorylated H3 (Ser10). Data are means ± SEM for $n = 4$ independent trials on tissue from different animals. Sampling points sharing the same letter over the histogram bars are not significantly different from each other but are significantly different ($P < 0.05$) from bars labeled with other letters.

hibernating *I. tridecemlineatus* was only 15% of the euthermic value, and Wu and Storey [28] documented inhibition of the mTOR signaling pathway and its ribosomal targets during torpor. These studies

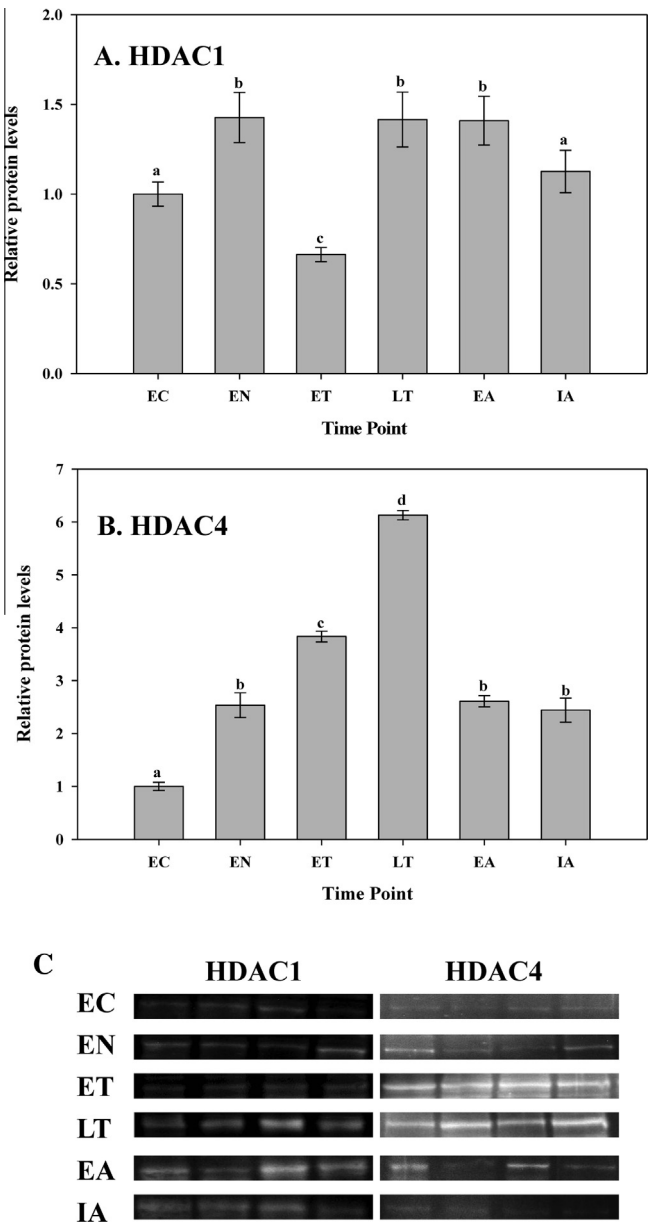

Fig. 2. Protein levels of histone deacetylases in euthermic controls and over the torpor–arousal cycle in *I. tridecemlineatus* brown adipose tissue. (A) Relative HDAC1 protein levels. (B) Relative HDAC4 protein levels. (C) Immunoblot images for HDAC1 and HDAC4. Other information as in Fig. 1.

however, did not allude to the role of global epigenetic mechanisms in restricting gene expression during hibernation.

DNA structure and accessibility in the nucleus are two important factors that determine whether proteins, such as transcription factors and RNA polymerase, can bind to and initiate transcription [13]. DNA segments are tightly wrapped around a four protein histone core forming a dense package which regulates gene expression. When acetylation or phosphorylation modifications are added to histone tails, the tightly wound chromatin unravels to permit the transcriptional machinery to bind to the exposed DNA [4,14]. Transcription is also regulated by reversible cysteine methylation of the DNA itself by DNA methyltransferase enzymes, subsequent methylation of DNA blocks the action of RNA polymerase. This study examined whether global mechanisms of transcriptional regulation are differentially expressed from euthermic controls and within the course of the torpor–arousal cycle in brown adipose tissue of hibernating thirteen-lined ground squirrels.

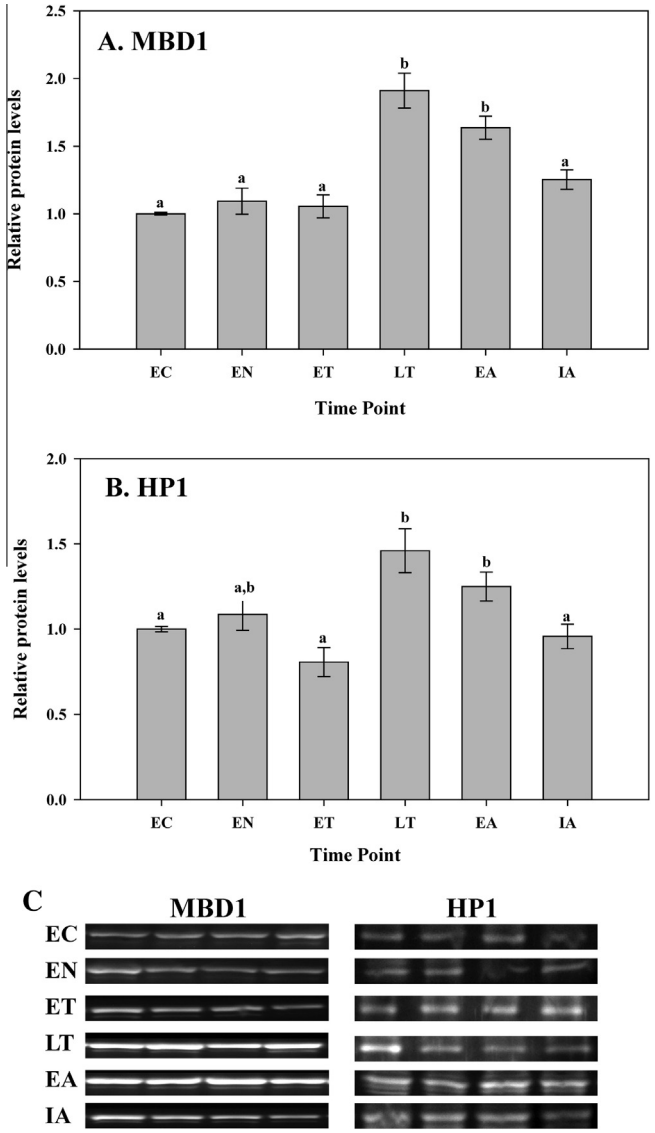

Fig. 3. Protein levels of co-repressor proteins in euthermic controls and over the torpor–arousal cycle in *I. tridecemlineatus* brown adipose tissue (A) Relative levels of MBD1 protein. (B) Relative levels of HP1. (C) Immunoblot images for MBD1 and HP1. Other information as in Fig. 1.

Our results show that histone H3 undergoes select post-translational modifications in ground squirrel BAT both between euthermic control and torpid groups and over the torpor–arousal cycle (Fig. 1). Total histone H3 protein levels did not change between euthermic controls and torpid animals, while the amount of acetylated H3 (Lys23) was reduced by about 50% during EN, ET and LT as well as EA and IA when compared with non-torpid EC. These findings suggest that acetylation levels are significantly reduced during torpor.

Phosphorylation of histone H3 also decreased significantly during EA stage to 63% of euthermic control values. These findings complement an earlier study of histone regulation in *I. tridecemlineatus* skeletal muscle that found unchanged total histone H3 levels, but a 40% reductions in both acetylation (Lys23) and phosphorylation (Ser10) of the protein during torpor [18]. A comparison of the two studies also hints at possible tissue specific programs of histone modifications (and heterochromatin formation) during torpor since skeletal muscle tissue showed reduced phospho-H3 levels in late torpor (LT), whereas BAT showed this in early arousal (EA).

The reduction in acetylated histone H3 (Lys23) in BAT during torpor, suggests the differential activity or regulation of HDACs that catalyze the reaction. There are two main families of HDAC proteins: the SIR2 family of NAD^+-dependent HDACs and the classical HDAC family comprising class I and class II HDACs. Class I HDACs (HDAC 1, 2, 3, and 8) are found almost exclusively in the nucleus, whereas class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) translocate to the nucleus from the cytoplasm under certain physiological conditions [6]. HDAC1 and HDAC4 represent class I and class II deacetylases, respectively, and protein levels of both were elevated in BAT of torpid ground squirrels (Fig. 2). Both HDAC proteins also increased in *I. tridecemlineatus* skeletal muscle during torpor and total HDAC activity in muscle was 1.8-fold higher in LT when compared to EC [18]. HDAC1 protein levels in BAT were elevated by ~1.4-fold in EN, LT and EA, whereas HDAC4 levels increased continuously from entrance (a 2.5-fold increase in EN) through to LT where levels peaked at 6-fold higher than control values. Levels of both HDACs declined again during arousal from torpor. This pattern of HDAC expression suggests a crucial role for HDACs in the regulated suppression of gene transcription during torpor and the very large increase in HDAC4 protein levels may indicate the particular importance of class II HDACs in transcriptional suppression in hibernators.

DNA methylation is perhaps one of the best studied chemical modification of genes and occurs by the covalent attachment of a methyl group at carbon 5 of the cytosine ring in the CpG context [26]. This method of gene silencing seems optimal as a widespread means to regulated metabolic depression as it can be accomplished rapidly by reversible enzymatic mechanisms, involving just a few proteins [26]. The silencing of DNA expression by methylation is achieved in two ways: (1) methylation of gene promoter regions directly hinders the binding of transcription factors, and (2) methylation promotes recruitment of co-repressors and proteins which reorganize the conformation of chromatin into its inactive form [22]. Analysis of ground squirrel BAT showed a 1.7-fold increase in global DNA methylation during LT which clearly indicates that this mechanism is an integral part of transcriptional suppression in hibernation.

The major group of methyl-DNA binding proteins is the methyl-CpG binding domain (MBD) family. MBD1 is the largest in the family of MBD proteins and belongs to a subfamily, which also includes MBD2, MBD3, MBD4 and MeCP2 [5]. The currently accepted mechanism of MBD1-dependent transcriptional inhibition is via the recruitment of HDACs to regions of methylated DNA and the subsequent deacetylation of adjacent histones, resulting in chromatin condensation [24]. Other studies suggest additional modes of action such as the physical displacement of transcription factors at methylated promoter sites [12,29]. MBD1 and HP1 proteins may also act in parallel, altering the DNA and chromatin architecture to produce transcriptional inhibition [8]. HP1 is known to both facilitate the formation of heterochromatin and silence specific genes by binding to their promoter regions [19]. HDACs remove acetyl-groups from histone proteins, which are then methylated at the Lys9 residue. HP1 recognizes the methylated regions through its chromo domain and heterochromatin formation is propagated by self-association along the DNA molecule [29]. This present study of ground squirrel BAT showed significant increases in both MBD1 and HP1 protein expression of about 1.9- and 1.5-fold, respectively, during LT compared to EC (Fig. 3). The similar pattern of expression of both of these chromatin-altering proteins suggests that MBD1 and HP1 may act together during torpor to promote a global reduction in mRNA synthesis.

Taken together, the results of this study present strong evidence for global suppression of gene transcription in BAT of hibernating thirteen-lined ground squirrels via the action of epigenetic controls. The rise in global DNA methylation and the increased

amounts of HP1 and MBD1 co-repressor proteins and HDAC enzymes along with the decrease in histone acetylation are all consistent with a regulated global silencing of the transcription of genes. This silencing is apparently carried out by a reorganization of DNA packaging, preventing the binding of transcription factors and members of the transcriptional complex. This evidence of direct transcriptional silencing during torpor is also supported by other data, namely a large increase in SUMO (small ubiquitin-related modifier) conjugation of proteins during torpor [15]. SUMOylation affects many proteins involved in gene expression, chromatin structure, signal transduction, and maintenance of the genome [10]. In particular, transcription factors are major targets and SUMOylation of these proteins has mainly negative effects on gene expression [9]. Inhibition of transcription factor action by SUMOylation would clearly go hand-in-hand with an inhibition of transcription factor binding as a result of enhanced chromatin packing brought about by the epigenetic modifications documented in this study.

Brown adipose tissue plays a vital physiological role during the arousal stage of the hibernation cycle when the body must be rewarmed to euthermic levels despite a low ambient temperature. Non-shivering thermogenesis by BAT is the first source of heat for this process and is supplemented once the body is partially rewarmed by an activation of shivering thermogenesis in skeletal muscle. It is unclear whether rates of gene transcription return to EC levels during the arousal process or whether only a selected group of genes are unregulated to carry out the necessary functions to initiate rewarming [3]. The present results indicate that inhibitory transcriptional controls largely remain in place during the arousal phase of the torpor cycle (i.e. while the body is rewarming in EA) since only HDAC4 levels returned to near control values during this stage. However, other parameters (levels of MBD1, HP1 and both HDACs) were suppressed again during the IA, although histone H3 acetylation levels remained low. This suggests that a general reactivation of gene expression can occur during interbout, supporting transcriptional activities needed to sustain the thermogenic function of BAT and replenish proteins that were damaged during long term torpor.

Acknowledgments

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