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The role of global histone post-translational modifications during mammalian hibernation

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Mammalian hibernators must cope with hypothermia, ischemia-reperfusion, and finite fuel reserves during days or weeks of continuous torpor. One means of lowering ATP demands during hibernation involves substantial transcriptional controls. The present research analyzed epigenetic regulatory factors as a means of achieving transcriptional control over cycles of torpor-arousal. This study analyzes differential regulation of select histone modifications (e.g. phosphorylation, acetylation, methylation), and identifies post-translational modifications on purified histones using mass spectrometry from thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*). Post-translational modifications on histone proteins were responsive to torpor-arousal, suggesting a potential mechanism to dynamically alter chromatin structure. Furthermore, proteomic sequencing data of ground squirrel histones identified lysine 19 and 24 acetylation on histone H3, while acetylation sites identified on H2B were lysine 6, 47, 110, and 117. The present study provides a new glimpse into the epigenetic mechanisms which may play a role in transcriptional regulation during mammalian hibernation.

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1. Introduction

Epigenetic regulatory mechanisms including DNA methylation and histone proteins are involved in an overwhelming number of physiological and pathological processes [22,28]. One particularly interesting model system for understanding epigenetic changes are mammalian hibernators because regulated metabolic suppression relies on coordinated decreases in energy-consuming processes such as transcription [9,21,33,42,51]. Hibernation generally consists of multiple bouts of deep torpor, either occurring on the order of days or weeks, with intermittent periods of arousal during which animals rewarm to euthermic body temperature (T_b) [48]. During this process, hibernators can achieve huge energy savings, involving profound behavioral and physiological changes that can be traced to important molecular "switches" that shut down metabolism and aid in meeting challenges associated with hypometabolism [5,41]. Consequently, the present research aimed at investigating the epigenetic factors which may contribute to global decreases in gene expression, thereby mediating energy sparing during mammalian hibernation. More specifically, this research focuses on epigenetic factors which are physically associated with the chromosomes on which they act through reversible histone modifications.

DNA is packaged in the nucleus via interactions with positively charged histone proteins organized into nucleosomes and forming the main building blocks of eukaryotic chromatin. DNA is wrapped around nucleosomes which are composed of an octamer core containing two copies of each of histone H2A, H2B, H3, and H4 and each nucleosome is separated by linker DNA which is organized by H1. Core histone proteins carry epigenetic information in post-translational modifications (PTMs) including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation [4]. PTMs occurring on histone proteins can influence histone:DNA and histone:histone interactions, especially those in the *N*- and *C*-terminal tails. Of the post-translational modifications present on histones proteins, acetylation is one of the best characterized and is almost

Abbreviations: amu, atomic mass unit; DTT, dithiothreitol; EA, early arousal; EC, euthermic cold room; EN, entrance; HDAC, histone deacetylase; IA, interbout arousal; LT, late torpor; PTM, post-translational modification; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIRT, sirtuin; T_b, body temperature.

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invariably associated with relaxed chromatin and the activation of transcription [37]. However, while changes in chromatin structure as a function of post-translational modifications can affect the accessibility of transcriptional proteins that interact with DNA regulatory sites, this accessibility is also influenced by other mechanisms. These can include, for example, positioning of the nucleosome or the expression level of DNA binding proteins. In most mammals, H2B is primarily acetylated at Lys5, 12, 15, and 20, H2A at Lys5, 9, 13, and 15, H3 at Lys9, 14, 18, 23, 27, and 56, and H4 at Lys5, 8, 12, 16, and 20 [7,43]. Additionally, phosphorylation at Ser10 of histone H3 is correlated with chromosome condensation [16].

While epigenetic changes occur in response to various stimuli, histone modifications can have a direct effect on the accessibility of chromatin to transcription factors thereby playing a role in gene expression programs. The data in the present study analyzes and compares relative changes of select histone modifications over the torpor-arousal cycle of hibernation. The present study seeks to build on evidence that thirteen-lined ground squirrel histone H3 modifications (phosphorylated Ser 10 and acetylated Lys 23) were significantly reduced in skeletal muscle during deep torpor [33]. These reductions are believed to be facilitated in part by histone deacetylases (HDACs), which have been shown to increase with respect to both enzyme activity and protein levels in skeletal muscle and brown adipose tissues of torpid ground squirrels [3,33]. Furthermore, a study has found that *I. tridecemlineatus* sirtuins (SIRTs), a family of protein deacetylases, is differentially regulated throughout bouts of torpor-arousal. In particular, skeletal muscle SIRT3 protein levels and total SIRT activity increased during torpor, suggesting a suppression in gene transcription during periods of hypometabolism [36]. In order to expand on these measurements, western blotting was used to assess Ac-H3K23 and p-H3S10 over a range of other time points in the torpor-arousal cycle. In addition, the effects of hibernation on additional acetylation sites on histone H3, H2A, H2B, and H4 were also assessed (H3 K9, 14, 18, 27, 56; H2A and H2B K5; H4 K8). In general, we predicted that deep torpor would be associated with chromatin condensation, which would be reversed during arousal from torpor. Finally, we were interested in the idea that specific histone amino acid substitutions and PTMs may be present in hibernating ground squirrels, as compared to non-hibernating mammals. In pursuit of this goal, we used a column-based histone purification technique and downstream mass spectrometry whereby we identified PTMs on histone proteins. These results warrant future studies to investigate the functional consequences of these unique histone signatures.

2. Material and methods

2.1. Animals

Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) were wild-captured and transported to the Animal Hibernation Facility, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD. Hibernation experiments were conducted by the laboratory of Dr. J.M. Hallenbeck, as previously described [30]. Thirteen-lined ground squirrels for the summer experimental condition were sampled during July and had been in the NIH colony since the previous August. They were maintained at room temperature and had hibernated the previous winter. Thirteen-lined ground squirrels were individually housed in shoebox cages in a holding room with an ambient temperature of 21 °C under a 12/12-h light/dark cycle. Animals were fitted with a sensor chip (IPTT-200; Bio Medic Data Systems, Seaford, DE) injected subcutaneously while the squirrels were anesthetized with 5% isofluorane. Animals were fed standard rodent diet and water *ad libitum* until they gained sufficient lipid stores to enter hibernation. To induce hibernation, animals were transferred to cold chambers at 4-6 °C and 60% humidity in cages containing wood shavings. Respiration rate and T_b were monitored over time to determine the stage of hibernation. Experiments were done during the winter months of December–March, and all animals (except summer animals) had been through a series of torpor-arousal bouts prior to sampling. Animals were sampled at different points over torpor-arousal cycles following the administration of 5% isofluorane and rapid decapitation. Tissue samples were quickly excised and immediately frozen in liquid nitrogen. This study, including animal housing and experimental protocols, were approved by the NINDS institutional animal care and use committee (IACUC; Permit number ASP 1223-05). Samples were delivered to Carleton University on dry ice and stored at −80 °C until use. Tissue samples were retrieved from the following conditions: (1) EC designates euthermic, cold room; these euthermic squirrels had a stable T_b (~37 °C) and high metabolic rate for at least three days. (2) EN designates entrance; animals that are in the entrance phase of the hibernation bout (T_b = 25-18 °C) characterized by decreasing T_b (at least two successive temperature readings showed a decreasing T_b). (3) LT designates late torpor; animals that have remained in the deep torpor phase of the hibernation bout for 5 days and have not begun a periodic arousal (T_b = 5–8 °C). (4) EA designates early arousal animals; characterized by an increased respiratory rate of more than 60 breaths per minute accompanied by a rising body temperature with sampling between T_b = 9–12 °C. (5) IA designates interbout arousal; animals were naturally aroused after the torpor phase of the hibernation bout and reached the respiratory rate, metabolic rate, and body temperature of fully aroused animals for up to 18 h after being in torpor for up to 5 days.

2.2. Total protein extraction

Samples of frozen skeletal muscle from five time points in the torpor-arousal cycle (EC, EN, LT, EA, IA) were separately extracted ($n = 4$) as previously described [29]. Skeletal muscle was chosen for the present study since it is important at multiple experimental conditions throughout the torpor-arousal cycle: during deep-torpor muscle must avoid atrophy during extended periods of inactivity and during arousal to euthermia shivering thermogenesis contributes to heat generation. Briefly, samples of skeletal muscle were quickly weighed, crushed into small pieces under liquid nitrogen, and then homogenized 1:2 w:v in ice-cold homogenizing buffer. Protein concentrations were quantified by the Coomassie blue dye-binding method, and were adjusted to a constant 10 μg/μL by addition of homogenizing buffer. Aliquots were combined 1:1 v/v with 2X SDS loading buffer and boiled. The final protein samples (5 μg/μL) were stored at −40 °C until use.

2.3. Western blotting

In order to ensure that signals are in the linear range of the detection system, optimization for amount of protein loaded, gel loading/transfer conditions, blocking conditions, antibody dilution, and detection method for each target of interest was performed. Equal amounts of protein from each biological replicate ($n = 4$) across 5 experimental conditions (i.e. EC, EN, LT, EA, IA) were loaded onto SDS-polyacrylamide gels or Tris-tricine gels and separated on a BioRad Mini Protean III apparatus. The data quantified depicts relative changes with each experimental condition compared against EC controls. To control for differences in gels during relative quantification, one time point ($n = 4$) was included on every gel/blot to which all samples from all other time points were standardized. Samples loaded on 15% Tris-tricine gels were run at 30 V for 1 h followed by ~2 h at 150 V. Other proteins were separated by

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