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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).

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