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Differential Expression of Selected Mitochondrial Genes in Hibernating Little Brown Bats, *Myotis lucifugus*

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ABSTRACT High rates of non-shivering thermogenesis by brown adipose tissue accompanied by additional shivering thermogenesis in skeletal muscle provide the powerful reheating of body organs that allows hibernating mammals to return from their state of cold torpor back to euthermic function. Previous studies have suggested that changes to brown adipose mitochondria occur during hibernation and are partially responsible for its capacity for non-shivering thermogenesis. The current study shows that selected mitochondrial enzyme activities are elevated and selected genes and proteins are induced during torpor in brown adipose tissue of the little brown bat, *Myotis lucifugus*. Cytochrome oxidase activity in brown adipose tissue was more than 3-fold higher during torpor than in euthermic animals. Transcript levels of mitochondria-encoded genes, *coxII* and *nad4*, were also 3–4-fold higher during torpor, as evidenced by northern blotting. By contrast, transcripts of these genes were unchanged in skeletal muscle during torpor. Protein levels of carnitine palmitoyl transferase-1β, an enzyme embedded in the outer membrane of the mitochondria that is the rate-limiting step enzyme in β-oxidation, were also elevated by 2-fold during torpor in brown adipose but were unchanged in skeletal muscle. Cloning and sequencing of a 624 bp segment of *cpt-1β* revealed a number of amino acid substitutions in the bat protein as compared to CPT-1β from other mammals; these may be beneficial for enzyme function at low body temperatures during torpor. This study provides further evidence for a key role of mitochondria in hibernation. *J. Exp. Zool. 305A: 620–630, 2006.*

Hibernation is a widespread adaptation for winter survival among many small mammals (and also in bears). Hibernators can be found in seven mammalian orders, being most common in the orders *Rodentia* and *Chiroptera* (Geiser, 2004; Heldmaier et al., 2004). By hibernating, animals can save up to 90% of the energy that would otherwise be consumed to remain euthermic over the winter (Wang and Lee, '96). During the hibernation season, animals enter long periods of deep torpor (lasting days to weeks) where metabolic rate can be as little as 1–5% of resting euthermic rate and body temperature (T_b) can fall to near 0°C (Geiser, 2004). Torpor is not continuous over the winter but is punctuated by brief interbout periods of arousal (often 12–24 hr) when animals rewarm to 37–38°C. Arousal is powered primarily by non-shivering thermogenesis in brown adipose tissue (Mejsnar and Jansky, '70; Foster, '84) and also, once the animal is partly rewarmed, by skeletal muscle shivering. Although hibernators appear to have abandoned body temperature control to track ambient, thermoregulation reappears if ambient temperature falls below 0°C and the animal is at risk of freezing. In this case, heat generation by brown adipose is activated and under very cold conditions, the thermogenic capacity of the tissue is probably increased, as evidence by elevated levels of mRNA for uncoupling protein (UCP) 1 in brown fat (Boyer et al., 1998).

Despite the strong overall suppression of metabolism in hibernating mammals, brown adipose tissue shows substantial metabolic activity during torpor. A variety of genes are up-regulated in brown adipose during torpor in bats and ground

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squirrels, including several involved in lipid metabolism such as fatty acid binding proteins (*a-fabp* and *h-fabp*) (Hittel and Storey, 2001, 2002a; Eddy and Storey, 2004), the peroxisome proliferator-activated receptor gamma (PPARγ) (Eddy and Storey, 2003; Eddy et al., 2005) and the mitochondria-encoded genes, cytochrome oxidase subunit 1 (*coxI*) and ATPase 6/8 (*atpase 6/8*) (Hittel and Storey, 2002b). Not only are these genes up-regulated, but brown fat does not show the suppression of the transcriptional machinery during torpor (Hittel and Storey, 2002a) that is seen in other organs, including liver (Knight et al., 2000), kidney (Hittel and Storey, 2002a) and brain (Frerichs et al., '98). The interplay between mitochondria and thermogenesis in brown adipose tissue is evidenced by elevated levels of UCPs (Nizielski et al., '95). UCPs are responsible for uncoupling electron transport from oxidative phosphorylation, thereby allowing energy that would otherwise be trapped as ATP to be released as heat. In hibernators, levels of the brown adipose tissue-specific *ucp1* rise during the late summer proliferation of brown fat. Expression levels of *ucp1* and *ucp2* transcripts in brown fat also increase during winter torpor if animals are exposed to ambient temperatures below 0°C (Boyer et al., '98). This indicates that thermogenic capacity can still be modified in the torpid animal when ambient conditions demand.

Recently, we have also identified the PPARγ co-activator, PGC-1α, as being up-regulated in brown adipose tissue during hibernation in both ground squirrels and bats (Eddy and Storey, 2003; Eddy et al., 2005). Although important as a cofactor in the PPARγ-mediated regulation of lipid metabolism, PGC-1α has a key function of its own as a mitochondrial biogenesis factor (Wu et al., '99) and likely plays a role in activating gene transcription of mitochondrial subunits (Hittel and Storey, 2002b). The current study addresses the coordinated up-regulation of mitochondrial genes and nuclear-encoded mitochondrial proteins, which enhance and optimize mitochondrial oxidative capacity of brown fat in the cold. We identified mitochondrial targets that are differentially expressed in brown fat from euthermic vs. hibernating little brown bats, *Myotis lucifugus*. We found higher enzymatic activity of cytochrome oxidase (COX) and higher expression of two more mitochondrially encoded genes, *coxII* and *nad4* (subunit 4 of NAD-ubiquinone oxidoreductase), in brown adipose tissue of hibernating animals as well as higher expression of the muscle isoform of carnitine palmitoyltransferase (mCPT-1), also known as CPT-1β. The CPT-1β isozyme was first found in skeletal muscle, but is also now known to be present in heart, brown adipose tissue and white adipose tissue (Esser et al., '96). By contrast, skeletal muscle, which also plays a role in thermogenesis, did not show changes in *coxII* or *nad4* transcripts or CPT-1β protein levels during torpor. The protein encoded by this gene is embedded in the mitochondrial outer membrane where it acts as the rate-limiting step in the long-chain fatty acid (LCFA) β-oxidation pathway. A partial amino acid sequence for bat *cpt-1β* was also obtained and compared with the sequence from other mammals in order to search for amino acid substitutions that are specific to the little brown bat and that may be advantageous for enzyme function at very low body temperatures.

MATERIALS AND METHODS

Animals

Little brown bats, *M. lucifugus*, of 7–8 g body mass were collected by Dr. D. Thomas on November 30, 1999 from a disaffected slate mine near Sherbrooke, Quebec where they had been hibernating since October (cave air temperature was 5°C). Collection aroused the bats and they remained aroused during transport to the Université de Sherbrooke. Upon arrival, ten bats were maintained under euthermic conditions at 23–24°C ambient temperature; these were kept awake for a total of 48 hr post-collection and were then euthanized by cervical dislocation. Ten others were placed in a cold room at 5°C and allowed to enter torpor; 10–12 hr were required for full torpor to be re-established with a body temperature close to ambient. Animals remained torpid until sampled 36–38 hr later; measured rectal temperatures at sampling were 5–6°C. Tissues from euthermic and hibernating animals were quickly excised, immediately frozen in liquid nitrogen, and then transported to Ottawa where they were stored at −80°C until analysis. Protocols for animal care meet the guidelines of the Canadian Council on Animal Care.

COX activity assay

A sample of 100 mg of frozen brown fat was suspended in 1 mL of homogenization buffer (100 mM Na_2HPO_4, pH 7.0, 0.2% w:v deoxycholate) and the sample was homogenized using 15 strokes in a pre-chilled Dounce homogenizer.

Homogenates were incubated on ice for 30 min and centrifuged at 10,000*g* for 10 min. The supernatant was collected and protein concentration was determined using the Coomassie blue dye-binding method with the BioRad prepared reagent. Reduced horse cytochrome *c* was prepared by mixing 10 μL of 1 M sodium ascorbate (pH 6) with 300 μL of a 40 mg/ml solution of horse heart type III cytochrome *c* (Sigma). The solution was mixed until it turned to a light red color indicating the reduction of cytochrome *c*. Reduced cytochrome *c* was separated from free ascorbate by running the sample through a Sephadex G-50 column. The COX assay was performed in microplates by mixing 0.2 ml of 25 mM Na_2HPO_4, pH 7.0, 0.2% deoxycholate and 6.25 μL of the reduced cytochrome *c*. The assay was started by adding 10 μL of a 1.5 mg/ml tissue extract and absorbance readings at 550 nm were taken at 15 sec intervals until no changes in absorbance were recorded. Activity was calculated from at least five points on the initial linear portion of the OD vs. time relationship and is reported as nmoles cytochrome *c* oxidized per min (Units) per mg of protein (U/mg protein).

Total RNA isolation and quality

Total RNA was isolated from brown fat and skeletal muscle samples using Trizol reagent (Invitrogen), according to manufacturer's instructions, and resuspended in diethylpyrocarbonate-treated water. RNA concentration was determined by absorbance at 260 nm and the ratio of absorbance at 260/280 nm was found to be between 1.8 and 2.0 indicating a sample of acceptable RNA purity.

Northern blotting

Aliquots of 15 μg of total RNA were electrophoresed on 1% denaturing formaldehyde agarose gels containing ethidium bromide (EtBr). The quality of RNA was determined by the relative amounts of 28S and 18S rRNA bands visualized and photographed under UV light. Blots were transferred overnight onto Hybond N nylon membrane in 10 × SSC (1 × SSC being 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Blots were then cross-linked in a UV crosslinker and baked at 80°C prior to probing. Blots were prehybridized in Church's buffer (0.25 M Na_2HPO_4, 0.25 M NaH_2PO_4, pH 7.5, 7% w:v SDS) at 55°C for at least 1 hr and then incubated overnight at 48°C with ^{32}P-labeled probes, prepared from rat cDNA clones purchased from the I.M.A.G.E. Consortium. Blots were washed the following day in 0.1 × SSC, 1% SDS and then exposed to X-ray film (X-OMAT AR, Kodak) and/or phosphor screens (BioRad). Quantification of *coxII* and *nad4* mRNA band densities was performed using Imagequant (Molecular Dynamics).

cDNA synthesis and PCR amplification of cpt-1β

A 30 μg aliquot of total RNA from brown adipose of hibernating *M. lucifugus* was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and following the manufacturer's protocol. Dilutions of the cDNA in water were prepared and were used to amplify *cpt-1β*. Primers used for amplification of *cpt-1β* were designed using the Primer Designer program, version 3.0 (Scientific and Educational Software) based on the consensus sequences of mammalian *cpt-1β*. The forward primer sequence was 5′-GTCGTCATCATGGCAACAGT-3′ and the reverse primer sequence was 5′-TGCCTGCAC GTCTGTATTCT-3′. PCR amplification consisted of the following cycles after an initial 2 min at 94°C: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, repeated 37 times followed by a final elongation step at 72°C for 5 min. After separation on an agarose gel and staining with EtBr, a single bad was visualized under UV light with a size of ~642 bp. This product was then sequenced by Canadian Molecular Research Services (Ottawa, ON). The sequence was confirmed as encoding *cpt-1β* by sequence comparison in BLAST. The sequence was deposited into GenBank and assigned the Accession Number DQ066455.

SDS-PAGE and immunoblotting

Frozen tissue samples were ground into small pieces in a mortar and pestle under liquid N_2. Approximately 100 mg of tissue was placed in 1 ml of homogenization buffer (100 mM MOPS, pH 7.8, 25 mM HEPES, pH 7.8, 25 mM β-glycerophosphate, 5 mM EDTA pH 8.0, 1 mM EGTA and 250 μM $NaVO_4$, pH 7.4) with 1 mM phenylmethylsulfonyl fluoride added immediately before homogenization. After centrifugation, supernatants were removed and soluble protein content was measured using the Coomassie blue dye-binding method and the BioRad prepared reagent. Supernatant samples were then diluted 1:1 in 2 × SDS sample buffer. Aliquots containing 15 μg of total protein were loaded into each lane of 10% gels.

Electrophoresis and transfer to PVDF membranes (Biotrace, PALL Life Sciences) was carried out as described previously (Hittel and Storey, 2002a).

mCPT-1 (CPT-1β) antibodies were a gift from Dr. Victor Zammit (Hannah Research Institute). Antibody dilutions of 1:1,000 v:v in 5% w:v non-fat dried milk prepared in TBST (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% v:v Tween 20) were used for all procedures. Secondary rabbit IgG HRP conjugated antibody was purchased from Cell Signaling. Blots were developed using the Western Lightning™ Chemiluminescense *Plus* (NEN, Perkin Elmer) system according to protocols provided with the system. Blots were briefly exposed to Kodak X-OMAT-AR film and developed according to manufacturer's protocols. X-ray images were quantified using densitometric analysis software (Imagequant, Molecular Imaging). After antibody reactions were complete, blots were restained with Coomassie blue to confirm equal loading.

Data and statistics

Ratios for torpor vs. aroused were calculated as follows. For Northern blotting analysis, the intensities of radiolabeled bands on northern blots were normalized against the intensity of the corresponding EtBr-stained 28S rRNA band in the same lane. For Western blotting analysis, band intensity of immunoreactive material was normalized against corresponding Coomassie stained protein bands in the same lane that did not change between torpor and arousal. Mean normalized band densities ± SEM were then calculated for aroused vs. torpid samples and significant differences between the groups were tested using the Student's *t*-test. The ratio torpor:arousal was plotted; error bars on the final histograms are the sum of SEM values for torpor and arousal trials.

RESULTS

COX activity in M. lucifugus

COX activity was measured in brown fat from euthermic and hibernating *M. lucifugus* by monitoring the oxidation of reduced horse cytochrome *c* at 550 nm; a graph showing typical assay results for extracts from aroused and torpid bats is shown in Figure 1a. Rate data were calculated from the initial linear portion of the relationship. Mean activities were 861 ± 140 U/mg protein in euthermic brown adipose and 3.4-fold higher at 2,931 ± 138 U/mg protein (means ± SEM, $n = 3$ independent trials, $P < 0.01$) in torpid bats (Fig. 1b).

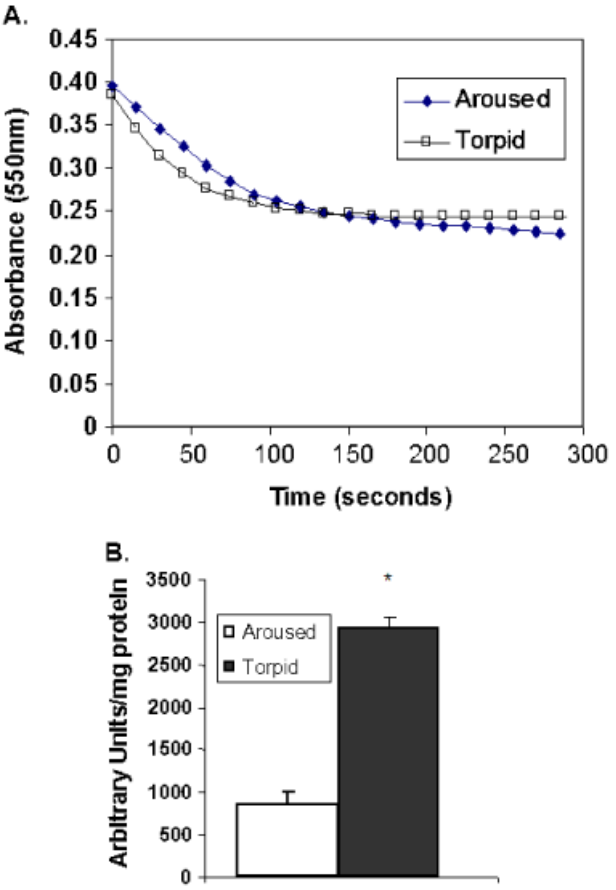

Fig. 1. (**A**) Typical assay of COX activity in extracts from brown adipose tissue of euthermic aroused and torpid *M. lucifugus*. The decrease in absorbance at 550 nm was monitored and the initial linear portion was used to quantify the rate of cytochrome *c* oxidase. (**B**) Mean COX activity (± SEM, $n = 3$ independent trials) in brown adipose of euthermic and torpid bats; the *y*-axis depicts arbitrary units of activity per mg soluble protein. Torpid and aroused values are significantly different, $^{*}P < 0.01$.

Northern blotting for selected mitochondrial genes

Because COX activity was higher in brown adipose tissue isolated from torpid bats, we next sought to identify changes in a mitochondrially encoded COX subunit to determine whether effects of hibernation were seen on multiple levels of COX organization. Figure 2 shows Northern blots detecting mRNA transcript levels of the *coxII* subunit as well as another mitochondria-encoded protein, *nad4*. Transcripts of both genes were significantly higher in brown fat from torpid bats as compared with euthermic, aroused animals.

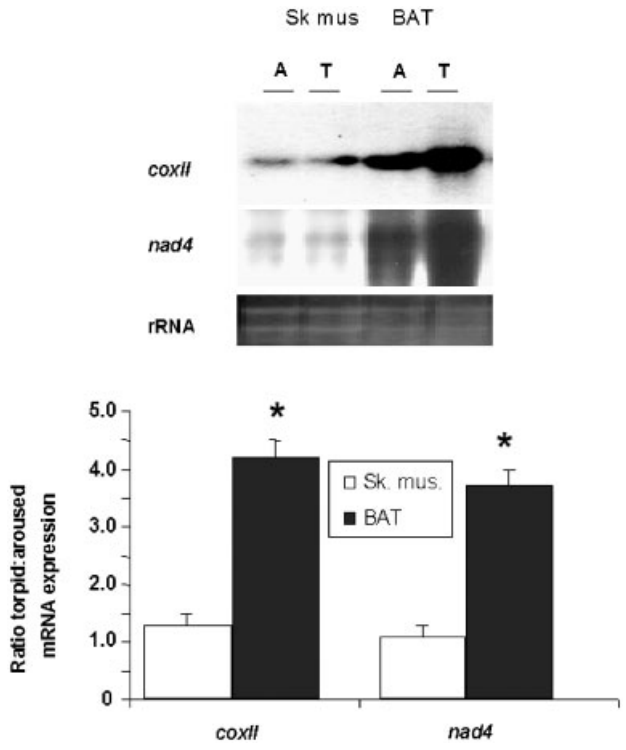

Fig. 2. Representative Northern blots showing mRNA transcript levels of mitochondrial-encoded *coxII* and *nad4* in skeletal muscle (Sk Mus) and brown adipose tissue (BAT) from euthermic aroused (A) and torpid (T) *M. lucifugus*. Corresponding EtBr-stained rRNA bands are also shown. Histograms show the ratio of mRNA transcript levels in torpor vs. arousal, means $\pm$SEM, $n = 3$ independent trials with mRNA isolated from different individuals. Torpid and aroused values are significantly different, $^{*}P < 0.05$.

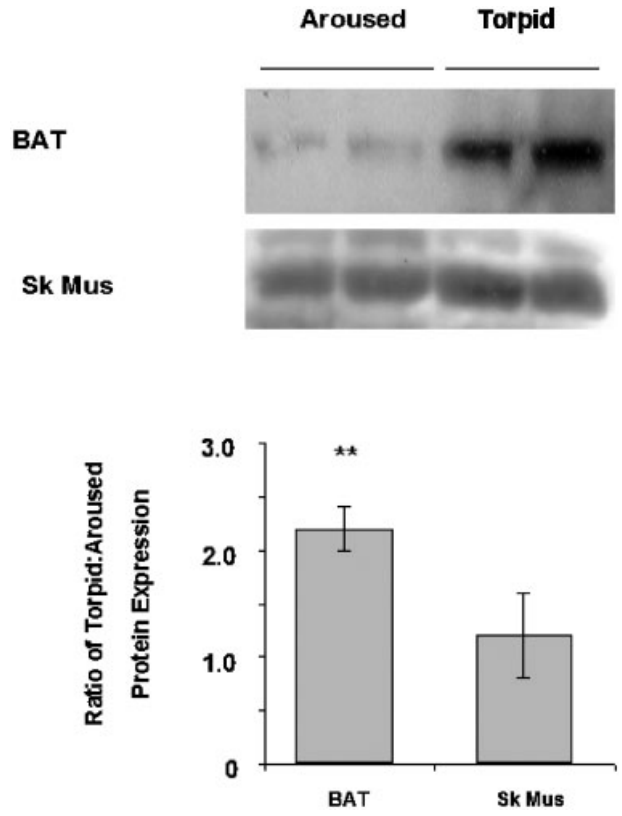

Fig. 3. Western blots showing CPT-1β protein content in brown adipose tissue (BAT) and skeletal muscle (Sk Mus) from aroused and torpid *M. lucifugus*. Aliquots of 15 μg of total protein were run in each lane. One immunoreactive band was detected at ~90 kDa, consistent with the known molecular weight of mammalian CPT-1β. Data are means $\pm$SEM, $n = 4$ independent trials. Torpid and aroused values are significantly different, $^{**}P < 0.01$.

Expression of *coxII* and *nad4* was normalized against 28S rRNA bands and levels were determined to be 4.2 ± 0.3 and 3.7 ± 0.4-fold higher in torpid bats ($P < 0.05$), respectively, compared with aroused animals. As a comparison, the same genes were probed in total RNA isolated from skeletal muscle, another thermogenic tissue in hibernators that displays high levels of mitochondria. Transcript levels of *coxII* and *nad4* showed no significant increase in muscle during torpor; the expression ratios torpor:euthermic were 1.3 ± 0.3 and 1.1 ± 0.4, respectively.

CPT-1β protein is up-regulated in hibernating brown adipose tissue

To further explore the role of mitochondria in hibernation, we assessed changes in CPT-1β protein levels. This transporter in the outer mitochondrial membrane gates the transfer of LCFAs into the organelle. Since most hibernator organs rely on the oxidation of LCFAs for ATP production during torpor (except brain) and since LCFAs are also the primary fuels of thermogenic organs, CPT-1β is critically important to hibernator energy metabolism. Protein extracts from brown adipose tissue and skeletal muscle were electrophoresed on 10% SDS-PAGE gels and immunoblotting showed only one strongly cross-reacting band at ~90 kDa, the correct subunit size for mammalian CPT-1β. The results showed a strong increase in CPT-1β protein content in brown fat during hibernation, a 2.1 ± 0.5-fold increase ($P < 0.001$) as compared with levels in euthermic brown fat. However, expression of CPT-1β in skeletal muscle was unaltered between euthermic and torpid states. Expression of CPT-1β was determined by normalization to blots stained with Coomassie Blue (data not shown) (Fig. 3).

cpt-1β gene and protein sequence

Transcript levels of *cpt-1β* were quantified in brown adipose tissue from euthermic and hibernating *M. lucifugus* using RT-PCR. Primers were designed from a consensus sequence for mammalian *cpt-1β* and expression was normalized against the expression of α-*tubulin* mRNA, a constitutively expressed gene that was amplified from the same samples. The results showed no significant difference in *cpt-1β* transcript levels between the euthermic and hibernating states (data not shown). RT-PCR generated a 624 bp product, which was sequenced. Figure 4 shows the nucleotide sequence along with its deduced amino acid sequence (Genbank accession number DQ066455). Nucleotide and amino acid comparisons with the sequences from other mammalian species confirmed that the PCR product encoded *cpt-1*β. The amplified segment represents 27% of the total CPT-1β protein. The 208 amino acids encoded in this segment are located in the N-terminal region of CPT-1β, which has a full length of 772 amino acid residues. Alignment of the partial bat CPT-1β amino acid sequence with the corresponding regions of CPT-1β from human, mouse and rat CPT-1β is shown in Figure 5. Over the amplified region, bat CPT-1β shared 86%, 84% and 81% identity with human, mouse and rat CPT-1β, respectively. CPT-1β from *M. lucifugus* contained several amino acid substitutions that were absent in the other mammalian CPT-1β sequences; these are shown in bold underlined in Figure 5. These included the substitution of a tyrosine residue for a cysteine residue at position 86 (position numbers are based on the human sequence) and substitutions of lysine and histidine residues for arginine and cysteine residues at positions 89 and 90, respectively. A valine residue was substituted for threonine residues at position 119. A glutamine

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GTCGTCATCATGGCAACAGTGGGTTCTTCCTACTGCAATGTGGACATCTCCAATGGGCTG
 V  V  I  M  A  T  V  G  S  S  Y  C  N  V  D  I  S  N  G  L

GTCTATTATATCCAGAAACACCTCCCGGAGAGGTATGTCCCCTACCAGACCCCACAGACA
 V  Y  Y  I  Q  K  H  L  P  E  R  Y  V  P  Y  Q  T  P  Q  T

CGGGCACTGGTCAGCATGGCCATCTTCTCCACGGGGGTTTGGATCGTGGGCATCTTCTTC
 R  A  L  V  S  M  A  I  F  S  T  G  V  W  I  V  G  I  F  F

TTCCGCCAAACCCTGAAGCTGCTGCTCTCCTACCATGGGTGGATGTTTGAGATGCATGGC
 F  R  Q  T  L  K  L  L  L  S  Y  H  G  W  M  F  E  M  H  G

CAGACCAGCCGCAGCACCAAAGTCTGGGCGGCCTGTGTCCGCCTTCTGTCCAGTGGGCGG
 Q  T  S  R  S  T  K  V  W  A  A  C  V  R  L  L  S  S  G  R

CCCATGCTGTACAGCTTTCAGACGTCCCTGCCCAAGCTTCCCGTGCCCAGCGTGCAAGCC
 P  M  L  Y  S  F  Q  T  S  L  P  K  L  P  V  P  S  V  Q  A

ACAATTCAACGGTACCTGGAGTCTGTGCGGCCCTTGTTGGATGACAAGAAGTATCAGCGC
 T  I  Q  R  Y  L  E  S  V  R  P  L  L  D  D  K  K  Y  Q  R

ATGGAGATACTAGCCAAGGAATTCCAGGACAAGACTGCCCCCAGGCTACAGAAGTACTTG
 M  E  I  L  A  K  E  F  Q  D  K  T  A  P  R  L  Q  K  Y  L

GTACTCAAGTCGTGGTGGGCAACTAACTATGTGAGTGACTGGTGGGAAGAATACATCTAC
 V  L  K  S  W  W  A  T  N  Y  V  S  D  W  W  E  E  Y  I  Y

CTTCGAGGCAGGAATCCCCTCATGGTGAACAGCAACTACTATGCGATGGACTTTGTGCTC
 L  R  G  R  N  P  L  M  V  N  S  N  Y  Y  A  M  D  F  V  L

ATCAAGAATACAGACGTGCAGGCA
 I  K  N  T  D  V  Q  A
```

Fig. 4. Nucleic acid sequence and deduced amino acid sequence of *M. lucifugus cpt-1β*. The sequence was deposited into Genbank and assigned the Accession number DQ066455.

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bat       ....................................      0
human     maeahqavafqftvtpdgvdfrlsrealkhvylsginswk     40
mouse     maeahqavafqftvtpdgvdfrlsrealrhiylsginswk     40
rat       maeahqavafqftvtpdgvdfrlsrealrhiylsginswk     40

bat       ....................VVIMATVGSSYCNVDIS     17
human     krlirikngilrgvypgsptswl----------f------     80
mouse     krlirikngilrgvypgsptswl--v------n--k----     80
rat       krlirikngilrgvypgsptswl--v------n--k----     80

bat       NGLVYYIQKHLPERYVPYQTPQTRALVSMAIFSTGVWIVG     57
human     l---sc--rc--qgcg----------l----------vt-    120
mouse     m---dc--rc-----ghfg----e--l--v-------at-    120
rat       m---hc--rc--t--gs-g----et-l--v-------at-    120

bat       IFFFRQTLKLLLSYHGWMFEMHGQTSRSTKVWAACVRLLS     97
human     ----------c----------k--nl-ri--m-i----    160
mouse     --------------------sk--ha--i--i------    160
rat       --l------------------sk--ha--i--i------    160

bat       SGRPMLYSFQTSLPKLPVPSVQATIQRYLESVRPLLDDKK    137
human     -rh---------------r-s----------------ee    200
mouse     -r------------------p---h---d--------ea    200
rat       -r------------------p---h---d--------ea    200

bat       YQRMEILAKEFQDKTAPRLQKYLVLKSWWATNYVSDWWEE    177
human     -y---l----------------------s---------    240
mouse     -y---t---------------------------------    240
rat       -f---s--------i------------------------    240

bat       YIYLRGRNPLMVNSNYYAMDFVLIKNTDVQA.........    208
human     -------s---------v--l----------arlgniiha    280
mouse     -v---s-s-------------------n---arlgnavha    280
rat       -v-----s-i--d--------------sq--arlgntvha    280
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Fig. 5. Multiple alignment showing the amino acid sequence of CPT-1β from little brown bat (*M. lucifugus*) aligned with the sequences for human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*) CPT-1β. GenBank accession numbers are DQ066455, NP_004368, NP_034078 and NP_037332, respectively. Dashes in the alignment represent amino acids that are identical with the little brown bat sequence; spacer dots are inserted when residues are not present in all sequences. Notable amino acid substitutions in the bat sequence are shown in bold and underlined.

substitution for a lysine residue occurred at position 144 and an arginine substitution for asparagine or histidine was seen at position 147. Interestingly, two adjacent lysine substitutions were found at positions 199 and 200, whereas in the human sequence, two glutamic acid residues occurred and the mouse and rat sequences have glutamate and alanine residues at the positions in question. Lastly, an asparagine substitution for a serine residue occurred at position 248.

DISCUSSION

Brown fat was first described over four centuries ago as a gland in hibernating mammals (Gressner, 1551), but the realization that it is found in all mammals and its importance in heat production is a relatively new finding (Smith, '61). Brown adipose tissue is extremely interesting from the standpoint of hibernation physiology and biochemistry as its primary function is non-shivering thermogenesis, fueled by the oxidation of extensive LCFA stores and the tremendously high content of mitochondria in the tissue (Hayward, '68; Mejsnar and Jansky, '70). Brown adipose fuels the initial rewarming phase of hibernation, allowing heart rate and breathing to increase quickly. Shivering thermogenesis by skeletal muscle also contributes to arousal and Postnikova et al. ('99) reported a 3-fold higher myoglobin content in skeletal muscle

of winter vs. summer ground squirrels that suggests an improved capacity for oxygen-based fuel catabolism by muscle during hibernation. Studies with other animal models of hibernation have frequently noted the up-regulation of mitochondrial genes when animals are exposed to a variety of stresses including cold temperatures (Martin et al., '93), suggesting a conserved mechanism of regulation.

The COX complex plays a crucial role in the electron transport chain as it catalyzes the final reduction of dioxygen to water (Gnaiger et al., '98). Given its location in this important cellular process and its known involvement in responses to ischemia/reperfusion injuries (Van Itallie et al., '93), COX activity was investigated in this study. The results showed a significant 3.4-fold increase in COX activity in brown adipose tissue from hibernating vs. euthermic bats. During thermogenesis by brown fat mitochondria, the electron transport chain is solicited to run at high rates but oxidative phosphorylation is uncoupled so that energy is released as heat. The electron transport chain in brown fat of hibernators is evidently important and this can explain the considerably higher COX activity found in the hibernating state.

To further investigate COX in hibernators, we focussed on gene regulation of one of the mitochondria-encoded subunits, *coxII*. The mitochondrial genome of vertebrates is transcribed as a polycistronic precursor RNA and is further processed into mRNA, tRNA and rRNA transcripts (Clayton, '91); several subunits of COX and of NADH-ubiquinone oxidoreductase are among the genes encoded. Previous studies from our lab identified major increases in the transcript levels of selected mitochondria-encoded genes during hibernation in ground squirrels including *nad2* in heart, liver and skeletal muscle of *Spermophilus lateralis* (Fahlman et al., 2000) and *cox1* and *atpase6/8* in brown fat, heart and kidney of *S. tridecemlineatus* (Hittel and Storey, 2002b). Consistent with the up-regulation of mitochondrial transcripts during hibernation, we also identified increased expression of PGC-1α, a major determinant in mitochondrial biogenesis (Wu et al., '99), in brown fat from hibernating *M. lucifugus* and *S. tridecemlineatus* (Eddy and Storey, 2003; Eddy et al., 2005). The present study shows that transcripts of *coxII* and *nad4* are both up-regulated in brown adipose of bats during the torpor phase of hibernation, consistent with the deduced role of PGC-1α in mitochondrial biogenesis and gene expression. The up-regulation of *coxII* during hibernation further suggests that the hibernation responsive increase in COX activity may be at least partially due to new protein synthesis when the animals enter torpor. Somewhat surprisingly, skeletal muscle, which also displays elevated PGC-1α expression during torpor in *M. lucifugus* (Eddy and Storey, 2003) showed no change in *coxII* and *nad4* transcript levels between aroused and torpid animals. This may be due to a relatively inactive transcriptional and translational machinery in muscle during torpor as opposed to brown fat, which remains metabolically active under the same conditions (Hittel and Storey, 2002a). Future studies on mitochondrial transcription and translation in skeletal muscle during the arousal and shivering thermogenesis stages of hibernation will provide further insight into this mechanism.

We further chose to link mitochondrial expression with fat metabolism during hibernation by investigating other areas of mitochondrial function. During hibernation, animals suppress carbohydrate catabolism via a number of mechanisms, including the reversible phosphorylation of key enzymes such as glycogen phosphorylase, phosphofructokinase, pyruvate kinase and pyruvate dehydrogenase (PDH) (Storey, '87, '97). The suppression of PDH activity is due to phosphorylation by PDH kinase 4, which is strongly up-regulated at gene level during hibernation (Buck et al., 2002). With the suppression of carbohydrate catabolism, animals switch to a lipid-based metabolism during torpor. There is an increase in lipolysis in white adipose tissue (Moreau-Hamsany et al., '88), increased expression of lipoprotein lipase and hormone sensitive lipase during the winter months (Wilson et al., '92), and elevated levels of FABPs in multiple organs to enhance intracellular lipid transport (Hittel and Storey, 2001; Eddy and Storey, 2003, 2004). CPT-1β is the protein responsible for transport of LCFAs into the mitochondria and is the rate-limiting step in fatty acid oxidation. The elevated CPT-1β protein content in *M. lucifugus* brown fat in the torpid versus aroused state is consistent with the demand for high rates of lipid oxidation in brown adipose tissue to support thermogenesis, both for low-level heating during torpor and high-level heating during arousal. Increased levels of H-FABP and A-FABP (Eddy and Storey, 2004) as well as CPT-1β in bat brown fat correlate with this need for elevated thermogenic capacity during both torpor and arousal. The lack of up-regulation

of CPT-1β protein in skeletal muscle is consistent with the results for *coxII* and *nad4* and suggests that skeletal muscle oxidative capacity is not modified over torpor-arousal cycles but is probably optimized before the hibernating season begins.

The increase in CPT-1β content in bat brown adipose tissue may be regulated by the protein kinase, Akt. Levels of the active form of Akt rose in brown adipose tissue during hibernation (Eddy and Storey, 2003) and Akt has been linked to the regulation of lipid metabolism in other mammals (Sul et al., 2000). It may appear that higher levels of CPT-1β in torpor could cause an increased utilization of fats, but this is not the case. Rather it increases the capacity for fatty acid oxidation under optimal conditions, and coupled with the up-regulation of mitochondrial transcripts and COX activity in brown fat during torpor, this suggests an emphasis on the mitochondria in preparation for arousal. However, because metabolic rate is strongly suppressed during hibernation (Storey, 2003; Storey and Storey, 2004) and because of the Q_{10} effect, which over a 30°C change in body temperature range should suppress LCFA transport rates by 8–10-fold, it is not likely that substantial fat loss occurs in torpor. The need for enhanced CPT-1β protein and mitochondrial proteins is more likely to serve the high rates of oxidation of fats during interbout arousals.

Sequence analysis of the deduced N-terminal polypeptide sequence of CPT-1β revealed a number of interesting amino acid substitutions present in the *M. lucifugus* protein. While the N-terminal region does not contain the catalytic domain, recent studies of both CPT-1α and CPT-1β have shown that this region is largely responsible for binding affinities to malonyl-CoA (Shi et al., '98, '99, 2000; Price et al., 2003). The immediate N-terminus, specifically, glutamate-3, valine-19, leucine-23 and serine-24, are essential for malonyl-CoA binding, but we speculate that the amino acid substitutions in other regions of the N-terminal region of *M. lucifugus* CPT-1β may act to alter protein conformation and thereby influence the binding of LCFAs at the low body temperatures during torpor. Selected amino acid substitutions have also been identified in the sequence of H-FABP, another lipid-binding transport protein, from both bats and ground squirrels (Hittel and Storey, 2001; Eddy and Storey, 2004). Both proteins showed lysine substitutions at key sites, K72 in bat H-FABP and K69 in the ground squirrel protein, and recent studies of rat FABP using site-directed mutagenesis have shown that lysine substitution at selected sites can increase the ability of the protein to take up fatty acids from the plasma membrane (Herr et al., '96). It would be interesting to determine whether one or all of the three lysine substitutions in bat CPT-1β similarly enhance the ability of CPT-1β to bind fatty acids, especially at low temperature. Further kinetic analysis of *M. lucifugus* CPT-1β via the construction of chimeric proteins containing the N-terminal region of *M. lucifugus* CPT-1β will be required to analyze this possibility. Additionally, sequence analysis of CPT-1β from other hibernating species may provide insight into other residues that are critical for low-temperature function.

We propose that an anticipatory mechanism occurs that enhances and coordinates the expression and oxidative and thermogenic capacity of mitochondria of brown fat in the cold in expectation of the need for high-level thermogenesis during arousal. This adaptive response is likely triggered and controlled by increased PGC-1α expression, which may also enhance mitochondrial numbers during hibernation. Interestingly, this mechanism that appears to be conserved in *M. lucifugus* (Eddy and Storey, 2003) and *S. tridecemlineatus* (Eddy et al., 2005) and would also explain the increased expression of mitochondrial subunits and UCPs seen in a variety of ground squirrel species during hibernation (Nizielski et al., '95; Boyer et al., '98; Fahlman et al., 2000; Hittel and Storey, 2002b). There has long been debate within the field as to whether hibernation has arisen independently numerous times throughout evolution or whether it is an ancestral ability that has subsequently been lost in most mammalian species. Broad analyses of the occurrence of mammalian heterothermy and of the instances of reptilian endothermy now favor the latter interpretation (Grigg et al., 2004). Our present results for *M. lucifugus* support this. The up-regulation of mitochondrial genes seen here, along with our previous studies on bats that identified A- and H-FABPs, PGC-1α, PPARα and PPARγ as up-regulated proteins during torpor, along with an opposite strong suppression of Akt (Eddy and Storey, 2003, 2004) exactly parallel the responses by ground squirrel organs during torpor (up-regulation of mitochondrial genes, A- and H-FABPs, PGC-1α, PPARα and suppression of Akt) (Geiser, '88; Hittel and Storey, 2001, 2002a; Cai et al., 2004; Hoehn et al., 2004; Eddy et al., 2005; Fleck and Carey, 2005). These strongly conserved molecular mechanisms for hibernation

support the viewpoint that torpor and hibernation are ancient cellular capacities that have been retained or lost in different mammalian lineages, depending on environmental pressures.

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