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Cloning and expression of hypoxia-inducible factor 1α from the hibernating ground squirrel, *Spermophilus tridecemlineatus*

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Abstract

Mammalian hibernation is associated with apnoic breathing patterns and a hypoxia–hypothermia connection has been suggested as part of the mechanism by which body temperature is reduced as animals enter torpor. Hence, we hypothesized that changes in the expression of the hypoxia inducible factor (HIF-1) may potentially be involved in regulating hibernation-responsive gene targets. The expression of the alpha subunit of HIF-1 was quantified at both gene and protein levels in four organs of the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*. Reverse transcription-PCR showed no change in *hif-1α* transcript levels in the liver, lung, skeletal muscle or brown adipose tissue of euthermic versus hibernating animals but HIF-1α protein levels were elevated by 60–70% in the two organs responsive for thermogenesis (brown adipose and skeletal muscle). Furthermore, assessment of DNA binding by HIF-1 in nuclear extracts from brown adipose revealed 6-fold higher levels in hibernator tissue than in euthermic controls suggesting increased expression of HIF-1 responsive genes during hibernation. The complete nucleotide sequence of *hif-1α* from ground squirrels, the first *hif-1α* sequence amplified from a hibernating mammal, was obtained using PCR amplification and 3′ and 5′ RACE. Amino acid sequence analysis revealed 90–95% identity with the HIF-1α protein from other mammals. Several unique amino acid sequence substitutions were identified that may affect protein conformation and could possibly function to counteract low temperature effects on HIF-1α conformation at near 0 °C body temperatures during torpor.

Keywords: Mammalian hibernation; Hypoxia-inducible factor 1; 13-Lined ground squirrel; Brown adipose tissue; Muscle; Thermogenesis

1. Introduction

To survive winter, many small mammals enter hibernation. In this state, metabolic rate is profoundly depressed, frequently to only 1–5% of the normal euthermic rate, and body temperature decreases to near ambient (often falling to 0–5 °C). The winter hibernation season consists of long periods of deep torpor (lasting days to weeks) interspersed by brief interbout periods (often 12–24 h) when the animals rewarm to 37–38 °C. Arousal relies on the heat generated by nonshivering thermogenesis in brown adipose tissue and shivering in skeletal muscle. By hibernating, animals can often save up to 90% of the energy that they would otherwise need to remain euthermic over the winter months [1]. Transitions to and from the torpid state are closely regulated by strong reversible controls (e.g. protein phosphorylation or dephosphorylation) on the rates of multiple energy-expensive metabolic processes as well as by the enhanced expression of selected genes whose protein products address specific needs of the animal in the hypometabolic, hypothermic state of torpor.

While hibernating both heartbeat and breathing rate are profoundly depressed and organ perfusion rates can drop to ~10% of normal [2], a level that would be considered severely ischemic under normal circumstances. Furthermore, many hibernating species breath irregularly while in torpor and show long periods of apnea so that tissues would experience a wide range of oxygen levels, potentially even

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hypoxic values. An ancient link between hypoxia and hypothermia occurs in mammals—core temperature falls when oxygen is limiting and hibernating species show a more pronounced drop in body temperature under hypoxia than do nonhibernators [3]. Indeed, this hypoxia–hypothermia connection is one of the mechanisms that has been proposed to help initiate and manage the fall in body temperature that occurs during entry into hibernation [3,4].

This potential link led us to wonder about the regulation of gene expression during entry into hibernation and the role that might be played by the hypoxia-inducible factor 1 (HIF-1), a transcription factor that responds to low oxygen. HIF-1 is known to up-regulate the transcription of a variety of genes that enhance hypoxia tolerance including those that improve the delivery of oxygen to tissues (e.g. vascular endothelial growth factor, and erythropoietin) and those that enhance ATP generation by anaerobic glycolysis (e.g. several glycolytic enzymes and glucose transporter isoform 1) [5–7]. HIF-1 is composed of α and β subunits and levels of the HIF-1α subunit are the limiting factor in net HIF-1 activity. When oxygen levels are high, HIF-1α is susceptible to the oxygen-dependent hydroxylation of two proline residues via the enzymatic activity of a prolyl-4-hydroxylase. Modification of these residues in the oxygen-dependent degradation domain targets the protein for rapid ubiquitinylation and degradation [8–10]. Under low oxygen conditions, however, the rate of proline hydroxylation is reduced and HIF-1α is stabilized and can move to the nucleus to form the HIF-1 heterodimer and stimulate transcription of genes.

The goal of this study was to investigate the potential relationship between HIF-1-mediated gene regulation and hibernation in tissues of thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. We proposed that the physiological conditions of hibernation may elevate HIF-1α transcript and/or protein levels in certain organs, leading to increased HIF-1 DNA binding activity to the hypoxia response elements (HRE) to increase the expression of selected HIF-1-regulated genes. HIF-1α mRNA and protein levels were quantified in four organs and HIF-1 DNA binding activity was assessed in brown adipose tissue. The full length HIF-1α sequence from ground squirrels, the first HIF-1α sequenced from a hibernating species, was retrieved and probed for key amino acid differences that could aid the function of the transcription factor under the low body temperature conditions of the hibernating state.

2. Materials and methods

2.1. Animals

Thirteen lined ground squirrels, *S. tridecemlineatus* (130–180 g), were captured by a licensed trapper (TLS Research, Michigan) and transported to the Animal Hibernation Facility (NIH, Bethesda, MD). Hibernation experiments were conducted by the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke) essentially as in [11]. Animals were kept on a fall day/night light cycle in shoebox cages maintained at 21 °C and fed ad libitum until they entered and finished the pre-hibernation phase of hyperphagia that maximizes lipid stores. A sensor chip was introduced under the squirrel skin and the body temperature of each animal was monitored electronically. When squirrels had reached a plateau weight gain of 220–240 g, they were randomly divided into two groups. One group (euthermic controls) was maintained under the same conditions as previously and body temperatures of the animals were confirmed as being 36–38 °C at the time of tissue sampling. The other group was placed in a dark chamber at 5–6 °C (food was withdrawn) to induce hibernation. Individuals settled into hibernation after different lengths of time but all were sampled on the same day after each individual had been hibernating for 2–5 days (as indicated by continuous body temperature readings of ~6 °C). Euthermic control animals were sampled on the same day. All animals were sacrificed by decapitation and tissues were excised, frozen immediately in liquid nitrogen and then transported to Ottawa on dry ice where they were then placed at −80 °C until use.

2.2. Total RNA isolation and quality

Total RNA was isolated from tissue samples using Trizol reagent (Gibco BRL), according to manufacturer's instructions, and resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA concentration was determined by absorbance at 260 nm and the ratio of absorbance at 260/280 nm was used as an indicator of RNA purity. RNA quality was also assessed by running samples on a 1.2% agarose denaturing gel and staining with ethidium bromide to reveal two sharp bands in every sample.

2.3. cDNA synthesis and PCR amplification of HIF-1α

A 30 μg aliquot of total RNA from brown adipose tissue of hibernating *S. tridecemlineatus* was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and following the manufacturer's protocol. Serial dilutions of the cDNA in water were prepared (10^{-1}–10^{-4}) and were used to amplify both *hif-1α* and α-tubulin. The primers used for amplification of *hif-1α* were designed using the Primer Designer program, version 3.0 (Scientific and Educational Software) based on the consensus sequences of mammalian *hif-1α*. The forward primer sequence was 5′-TGCTCATCAGTTGCCACTTC-3′ and the reverse primer sequence was 5′-GTACTGTC-CTGTGGTGACTT-3′. For a control gene, α-tubulin was amplified with forward (5′-AAGGAAGATGCTGCCAA-TAA-3′) and reverse (5′-GGTCACATTTCACCATCTG-3′) primers. The PCR reaction was performed by mixing 5 μL of each cDNA dilution with 1.25 μL of primer mixture

(0.5 μM forward and 0.5 μM reverse), 15 μL of sterile water, 2.5 μL of 10× PCR buffer (Invitrogen), 1.25 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs and 0.125 μL of Taq Polymerase (Invitrogen) for a total volume of 25 μL. The cycles performed for amplification consisted of an initial step of 2 min at 94 °C, followed by 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min repeated 37 times; the final step was at 72 °C for 2 min. PCR products were separated on a 1.0% agarose gel. The gel was prepared by adding 3 g of agarose to 300 mL of 1× TAE buffer prepared by mixing 6 mL of 50× TAE buffer (242 g Tris base, 57.1 mL concentrated acetic acid, 100 mL of 0.5 M EDTA in 1 L water, adjusted to pH 8.5) with 294 mL of DEPC-treated water. Ethidium bromide (0.3 mg/300 mL) was added in the solution and the mixture was heated. The heated solution was then poured in a gel tray and the gel was allowed to cool down and solidify. A 10 μL aliquot of the PCR product was mixed with 2 μL of 6× blue/orange loading dye (Promega, USA) and the solution was loaded on the 1% agarose gel. The gel was run in 1× TAE buffer. After separation, the bands were visualized with ethidium bromide on a UV box. The bands from the most dilute cDNA sample were used for quantification purposes to make sure that the products had not reached amplification saturation. A *hif-1α* fragment of ~800 bp was retrieved and sequenced by Canadian Molecular Research Services (Ottawa, ON). The sequence was confirmed as encoding HIF-1α by sequence comparison in BLAST.

The 3′ end of *hif-1α* was amplified using the BD Biosciences SMART RACE kit (Clontech, USA). The cDNA was amplified using a forward primer with the sequence 5′-CTTGTGAGGAAACTTCTGGATGC-TGGTGA-3′. The protocol used was as supplied by the manufacturer. The annealing temperature was 65 °C and a product of ~2000 bp was obtained and sequenced. The 5′ end was amplified using the same kit. The primer used was 5′-GCAAGCATCCTATACTGTCCTGTGGTGAC-3′. Annealing temperature was 63 °C and a PCR product of ~900 bp was obtained and sequenced. Sequences were assembled and the complete transcript of *S. tridecemlineatus hif-1α* was obtained and then submitted to Genbank.

2.4. Western blotting

Frozen tissue samples (~500 mg) were homogenized in 2 mL of buffer containing 100 mM MOPS, 25 mM HEPES, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM EGTA and 250 μM $NaVO_4$, adjusted at pH 7.4, with 1 mM phenylmethylsulfonyl fluoride (PMSF) added immediately before homogenization. After centrifugation at 10,000 × *g* for 10 min at 4 °C, supernatants were collected and soluble protein concentrations were determined using the Coomassie blue dye-binding method and the BioRad prepared reagent (BioRad, Hercules, CA). SDS-polyacrylamide gel electrophoresis and blotting to polyvinylidene difluoride membranes was carried out essentially as in [12] with 10% gels (5% stacking gel), 20 μg of protein per well, and electrophoresis at 200 V for 45 min. Wet transfer of proteins onto membranes was made using a transfer buffer solution containing 25 mM Tris (pH 8.5), 192 mM glycine and 10% v/v methanol at 4 °C for 1.5 h at 300 mA. Following transfer, the PVDF membrane was blocked for 1 h in TBST (50 mM Tris–HCl pH 6.8, 150 mM NaCl, 0.05% v/v Tween 20) with 2.5% w/v powdered skim milk. This was decanted and then membranes were incubated overnight at 4 °C with primary antibody (polyclonal chicken anti-HIF-1α, kindly provided by Dr. M. Gassmann, Physiologisches Institut, Universitat Zuerich-Irchel, Switzerland) at a 1:500 v/v dilution in 5 mL of TBST. Subsequently, the membrane was incubated with HRP-linked anti-chicken IgG secondary antibody (1:2000 v/v dilution) in TBST for 1 h and then blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's protocol. Bands were visualized using a Syngene (BioRad, Hercules, CA) and band intensities were quantified using the Gene Tools program. Preliminary trials used two-dimensional electrophoresis to confirm that the chicken antibody cross-reacted with only a single protein in hibernator tissues, with the appropriate molecular mass and isoelectric point for HIF-1α.

2.5. DNA binding assay

Nuclear extracts were prepared from brown adipose tissue. Briefly, tissue samples were disrupted using a Dounce homogenizer in a homogenization buffer (10 mM HEPES, 10 mM KCl, 10 mM EDTA, 1 mM DTT, pH 7.9) with 1 mM PMSF added prior to homogenization. Samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants were discarded and pellets were resuspended in an extraction buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 50% v/v glycerol, 1 mM DTT, pH 7.9). Tubes containing samples were put on ice horizontally on a rocking platform for 1 h. Samples were then centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants containing nuclear extracts were collected and protein concentration was measured with the BioRad assay. Aliquots containing an equal amount of protein from each sample were then used to assess the amount of binding by HIF-1 to its response element using a DNA binding assay from ActiveMotif. This is an ELISA-type assay that uses 96-well microplates that have been coated previously with the oligonucleotide corresponding to the hypoxia response element (HRE) that is recognized by HIF-1. Binding assays were performed according to the manufacturer's protocol. HIF-1 binding was carried out for 1 h at 21 °C with mild agitation on a rocking platform at 100 rpm. The plates were then washed three times with phosphate buffer (10 mM phosphate buffer, pH 7.5, 50 mM NaCl, 0.1% Tween-20) to remove unbound transcription factors. HIF-1α specific primary antibody diluted 1:1000 v/v in a washing buffer was then added to each well and incubated for 1 h at 21 °C without agitation. The wells were then washed three times

with washing buffer and the secondary antibody HRP-linked anti-mouse IgG was added to the wells in a 1:1000 v/v dilution in washing buffer for 1 h at 21 °C. The wells were then washed four times with washing buffer and the manufacturer's developing solution was then added. After developing for 10 min in the dark, the stop solution was added and color development was quantified by absorbance readings at 460 nm.

2.6. Quantification and statistics

RT-PCR and Western bands were scanned and densitometric analysis was performed using GeneTools (SynGene). RT-PCR bands for *hif-1α* mRNA were normalized relative to RT-PCR bands of α-tubulin run from the same cDNA reaction. The band intensities of immunoreactive material on Western blots were first normalized against three Coomassie stained protein bands that did not appear to change between the euthermic and hibernating states. Mean normalized band densities ± S.E. were then calculated for samples from hibernating versus euthermic animals and significant differences between the groups were tested using the Student's *t*-test. The ratio hibernating:euthermic was calculated and plotted; error bars on the final histograms are the sum of S.E. values for hibernating and euthermic trials.

```
squirrel    MEGAGGTNDKKKI.SSERRKEKSRDAARSRRSKESEVFYELAHQLPLPHXVSSHLDKASVMRLTISYLLVRKLLDAGDLD 79
human       ------a------.-----------------------------------n------------------r----------- 79
mouse       ------e-e---.m----------------------------------n------------------r--------g-- 79
rat         ------e-e--nrm----------------------------------n------------------r-----g----- 80

squirrel    IEDEMKAQMNCFYLKALDGFVMVLTDDGDMIYISDNVNKYMGLTQFELTGHSVFDFTHPCDHEEMREMLTHRNGPVKKGK 159
human       ---d-------------------------------------------------------------------l----- 159
mouse       s--------d--------------------v-----------------------------------------r--- 159
rat         ------------------------------------------------------------------------r--- 160

squirrel    EQNTQRSFFLRMKCTLTSRGRTMNIKSATWKVLHCTGHIHVYDTNSNQSQCGYKKPPMTCLVLICEPIPHPSNIEIPLDS 239
human       ------------------------------------------------p------------------------------ 239
mouse       -l----------------------------------------------p------------------------------ 239
rat         ---------------------------------------------s---p------------------------------ 240

squirrel    KTFLSRHSLDMKFSYCDERITELMGYEPEELLGRSIYEYYHALDSDHLTKTHHDMFTKGQVTTGQYRMLAKRGGYVWVET 319
human       ------------------------------------------------------------------------------- 319
mouse       ------------------------------------------------------------------------------- 319
rat         ------------------------------------------------------------------------------- 320

squirrel    QATVIYNTKNSQPQCIVCVNYVVSGIIQHGLIFSLQQTECVLKPVESSDMKMTQLFTKVESEDTSSLFDKLKKEPDALTL 399
human       ----------------------------d-------------------------------------------------- 399
mouse       ----------------------------d---------s------------------------c--------------- 399
rat         ----------------------------d---------s------------------------c--------------- 400

squirrel    LAPAAGDTIISLDFGSNDTETEDQQLEEVPLYNDVMFPSSSEKLQNINLAMSPLPASETPKPLRSSADPALNQEVALKLE 479
human       --------------------d--------------l--pn--------------ta----------------------- 479
mouse       ----------------d----------d------------n---.----------s----------------------- 478
rat         ----------------d----------d------------n---.----------------------------------- 479

squirrel    PNPESLELSFTMPQIQDQPASPSDGSTRQSSPE.............PNSPSEYCFDVDSDMVNVFKLELVEKLFAEDTE 545
human       ------------------tp-------------.............---------y-------e--------------- 545
mouse       ss----g--------------------------rllqenvntpnfsq---------------------------------- 558
rat         ss----g-----------------------------.....---------------------------------------- 545

squirrel    AKNPFSTQDTDLDLEMLAPYIPMDDDFQLRSFDQLSPLESSSSSPQSV...STISVFQQTQIQEPTIN..TTTATTDELK 620
human       ----------------------------------------a--e-aspq--vt----------a-at---------- 625
mouse       ----------------------------------------n-p--p-mstvtgfqqt-lqkptitatatt..------s- 636
rat         ------a----------------------------------n-p--p--stvtgfqqt-lqkptitvtata..------s- 623

squirrel    TVTKDDMEDIKILIASWSPTHAPKETTSATTSSYNDTQSRTASPNRAGKEVIEQTEKSHPRSPNVVSVTLSQRNTVPEEE 700
human       -----r----------p----ih-------s-p-r--------------g---------------l--a----t------ 705
mouse       -e---nk---------p-s-qv-q---t-ka-a-sg-h------d----r-----d-a----lkl.-a--n--------- 715
rat         a----ni---------pps-qv-q-m-t-ka-a-sg-h------d----r---k-d-a----l-l.----n--------- 702

squirrel    LNPKILALQNAQKKAKMEQDGSLFQAVGIGTLLQQPDDRATTTSLSWKRVKGCKSSEQNGMEQKTIILIPSDLACRLLGQ 780
human       ------------r-r---h--------------------h-a------------------------------------- 785
mouse       ----ti-s----r-r---h-------a---------g-c-p-m---------fi------t------------------- 795
rat         ----ti------r-r---h-------a---------g---p-m---------yi----d-------f------------- 782

squirrel    SMDESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRALDQVN  821
human       ------------------------------------------  826
mouse       ------------------------------------------  836
rat         ------------------------------------------  823
```

Fig. 1. Multiple alignment showing the amino acid sequence of HIF-1α from thirteen-lined ground squirrel (*S. tridecemlineatus*) aligned with the sequences for human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*) HIF-1α. Genbank accession numbers are AY713478, NP_001521, NP_034561 and NP_077335, respectively. Dashed lines in the alignment represent amino acids that are identical with the ground squirrel sequence; spacer dots are inserted when residues are not present in all sequences. Unique amino acid substitutions in the ground squirrel sequence are shown in bold, underline.

3. Results

3.1. cDNA cloning of *hif-1α*

Using RT-PCR and the primers derived from the consensus sequence of *hif-1α* from other mammalian species, a PCR product of 796 bp was retrieved from the total RNA prepared from brown adipose tissue of hibernating *S. tridecemlineatus*. The product was confirmed as encoding a portion of the *hif-1α* sequence and then 5′ and 3′ RACE were used to extend the sequence and a final assembled sequence of 2466 bp was achieved. The ground squirrel *hif-1α* sequence was submitted to GenBank with accession number AY713478. Fig. 1 shows the translated amino acid sequence of ground squirrel HIF-1α aligned with the sequences for human, mouse and rat HIF-1α. The ground squirrel protein contained 821 amino acids compared with 823–836 for the other species. The amplified ground squirrel HIF-1α was 95% identical to the human sequence and showed 90% identity with the mouse or rat sequences. Ground squirrel HIF-1α contained a few unique amino acid substitutions that were not seen in non-hibernating mammals (shown in bold, underline in Fig. 1). Multiple sequencing runs used during the course of assembling the full sequence (both initial sequencing of the PCR product and sequencing of 5′ and 3′ RACE segments) showed that these substitutions were not artifacts due to sequencing errors. These included the substitution of a hydrophobic leucine for a basic arginine residue at position 68, a glycine substitution for aspartic acid at residue 349, and two serine substitutions, one for proline at position 208 and one for asparagine at position 440. In addition, there was a tryptophan substitution for proline at position 637 and a glutamine substitution for histidine at position 719. Finally, two arginine residues at positions 713 and 715 were changed to lysine and alanine in the ground squirrel sequence, respectively.

3.2. *hif-1α* gene expression in *S. tridecemlineatus*

Transcript levels of *hif-1α* were assessed in four tissues (brown adipose, skeletal muscle, liver and lung) from euthermic and hibernating *S. tridecemlineatus* using RT-PCR. Primers for *hif-1α* were designed from a consensus

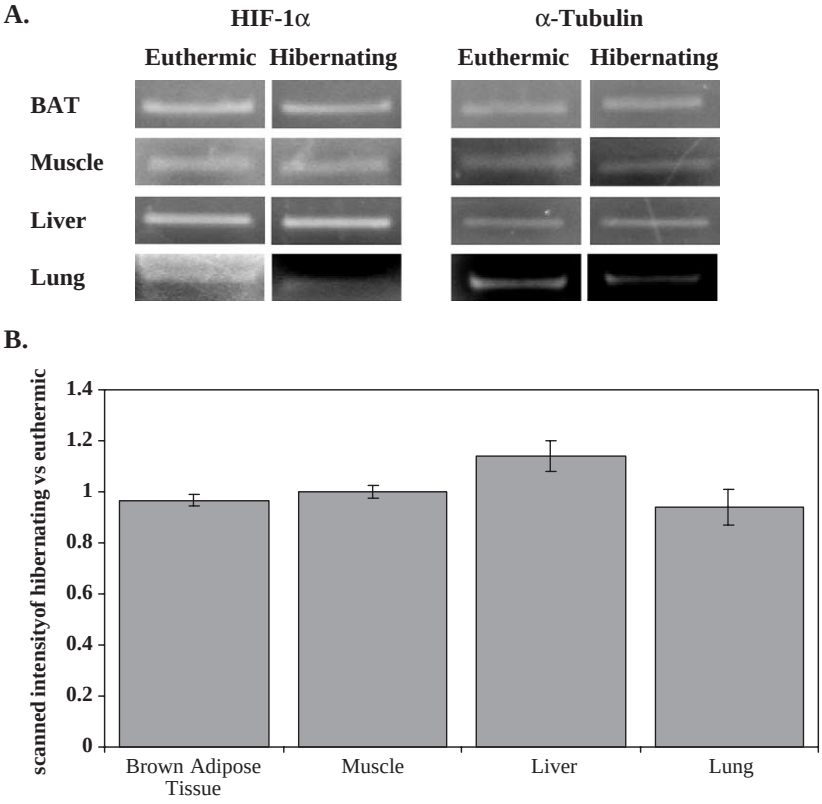

Fig. 2. (A) Effect of hibernation on the mRNA levels in four tissues of thirteen-lined ground squirrels. *hif-1α* transcripts in each sample were amplified by RT-PCR and PCR products were separated on agarose gels, stained with ethidium bromide and visualized under UV light using a Syngene. Paired tubes amplified α-tubulin transcripts in the same samples and *hif-1α* band intensities were normalized against the corresponding paired α-tubulin intensity for the same sample. Band sizes were 796 bp for *hif-1α* and 616 bp for α-tubulin. BAT is brown adipose tissue. (B) Histogram showing the ratio of normalized PCR product levels in tissues from hibernating versus euthermic animals ground squirrels. Values are means ± S.E. for $n = 3$ independent trials on tissue from different animals. Statistical analysis using the Student's *t*-test showed no significant difference in *hif-1α* transcript levels between the hibernating and euthermic situations in any tissue.

sequence made from human, mouse and rat sequences and were used to amplify mRNA from ground squirrel tissues. Fig. 2 shows the relative levels of *hif-1α* mRNA expression in each organ. Alpha-tubulin mRNA, a constitutively expressed gene, was also amplified from the same samples and *hif-1α* transcript levels were normalized against the tubulin transcript level in each sample. Fig. 2B shows the ratio of normalized *hif-1α* transcript levels in tissues from hibernating versus euthermic animals. Transcript levels of *hif-1α* did not change significantly during hibernation in any of the four tissues analyzed.

3.3. HIF-1α protein levels in S. tridecemlineatus

HIF-1α protein levels were assessed via Western blotting in four tissues of euthermic and hibernating *S. tridecemlineatus* (Fig. 3). The polyclonal chicken antibody cross-reacted with a single protein band of ~110 kDa that corresponded with the known size of the HIF-1α protein in other mammals. Fig. 3B shows the ratio of HIF-1α protein levels in hibernating versus euthermic situations for brown adipose tissue, skeletal muscle, liver and lung. HIF-1α protein content was significantly higher ($P<0.05$) in both brown adipose tissue and muscle of hibernating *S. tridecemlineatus*; values were 1.70- and 1.55-fold higher than in tissues from euthermic animals, respectively. HIF-1α protein levels did not change significantly in either liver or lung during hibernation.

3.4. HIF-1 DNA binding activity in S. tridecemlineatus

Activated HIF-1α translocates to the nucleus, dimerizes with HIF-1β and stimulates the transcription of genes, whereas inactive HIF-1α does not translocate or dimerize with HIF-1β. Changes in HIF-1 DNA binding activity between the euthermic and hibernating states were assessed in nuclear extracts from brown adipose tissue of *S. tridecemlineatus*. Mean absorbances at 460 nm corresponding to HIF-1 DNA binding activity were 0.056 ± 0.005 ($n=3$ independent trials) for extracts from euthermic animals and 5.6-fold higher at 0.311 ± 0.048 ($n=3$; $P<0.05$) in extracts from hibernating animals.

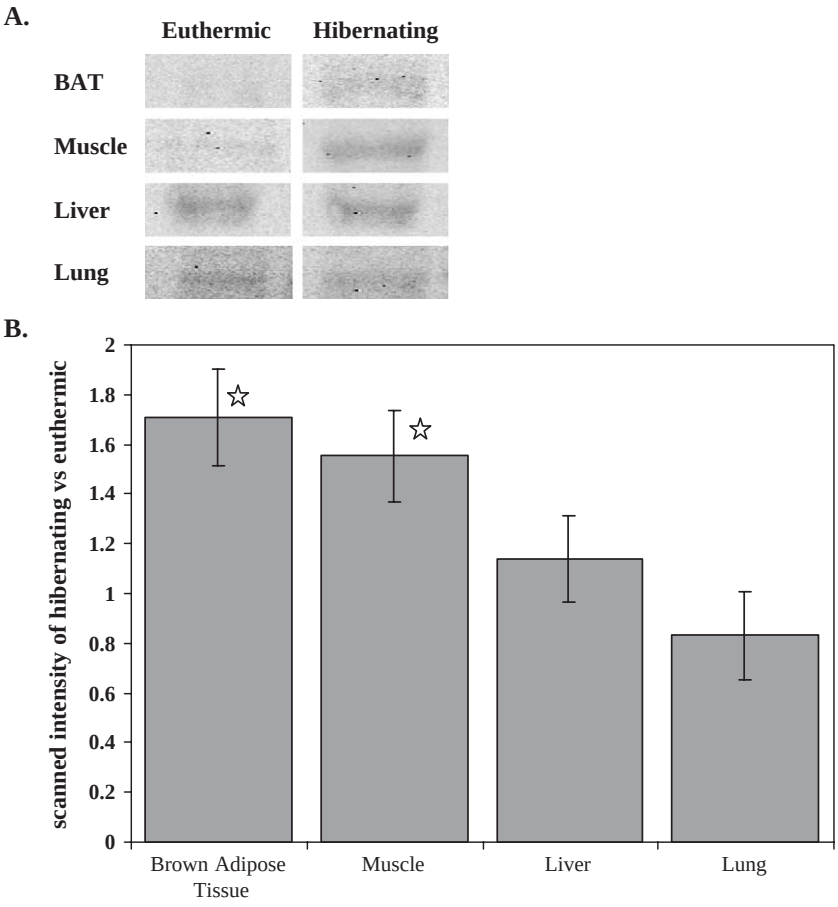

Fig. 3. (A) HIF-1α protein expression in four tissues of hibernating versus euthermic *S. tridecemlineatus*. Representative Western blots show expression of the HIF-1α protein band at 110 kDa. (B) Histogram showing relative HIF-1α protein expression in organs from hibernating versus euthermic squirrels. Data are means ± S.E. for $n=3$ independent trials on tissue from different animals. ☆Values are significantly different from the corresponding euthermic control value, $P<0.05$.

4. Discussion

Soon after a hypoxia-inducible transcription factor was identified, studies showed that while HIF-1α protein levels increased in hypoxia, *hif-1α* mRNA transcript levels remained largely unchanged [13]. Indeed, subsequent studies suggested that HIF-1α levels were regulated primarily at the protein level by post-translational modifications of the protein via phosphorylation [14] and hydroxylation [15] which play roles in HIF-1α activation and degradation, respectively. The modulation of HIF-1α protein levels with respect to oxygen levels is derived from oxygen-dependent hydroxylation of two proline residues on HIF-1α that target it for degradation. Under low oxygen conditions this process is inhibited so that HIF-1α protein levels rise allowing increased formation of HIF-1 dimer and enhanced HIF-1 binding activity to the hypoxia response element (HRE) of various genes [16]. The object of this study was to probe the potential link between HIF-1 and hibernation. Arguments have been made that suggest that this link may be present: (a) an interaction seems to exist between hypoxia and hypothermia that could participate in the mechanism of metabolic rate depression observed in hibernators, and (b) a number of hypoxia-related genes are up-regulated in various tissues of hibernating mammals [4].

The present study analyzed HIF-1α gene and protein expression in four main organs of hibernating ground squirrels (brown adipose, skeletal muscle, liver and lung). The levels of *hif-1α* mRNA remained constant in all four organs of hibernators compared with euthermic controls but HIF-1α protein content rose significantly during hibernation in two organs. The increase was 1.70-fold in brown adipose tissue and 1.55-fold in muscle. This could result from a more active translation of *hif-1α* transcripts during torpor. Indeed, despite a strong general suppression of translation during hibernation, the translation of a selected few proteins that aid hibernation is strongly increased [4,17]. HIF-1α may be one of these. Alternatively, apnoic breathing patterns in torpor would create conditions of declining oxygen levels in ground squirrel organs during most of a torpor bout, compared with high and constant oxygenation during euthermia, and this could lead to enhanced stability of HIF-1α in torpor or HIF-1α protein stability might also be improved by other mechanisms such as an effect of low body temperatures on the relative rates of HIF-1α synthesis versus degradation. A relative reduction in HIF-1α degradation during hibernation might be suspected but, interestingly, results from cDNA array screening in our laboratory (using human 19 K gene chips from the Ontario Cancer Institute) have revealed that prolyl hydroxylase transcript levels are up-regulated in tissues from hibernating animals. This suggests that the whole oxygen sensing mechanism may be up-regulated during hibernation, at least in the thermogenic organs that need to conduct high rates of aerobic lipid oxidation for heat production during arousal: i.e. brown adipose that conducts nonshivering thermogenesis and skeletal muscle that contributes shivering thermogenesis. Indeed, this interpretation is also supported by the observed 6-fold increase in HIF-1 DNA binding capacity in brown adipose from hibernating animals which strongly indicates that multiple genes under HIF-1 control are also up-regulated during hibernation. The differential between the 6-fold increase in DNA binding activity of HIF-1 and the 1.7-fold increase in HIF-1α protein levels in brown adipose suggests that an additional factor(s) is involved in HIF-1 activation in hibernation. This factor may be protein phosphorylation. The phosphorylation of HIF-1α by p42/p44 mitogen-activated protein kinases (MAPKs; also known as ERK2 and ERK1, respectively) leads to increased transcriptional activity of the HIF-1 complex [18]. Furthermore, p42/p44 MAPKs also phosphorylate the p300/CBP co-activator proteins that aid HIF-1 binding to DNA [19]. New studies in our lab have shown that ERK2 activity is strongly increased in skeletal muscle of hibernating ground squirrels [20], with muscle being the other tissue that, like brown adipose, showed increased HIF-1α in hibernation. Although the status of ERK activation in brown adipose during hibernation is not yet known, it is tempting to speculate that the ERK-mediated phosphorylation of HIF-1α might account for the strong increase in DNA binding to the HRE by HIF-1 in brown adipose of hibernating animals.

Interestingly, another mechanism of HIF-1 activation has recently been demonstrated and this may be important in torpor. The argument can be made that hibernators are not hypoxic; oxygen levels drop over the course of apnoic periods but metabolism remains firmly lipid-based both in torpor and during arousal which suggests that oxygen is never limiting to cellular energy metabolism. Then how and why is HIF-1 activated? New research has shown that HIF-1 can also be activated under normoxic conditions to mediate biological functions unrelated to hypoxia defense [18]. A non-hypoxic activation of HIF-1 has been demonstrated as a response to various growth factors, cytokines, vascular hormones and viral proteins [18]. Furthermore, in this situation, activation does not appear to result from HIF-1α stabilization (e.g. hydroxylation vs. phosphorylation) but rather from an increase in HIF-1α protein translation. This could also be the mechanism of HIF-1α increase in hibernator tissues. To date, only a few gene targets of non-hypoxic HIF-1 activation are known and so it is not yet possible to suggest a potential function for non-hypoxic HIF-1 activation in hibernation. Nonetheless, the present study, by showing that HIF-1α levels are elevated in hibernation and HIF-1 DNA binding is enhanced, strongly indicates that a suite of HIF-1-regulated genes are up-regulated during torpor in selected tissues (at least brown adipose and skeletal muscle). The identification of the genes involved and their functions in hibernation is the next challenge.

The complete sequence of ground squirrel HIF-1α was obtained and analyzed. The ground squirrel protein showed 90–95% identity with the sequences for nonhibernating mammals (human, mouse, rat) with most of the variation in the ground squirrel protein, compared with the others. Specific functional groups that characterize HIF-1α from other species were present. For example, the two proline residues of the oxygen-dependent domain, Pro402 and Pro564, that are hydroxylated in order to target HIF-1α for degradation [21] were conserved in *S. tridecemlineatus*. Considerable variability between species was seen in the C-terminal region. In particular, four unique sequence changes occurred in the hibernator protein (residues 637, 713, 715 and 719) within the inhibitory domain (amino acids 576–785) that separates the two C-terminal transactivation domains [22]. For example, the replacement of the proline residue at 637 with a tryptophan could cause a significant change in conformation because proline residues are commonly found at turns. These four substitutions in the inhibitory domain could be important in adjusting protein conformation/function with respect to temperature change and influencing the transactivation ability of HIF-1 (e.g. transactivation domains bind with the co-activator CBP/p300) under the low body temperatures of the hibernating state. On the other hand, no changes were found in the key amino acid residues that are responsible for HIF-1α binding to the HRE: Ser22, Ala25, Arg30 [23]; and only one unique substitution (arginine to leucine at residue 68) occurred within the N-terminal bHLH domain. The fact that this HRE-binding region is conserved in a hibernator as compared with other non-hibernating mammals highlights the importance of a conserved DNA binding region and suggests that no modifications to DNA binding capacity are needed to support HIF-1 function at the low body temperatures of the hibernating state. In the region associated with the PAS domain of HIF-1α, amino acids 106–526, three unique substitutions were observed (proline to serine at 208, aspartic acid to glycine at 349, and asparagine to serine at 440). Due to their small size, glycine residues are also known to be located at turns in a protein and hence both the loss of the proline at 208 and the gain of the glycine at 349 could contribute to conformational changes to better sustain dimerization ability with HIF-1β at low body temperatures.

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References

[1] L.C.H. Wang, T.F. Lee, in: M.J. Fregley, C.M. Blatteis (Eds.), Handbook of Physiology: Environmental Physiology, Section 4, vol. 1, Oxford University Press, New York, 1996, pp. 507–532.
[2] K.U. Frerichs, C. Kennedy, L. Sokoloff, J.M. Hallenbeck, Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia", J. Cereb. Blood Flow Metab. 14 (1994) 193–205.
[3] R.C.H. Barros, M.E. Zimmer, L.G.S. Branco, W.K. Milsom, Hypoxic metabolic response of the golden-mantled ground squirrel, J. Appl. Physiol. 91 (2001) 603–612.
[4] K.B. Storey, Mammalian hibernation. Transcriptional and translational controls, Adv. Exp. Med. Biol. 543 (2003) 21–38.
[5] P.J. Ratcliffe, J.F. O'Rourke, P.H. Maxwell, C.W. Pugh, Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression, J. Exp. Biol. 201 (1998) 1153–1162.
[6] G.L. Semenza, HIF-1: mediator of physiological and pathophysiological responses to hypoxia, J. Appl. Physiol. 88 (2000) 1474–1480.
[7] G. Hopfl, O. Ogunshola, M. Gassmann, Hypoxia and high altitude. The molecular response, Adv. Exp. Med. Biol. 543 (2003) 89–115.
[8] L.E. Huang, J. Gu, M. Schau, H.F. Bunn, Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7987–7992.
[9] P.H. Maxwell, M.S. Wiesener, G.W. Chang, S.C. Clifford, E.C. Vaux, M.E. Cockman, C.C. Wykoff, C.W. Pugh, E.R. Maher, P.J. Ratclife, The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis, Nature (London) 399 (1999) 271–275.
[10] U.R. Jewell, I. Kvietikova, A. Scheid, C. Bauer, R.H. Wenger, M. Gassmann, Induction of HIF-1alpha in response to hypoxia is instantaneous, FASEB J. 15 (2001) 1312–1314.
[11] Y. Chen, M. Matsushita, A.C. Nairn, Z. Damuni, D. Cai, K.U. Frerichs, J.M. Hallenbeck, Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels, Biochemistry 40 (2001) 11565–11570.
[12] J.N.A. De Croos, J.D. McNally, F. Palmieri, K.B. Storey, Up-regulation of the mitochondrial phosphate carrier during freezing in the wood frog *Rana sylvatica*: potential roles of transporters in freeze tolerance, J. Bioenerg. Biomembranes 36 (2004) 229–239.
[13] R.H. Wenger, I. Kvietikova, A. Rolfs, M. Gassmann, H.H. Marti, Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level, Kidney Int. 51 (1997) 560–563.
[14] G.L. Wang, B.H. Jiang, G.L. Semenza, Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1, Biochem. Biophys. Res. Commun. 216 (1995) 669–675.
[15] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, Av. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation, Science 292 (2001) 468–472.
[16] G.L. Wang, G.L. Semenza, Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia, J. Biol. Chem. 268 (1993) 21513–21518.
[17] D. Hittel, K.B. Storey, The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*), Arch. Biochem. Biophys. 401 (2002) 244–254.
[18] M.A. Dery, M.D. Michaud, D.E. Richard, Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators, Int. J. Biochem. Cell Biol. 37 (2005) 535–540.
[19] N. Sang, D.P. Stiehl, J. Bohensky, I. Leshchinsky, V. Srinivas, J. Caro, MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300, J. Biol. Chem. 278 (2003) 14013–14019.
[20] J.A. MacDonald, K.B. Storey, Mitogen-activated protein kinases and selected downstream targets display organ-specific responses in the hibernating ground squirrel, Int. J. Biochem. Cell Biol. 37 (2005) 679–691.

[21] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin Jr., HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing, Science 292 (2001) 464–468.
[22] B.H. Jiang, J.Z. Zheng, S.W. Leung, R. Roe, G.L. Semenza, Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension, J. Biol. Chem. 272 (1997) 19253–19260.
[23] G. Michel, E. Minet, D. Mottet, J. Remacle, C. Michiels, Site-directed mutagenesis studies of the hypoxia-inducible factor-1alpha DNA-binding domain, Biochim. Biophys. Acta 1578 (2002) 73–83.