Archives of Biochemistry and Biophysics 477 (2008) 77–85

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Coping with the stress: Expression of ATF4, ATF6, and downstream targets in organs of hibernating ground squirrels

Hapsatou Mamady, Kenneth B. Storey *

Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ont., Canada K1S 5B6

ARTICLE INFO

Article history:
Received 29 February 2008
and in revised form 16 May 2008
Available online 25 May 2008

Keywords:
Activating transcription factor
Unfolded protein response
Endoplasmic reticulum stress
PERK
CREB
Spermophilus tridecemlineatus
Torpor

ABSTRACT

Perturbation of the endoplasmic reticulum (ER) protein folding apparatus via any one of several environmental or metabolic stresses rapidly triggers a complex program of cellular responses that is termed the unfolded protein response (UPR). Stresses that trigger this response in mammals can include low temperature, hypoxia, ischemia, and oxidative stress. All of these can be natural features of mammalian hibernation, and hence the UPR might be integral to long term survival in a state of cold torpor. The present study analyzes changes in gene and/or protein expression of multiple markers of the UPR in tissues of euthermic (control) versus hibernating ground squirrels, *Spermophilus tridecemlineatus*. Immunoblot analysis of ATF4 protein expression revealed strong increases of 1.9- to 2.5-fold in brown adipose tissue, skeletal muscle, and brain during hibernation. However, transcript levels of *atf4* were unchanged or lowered which suggests that ATF4 protein levels were regulated at the translational level. Subcellular localization studies showed that ATF4 translocated into the nucleus during hibernation, as did its cofactor, the phosphorylated form of CREB-1, which rose by 25- to 39-fold in nuclear extracts of brain and skeletal muscle of torpid animals. The responses of other proteins involved in the UPR including p-PERK, ATF6, GADD153, and GADD34 were also evaluated. The data suggest that ATF4 up-regulation may play an important role in coordinating gene expression responses that support the hibernating phenotype.

A variety of physiological stresses and pathologies have in common the accumulation of misfolded proteins in cells. This is collectively called endoplasmic reticulum (ER)[1] stress and elicits the activation of a complex signal transduction cascade known as the *U*nfolded *P*rotein *R*esponse (UPR) as well as the ER associated protein degradation (ERAD) pathway [1]. Conditions that can invoke the UPR include hypothermia, ischemia, hypoxia, depletion of ER calcium stores, reductive, and oxidative stress and inhibition of N-linked glycosylation in the ER [2]. Mammalian cells respond to ER stress by attenuating protein translation and activating the UPR. The UPR coordinates a program of modified transcription and translation that includes the preferential up-regulation of genes encoding ER-resident chaperones, factors that regulate the metabolism and redox environment of the cell, and proteins of the ERAD pathway [2].

Hypothermia, ischemia, and hypoxia are serious stresses for most mammals but conditions that are quite comparable to these stresses occur naturally in hibernating mammals. Hibernators spend days or weeks in a state of cold torpor when core body temperature (T_b) is often reduced to near 0 °C, all physiological functions (e.g. heartbeat, breathing rate, kidney perfusion) are profoundly suppressed, organs are hypoperfused and metabolic rate typically falls to <5% of the euthermic resting rate [3]. Yet after long periods of torpor, animals arouse back to euthermia ($T_b \sim 37$ °C) without any apparent metabolic injuries. We wondered, then, whether the UPR would be a natural component of hibernation, triggering actions including enhanced chaperone levels and suppression of translation that could be beneficial to long term life in a state of cold torpor. In a previous study we found that both mRNA transcript and protein levels of the glucose-regulated protein 78 (GRP78), one of the most abundant ER chaperones and a central regulator of the ER stress–response [4], were strongly up-regulated in organs of hibernating thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), as compared with euthermic animals [5]. Hence, we had initial evidence that the UPR is a key component of cell preservation during torpor.

* Corresponding author. Fax: +1 613 520 3749.
E-mail address: kenneth_storey@carleton.ca (K.B. Storey).

[1] *Abbreviations used:* ER, endoplasmic reticulum; UPR, Unfolded Protein Response; ERAD, ER associated protein degradation; GRP78, glucose-regulated protein 78; IRE1, inositol-requiring 1 kinase; PERK, PKR-like endoplasmic reticulum kinase; ATF6, activating transcription factor 6; p-PERK, phosphorylated PERK; eIF2α, α-subunit of the eukaryotic initiation factor-2; ERSE, ER stress–response elements; ATF4, activating transcription factor 4; ATF/CREB, activating transcription factor/cAMP response element binding proteins; GADD153, growth arrest and DNA damage-inducible protein 153; C/EBP, CCAAT/Enhancer Binding Protein homologous; PVDF, polyvinylidene difluoride; TBST, Tris buffered saline containing Tween 20; ECL, enhanced chemiluminescence; serpins, serine protease inhibitors; bZIP, basic leucine zipper.

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.05.006

GRP78 regulates the UPR via its ability to control the activation of three transmembrane ER stress sensors: inositol-requiring 1 kinase (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) [4]. Upon ER stress, these signaling molecules dissociate from GRP78 and undergo posttranslational modifications that activate them. Phosphorylated PERK (p-PERK) inhibits ribosomal protein translation via phosphorylation of the α-subunit of the eukaryotic initiation factor-2 (eIF2α); the result is a decrease in the amount of client protein entering the ER [6,7]. Activation of IRE1 and ATF6 up-regulates the transcription of genes encoding ER chaperones and proteins involved in apoptosis [2,8]. In response to stress, inactive 90 kDa ATF6 translocates from the ER to the Golgi where it is converted to its active 50 kDa form via sequential cleavage by site-1 and site-2 proteases, S1P and S2P [9]. The active ATF6-50 kDa then moves into the nucleus, forms complexes with co-activators, and binds to the ER stress–response elements (ERSE) in genes such as *grp78*, *grp94*, *PDI*, and *gadd153* to induce their transcriptional activation [10].

Multiple studies have reported that phosphorylation of eIF2α enhances expression of the activating transcription factor 4 (ATF4), a member of the activating transcription factor/cAMP response element binding proteins (ATF/CREB) family [6,11,12]. In turn, ATF4 activates both pro-survival and pro-apoptotic genes that participate in the UPR. For example, ATF4 forms a complex with its nuclear co-activator, the phosphorylated form of CREB1, to bind to the promoter region of the pro-survival gene, *grp78* [5,11]. One of the pro-apoptotic genes that is downstream of ATF4 is the growth arrest and DNA damage-inducible protein 153 (GADD153), also known as CHOP, a member of the CCAAT/Enhancer Binding Protein homologous (C/EBP) family of transcription factors. GADD153 responds to a number of stresses including impaired protein folding in the ER, reactive oxygen species, hypoxia, and nutrient deprivation [13]. Another downstream gene regulated by ATF4 is GADD34 which is known to associate with protein phosphatase 1 to dephosphorylate phospho-eIF2α in a feedback regulatory loop that promotes translational recovery [14].

Based on our prior demonstration of the up-regulation of GRP78 in hibernating ground squirrels, we hypothesized that the UPR was activated during entry into torpor. The present study analyzes the organ-specific responses of selected signaling proteins that participate in the UPR; these included p-PERK, ATF6 (50 kDa), ATF4, CREB-1, p-CREB1, GADD153, and GADD34. An extended analysis of ATF4 also documented hibernation-responsive changes in both mRNA transcript and protein levels of this transcription factor and assessed sequence differences in the hibernator protein that may be useful for low temperature function of ATF4. The data indicate that ATF4 is induced at the translational level during torpor and moves into the nucleus where, in complex with greatly elevated levels of its co-activator p-CREB1, it mediates an integrated stress–response to protect cells during torpor.

Materials and methods

Tissue sample collection

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were captured in the autumn in Illinois and shipped to an animal housing facility on the National Institutes of Health campus (Bethesda, MD) where hibernation experiments were conducted using the protocol as previously reported [5], approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stoke. All animal experiments were as described previously [5]. Individuals that had settled into hibernation were sampled after 2–5 days of continuous torpor, as evidence by continuous low T_b values of ~6 °C as detected by a sensor chip that had previously been introduced under the skin. Animals that had not entered torpor after at least 3 days in the cold room and that showed continuous high T_b (36–38 °C) were sampled as euthermic controls. All animals were sacrificed by decapitation and tissue samples of euthermic and deeply hibernating animals were quickly excised, flash frozen in liquid nitrogen, packed in dry ice and air-freighted to Carleton University where they were stored at −80 °C until use.

Total RNA extraction and RT-PCR analysis

Total RNA was isolated from 100 mg tissue samples and cDNA was reverse transcribed from 15 μg of total RNA, as described previously [5]. The resulting cDNA samples were serially diluted and *atf4* was amplified in each sample using reverse transcription (RT)-PCR according to standard protocols. The reaction conditions were as follows: 94 °C pre-heat for 3 min, followed by 35 cycles of 95 °C for 30 s (denaturation), 57 °C for 30 s (annealing) and 72 °C for 30 s (elongation), then a last step of 7 min of 72 °C (final elongation). Primers for *atf4* were designed based on the human a*tf4* sequence (GenBank Accession No. NM_182810) and according to a consensus sequence of a*tf4* from other mammalian species. The nucleotide sequences of the a*tf4* primer pair were 5′-GGAGGTGGCCAAGCACTTCA-3′ and 5′-CTTCTG GCGGTACCTAGTGG-3′. As an internal control, the housekeeping gene α-tubulin was also amplified from the same samples using the primers 5′-AAGGAAG ATGCTGCCAATAA-3′ and 5′-GGTCACATTTCACCATCTG-3′. PCR products were separated on a 1% agarose gel, stained with ethidium bromide, scanned and quantified using the UV transilluminator option of the ChemiGenius imaging system and GeneTools software (Syngene, MD, USA). RT-PCR of *atf4* from BAT generated a product of 781 bp that was sequenced by CORTEC (Kingston, ON, Canada). The resulting sequence was verified as encoding the expected gene using the program BLASTN at the NIH. The 3′ end was amplified as previously described [5], using a Takara 3′ RACE kit (Promega). The gene specific primer used was: 5′-AACCATGCCAG ATGAGCTTT-3′. The 3′ RACE generated a fragment of 889 bp, verified to encode the *atf4* gene. The processed and assembled sequence was submitted to Genbank (Accession No. DQ324000). The deduced amino acid sequence was also verified to be ATF4 via the BLASTP program.

Preparation of protein extracts and SDS–PAGE

Tissue samples were homogenized (1:2 w/v) in 20 mM Hepes buffer, pH 8–8.5, containing 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, and 10 mM β-glycerophosphate. Immediately prior to homogenization, 1 mM phenylmethylsulfonyl fluoride and 1 μL of protease inhibitor cocktail (Sigma) were added. After a 15 min centrifugation at 15,000*g* at 4 °C, supernatants were removed and soluble protein concentrations were determined using the Coomassie blue dye-binding method and the BioRad prepared reagent (BioRad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Aliquots of supernatant were then mixed 1:1 v/v with 2× SDS–PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, and 10% v/v 2-mercaptoethanol), boiled and stored at −20 °C until use. Equal amounts of protein (10–20 μg) from control and hibernating extracts were then loaded on 8% or 12% SDS–polyacrylamide gels (depending on the molecular mass of the protein under investigation), and separated for 1 h at 200 V using a 1× running buffer (3 g Tris base, 18.8 g glycine, and 1 g SDS per liter, pH ~ 8.3). Proteins were then electroblotted (70 V at 4 °C for 90 min) onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp.) by wet transfer with pre-chilled solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 20% v/v methanol.

Western blotting

After the transfer of proteins, membranes were blocked for 30–60 min in Tris buffered saline containing Tween 20 (TBST: 20 mM Tris base, 140 mM NaCl, 0.1% v/v Tween 20) with 2.5% non-fat dried milk added. The blots were then incubated at 4 °C overnight with a primary antibody in diluted TBST. The antibodies used were rabbit polyclonal antibodies against ATF4 diluted 1:500 and goat polyclonal antibodies against ATF6 diluted 1:100 (both from Santa Cruz Biotechnology Inc.), rabbit polyclonal antibodies recognizing the phosphorylated form of PERK diluted 1:1000 (Cell Signaling), rabbit polyclonal antibodies against CREB-1 and phospho-CREB-1 diluted 1:250 (both from Santa Cruz Biotechnology Inc.), mouse monoclonal antibodies against GADD153 diluted 1:1000 (Stressgen), and rabbit antibodies against GADD34 diluted 1:300 (Santa Cruz Biotechnology Inc.). After incubation, blots were washed several times with TBST and then incubated at room temperature for 2 h with a secondary antibody, anti-rabbit and anti-mouse (Cell Signaling) or anti-goat (Santa Cruz Biotechnology Inc.) IgG conjugated to horseradish peroxidase at a 1:2000 dilution. Detection of signal on the PVDF membrane was done using an enhanced chemiluminescence (ECL) system as recommended by the manufacturer (Pierce). The membrane was scanned using the ChemiGenius (Syngene, MD, USA) and the resulting image was analyzed with the associated Gene Tools software. After immunoblots were scanned and quantified, the membrane was stained with Coomassie blue and rescanned to confirm equal loading of proteins in the lanes. Three sharp bands that were present in all samples and that were distinctly separated from the molecular weight of the immunoreactive band were then chosen and scanned in each lane and their densities were summed. The intensity of the immunoreactive band in each lane was then normalized against the summed intensity of the Coomassie stained bands.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts of brain, heart and muscle were prepared using the method described previously [5]. Briefly, 0.5 g of tissue was disrupted in 1 mL homogenization buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM DTT, and 1.5 μL protease inhibitor cocktail [Sigma]) at 4 °C using a Dounce homogenizer. Nuclei were pelleted by centrifugation at 8000*g* for 10 min. The supernatant, which represents the cytoplasmic extract, was removed and the pellet was washed with the same buffer before being resuspended in 150 μL of extraction buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10% v/v glycerol, 10 mM DTT, and 1.5 μL protease inhibitor cocktail) and mixed at 4 °C for 1 h with gentle shaking. After centrifugation at 10,000*g* for 10 min, the supernatant (nuclear extract) was collected. Protein concentration in each fraction was determined and aliquots containing 10–20 μg were mixed with 2× SDS–PAGE sample buffer and prepared for electrophoresis as described above. Proteins were separated on 10% SDS–PAGE minigels at 180 V for 45 min, transferred onto PVDF membranes, and then used for immunoblotting with the ATF6, ATF4, or p-CREB-1 antibodies, as described above. Western blotting with anti-histone H3 antibody was performed as described previously [5] to confirm the integrity of the nuclei when separated from the cytoplasmic fraction; histone H3 band was found in the nuclear extracts and not in the cytoplasmic fractions.

Statistics

Western blot band intensities were normalized against the summed intensities a group of three Coomassie stained protein bands that did not show variation between euthermic and hibernating states and that were distant on the blot from the protein of interest. RT-PCR bands of *atf4* were normalized against band intensities for α-tubulin amplified from the same cDNA reaction. Statistical testing for differences between normalized band intensities from euthermic and hibernating samples used the Student's *t*-test with significance levels of $P < 0.05$ or $P < 0.01$. The data shown in histograms are means ± SEM, from a minimum of $n = 3$ independent trials using tissue samples from different animals.

Results

PERK and ATF6 protein expression

Proximal events for the UPR include the activation of ER-resident signaling molecules such as PERK and ATF6 in order to initiate transcriptional and translational programs that alleviate ER stress. To investigate how these systems respond in hibernation, protein levels of p-PERK and ATF6 were assessed in selected tissues of euthermic versus hibernating squirrels. Levels of p-PERK were evaluated only in brain where cross-reaction of p-PERK antibody with a single protein band of ~170 kDa was seen in both euthermic and hibernating states (Fig. 1A). However, densitometry analysis found no difference in p-PERK levels in brain between the two states. Antibodies raised against ATF6 detected a 50 kDa protein in ground squirrel tissues. Analysis of brain and heart found no difference in ATF6 levels between hibernating and euthermic animals but ATF6 expression in skeletal muscle of hibernating squirrels was 1.20 ± 0.01-fold higher and significantly different ($P < 0.05$) than in euthermia (Fig. 1B).

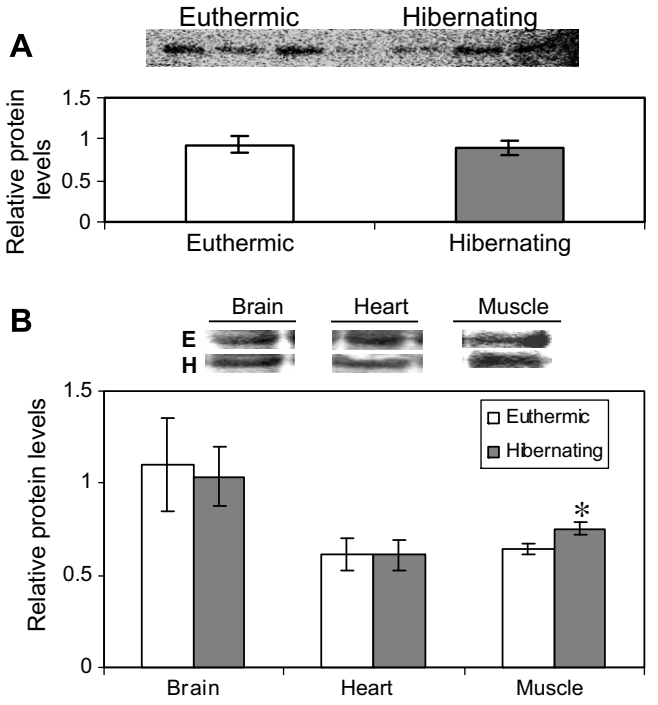

Fig. 1. (A) Immunoblot analysis of p-PERK levels in brain of euthermic and hibernating ground squirrels, as assessed on 8% SDS–polyacrylamide gels. (B) Comparable analysis of ATF6 levels in three tissues from euthermic (E) and hibernating (H) ground squirrels; separation was on 12% SDS–PAGE gels. Histograms for both p-PERK and ATF6 show mean normalized protein levels (±SEM, $n = 3$ independent trials). *Significantly different from the corresponding euthermic value using the Student's *t*-test (two-tailed), $P < 0.05$.

Effect of hibernation on ATF4 protein expression

Fig. 2 shows the protein levels of ATF4 in six tissues of euthermic and hibernating ground squirrels. Western blot analysis showed a cross-reacting band at ~40 kDa, consistent with the known molecular weight of mammalian ATF4. ATF4 protein levels increased significantly during hibernation in BAT, brain and skeletal muscle by 2.51 ± 0.09-, 1.88 ± 0.05-, and 2.48 ± 0.09-fold, respectively. By comparison, ATF4 levels were reduced in heart during hibernation to 56 ± 1% of the control level. ATF4 levels were unchanged in kidney and liver.

Atf4 sequence and transcript levels

Using forward and reverse primers designed from the human *atf4* sequence (GenBank Accession No. NM_182810) but with a consensus sequence of *atf4* from mouse and rat (NM_009716 and NM_024403, respectively), a 781 bp cDNA was amplified from ground squirrel BAT. After nucleotide sequencing, the cDNA was shown to encode ATF4. Next, a gene specific primer was designed from the partial *atf4* sequence and was used in 3′ RACE to extend and amplify the 3′ end of the ground squirrel *atf4* sequence. The final assembled ground squirrel *atf4* cDNA sequence of 1032 bp encoded an amino acid sequence of 313 residues, including the C terminus of the protein; this corresponded to ~89% of the full human ATF4 sequence (351 amino acids). The ground squirrel *atf4* sequence was submitted to GenBank with Accession No. DQ324000. Fig. 3 shows the partial ground squirrel ATF4 sequence aligned with the amino acid sequences of human, mouse and rat ATF4. The ground squirrel sequence was 85% identical with human ATF4 and 81% identical with the mouse and rat protein. Ground squirrel ATF4 contained 19 unique amino acid substitutions that replaced amino acids that were conserved in the three nonhibernators; these residues are shown in bold underline in Fig. 3.

To determine whether the increase in ATF4 protein levels in selected tissues during hibernation resulted from increased gene expression, we quantified *atf4* mRNA transcripts in six tissues of euthermic versus hibernating ground squirrels using relative RT-PCR (Fig. 4). An internal control, α-tubulin, was amplified in tandem; we have previously shown that α-tubulin transcript levels are unchanged in ground squirrel tissues between euthermic and hibernating states. Relative transcript levels of *atf4* increased significantly by 2.32 ± 0.16-fold ($P < 0.01$) in liver during hibernation but decreased significantly to just 52 ± 2% ($P < 0.01$) of the euthermic value in heart of hibernating squirrels. *Atf4* transcript levels remained stable in other tissues.

Effect of hibernation on CREB1 and p-CREB1 protein expression

Since ATF4 protein levels were up-regulated in selected tissues (BAT, brain, muscle) of hibernating ground squirrels, we next eval-

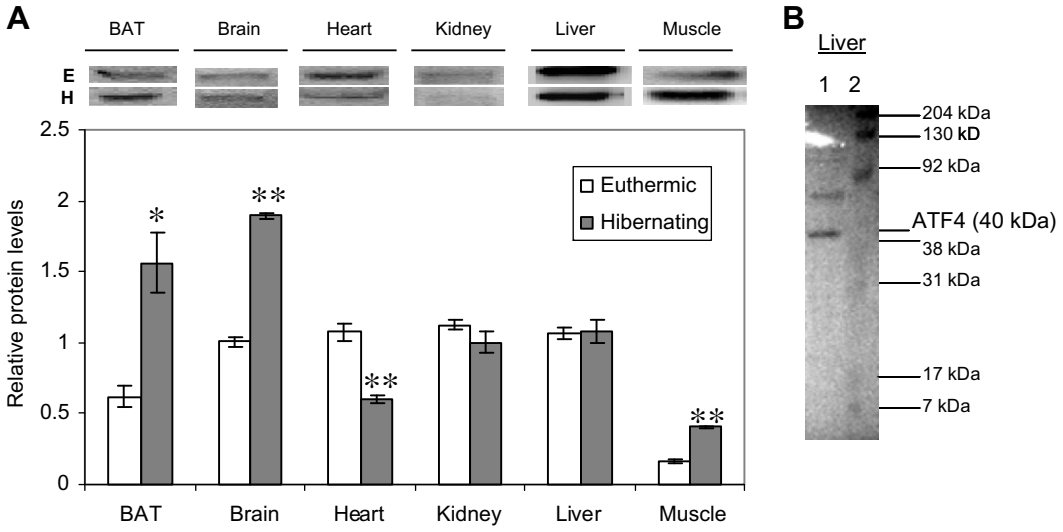

Fig. 2. (A) ATF4 protein levels in six tissues from euthermic (E) and hibernating (H) ground squirrels. Upper panel shows representative Western blots of ATF4 whereas the histogram shows mean ATF4 levels (±SEM, $n = 3$ independent trials). BAT is brown adipose tissue. *Significantly different from the corresponding euthermic value using the two-tailed Student's t-test, $P < 0.05$; **$P < 0.01$. (B) Western blot showing the band corresponding to ATF4 protein at ~40 kDa (lane 1) along with Kaleidoscope protein standards (Bio-Rad Laboratories) (lane 2).

```
Squirrel   ....................................EVAKHFKPHGFSGDKAKAGSSEWLAV.DGLVDASDTGKEDAF     41
Human      mtemsflssevlvgdlmspfdqsglgaeeslgllddyl------------s-------------.----sp-nns-----     79
Mouse      mtemsflnsevlagdlmspfdqsglgaeeslgllddyl-----l------s-..-------p-md---as----------     78
Rat        mtemsflnsevlagdlmspfdqsglgaeeslgllddyl------------s-..---------m.----s----------     77

Squirrel   SGTDWMLEKMDLKEFDFDALLSIDDLETMPDELLATFYDTCDLFPPLVQETNKEPPQVVNLIGHLPESSKT.DQVAPFTF    120
Human      ----------------l----g---------d--t-ld------a--------q---t--p-------ltkp--------    159
Mouse      --------------------frm-----------t-ld------a------------t--p-------likv--------    158
Rat        --------------------frm-------------ld------a------------t--p-------vikv--a-----    157

Squirrel   FQPLSLSPGDLSSTQDHSFSLELGSEVDISEGERKPDSAAYGTMIPQCVKEEDGPSDNDSGICMSPESYLGSPQHSPSVS    200
Human      l---p----v----p-------------t--d----yt--va-----i----t-----------------------tr    239
Mouse      l--fpc---v----pe----------------d--------i-l--p------t-----------------------t-    238
Rat        l---pc---f----p----------------d--------i-lt-------t---s---------------------t-    237

Squirrel   KGSPNVSLPSPGGPCGSNRPKPYDPPGDKMVIAKVKGEKLDKKLKKMEQNKTAATRYRQKERAEQEALTGECIELEKKNE    280
Human      .----r------vl---a---------e---a---------------------------k-----------k-------    318
Mouse      .ra-pdn------sr--p---------vslt.----t----------------------k-----------k-------    316
Rat        .ra-pd------v-r--.---------vsvt.----t----------------------k-----------k-------    314

Squirrel   ALKEKADSLAKEIQYLKDLIEEVRKARGEKKGP          313
Human      ----r-----------------------k-rv-          351
Mouse      ----------------------------k-rv-          349
Rat        ----------------------------k-rv-          347
```

Fig. 3. Ground squirrel (*Spermophilus tridecemlineatus*) ATF4 partial amino acid sequence (GenBank Accession No. DQ324000) aligned with human (*Homo sapiens*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*) sequences (GenBank Accession Nos. NM_182810, BC085169 and NM_024403, respectively). Unique amino acid substitutions in the squirrel sequence are indicated in bold underline. Dashes (-) represent amino acids in the human, mouse or rat sequences that are identical with the squirrel sequence. Periods are present in the alignment to indicate where an amino acid is not present in the coding region of one species.

uated levels of its nuclear co-factor CREB1, a member of the ATF/CREB family that forms an *in vivo* complex with ATF4 upon ER stress. The CREB1 antibody cross-reacted with a single band at ~43 kDa in ground squirrel extracts, reflecting the known molecular weight of the mammalian protein. Fig. 5 shows representative Western blots of total CREB1 protein and phosphorylated (Ser133) active protein and the histogram shows the mean normalized amount of p-CREB1 (Ser133) in tissues from euthermic and hibernating squirrels. Total CREB1 protein did not change during torpor in four tissues but fell in kidney and muscle of hibernating squirrels to 87% and 52% of the euthermic values, respectively. By contrast, levels of p-CREB1 increased significantly in all six organs of hibernating squirrels, particularly in brain and kidney where the calculated ratio in hibernating versus euthermic situations was 7.44 ± 0.22 ($P < 0.01$) and 15.8 ± 0.5 ($P < 0.01$), respectively. The hibernating:euthermic ratio was 4.28 ± 0.19 ($P < 0.05$), 3.58 ± 0.14 ($P < 0.05$), 2.07 ± 0.05 ($P < 0.01$), and 2.57 ± 0.09 ($P < 0.01$) for BAT, heart, liver, and muscle, respectively.

Distribution of ATF6, ATF4, and p-CREB1 in subcellular fractions of tissues

To determine whether the transcription factors moved into the nucleus during hibernation, nuclear and cytoplasmic fractions

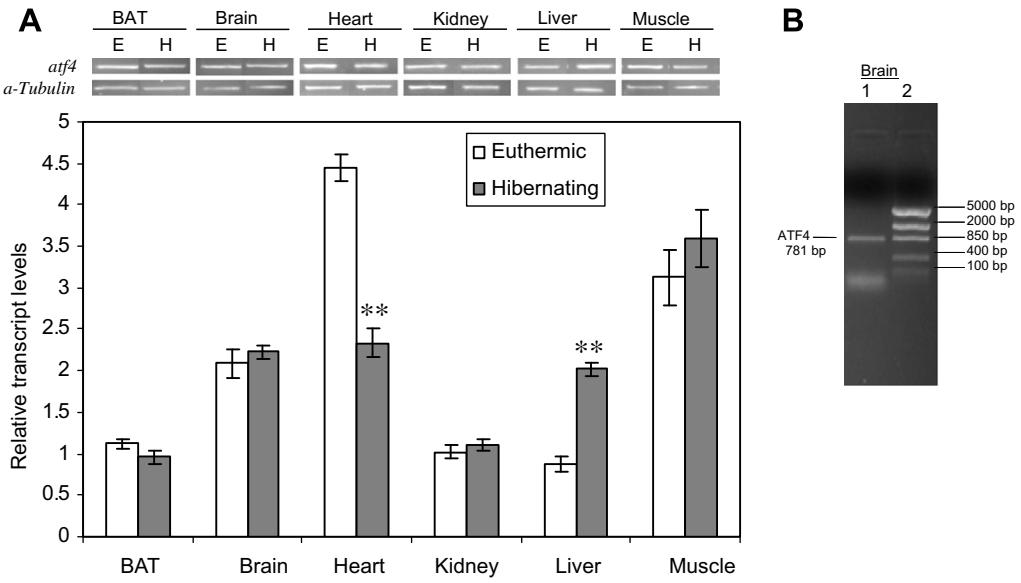

Fig. 4. (A) Effect of hibernation on *atf4* mRNA levels in six tissues of thirteen-lined ground squirrels. Upper panel shows representative PCR product levels on agarose gels stained with ethidium bromide. The highest dilutions of *atf4* (10^{-4} or 10^{-5}) and α-tubulin (from the same dilution) that gave visible PCR product bands were used for quantification. Band densities for *atf5* were normalized against the corresponding band densities for α-tubulin from the same sample. The histogram shows mean normalized PCR product levels (±SEM, $n = 3$–4 independent trials). **Significantly different from the corresponding euthermic value, $P < 0.01$. (B) Agarose gel showing the band corresponding to *atf4* cDNA (781 bp) from squirrel brain (lane 1) together with FastRuler DNA ladder (Fermentas) (lane 2).

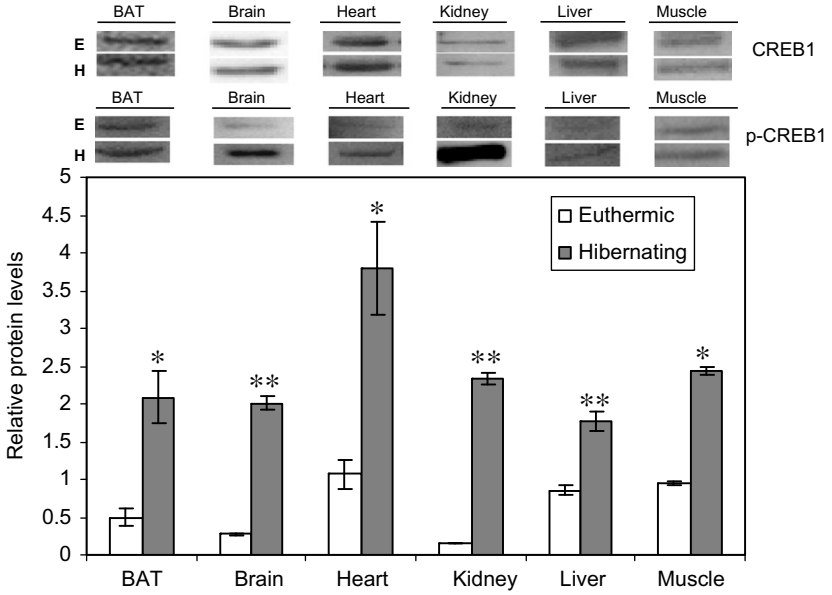

Fig. 5. CREB-1 and phosphorylated CREB-1 levels in tissues from euthermic (E) and hibernating (H) ground squirrels. The upper panel shows representative Western blots. The histogram shows mean p-CREB-1 levels (±SEM, $n = 4$ independent trials on samples from different animals). *,**Significantly different from the corresponding euthermic value as determined by the Student's *t*-test, $P < 0.05$ and $P < 0.01$, respectively.

were isolated from brain, heart, and skeletal muscle and the distribution of the three transcription factors was assessed by Western blotting. Fig. 6 shows results for ATF6. In heart, the amount of nuclear ATF6 increased by 1.45-fold ($P < 0.05$) during hibernation as compared with euthermia but ATF6 distribution in brain was unaltered between euthermic and hibernating states. In skeletal muscle levels of ATF6 in the nucleus remained stable during torpor but increased significantly by 1.47-fold ($P < 0.05$) in the cytoplasm. Fig. 7 shows the comparable subcellular distributions of ATF4 and p-CREB1 (Ser133) in brain and muscle. ATF4 protein content increased significantly in both cytoplasmic and nuclear fractions from brain (by 1.46-fold and 1.17-fold, respectively, $P < 0.01$) and muscle (by 1.45-fold and 1.22-fold, respectively, $P < 0.05$) during hibernation (Fig. 7A). Very large significant increases in p-CREB1 content occurred in both fractions during torpor, particularly in the nuclear fraction (Fig. 7B). The fold increases were 7.66- and 38.6-fold ($P < 0.01$) in cytoplasmic and nuclear fractions from brain, respectively, and 3.80- and 25.2-fold in skeletal muscle ($P < 0.05$).

GADD153 and GADD34 protein levels

Protein levels of the transcription factors GADD153 (CHOP) and GADD34 were also assessed via Western blotting in three tissues of

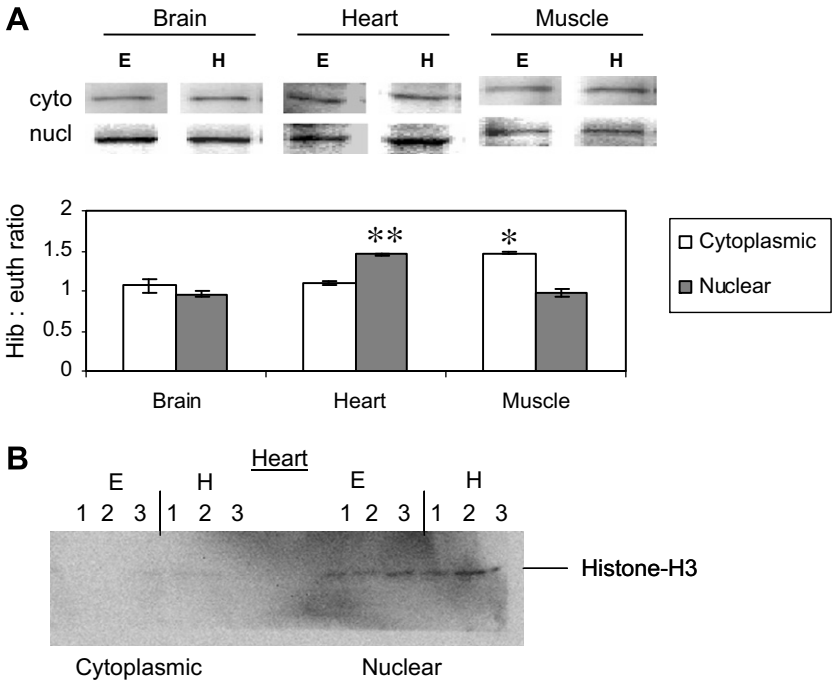

Fig. 6. (A) Analysis of ATF6 (50 kDa) protein distribution between cytoplasmic and nuclear fractions in three tissues of ground squirrels. The upper panel shows representative Western blots detecting ATF6 in nuclear and cytoplasmic extracts from euthermic (E) and hibernating (H) animals. The histogram shows the hibernating:euthermic ratio for ATF6 levels in cytoplasmic and nuclear fractions from each tissue. Data are means ± SEM, $n = 3$ independent determinations. Although data are plotted as hibernating:euthermic ratios for convenience, statistical testing was conducted using the individual values for euthermic and hibernating samples; subsequently ratios were calculated and plotted. Asterisks (*,**) show hibernating values that are significantly different from the corresponding euthermic value, $P < 0.05$ and $P < 0.01$, respectively. (B) Distribution of a known nuclear protein (Histone H3) between cytoplasmic and nuclear extracts of heart from euthermic and hibernating ground squirrels.

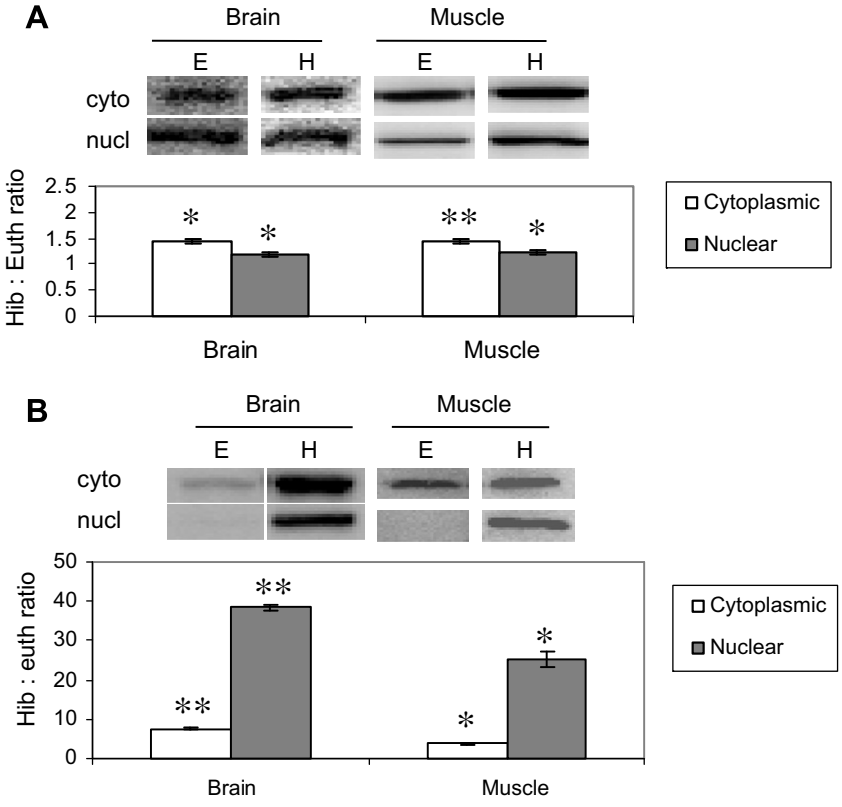

Fig. 7. Distribution of the transcription factors (A) ATF4 and (B) p-CREB1 between cytoplasmic and nuclear fractions of brain and skeletal muscle from euthermic and hibernating ground squirrels. Other information as in Fig. 6.

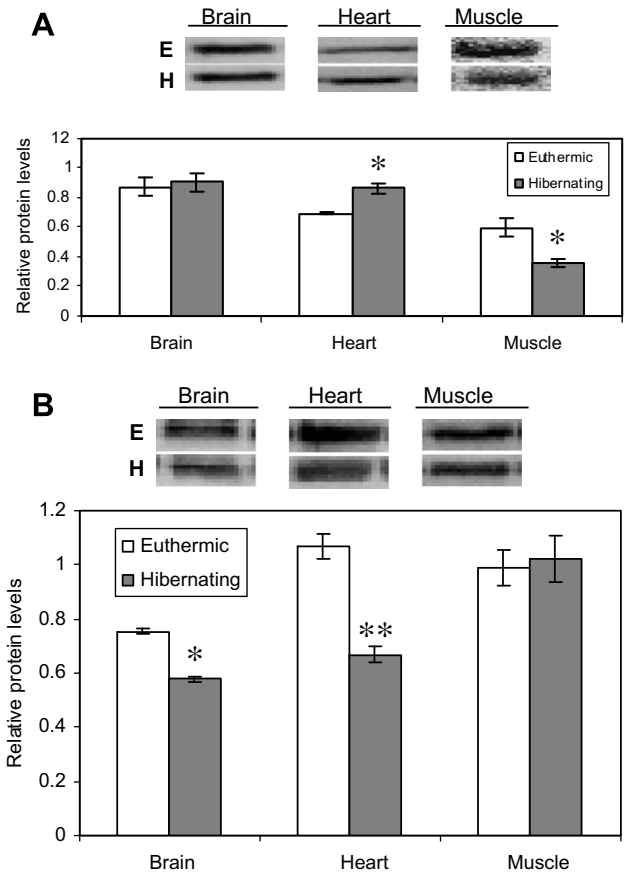

Fig. 8. Analysis of (A) GADD153 and (B) GADD34 protein levels in tissues from euthermic (E) and hibernating (H) ground squirrels. Aliquots (15 μg) of total protein extracts were separated on 10% SDS–polyacrylamide gels. Representative Western blots are shown along with histograms showing mean relative protein levels (±SEM, $n = 3$ independent trials). *,**Significantly different from the corresponding euthermic value using the Student's t-test, $P < 0.05$ and $P < 0.01$, respectively.

euthermic and hibernating ground squirrels. The rabbit polyclonal antibody raised against GADD34 cross-reacted with a single protein band of ~82 kDa that corresponded with the known size of the GADD34 protein in other mammals. The mouse monoclonal antibody against GADD153 cross-reacted with a single protein band of ~30 kDa as found in other mammals. GADD153 levels responded differently in each organ during hibernation (Fig. 8A). Levels increased significantly by 1.25-fold in heart during hibernation but decreased to 61% of the euthermic value in muscle and were unchanged in brain. During hibernation, levels of GADD34 were unchanged in muscle but decreased significantly to 77% ($P < 0.05$) in brain and 63% ($P < 0.01$) in heart of the euthermic control value (Fig. 8B).

Discussion

Much research has established that perturbations of ER function lead to the accumulation of unfolded proteins in the ER lumen, which triggers the UPR and initiates responses that facilitate recovery. These include (a) reducing folding demand by suppressing translation to lower the input of nascent proteins, (b) increasing the clearance of slowly folding or misfolded proteins by enhancing the ERAD pathway, and (c) increasing folding capacity by synthesis of more chaperones and foldases [1,4,11]. Hibernators deal naturally with a variety of conditions that would cause ER stress in non-hibernating mammals; these include a profound suppression of all metabolic functions, energy restriction, a drop in core T_b to near 0 °C, hypoperfusion of selected organs, and pronounced oxidative stress during arousal from torpor (when O_2 consumption increases massively to support thermogenesis) [15]. Clearly, rates of overall protein production are strongly reduced as part of torpor [16] but various hibernation-responsive proteins are up-regulated. Hence, the protein folding machinery in the ER must not only remain intact and functional but, if needed, be optimized for function at low T_b values. We wondered how ground squirrels deal with the potential disruption of ER function that could arise during entry into, arousal from, and/or sustained cold torpor and whether activation of the UPR contributed to ER stability during long term torpor. Other defensive mechanisms are known to contribute to hibernation success including elevated antioxidant defenses and increased levels of heat shock proteins and serine protease inhibitors (serpins) [16–21].

We recently showed that one of the key components of the UPR, the up-regulation of GRP78, occurs during hibernation in BAT and brain of thirteen-lined ground squirrels [5]. Both *grp78* mRNA and GRP78 protein were elevated and this suggested that added protection of ER function is needed during cold torpor. Since GRP78 production is a key biomarker for the onset of the UPR, we turned our attention in the present study to an analysis of some of the other proteins that are involved in the UPR and evaluated their expression during hibernation.

Phosphorylation of the ribosomal initiation factor eIF2α blocks delivery of the initiating Met-tRNA to the ribosomal preinitiation complex and studies with multiple ground squirrel tissues, including brain, have shown that the amount of phospho-eIF2α increases strongly during hibernation as one of the mechanisms of metabolic rate depression [15,16,22,23]. The phosphorylated form of PERK (p-PERK) has been implicated in the inhibition of translation by phosphorylating eIF2α at Ser-51 [24]. However, our analysis of p-PERK expression levels in ground squirrel brain found no difference between euthermic and hibernating states (Fig. 1A) which suggests that p-PERK is not the kinase responsible for eIF2α inactivation during torpor, at least in brain. It is known that, eIF2α can be phosphorylated by other protein kinases such as GCN2, PKR, and HRI so one of these may mediate this action during hibernation [25].

A second signaling molecule involved in the UPR is the transcription factor ATF6. It is constitutively expressed as a 90 kDa protein, which is converted to a 50 kDa active protein in ER-stressed cells and moves into the nucleus to induce transcription of downstream genes including *grp78*, *grp94*, *gadd153*, and *XBP-1* [26–28]. Our data show that overall protein levels of ATF6-50 kDa increased slightly by 20% in skeletal muscle during hibernation whereas no change in ATF6-50 kDa expression occurred in brain and heart (Fig. 1B). However, the critical determinant of ATF6-50 kDa function is whether the transcription factor moves into the nucleus during torpor. Analysis of cytoplasmic versus nuclear fractions showed elevated nuclear content of ATF6-50 kDa (by 1.45-fold) in heart during hibernation but ATF6 distribution was unaltered in brain and in skeletal muscle. Hence, for muscle, the observed increase in total ATF6-50 kDa (Fig. 1) appeared to lead mainly to an enhanced cytoplasmic content of the transcription factor (Fig. 6). The greater amount of ATF6-50 kDa that is translocated into the nuclear fraction of heart during hibernation suggests enhanced expression of genes under the control of ATF6. Heart has a critical function to play in hibernation because blood pumping has to be maintained throughout torpor; indeed, although heart rate is extremely low in torpor, peripheral resistance increases substantially and to compensate for this the force of contraction actually increases [29]. It is not surprising, then, that myosin restructuring occurs in heart during hibernation, involving the hibernation-responsive up-regulation of selected myosin isoforms [21]. In skeletal muscle, the elevated content of ATF6-50 kDa in the cytoplasm

indicates that there is perhaps no change in ATF6-mediated gene expression during torpor but high cytoplasmic ATF6-50 kDa might be put to use when ground squirrels arouse from torpor (a process that can be measured in minutes for small hibernators) in order to support a rapid increase in gene expression at that time.

The main pathway of the UPR that was investigated in this study is the PERK/eIF2α/ATF4 pathway. Evidence has been presented that ATF4 requires eIF2α phosphorylation for its translation under stress conditions including oxidative stress in nonhibernating mammals [7]. ATF4 messenger RNA is an important mammalian transcript which is translated by eIf2α (P)-mediated bypass scanning of 5′ upstream open-reading frames [7,30]. Despite the fact that p-PERK levels did not change during hibernation in brain (Fig. 1A) and kidney (data not shown), high levels of phospho-eIF2α have been reported in tissues of hibernating ground squirrels [31,32]. Thus, an assessment of ATF4 expression levels in ground squirrel tissues was justified (Fig. 2). The data show that ATF4 was markedly elevated by 1.9- to 2.5-fold in BAT, brain and muscle during hibernation. These results suggest an important role for ATF4 in torpor and implicate the genes that are downstream of ATF4 as having potential roles in the hibernating phenotype. In order to determine whether the observed elevation of ATF4 protein levels resulted from increased transcription of the *atf4* gene, we quantified *atf4* mRNA levels in six tissues of euthermic versus hibernating ground squirrels. We amplified a 781 bp cDNA from ground squirrel BAT and verified that it encoded ATF4. RT-PCR analysis indicated no significant differences in *atf4* message levels between the two states in BAT, brain, kidney or muscle but *atf4* mRNA increased by 2.32-fold in liver and decreased in heart by 52% during torpor. However, despite the high levels of *atf4* message in liver, protein levels were unchanged in this organ between euthermic and hibernating conditions. Overall, then, the data suggest that the elevation of ATF4 protein in selected organs during hibernation does not occur at the transcriptional level but is a result of enhanced *atf4* translation. The reduced level of *atf4* mRNA in heart correlates with the decreased level of ATF4 protein in the same organ.

The partial ground squirrel *atf4* sequence that was retrieved encoded ~89% of the ATF4 protein (including the C-terminal) as compared with the full sequence from other mammals (Fig. 3). The squirrel ATF4 protein sequence showed high overall identity (82–86%) with the human, rat and mouse sequences. However, there were 19 unique amino acid substitutions that replaced amino acids that were fully conserved in the sequences from the nonhibernators. Unlike the situation for the majority of mammals, proteins in hibernating species must be able to function at both euthermic (37 °C) and hibernating (near 0 °C) T_b values. Maintenance of functional integrity over this wide temperature change may be aided by changes in amino acid sequence that could provide better support for protein function at low temperature. This could include substitutions that add greater flexibility to protein conformation or that sustain binding capacities for functional sites; e.g. several substitutions in the basic leucine zipper (bZIP) domain at the carboxyterminus of ground squirrel ATF4 could modulate its binding to DNA at low T_b.

CREB1, which is phosphorylated in response to ER stress, forms a complex in vivo with ATF4 to induce transcription of a set of downstream genes including *grp78* [11]. Total CREB1, as well as the amount of its active, phosphorylated form were measured in ground squirrel tissues (Fig. 5). Total CREB1 content did not change during torpor in four tissues and fell in the other two but p-CREB1 (Ser133) increased significantly in all organs tested during hibernation, rising by 2- to 16-fold. This indicates that ER stress occurring during hibernation induces the phosphorylation of CREB1 and mediates transcriptional activation of downstream genes via this route. These data are also consistent with studies on another hibernator that found 2- to 7-fold higher levels of phospho-CREB1 during hibernation in organs of Richardson's ground squirrels (*S. richardsonii*) [33].

Elevated levels of both ATF4 and p-CREB1 in selected organs of hibernators implicate their importance but both need to migrate to the nucleus to influence gene expression. Hence, we analyzed the distribution of these transcription factors between cytoplasmic and nuclear fractions of brain and skeletal muscle in euthermic and hibernating squirrels. These studies showed moderate, but significant, elevation of nuclear ATF4 content and extremely large increases (25- to 39-fold) in nuclear p-CREB1 abundance during hibernation (Fig. 7). As a co-activator of ATF4, the translocation of p-CREB1 into nuclei during hibernation could strongly impact the amount of ATF4·p-CREB1 complex present, particularly if the amount of nuclear p-CREB1 was typically a limiting factor in complex formation. The result would be activation of genes under the control of these transcription factors. Hence, elements of the UPR that provide protection to ER proteins during hibernation (such as enhanced GRP78 expression) appear to be under the control of ATF4 and p-CREB1 transcription factors.

Furthermore, our Western blot analysis of GADD proteins revealed differential expression in heart: GADD153 increased and GADD34 decreased. Further work remains to be done to determine whether the elevated levels of GADD153 in heart leads to some amount of apoptosis during hibernation. The decrease in GADD34, which plays a role in recruiting protein phosphatase 1 to dephosphorylate eIF2α, is consistent with maintaining high levels of phospho-eIF2α in hibernator tissues. This response by GADD34 might be rapidly reversed during the brief periods of arousal when T_b rises again to euthermic values. Thus, GADD34 could represent the intermediate signal for the switch from global translation inhibition to the resumption of normal translation rates as animals arouse from torpor. This is consistent with previous studies [12,14] showing that GADD34 forms a negative feedback regulatory loop and that its up-regulation mediates eIF2α dephosphorylation followed by inhibition of ATF4 expression and, thereby, mediates cellular recovery after ER stress.

In summary, the results of the present study indicate that expression of ATF4 increased in BAT, brain, and skeletal muscle during hibernation, indicating ER stress, despite unchanged levels of p-PERK, one of the eIF2α kinases. The increase in ATF4 protein appears to be due to enhanced translation of *atf4* message rather than increased transcription of the *atf4* gene. Protein synthesis is strongly suppressed in torpor and one mechanism that would contribute to this is the reduced levels of GADD34 that would help to keep eIF2α in the phosphorylated inactive state during torpor. ATF4 involvement in the ER stress–response links both branches of the UPR with the induction of pro-apoptotic GADD153 protein and pro-survival proteins such as GRP78 [5]. ATF4, accompanied by its nuclear cofactor p-CREB1 may mediate the adaptive gene expression responses to ER stress during hibernation. By understanding the patterns of gene expression in the UPR pathway during mammalian hibernation, we can gain knowledge of both the key metabolic problems faced by different organs during torpor and the natural solutions that have been developed. Interestingly, a recent study on ER stress and oxidative stress has linked the ATF4 and Nrf2 signaling pathways, suggesting that the phosphorylated form of Nrf2 might also participate in ER stress signaling by promoting expression of genes encoding antioxidants as well as genes implicated in cell growth, protein folding and cell survival [8].

Acknowledgments

We are grateful to Dr. J. Hallenbeck, National Institute of Neurological Disorders and Stroke, Bethesda, MD, for providing samples

of ground squirrel tissues. We thank J.M. Storey for helpful discussions and editorial review of the manuscript. This research was supported by the Natural Sciences and Engineering Research Council of Canada and by the Canada Research Chairs program.

References

[1] M. Schröder, R.J. Kaufman, Ann. Rev. Biochem. 74 (2005) 739–789.
[2] D.T. Rutkowski, R.J. Kaufman, Trends Cell Biol. 14 (2004) 20–28.
[3] L.C.H. Wang, T.F. Lee, in: M.J. Fregley, C.M. Blatteis (Eds.), Handbook of Physiology: Environmental Physiology, vol. 1, Oxford University Press, New York, 1996, pp. 507–532 (Section 4).
[4] A.S. Lee, Methods 35 (2005) 373–381.
[5] H. Mamady, K.B. Storey, Mol. Cell. Biochem. 292 (2006) 89–98.
[6] H.-Y. Jiang, R.C. Wek, J. Biol. Chem. 260 (2005) 14189–14202.
[7] H.P. Harding, I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, D. Ron, Mol. Cell 6 (2000) 1099–1108.
[8] S.B. Cullinan, J.A. Diehl, Int. J. Biochem. Cell Biol. 38 (2006) 317–332.
[9] J. Shen, R. Prywes, J. Biol. Chem. 279 (2004) 43046–43051.
[10] T. Okada, H. Yoshida, R. Akazawa, M. Negishi, K. Mori, Biochem. J. 366 (2002) 585–594.
[11] S. Luo, P. Baumeister, S. Yang, S.F. Abcouwer, A.S. Lee, J. Biol. Chem. 378 (2003) 37375–37385.
[12] J.D. Blais, V. Philipenko, M. Bi, H.P. Harding, D. Ron, C. Koumenis, B.G. Wouters, J.C. Bell, Mol. Cell. Biol. 24 (2004) 7469–7482.
[13] X.Z. Wang, B. Lawson, J.W. Brewer, H. Zinszner, A. Sanjay, L.J. Mi, R. Boorstein, G. Kreibich, L.M. Hendershot, D. Ron, Mol. Cell. Biol. 16 (1996) 4273–4280.
[14] Y. Ma, L.M. Hendershot, J. Biol. Chem. 278 (2003) 34864–34873.
[15] K.B. Storey, Adv. Exp. Med. Biol. 543 (2003) 21–38.
[16] K.B. Storey, J.M. Storey, J. Exp. Biol. 210 (2007) 1700–1714.
[17] B. Buzadzic, M. Spasic, Z.S. Saicic, R. Radojicic, V.M. Petrovic, B. Halliwell, Free Radic. Biol. Med. 9 (1990) 407–413.
[18] K.L. Drew, O. Toien, P.M. Rivera, M.A. Smith, G. Perry, M.E. Rice, Comp. Biochem. Physiol. C 133 (2002) 483–492.
[19] S.F. Eddy, J.D. McNally, K.B. Storey, Arch. Biochem. Biophys. 435 (2005) 101–111.
[20] K.B. Storey, J.M. Storey, Biol. Rev. 79 (2004) 207–233.
[21] K. Lee, J.Y. Park, W. Yoo, T. Gwag, J.W. Lee, M.W. Byun, I. Choi, J. Cell. Biochem. 104 (2008) 642–656.
[22] D. Hittel, K.B. Storey, J. Exp. Biol. 205 (2002) 1625–1631.
[23] K.U. Frerichs, C. Kennedy, L. Sokoloff, J.M. Hallenbeck, J. Cereb. Blood Flow Metab. 14 (1994) 193–205.
[24] C. Koumenis, N.C. Denko, M. Koritzinski, S. Rastani, A. Diehl, N. Sonenberg, A. Koromilas, B.G. Wouters, Mol. Cell. Biol. 22 (2002) 7405–7416.
[25] M.J. Clemens, Prog. Mol. Subcell. Biol. 27 (2001) 57–89.
[26] Y. Wang, J. Shen, N. Arenzana, W. Tirasophon, R.J. Kaufman, R. Prywes, J. Biol. Chem. 275 (2000) 27013–27020.
[27] H. Yoshida, T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi, K. Mori, Mol. Cell. Biol. 20 (2000) 6755–6767.
[28] J. Shen, R. Prywes, Methods 35 (2005) 382–389.
[29] L.C.H. Wang, in: L.C.H. Wang (Ed.), Advances in Comparative and Environmental Physiology, vol. 4, Springer, Heidelberg, 1989, pp. 361–401.
[30] D.J. DeGracia, R. Kumar, C.R. Owen, G.S. Krause, B.C. White, J. Cereb. Blood Flow Metab. 22 (2002) 127–141.
[31] K.U. Frerichs, C.B. Smith, M. Brenner, D.J. DeGracia, G.S. Krause, L. Marrone, T.E. Dever, J.M. Hallenbeck, Proc. Natl. Acad. Sci. USA 95 (1998) 14511–14516.
[32] D. Hittel, K.B. Storey, Arch. Biochem. Biophys. 401 (2002) 244–254.
[33] J.A. MacDonald, K.B. Storey, Intl. J. Biochem. Cell Biol. 37 (2005) 679–691.