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Up-regulation of the endoplasmic reticulum molecular chaperone GRP78 during hibernation in thirteen-lined ground squirrels

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Abstract

Hibernating mammals endure conditions of low body temperature and oxidative stress that would be highly injurious to humans and most other mammals. Stress conditions frequently trigger the production of molecular chaperones; in the endoplasmic reticulum the glucose-regulated protein-78 (GRP78) helps to minimize protein misfolding under stress. The present study evaluated the GRP78 response in seven organs of hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. Transcript levels of *grp78*, assessed by RT-PCR, were significantly higher (3.5- to 4.1-fold) in brown adipose tissue and brain of hibernating squirrels compared with euthermic control animals but remained low or stable in all other tissues. GRP78 protein content, assessed by Western blotting, was also elevated in brown adipose and brain during hibernation by 1.4–1.6 fold. A 2490 bp cDNA sequence was retrieved that contained the full length open reading frame of ground squirrel *grp78* and the translated protein sequence of 654 amino acids shared 98–99% identity with GRP78 from other mammalian sources. Selected specific amino acid substitutions were found in the ground squirrel sequence that may aid GRP78 function under the near 0 °C body temperatures of the hibernating state. Electrophoretic mobility shift and supershift assays showed that the activating transcription factor, ATF4, binds to the promoter region of the *grp78* gene in ground squirrel brain and may be responsible for *grp78* up-regulation during hibernation. Changes in *grp78* gene and protein expression appear to aid stress tolerance in two highly oxygen-dependent organs that are critical to whole animal survival during hibernation. (Mol Cell Biochem **292:** 89–98, 2006)

Key words: *Spermophilus tridecemlineatus*, torpor, glucose-regulated protein 78, activating transcription factor 4, unfolded protein response, endoplasmic reticulum stress

Introduction

Mammalian hibernation is an energy-conservation strategy that includes biochemical, physiological and behavioral adjustments that allow animals to live for extended periods of time at very low metabolic rates (often 1–5% of normal) and low core body temperatures (often near 0 °C) [1]. Hibernation consists of extended periods of cold torpor interspersed with brief periods of arousal (12–24 h) back to euthermia (∼37 °C body temperature). Vital functions drop to very low levels during torpor; for example, heartbeat and breathing rate are profoundly depressed and organ perfusion rates can drop to ∼10% of normal [2]. Mechanisms that support hibernation include a profound suppression of overall metabolic rate, a reorganization of cell priorities for ATP use to preferentially turn down many nonessential ATP-consuming cell functions,

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and enhanced expression of selected genes whose products aid long term survival in the torpid state [3, 4]. One of the ATP-consuming functions that is suppressed in hibernator organs is protein synthesis; this is accomplished by mechanisms including polysome disaggregation and reversible phosphorylation control of ribosomal initiation and elongation factors [3, 5]. However, selective synthesis of some proteins continues; for example, increased expression of multiple proteins involved in lipid transport/catabolism and mitochondrial energy metabolism has been documented at both gene and protein levels during hibernation [3–7].

Protein synthesis during hibernation occurs under cellular conditions that are very different from the normal situation for mammals. For example, body temperature (Tb) can vary over at least 35 °C. Temperature change not only alters reaction rates but also affects the strength of the weak bonds that are critical to the conformation of macromolecules, the conformational changes that occur during ligand binding and catalysis in enzymes, and various macromolecular interactions such as protein-protein, protein-DNA, and protein-membrane binding. Such effects of low temperature could alter critical steps in the synthesis and processing of new proteins. Arousal from torpor also generates significant oxidative stress associated with the tremendously rapid increase in oxygen uptake and oxygen consumption that is needed to fuel thermogenesis [8, 9]. These stresses in nonhibernating mammals can cause protein misfolding and saturation of the folding pathways in the endoplasmic reticulum (ER) creating a condition called the unfolded protein response (UPR) [10]. Hence, we predicted that hibernators would need to have mechanisms to minimize protein misfolding during protein synthesis under the unusual cellular conditions of the hibernating state. One of these mechanisms could be the enhanced production of molecular chaperones such as the 78 kDa glucose regulated protein (GRP78).

GRP78 is a constitutively expressed protein that is a member of the heat shock protein 70 family of molecular chaperones. It resides in the ER and is involved in the folding and assembly of newly synthesized proteins [11–13]. GRP78 also has an anti-apoptotic function to prevent ER stress-induced cell death [12] and it is used as a biomarker for the onset of the UPR [13]. The present study examines both mRNA and protein expression of GRP78 in seven organs of hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*, documenting strong hibernation-responsive up-regulation of the protein in two key oxygen-sensitive organs – brain and brown adipose tissue (BAT). We further examined the transcriptional control of ground squirrel *grp78*, providing evidence that transcriptional activation of the *grp78* promoter during hibernation may be linked to the activating transcription factor 4 (ATF4).

Materials and methods

Animal experiments and tissue collection

Thirteen lined ground squirrels, *Spermophilus tridecemlineatus* (130–180 g), were captured by a licensed trapper (TLS Research, Michigan) and transported to the Animal Hibernation Facility at the National Institute of Neurological Disorders and Stroke (NIH, Bethesda, MD). All animal experiments were conducted by the laboratory of Dr. J.M. Hallenbeck using their standard protocols [14] that are approved by the NIH Institutional Animal Care and Use Committee. Briefly, 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 Tb of each animal was monitored electronically. When squirrels had reached a plateau weight of 220–240 g, they were placed in their cages containing wood shavings into a cold chamber at 4 °C and 60% humidity. The hibernaculum was kept in constant darkness, except for a photographic red safe light (3–5 lux), and could be entered only through a darkened anteroom. Noise within the chamber was kept to a minimum. 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 Tb readings of ∼6 °C). Animals that had not entered torpor after at least 3 days in the cold room and that showed continuous high Tb (36–38 °C) were sampled as controls. All animals were killed by decapitation and tissues were excised, frozen immediately in liquid nitrogen and transported to Ottawa on dry ice where they were placed at −80 °C until use.

Total RNA extraction

All materials and solutions were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and autoclaved prior to use. Total RNA was isolated from tissues of control and hibernating ground squirrels using Trizol™ reagent (Invitrogen), essentially following manufacturer's instructions. Briefly, 100 mg of tissue was homogenized in 1 mL Trizol using a Polytron homogenizer followed by the addition of 200 μL of chloroform and then centrifugation at 10,000 $\times$ g for 15 min at 4 °C. The upper aqueous phase that contains total RNA was removed to a fresh tube and RNA was precipitated by adding 500 μL of isopropanol followed by incubation at room temperature for 10 min. After a second centrifugation at 10,000 $\times$ g for 15 min at 4 °C, the total RNA pellet was washed with 1 mL of 70% ethanol and then centrifuged as above. The supernatant was removed and the pellet was air-dried for 10–15 min. A volume of

70 μL DEPC-treated water was added to resuspend the RNA pellet. Three samples of each tissue were separately extracted. The quality of total RNA was judged based on the ratio of absorbances at 260 nm and 280 nm. In addition, all RNA samples were assessed using 1% formaldehyde agarose gel electrophoresis with ethidium bromide staining to check for the integrity of 18S and 28S ribosomal RNA (rRNA) bands.

First strand cDNA synthesis and PCR amplification

Approximately 10–15 μg of total RNA was diluted with DEPC water to 10 μL final volume and used to synthesize cDNA. Briefly, 1 μL of 200 ng/μL oligo-dT (5′-TTTTTTTTTTTTTTTTTTTTTV-3′; V = A or G or C) (Sigma Genosys) that forms a hybrid with the polyA tail of mRNA was added to the RNA sample and placed in a 68 °C water bath for 5 min. The mixture was then chilled rapidly on ice and 4 μL 5X first strand buffer, 2 μL 10 mM DTT, 1 μL and 1 μL reverse transcriptase enzyme Superscript II (all reagents from Invitrogen) were added for a total volume of 19 μL. The mix was incubated at 42 °C for 45 min then held at 4 °C. The resulting cDNAs from both control and hibernating samples were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) and amplified by polymerase chain reaction (PCR) with *grp78* primers that were designed from the consensus sequence of mammalian *grp78*, using the Primer Designer program, version 3.0 (Scientific and Educational Software). The nucleotide sequences of the *grp78* primer pairs were 5′-AAGAACGGCCGCGTGGAGAT-3′ and 5′-CTCTTATCCAGGCCATAATGC-3′. The PCR reaction of 25 μL final volume was composed by mixing 14.37 μL of sterile water, 5 μL of diluted cDNA, 1.25 μL of primer mixture (0.5 μM forward and 0.5 μM reverse), 2.5 μL of 10X PCR buffer (Invitrogen), 1.25 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs and 0.13 μL of *Taq* Polymerase (Invitrogen). The PCR started with an initial step of 3 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C. The final step was 72 °C for 7 min. The housekeeping gene α-tubulin was amplified as an internal control; the primers used for this were forward (5′-AAGGAAGATGCTGCCAATAA-3′) and reverse (5′-GGTCACATTTCACCATCTG-3′). PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and the intensity of the most dilute cDNA sample that was visible was used for quantification purposes to make sure that the products had not reached their amplification saturation. Quantification was performed using a ChemiGenius Bio Imaging System and its associated Gene Tools software (Syngene, MD, USA).

Rapid amplification of cDNA ends (5′ and 3′ RACE)

A partial cDNA sequence of ground squirrel *grp78* (465 bp) was obtained by RT-PCR. To retrieve the full cDNA sequence, the technique of RACE was used. The 5′ end of *grp78* was amplified using two anti-sense gene specific primers (GSP) designed from the partial sequence. The nucleotide sequences of the primers were: 5′-GGTCACATTTCACCATCTG-3′ for GSP1 and 5′-GAGGTGAGCTGCTTCTTGACTGTATC-3′ for GSP2. The protocol used was as described by Gibco BRL with a few modifications. Briefly, 1 μg of total RNA from BAT of hibernating ground squirrels was reverse transcribed to cDNA in a sterile microcentrifuge tube containing the following in a final volume of 25 μL: 2.5 μL of GSP1 (1 μM), 2.5 μL of 10X PCR buffer, 2.5 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTPs and 1 μL of reverse transcriptase MMLV-RT (Invitrogen). After an incubation of 50 min at 42 °C, the tube was placed at 70 °C for 15 min to denature the reverse transcriptase. The mixture was collected from the side of the tube by brief spinning and then 1 μL of 2 U/μL RNase H was added followed by incubation for 30 min at 37 °C and then placing the sample on ice. To purify the cDNA, 2.5 μL of 3 M sodium acetate pH 5.3 and 25 μL isopropanol (Sigma) were added and the mixture was incubated for 2 h at –20 °C followed by centrifugation at 10,000 × *g* for 20 min, and retrieval of the pellet which was resuspended in sterile water. A dC tail was then added onto the cDNA by incubation with 2 mM dCTP and a terminal deoxynucleotidyl-terminal transferase according to the manufacturer's protocol (Invitrogen). Next, PCR was performed using the dC-tailed cDNA with GSP2 and a universal primer AAP (5′-GGCCACGCGTCGACTAGTACGGGGGGGGGG-3′) (Sigma Genosys). Cycles for amplification were 3 min at 94 °C for the initial step followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min with a final step of 72 °C for 7 min. The resulting PCR product was diluted 100 times with sterile water and 2.5 μL was used in a second round of PCR as described above with *grp78* gene specific primer and the universal primer AUAP (5′-GGCCACGCGTCGACTAGTAC- 3′) (Sigma Genosys). The PCR products were separated on a 1% agarose gel to confirm the amplification of a cDNA with the expected size.

The 3′ end was amplified using a Takara 3′ RACE kit (Promega). Total RNA (1 μg) was reverse transcribed into cDNA according to the manufacturer's instructions, using an oligo-dT-3 sites primer to initiate the first strand synthesis. The resulting cDNA was amplified with *grp78* gene specific primer (5′-CAGCTGCTATTGCTTATGGCCTGGAT-3′) and the 3-sites adaptor primer (Promega). After performing 35 cycles of PCR (94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min) and a final extension at 72 °C for 7 min, the PCR products were separated on a 1% agarose gel and analyzed as above.

Sequencing

RT-PCR and RACE products were sequenced by either Canadian Molecular Research Services (Ottawa, ON) or by CORTEC (Kingston, ON). Sequences were verified as encoding the *grp78* using the program BLASTN (http://www.ncbi.nlm.nih.gov/blast) at the NIH. The deduced amino acid sequences were also verified as being GRP78 by using the program BLASTP at the same site. The sequence was submitted to GenBank with the accession number DQ166628.

Protein extraction and Western blot analysis

Western blotting was used to examine the levels of GRP78 protein in tissue samples from euthermic and hibernating animals. Cell-free extracts were prepared by homogenizing frozen tissue samples (500 mg) using a Polytron homogenizer and 1 mL of homogenizing buffer containing 20 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 10 mM ß-glycerophosphate, 1% v/v Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. After a 15 min centrifugation at 10,000 × *g*, supernatants were removed and soluble protein levels were determined using the BioRad protein assay (BioRad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Aliquots of supernatant were then mixed 1:1 v/v with 2X SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 4% w/v SDS, 20% v/v glycerol, 0.4% w/v bromophenol blue) and 10% v/v 2-mercaptoethanol, boiled and stored at −20 °C until use. Equal amounts of protein from control and hibernating lysates (10–30 μg depending on the experiment) were then layered on 10 or 12% SDS polyacrylamide gels (10% or 12% acrylamide, 0.4 M Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.04% TEMED), covered with a 5% stacking gel (5% acrylamide, 0.13 M Tris (pH 6.8), 0.1% SDS, 0.1% APS, 0.1% TEMED) and separated at 180 V for 1 h. Kaleidoscope prestained molecular mass markers (Bio-Rad) were used to estimate the size and positions of proteins on the gel. Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Pall) by wet transfer with pre-chilled solution containing 25 mM Tris (pH 8.5), 192 mM glycine, and 20% v/v methanol at 4 °C for 2 h at 70 V. After the transfer of proteins, membranes were blocked for 1 h in Tris buffered saline containing Tween-20 (TBST: 20 mM Tris base, 140 mM NaCl, 0.05% v/v Tween-20) with 2.5% non-fat dried milk added. The blots were then incubated at 4 °C overnight with primary antibody diluted 1:20000 v/v in TBST; the primary was rabbit anti-GRP78 (BiP) polyclonal antibodies (Stressgen). After incubation, blots were washed several times with TBST and then incubated at room temperature for 2 h with a 1:2000 v/v dilution of secondary antibody, anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling). 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 and the resulting image was analyzed with Gene Tools software (Syngene, MD, USA). Equal protein loading was confirmed by Coomassie blue staining of the membrane.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from brain of euthermic and hibernating ground squirrels were prepared using a slight modification of the method described by Dignam *et al*. [15]. Briefly, 0.5 g of tissue was disrupted at 4 °C 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]) 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 gently shaking. After centrifugation at 10,000 × *g* for 10 min the supernatant (nuclear extract) was collected. Protein concentration in the nuclear fraction was determined by the Bio-Rad protein assay. Since transcription factors are known to move inside the nucleus where they activate downstream genes, the separation of nuclear and cytoplasmic fractions is critical. To confirm the separation of these two fractions, 10 μg of protein from cytoplasmic and nuclear extracts of both euthermic and hibernating animals were separated by SDS-PAGE and then gels were subjected to Western blotting with anti-histone H3 antibody (Cell Signaling) at a dilution of 1/1000, followed by anti-rabbit secondary antibody as above. The histone H3 band was found only in the nuclear extracts and not in the cytoplasmic extracts, thereby confirming the integrity of the nuclei when separated from the cytoplasmic fraction (data not shown).

The electrophoretic mobility shift assay was performed as described by Roy and Lee [16] with slight modifications and using radiolabeled synthetic oligonucleotides (probe) containing a sequence corresponding to a *cis*-acting element, the ATF4 binding site (5′-TGACGTGA-3′) in the *grp78* promoter [17]. The oligonucleotides used for probing were designed from the rat *grp78* promoter with sequences as follows (ATF4 binding site is underlined): 5′-CGCGTACCAGTGACGTGAGTTGCGGAGG-3′ and the complementary strand 5′-CCTCCGCAACTCACGTCA-

CTGGTACGCG-3′. Mutant probes were: 5′-CGCGTA-CCAGGTCGACTCGTTGCGGAGG-3′ and 5′-CCTCCGC-AACGAGTCGACCTGGTACGCG-3′ (mutated ATF4 binding site underlined). The above oligonucleotides were annealed into double-stranded form and were ^{32}P 5′-end-labeled with T4 polynucleotide kinase (New England Biolabs), using 5 μL/reaction of 10 mCi/mL ^{32}P-γ-dATP (Amersham). Prior to starting the binding reaction, the nuclear extract was digested with restriction enzymes that do not cut inside the promoter region used, *EcoRI* (Gibco BRL) and *SmaI* (New England Biolabs), to shear the genomic DNA contained in the nuclear extract. Equal amounts (20 μg) of digested nuclear extract from brain of euthermic and hibernating squirrels were incubated for 5 min at room temperature in 20 μL of binding reaction with final concentrations of reagents: 50 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.5 mM dithiothreitol, 5% v/v glycerol and 0.3 μg poly(dIdC). For specific and nonspecific competition with the labeled probe, 10X more unlabeled probe or mutant probe, respectively, were added to the binding reaction mixture before incubation. Then 160 ng of double stranded, radiolabeled oligonucleotide probe was added to the reaction mixture and incubated for a further 20 min at room temperature. Antibodies against CREB-1, a cofactor of ATF4) as well as Bcl-2 (used as negative control) were purchased from Santa Cruz Biotechnology. The antibodies were then added to each reaction and incubated at room temperature for another 20 min. DNA–protein complexes were separated from unbound DNA probe by electrophoresis on native 5% polyacrylamide gel at 300 V (∼25 mA) in 45 mM Tris/borate (pH 8.0) containing 1 mM EDTA. The resolved gel was dried and exposed to a phosphor screen (BioRad).

Statistical analysis

RT-PCR ($n = 3$) and Western Blotting ($n = 3$–5) data were gathered from independent tissue extracts from different animals, a total pool size of $n = 6$ individuals being available for each treatment group. RT-PCR bands for *grp78* were normalized relative to α-tubulin bands obtained from the same cDNA reaction whereas Western blot bands specific to GRP78 protein were normalized against three Coomassie stained protein bands that did not change in intensity between euthermic and hibernating conditions. Data are expressed as means with corresponding SEM. Statistical testing of normalized band intensities from euthermic versus hibernating samples used the Student's *t*-test with significance levels of $P < 0.05$. The ratio hibernating/euthermic was then calculated and plotted in histograms. Error bars shown on histograms are the sum of SEM values for euthermic and hibernating trials.

Results

Analysis of grp78 transcript levels

Using forward and reverse primers designed from a consensus sequence of human, mouse and rat *grp78* (GenBank accession numbers: NM_005347, NM_022310 and NM_013083 respectively), a 465 bp cDNA was amplified from BAT of ground squirrels. After nucleotide sequencing, the cDNA was shown to encode *grp78*; the nucleotide sequence shared 94% identity with human *grp78* cDNA and its deduced amino acid sequence was 99% identical with human GRP78 protein.

The primers were then used in RT-PCR to assess *grp78* mRNA levels in seven tissues (BAT, brain, heart, kidney, liver, lung, skeletal muscle) of euthermic versus hibernating ground squirrels. Figure 1A shows *grp78* mRNA expression in each organ together with expression levels of α-tubulin. Preliminary studies in our laboratory showed that α-tubulin mRNA levels were unchanged in 13-lined ground squirrel tissues between euthermic and hibernating states. Transcript levels of *grp78* were normalized against α-tubulin amplified from the same samples, expression ratios were calculated, and mean ratios in hibernation versus euthermia are shown in Figure 1B. Transcript levels of *grp78* increased significantly ($P < 0.05$) by 3.5-fold in BAT and 4.1-fold in brain of hibernating squirrels compared with euthermic controls but remained stable in all other tissues.

Cloning of S. tridecemlineatus grp78 cDNA

To retrieve the full cDNA sequence of ground squirrel *grp78*, GSPs were designed from the 465 bp partial *grp78* cDNA sequence and used in 5′ and 3′ RACE to extend and amplify the remaining portions of the ground squirrel *grp78* sequence. A final assembled cDNA sequence of 2490 bp contained the full open reading frame (ORF) of ground squirrel *grp78* cDNA and was submitted to GenBank with accession number DQ166628. Translation of the ORF revealed a protein of 654 amino acids as in human and rat GRP78 (mouse GRP78 has 655 amino acids). Figure 2 shows the alignment of ground squirrel GRP78 with the amino acid sequences of human, mouse and rat GRP78. The squirrel protein was 99% identical to the human protein and 98% identical to mouse and rat GRP78. Four amino acid substitutions were evident in the squirrel sequence as compared with the other mammalian species (residues shown in bold underline in Figure 2). These substitutions were a valine at position 8 compared with methionine or alanine in the other species, a hydrophilic serine substitution at position 451 replacing a hydrophobic phenylalanine in the other sequences, and two substitutions of arginine at positions 554 and 651 replacing lysine in the other sequences.

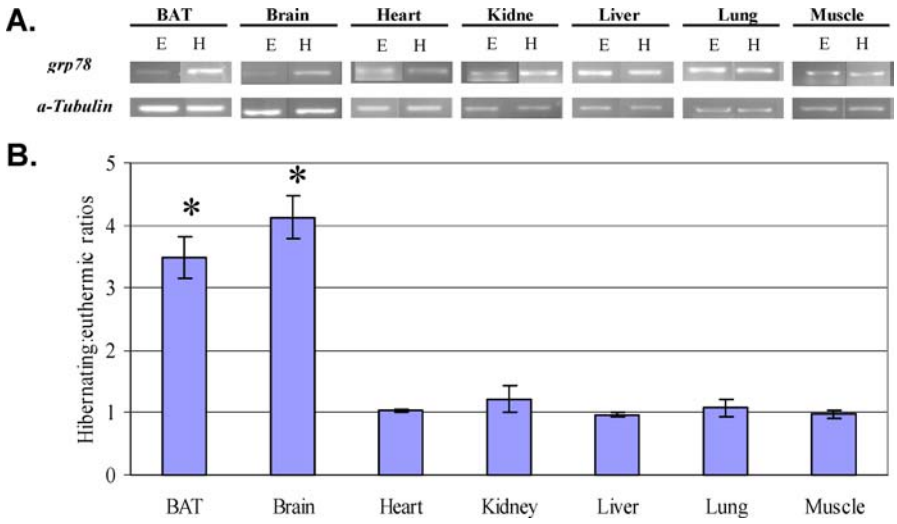

Fig. 1. Effect of hibernation on *grp78* mRNA levels in seven tissues of thirteen-lined ground squirrels. (A) Representative PCR product bands amplified from mRNA samples from tissues of euthermic and hibernating squirrels. (B) Histogram showing the mean ratio of normalized PCR product levels (relative to α-tubulin) in tissues from hibernating versus euthermic ground squirrels. The highest dilutions for *grp78* (10^{-4} or 10^{-5}) and α-tubulin (from the same sample tube) which gave visible PCR product bands were chosen for quantification. Data are means $\pm$ S.E.M., $n = 3$ independent trials. BAT is brown adipose tissue; muscle is hind leg skeletal muscle; E – euthermic, H – hibernating. * – Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

GRP78 protein levels

Western blotting assessed GRP78 expression in ground squirrel tissues using a rabbit anti-GRP78 polyclonal antibody raised against rat GRP78 synthetic peptide (residue 645–654 at the C-terminal). On one-dimensional SDS-PAGE gels, all tissues showed a single band, corresponding to the known molecular mass (78 kDa) of GRP78. The specificity of the GRP78 antibody was further confirmed with two-dimensional electrophoresis which resulted in antibody cross-reaction with only a single spot on the gel (data not shown) corresponding with the known molecular mass and isoelectric point (pI = 5.0) of mouse GRP78 [18]. Figure 3A shows representative Western blots of GRP78 protein levels in seven tissues of ground squirrels and Figure 3B shows the mean ratio hibernating:euthermic in each tissue. During hibernation, GRP78 levels were significantly elevated in BAT and brain, by 1.57- and 1.37-fold, respectively, whereas protein content significantly decreased in heart and liver to 81- and 58% of the euthermic value, respectively. The results for BAT and brain were consistent with the transcriptional up-regulation shown in Figure 1B.

Binding of ATF4 to the grp78 promoter

ATF4 is a transcription factor that is known to regulate *grp78* expression in other systems. To determine whether ATF4 might also be involved in the hibernation-responsive expression of *grp78*, the interaction of ATF4 with the *grp78* promoter was investigated in gel mobility shift assays using synthetic radiolabeled oligonucleotides containing the ATF/CRE site, the binding domain of ATF4. Figure 4A shows that the ^{32}P-labeled probe bound to three major protein complexes on the gel (I, II and III) as seen in lanes 2 and 3. Competition experiments were used to determine which complex was specific for ATF4 binding. When unlabeled wild type probe was used as a competitor, ^{32}P-labeling of complex I and II disappeared (lane 5). However, when mutant probe (containing a mutated ATF4 binding site) was used only complex II was out-competed (lane 4). From this we concluded that only complex I represented the specific ATF4/*grp78* complex. None of the competitors inhibited the formation of complex III which was present in all the lanes. Furthermore, to confirm that complex I was specific to ATF4 binding, a supershift assay was performed with an antibody directed against a nuclear cofactor of ATF4, CREB-1. Previous studies have provided evidence that CREB-1 forms an *in vivo* complex with ATF4 to achieve the activation and regulation of genes [17]. Anti-Bcl2 antibody was used to control for nonspecific binding in the supershift assay. Figure 4B shows a shifted band of complex I (lanes 3 and 4) at a position of slower mobility in the presence of anti-CREB1 antibody. In contrast, no supershift was observed with anti-Bcl2 antibody (lanes 5 and 6).

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Squirrel   .MKLSLVAAVLLLLCAARAEEEDKKEDVGTVVGIDLGTTYSCVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDA    79
Human      .--------m----s-------------------------------------------------------------------    79
Mouse      m--ftv---a----g-v-----------------------------------------------------------------    80
Rat        .--ftv---a------v-----------------------------------------------------------------    79

Squirrel   AKNQLTSNPENTVFDAKRLIGRTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVLTKMKETAEA   159
Human      ---------------------------------------------------------------------------------   159
Mouse      ---------------------------------------------------------------------------------   160
Rat        ---------------------------------------------------------------------------------   159

Squirrel   YLGKKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDN   239
Human      ---------------------------------------------------------------------------------   239
Mouse      ---------------------------------------------------------------------------------   240
Rat        ---------------------------------------------------------------------------------   239

Squirrel   GVFEVVATNGDTHLGGEDFDQRVMEHFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKRALSSQHQARIEIESFYEGEDFS   319
Human      ---------------------------------------------------------------------------------   319
Mouse      ------------------------------------------------------------------------f------   320
Rat        ------------------------------------------------------------------------f------   319

Squirrel   ETLTRAKFEELNMDLFRSTMKPVQKVLEDSDLKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAA   399
Human      ---------------------------------------------------------------------------------   399
Mouse      ------------------------e--------------------------------------------------------   400
Rat        ---------------------------------------------------------------------------------   399

Squirrel   VQAGVLSGDQDTGDLVLLDVCPLTLGIETVGGVMTKLIPRNTVVPTKKSQISSTASDNQPTVTIKVYEGERPLTKDNHLL   479
Human      ----------------------------------------------------f----------------------------   479
Mouse      ----------------------------------------------------f----------------------------   480
Rat        ----------------------------------------------------f----------------------------   479

Squirrel   GTFDLTGIPPAPRGVPQIEVTFEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVNDAEKFAEEDKRLKERI   559
Human      ---------------------------------------------------------------------------k-----   559
Mouse      ----------------------------i----------------------------------------------k-----   560
Rat        ---------------------------------------------------------------------------k-----   559

Squirrel   DTRNELESYAYSLKNQIGDKEKLGGKLSSEDKETMEKAVEEKIEWLESHQDADIEDFKAKKKELEEIVQPIISKLYGSAG   639
Human      ---------------------------------------------------------------------------------   639
Mouse      ---------------------------------------------------------------------------------   640
Rat        ----------------------------p----------------------------------------------------   639

Squirrel   PPPTGEEDTSERDEL                                                                     654
Human      ---------a-k---                                                                     654
Mouse      ----------k---                                                                      655
Rat        ----------k---                                                                      654
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Fig. 2. Ground squirrel (*Spermophilus tridecemlineatus*) GRP78 full length amino acid sequence (GenBank accession number DQ166628) aligned with human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*) sequences (Genbank accession numbers NP_005338, NP_071705 and NP_037215, 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 of the species.

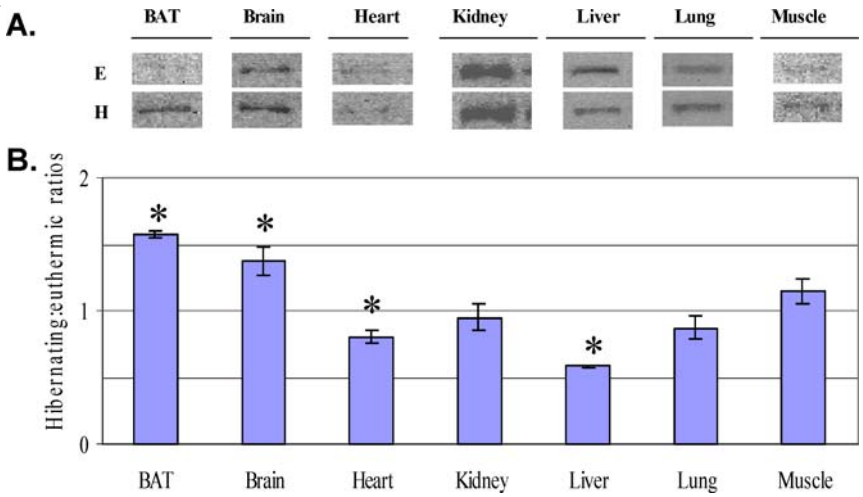

Fig. 3. (A) Representative Western blots showing GRP78 protein levels in seven tissues from euthermic (E) and hibernating (H) ground squirrels. (B) Histogram showing the relative levels of GRP78 protein in tissues from hibernating versus euthermic animals. Data are means ± S.E.M., $n = 3$–5 independent trials. * - Mean value for hibernating sample is significantly different from the corresponding euthermic value, $P < 0.05$.

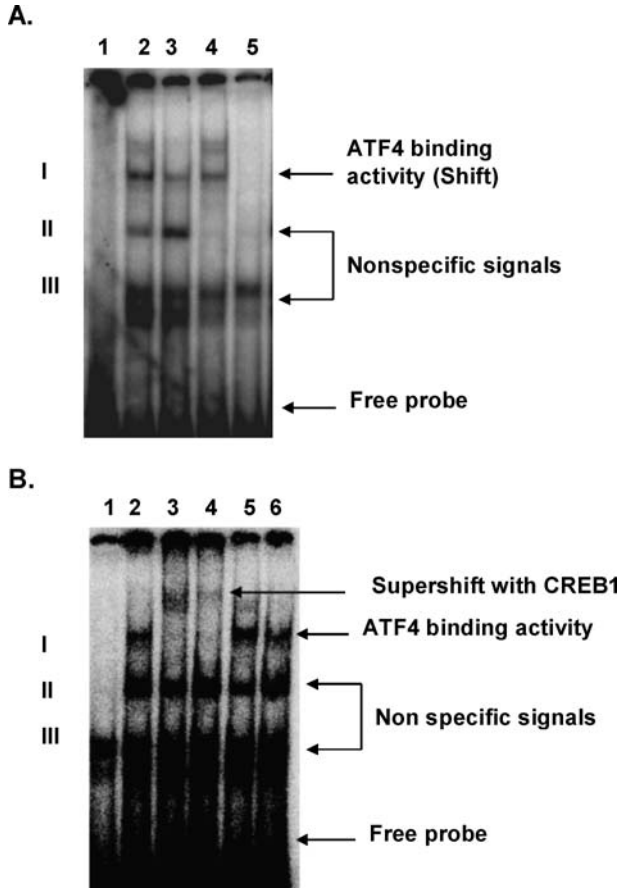

Fig. 4. Electrophoretic mobility shift and supershift assays using nuclear extracts from brain of 13-lined ground squirrels. The ATF/CRE probe was designed from the rat *grp78* promoter and contains the binding domain for the transcription factor ATF4. (A) Electrophoretic mobility shift: Lane 1, free probe; Lanes 2 and 3, nuclear extracts from hibernating and euthermic animals, respectively, with no competitor; Lanes 4 and 5, nuclear extracts from hibernating squirrels preincubated with competitors before adding the labeled probe (lane 4: mutant probe for nonspecific competition, lane 5: wild type unlabeled probe for specific competition). (B) Gel mobility shift and supershift assays. Lanes 1 and 2, nuclear extract from hibernating squirrels incubated with unlabeled wild type probe and no competitor, respectively. In Lanes 3 and 5 (hibernating nuclear extract) and Lanes 4 and 6 (euthermic nuclear extract) 1 μg of anti-CREB1 antibody (lanes 3 and 4) or anti-Bcl2 antibody (lanes 5 and 6) were added and incubated for 20 min before adding labeled probe.

Discussion

The accumulation of unfolded or misfolded proteins triggers the UPR, which then acts to release cells from the stress condition [10, 19]. The UPR encompasses a number of complex signal transduction cascades, responsible for various cytoprotective measures, that are undertaken when cells are experiencing ER stress [10]. GRP78 accumulation is an integral part of the UPR [13] and various lines of evidence indicate that GRP proteins have a neuroprotective role against excitotoxicity and apoptosis by suppressing oxidative stress and stabilizing calcium homeostasis [20]. The up-regulation of GRP78 in two key organs, BAT and brain, during hibernation provides presumptive evidence that ground squirrel organs may undergo ER stress during hibernation. Gene expression was strongly increased with *grp78* mRNA transcript levels being 3.5- and 4.1-fold higher, respectively, in BAT and brain of hibernators, compared with euthermic controls, whereas GRP78 protein levels also rose significantly by 1.57- and 1.37-fold, respectively. Interestingly, in a study on hibernating bats (*Rhinolopus ferrumequinum*), Lee *et al.* [20] found that GRP78 protein levels increased by 1.7-fold in bat brain within the first 30 min of arousal, compared with levels in the torpid state. Combined with our data, this may indicate that the elevation of *grp78* transcript levels that is initiated during entry into torpor supports GRP78 protein synthesis not only during torpor but also further enhances GRP78 production during arousal. This may be a valuable mechanism for ensuring that GRP78 chaperone levels are optimally elevated to deal with ER stress during arousal. ER stress may arise particularly from oxidative stress during arousal and other studies have shown that hibernators show adaptations of antioxidant defense mechanisms including have high constitutive activities of antioxidant enzymes and metabolites and up-regulation of selected antioxidant enzymes during torpor [8, 9, 21–23].

It is interesting that GRP78 up-regulation during hibernation was confined to two organs of ground squirrels that play essential roles in survival during hibernation, brain and BAT. Brain must maintain its sensing and signaling capacities over Tb values that can range from 0 to 37 °C. BAT is the key organ that is responsible for rewarming the animal back to euthermic Tb during arousal from torpor. BAT also provides low level heating of the body during torpor if ambient temperature drops below 0 °C. Hence, unlike most other tissues of hibernators, BAT maintains high metabolic activity during hibernation. Multiple hibernation-responsive proteins are synthesized and the ribosomal protein synthesis machinery is not suppressed in BAT during torpor as it is in other organs [5, 24]. This allows adjustments to be made to the thermogenic capacity of the organ in response to ambient environmental conditions; for example, if ambient air temperature in the burrows of ground squirrels falls below 0 °C, the thermogenic capacity of BAT is enhanced by upregulation and increased synthesis of the mitochondrial uncoupling protein 1 (UCP 1) [25]. It is notable, therefore, that the two organs that are most critical to survival at low Tb during torpor are the ones that show GRP78 up-regulation during torpor – the brain that detects Tb (in the hypothalamus) and determines the timing of periodic arousals and the BAT that provides both low level heating to prevent freezing and high level thermogenesis to power arousal.

Previous work on GRP78 has shown that the protein sequence contains four evolutionarily-conserved hydrophobic domains that form transmembrane helices with domain I matching the leader peptide that targets GRP78 to the ER [26, 27]. Our results show that the ground squirrel GRP78 sequence, as compared with other mammalian sequences, has a substitution in the region equivalent to domain I at the N-terminal; a valine residue (high hydrophobicity value: 4.2) at position 9 replacing methionine (low hydrophobicity value: 1.9) in the human sequence or alanine (low hydrophobicity value: 1.8) in the mouse and rat sequences. This may suggest a stronger anchoring of squirrel GRP78 in the ER. GRP78 resides as an integral transmembrane protein to perform its folding function via the carboxyterminus within the ER lumen, coordinating the sequential binding and release of protein substrates [12]. Indeed, the carboxyterminal of the ground squirrel GRP78 sequence contains the sequence RDEL at position 651 which matches the ER target sequence, the common retention signal for ER proteins [28, 29], identified using the database of protein families and domains (PROSITE). Arginine (R) replaces the similar amino acid lysine that occurs in the signal sequence of the other mammalian species (Figure 2). Moreover, it has been shown by Ramachandra *et al.* [26] that in addition to the carboxyterminal function of GRP78, the N-terminus is within the cytoplasm and this interacts with cytosolic components via its ATP binding domain. At that N-terminus, GRP78 plays an anti-apoptotic function by interacting with caspase-7 and caspase-12 both *in vivo* and *in vitro* to block caspase activation. This may also be an important feature of GRP78 action in 13-lined ground squirrel organs – the inhibition of apoptosis that would normally be triggered by hypothermia or oxidative stresses in nonhibernating species. The four unique amino acid substitutions in ground squirrel GRP78 as compared with nonhibernating mammals may also help to maintain optimal GRP78 function in the ER over the wide range of Tb values ($\sim$0 °C to 37 °C) experienced by hibernators. Temperature has major effects on protein conformation and protein-protein binding interactions and selected substitutions in the GRP78 sequence may ensure that the important function of GRP78 as a chaperone is unimpaired in organs during cold torpor. Other proteins that are typically highly conserved in mammals, such as fatty acid binding protein (the intracellular lipid transporter), also show selected amino acids substitutions in hibernator sequences that appear to modify protein conformation for enhanced protein function at low Tb [30, 31].

A large amount of work has established that, in addition to its folding function and its ability to block the apoptotic process, GRP78 binds to three transducers of ER stress signaling to maintain them in an inactive form: the activating transcription factor 6 (ATF6), the serine/threonine endoribonuclease IRE1, and the serine/threonine kinase PERK [10, 32, 33]. These sensors are activated when they are released from GRP78 under ER stress. The resulting effects of their activation include an up-regulation of genes encoding proteins involved in the secretory pathways (ER resident chaperones), proteins involved in ER-associated protein degradation and a shut down of overall protein synthesis. In addition, it has been reported that under ER stress, the pathway that is activated most rapidly is translational repression mediated by PERK which phosphorylates its direct substrate, the α subunit of the eukaryotic initiation factor 2 (eIF2α) [32]. Studies by our lab and others have shown that during hibernation, eIF2α is phosphorylated to block the delivery of the initiating Met-tRNA to the ribosomal preinitiation complex [5, 24, 34]. Furthermore, the phosphorylation of eIF2α was found to induce the translation of ATF4 which has been shown to bind to the promoter of several genes involved in the UPR including *grp78* [17]. ATF4 is known to bind to the *grp78* promoter to activate its transcription in response to hypoxia and other ER stress (such as those induced by thapsigargin and tunicamycin) [17]. Hence, we examined whether ATF4 could also regulate *grp78* in ground squirrels. Electrophoretic mobility shift and supershift assays demonstrated that ATF4 can bind to the promoter region of ground squirrel *grp78* to activate its transcription. Hence, ATF4 could be the transcription factor responsible for *grp78* up-regulation during hibernation.

The supershift assay made use of a functional interaction with a previously established DNA binding domain at the CRE/ATF site designed from the rat *grp78* promoter [17, 35]. Figure 4B (lanes 3 and 4) provides evidence that complex I is specific to ATF4 since the complex I band shifted to a position of slower mobility in the presence of the anti-CREB1 antibody in the supershift assay. Complex II and complex III were determined to be nonspecific signals created by the binding of ubiquitous factors that have general affinity for DNA and that are present in high abundance in cell extracts, as previously reported [35].

Overall, these data show a coordinated increase in both *grp78* mRNA and GRP78 protein levels in BAT and brain of hibernating squirrels compared to euthermic controls and this appears to be physiologically relevant to survival during hibernation. The up-regulation of GRP78 upon ER stress suggests its pivotal role in correctly folding hibernation-responsive proteins, maintaining cellular homeostasis, balancing between cell survival and apoptosis, and suggests an important protective response used by cells when they enter the hypometabolic, low Tb state of hibernation. The activation of *grp78* expression during hibernation may be mediated by ATF4 although other transcription factors including ATF6 and IRE1/XBP1 can also regulate the gene. Studies have only recently begun to identify the transcription factors involved in hibernation-responsive gene expression and much more work remains to be done. To date, for example, the up-regulation of

some proteins/enzyme involved in lipid catabolism and mitochondrial energy production have been linked with elevated levels of PPARγ and PGC-1 in hibernator organs [6, 7].

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References

1. Wang LCH, Lee TF: Torpor and hibernation in mammals: metabolic, physiological and biochemical adaptations. In: MJ Fregley, CM Blatteis CM (eds). Handbook of Physiology: Environmental Physiology. Section 4, Oxford University Press, New York, 1: 507–532, 1996
2. Frerichs KU, Kennedy C, Sokoloff L, Hallenbeck JM: Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia". J Cereb Blood Flow Metab 14: 193–205, 1994
3. Storey KB: Mammalian hibernation: transcriptional and translational controls. Adv Exp Med Biol 543: 21–38, 2003
4. Storey KB: Cold, ischemic organ preservation: lessons from natural systems. J Invest Med 52: 315–322, 2004
5. Storey KB, Storey JM: Metabolic rate depression in animals: transcriptional and translational controls. Biol Rev 79: 207–233, 2004
6. Eddy SF, Storey KB: Up-regulation of fatty acid-binding proteins during hibernation in the little brown bat, *Myotis lucifugus*. Biochim Biophys Acta 1676: 63–70, 2004
7. Eddy SF, Morin P, Storey KB: Cloning and expression of PPARγ and PGC-1α from the hibernating ground squirrel, *Spermophilus tridecemlineatus*. Mol Cell Biochem 269: 175–182, 2005
8. Barja de Quiroga G: Brown fat thermogenesis and exercise: two examples of physiological oxidative stress? Free Radic Biol Med 13: 325–340, 1992
9. Carey HV, Frank CL, Seifert JP: Hibernation induces oxidative stress and activation of NF-kappaB in ground squirrel intestine. J Comp Physiol B 170: 551–559, 2000
10. Schroder M, Kaufman RJ: The mammalian unfolded protein response. Annu Rev Biochem 74: 739–789, 2005
11. Kozutsumi Y, Segal M, Normington K, Gething M-J, Sambrook J: The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature 332: 462–464, 1988
12. Gething M-J: Role and regulation of the ER chaperone BiP. Cell Devel Biol 10: 465–472, 1999
13. Lee AS: The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 26: 504–510, 2001
14. Chen Y, Matsushita M, Nairn AC, Damuni Z, Cai D, Frerichs KU, Hallenbeck JM: Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. Biochemistry 40: 11565–11570, 2001
15. Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nuc Acids Res 11: 1475–1489, 1983
16. Roy B, Lee AS: The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. Nuc Acids Res 27: 1437–1443, 1999
17. Luo S, Baumeinster P, Yang S, Abcouwer SF, Lee AS: Induction of GRP78/BiP by translational block. Activation of the Grp78 promoter by ATF4 through an upstream ATF/CRE site independent of the endoplasmic reticulum stress elements. J Biol Chem 378: 37375–37385, 2003
18. Calvert ME, Digilio LC, Herr JC, Coonrod SA: Oolemmal proteomics – identification of highly abundant heat shock proteins and molecular chaperones in the mature mouse egg and their localization on the plasma membrane. Reprod Biol Endocrinol 1: 27–37, 2003
19. Patil C, Walter P: Intracellular signalling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr Opin Cell Biol 13: 349–355, 2001
20. Lee M, Choi I, Park K: Activation of stress signaling molecules in bat brain during arousal from hibernation. J Neurochem 82: 867–873, 2002
21. Eddy SF, McNally JD, Storey KB: Up-regulation of a thioredoxin peroxidase-like protein, proliferation associated gene, in hibernating bats. Arch Biochem Biophys 435: 101–111, 2005
22. Drew KL, Toien O, Rivera PM, Smith MA, Perry G, Rice ME: Role of the antioxidant ascorbate in hibernation and warming from hibernation. Comp Biochem Physiol C 133: 483–492, 2002
23. Osborne PG, Hashimoto M: Brain antioxidant levels in hamsters during hibernation, arousal and cenothermia. Behav Brain Res 168: 208–214, 2006
24. Hittel D, Storey KB: The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*). Arch Biochem Biophys 401: 244–254, 2002
25. Boyer BB, Barnes BM: Molecular and metabolic aspects of mammalian hibernation. Bioscience 49: 713–724, 1999
26. Ramachandra KR, Changhui M, Baumeister P, Austin RC, Kaufman RJ, Lee AS: Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 278: 20915–20924, 2003
27. Wooden SK, Lee AS: Comparison of the genomic organizations of the rat grp78 and hsc73 gene and their evolutionary implications. DNA Seq 3: 41–48, 1992
28. Pelham HRB: The retention signal for soluble proteins of the endoplasmic reticulum. Trends Biochem Sci 15: 483–486, 1990
29. Munro S, Pelham HRB: A C-terminal signal prevents secretion of luminal ER proteins. Cell 48: 899–907, 1987
30. Hittel D, Storey KB: Differential expression of adipose and heart type fatty acid binding proteins in hibernating ground squirrels. Biochim Biophys Acta 1522: 238–243, 2001
31. Eddy SF, Storey KB: Up-regulation of fatty acid-binding proteins during hibernation in the little brown bat, *Myotis lucifugus*. Biochim Biophys Acta 1676: 63–70, 2004
32. Rutkowski DT, Kaufman RJ: (2004) A trip to the ER: coping with stress. Trends Cell Biol 14: 20–28, 2001
33. Lee AS: The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35: 373–381, 2005
34. Frerichs KU, Smith CB, Brenner M, DeGracia DJ, Krause GS, Marrone L, Dever TE, Hallenbeck JM: Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. Proc Natl Acad Sci USA 95: 14511–14516, 1998
35. Alexandre S, Nakaki T, Vanhamme L, Lee AS: A binding site for the cyclic adenosine 3′, 5′-monophosphate-response element binding protein as a regulatory element in the *grp78* promoter. Mol Endocrinol 5: 1862–1878, 1991