Heme oxygenase expression and Nrf2 signaling during hibernation in ground squirrels[1]

Zhouli Ni and Kenneth B. Storey

Abstract: Mammalian hibernation is composed of long periods of deep torpor interspersed with brief periods of arousal in which the animals, fueled by high rates of oxygen-based thermogenesis in brown adipose tissue and skeletal muscle, power themselves back to euthermic (~37 °C) body temperatures. Strong antioxidant defences are important both for long-term cytoprotection during torpor and for coping with high rates of reactive oxygen species generated during arousal. The present study shows that the antioxidant enzyme heme oxygenase 1 (HO1) is strongly upregulated in selected organs of thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) during hibernation. Compared with euthermic controls, HO1 mRNA transcript levels were 1.4- to 3.8-fold higher in 5 organs of hibernating squirrels, whereas levels of the constitutive isozyme HO2 were unchanged. Similarly, HO1 protein levels increased by 1.5- to 2.0-fold in liver, kidney, heart, and brain during torpor. Strong increases in the levels of the Nrf2 transcription factor and its heterodimeric partner protein, MafG, in several tissues indicated the mechanism of activation of hibernation-responsive HO1 gene expression. Furthermore, subcellular distribution studies with liver showed increased nuclear translocation of both Nrf2 and MafG in torpid animals. The data are consistent with the suggestion that Nrf2-mediated upregulation of HO1 expression provides enhanced antioxidant defence to counter oxidative stress in hibernating squirrels during torpor and (or) arousal.

Key words: Spermophilus tridecemlineatus, torpor, antioxidant defence, cytoprotection, MafG transcription factor, gene expression, protein levels.

Résumé : L'hibernation des mammifères se compose de longues périodes de léthargie profonde entrecoupées de brèves périodes de réveil pendant lesquelles les animaux retrouvent une température corporelle euthermique (~37 °C) par une thermogenèse active basée sur la consommation d'oxygène dans le tissu adipeux brun et le muscle squelettique. Des défenses antioxydantes solides sont nécessaires tant pour assurer une cytoprotection tout au long de la léthargie que pour faire face à la forte production d'espèces réactives de l'oxygène au cours des périodes de réveil. La présente étude montre que, durant l'hibernation, l'enzyme antioxydante, hème oxygénase 1 (HO1), augmente considérablement dans certains organes des spermophiles rayés (*Spermophilus tridecemlineatus*). Comparés aux taux observés chez les témoins euthermiques, les taux d'ARNm transcrits de HO1 ont été d'un facteur 1,4–3,8 plus élevés dans cinq organes des spermophiles hibernants, alors que les taux de l'isoenzyme constitutive HO2 sont demeurés stables. Par ailleurs, les taux de la protéine HO1 ont augmenté d'un facteur 1,5–2,0 dans le foie, le rein, le cœur et le cerveau durant la léthargie. De fortes augmentations des taux du facteur de transcription Nrf2 et de sa protéine partenaire hétérodimère, MafG, dans plusieurs tissus ont exprimé le mécanisme d'activation de l'expression du gène HO1 sensible à l'hibernation. De plus, des examens de la distribution subcellulaire dans le foie ont montré une augmentation de la translocation nucléaire de Nrf2 et de MafG chez les animaux en léthargie. Les résultats sont en accord avec l'hypothèse qui veut qu'une augmentation de l'expression de HO1 véhiculée Nrf2 fournit une défense antioxydante accrue contre le stress oxydatif dans les spermophiles hibernants durant les périodes de léthargie ou de réveil, ou durant les deux périodes.

Mots-clés : Spermophilus tridecemlineatus, léthargie, défense antioxydante, cytoprotection, facteur de transcription MafG, expression génique, taux de protéines.

[Traduit par la Rédaction]

Received 31 May 2009. Accepted 2 November 2009. Published on the NRC Research Press Web site at cjpp.nrc.ca on 9 April 2010.

Z. Ni[2] and K.B. Storey.[3] Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.

[1]This article is one of a selection of papers published in a Special Issue on Oxidative Stress in Health and Disease.

[2]Present address: Department of Developmental Biology, Howard Hughes Medical Institute, 318 Campus Drive W200, Stanford University School of Medicine, Stanford, CA 94305, USA.

[3]Corresponding author (e-mail: kenneth_storey@carleton.ca).

Introduction

Hibernation is a critical winter survival strategy for many small mammals. By entering a state of torpor and allowing their body temperature (Tb) to drop to near-ambient levels, animals can save about 90% of the energy that would otherwise be needed to remain euthermic over the winter months (Wang and Lee 1996). Accumulating evidence indicates that good antioxidant defences are important to hibernation success. These defences not only provide cytoprotection over weeks of torpor, but, critically, also defend against high rates of reactive oxygen species (ROS) generation during periods of intermittent arousal, in which huge surges in oxygen consumption power thermogenesis to rewarm the body.

Indeed, the rapid consumption of plasma ascorbate during the arousal process attests to the ROS challenge presented (Drew et al. 2002; Osborne and Hashimoto 2006). Activities, protein levels, and (or) mRNA expression levels of various antioxidant enzymes are also elevated in selected tissues during hibernation, increasing antioxidant defence capacity (Buzadzić et al. 1990; Storey 2003, 2006; Carey et al. 2003; Eddy et al. 2005; Morin and Storey 2007; Page et al. 2009).

Upregulation of a range of antioxidant defence genes in response to environmental stress and industrial toxins is mediated via the action of a basic leucine zipper transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) (Alam et al. 1999; Owuor and Kong 2002) that binds to the antioxidant response element (ARE) in the promoter region of antioxidant genes. In a recent study, we reported increased levels of Nrf2 protein in some organs of thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*, during hibernation (Morin et al. 2008). A strong correlation was seen in the heart during hibernation between increased *nrf2* mRNA transcript levels, elevated Nrf2 protein, and enhanced levels of 3 antioxidant enzymes under Nrf2 control: aflatoxin aldehyde reductase (AFAR), Cu and Zn superoxide dismutase, and heme oxygenase 1 (HO1). The present study takes a closer look at the tissue-specific responses and regulation of HO1 in hibernating *S. tridecemlineatus*.

Heme oxygenase catalyzes the first and rate-limiting step of heme degradation, producing CO, biliverdin, and ferrous iron (Tenhunen et al. 1968). Two main isoforms exist. HO1 is the inducible form, and HO2 is constitutive (Maines 1988). Biliverdin and its catabolite, bilirubin, have antioxidant properties with demonstrated beneficial effects in a number of disease and injury states, including ischemia–reperfusion, organ transplantation, graft rejection, and autoimmune inflammation (Öllinger et al. 2007; Idriss et al. 2008; Soares and Bach 2009). HO1 is induced by numerous stresses, including accumulation of heme (Maines 1988), UV light, heat shock (Keyse and Tyrrell 1989), sulfhydryl reactive reagents (Applegate et al. 1991), heavy metals, anoxia, hypoxia, hyperoxia (Lee et al. 1997; Lee et al. 2000), and ischemia (Semenza 2000). A common feature of these stress inducers is their ability to generate ROS, suggesting that HO1 provides antioxidant defence (Idriss et al. 2008).

The present study documents the hibernation-responsive upregulation of HO1 at both the mRNA and protein levels in several tissues of thirteen-lined ground squirrels and links this to Nrf2 signaling. Nrf2 distribution between the free 57 kDa protein and the inactive actin-bound 100 kDa state (Nrf2 is sequestered in the cytoplasm by Keap1 protein that can be bound to the actin cytoskeleton) was evaluated. We also oxamined hibernation-responsive changes in the levels of the partner protein, MafG, that forms a heterodimer with Nrf2 to bind to the ARE of the *HO1* gene.

Materials and methods

Animal treatments

Animal studies were conducted in the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, USA) under a protocol approved by the NINDS Animal Care and Use Committee and meeting the guidelines of the NIH *Guide for the Care and Use of Laboratory Animals*; conditions are described in greater detail by Morin et al. (2008). Briefly, thirteen-lined ground squirrels were acquired in late summer (initial body mass 130–180 g), housed at 21 °C under an autumn 12 h light : 12 h dark cycle, and fed ad libitum until they reached a plateau weight of 220–240 g. Some animals were then placed in a cold chamber at 4–5 °C in constant darkness and entered torpor within several days. Animals were sampled after 2–5 days of continuous torpor as indicated by a constant low core Tb of approximately 5 °C (measured via a subcutaneously implanted thermal transmitter). Other squirrels were maintained as euthermic controls at 21 °C and were sampled on the same dates as the hibernating animals. Animals were killed by decapitation and tissue samples were removed quickly, frozen immediately in liquid nitrogen, air-freighted to Carleton University on dry ice, and stored at –80 °C until use.

PCR analysis of HO1 and HO2 mRNA transcript levels

RNA preparation

Total RNA was isolated from tissues of control and hibernating ground squirrels (n = 3 or 4 separate preparations from different animals for each condition) using Trizol reagent (Invitrogen), following manufacturer's instructions as per Mamady and Storey (2006). RNA was separated on a 1.2% denaturing formaldehyde agarose gel, and high quality was confirmed by the presence of sharp 28S and 18S rRNA bands. RNA concentration was quantified spectrophotometrically by measuring absorbance at 260/280 nm. RNA was stored at –80 °C until use.

First-strand cDNA synthesis

A 15 μg aliquot of total RNA from each sample was used for first-strand synthesis as described by Mamady and Storey (2006). The cDNA stock solution was stored at –20 °C, and serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were prepared before performing PCR.

PCR using degenerate primers

Degenerate primers for *HO1* and *HO2* were designed from consensus sequences based on the human, mouse, rat, rabbit, and pig sequences using DNAman (version 4.11) and Primer Designer (version 3.0). Primers were synthesized by Sigma Genosys with the following sequences: *HO1* forward (5′-TGAAGGAGGCCACCAAGGAG-3′) and reverse (5′-GTTGAGCAGGAASGCRGTCTT-3′); *HO2* forward (5′-ATGGABCGCAAYAAGGAC-3′) and reverse (5′-TAGTACCAGGCCAAGAGTCC-3′). PCR reactions were carried out in a mixture containing 5 μL 10^{-2} cDNA dilution, 15 μL H_2O, 2.5 μL 10× PCR Rxn buffer (without $MgCl_2$), 1.25 μL 50 mmol/L $MgCl_2$, 0.5 μL 10 mmol/L dNTPs, 0.125 μL *Taq* DNA polymerase (all Invitrogen), and 2.5 μL 30 nmol/mL forward and reverse primer mixture (1:1). A Mastercycler gradient PCR machine (Eppendorf) was used to provide 35 cycles as follows: denaturation at 95 °C for 30 s, primer annealing for 30 s at optimum temperature (68° and 65 °C for *HO1* and *HO2*, respectively), and primer extension at 72 °C for 30 s. PCR products were separated on a 0.8% agarose / ethidium bro-

mide gel, and bands were imaged and quantified using the ChemiGenius Bio imaging system with GeneTools software (Syngene, Frederick, USA). PCR products were sequenced by Canadian Molecular Research Services (Ottawa, Canada) or by Cortec (Kingston, Canada). Nucleotide and deduced amino acid sequences were verified as being the expected products using the programs BlastN and BlastP at the NIH.

Semiquantitative PCR

Primers were then revised to be ground squirrel-specific with the following sequences: *HO1* forward (5′-TCCAAGCTGGTGATGGTGTC-3′); *HO1* reverse (5′-GCTTGAACTTGGTGGCACTG-3′); *HO2* forward (5′-CTGACCAAGGACATGGAGTA-3′); and *HO2* reverse (5′-CTTGCTGAGCAGCATAGAAG-3′). The housekeeping gene, *α-tubulin*, was also amplified with forward (5′-AAGGAAGATGCTGCCAATAA-3′) and reverse (5′-GGTCACATTTCACCATCTG-3′) primers.

Paired PCR reactions were performed with each set of cDNA dilutions, one to evaluate the quantity of the cDNA of interest (*HO1* or *HO2*) and the other to quantify the housekeeping gene *α-tubulin*. A tube with reaction mix and *α-tubulin* primers but with no cDNA was used as a negative control, whereas a tube with reaction mix and *α-tubulin* primers but with total RNA was used as a control to exclude genomic contamination. Reaction mixtures and cycling conditions were as above. PCR products were separated in 0.8% agarose / ethidium bromide gels, which were then imaged and quantified using the ChemiGenius. To be sure that the PCR products had not reached amplification saturation, the lowest dilutions that gave visible bands were quantified. HO1 or HO2 band intensity was normalized against the corresponding α-tubulin band density amplified from the same sample.

Western blotting

Preparation of tissue extracts

Samples of frozen tissues (n = 4 from different animals for each condition) were homogenized 1:10 *w*/*v* in buffer containing 250 mmol/L sucrose, 25 mmol/L Hepes, 25 mmol/L KCl, 1 mmol/L EGTA, and 1 mmol/L EDTA with protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), added immediately before homogenization. Samples were centrifuged at 5 000 rpm for 5 min at 4 °C in a Biofuge 15 (Baxter Canlab), supernatant was collected, and soluble protein concentration was determined by using the Coomassie blue dye-binding method with bovine serum albumin as the standard and the Bio-Rad commercial reagent.

SDS–polyacrylamide gel electrophoresis

Electrophoresis was carried out essentially as in Mamady and Storey (2006). Supernatant was mixed 1:1 *v*/*v* with 2× SDS–PAGE sample buffer (100 mmol/L 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. Aliquots containing 10 μg protein from euthermic and hibernating samples were loaded onto 7.5%–15% gels (depending on subunit molecular mass of the protein) and followed by electrophoresis for approximately 1 h at 200 V using the Bio-Rad Mini-Protean 3 system. Proteins were then blotted to polyvinylidene difluoride (PVDF) membranes by wet transfer at 4 °C for 2 h at 70 V or 30 V overnight.

Immunoblotting and protein visualization

PVDF membranes were rinsed with TBST buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.05% *v*/*v* Tween 20) and then incubated in blocking buffer (TBST with 5% non-fat dry milk) for 30 min at 21 °C. The blot was then placed in 5 mL TBST containing rabbit polyclonal antibodies against the protein of interest and incubated at 21 °C for 2 h or at 4 °C overnight. For HO1, the antibody was rabbit anti-HO1 (1:2000 *v*/*v* dilution; SPA-896, Stressgen); a duplicate blot was incubated with TBST containing HO1 antibody and 10 μg HO1 immunizing peptide (SPT-896E, Stressgen) as a negative control. Rabbit anti-Nrf2 and anti-MafG were gifts from Dr. John Hayes, Biomedical Research Centre, University of Dundee, and were used at 1:2000 and 1:1000 *v*/*v*, respectively. Mouse anti-actin antibodies (JLA20, University of Iowa) were used at 1:2000 *v*/*v*. After incubation, blots were washed 3–5 times in TBST (5 min, 21 °C) and then incubated in 5 mL TBST solution containing secondary antibody overnight at 4 °C. Secondary antibodies were horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (H and L chains) for HO1, Nrf2, and MafG, and HRP-linked horse anti-mouse IgG (H and L) for actin (all from Cell Signaling; used at 1:2000 *v*/*v*). Target proteins were visualized by enhanced chemiluminescence using SuperSignal West Pico chemiluminescent substrate (Pierce), and bands were scanned and quantified with ChemiGenius. Equal loading of proteins was confirmed by subsequent Coomassie blue staining of the blots.

Nuclear versus cytoplasmic fractionation

Nuclear extracts were prepared using a slight modification of the method described by Dignam et al. (1983). Briefly, samples (~0.5 g) of frozen tissue were crushed under liquid nitrogen and homogenized using a Dounce homogenizer in 1 mL hypotonic buffer containing 10 mmol/L Hepes, pH 7.9, 10 mmol/L KCl, 10 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), and 10 μL of a protease inhibitor mixture (1 mmol/L PMSF, 1 μg/mL aprotinin, 0.01 mg/mL pepstatin A, and 1 μg/mL antipapain). Lysates were centrifuged at 10 000*g* for 10 min at 4 °C. Supernatant was removed as the cytoplasmic fraction. Pellets containing crude nuclei were resuspended in 200 μL extraction buffer (20 mmol/L Hepes, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 1.5 μL protease inhibitor) and incubated for 1 h on ice with gentle rocking. Samples were centrifuged at 10 000*g* for 10 min at 4 °C, and supernatants representing the nuclear faction were collected. Fractions were stored at –80 °C until use. SDS–PAGE and immunoblotting were performed as above using 12% gels; antibody dilutions were 1:5000 *v*/*v* for anti-Nrf2 and 1:2000 *v*/*v* for anti-MafG. Western blotting was also conducted with anti-histone H3 antibody as described previously (Mamady and Storey 2006) to confirm the separation of the nuclear versus cytoplasmic fractions. Bands for histone H3 band were found only in the nuclear extracts and not in the cytoplasmic fractions (data not shown).

Data quantification and statistics

PCR product bands were normalized against bands for α-tubulin amplified from the same cDNA sample. Western blot band intensities were standardized against a group of 3 Coomassie-stained protein bands that did not show variation between the different experimental states and were not located close to the protein bands of interest. Mean normalized band intensities were then calculated (± SE, $n = 3$ or 4 independent preparations of cDNA or $n = 4$ separate protein extracts, all from different animals) for euthermic and hibernating samples and the Student's t test was used to test for significant differences between the 2 groups. The ratio of hibernating to euthermic levels was then calculated and plotted in histograms; error bars show the sum of SE values for euthermic and hibernating trials.

Results

HO1 and HO2 gene expression

Degenerate primers were used to amplify PCR products that were sequenced and confirmed via BlastN as encoding HO1 and HO2. Species-specific primers were then produced and used for relative quantitative PCR to examine transcriptional levels of HO1 and HO2. Transcripts of both genes were amplified from serial dilutions of cDNA (10^{-1} to 10^{-5}) prepared from RNA of 8 tissues. The housekeeping gene, *α-tubulin*, was also amplified from each sample and used for normalization. Figure 1 shows an example of PCR products amplified from kidney and separated on agarose / ethidium bromide gels. Band densities were quantified and normalized against α-tubulin expression; mean values were compared between hibernating and euthermic states. The histograms in Fig. 1B show that during hibernation, HO1 mRNA levels rose significantly in 5 organs: by 3.8-fold in brain, 2.8-fold in lung, 2.4-fold in liver, 2.15-fold in skeletal muscle, and 1.4-fold in kidney. In contrast, HO1 mRNA levels fell in heart and were unchanged in brown adipose tissue (BAT); we were unable to amplify HO1 from white adipose tissue (WAT).

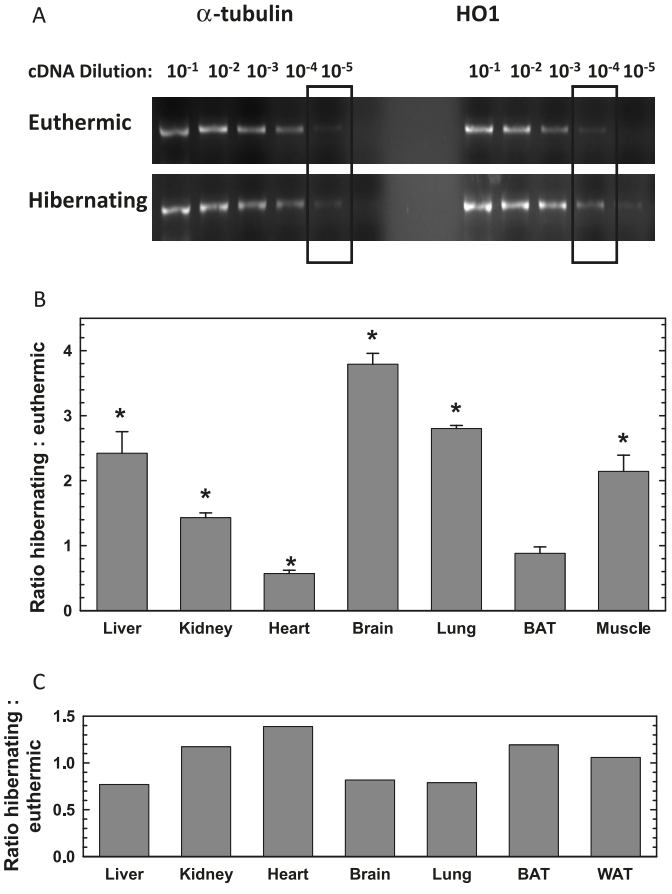
The equivalent analysis of HO2 successfully amplified transcripts from 7 ground squirrel organs. However, no significant differences in HO2 mRNA expression were found between euthermic and hibernating states for any tissue (Fig. 1C), which is consistent with the status of mammalian HO2 as a constitutive enzyme.

Western blotting analysis of HO1 expression

Immunoblotting was used to study HO1 translational regulation. A polyclonal antibody raised against human HO1 (residue 1–30) cross-reacted with a band on Western blots at 32 kDa that corresponds with the known molecular mass of mammalian HO1. Furthermore, when parallel gels were incubated with antibody that was pre-exposed to the HO1 immunizing peptide (human HO1 synthetic peptide, amino acids 1–30), immunoreactivity at the 32 kDa band was abolished (data not shown); this confirmed that the 32 kDa band detected in squirrel samples was HO1.

Figure 2 shows representative Western blots of HO1 protein in 7 ground squirrel tissues; histograms plot the ratio of hibernating to euthermic protein levels. HO1 levels were significantly elevated in 4 organs during hibernation: by 1.5-fold in brain and kidney and by 1.9-fold in liver and heart. The results for brain, kidney, and liver were consistent with the transcriptional upregulation shown in Fig. 1.

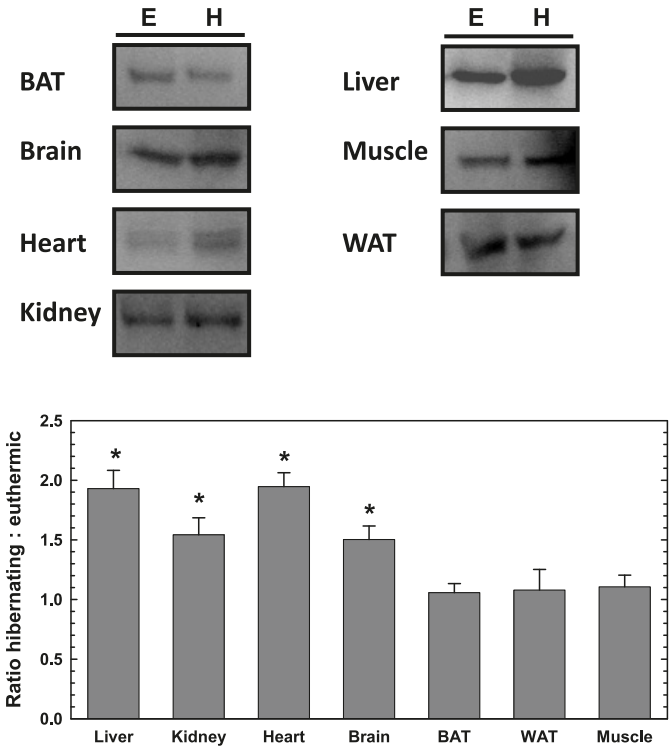
Fig. 1. Analysis of HO1 and HO2 mRNA transcript levels in ground squirrel organs during hibernation, as determined by semi-quantitative PCR. The lowest dilutions of cDNA stock that gave visible PCR product bands (10^{-3} to 10^{-5} for different tissues) were chosen for quantification of band densities. (A) An example of HO1 amplification from kidney cDNA. For this organ, bands from 10^{-4} and 10^{-5} dilutions were quantified for HO1 and α-tubulin, respectively (these bands are boxed in the figure). HO1 band densities were normalized against the corresponding α-tubulin band density for the same sample. (B) Ratios of normalized hibernating versus euthermic values for HO1; data are means from $n = 3$ or 4 independent determinations on cDNA samples prepared from tissue samples of different animals. (C) Ratios of normalized hibernating versus euthermic values for HO2; data are means, $n = 2$. BAT and WAT are brown and white adipose tissue, respectively. *, Significant at $p < 0.05$ vs. corresponding euthermic value, by Student's t test.



Analysis of Nrf2, MafG, and actin protein expression in ground squirrel tissues

A common mechanism of HO1 gene regulation is activation of transcription via the ARE in response to Nrf2 transcription factor signaling (Alam et al. 1999, Owuor and Kong 2002). Previous studies showed that Nrf2 was detected in 57 kDa and 100 kDa forms on immunoblots and that the 100 kDa form was a complex of Nrf2 and actin (Kang et al. 2002). Nrf2 was detected in 2 bands in ground squirrel tis-

Fig. 2. HO1 protein levels in tissues from euthermic and hibernating ground squirrels, as assessed by Western immunoblotting. (A) Representative Western blots are shown for tissues from euthermic (E) versus hibernating (H) ground squirrels. (B) Values were normalized to α-tubulin, and ratios were plotted for hibernating versus euthermic conditions (values are means, $n = 4$ samples from different animals). A ratio of 1 denotes no difference between euthermic and hibernating samples. *, Significant at $p < 0.05$ vs. corresponding euthermic value, by Student's t test.

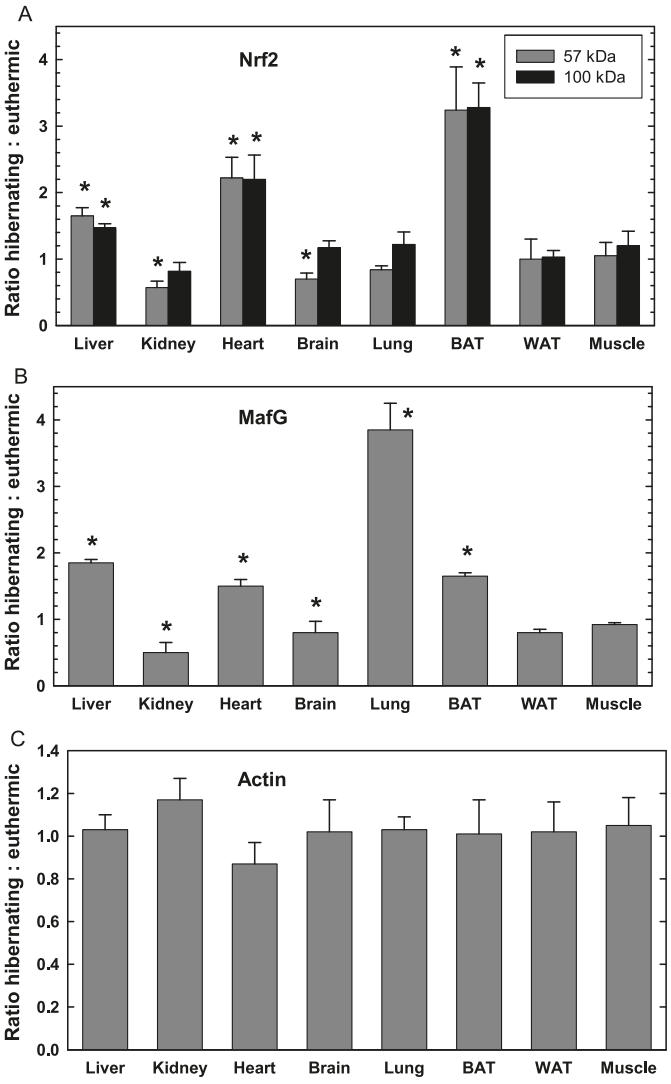


sues. One-dimensional gels showed immunoreactive bands at both 57 and 100 kDa. Two-dimensional gels resolved 2 substantial immunoreactive spots (data not shown). One spot was detected at 57 kDa with an isoelectric point (p*I*) of approximately 4.5, consistent with the known mass of Nrf2 and the theoretical p*I* value of 4.67 calculated by Swiss-2D PAGE for human Nrf2 (GenBank accession number Q16235). Another spot was detected at 100 kDa with a p*I* of approximately 5.4, the putative Nrf2–actin complex.

Figure 3A shows the relative levels of Nrf2 protein in tissues from hibernating versus euthermic ground squirrels. During hibernation, the content of the 57 kDa Nrf2 protein increased significantly in liver, heart, and BAT by 1.6-, 2.2-, and 3.2-fold, respectively. Nrf2 protein content in the 100 kDa band also increased significantly in these 3 tissues by nearly equivalent amounts, so that the total amount of Nrf2 in cells was clearly strongly elevated in tissues during torpor. In contrast, the amount of 57 kDa Nrf2 protein decreased significantly in brain and kidney to 70% and 60% of the euthermic value, respectively, but the 100 kDa protein was unaffected. Neither protein form was affected by hibernation in lung, muscle, or WAT.

The small Maf proteins act as obligatory heterodimeric partner molecules with Nrf2 for binding to the ARE. Western blot analysis of MafG in ground squirrel tissues revealed

Fig. 3. Effect of hibernation on the levels of proteins associated with the Nrf2 signal transduction pathway in ground squirrel tissues. (A) Relative levels of Nrf2 in the 57 kDa and 100 kDa protein bands, (B) MafG protein levels, and (C) actin protein levels. Values are means, $n = 4$ samples from different animals. *, Significant at $p < 0.05$ vs. corresponding euthermic value, by Student's t test.



that the content of MafG protein during hibernation was elevated in BAT, heart, liver, and lung by 1.6-, 1.5-, 1.8-, and 3.8-fold, respectively (Fig. 3B). Levels of MafG were unaffected in muscle and WAT and decreased significantly in brain and kidney. In all organs except lung, the pattern of change in MafG correlated well with the results for 57 kDa Nrf2 protein.

Western blotting was also used to analyze the protein expression profile of actin in ground squirrel tissues. The 43 kDa actin band did not change in intensity during hibernation in any of the 8 tissues analyzed (Fig. 3C). However, given that the Nrf2 antibody cross-reacted with bands at both 57 kDa and 100 kDa, we reasoned that the actin antibody should also show a double band pattern. Figure 4 shows results for brain and heart. An actin band was detected at the expected molecular mass of 43 kDa band and

Fig. 4. (A) Western blots using actin antibody to detect the putative 100 kDa complex with Nrf2 in tissue extracts from heart and brain of ground squirrels. Free actin is shown at 43 kDa and a putative complex at 100 kDa in representative samples from euthermic (E) versus hibernating (H) animals. (B) Relative amounts of putative actin–Nrf2 complex in hibernating versus euthermic squirrels. Values are means, $n = 4$ samples from different animals. *, Significant at $p < 0.05$ vs. corresponding euthermic value, by Student's t test.

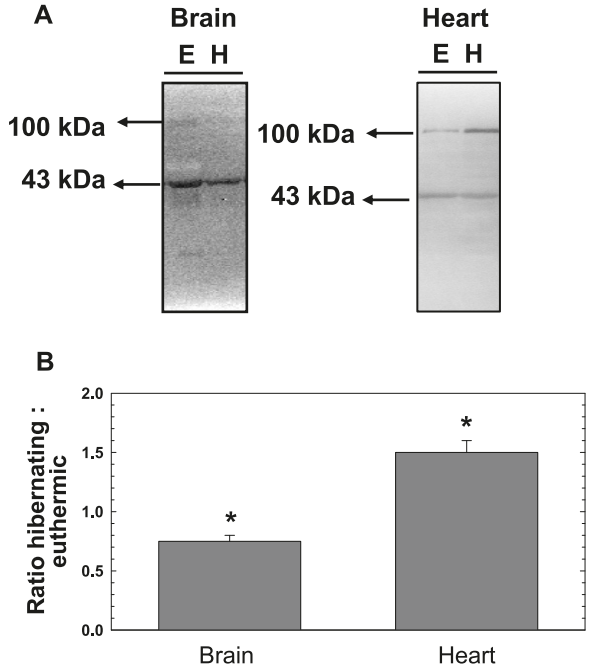
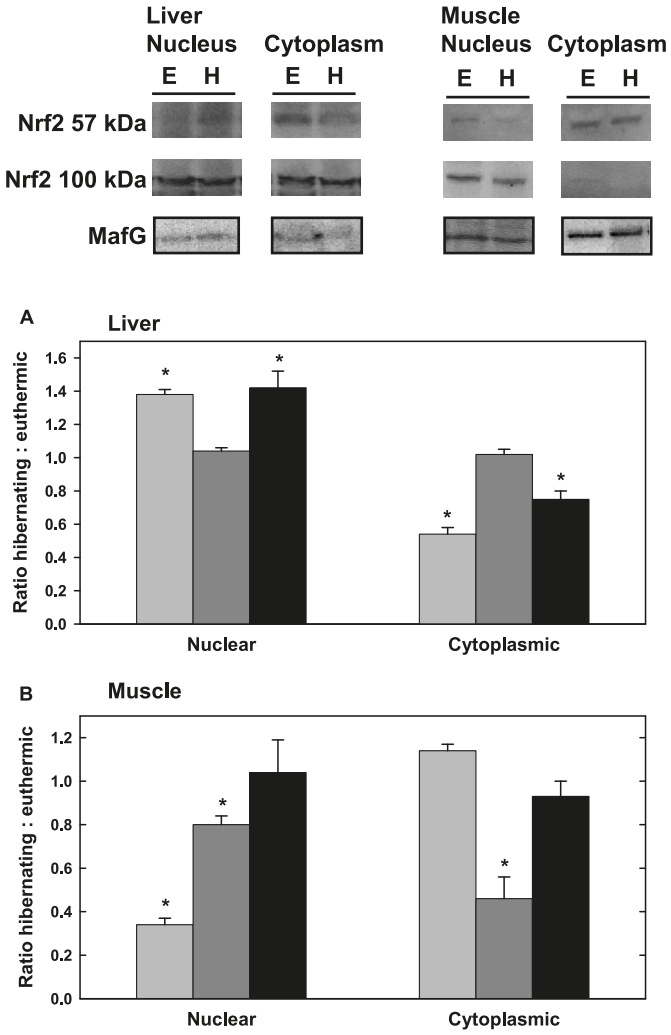


Fig. 5. Protein distribution between nuclear and cytoplasmic fractions in ground squirrel organs. Representative Western blots show Nrf2 57 and 100 kDa and MafG bands in nuclear and cytoplasmic fractions from liver and skeletal muscle of euthermic (E) and hibernating (H) ground squirrels. Histograms show ratio of hibernating versus euthermic protein distribution in liver (A) and muscle (B) for Nrf2 57 kDa (light grey), Nrf2 100 kDa (dark grey), and MafG (black). Values are means, $n = 4$ samples from different animals. *, Significant at $p < 0.05$ vs. corresponding euthermic value, by Student's t test.



another was found at 100 kDa, which could be the putative complex with Nrf2. In both tissues, the relative intensities of the 100 kDa band changed during hibernation. In heart, the relative amount of the 100 kDa putative protein complex increased significantly by 1.5-fold during hibernation, whereas in brain, the relative intensity of 100 kDa protein bands decreased to 75% ($p < 0.05$) of the euthermic level.

Nuclear translocation of Nrf2 and MafG in liver and muscle

To trigger gene expression, transcription factors must be present in the nucleus, and regulation of their translocation from cytoplasm to nucleus is a common mechanism that controls transcription factor action. To determine whether Nrf2 and its binding partner MafG moved into the nucleus during hibernation, nuclear and cytoplasmic fractions were isolated from liver and skeletal muscle and the distribution of Nrf2 and MafG in these fractions was analyzed by immunoblotting (Fig. 5).

In liver, the relative amount of 57 kDa Nrf2 in the nuclear fraction increased significantly by 1.4-fold during hibernation, while cytoplasmic levels decreased by 50% (Fig. 5A). This indicates that the 57 kDa Nrf2 protein translocates from the cytoplasm into the nucleus during hibernation. By contrast, the amount of 100 kDa Nrf2 was stable in both nucleus and cytoplasm during hibernation. The distribution of MafG in liver paralleled that of 57 kDa Nrf2; the amount of MafG in the nuclear fraction increased significantly by 1.4-fold during hibernation, but levels were reduced in the cytoplasm to 75% of the euthermic level.

In muscle, a different situation occurred. Nuclear 57 kDa Nrf2 content decreased significantly during hibernation to just 30% of the euthermic value, but the amount of cytoplasmic 57 kDa Nrf2 remained stable (Fig. 5B). Nuclear levels of the 100 kDa Nrf2 also decreased during hibernation to 80% of the euthermic value, while cytoplasmic levels dropped to 40%. MafG levels remained constant during hibernation in both nuclear and cytoplasmic compartments.

Discussion

Exposure of mammalian cells to oxidative stimuli induces HO1, and in various systems of tissue injury, HO1 induction

has been shown to confer protection while its abrogation accelerates injury. Protection has been linked with the antioxidant actions of biliverdin and bilirubin (Öllinger et al. 2007; Idriss et al. 2008; Soares and Bach 2009). The present study shows that HO1 is enhanced in multiple organs of ground squirrels during hibernation and this suggests that HO1 contributes to natural cytoprotection during torpor. These data, therefore, both confirm a role for HO1 in situations of natural oxidative stress challenge and support the demonstrated antioxidant actions of HO1 in pathophysiological conditions. The actions of HO1 may contribute to sustaining long-term viability of animals in the torpid state. They may also play a role in combating oxidative stress during arousal when oxygen levels and oxygen consumption rise dramatically over short periods of time as animals power themselves back to euthermia by means of high rates of lipid-fueled thermogenesis in BAT and skeletal muscle. Indeed, recent studies have shown that elevated levels of biliverdin or bilirubin are associated with hibernation: plasma bilirubin rose 5-fold in torpid jerboas (Mountassif et al. 2007), and bilirubin levels increased 5-fold in bile in hibernating ground squirrels (Baker and van Breukelen 2009). Compared with squirrels entering hibernation or in full torpor, biliverdin in ground squirrel liver was 15- to 20-fold higher both during arousal (Tb 20–25 °C) and during the interbout periods before animals re-entered torpor (>2 h at Tb >34 °C) (Nelson et al. 2009). The findings of the last study suggest 2 possibilities: (*i*) a burst of heme degradation by liver during the arousal period may be one of the regenerative mechanisms that take place during the euthermic interbout, or (*ii*) targeted production of biliverdin has a positive action in hibernation and (or) arousal. Our data on HO1 upregulation during hibernation argue for the latter and suggest that a positive action of HO1 in producing biliverdin (with subsequent bilirubin formation by biliverdin reductase) is an antioxidant defence mechanism for the hibernator.

Specific upregulation of HO1 during hibernation occurred at both the gene and protein levels in ground squirrel tissues. HO1 protein levels increased significantly by 1.5- to 2-fold in 4 organs of ground squirrels during hibernation (Fig. 2) and HO1 mRNA levels were strongly upregulated by 1.5- to 3.5-fold in 5 organs (Fig. 1). Changes in HO1 mRNA and protein levels correlated well in brain, kidney, and liver of hibernating squirrels, suggesting that mRNA substrate availability is important in determining the synthesis of HO1 protein. Upregulation of HO1 mRNA transcripts during torpor could also set the stage for a subsequent rapid increase in HO1 protein synthesis when animals enter the arousal phase and inhibitory controls on global protein synthesis are released (Storey 2003). By contrast, mRNA transcript levels of HO2 did not change during hibernation, which is consistent with the constitutive nature of this isozyme.

HO1 catalyzes the breakdown of heme to CO, biliverdin, and free iron. If high biliverdin (and bilirubin) levels are cytoprotective during hibernation and (or) arousal, as is argued by the hibernation-responsive upregulation of HO1, then a mechanism is needed to protect cells from the prooxidant effects of iron. In other systems, the iron released through HO1 action stimulates the synthesis of ferritin (an iron storage protein), which provides an iron detoxification mechanism that contributes to the long-term cytoprotection observed after HO induction (Soares and Bach 2009). Notably, DNA array screening identified the ferritin gene as upregulated during hibernation, along with several antioxidant enzymes (Storey 2006).

Nrf2 pathway and regulation of the HO1 gene

A variety of stresses that cause oxidative damage trigger the upregulation of antioxidant defence enzymes mediated by the Nrf2 transcription factor. Indeed, Nrf2-deficient cells have been shown to be hypersensitive to oxidative stresses (Itoh et al. 1997). In partnership with a small Maf protein, the Nrf2–Maf heterodimer binds to the ARE in the promoter region of antioxidant genes. Apart from HO1, a variety of other antioxidant enzymes are known to be upregulated during hibernation (including glutathione *S*-transferases (GSTs), SOD, and peroxiredoxins), many of which are regulated by Nrf2 (Buzadzić et al. 1990; Storey 2003, 2006; Carey et al. 2003; Eddy et al. 2005; Morin and Storey 2007; Page et al. 2009). For example, in thirteen-lined ground squirrels, levels of peroxiredoxin 1, which is Nrf2-regulated (Ishii and Yanagawa 2007), were 4- and 13-fold higher in BAT and heart, respectively, during hibernation than during euthermia (Morin and Storey 2007).

Our results for Nrf2 and MafG show coordinated responses by these 2 transcription factor proteins. Levels of both increased significantly in liver, heart, and BAT during hibernation, whereas both fell in brain and kidney (Fig. 3). Increased levels of 57 kDa Nrf2 protein in ground squirrel heart during hibernation correlated with elevated protein contents of Cu,Zn SOD, AFAR1, and peroxiredoxin antioxidant enzymes (Morin and Storey 2007; Morin et al. 2008), as well as with HO1 in the present study. In liver, the evidence for Nrf2–MafG regulation of HO1 was particularly strong: elevated levels of Nrf2 (1.6-fold) and MafG (1.8-fold) during hibernation correlated with increased HO1 mRNA (2.4-fold) and protein (1.9-fold). Furthermore, both 57 kDa Nrf2 and MafG showed translocation into the nuclear fraction of hibernator liver (~1.4-fold enhancement) whereas the relative content of both proteins in cytoplasmic fractions was strongly reduced (to 50% and 75% of euthermic values).

Skeletal muscle and WAT showed no changes in the protein levels of HO1, Nrf2, or MafG, and nuclear 57 kDa Nrf2 was strongly reduced during hibernation, which suggests that enhanced HO1 is not needed in these tissues, at least during torpor itself. Notably, however, HO1 mRNA levels were elevated in skeletal muscle, which could provide for enhanced HO1 synthesis during arousal. In other organs, although Nrf2 and MafG levels were generally correlated, the HO1 response did not correlate well. In brain and kidney, for example, HO1 mRNA and protein contents were elevated but Nrf2 and MafG decreased. This suggests that another mechanism of transcriptional upregulation of HO1 may occur in these organs. For example, HO1 is also known to respond to the hypoxia-inducible factor 1 (HIF-1) transcription factor (Lee and Andersen 2006) that is upregulated in hibernation in some organs (Morin and Storey 2005).

Nrf2 is maintained in an inactive form in the cytoplasm by interaction with the Keap1 protein, which is immobilized by binding to filamentous actin and (or) myosin VIIa of the

cytoskeleton (Li and Kong 2009). The current model for Nrf2 regulation posits that Nrf2 binding to Keap1 positions Nrf2 so that it can be ubiquitinated and degraded by proteolysis; hence, Keap1 promotes rapid turnover and low resting levels of Nrf2. However, under oxidative stress, modification of Keap1 cysteine residues changes its conformation so that Nrf2 can bind to Keap1 but is not correctly positioned to be ubiquitinated (Li and Kong 2009). As a result Keap1 becomes saturated with Nrf2 and free Nrf2 levels then rise rapidly and activate transcription of selected genes. Given the interaction of Keap1 with actin and previous reports of Nrf2 presence in a 100 kDa protein complex with actin (Kang et al. 2002), we undertook an analysis of Nrf2 interaction with actin in ground squirrel tissues. In all 8 tissues, a portion of the Nrf2 protein was detected in 100 kDa bands, presumably representing the Keap1-bound, actin-associated fraction of Nrf2. The expression patterns of the 57 kDa free Nrf2 and the 100 kDa Nrf2 complex correlated in all organs except in kidney and brain, in which the 57 kDa protein was reduced during hibernation whereas the 100 kDa band remained stable. The denaturing conditions of the SDS–PAGE typically disrupt protein–protein interactions. Since this was not the case for the putative Nrf2–actin pair, this suggests that binding of Nrf2 in the actin complex has a covalent component. Indeed, binding may be due to protein phosphorylation, since studies by Kang et al. (2002) showed that treatment of the 100 kDa Nrf2 with a phosphatase released a 57 kDa Nrf2. To analyze this complex further, we used an actin antibody that detected 43 kDa (actin) in all tissues and confirmed a 100 kDa actin-containing band in brain and heart that may represent the complex. During hibernation, levels of the 100 kDa putative actin complex increased by 1.5-fold in heart, but were reduced to 75% of the control value in brain. The results in heart paralleled those seen for 100 kDa Nrf2 during hibernation, but in brain, the 100 kDa Nrf2 band did not change.

In summary, the data show that HO1 was upregulated at both transcriptional and translational levels in tissues of ground squirrels during hibernation. This suggests a significant role for HO1 in antioxidant defence in hibernator organs, consistent with the known antioxidant actions of biliverdin and bilirubin. The data also provide strong evidence for the regulatory role of the Nrf2–MafG transcription factors in the hibernation-responsive regulation of HO1.

Acknowledgements

We thank Dr. J.M. Hallenbeck, NINDS, NIH, Bethesda, Maryland, for supplying us with samples of ground squirrel tissues, and Dr. John D. Hayes, University of Dundee, for providing the Nrf2 and MafG antibodies. Thanks also to J.M. Storey for editorial review of the manuscript. Supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada; K.B.S. holds the Canada Research Chair in Molecular Physiology.

References

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A.M., and Cook, J.L. 1999. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J. Biol. Chem. **274**(37): 26071–26078. doi:10.1074/jbc.274.37.26071. PMID:10473555.

Applegate, L.A., Luscher, P., and Tyrrell, R.M. 1991. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. Cancer Res. **51**(3): 974–978. PMID:1988141.

Baker, J.A., and van Breukelen, F. 2009. Bile constituents in hibernating golden-mantled ground squirrels (*Spermophilus lateralis*). Comp. Hepatol. **8**(1): 2. doi:10.1186/1476-5926-8-2. PMID: 19470180.

Buzadzić, B., Spasić, M., Saicić, Z.S., Radojicić, R., Petrović, V.M., and Halliwell, B. 1990. Antioxidant defenses in the ground squirrel *Citellus citellus*. 2. The effect of hibernation. Free Radic. Biol. Med. **9**(5): 407–413. doi:10.1016/0891-5849(90)90017-D. PMID:2292435.

Carey, H.V., Andrews, M.T., and Martin, S.L. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol. Rev. **83**(4): 1153–1181. PMID:14506303.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**(5): 1475–1489. doi:10.1093/nar/11.5.1475. PMID:6828386.

Drew, K.L., Tøien, Ø., Rivera, P.M., Smith, M.A., Perry, G., and Rice, M.E. 2002. Role of the antioxidant ascorbate in hibernation and warming from hibernation. Comp Biochem Physiol C Toxicol Pharmacol, **133**(4): 483–492. doi:10.1016/S1532-0456(02)00118-7. PMID:12458177.

Eddy, S.F., McNally, J.D., and Storey, K.B. 2005. Up-regulation of a thioredoxin peroxidase-like protein, proliferation-associated gene, in hibernating bats. Arch. Biochem. Biophys. **435**(1): 103–111. doi:10.1016/j.abb.2004.11.020. PMID:15680912.

Idriss, N.K., Blann, A.D., and Lip, G.Y. 2008. Hemoxygenase-1 in cardiovascular disease. J. Am. Coll. Cardiol. **52**(12): 971–978. doi:10.1016/j.jacc.2008.06.019. PMID:18786476.

Ishii, T., and Yanagawa, T. 2007. Stress-induced peroxiredoxins. Subcell. Biochem. **44**: 375–384. doi:10.1007/978-1-4020-6051-9_18. PMID:18084904.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun. **236**(2): 313–322. doi:10.1006/bbrc.1997.6943. PMID:9240432.

Kang, K.W., Lee, S.J., Park, J.W., and Kim, S.G. 2002. Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. Mol. Pharmacol. **62**(5): 1001–1010. doi:10.1124/mol.62.5.1001. PMID:12391262.

Keyse, S.M., and Tyrrell, R.M. 1989. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc. Natl. Acad. Sci. U.S.A. **86**(1): 99–103. doi:10.1073/pnas.86.1.99. PMID:2911585.

Lee, D.W., and Andersen, J.K. 2006. Role of HIF-1 in iron regulation: potential therapeutic strategy for neurodegenerative disorders. Curr. Mol. Med. **6**(8): 883–893. doi:10.2174/156652406779010849. PMID:17168739.

Lee, P.J., Jiang, B.H., Chin, B.Y., Iyer, N.V., Alam, J., Semenza, G.L., and Choi, A.M. 1997. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J. Biol. Chem. **272**(9): 5375–5381. doi:10.1074/jbc.272.9.5375. PMID:9038135.

Lee, P.J., Camhi, S.L., Chin, B.Y., Alam, J., and Choi, A.M. 2000. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. Am. J. Physiol. Lung Cell. Mol. Physiol. **279**(1): L175–L182. PMID:10893216.

Li, W., and Kong, A.N. 2009. Molecular mechanisms of Nrf2-

mediated antioxidant response. Mol. Carcinog. **48**(2): 91–104. doi:10.1002/mc.20465. PMID:18618599.

Maines, M.D. 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. **2**(10): 2557–2568. PMID:3290025.

Mamady, H., and Storey, K.B. 2006. Up-regulation of the endoplasmic reticulum molecular chaperone GRP78 during hibernation in thirteen-lined ground squirrels. Mol. Cell. Biochem. **292**(1-2): 89–98. doi:10.1007/s11010-006-9221-8. PMID: 16788740.

Morin, P., Jr., and Storey, K.B. 2005. Cloning and expression of hypoxia-inducible factor 1alpha from the hibernating ground squirrel, *Spermophilus tridecemlineatus*. Biochim. Biophys. Acta, **1729**(1): 32–40. PMID:15811624.

Morin, P., Jr., and Storey, K.B. 2007. Antioxidant defense in hibernation: cloning and expression of peroxiredoxins from hibernating ground squirrels, *Spermophilus tridecemlineatus.* Arch. Biochem. Biophys. **461**(1): 59–65. doi:10.1016/j.abb.2007.01.035. PMID:17362873.

Morin, P., Jr., Ni, Z., McMullen, D.C., and Storey, K.B. 2008. Expression of Nrf2 and its downstream gene targets in hibernating 13-lined ground squirrels, *Spermophilus tridecemlineatus*. Mol. Cell. Biochem. **312**(1-2): 121–129. doi:10.1007/s11010-008-9727-3. PMID:18327701.

Mountassif, D., Kabine, M., Latruffe, N., and El Kebbaj, M.S. 2007. Prehibernation and hibernation effects on the D-3-hydroxybutyrate dehydrogenase of the heavy and light mitochondria from liver jerboa (*Jaculus orientalis*) and related metabolism. Biochimie, **89**(8): 1019–1028. doi:10.1016/j.biochi.2007.04.001. PMID:17531367.

Nelson, C.J., Otis, J.P., Martin, S.L., and Carey, H.V. 2009. Analysis of the hibernation cycle using LC-MS-based metabolomics in ground squirrel liver. Physiol. Genomics, **37**(1): 43–51. doi:10.1152/physiolgenomics.90323.2008. PMID:19106184.

Öllinger, R., Wang, H., Yamashita, K., Wegiel, B., Thomas, M., Margreiter, R., and Bach, F.H. 2007. Therapeutic applications of bilirubin and biliverdin in transplantation. Antioxid. Redox Signal. **9**(12): 2175–2185. doi:10.1089/ars.2007.1807. PMID: 17919067.

Osborne, P.G., and Hashimoto, M. 2006. Brain antioxidant levels in hamsters during hibernation, arousal and cenothermia. Behav. Brain Res. **168**(2): 208–214. doi:10.1016/j.bbr.2005.11.007. PMID:16343656.

Owuor, E.D., and Kong, A.N. 2002. Antioxidants and oxidants regulated signal transduction pathways. Biochem. Pharmacol. **64**(5-6): 765–770. doi:10.1016/S0006-2952(02)01137-1. PMID: 12213568.

Page, M.M., Peters, C.W., Staples, J.F., and Stuart, J.A. 2009. Intracellular antioxidant enzymes are not globally upregulated during hibernation in the major oxidative tissues of the 13-lined ground squirrel *Spermophilus tridecemlineatus*. Comp. Biochem. Physiol. A Mol Integr Physiol., **152**(1): 115–122. doi:10.1016/j.cbpa.2008.09.032. PMID:18948223.

Semenza, G.L. 2000. Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. J. Clin. Invest. **106**(7): 809–812. doi:10.1172/JCI11223. PMID:11018065.

Soares, M.P., and Bach, F.H. 2009. Heme oxygenase-1: from biology to therapeutic potential. Trends Mol. Med. **15**(2): 50–58. doi:10.1016/j.molmed.2008.12.004. PMID:19162549.

Storey, K.B. 2003. Mammalian hibernation. Transcriptional and translational controls. Adv. Exp. Med. Biol. **543**: 21–38. PMID: 14713112.

Storey, K.B. 2006. Gene hunting in hypoxia and exercise. Adv. Exp. Med. Biol. **588**: 293–309. doi:10.1007/978-0-387-34817-9_24. PMID:17089897.

Tenhunen, R., Marver, H.S., and Schmid, R. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc. Natl. Acad. Sci. U.S.A. **61**(2): 748–755. doi:10.1073/pnas.61.2.748. PMID:4386763.

Wang, L.C.H., and Lee, T.F. 1996. Torpor and hibernation in mammals: metabolic, physiological and biochemical adaptations. *In* Handbook of physiology: environmental physiology. *Edited by* M.J. Fregley and C.M. Blatteis. Oxford University Press, Oxford. pp. 507–532.