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# Expression of Nrf2 and its downstream gene targets in hibernating 13-lined ground squirrels, *Spermophilus tridecemlineatus*

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**Abstract** Mammalian hibernation is associated with wide variation in heart rate, blood flow, and oxygen delivery to tissues and is used as a model of natural ischemia/reperfusion. In non-hibernators, ischemia/reperfusion is typically associated with oxidative stress but hibernators seem to deal with potential oxidative damage by enhancing antioxidant defenses in an anticipatory manner. The present study assesses the role of the Nrf2 transcription factor in the regulation of antioxidant defenses during hibernation. Nrf2 mRNA and protein expression were enhanced in selected organs of 13-lined ground squirrels, *Spermophilus tridecemlineatus* during hibernation. Furthermore, Nrf2 protein in heart was elevated by 1.4–1.5 fold at multiple stages over a torpor–arousal bout including during entry, long term torpor, and early arousal. Levels returned to euthermic values when squirrels were fully aroused in interbout. Protein levels of selected downstream target genes under Nrf2 control were also measured via immunoblotting over the torpor–arousal cycle in heart. Cu/Zn superoxide dismutase and aflatoxin aldehyde reductase levels increased significantly during entry into torpor and then gradually declined falling to control levels or below in fully aroused animals. Heme oxygenase-1 also showed the same trend. This suggests a role for Nrf2 in regulating the antioxidant defenses needed for hibernation success. Heart *nrf2* was amplified by PCR and sequenced. The deduced amino acid sequence showed high identity with the sequence from other mammals but with selected unique substitutions (e.g., proline residues at positions 111 and 230) that might be important for conformational stability of the protein at near 0°C body temperatures in the torpid state.

**Keywords** Oxidative stress · Antioxidant defense · Ischemia resistance · Torpor–arousal cycle · NF-E2-related factor-2 · Superoxide dismutase · Heme oxygenase · Aflatoxin aldehyde reductase · Heart

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## Introduction

In order to survive winter, many small mammals use hibernation. The winter hibernation season consists of long periods of deep torpor (lasting days to weeks) interspersed by brief interbout periods (often 12–24 h) when animals rewarm to 37–38°C. During torpor, metabolic rate is profoundly depressed, frequently to only 1–5% of the normal euthermic rate, and core body temperature (Tb) decreases to near ambient (often falling to 0–5°C). By hibernating, small mammals can often save up to ~90% of the energy that they would otherwise need to remain euthermic over the winter months [1]. Transitions to and from the torpid state are closely regulated by strong reversible controls (e.g., protein phosphorylation or dephosphorylation) on the rates of multiple energy-expensive metabolic processes as well as by the enhanced expression of selected genes whose protein products address specific needs of the animal in the hypometabolic, hypothermic state of torpor [2, 3].

Hibernating mammals show a strong reduction of heart rate and blood flow during torpor. These conditions would be considered severely ischemic for non-hibernating species. However, studies with Arctic ground squirrels found no evidence of hypoxia during torpor itself but measurement of blood oxygen content indicated global hypoxia

conditions occurred during the arousal period caused by a demand for oxygen that outstripped delivery capacity [4]. Thus, the torpor–arousal cycle has features of an ischemia-reperfusion cycle which is known to cause oxidative stress in other systems (e.g., human heart attack or stroke). Indeed, because of this hibernating mammals are being used as a stroke model system [5]. Another factor that can contribute to the generation of reactive oxygen species (ROS) in the hibernator is the altered composition of lipid reserves that is necessary to maintain lipid fluidity at low Tb values. For optimal hibernation, lipid depots must contain elevated levels of polyunsaturated fatty acids (PUFAs) such as linoleic acid [6] but PUFAs are very susceptible to free radical attack leading to autoxidation and the generation of lipid peroxide radicals [7]. Hence, because of these factors that increase susceptibility to ROS damage during the hibernating season, mammalian hibernators must set up efficient antioxidant defenses to deal with wide variations in ROS generation over torpor–arousal cycles.

Regulation of antioxidant defenses is under the control of specific transcription factors that regulate gene expression, one of the prominent signal transduction pathways being the Nrf2/ARE pathway. The NF-E2-related factor-2 (Nrf2) is a basic leucine zipper transcription factor that binds to a cytoplasmic repressor protein, Keap1, or to different nuclear binding partners. When bound to Keap1 and sequestered in the cytoplasm, Nrf2 is unable to stimulate transcription. Nrf2 can also be directed towards degradation by the proteasome via its interaction with Keap1 [8]. However, under oxidative stress conditions, Nrf2 is released from Keap1 and translocates into the nucleus [9] where it dimerizes with proteins such as the small Mafs, Jun and activating transcription factor-4 (ATF-4) [10–12]. The complex then binds to the antioxidant response element (ARE) in the promoter region of genes that respond to oxidative stress. Well-known Nrf2-responsive antioxidant genes include thioredoxin and GSTs [13, 14], Cu/Zn superoxide dismutase (Cu/Zn SOD) [15], and heme oxygenase-1 (HO-1) [16]. Furthermore, the enzyme aflatoxin aldehyde reductase (AFAR1) is an aldo-keto reductase that is involved in the detoxification of various aldehydes and ketones including the environmental carcinogen aflatoxin $B_1$ [17]. A recent study has shown that the enzyme also contains multiple AREs in its promoter region [18].

The potentially harmful oxidative stress conditions that hibernators may experience over the course of torpor–arousal cycles led us to hypothesize that the Nrf2 pathway, well-known to be involved in ROS detoxification in mammals, would play a role in providing the hibernator with the necessary antioxidant defenses required to deal with hibernation-related oxidative stress. This could include an increase in Nrf2 levels during hibernation and increased expression of downstream gene products that are under Nrf2 control such as Cu/Zn SOD and HO-1. The present study of 13-lined ground squirrels, *Spermophilus tridecemlineatus*, substantiates this hypothesis. Nrf2 gene and protein expression were elevated during torpor and this correlated with a significant increase in the expression of downstream antioxidant enzymes that are known targets of Nrf2.

## Materials and methods

### Animals

Thirteen-lined ground squirrels (130–180 g) were captured by a licensed trapper (TLS Research, Michigan) and transported to the Animal Hibernation Facility (NIH, Bethesda, MD). Hibernation experiments were conducted by the laboratory of Dr. J. M. Hallenbeck (National Institute of Neurological Disorders and Stroke). 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. Tb was measured with an Implantable Programmable Temperature Transponder IPTT-200 (Bio Medic Data Systems). In order to implant the temperature transmitters, ground squirrels were anesthetized with 5% isofluorane and then the transmitter was injected subcutaneously into the middle of the back using a sterile disposable syringe [19]. When squirrels had reached a plateau weight gain of 220–240 g, they were randomly divided into groups for one of two protocols. Initial trials to characterize organ-specific expression of *nrf2* compared two groups. Euthermic controls were maintained under the same conditions as previously and Tb was confirmed as 36–38°C at the time of sampling. The other group was placed in a dark chamber at 4°C. Most animals entered deep torpor within 3–8 days and were sampled after each individual had been hibernating for 2–5 days (as indicated by continuous Tb readings of $\sim$6°C). Euthermic control animals were sampled on the same days as the hibernators. All animals were anesthetized and then sacrificed by decapitation. Tissues were excised, frozen immediately in liquid nitrogen and then transported to Ottawa on dry ice where they were stored at $-$80°C until use.

In subsequent studies, pre-hibernation treatment was the same but animals were sampled at multiple time points over the course of torpor and arousal in a 4°C dark cold room. In this protocol, all animals were transferred into the cold room at the start of the experimental course. The experimental groups sampled were: (1) active in the cold room (ACR); these euthermic squirrels had not yet entered torpor after 3 days in the cold as determined by Tb

readings, (2) early entrance into torpor with falling Tb (ENT) (Tb = 31–12°C, >14 h in the active state after full arousal from a torpor phase of at least 5 days), (3) early in the torpid state (<24 h) with a stable Tb of ~5–7°C (HIB-E), (4) later in the torpid state (at least 3 d continuous torpor) with stable Tb of ~5–7°C (HIB-L), (5) early in arousal with rising Tb (AR-E) (Tb = 9–12°C), and (6) fully aroused in interbout with Tb back at 37°C for at least 18 h before sampling (AR). Animals in the aroused groups had been in torpor continuously for at least 3 d.

### Total RNA isolation and quality assessment

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

### cDNA synthesis and PCR amplification of Nrf2

A 30 μg aliquot of total RNA from heart of hibernating squirrels was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and following the manufacturer's protocol. Serial dilutions of the cDNA in water were prepared ($10^{-1}$–$10^{-4}$) and were used to amplify both Nrf2 and α-tubulin. The primers used for amplification of Nrf2 were designed using the Primer Designer program, v3.0 (Scientific and Educational Software) based on the consensus sequences of mammalian Nrf2. The forward primer sequence was 5′-TCCCAGGT TGCCCACAT-3′ and the reverse was 5′-AATGCCRGAG TCAGARTC-3′. As a control gene, α-tubulin was amplified with forward (5′-AAGGAAGATGCTGCCAATAA-3′) and reverse (5′-GGTCACATTTCACCATCTG-3′) primers. The PCR was performed by mixing 5 μl of each cDNA dilution with 1.25 μl of primer mixture (0.5 μM forward and 0.5 μM reverse), 15 μl of sterile water, 2.5 μl of 10× PCR buffer (Invitrogen), 1.25 μl of 50 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, and 0.125 μl of *Taq* Polymerase (Invitrogen) for a total volume of 25 μl. The cycles performed for amplification consisted of an initial step of 2 min at 94°C, followed by 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min repeated 37 times; the final step was at 72°C for 2 min. PCR products were separated on a 1.0% agarose gel. The gel was prepared by adding 3 g of agarose to 300 ml of 1× TAE buffer prepared by mixing 6 ml of 50× TAE buffer (242 g Tris base, 57.1 ml concentrated acetic acid, 100 ml of 0.5 M EDTA in 1 l water, adjusted to pH 8.5) with 294 ml of DEPC treated water. Ethidium bromide (0.3 mg/300 ml) was added in the solution and the mixture was heated. The heated solution was then poured in a gel tray and the gel was allowed to cool and solidify. A 10 μl aliquot of PCR product was mixed with 2 μl of 6× blue/orange loading dye (Promega) and the solution was loaded on the 1% agarose gel. The gel was run in 1× TAE buffer. After separation, the bands were visualized with ethidium bromide on a UV box. The bands from the most dilute cDNA sample were used for quantification purposes to make sure that the products had not reached amplification saturation. A *nrf2* fragment of 717 bp was found and was excised and sequenced by Canadian Molecular Research Services (Ottawa, ON). The sequence was confirmed as encoding Nrf2 by sequence comparison in BLAST.

### Western blotting

Frozen tissue samples (~500 mg) were homogenized in 2 ml of buffer containing 100 mM MOPS, 25 mM HEPES, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM EGTA, and 250 μM $Na_3VO_4$, adjusted at pH 7.4, with 1 mM phenylmethylsulphonyl fluoride added immediately before homogenization. After centrifugation at 10,000*g* for 10 min at 4°C, supernatants were collected and soluble protein concentrations were determined using the Coomassie blue dye-binding method and the BioRad prepared reagent (BioRad, Hercules, CA). SDS-polyacrylamide gel electrophoresis and blotting to polyvinylidene difluoride membranes was carried out essentially as in Morin and Storey [20] with 10% gels (5% stacking gel), 20 μg of protein loaded per well, and electrophoresis at 200 V for 45 min. Wet transfer of proteins onto PVDF membranes was made using a transfer buffer solution containing 25 mM Tris (pH 8.5), 192 mM glycine and 10% v/v methanol at 4°C for 1.5 h at 0.3 mA. Following transfer, membranes were blocked for 1 h in TBST (50 mM Tris–HCl pH 6.8, 150 mM NaCl, 0.05% v/v Tween 20) with 2.5% w/v powdered skim milk. This was decanted and then membranes were incubated overnight at 4°C with primary antibodies. Antibodies specific for mammalian Nrf2 were purchased from Santa Cruz Biotechnologies and used at a 1:200 v:v dilution in TBST. Antibodies specific to Cu/Zn SOD and HO-1 were purchased from Stressgen and used at dilutions of 1:4000 and 1:2000, respectively. AFAR1 antibody was a gift from Dr. John D. Hayes, University of Dundee and was used at a 1:1000 dilution. Subsequently, membranes were incubated with HRP-linked anti-rabbit IgG secondary antibody (1:2000 v:v dilution) in TBST for 1 h and then blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's protocol. Bands were visualized using a ChemiGenius Bio Imaging System (SynGene, MD,

USA) and band densities were quantified using the associated Gene Tools software.

### Quantification and statistics

Bands corresponding to *nrf2* PCR product were normalized against bands for α-tubulin amplified from the same cDNA sample. Western blot band intensities were standardized against a group of three 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. Significant differences between the groups were assessed using GraphPad InStat 3 software and either the Student's *t*-test for the euthermic versus hibernating data or analysis of variance followed by the Dunnett's test for the hibernation cycle data.

## Results

### cDNA cloning of Nrf2

Using RT-PCR and primers derived from the consensus sequence of *nrf2* from other mammalian species, a PCR product of 717 bp was retrieved from total RNA prepared from heart of hibernating ground squirrels. The product was confirmed as encoding a portion of the *nrf2* sequence and the sequence was submitted to GenBank with accession number DQ328859. Figure 1 shows the partial translated amino acid sequence of ground squirrel Nrf2 aligned with the sequences for the human, mouse and rat protein. The full Nrf2 sequence has 605 residues in humans and 597 residues in mice and rats whereas the amplified portion of *S. tridecemlineatus* Nrf2 encoded 239 amino acids residues, corresponding to ~40% of the full sequence. Ground squirrel Nrf2 was quite similar to Nrf2 from other mammals and shared 87.9, 77.4, and 78.3% identity to human, mouse and rat Nrf2, respectively, over the amplified region. Ground squirrel Nrf2 contained a few unique amino acid substitutions that were not seen in non-hibernating mammals; these are shown in bold underline in Fig. 1. These included substitutions of two proline residues at positions 111 and 230 as compared with the human sequence. An asparagine residue replaced a tyrosine residue at position 226 and a tyrosine was substituted for a histidine at position 227.

### Nrf2 gene expression

Levels of *nrf2* mRNA transcripts were measured for three tissues of euthermic and hibernating ground squirrels: brown adipose tissue (BAT), heart and lung. Using RT-PCR and the primers for *nrf2* and alpha-tubulin, a constitutively expressed gene, the relative levels of both transcripts were assessed in each organ. Levels of *nfr2* transcripts were normalized against the tubulin transcript level in each sample. Figure 2b shows the ratio of normalized *nrf2*

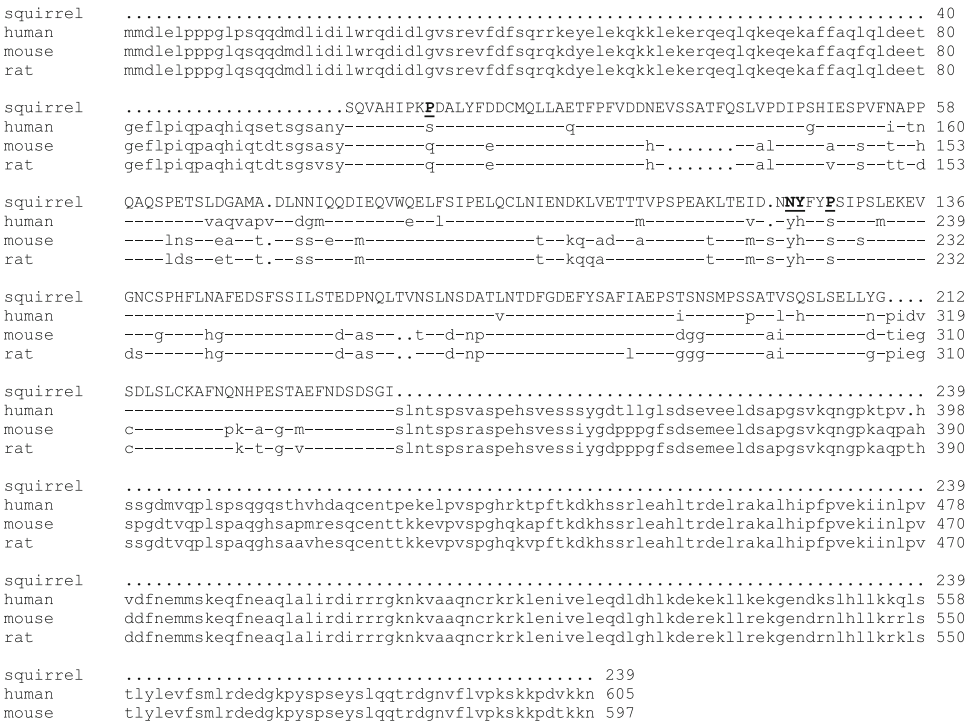


**Fig. 1** Partial amino acid sequence (239 residues) of Nrf2 from 13-lined ground squirrels (*S. tridecemlineatus*) compared to Nrf2 sequences of human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*); Genbank accession numbers for the four species are DQ328859, NP_006155, NP_035032 and O54968, respectively. The full length protein is 605 amino acids for human Nrf2 and 597 amino acids for mouse and rat Nrf2. Dashed lines in the alignment represent amino acids that are identical with the ground squirrel sequence; spacer dots are inserted when residues are not present in all sequences. Selected substitutions in the *S. tridecemlineatus* sequence are shown in bold underline

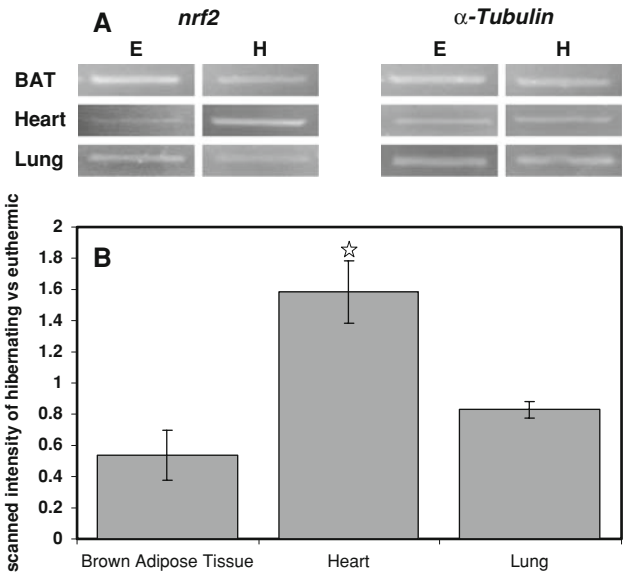


**Fig. 2** Effect of hibernation on *nrf2* mRNA levels in three tissues of 13-lined ground squirrels. (**a**) Representative bands on agarose gels show PCR product levels amplified from ground squirrel cDNA. Paired tubes amplified *nrf2* and *α-tubulin* transcripts from the same samples and *nrf2* band intensities were normalized against the corresponding *α-tubulin* intensity for the same sample. Band sizes were 717 bp for *nrf2* and 616 bp for *α-tubulin*. BAT is brown adipose tissue. (**b**) Histogram showing the ratio of normalized PCR product levels in tissues from hibernating (2–5 days of continuous torpor in the 4°C cold room with Tb readings of ~6°C) versus euthermic (at 21°C with Tb 36–38°C) ground squirrels. Values are means ± SEM for $n = 3$ independent trials on tissue from different animals. *, Hibernating values are significantly different from the corresponding euthermic control, $P < 0.05$

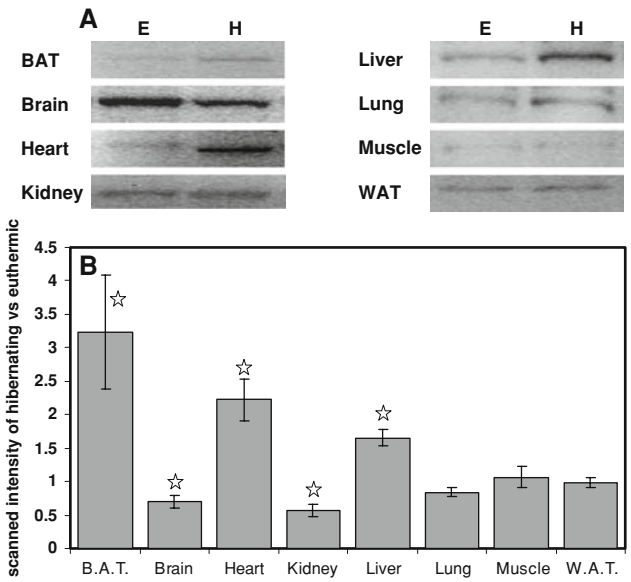


**Fig. 3** Nrf2 protein levels in eight tissues of 13-lined ground squirrels. (**a**) Representative Western blots show expression of the Nrf2 band at 57 kDa. (**b**) Histogram shows mean relative protein expression in tissues from hibernating versus euthermic ground squirrels; animal conditions are as in Fig. 2. Data are means ± SEM, $n = 3$ independent trials on tissue from different animals. *, Hibernating values are significantly different from the corresponding euthermic control, $P < 0.05$

transcript levels in the tissues. Relative levels of *nfr2* transcripts changed significantly only in heart, with a 1.6-fold increase in transcript levels during hibernation.

## Nrf2 protein expression

Nrf2 protein levels were measured by immunoblotting. Western blot analysis of Nrf2 in ground squirrel tissues revealed that the content of the 57 kDa Nrf2 protein increased significantly in BAT, heart and liver during hibernation (by 3.2-, 2.2- and 1.6-fold, respectively, $P < 0.05$) (Fig. 3). Interestingly, Nrf2 protein levels decreased in brain and kidney of hibernating animals to 70 and 60% of the euthermic values, respectively ($P < 0.05$).

## Protein levels of Nrf2 and downstream targets in hibernating heart

The above data for both *nrf2* mRNA and Nrf2 protein levels indicated that changes in this transcription factor are important in heart during hibernation. In order to further explore Nrf2 expression in heart, we measured Nrf2 protein levels at six different points over the course of the hibernation cycle: (1) euthermic squirrels that were active in the cold room (ACR) and had not yet entered hibernation, (2) early entrance into torpor with falling Tb (ENT), (3) early in the torpid state (<24 h) with a stable Tb at ~5–7°C (HIB-E), (4) later in the stable torpid state (at least 3 d) with Tb at ~5–7°C (HIB-L), (5) early in arousal with rising Tb (AR-E), and (6) fully aroused in interbout with Tb back at 37°C (AR) (Fig. 4). Nrf2 protein in heart of ground squirrels entering hibernation rose significantly to levels that were 1.36-fold higher than the values in ACR controls ($P < 0.05$). Amounts remained elevated throughout torpor and early arousal (by 1.3 to 1.5-fold compared with ACR) but declined back to control levels in fully aroused animals.

In order to determine whether elevated Nrf2 during hibernation altered the expression of genes that are under Nrf2 control in other mammals, levels of three proteins were assessed in heart over the same time course of hibernation (Fig. 4). AFAR1 protein levels in ground squirrel heart increased significantly during entrance into hibernation by 1.43-fold as compared with ACR animals ($P < 0.05$). Levels then declined gradually back to euthermic values. Cu/Zn SOD levels also rose during entrance into hibernation by 1.5-fold ($P < 0.05$) and remained high during hibernation. Levels decreased significantly when squirrels were fully aroused from torpor;

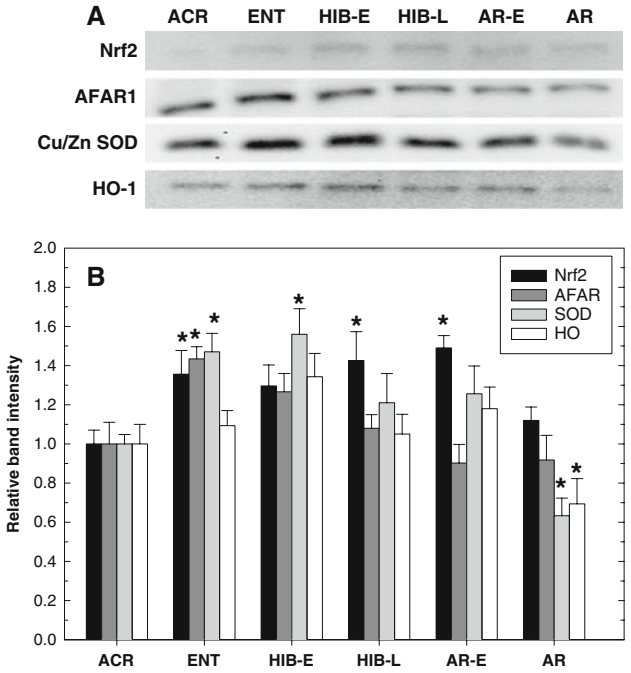


**Fig. 4** Protein expression of Nrf2, AFAR1, Cu/Zn SOD and HO-1 in heart of *S. tridecemlineatus*. (**a**) Representative Western blots showing single strong cross-reacting bands at 57, 40, 19 and 32 kDa, respectively, the expected monomeric molecular masses of Nrf2, AFAR1, Cu/Zn SOD and HO-1 in other mammals. (**b**) Histogram showing relative protein expression in squirrels held in a 4°C cold room and sampled from different physiological conditions (fully defined in the Materials and Methods): active in the cold room (ACR), entrance into hibernation (ENT), early-hibernation (Hib-E), late-hibernation (Hib-L), early-arousal (Ar-E), and fully aroused (AR). Data for standardized band intensities are expressed relative to the corresponding values for ACR. Data are means ± SEM, $n = 3$–7 independent trials on heart samples from different animals. *, Value is significantly different from the corresponding ACR value, $P < 0.05$

heart SOD levels in the AR group were just 63% of the ACR value ($P < 0.05$). HO-1 protein showed an increasing trend during hibernation (although not significant) but, like SOD, HO-1 decreased significantly in fully aroused animals (AR), falling to 69% of the ACR value ($P < 0.05$).

## Discussion

The potential for oxidative stress during the different hibernation stages has been assessed previously. Most of the work performed to date has focused on the levels and/or the activity of antioxidant enzymes and markers of antioxidative defense over the hibernation time course. This study adds to this previous work by measuring the levels of the Nrf2 transcription factor and of its target genes at different time points over the torpor–arousal cycle. Evidence that oxidative stress occurs during hibernation has been produced previously from studies with intestine of 13-lined ground squirrels [21]. Studies with black bears have also highlighted increases in a marker for lipid peroxidation during hibernation [22]. Indirect evidence that oxidative stress occurs comes from several studies of antioxidant defense mechanisms which have shown specific elevation during hibernation of the activities of selected antioxidant enzymes including glutathione peroxidase in ground squirrels (*Citellus citellus*) [23], peroxiredoxin in both bats (*Myotis lucifugus*) and ground squirrels (*S. tridecemlineatus*) [24, 25], and serum catalase and superoxide dismutase-like activity in hamsters (*Mesocricetus auratus*) [26, 27]. Furthermore, plasma ascorbate levels in Arctic ground squirrels (*Spermophilus parryii*) rose by 3–5 fold during each torpor bout and were then rapidly depleted during arousal, the highest rate of decrease correlating with the time when oxygen consumption was maximal during thermogenesis [28]. Microanalysis of tissue and extracellular fluid in brain of hamsters also showed depletion of ascorbate during arousal, consistent with oxidative stress in this stage of the hibernation cycle [29].

Clearly, then, hibernators need well-developed antioxidant defenses and this would predictably include increased expression of genes coding for antioxidant enzymes. One way to stimulate the antioxidant response is through activation of transcription factors that regulate these genes. The present study examines the expression of the transcription factor Nrf2. Many studies have shown the importance of this transcription factor in protecting cells against oxidative stress. For example, studies using Nrf2 knockout mice showed that expression of several detoxification enzymes was strongly reduced in the knockout strain [30]. Furthermore, hyperoxia-induced levels of mRNA for antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and HO-1 were much lower in Nrf2 knockout mice than in control mice, linking Nrf2 to the antioxidant defenses needed to deal with hyperoxia-associated ROS production [31]. Hence, Nrf2 and its downstream enzymes could be good indicators of current or anticipated oxidative stress in hibernator tissues.

The Nrf2 transcription factor from ground squirrel heart was partially cloned in the N-terminal region of the protein. The amplified fragment was 239 amino acids long and started at position 103 as compared with the human Nrf2 sequence. Several amino acids substitutions were identified when the ground squirrel partial Nrf2 sequence was compared to Nrf2 sequences from non-hibernating mammals. The amplified Nrf2 segment from ground squirrels contained two key domains, Neh4 (within residues 98–156) and Neh5 (within residues 153–227), which have both been shown to act synergistically to allow Nrf2 transactivation through CREB binding [32]. Deletion of these domains leads to a severe disruption in Nrf2 transactivation activity. It has also been demonstrated that phosphorylation of

residues within these domains by mitogen-activated kinase family members, ERK and/or JNK, leads to a positive regulation of Nrf2 transactivational activity [33]. Only minor modifications were seen in the Neh4 and Neh5 domains of the ground squirrel protein suggesting that the transactivation process is largely conserved in the hibernator. However, two changes of interest occurred near these regions; the substitutions of proline residues for a serine/arginine residue at position 111 and for a serine residue at position 230. These modifications, along with the other substitutions that were not exclusive to the ground squirrel, could result in a conformational change to the hibernator Nrf2 protein that might aid its transcriptional activity at low body temperatures.

Levels of *nrf2* mRNA transcripts were measured in three ground squirrel tissues using RT-PCR. Transcript levels were significantly elevated only in heart during hibernation (by 1.6-fold) whereas amounts in other tissues appeared to be down-regulated. Previous studies have been contradictory as to whether *nrf2* transcript levels rise under oxidative stress conditions. Whereas Cho et al. [31] reported an induction of *nrf2* transcripts by 2.0- and 2.6-fold in lungs of mice subjected to 48 and 72 h of hyperoxia, a study by Papaiahgari et al. [34] had contrary results. Data gathered here seems to agree with the former study since *nrf2* gene induction was observed. Nrf2 protein levels were then measured in ground squirrel tissues by immunoblotting. Levels were significantly elevated in BAT, heart and liver (3.2-, 2.2- and 1.6-fold, respectively) during hibernation but decreased significantly in ground squirrel brain and kidney.

Due to the concomitant increase of Nrf2 mRNA and protein levels in ground squirrel heart during hibernation, Nrf2 protein levels in this organ were assessed at multiple time points over a hibernation cycle. Significantly elevated levels of this transcription factor were found over heterothermic portions of the time course: entry into torpor, late torpor, and arousing from torpor (Fig. 4). Levels returned to near control values only after animals were fully aroused. Reports have suggested that Nrf2 protein levels increase under oxidative stress [35, 36]. Hence, the general elevation of Nrf2 protein over the torpor portions of the hibernation cycle suggests that the antioxidant defenses of hibernator heart may also be enhanced during this time.

In order to assess this idea, three enzymes that have ARE binding sites in their promoter regions were chosen for study: Cu/Zn SOD, AFAR1 and HO-1. These enzymes all play important roles in the detoxification of ROS (SOD, HO-1) or xenobiotic carbonyl compounds (AFAR1) and they could all have roles in the protection against oxidative stress in hibernators. HO-1 is an inducible enzyme that is involved in heme degradation and the subsequent production of biliverdin, a known antioxidant [37]. The enzyme is activated under many conditions that lead to ROS generation such as anoxia, hypoxia, and ischemia [37, 38]. Control of HO-1 involves transcription factors including HIF-1 and Nrf2 [16, 38]. Cu/Zn SOD is a cytoplasmic enzyme that catalyzes the breakdown of harmful superoxide into hydrogen peroxide and water. It is involved in the regulation of ROS generation and the Cu/Zn SOD gene is up-regulated in response to oxidative stress. Cu/Zn SOD can also help to reduce cellular injury following reperfusion of tissues that were previously ischemic [39]. Since Nrf2 was elevated over the hibernation time course, protein levels of these downstream enzymes were also measured over the same time course to see if they correlated with Nrf2 levels. The data showed that Cu/Zn SOD and AFAR1 protein levels increased significantly in parallel with elevated Nrf2 levels during entry into torpor whereas HO-1 protein levels also showed an upward trend (Fig. 4). This suggests that induction of synthesis of these antioxidant proteins is triggered as one of the first events when animals begin to suppress metabolic rate. This highlights the possibility that hibernators increase antioxidant defenses as an anticipatory or preparatory response in order to deal with the oxidative stress that occurs during prolonged torpor and arousal. Such a preparation for oxidative stress by elevation of antioxidant defenses has been proposed previously for other situations of hypometabolism/arousal such as during estivation in land snails [40]. It is interesting that none of the three downstream proteins remained significantly elevated in heart during early arousal (although Nrf2 was), a time when oxidative stress should be high due to the high rates of oxygen-based thermogenesis needed to return to euthermic Tb [28, 29]. Hence, it is possible that these particular Nrf2 regulated enzymes actually have their most prominent functional role during the torpor period itself. Various defensive mechanisms are needed to ensure metabolic stability and viability over the long term during torpor, since the potential to degrade oxidatively damaged proteins and/or synthesize new proteins is strongly suppressed during torpor as part of the general suppression of ATP expenditure [3]. However, given that Nrf2 levels remain elevated during early arousal, it is probable that additional gene targets under Nrf2 control may remain elevated and/or be specifically up-regulated during the arousal phase. One of these may be peroxidredoxin (Prdx). A recent report documented Nrf2 control over Prdx1 in response to hypoxia/reoxygenation [41]. Our analysis of Prdx isozymes in 13-lined ground squirrels showed strong hibernation responsive up-regulation of 3 Prdx isozymes in heart including a 13-fold increase in Prdx1 protein over euthermic levels [25]. Nrf2 may be the transcription factor responsible.

Overall, these results document an up-regulation of the Nrf2 transcription factor in heart and selected other organs

during hibernation suggesting that gene targets under Nrf2 control have important roles to play in hibernation success. This is substantiated by parallel increases during hibernation in Cu/Zn SOD and AFAR1 protein levels, as well as peroxiredoxins [25], all known Nrf2 gene targets. We conclude that a Nrf2-mediated up-regulation of multiple antioxidant enzymes is a key preventative defense against oxidative stress occurring over the torpor–arousal cycle.

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