Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 461 (2007) 59–65
ABB
www.elsevier.com/locate/yabbi

Antioxidant defense in hibernation: Cloning and expression of peroxiredoxins from hibernating ground squirrels, *Spermophilus tridecemlineatus*

Pier Jr Morin *, Kenneth B. Storey

Institute of Biochemistry and Department of Chemistry, College of Natural Sciences, Carleton University, 1125 Colonel By Drive, Ottawa, Ont., Canada K1S 5B6

Received 27 November 2006, and in revised form 28 January 2007
Available online 23 February 2007

Abstract

Mammalian hibernation is characterized by prolonged torpor bouts interspersed by brief arousal periods. Adequate antioxidant defenses are needed both to sustain cell viability over weeks of deep torpor and to defend against high rates of oxyradical formation associated with massive oxygen-based thermogenesis during arousal. The present study shows that up-regulation of peroxiredoxins contributes to antioxidant defense during torpor in thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. Expression levels of three isozymes of the 2-Cys peroxiredoxin (Prdx) family were quantified by Western blotting, the results showing 4.0- and 12.9-fold increases in Prdx1 protein in brown adipose tissue (BAT) and heart, respectively, during hibernation compared with euthermia. Comparable increases in Prdx2 were 2.4- and 3.7-fold whereas Prdx3 rose by 3.1-fold in heart of torpid animals. Total 2-Cys peroxiredoxin enzymatic activity also rose during hibernation by 1.5-fold in heart and 3.5-fold in BAT. Furthermore, RT-PCR showed that *prdx2* mRNA levels increased by 1.7- and 3.7-fold in BAT and heart, respectively, during hibernation. A partial nucleotide sequence of *prdx2* from ground squirrels was obtained by PCR amplification, the deduced amino acid sequence showing 96–97% identity with Prdx2 from other mammals. Some unique amino acid substitutions were identified that might contribute to stabilizing Prdx2 conformation at the near 0 °C body temperatures during torpor.

Keywords: Oxidative stress; Thermogenesis; Torpor; Thioredoxin peroxidase; Mammalian hibernation; Brown adipose tissue; Heart

Many small mammals use hibernation to survive the winter. The hibernating season is characterized by long periods of torpor (a few days to several weeks) interspersed by brief periods of interbout euthermia when the animal rewarms to body temperatures of 37–38 °C. During torpor, metabolic rate may be reduced to just 1–5% of the normal euthermic rate and core body temperature can fall to 0–5 °C. By hibernating, ground squirrels can save nearly 90% of the energy that they would otherwise require to remain euthermic over the winter months [1]. Entrance into and arousal from torpor are tightly regulated, the molecular mechanisms involved including reversible phosphorylation of key enzymes and functional proteins to coordinate the suppression of energy-expensive metabolic processes as well as the selected expression of genes whose protein products address specific needs during torpor [2–4].

One need that the hibernator has is to protect its cells against oxidative damage occurring both over the long term while in torpor and as a result of the huge increase in oxygen consumption that occurs during arousal. Previous studies on brown adipose tissue (BAT)[1] of rats have shown that prolonged exposure to cold leads to an activation of antioxidant defenses, presumably to deal with high

* Corresponding author. Fax: +1 613 520 2569.
E-mail address: pierjr.morin@fmi.ch (P.J. Morin).

[1] *Abbreviations used:* BAT, brown adipose tissue; ROS, reactive oxygen species; NF, nuclear factor; Prdx, peroxiredoxin; DEPC, diethylpyrocarbonate; PMSF, phenylmethylsulphonyl fluoride; Trx, thioredoxin.

0003-9861/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.abb.2007.01.035

rates of reactive oxygen species (ROS) generation associated with the very high oxygen consumption by this organ during the uncoupled respiration that provides thermogenesis [5]. Lipid-fueled thermogenesis by BAT of hibernators similarly results in a huge increase in oxygen consumption, typically overshooting normal euthermic rates to raise the body temperature (T_b) back to euthermic values within minutes. The potential for ROS generation during this time is very high and requires that hibernators are protected with well-developed antioxidant defenses. Indeed, studies of hibernator BAT provided some of the first examples of adaptive change in antioxidant enzyme activities in response to physiological oxidative stress [6,7].

Recently, a new group of antioxidant enzymes has been discovered—the peroxiredoxins. These intracellular enzymes reduce and detoxify a wide range of hydroperoxides, typically using thioredoxin as the electron donor (Fig. 1) [8,9]. Peroxiredoxins are actually major components of cells, representing about 0.1–0.8% of the total soluble protein fraction in most mammalian cell types [10]. The six known family members in mammals are divided into two classes, 1-Cys and 2-Cys peroxiredoxins, depending on the number of cysteine residues involved in catalyzing the reaction [11] and recent data suggests that the 2-Cys peroxiredoxin subclass can be further divided into two more categories based on mechanistic and structural differences. Studies have shown that peroxiredoxins can counteract oxidative stress arising from a variety of sources in different organisms [12–14]. Apart from their role in antioxidant defense in the detoxification of hydroperoxides (in the presence of thioredoxin and thioredoxin reductase) [10], recent studies have also shown strong evidence that H_2O_2 is involved in intracellular signal transduction and that peroxiredoxins have a role in this signaling function [15,16]. For example, studies have shown that (a) transient elevations of intracellular H_2O_2 occur in response to various cytokines and peptide growth factors, (b) elevated H_2O_2 affects the function of various protein kinases and phosphatases, transcription factors, and G proteins, and (c) inhibition of H_2O_2 generation results in a complete blockage of signaling by various growth factors [15]. Studies that have analyzed H_2O_2 generation as a result of membrane receptor activation have shown that 2-Cys peroxiredoxins not only eliminate the intracellular H_2O_2 produced during receptor activation but also prevent further downstream signaling events from occurring [17]. Overexpression of peroxiredoxins could ultimately also suppress the transcriptional activity of the nuclear factor (NF)-κB transcription factor [18]. This ability of peroxiredoxins to affect transcriptional activity made them an even more interesting set of enzymes to study in the hibernator model.

Given the importance of antioxidant defenses to successful hibernation, we reasoned that hibernation-responsive changes in peroxiredoxins may be part of the adaptive strategy that provides defense against oxidative stress for the hibernating mammal. The present study examines the responses to hibernation by peroxiredoxin 1, 2 and 3 (Prdx1, Prdx2, Prdx3), all 2-Cys subfamily members, in BAT and heart of thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. Western blotting was used to quantify Prdx protein levels in tissue samples from euthermic versus torpid animals, a partial *prdx2* sequence was amplified and used to assess *prdx2* transcript levels by RT-PCR, and total 2-Cys peroxiredoxin enzymatic activity was measured. The data highlight the importance of peroxiredoxins in the defense mechanisms for dealing with oxidative stress in hibernator organs and their potential involvement in intracellular signaling over torpor-arousal cycles.

Materials and methods

Animals

Thirteen lined ground squirrels, *S. tridecemlineatus* (130–180 g) were captured by a licensed trapper (TLS Research, Michigan) and transported to the Animal Hibernation Facility (NIH, Bethesda, MD). Hibernation experiments were conducted by the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke). 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. Body temperature (T_b) was measured with an Implantable Programmable Temperature Transponder IPTT-200 (Bio Medic Data Systems). To implant the temperature transmitters, the ground squirrels were first anaesthetized with 5% isofluorane

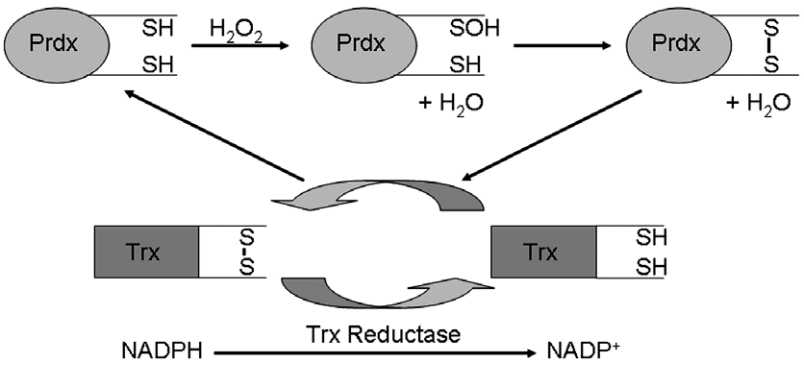

Fig. 1. The 2-Cys peroxiredoxin (Prdx) enzymatic reaction showing catabolism of H_2O_2 by reduced Prdx and the conversion of oxidized Prdx back to the reduced form by thioredoxin (Trx) with the aid of NADPH-dependent thioredoxin reductase. This coupled enzyme system was used to assay Prdx activity. Figure is modified from Kim et al., 2005.

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 two groups. One group (euthermic controls) was maintained under the same conditions as previously and body temperatures of the animals were confirmed as being 36–38 °C at the time of tissue sampling. The other group was placed in a dark chamber at 5–6 °C to induce hibernation. Most of the animals entered a state of deep torpor within 3–8 days [20] and were sampled after each individual had been hibernating for 2–5 days (as indicated by continuous body temperature readings of ~6 °C). Euthermic control animals were sampled on the same day as the hibernators. All animals were sacrificed by decapitation and tissues were excised, frozen immediately in liquid nitrogen and then transported to Ottawa on dry ice where they were then placed at −80 °C until use.

Total RNA isolation

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 prdx2

A 30 μg aliquot of total RNA from heart of hibernating *S. tridecemlineatus* was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and following the manufacturer's protocol. Serial dilutions of the cDNA in water were prepared (10^{-1}–10^{-4}) and were used to amplify both *prdx2* and α-tubulin. The primers used for amplification of *prdx2* were designed using the Primer Designer program, version 3.0 (Scientific and Educational Software) based on the consensus sequences of mammalian *prdx2*. The forward primer sequence was 5′-CKGTSGACTCTCAGTTCA-3′ and the reverse primer sequence was 5′-TATTCCTTGCTGTCRTCCAC-3′. For 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, 63 °C for 1 min, and 72 °C for 1 min repeated 37 times; the final step was at 72 °C for 2 min. PCR products were separated on a 1.0% agarose gel. The gel was prepared by adding 3 g of agarose to 300 mL of 1× TAE buffer prepared by mixing 6 mL of 50× TAE buffer (242 g Tris base, 57.1 mL concentrated acetic acid, 100 mL of 0.5 M EDTA in 1 L water, adjusted to pH 8.5) with 294 mL of DEPC treated water. Ethidium bromide (0.3 mg/300 mL) was added in the solution and the mixture was heated. The heated solution was then poured in a gel tray and the gel was allowed to cool down and solidify. A 10 μL aliquot of the PCR product was mixed with 2 μL of 6× blue/orange loading dye (Promega, USA) and the solution was loaded on the 1% agarose gel. The gel was run in 1× TAE buffer. After separation, the bands were visualized with ethidium bromide on a UV box. The bands from the most dilute cDNA sample were used for quantification purposes to make sure that the products had not reached amplification saturation. A *prdx2* fragment of 303 bp was retrieved and sequenced by Canadian Molecular Research Services (Ottawa, Ont.). The sequence was confirmed as encoding *prdx2* 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 $NaVO_4$, adjusted at pH 7.4, with 1 mM phenylmethylsulphonyl fluoride (PMSF) added immediately before homogenization. After centrifugation at 10,000*g* for 10 min at 4 °C, supernatants were collected and soluble protein concentrations were determined using the Coomassie blue dye-binding method and the Bio-Rad prepared reagent (Bio-Rad, Hercules, CA). Samples were then diluted to the desired concentration in sample buffer containing 100 mM Tris–HCl (pH 6.8), 4% SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, and 0.2% w/v bromophenol blue. Samples were boiled for 5 min, cooled and then frozen at −20 °C until use. SDS–polyacrylamide gel electrophoresis used 10% gels (5% stacking gel), 20 μg of protein per well, and electrophoresis at 200 V for 45 min. Proteins were then wet transferred onto polyvinylidene difluoride membranes 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, the PVDF membrane was blocked for 1 h in TBST (50 mM Tris–HCl, pH 6.8, 150 mM NaCl, 0.05% v/v Tween 20) with 2.5% w/v powdered skim milk. This was decanted and then membranes were incubated overnight at 4 °C with primary antibodies (polyclonal rabbit antibodies raised against human Prdx1, Prdx2 or Prdx3 from LabFrontier, Korea) at a 1:2000 v:v dilution in 5 mL of TBST. 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 (Syngene, MD, USA) and band intensities were quantified using the associated GeneTools software.

Peroxiredoxin activity assay

The assay was performed as described in Kim et al. [21] using the coupled enzyme system in Fig. 1. Samples of frozen tissue were homogenized 1:10 w:v in lysis buffer (20 mM Hepes buffer, pH 7.0, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1 % v/v Triton X-100) with a few crystals of PMSF added immediately before homogenization. Samples were centrifuged at 10,000*g* for 15 min at 4 °C and supernatants were collected, stored on ice and assayed for 2-Cys peroxiredoxin activity. A pre-reaction cocktail was prepared that contained 50 mM Hepes–NaOH (pH 7.0), 1 mM EDTA, 200 μM NADPH, 1.5 μM *Escherichia coli* thioredoxin and 0.8 μM thioredoxin reductase (kindly provided by Dr. Sylke Muller, University of Glasgow). Then 100 μM H_2O_2 and 15 μL of enzyme extract were added into reaction wells on a 96-well microplate. The reaction was started by adding 180 μL of the pre-reaction cocktail to the wells and NADPH oxidation was monitored for 30 min. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min at 21 °C.

Results

cDNA cloning of peroxiredoxin 2

Using RT-PCR and primers derived from the consensus sequence of *prdx2* from other mammalian species, a PCR product of 303 bp was retrieved from total RNA prepared from heart of hibernating *S. tridecemlineatus*. The product was confirmed as encoding a portion of the *prdx2* sequence and the sequence was submitted to GenBank with Accession No. DQ201844. Fig. 2 shows the amino acid sequence of ground squirrel Prdx2 aligned with the sequences for the human, mouse and rat protein. The full mammalian Prdx2 sequence has 198 residues whereas the amplified portion of *S. tridecemlineatus* Prdx2 encoded 101 amino acids residues, corresponding to 51% of the full sequence. Ground squirrel Prdx2 contained three amino acid substitutions that were not seen in non-hibernating rodents (shown in

```
S. tridecemlineatus  .....                                    0
R. norvegicus        masgnahigkpapdftgtavvdgafkeiklsdyrgkyvvl  40
M. musculus          ******q***s*****a*******************d*   40
H. sapiens           ******r*********a**********v*****k******  40

S. tridecemlineatus  .....                                    0
R. norvegicus        ffypldftfvcpteiiafsdhaedfrklgcevlgvsvdsq  80
M. musculus          ****************************r***********  80
H. sapiens           ******************nr******************  80

S. tridecemlineatus  ..........KEGGLGPLNIPLLADVTRSLSHNYGVLKS  29
R. norvegicus        fthlawintpr----------------k---q------n  120
M. musculus          **********-----------------k----------n  120
H. sapiens           **********-----------------r--ed-----t  120

S. tridecemlineatus  DEGIAYRGLFIIDGKGVLRQITVNDLPVGRSVDEALRLVQ  69
R. norvegicus        ------------a-------------------------  160
M. musculus          ---v---------as-------------------------  160
H. sapiens           ----------------------------------------  160

S. tridecemlineatus  AFQYTDEHGEVCPAGWKPGSDTIKPNVDDSKE......  101
R. norvegicus        --------------------------------yfskhn  198
M. musculus          --------------------------------******  198
H. sapiens           --------------------------------******  198
```

Fig. 2. Partial amino acid sequence (101 residues) of Prdx2 from the 13-lined ground squirrel *S. tridecemlineatus* compared to Prdx2 sequences of human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*); GenBank accession for the four species are DQ201844, NP_005800, NP_035693, and NP_058865, respectively. Note that the full length protein is 198 amino acids. Dashes replace amino acid residues that are identical with *S. tridecemlineatus*; * replace amino acids that are identical with *R. norvegicus;* spacer dots indicate residues that are missing in the ground squirrel sequence. Selected substitutions in the *S. tridecemlineatus* sequence are shown in bold underline.

bold underline in Fig. 2). These were the substitutions of an arginine residue for a lysine residue at position 109 of the rat sequence, a serine substitution for asparagine at position 120 and a glycine for alanine substitution at position 134.

Peroxiredoxin 2 gene expression in ground squirrel tissues

Transcript levels of *prdx2* mRNA were assessed in BAT and heart from euthermic and hibernating *S. tridecemlineatus* using RT-PCR. Primers for *prdx2* were designed from a consensus sequence made from human, mouse and rat sequences. Fig. 3 shows the relative *prdx2* mRNA expression in each organ. α-Tubulin mRNA, a constitutively expressed gene, was also amplified from the same samples and *prdx2* transcript levels were normalized against the tubulin transcript level in each sample. Fig. 3b shows the ratio of normalized *prdx2* transcript levels in hibernation versus euthermia. *prdx2* transcript levels increased significantly during hibernation in both organs; levels were 1.7- and 3.7-fold higher in BAT and heart, respectively, from hibernating versus euthermic animals ($P < 0.05$).

Peroxiredoxin protein levels

Protein levels of peroxiredoxin isozymes 1, 2 and 3 were measured by immunoblotting (Fig. 4). Each antibody crossreacted with only a single protein band at 23, 25 or 28 kDa for Prdx1, Prdx2 and Prdx3, respectively, which corresponds to the molecular weight of these enzymes from other mammalian sources. Fig. 4b shows the ratio of Prdx1, Prdx2 and Prdx3 protein in BAT and heart from hibernating versus euthermic ground squirrels. Prdx1 and Prdx2 protein content increased significantly in both tissues of hibernating animals, compared with euthermic ground squirrels ($P < 0.05$). Prdx1 protein content rose by 4.0- and 12.9-fold in BAT and heart, respectively, during hibernation, whereas Prdx2 protein was 2.4- and 3.7-fold higher in the same tissues. Prdx3 protein levels increased significantly in heart during hibernation by 3.1-fold ($P < 0.05$).

2-Cys peroxiredoxin activity assay

Total 2-Cys peroxiredoxin activity was assayed in ground squirrel BAT and heart using a spectrophotometric assay that follows the decrease in absorbance at 340 nm due to NADPH oxidation. Peroxiredoxin catabolism of H_2O_2 oxidizes thioredoxin which is then regenerated via thioredoxin reductase with the consumption of NADPH. Mean enzymatic activity in BAT was 0.45 ± 0.18 mU/g fresh weight in samples from euthermic ground squirrels and significantly higher, 1.52 ± 0.14 mU/gfw in samples from hibernating animals, a 3.4-fold increase (both $n = 3$, $P < 0.05$). In heart, activity increased significantly by

1.5-fold during hibernation, from 1.96 ± 0.09 mU/gfw in euthermia to 2.96 ± 0.16 mU/gfw in hibernation (both $n = 3$, $P < 0.05$).

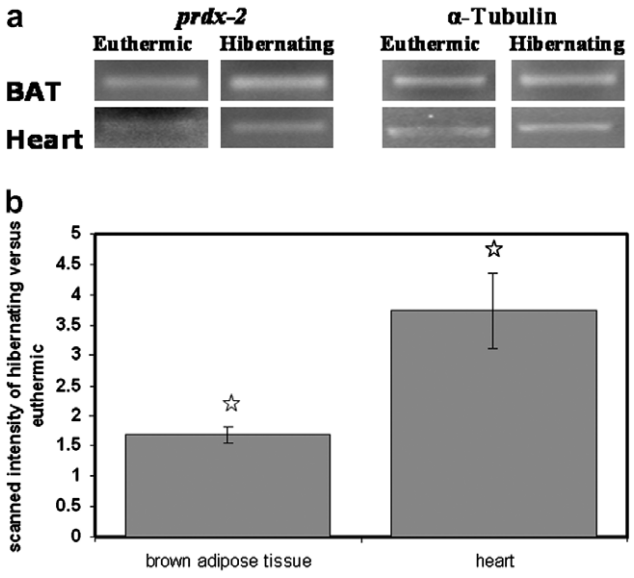

Fig. 3. (a) Effect of hibernation on *prdx2* mRNA levels in brown adipose tissue and heart of 13-lined ground squirrels. Transcripts in each sample were amplified by RT-PCR and products were separated on agarose gels, stained with ethidium bromide and visualized under UV light using a ChemiGenius (SynGene, MD, USA). Paired tubes amplified α-tubulin transcripts from the same samples and *prdx2* band intensities were normalized against the corresponding paired α-tubulin intensity for the same sample. Band sizes were 303 bp for *prdx2* and 616 bp for α-tubulin. (b) Histogram showing the ratio of normalized PCR product levels in tissues from hibernating versus euthermic ground squirrels. Data are means ± SEM for $n = 3$ independent trials. *Hibernating values are significantly different from the corresponding euthermic control, $P < 0.05$.

Discussion

Cycles of torpor-arousal result in huge changes in tissue oxygenation and oxygen consumption by hibernator tissues that could also cause wide variation in ROS generation. ROS are highly damaging to biomacromolecules and, hence, effective antioxidant defense mechanisms are needed. Indeed, early reports showed an increase in the activities of superoxide dismutase and glutathione peroxidase, two major antioxidant enzymes, in arousing hibernators [6] and a new study on hamsters found elevated extracellular catalase during arousal [22]. Levels of the metabolite antioxidant, ascorbate, also rise 3–5-fold in plasma during hibernation but are rapidly depleted when oxygen consumption peaks during arousal [23]. Osborne and Hashimoto [24] found similar ascorbate consumption in brain striatal extracellular fluid during arousal. Studies with ground squirrel intestine have also shown that a redox-sensitive transcription factor is up-regulated during both short and long torpor [25]. New studies have also shown that organs of hibernating bats (*Myotis lucifugus*) also adjust antioxidant defenses; both message and protein levels of Prdx1 were up-regulated during hibernation and other oxidative stress markers, p-IκB-α (Ser 32) and p-HSP27 (Ser 78/82), were elevated in heart and skeletal muscle during hibernation [26].

Peroxiredoxins have been linked to an oxidative stress response in a variety of situations and the data in this paper further support their role in antioxidant defense during hibernation. Besides responding to oxidative stress, 2-Cys peroxiredoxins have also been linked with intracellular signaling [27]. Increased levels of peroxiredoxins seem to inhibit certain pathways. Such is the case of Prdx2 and the platelet-derived growth factor (PDGF) receptor. Reports

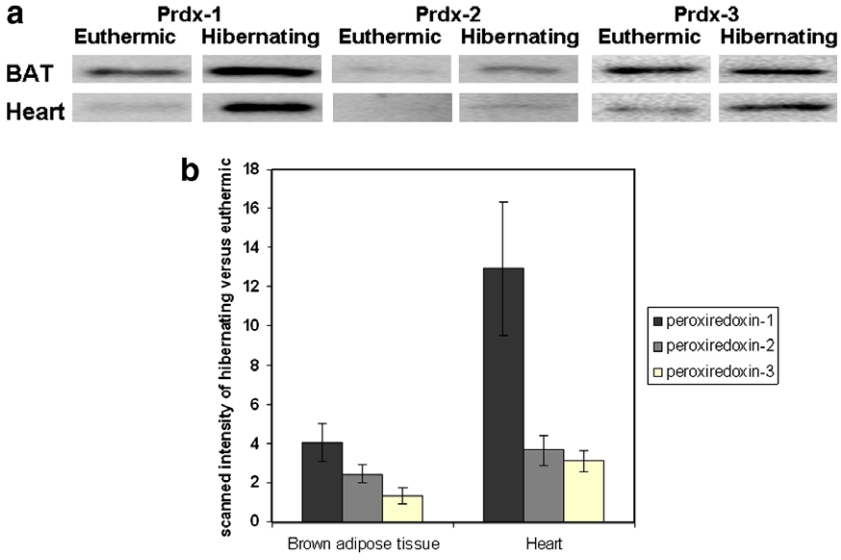

Fig. 4. (a) Expression of 2-Cys Prdx isozymes in brown adipose tissue and heart of hibernating versus euthermic *S. tridecemlineatus.* Representative Western blots show expression of the Prdx1, Prdx2 and Prdx3 protein bands at 23, 25 and 28 kDa, respectively. (b) Histogram shows mean relative protein expression in tissues from hibernating versus euthermic squirrels. Data are means ± SEM, $n = 3$ independent trials on tissue from different animals. *Hibernating values are significantly different from the corresponding euthermic control value, $P < 0.05$.

have shown that increased levels of Prdx2 can successfully inhibit the initiation of the signaling cascade that would normally originate from this receptor [28]. Studies using *prdx2* gene knockout have also previously reported activation of JNK and p38 MAP kinase pathways [29]. These kinases are differentially activated during hibernation. A strong increase in JNK activity combined with a decrease in ERK activity was reported in the arousing bat brain [30], whereas changes in MAP kinases activities were reported in two species of hibernating ground squirrels [31,32]. Hence, modification of Prdx levels in hibernator organs could also result in changes in signal transduction and cellular responsiveness to growth factors and other signals at different stages of the hibernation cycle (euthermia-entry-torpor-arousal).

It is well-known that thermogenesis in brown fat can trigger oxidative stress due to the sudden increase in oxygen consumption and the generation of ROS that ensues [7]. Heart is also known to undergo oxidative stress during cycles of ischemia and reperfusion [33]. Hibernating mammals, which have to deal with multiple bouts of torpor and arousal (and possibly associated ischemia and reperfusion as well), would also be confronted with cycles of ROS generation and therefore require substantial antioxidant defenses in these two tissues. Transcript levels of *prdx2* were measured in BAT and heart of hibernating ground squirrels. *Prdx2* transcripts were up-regulated in both BAT and heart (by 1.7- and 3.7-fold, respectively) in torpid squirrels as compared with euthermic controls (Fig. 3). Furthermore, Western blotting with a Prdx2 antibody confirmed that the transcriptional up-regulation led to significant increases in Prdx2 protein levels in these tissues, by 2.4- and 3.7-fold in BAT and heart, respectively (Fig. 4). These data suggest the importance of Prdx2 in the oxidative stress response associated with hibernation.

Prdx2 is a member of the 2-Cys peroxiredoxin family and to determine if the Prdx2 response was a specific or a general response by the whole family, we also evaluated the protein levels of other 2-Cys Prdx family members. Western blotting confirmed that a general elevation of 2-Cys Prdxs occurred during hibernation. Thus, Prdx1 protein content increased significantly during torpor by 4.0- and 12.9-fold in BAT and heart, respectively, whereas Prdx3 protein rose by 3.1-fold in heart (Fig. 4). This elevation of Prdx1 protein correlates with our previous work on hibernating bats that showed that levels of the protein PAG (now known as Prdx1) rose by ~2.0-fold in bat heart during hibernation [26]. Furthermore, studies performed with bovine aortic endothelial cells submitted to different oxidative stresses showed comparable increases of Prdx3 protein under stress [34]. Hence, it appears that up-regulation of 2-Cys Prdx family members is an integral response to hibernation, and given that these proteins are known to respond to oxidative stress in other mammalian systems, we can postulate that their up-regulation in hibernation is due to either direct or anticipated oxidative stress associated with torpor-arousal cycles. Indeed, an anticipatory enhancement of antioxidant defenses is a commonly seen adaptive response by species that endure frequent bouts of oxidative stress as a result of exposure to various environment stresses [35]. It should be noted, however, that although elevated Prdx in BAT and heart clearly serves ground squirrel hibernation, the trigger for Prdx up-regulation cannot be discerned from the present data. Prdx may be responsive to entry into torpor or to cold exposure (transfer into the cold room) or to both factors. Since thermogenesis by BAT is clearly enhanced in non-hibernating rodents in the cold [36], it is possible that Prdx is cold-induced as part of the antioxidant defenses supporting thermogenic capacity but further enhancement could occur during torpor and/or during arousal. When the opportunity arises, it will be valuable to assess the patterns of antioxidant defense responses, including Prdx, at multiple time points over cycles of torpor and arousal from hibernation, as well as in warm- and cold-acclimated animals.

Increased levels of Prdx protein in ground squirrel tissues during torpor does not necessarily mean that the enzyme is more active since the activities of enzymes can be modified in many different ways. For example, it has been shown that Prdx can be overoxidized and inactivated when dealing with oxidative stress [37]. This phenomenon was subsequently shown to be reversible in radiolabelling studies [38]. Hence, to confirm that elevated Prdx protein resulted in increased enzymatic activity, we quantified total 2-Cys activity in brown adipose and heart. In both cases, a significant increase in 2-Cys Prdx activity was found during torpor with 3.4- and 1.5-fold increases in brown adipose and heart, respectively, as compared with euthermia. Thus, the hibernation-responsive gene and protein up-regulation of Prdx seen in ground squirrel organs were paralleled by increased Prdx activity. This increase in Prdx activity supports the potential importance of peroxiredoxins in antioxidant defense during hibernation. This activity is most likely directed towards H_2O_2 detoxification, but elevated Prdx activity could also have a signaling function during torpor. Studies performed with Prdx2 gene knockouts have revealed that deletion of Prdx2 can affect the activity of a variety of protein kinases [29]. Previous work from our laboratory has revealed the importance of signaling cascades via protein kinase activities in hibernating Richardson's ground squirrels, *Spermophilus richardsonii* [31]. Therefore, the differential regulation of 2-Cys peroxiredoxins might also be used by hibernators to tightly regulate protein kinase cascades that are sensitive to hydrogen peroxide signals during torpor.

Analysis of the putative protein sequence of ground squirrel Prdx2 revealed strong identity with the protein from non-hibernating mammals (rat, mouse, human) (Fig. 2). Of particular importance, the cysteine residue in the C-terminal region, a feature of 2-Cys Prdx family members [27], was conserved in the ground squirrel protein. However, three specific substitutions were noted when the ground squirrel sequence was compared with other

rodents: substitution of an arginine residue for a lysine occurred position 109, serine was substituted for asparagine at position 120, and a glycine replaced alanine at position 134. Selected amino acid substitutions in the sequences of ground squirrel proteins have been identified in several cases, for example, in fatty acid binding proteins [39], and these appear to cause protein conformational changes that benefit low temperature function in the torpid animal. The substitutions in ground squirrel Prdx2, as compared with the rat or mouse enzyme, might also be important for sustained function of the enzyme at low T_b but since these substitutions were not seen when human and ground squirrel Prdx2 were compared, the purpose of these amino acid changes cannot yet be determined.

In conclusion, the strong increase in both *prdx2* transcript and Prdx2 protein expression in heart and BAT suggests a key role for this antioxidant enzyme in hibernation. Furthermore, the concurrent increase in Prdx1 and Prdx3 protein, as well as the strong increase in total 2-Cys Prdx activity during torpor, suggests that the entire 2-Cys Prdx family is important in hibernation success. These data further reinforce the fact that small hibernators such as thirteen-lined ground squirrels are confronted with oxidative stress over torpor-arousal cycles and must up-regulate their antioxidant defenses to prevent oxidative injuries. Furthermore, peroxiredoxin activity might also have a role in the reversible control of selected signaling pathways during torpor.

Acknowledgments

We thank Dr. J.M. Hallenbeck, National Institute of Neurological Disorders and Stroke, N.I.H., Bethesda, MD for providing ground squirrel tissues and 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

[1] L.C.H. Wang, T.F. Lee, in: M.J. Fregley, C.M. Blatteis (Eds.), Handbook of Physiology: Environmental Physiology, Torpor and Hibernation in Mammals: Metabolic, Physiological, and Biochemical Adaptations, Oxford University Press, New York, 1996, pp. 507–532.
[2] H.V. Carey, M.T. Andrews, S.L. Martin, Physiol. Rev. 83 (2003) 1153–1181.
[3] K.B. Storey, J.M. Storey, Biol. Rev. Camb. Philos. Soc. 79 (2004) 207–233.
[4] K.M. Brauch, N.D. Dhruv, E.A. Hanse, M.T. Andrews, Physiol. Genomics 23 (2005) 227–234.
[5] M.B. Spasic, Z.S. Saicic, B. Buzadzic, B. Korac, D. Blagojevic, V.M. Petrovic, Free Rad. Biol. Med. 15 (2003) 291–299.
[6] B. Buzadzic, M. Spasic, Z.S. Saicic, R. Radojicic, V.M. Petrovic, B. Halliwell, Free Radic. Biol. Med. 9 (1990) 407–413.
[7] G. Barja de Quiroga, Free Radic. Biol. Med. 13 (1992) 325–340.
[8] B. Hofmann, H.J. Hecht, L. Flohe, Biol. Chem. 383 (2002) 347–364.
[9] S.G. Rhee, H.Z. Chae, K. Kim, Free Radic. Biol. Med. 38 (2005) 1543–1552.
[10] H.Z. Chae, H.J. Kim, S.W. Kang, S.G. Rhee, Diabetes Res. Clin. Pract. 45 (1999) 101–112.
[11] H.Z. Chae, K. Robison, L.B. Poole, G. Church, G. Storz, S.G. Rhee, Proc. Natl. Acad. Sci. USA 91 (1999) 7017–7021.
[12] H.S. Kim, S.W. Kang, S.G. Rhee, L.B. Clerch, Am. J. Physiol. 280 (2001) L1212–L1217.
[13] A. Mitsumoto, Y. Takanezawa, K. Okawa, A. Iwamatsu, Y. Nakagawa, Free Radic. Biol. Med. 30 (2001) 625–635.
[14] S.E. Akerman, S. Muller, Mol. Biochem. Parasitol. 130 (2003) 75–81.
[15] S.G. Rhee, T.S. Chang, Y.S. Bae, S.R. Lee, S.W. Kang, J. Am. Soc. Nephrol. 14 (2003) S211–S215.
[16] Z.A. Wood, E. Schroder, J. Robin Harris, L.B. Poole, Trends Biochem. Sci. 28 (2003) 32–40.
[17] H. Kim, T.H. Lee, E.S. Park, J.M. Suh, S.J. Park, H.K. Chung, O.Y. Kwon, Y.K. Kim, H.K. Ro, M. Shong, J. Biol. Chem. 275 (2000) 18266–18270.
[18] S.W. Kang, H.Z. Chae, M.S. Seo, K. Kim, I.C. Baines, S.G. Rhee, J. Biol. Chem. 273 (1998) 6297–6302.
[19] Y.J. Lee, S.I. Miyake, H. Wakita, D.C. McMullen, Y. Azuma, S. Auh, J.M. Hallenbeck, J. Cereb. Blood Flow Metabol. (2006), Epub. Sept. 6.
[20] E.Z. Yu, J.M. Hallenbeck, D. Cai, R.M. McCarron, Brain Res. Mol. Brain Res. 102 (2002) 9–17.
[21] J.A. Kim, S. Park, K. Kim, S.G. Rhee, S.W. Kang, Anal. Biochem. 338 (2005) 216–223.
[22] H. Ohta, I. Okamoto, T. Hanaya, S. Arai, T. Ohta, S. Fukuda, Comp. Biochem. Physiol. C 143 (2006) 484–491.
[23] K.L. Drew, O. Toien, P.M. Rivera, M.A. Smith, G. Perry, M.E. Rice, Comp. Biochem. Physiol. C 133 (2002) 483–492.
[24] P.G. Osborne, M. Hashimoto, Behav. Brain Res. 168 (2006) 208–214.
[25] H.V. Carey, C.L. Frank, J.P. Seifert, J. Comp. Physiol. B 170 (2000) 551–559.
[26] S.F. Eddy, J.D. McNally, K.B. Storey, Arch. Biochem. Biophys. 435 (2005) 103–111.
[27] S.W. Kang, S.G. Rhee, T.S. Chang, W. Jeong, M.H. Choi, Trends Mol. Med. 11 (2005) 571–578.
[28] M.H. Choi, I.K. Lee, G.W. Kim, B.U. Kim, Y.H. Han, D.Y. Yu, H.S. Park, K.Y. Kim, J.S. Lee, C. Choi, Y.S. Bae, B.Y. Lee, S.G. Rhee, S.W. Kang, Nature 435 (2005) 347–353.
[29] K.W. Kang, T.S. Chang, T.H. Lee, E.S. Kim, D.Y. Yu, S.G. Rhee, J. Biol. Chem. 279 (2004) 2535–2543.
[30] M. Lee, I. Choi, K. Park, J. Neurochem. 82 (2002) 867–873.
[31] J.A. MacDonald, K.B. Storey, Int. J. Biochem. Cell Biol. 37 (2005) 679–691.
[32] X. Zhu, M.A. Smith, G. Perry, Y. Wang, A.P. Ross, H.W. Zhao, J.C. Lamanna, K.L. Drew, J. Neurosci. Res. 80 (2005) 862–868.
[33] N. Marczin, N. El-Habashi, G.S. Hoare, R.E. Bundy, M. Yacoub, Arch. Biochem. Biophys. 420 (2003) 222–236.
[34] M. Araki, H. Nanri, K. Ejima, Y. Murasato, T. Fujiwara, Y. Nakashima, M. Ikeda, J. Biol. Chem. 274 (1999) 2271–2278.
[35] M. Hermes-Lima, J.M. Storey, K.B. Storey, in: K.B. Storey, J.M. Storey (Eds.), Cell and Molecular Responses to Stress, Antioxidant Defenses and Animal Adaptation to Oxygen Availability During Environmental Stress, Elsevier Press, Amsterdam, 2001, pp. 263–287.
[36] M. Florez-Duquet, B.A. Horwitz, R.B. McDonald, Am. J. Physiol. 274 (1998) 196–203.
[37] T. Rabilloud, M. Heller, F. Gasnier, S. Luche, C. Rey, R. Aebersold, M. Benahmed, P. Louisot, J. Lunardi, J. Biol. Chem. 277 (2002) 19396–19401.
[38] H.A. Woo, H.Z. Chae, S.C. Hwang, K.S. Yang, S.W. Kang, K. Kim, S.G. Rhee, Science 300 (2003) 653–656.
[39] D. Hittel, K.B. Storey, Biochim. Biophys. Acta 1522 (2001) 238–243.