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Differential expression of mitochondria-encoded genes in a hibernating mammal

Dustin S. Hittel and Kenneth B. Storey*

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

*Author for correspondence (e-mail: kenneth_storey@carleton.ca)

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Summary

A cDNA library constructed from kidney of the thirteen-lined squirrel, *Spermophilus tridecemlineatus*, was differentially screened for genes that were upregulated during hibernation. A clone encoding cytochrome *c* oxidase subunit 1 was found and confirmed to have been upregulated by northern blotting. Differential expression of *Cox1* mRNA occurred in multiple organs during hibernation; in hibernating animals transcript levels were twofold higher in kidney and fourfold higher in heart and brown adipose tissue than in euthermic animals, but were unchanged in skeletal muscle. Transcript levels of mitochondrial-encoded ATP synthase 6/8 were similarly upregulated in these tissues whereas transcript levels of the nuclear encoded subunits *Cox4* and ATP synthase α did not change during hibernation. Immunoblot analysis revealed a 2.4-fold increase in Cox 1 protein and a slight decrease in Cox 4 protein in kidney of hibernating squirrels, compared with euthermic controls. Hibernating mammals may increase the expression of the mitochondrial genome in general, and *Cox1* specifically, to prevent or minimize the damage to the electron transport chain caused by the cold and ischemia experienced during a hibernation bout.

Key words: *Spermophilus tridecemlineatus*, hibernation, ischemia, kidney, cDNA library.

Introduction

Many small mammals living in seasonally cold climates are faced with difficult choices. A huge metabolic energy consumption is needed to defend body temperature against the winter cold while at the same time the animals are challenged with the scarcity or absence of food to provide this energy (Boyer and Barnes, 1999; Storey, 2000). Hibernation provides the solution to this dilemma, allowing animals to profoundly depress their metabolic rate (often to <5 % of the euthermic resting rate) and drop their body temperature (T_b) to near ambient in order to greatly extend the time that their body fuel reserves can support survival (Frerichs et al., 1998; Hochachka, 1986; Storey, 2000). The organs of most mammals sustain significant metabolic injuries during ischemia and at low body temperatures, but hibernators routinely suppress their T_b, heart rate and breathing for several weeks at a time (Frerichs et al., 1998; Storey, 2000). Researchers are therefore interested in mammalian hibernation as a natural model for tolerance to ischemia, in light of the poor performance of therapies based upon (non-hibernating) animal ischemia models (Frerichs et al., 1998). Investigating how animals survive this seemingly lethal combination of low temperature and ischemia is also attractive to those who research the long-term storage of organs at low temperatures for transplantation (Green, 2000; Zancanaro et al., 1999).

Protein phosphorylation is believed to be a primary agent by which hibernating animals make ‘acute’ metabolic adjustments (Boyer and Barnes, 1999; Storey, 2000). Many of the enzymes involved in ATP production (e.g. pyruvate dehydrogenase, phosphofructokinase) (Brooks and Storey, 1992; Storey, 1997) as well as ATP consumption (Na/K-ATPase) (Bennis et al., 1995; MacDonald and Storey, 1999) are reversibly inhibited by protein phosphorylation during hibernation. Increasingly, however, the importance of differential expression of genes in the expression and maintenance of the hibernation phenotype has been demonstrated (Srere et al., 1992). Several genes have been identified that are preferentially or increasingly expressed in the tissues of hibernating mammals. These include α2-macroglobulin (Srere et al., 1992) in the liver, pyruvate dehydrogenase kinase isozyme 4, pancreatic lipase and mitochondrial-encoded genes in the heart (Andrews et al., 1998), and a variety of genes in other tissues (Boyer and Barnes, 1999). The protein products of these genes affect a variety of adaptive changes in the tissues and organs of hibernating animals that allow them to survive this extreme physiological state.

A 100-fold decrease in heart rate reduces the mean arterial pressure by 60 % in hibernating ground squirrels (Anderson et al., 1990; Harlow and Braun, 1995). This dramatically reduces the rate of glomerular microfiltration by kidney and therefore the rate of urine production. Because of the greatly reduced or ‘trickle’ blood flow during hibernation it is likely that the kidney and other organs become profoundly ischemic (Frerichs

et al., 1998). Infrequent arousals during hibernation cause a rapid warming and reperfusion of the kidney, and the immediate restoration of blood filtration and urine production (Zancanaro et al., 1999). These physiological extremes endured by the kidney during hibernation are associated with significant adjustments to metabolic rate and glomular ultrastructure (Harlow and Braun, 1995). It is likely that this adaptation is in part, the result of differential gene expression and it is our contention that the hibernating kidney is an excellent model for natural resistance to ischemia and hypothermia.

In the present study we investigated the role of differential gene expression in supporting hibernation in the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*. The differential screening of a cDNA library constructed from the kidney of hibernating squirrels revealed a gene that is upregulated during hibernation. This gene was identified as the mitochondrial-encoded cytochrome *c* oxidase subunit 1 (*Cox1*). Northern and western blot analysis of kidney and other tissues revealed significant and tissue-specific changes in mRNA and protein levels of mitochondrial encoded and nuclear encoded subunits of the electron transport chain during hibernation.

Materials and methods

Animals

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus* Mitchill) weighing 120–180 g were captured in Illinois during August by a US Department of Agriculture-licensed trapper (TLC Research, Batlett, IL, USA) and shipped by air to the animal housing facility of the National Institutes of Health campus (Bethesda, MD, USA) (Frerichs et al., 1998). Initially, animals were housed in a holding room in individual shoebox cages maintained at 22–24 °C, with 60 % relative humidity and a 12 h:12 h light/dark cycle; free access was given to food and water. Individuals were weighed weekly to determine when they entered and finished the pre-hibernation phase of hyperphagia that maximized body lipid reserves prior to hibernation. When animals had exhibited a rapid increase in body mass (220–240 g), they were placed in a dark chamber at 5–6 °C and 60 % humidity to facilitate hibernation. Some animals were not placed in the hibernaculum at this time but were killed (by barbituate overdose) as controls; the tissues were rapidly excised and flash frozen in liquid nitrogen. After placement in the hibernaculum, most animals entered a state of deep torpor within 3–8 days. Animals that failed to enter hibernation within the 8 days were removed and immediately killed, and are referred to as being cold-adapted. Animals were subjected to experiments as hibernators after 1–7 days of continuous torpor. Tissue samples were transported to Ottawa on dry ice where the remaining experiments were performed.

RNA preparation and cDNA library construction

All materials and solutions used for RNA isolation were treated with 0.1 % v/v diethylpyrocarbonate (DEPC) and subsequently autoclaved. Total RNA was isolated from tissues of hibernating and euthermic ground squirrels using Trizol solution (Gibco-BRL), following the manufacturer's protocol. Poly(A)$^+$ RNA was isolated from total RNA using an oligo(dT)-cellulose column (Quiagen, Valencia, CA, USA). The concentration of the poly(A)$^+$ RNA was determined spectrophotometrically at 260 nm. Poly(A)$^+$ RNA from the kidneys of hibernating animals was used to construct a cDNA library using cDNA library and Uni-ZAP unidirectional cloning kits from Stratagene (La Jolla, CA, USA), following the manufacturer's instructions.

Differential screening of the cDNA library

^{32}P-labeled single-stranded cDNA probes were synthesized from poly(A)$^+$ RNA isolated from kidney of hibernating and euthermic control animals. Into an autoclaved, DEPC-treated, 1.5 ml microfuge tube, 2 µl poly(A)$^+$ RNA template (about 1 µg) and 6 µl DEPC ddH_2O were added. The mixture was heated at 65 °C for 5 min and then 5 µl of 5× 1st strand buffer (Gibco-BRL), 1.5 µl dNTPs without dCTP (5 mmol l^{-1} for each nucleotide), 1 µl oligo dT primer (200 ng µl^{-1}, NEB, Beverly, MA, USA), 1 µl RNasin (5 U µl^{-1}, Promega, Madison, WI, USA) and 2.5 µl dithiothreitol (0.1 mol l^{-1}, Gibco-BRL) were added. The reaction was mixed well and the primers were allowed to anneal to the poly(A)$^+$ RNA at room temperature for 10 min. Following this, 1 µl M-MLV reverse transcriptase (200 U µl^{-1}, Gibco-BRL), and 5 µl [α-^{32}P]dCTP (3000 Ci mmol^{-1}, 110 TBq mol^{-1}; Amersham) were added to the reaction, and the mixture was incubated at 37 °C for 1 h. The RNA was degraded by adding 1 µl EDTA (0.5 mol l^{-1}), 1 µl sodium dodecyl sulfate (10 % w/v), and 3 µl NaOH (3 mol l^{-1}) to the reaction and the mixture was incubated at 68 °C for 30 min. Next, the probe was cooled to room temperature followed by the addition of 10 µl Tris-HCl (1 mol l^{-1}, pH 7.4) and 3 µl HCl (2 mol l^{-1}). Finally, the probe was passed through a Sephadex G-50 column equilibrated in TE buffer, pH 8 (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, made with DEPC-treated water) and then brought to a final volume of 500 µl with TE buffer; a 2 µl portion was removed for scintillation counting. Approximately 10^6 disints min^{-1} of probe per ml of hybridization solution was used for hybridization of the plaque lifts.

For primary screening, approximately 35 000 plaques per plate were grown on 10 agar plates. Two lifts were made from each plate using nylon membranes (Amersham). The membranes were UV-crosslinked, and allowed to air dry. The lifts were then hybridized with ^{32}P-labeled, single-stranded cDNA probes made from kidney of either hibernating or euthermic animals, in a hybridization incubator (LAB-Line Instruments) using Denhardt's hybridization solution with 50 % formamide (Fahlman et al., 2000). Plaques showing a stronger signal with the probe from hibernated kidney compared with euthermic kidney were retrieved and subjected to two more rounds of screening to confirm the stronger signal and purify the clones. After tertiary screening, purified clones in Bluescript plasmid vectors were rescued by *in vivo* excision using Exassist as the helper phage.

Northern hybridization analysis

Northern hybridization was used to confirm the upregulation of the putative clones in the kidney of hibernating ground squirrels and to determine transcript abundance in other organs (heart, brown adipose, skeletal muscle). Total RNA was isolated from tissue samples using Trizol (Gibco-BRL), separated in a formaldehyde-agarose gel using 16 μg of total RNA per lane, and then blotted onto a Nytran membrane by capillary action. The quality of total RNA was assessed by the identification of well-defined 18S and 28S ribosomal bands. The DNA inserts from the tertiary screened clones were cut from the plasmid vector using *Bam*HI and *Xho*I and separated on a 1 % agarose gel run in 40 mmol l^{-1} Tris-acetate, 2 mmol l^{-1} EDTA, pH 8.5. The inserts were purified and labeled with ^{32}P using a random primer procedure. Northern blots were hybridized at 44 °C, using labeled probes (8×10^6 disints min^{-1} per 10 ml hybridization solution) and a sodium phosphate hybridization solution. After hybridization the blots were washed with increasing stringency and then placed into autoradiography cassettes with X-ray film (X-Omat, AR, Kodak). Prior to hybridization the northern blots were stained with Methylene Blue (0.03 % w/v) in ddH_2O to visualize ribosomal RNA bands, and destained in ddH_2O overnight.

Transcript levels were quantified by scanning the X-ray autoradiogram using a Scan Jet 3C scanner with DeskScan II V2.2 program (Hewlett Packard) and an Imagequant V3.22 program (Innovative Optical Systems Research). The ribosomal bands of the stained northern blots were quantified and these values were used to evaluate any differences in loading between lanes. RNA transcript sizes were estimated from a plot of RNA molecular mass (Gibco-BRL standards) *versus* migration distance in the formaldehyde gel.

DNA sequencing and analysis

Isolated clones were sequenced by Canadian Molecular Research Services (CMRS) Inc. (Ottawa, Ontario, Canada) using an automated DNA sequencing procedure, and a translation program (EditSeq, DNASTAR, Inc.) was used to define the putative protein sequence. The nucleotide sequence and the six possible open reading frames for each clone were loaded into a Blast program at NCBI for a similarity search in GenBank.

Western blotting

Samples of frozen tissues from euthermic and hibernating ground squirrels were crushed under liquid nitrogen, weighed and then homogenized at 1:3 w/v dilution in Buffer A (1 % SDS, 1 % 2-mercaptoethanol, 50 mmol l^{-1} Tris, pH 6.8, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} sucrose). Soluble protein content was measured with the Bio-Rad (Hercules, CA, USA) Coomassie Blue dye binding reagent using a microplate reader. An equal amount of protein from each sample was then boiled in 2× SDS-PAGE loading buffer, centrifuged for 1 min at 8200 ***g*** and loaded onto two identical 12 % SDS-PAGE mini-gels run at 150 V for 45 min. Bio-Rad kaleidoscope prestained markers were used to track the progress of the proteins through the gel. Protein samples, size-fractionated on 12 % SDS-PAGE gels, were transferred to a PVDF membrane by electroblotting. These membranes were blocked and incubated with a 1:1000 dilution of a mouse monoclonal antibody against either subunit 1 or subunit 4 of bovine cytochrome *c* oxidase (Molecular Probes, Eugene, OR, USA). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Molecular Probes) and developed with colorimetric immunoblot-staining reagents (Bio-Rad).

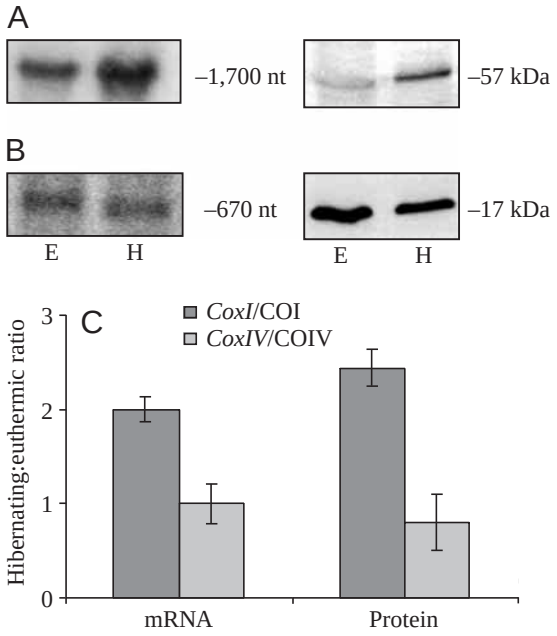

Fig. 1. Northern blot hybridization profiles and western blot profiles of *CoxI* (A) and *CoxIV* (B) genes and their protein products, respectively, in euthermic (E) and hibernating (H) ground squirrel kidneys. (C) Ratios of scanned band intensities for hibernating *versus* control values (means ± S.E.M., *N*=3 separately run northern and western blots; each blot contained RNA or protein, respectively, prepared from separate animals).

Results

A cDNA library was constructed with poly(A)$^+$ RNA isolated from the kidneys of hibernating ground squirrels. Approximately 10^6 plaques were differentially screened using ^{32}P-labeled single-stranded cDNA probes synthesized from poly(A)$^+$ RNA isolated from the kidneys of hibernating *versus* euthermic animals. Two clones showing a stronger signal in the kidney of hibernators were isolated after a second round of differential screening. Clones 9.1 and 13.2 were subsequently identified as the same clone by cross-hybridization experiments (data not shown). Clone 9.1 was used for further studies as it contained the longest insert. Northern blot analysis using the ^{32}P-labeled cDNA insert from clone 9.1 confirmed that mRNA transcripts of this gene were upregulated during hibernation in the kidney (Fig. 1A) and bound strongly to a band of approx. 1700 nucleotides (nt).

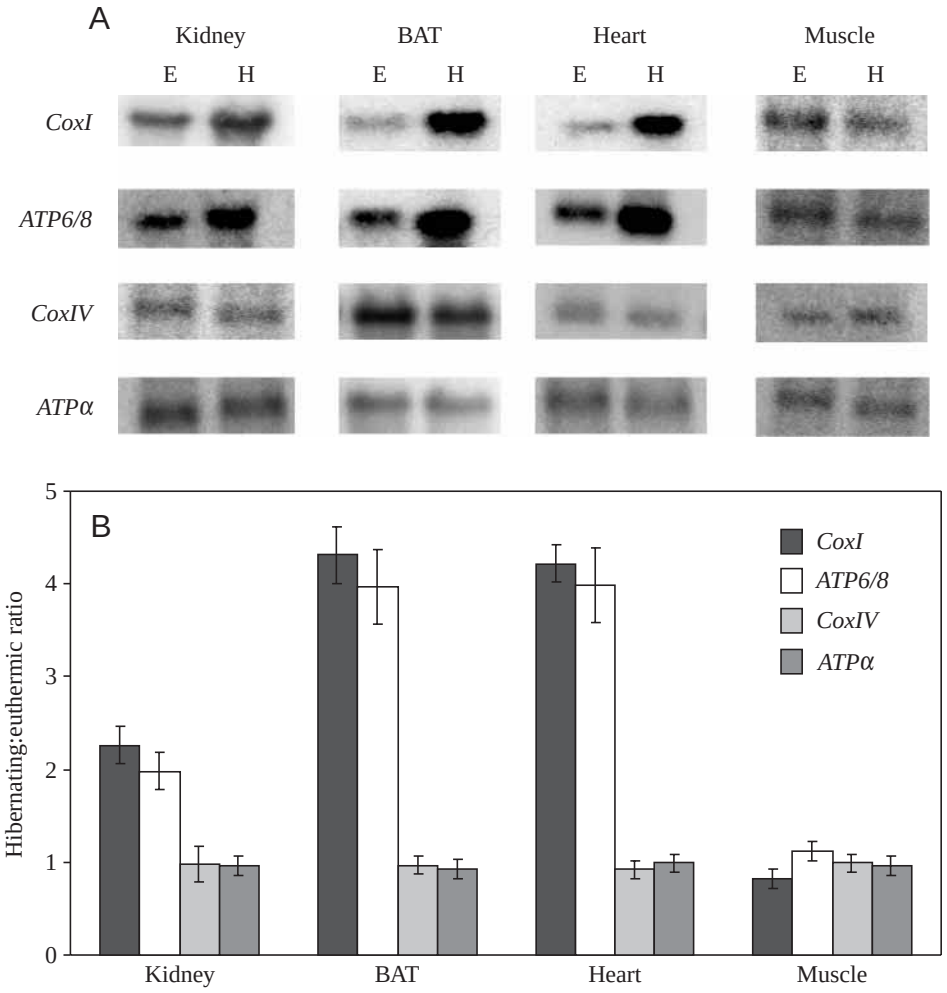

Fig. 2. Northern blots (A) and analysis (B) of three mitochondrial encoded and two nuclear encoded genes whose protein products include subunits of complex IV (cytrochrome *c* oxidase) and V (ATP synthase) of the mitochondrial electron transport chain, in four tissues from euthermic and hibernating ground squirrels. Northern blots of total RNA ($16\,\mu g\,lane^{-1}$) were scanned, then band intensities quantified and normalized to their corresponding 28S ribosomal bands. The ratios of normalized band intensities were then calculated. Values are means ± S.E.M., *N*=3 separately run northern blots; each blot contained RNA prepared from separate animals.

DNA sequence analysis of clone 9.1

Sequencing of clone 9.1 produced a 1604 nt cDNA (GenBank Accession No. AF330007) that was identified as encoding the gene (*Cox1*) for the mitochondrial-encoded subunit 1 of cytochrome *c* oxidase (COI). Analysis of the putative amino acid sequence of ground squirrel COI showed that it encoded 508 of the 513 amino acids of the full sequence with an intact C terminus. A comparison of the deduced amino acid sequence of the COI protein with those from other taxa revealed a 90.5 % amino acid identity with human and 98 % with COI from the Eurasian red squirrel (Reyes et al., 2000).

CoxI and CoxIV expression in the kidney

A plasmid containing the cDNA of mouse cytochrome *c* oxidase subunit IV (IMAGE ID# 535611), a nuclear encoded subunit of the protein, was purchased from Research Genetics (Huntsville, AL, USA). This cDNA insert was ^{32}P-labelled and used to probe a northern blot of kidney mRNA from euthermic and hibernating squirrels. The *CoxIV* probe bound to a band of approx. 670 nt (Fig. 1B) that is identical in size in most species described (Carter and Avadhani, 1991). The relative levels of *Cox1* mRNA increased 2.04±0.13-fold (mean ± S.E.M., *N*=3) in kidney from hibernators compared with controls, whereas the levels of *Cox4* mRNA were relatively unchanged (Fig. 1C). Western blot analysis revealed a 2.4±0.2-fold (mean ± S.E.M., *N*=3) increase in the amount of immunoreactive Cox 1 protein (COI) (Fig. 1A) and a small (approx. 12 %) decrease in the amount of immunoreactive Cox 4 protein (COIV) (Fig. 1B) in hibernating *versus* euthermic kidney (Fig. 1C).

Tissue-specific expression of mitochondrial and nuclear encoded genes

The vertebrate mitochondrial genome is transcribed as a large polycystronic transcript that is subsequently processed into distinct mRNA, tRNA and rRNAs (Clayton, 1991). The mRNAs tend to encode the core subunits of the proteins of the mitochondrial electron transport chain whereas the rRNAs and tRNAs constitute the RNA component of the mitochondrial translational machinery. Mitochondrial transcription proceeds, therefore, in an ‘all or none’ fashion, meaning if one transcript increases they all increase (Clayton, 1991). Because of the dichotomy of response to hibernation by the mitochondrial encoded *Cox1* and the nuclear encoded *Cox4* gene, we decided to examine the transcript levels of an additional mitochondrial encoded and nuclear encoded gene in parallel studies. Northern blot analysis of *Cox1*, *Cox4* and a previously identified ground

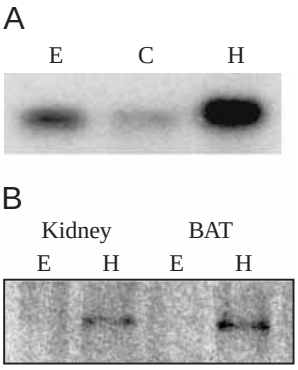

Fig. 3. Northern blot of *Cox1* transcript levels in euthermic (E), cold-adapted (C) and hibernating (H) 13-lined ground squirrel kidney (A), and an additional band (approx. 3.0 kb), which crossreacts with the *Cox1* probe in hibernating kidney and brown adipose tissue (B).

squirrel *ATPase 6/8* bicistronic mRNA (GenBank Accession no. AF362073) (D. S. Hittel and K. B. Storey, unpublished) and *ATPα* (I.M.A.G.E. ID# 3385828), a nuclear encoded subunit of the mitochondrial ATP synthase, are shown in Fig. 2A. During hibernation, *Cox1* transcript levels increased 2.0±0.13-fold in kidney, 4.51±0.5-fold in brown adipose tissue (BAT), 4.22±0.2-fold in heart and decreased slightly (18 %) in skeletal muscle. Transcript levels of *ATP6/8* also increased (1.98±0.2-fold) in kidney, BAT (3.97±0.3-fold) and heart (3.98±0.4-fold) of hibernators but did not change in skeletal muscle (Fig. 2B). The transcript levels of the nuclear encoded *Cox4* and *ATPa* did not change significantly in any of the hibernating tissues examined.

Cold-adapted animals

The transcript levels of *CoxI* were appreciably lower in cold-adapted animals compared to kidneys from euthermic controls (Fig. 3A). Although the functional significance of this observation is not known, it correlates with the inability of these animals to enter torpor.

Mitochondrial polycistronic precursors

Northern blot analysis of *CoxI* expression revealed the presence of an approx. 3 kb band in hibernating kidney and BAT (Fig. 3B), which probably represents an unprocessed mitochondrial RNA precursor. The presence of these precursors has been previously noted in anoxic turtle heart (Cai and Storey, 1996) and ischemic kidney (Van Itallie et al., 1993), where they are believed to indicate an increase in the transcription of the mitochondrial genome or the suppression of mitochondrial RNA processing machinery.

Discussion

The profound decrease in body temperature and cardiac output experienced by most hibernating mammals renders their organs both hypothermic and ischemic (Frerichs et al., 1998). In addition, periodic ‘interbout’ arousals during the hibernation season lead to a rapid rewarming and reperfusion of these same organs (Green, 2000). Surprisingly, the organs of hibernating mammals suffer no apparent damage after months of this cold ischemia/warm reperfusion regime (Zancanaro et al., 1999).

The kidney is one of the most frequently transplanted organs, yet ischemic damage caused before and during transplantation surgery is still a major cause of post-operative morbidity and mortality (Zancanaro et al., 1999; Green, 2000). The biochemical mechanisms used by hibernating mammals to endure these physiological extremes could be applied to the storage and preservation of organs destined for transplantation.

Cytochrome *c* oxidase (COX) is a 13-subunit complex spanning the inner mitochondrial membrane and responsible for the terminal reduction of dioxygen to water in the electron transport chain (Gnaiger et al., 1998; Napiwotzki and Kadenbach, 1998). Given this essential role in the electron transport chain, and its sensitivity to ischemia/reperfusion (IR) damage (Van Itallie et al., 1993; Montagna et al., 1998; Cochrane et al., 1999), it is not surprising that expression levels of cytochrome *c* oxidase subunits should respond to stress. An increase in the expression of the mitochondrial genome is known to occur in response to a wide variety of oxidative and cold stresses (Immaculada et al., 1993; Storey, 2000). Research in our laboratory has demonstrated an increase in the transcript levels of *Cox1* and other mitochondrial encoded genes in hibernating ground squirrels (Fahlman et al., 2000) and anoxic turtles (Cai and Storey, 1996). This indicates perhaps, a common mitochondrial response to anoxia and/or cold stress.

The mitochondria-encoded gene *Cox1* was recovered from the differential screening of a cDNA library made from kidney of hibernating *S. tridecemlineatus*. This gene encodes the large transmembrane, oxygen binding subunit of cytochrome *c* oxidase (Complex IV) (Poyton, 1999). The three core catalytic units COX I, II and III are trans-membrane proteins encoded by the mitochondrial genome, while the remaining 10 subunits are nuclear encoded and expressed in a tissue-specific manner (Lenka et al., 1998). The expression of nuclear and mitochondrial subunits of the mitochondrial respiratory chain is thought to be highly coordinated. We therefore investigated the expression of a nuclear encoded subunit of cytochrome *c* oxidase, *CoxIV*, which is believed to regulate COX activity according to the extramitochondrial ATP/ADP ratio (Napiwotzki and Kadenbach, 1998). The increased levels of *CoxI* mRNA and COI protein (Fig. 1A) in the hibernating kidney were concomitant with a slight decrease in the amount of immunoreactive COIV protein (Fig. 1B), suggesting perhaps a shift in the subunit ratio (normally 1:1) of the enzyme. Increasing the amount of COI protein may prevent or limit the damage caused to the cytochrome *c* oxidase complex by ischemia and cold.

The vertebrate mitochondrial genome is composed of 13 tightly packed open reading frames (ORFs) and two ribosomal RNAs that are punctuated by tRNA clusters (Clayton, 1991). Transcription of the mitochondrial genome produces several large polycistronic RNAs, which are subsequently processed into distinct coding and non-coding molecules. The stability and subsequent translation of the protein-coding mRNAs is regulated in a tissue-specific manner, yet poorly understood (Clayton, 1991). Two commercially available mouse (*Mus musculus*) mitochondrial cDNAs were used to probe northern blots of BAT, kidney, heart and skeletal muscle from

euthermic (active) and hibernating ground squirrels (Fig. 2A). Transcript levels of mitochondria-encoded genes were significantly increased (>fourfold) in hibernating BAT and heart, slightly elevated (>twofold) in kidney, and unchanged or slightly decreased in skeletal muscle. Previous studies have also demonstrated an increase in expression of selected mitochondrial transcripts in BAT during cold exposure and in the hearts of hibernating ground squirrels. Brown adipose tissue depots flank the major organs and arteries of hibernating mammals (Wang and Lee, 1996). The mitochondria in BAT are highly uncoupled and this allows them to generate heat by the futile cycling of protons across the inner mitochondrial membrane (Boyer and Barnes, 1999). The increased expression of the mitochondrial genome in hibernating BAT is believed to enhance the electron transport and thus, the thermogenic capacity of this tissue. Interestingly, the upregulation of the mitochondrial genome in BAT does not increase the levels of all 13 protein products (Clayton, 1991). Specifically, the levels of ATP synthase in BAT mitochondria are quite low, since the primary role of BAT is to generate heat by futile proton cycling. This, in contrast to the relatively high levels of *ATPase 6/8* and *ATP*α mRNA in BAT (Fig. 2A), suggests a high degree of post-transcriptional control (Tvrdik et al., 1992). The increased expression of the mitochondrial genome in hibernating hearts, along with other adaptive changes (Fahlman et al., 2000), may contribute to the continued functioning of the heart at extremely low body temperatures. The upregulation of mitochondrial transcripts has been observed in freezing and anoxia-tolerant species investigated in our laboratory (Cai and Storey, 1996). The transcript levels of nuclear encoded mitochondrial genes did not change in any of the hibernating organs studied (Fig. 2).

The increased expression of mitochondrial encoded but not nuclear encoded genes in ground squirrel kidney, BAT and heart suggests a global mitochondrial response associated with the expression of the hibernation phenotype. Interestingly, a significant decrease in the transcript level of *Cox1* was observed in the kidney of cold-adapted ground squirrels (Fig. 3A). This lack of upregulation of the expression of the mitochondrial genome may be one of the factors that prevents these animals from entering hibernation.

We propose that kidneys of hibernating ground squirrels ‘anticipate’ the metabolic lesions known to occur at complex IV during ischemia (Montagna et al., 1998) by overproducing those subunits that are particularly sensitive to ischemia/reperfusion (IR) damage. In so doing they may limit the damage to complex IV that would have otherwise compromised the viability of the organ and the animal. In animal models of renal IR damage, there is an increase in the expression of mitochondrial encoded genes, presumably to repair some damage that has been done to the organ (Van Itallie et al., 1993). Despite this effort, complex IV activity drops significantly after the IR insult and the organ suffers significant, sometimes fatal injury. This same study demonstrated an increase in the amount of *CoxII* mRNA as well unprocessed mitochondrial RNA precursors in response to renal IR. The presence of these precursors was also shown in hibernating tissues where the mRNA levels of *CoxI* increases (Fig. 3B). The presence of these precursors indicates either that there is a breakdown in the mitochondrial RNA processing machinery, or that there is an increase in the expression of the mitochondrial genome (Van Itallie et al., 1993).

Ischemic preconditioning is a technique initially developed to enhance the preservation time of transplanted hearts, but has since been extended to other organs including the liver, muscle and, more recently, the kidney (Cochrane et al., 1999). A preconditioned organ is made briefly ischemic to ameliorate the damage caused by a subsequent extended period of ischemia. Prior to entering hibernation, ground squirrels experience ‘test drops’ during which they briefly depress their metabolic rate (blood pressure, breathing, heart rate, body temperature) and then rapidly return to euthermy (Wang and Lee, 1996). These test drops may represent an innate subacute ischemic preconditioning program, which prepares hibernator organs for an extended bout of torpor using pre-existing stress response pathways (one of which is increased mitochondrial expression) to prevent widespread cellular damage.

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