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Hibernation induces oxidative stress and activation of NF-κB in ground squirrel intestine

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Abstract Dramatic changes in blood flow occur during torpor-arousal cycles in mammalian hibernators that could increase the risk of oxidative stress to sensitive tissues. We used 13-lined ground squirrels (*Spermophilus tridecemlineatus*) to determine the effect of hibernation on lipid peroxidation and expression of stress-activated signaling pathways in the intestine, a tissue highly susceptible to ischemia-reperfusion injury. Compared with summer-active squirrels, levels of the mitochondrial stress protein GRP75 were consistently higher in intestinal mucosa of hibernators in each of five hibernation states (entrance, short-bout torpid, long-bout torpid, arousal and interbout euthermia). The redox-sensitive transcription factor, nuclear factor-κB (NF-κB), was strongly activated in each hibernation state compared with summer squirrels except for squirrels during an arousal from torpor. In contrast, NF-κB activation in brown adipose tissue (BAT) was low in active and hibernating squirrels regardless of season. Levels of conjugated dienes (products of lipid peroxidation) were higher in intestine of hibernators entering torpor and early in a torpor bout compared with summer squirrels. Conjugated diene levels were also higher in short-bout torpid vs arousing squirrels. The results suggest that the intestinal mucosa is vulnerable to oxidative stress during the hibernation season and in response may activate cellular defense pathways that help minimize severe oxidative damage induced by torpor-arousal cycles.

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Abbreviations *BAT* brown adipose tissue · *DTT* dithiothreitol · *ERM* ezrin-radixin-moesin · *GRP75* glucose-regulated protein 75 · *HSP70* heat shock protein 70 · *NF-κB* nuclear factor-κB · *PMSF* phenylmethanesulfonyl fluoride · *ROS* reactive oxygen species

Introduction

Mammalian hibernation is a highly regulated suite of events that provides significant conservation of energy stores during periods of high metabolic demand and minimal food resources. The inability of nonhibernating species to survive conditions like prolonged hypothermia and metabolic depression suggests that hibernators must overcome significant physiological risks to insure survival during and after the hibernation season. One mechanism that might reduce such risks at the cellular level is seasonal induction of stress or "heat shock" proteins. These evolutionarily ancient molecules are induced by the presence of misfolded proteins and, by acting as molecular chaperones, reduce aggregation of nonnative proteins within cells (Feder and Hofmann 1999; Welch 1992).

We recently reported that levels of a mitochondrial stress protein, glucose-regulated protein 75 (GRP75), were increased in liver, muscle and intestinal tissues of torpid 13-lined ground squirrels (*Spermophilus tridecemlineatus*) compared with summer-active squirrels (Carey et al. 1999). GRP75 [also known as mitochondrial heat shock protein 70 (HSP70)] facilitates proper import and folding of nuclear-encoded, mitochondrial-resident proteins (Bhattacharyya et al. 1995; Mizzen et al. 1989). In addition to its important constitutive role in protein import, there is evidence from cell culture and whole animal studies that GRP75 is stress-inducible, particularly in response to conditions that interfere with

mitochondrial function, such as energy depletion, Ca^{2+} imbalance or oxidative stress (Hadari et al. 1997; Mizzen et al. 1989; Massa et al. 1995; Wu et al. 1999). Of these, oxidative stress may be a particularly relevant stimulus for GRP75 induction during hibernation. Mammalian hibernators require a minimum level of polyunsaturated fatty acids (PUFAs), specifically linoleic acid, for optimal hibernation patterns (Geiser 1990; Geiser and Kenagy 1987, 1993; Florant et al. 1993; Thorp et al. 1994), and this is reflected by high levels of linoleic acid in depot fats prior to the onset of hibernation (Florant et al. 1990; Frank 1992). However, diets high in PUFAs also increase the risk of oxidative damage of membrane and storage lipids because of the ease with which they undergo autoxidation (lipid peroxidation) compared with saturated or monounsaturated fatty acids (Gunstone 1996). Lipid peroxidation leads to self-sustaining chain reactions between PUFAs and reactive oxygen species (ROS) that can produce high levels of lipid peroxides and hydrocarbon polymers, both of which are toxic to cells (Gunstone 1996). A second factor that increases the risk of oxidative stress during hibernation is the profound changes in blood flow that accompany both entrance into torpor and arousal to euthermia (Bullard and Funkhouser 1962), which may induce ischemia/reperfusion injury to susceptible tissues (McCord 1985).

The present study examined the potential role of oxidative stress in seasonal induction of GRP75 in intestinal tissues of 13-lined ground squirrels. Intestine was chosen because GRP75 was strongly induced in this tissue in torpid compared with the summer-active squirrels (Carey et al. 1999), although its expression in other activity states during the hibernation season was not examined. Thus, we first asked whether GRP75 expression differed among summer-active squirrels and squirrels in five activity states within the hibernation season. Second, we asked whether the increase in intestinal GRP75 expression in hibernators was associated with indicators of oxidative stress. To do this we measured concentrations of conjugated dienes, which are intermediate by-products of lipid peroxidation that accumulate when the generation of ROS (primarily of mitochondrial origin) is not fully compensated for by cellular antioxidant defenses. We also measured seasonal changes in activation of the redox-sensitive transcription factor, nuclear factor-κB (NF-κB). NF-κB is normally retained in the cytoplasm in an inactive state by binding to an inhibitor protein, IκB. In response to a wide variety of stimuli associated with stress or injury NF-κB is translocated to the nucleus where it binds to promoter elements on responsive genes (Bowie and O'Neill 2000; Mercurio and Manning 1999; Baldwin 1996; Dalton et al. 1999). This induces expression of a variety of gene products, many of which are involved with immune and inflammatory pathways, as well as cellular growth, differentiation and apoptosis (Baldwin 1996; Jobin et al. 1998; Bowie and O'Neill 2000; Ghosh et al. 1998).

Materials and methods

Animals and tissue collection

Adult, nonlactating 13-lined ground squirrels trapped in the vicinity of Madison, Wisconsin, had free access to water and food (Purina rodent chow #5001, supplemented with sunflower seeds), and were held in rooms maintained at 22 °C with a light cycle of 12L:12D. Previous experiments with captive *Spermophilus lateralis* revealed that the amount of linoleic acid stored in white adipose tissue (WAT) was directly proportional to the level of this PUFA present in the diet (Frank et al. 1998) and that this relationship was described by the equation $y = 0.77x$ (where y is WAT linoleic acid content in % and x is diet linoleic acid content in mg/g). Linoleic acid contents of WAT samples collected from *S. tridecemlineatus* were therefore used to estimate dietary linoleic acid contents in this study. *S. tridecemlineatus* diets had a mean (±SE) linoleic acid content of 77.8 ± 1.5 mg/g, which is within the range of linoleic acid contents observed in natural *S. lateralis* food plants (Frank et al. 1998). Squirrels were given ivermectin before introduction to the animal facility to eliminate nematode parasites. Summer-active squirrels were killed from June through early August, had been in captivity for at least 6 weeks and were fed ad libitum until used. In early-mid September squirrels were lightly anesthetized with methoxyfluorane and then administered a subcutaneous injection of an anesthetic cocktail (32 mg/kg ketamine, 10.5 mg/kg acepromazine and 63 mg/kg xylazine). After laparotomy telemetry devises (Minimitter, Sunriver, Ore., USA) for continuous monitoring of T_b were inserted into the abdominal cavity. After squirrels were fully recovered from surgery they were returned to the 22 °C room for at least 2 weeks before being placed in a temperature-controlled room maintained at 5 °C in constant darkness. Food and water were removed when squirrels showed regular torpor bouts. Sampling of hibernators began at least 6 weeks into the hibernation season. Hibernators were sacrificed in one of five categories: ACT, summer-active squirrels ($T_b \approx 37$ °C); EN, entrance into torpor ($T_b \approx 25$ °C); SB, short-bout torpid (≤48 h in deep torpor, $T_b \approx 4$ °C); LB, long-bout torpid (≥7 days in deep torpor, $T_b \approx 4$ °C); AR, arousing from torpor ($T_b \approx 25$ °C); and IBA, during interbout arousal ($T_b \approx 36$ °C). The IBA animals were killed at least 4–5 h after they reached euthermia. After decapitation, mucosa from mid-small intestine and samples of intrascapular brown adipose tissue (BAT) were removed, frozen immediately in liquid N_2 and stored at −80 °C until analyzed.

Stress protein analysis

Gel electrophoresis and immunoblotting were carried out as described previously (Carey and Martin 1996). Mucosal tissues were homogenized in 0.25 M sucrose, 3 mM HEPES, 0.5 mM EGTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4. For BAT, the buffer was 10 mM HEPES, 1% triton X-100, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM PMSF, pH 7.5. All homogenates were centrifuged (15 min, 5000 *g*) and the supernatant containing cellular proteins was collected. Equivalent amounts of protein were loaded into 10% SDS-PAGE gels (50 μg for mucosa and 5 μg for BAT). After transfer of proteins to nitrocellulose, blots were incubated in a TBST (tris-buffered saline plus 0.05% Tween 20) solution containing 1% bovine serum albumin/1% nonfat dry milk to block nonspecific binding sites. Blots were then incubated overnight with anti-GRP75 antibodies (StressGen, Victoria, B.C., Canada) at dilutions determined in preliminary experiments to produce protein bands within the linear range of the film. To control for transfer efficiency among blots, control samples of activated HeLa cell lysate (StressGen, Victoria, B.C., Canada) were loaded on the same gels that contained ground squirrel tissues samples. Protein bands were detected with enhanced chemiluminescence and signals were quantified by scanning densitometry. Signals from tissue samples were normalized to the optical density obtained for the HeLa cell lysate on the same blot and then

expressed per μg of total protein loaded. This allowed comparison among animals from different activity states using blots processed on different days.

Lipid peroxidation

Oxidative stress in intestinal mucosa was estimated by measuring contents of conjugated dienes, which are chemical intermediates produced during the process of lipid peroxidation. Lipids were extracted from each sample (Corongiu and Milia 1983), dissolved in hexane, and measured at wavelengths ranging from 220 to 240 nm using double-derivative spectroscopy (Corongiu and Milia 1983). Content of conjugated dienes is the most reliable method of estimating the level of lipid peroxidation rate in vivo provided that the diet (and thus tissues) do not contain artificial isomers of linoleic acid in the "trans" form (natural PUFAs are always in the "cis" configuration) (Halliwell and Chirico 1993). Preliminary analyses of both the diets and tissues detected no trans-linoleic acid (nor any other "trans" isomers), thus the measurement of conjugated dienes in this study represents an accurate estimate of lipid peroxidation rate. Conjugated diene contents were calculated as double-derivative absorbance units per gram of tissue lipid (d^2A/dA per g lipid).

Preparation of nuclear proteins

Frozen pieces of small intestine or BAT (150–200 mg) were placed in 1.5 ml microfuge tubes and thawed on ice; 0.5 ml of buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% triton X-100, 0.5 mM dithiothreitol (DTT), 1× protease inhibitor cocktail (Calbiochem, San Diego, Calif., USA)] were placed into each tube. Tissues were chopped with small scissors, left on ice for 30 min, homogenized with a Dounce homogenizer (10–20 strokes) and centrifuged at 5000 *g* at 4 °C for 10 min. The pellet was resuspended in 200 μl buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1× protease inhibitor cocktail), incubated on ice for 30 min and centrifuged at 15,000 *g* at 4 °C for 30 min. The supernatant containing nuclear proteins was moved into a fresh tube, frozen immediately and stored at −80 °C. Protein concentration was determined using the BioRad kit (Hercules, Calif., USA).

Gel shift assays

An NF-κB oligonucleotide probe (5AGT TGA GGG GAC TTT CCC AGG C-3) was labeled with ^{32}P-ATP using T4 polynucleotide kinase (Promega, Madison, Wis., USA). Ten micrograms of nuclear proteins were preincubated with buffer [20 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10 μg/ml poly (dI-dC), 5% glycerol] on ice for 10 min before addition of radiolabeled oligonucleotide probe. The cocktail was then incubated for 20 min at room temperature. Protein/DNA complexes were resolved on precast 5% nondenaturing tris-borate gels (Bio-Rad, Hercules, Calif., USA) and run in 0.5 × TBE (45 mM tris-HCl, 45 mM boric acid, 1 mM EDTA) for ≈1 h at constant current (100 mA). The specificity of the binding reaction was determined by preincubating samples with either 100-fold molar excess of unlabeled oligonucleotide probe or anti-NF-κB antibodies (anti-p65 or anti-p50; Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Gels were transferred to Whatman 3 M paper, dried at 50 °C for 3–4 h, and exposed to photographic film. Signal densities of samples on autoradiographs were measured by scanning densitometry and normalized to the signal obtained from a recombinant NF-κB p50 protein (Promega, Madison Wis., USA) run on the same gel.

Statistics

Data were analyzed by *t*-test or analysis of variance using the SAS General Linear Model. If ANOVA analyses were significant, pairwise comparisons were carried out with the least square means procedure (SAS 1985). Significance was set at $P \leq 0.05$. Data are presented as means ± SE.

Results

GRP75 levels

The abundance of GRP75 in intestinal mucosa was significantly greater in each of the five hibernation states compared with summer-active (SA) squirrels (Fig. 1). Hibernators as a group had significantly higher levels of GRP75 than in active squirrels, and within the hibernation season, GRP75 levels in arousing (AR) squirrels were significantly lower than those in long-bout torpid (LB) squirrels.

Lipid peroxidation

Levels of conjugated dienes were measured in intestinal mucosa as an index of lipid peroxidation. Compared with summer-active squirrels, conjugated dienes were higher in animals entering torpor and in short-bout hibernators (Fig. 2), and were higher in all hibernators when combined as one group (Fig. 2). Within the

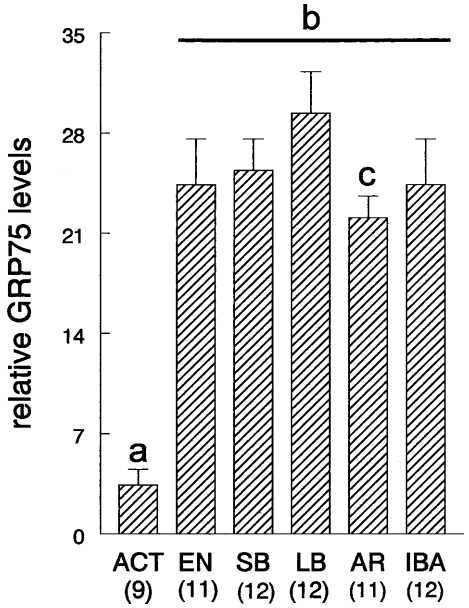

Fig. 1 GRP75 levels in ground squirrel intestine. Data are means ± SE and samples sizes are in parentheses. Optical density values for each sample were normalized to the optical density for a positive control (HeLa cell lysate) run on the same blot and then expressed per μg of total protein loaded (50 μg). *a* values in active squirrels are lower than in each hibernation group ($P < 0.001$), *b* values in active squirrels are lower than in all hibernators as a group ($P < 0.001$), *c* values in arousing squirrels are lower than in long-bout torpid squirrels ($P < 0.05$). *ACT* summer-active squirrels, *EN* entering torpor, *SB* ≤48 h in torpor, *LB* ≥7 days in torpor, *AR* arousing from torpor, *IBA* during interbout arousal

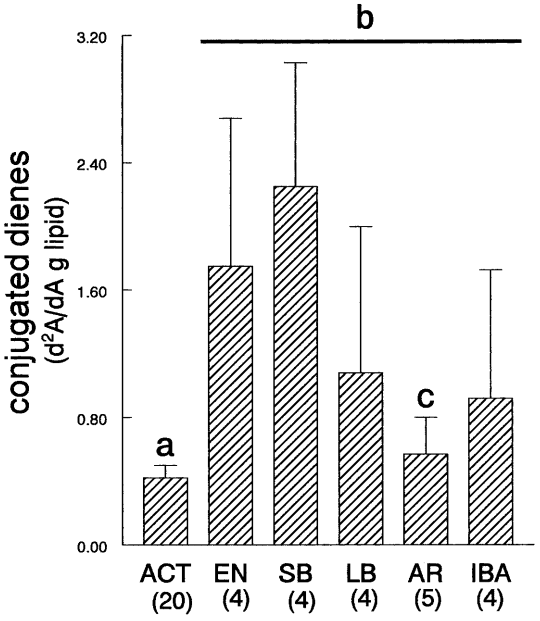

Fig. 2 Conjugated diene levels in ground squirrel intestine. Data are means ± SE and samples sizes are in parentheses *a* values in active squirrels are significantly lower than in entrance and short-bout torpid squirrels ($P < 0.01$), *b* values in hibernators as a group are significantly greater than in active squirrels ($P < 0.05$), *c* values in arousing squirrels are significantly lower than in short-bout torpid squirrels ($P < 0.05$). Abbreviations as in Fig. 1

hibernation season conjugated dienes were greater in short-bout vs arousing squirrels.

NF-κB activation

Results of a typical gel shift assay using nuclear proteins isolated from intestinal mucosa of three short-bout hibernators and six summer squirrels are shown in Fig. 3A. DNA binding activity of NF-κB was low or moderate in summer-active ground squirrels, but strongly induced in intestinal mucosa of hibernators. Densitometric analysis of the data indicated that with the exception of the AR group, NF-κB activation was greater in each of the hibernation states compared with summer-active squirrels, and when all hibernators were combined mean NF-κB activation was significantly greater than in summer (Fig. 3B). To determine the NF-κB subunits that bound to the probe, nuclear proteins were preincubated in anti-p65 or anti-p50 antibodies prior to addition of radiolabeled NF-κB probe (Fig. 3C). The NF-κB signals from one long-bout torpid and one interbout aroused squirrel were supershifted nearly completely by anti-p50 antibody (lanes 2) and were supershifted to a lesser extent by anti-p65 antibody (lanes 3). When both antibodies were used the signal was fully supershifted (lanes 4), which suggests that NF-κB proteins in ground squirrels are similar to those described for other mammals and consist of p50 homodimers (De Plaen et al. 1998; Savkovic et al. 1997) and possibly p50/p65 heterodimers (Rogler et al. 2000; Neurath et al. 1998). The specificity of the NF-κB oligonucleotide probe was further demonstrated by the loss of signal when nuclear proteins were preincubated in an excess of nonradiolabeled probe (lanes 5). As expected, NF-κB signals from nuclear proteins of a summer-active squirrel run on the same gel were much weaker than those for the hibernators. When the autoradiograph was developed further, results similar to those for the hibernators were observed with regard to supershift pattern and competition with cold probe (data not shown).

To determine if the hibernation-induced activation of NF-κB occurs in other nonintestinal tissues, we carried out gel shift assays with similar amounts of BAT and intestinal nuclear proteins and resolved the DNA-protein complexes on the same gel. The results showed that NF-κB activation in BAT was barely detectable at film development times that revealed very strong signals for the intestine (Fig. 3D). Quantitation of NF-κB signals in BAT from autoradiographs that were developed even longer revealed no significant differences among any of the active or torpid states (data not shown). Thus, in contrast to the intestine, basal levels of NF-κB DNA binding activity in BAT are very low and do not appear to vary throughout the year.

Discussion

Hibernation is accompanied by extreme changes in physiology. Body temperature, metabolic rate, heart rate, respiration and other parameters fall during torpor to levels that would be lethal for mammals that do not hibernate (Lyman et al. 1982). Moreover, their restoration to near-normal levels during arousal occurs with a speed that may significantly challenge the cellular and systemic regulatory pathways typically found in non-hibernators. The ease with which hibernators survive seasonal cycles of torpidity and arousal suggests that molecular, cellular and higher-order mechanisms have evolved to successfully manage these events and permit survival during and after the hibernation season. The results of this study suggest that induction of a stress protein, GRP75, in the gut during hibernation may be one such mechanism. A previous report also showed that GRP75 was expressed at higher levels in enterocytes of torpid 13-lined ground squirrels compared with summer-active squirrels (Carey et al. 1999), although it was not clear from that study whether the seasonal upregulation of GRP75 expression was restricted to periods of torpor. The present results suggest that intestinal GRP75 expression is upregulated in five phases of torpor-arousal cycles compared with summer-active squirrels. The one significant difference noted within the hibernation season was a lower level of GRP75 expression in arousing squirrels compared with long-bout torpid squirrels.

GRP75, a member of the HSP70 family of heat shock proteins, is constitutively expressed in mitochondria

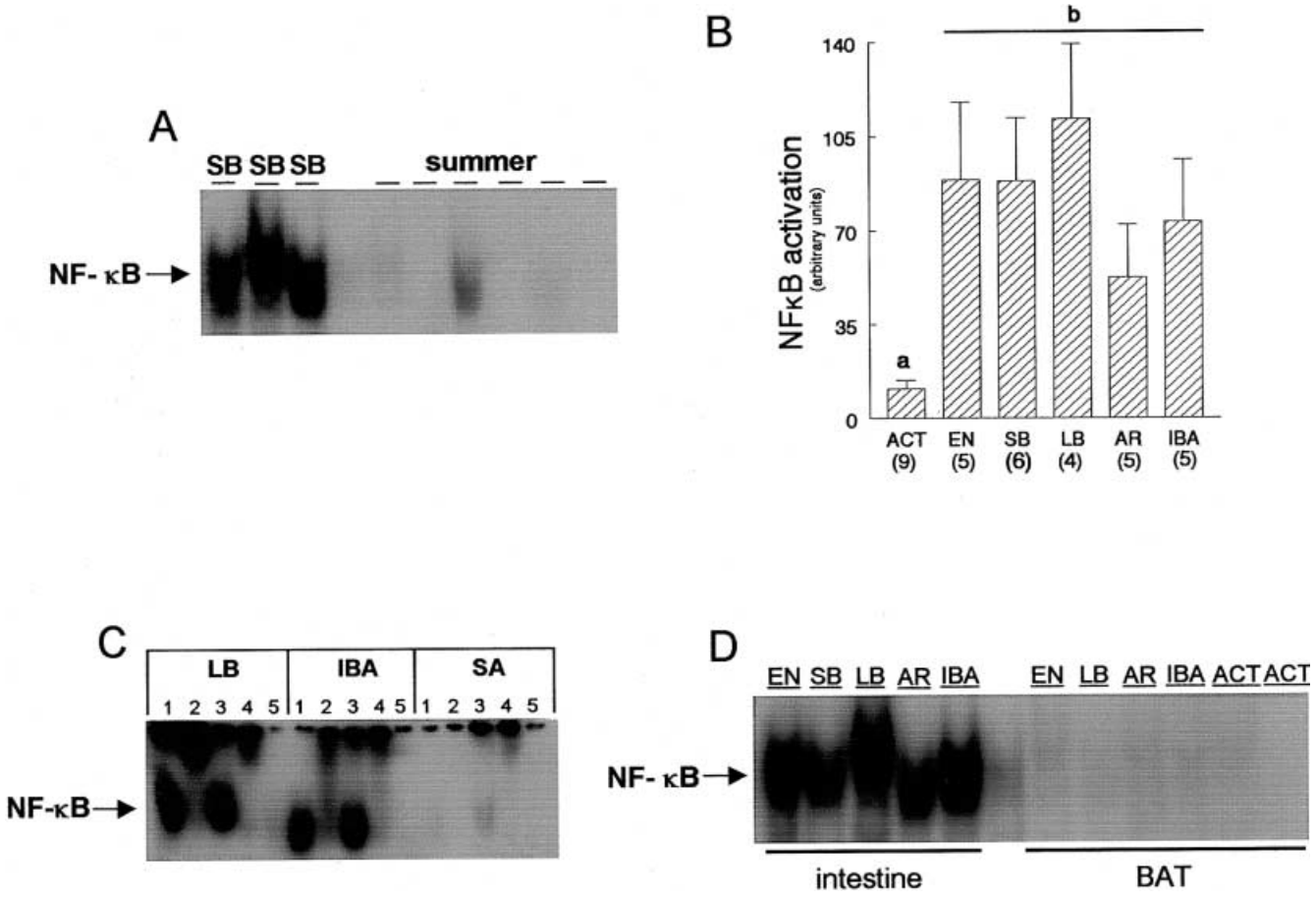

Fig. 3A–D NF-κB activation in ground squirrel intestine. **A** Representative autoradiograph from gel shift experiment using nuclear proteins from three short-bout hibernators (*SB*) and six summer squirrels (*ACT*) (lanes indicated by *lines*). **B** Densitometeric analysis of NF-κB activation. Shown are means ± SE in arbitrary units for NF-κB signals normalized to signals from control NF-κB protein run in the same gel. *a* values in active animals are significantly less than in *EN*, *SB*, *LB* and *IBA* groups ($P < 0.05$), *b* as a group hibernators have higher values than in active squirrels ($P < 0.001$). Abbreviations as in Fig. 1. **C** NF-κB signals from one long-bout, one interbout arousal and one summer-active squirrel (*lanes 1*) are supershifted by preincubation of nuclear proteins with anti-p50 antibodies (*lanes 2*), partially supershifted by anti-p65 (*lanes 3*), and fully supershifted by preincubation with both antibodies (*lanes 4*). Complete loss of signal occurs when nuclear proteins are preincubated with excess cold NF-κB probe (*lanes 5*). **D** Comparison of NF-κB activation in intestine from five hibernators and BAT from six squirrels. Abbreviations as in Fig. 1

where it participates in translocation and folding of proteins within the mitochondrial matrix (Bhattacharyya et al. 1995; Mizzen et al. 1989). One mechanism for upregulation of GRP75 would therefore be an increase in synthesis and translocation of mitochondrial proteins. This mechanism is unlikely to account for our findings, because we showed previously that total intestinal protein decreased during hibernation compared with summer-active values (Carey 1990). Furthermore, with the exception of the membrane-cytoskeletal linking protein, moesin, which is increased in enterocytes of torpid hibernators (Gorham et al. 1998), expression of several enterocyte proteins decreased or remained the same during hibernation (Carey and Martin 1996). The lack of seasonal change in another mitochondrial stress protein, HSP60, in intestine also argues against a general increase in mitochondrial protein synthesis as a mechanism for upregulation of GRP75 expression in hibernators (Carey et al. 1999). Discordant patterns of expression among different stress proteins during torpor and euthermia have been observed in other ground squirrel species (Bitting et al. 1999).

Another mechanism for upregulation of GRP75 in intestine of hibernators is increased synthesis in response to cellular stress. Unlike the related protein HSP70, which is strongly induced by thermal stress, induction of GRP75 is thought to be less heat-inducible and more closely associated with metabolic alterations, such as those induced by glucose depletion, perturbations in calcium homeostasis, anoxia and oxidative stress (Hadari et al. 1997; Mizzen et al. 1989; Shen et al. 1987; Bruschi et al. 1998). For example, in intact animals GRP75 was induced by focal or global brain ischemia (Massa et al. 1995) and by ozone exposure in respiratory epithelia (Wu et al. 1999), both of which are associated with oxidative damage to cellular structures including mitochondria. Thus, we examined the possibility that the seasonal change in intestinal GRP75 levels in ground squirrels reflected oxidative stress to the intestinal mucosa during the hibernation season. Our reasoning was

based in part on the cardiovascular changes that occur during this time. As animals enter torpor, blood flow is shunted from nonessential areas like the gut to maintain flow to other organs such as heart, diaphragm and BAT. This causes a reduction in intestinal blood flow to only 5% of normal rates (Bullard and Funkhouser 1962). In addition, the gut is one of the last organs to be reperfused with blood during periodic arousals (Bullard and Funkhouser 1962). These changes in splanchnic blood flow during torpor-arousal cycles have the potential to induce significant damage to the gut, because the intestinal mucosa is one of the most sensitive tissues to ischemia/reperfusion injury (Hall et al. 1999; Granger and Korthuis 1995; McCord 1985). Both ischemia alone (LeGrand and Aw 1997) and reperfusion after ischemic events (McCord 1985) can induce oxidative damage to the mucosa as reflected by increased lipid peroxidation, alterations in glutathione redox balance, increased capillary permeability, mucosal lesions, mitochondrial dysfunction and increased enterocyte apoptosis and necrosis (Henninger et al. 1995; Aw 1999; McCord 1985). In our study levels of conjugated dienes in intestine were significantly higher in two of the five hibernation states (entrance and short-bout torpor) compared with levels in summer-active squirrels, and hibernators as a group had higher levels than in summer. Because conjugated dienes are produced during the initial stages of peroxidation of PUFAs, their presence in biological tissues reflects oxidative stress. Chain reactions of lipid peroxidation can lead to cellular dysfunction through a variety of destructive events, including damage to membrane proteins and phospholipids (Refsgaard et al. 2000; Halliwell and Chirico 1993). It should be noted that the greatly reduced metabolism of deep torpor likely reduces the degree of oxidative stress due to Q_{10} effects on reactions that generate ROS. However, cumulative damage to cellular lipids by ROS can still occur at the low T_b of torpor, albeit at reduced rates, whereas conventional antioxidant enzyme systems may not be active at these temperatures. Thus, these results suggest that some aspect of torpor-arousal cycles can potentially damage cellular lipids and other macromolecules via oxidative stress.

The idea that hibernators may be at risk for oxidative stress is supported by other studies using nonintestinal tissues. For example, in ground squirrels levels of some antioxidant enzymes and the ROS scavenger ascorbate are increased during hibernation in BAT, liver and plasma (Buzadzic et al. 1990), and ascorbate concentrations in plasma and cerebrospinal fluid are significantly elevated during torpor and fall to pre-hibernation levels upon arousal (Drew et al. 1999). These effects presumably represent adaptive mechanisms to counter increased oxidant generation during torpor-arousal cycles. In addition, free-ranging ground squirrels may reduce oxidative damage in adipose tissues during the hibernation season by restricting their intake of polyunsaturated fatty acids (Frank et al. 1998), which are particularly susceptible to lipid peroxidation (Gunstone 1996; Refsgaard et al. 2000). There is also good evidence from nonmammalian vertebrates and invertebrates that antioxidant defense mechanisms are activated during, or in response to, periods of metabolic depression (Grundy and Storey 1998; Hermes-Lima et al. 1998). In fact, our results for hibernating ground squirrels are similar in several respects to those found during estivation in the desert toad *Scaphiopus*, (Grundy and Storey 1998). Compared with active toads, gut tissues of estivating toads had higher levels of conjugated dienes and there was a shift in glutathione redox balance to a more oxidized form (Grundy and Storey 1998). Preliminary studies suggest the intestinal glutathione system responds in a similar way to hibernation in ground squirrels (Carey et al. 2000).

The increase in activation of the transcription factor, NF-κB, in ground squirrel intestine during hibernation also supports the view that this tissue is subject to some level of oxidative stress during torpor-arousal cycles. NF-κB is a critical transcription factor that mediates expression of a variety of gene products in response to cellular stress, including infection, UV radiation, proinflammatory cytokines and oxidative stress (Rogler et al. 2000; Neurath et al. 1998; Ghosh et al. 1998; Mercurio and Manning 1999; Baldwin 1996). Although the relationship between oxidative stress and NF-κB is still under intense investigation (Li and Karin 1999), there is good evidence that ROS and their metabolites, including lipid peroxides, can activate NF-κB, due in part to their ability to alter cellular redox balance (Bowie and O'Neill 2000; Dalton et al. 1999; Mercurio and Manning 1999). Many of the gene products produced by NF-κB transcriptional activity are associated with immune and inflammatory pathways, including cell adhesion molecules and cytokines that mediate leukocyte proliferation, activation and trafficking to sites of infection or damage (Ghosh et al. 1998). Indeed, the degree to which NF-κB is activated in ground squirrel intestine during hibernation is typically only observed in response to inflammatory, infectious or ischemic events (Rogler et al. 2000; Neurath et al. 1998; De Plaen et al. 1998). Although the gene products induced by NF-κB during hibernation are yet to be identified, there are indications that some of the cellular pathways mediated by NF-κB transcriptional regulation are enhanced during hibernation. For example, there is a dramatic change in the intestinal mucosal immune system during hibernation, characterized by an increase in numbers of lymphocytes and plasma cells in the lamina propria as well as a marked increase in intraepithelial lymphocytes that reside between adjacent enterocytes (Shivatcheva and Hadjioloff 1987; Fichtelius and Jaroslow 1969) (Carey et al. 2000). This change is associated with some aspect of torpor-arousal cycles because it is not seen in squirrels that (for unknown reasons) do not hibernate in the fall and remain continuously active, even though they are housed at low ambient temperatures (H. Carey, personal observations). The possibility that hibernation-induced changes in the intestinal immune system are

related to oxidative stress to the mucosa is intriguing because of recent studies that implicate a subset of intraepithelial lymphocytes in the repair of stressed or damaged enterocytes (Groh et al. 1998; Kagnoff 1998; Boismenu and Havran 1994). Interestingly, despite our evidence for oxidative stress and immune activation in the gut during hibernation, the intestinal epithelium remains remarkably healthy. There is atrophy of the mucosa as the hibernation season progresses, as indicated by reductions in villus height and crypt depth (Carey 1990) that is probably due in part to the absence of food intake during this time. Yet, there are no obvious signs of tissue necrosis, edema, changes in villus:crypt ratio or a reduction in transport function when tissues are warmed to 37 °C (Carey 1990, 1992; Carey and Sills 1992, 1996). There are, however, two indications of altered epithelial physiology, both of which are associated with oxidative stress to the gut in other species (Grisham et al. 1990; Darmon et al. 1993): increased ionic permeability and enhanced responses to agents that induce intestinal secretion (Carey 1992). Whether epithelial dysfunction during hibernation is ameliorated in part by protective molecules released from mucosal immune cells is unknown. However, the possibility that some aspect of the hibernation cycle involves changes in leukocyte trafficking is supported by recent studies (Yasuma et al. 1997). Those authors showed that plasma from hibernating ground squirrels induced a dose-dependent increase in the adherence of monocytes to endothelial cells isolated from rat cerebral microvasculature. Plasma from hibernators also increased expression of the cell surface adhesion molecule ICAM-1 (Yasuma et al. 1997), which is also a key activator of leukocyte adherence to enterocytes (Jobin et al. 1998). These effects occurred to a significantly greater extent than did plasma from nonhibernating squirrels (Yasuma et al. 1997). Although the mechanistic basis for these effects was not identified, the results suggest that one or more circulating factors (e.g., cytokines or chemokines) may be elevated during hibernation and alter leukocyte-effector cell interactions.

The strong activation of NF-κB observed in the intestine of hibernators does not occur in all tissues, because NF-κB activation in BAT was very low in all of the activity states we studied. This observation is interesting, because elevated levels of certain antioxidant enzymes in BAT during the hibernation season are thought to be a compensatory response to the rapid increase in BAT mitochondrial respiration during arousals from torpor, which may increase the risk of excessive accumulation of ROS (Buzadzic et al. 1990). Thus, the enhanced levels of antioxidant enzymes in BAT during hibernation may be sufficient to minimize changes in redox balance that are necessary to activate NF-κB. Other factors that may reduce the risk of oxidative stress to BAT compared with other splanchnic organs like the gut include the relatively higher blood flow to this tissue that occurs both during torpor and arousal (Bullard and Funkhouser 1962), as well as high levels of the uncoupling protein, UCP1, which has been suggested to reduce $O_2^{\bullet -}$ generation in mitochondria by increasing H^+ leak across the inner mitochondrial membrane (Skulachev 1998). In this regard, it is interesting to note that GRP75 levels, although constitutively high in BAT, are not affected by torpor (Carey et al. 1999), which may reflect the relatively lower accumulation of ROS in this tissue. Taken together, these results further support the idea that the intestine is particularly susceptible to oxidative stress during hibernation and, in response, may possess tissue-specific defense mechanisms.

We were unable to determine precisely which aspect of torpor-arousal cycles leads to oxidative stress and induction of stress-responsive signaling pathways during hibernation, possibly due to the variability inherent in measurements using wild-caught animals. Yet, despite the few significant differences in levels of GRP75, conjugated dienes or NF-κB activation among the five hibernation states, there was a consistent trend for higher values for all three parameters in animals entering torpor or in deep torpor, and lower values during arousal or interbout euthermia. This pattern could be relevant in light of studies that suggest that ischemia or hypoxia alone (in the absence of reperfusion) can generate sufficient ROS to induce oxidative stress (Vanden Hoek et al. 1998; Chandel et al. 1999; LeGrand and Aw 1998; Noda et al. 1998) and can activate signaling pathways and transcriptional events that provide cellular protection from stressful events (Vanden Hoek et al. 1998; Chandel et al. 1999; LeGrand and Aw 1998; Noda et al. 1998). Confirmation of this idea awaits more detailed analyses of these and other parameters related to oxidative stress-induced signaling during the hibernation season. However, the changes reported here for intestinal tissues of hibernators may represent an integrated response that uses a moderate level of oxidative stress, generated during one portion of the hibernation cycle, to activate defensive pathways that protect this highly vulnerable tissue from more severe damage that might occur during a later phase of the cycle (Hermes-Lima et al. 1998).

Knowledge of the mechanisms used by mammals to minimize tissue injury during endogenous oxidative stress may lead to novel approaches to minimize stress-induced damage due to natural or artificial conditions that place mammals at the extremes of their normal physiology. For example, oxidative stress is an important factor in the deterioration of tissues stored in the cold before transplant, and administration of antioxidants to cold storage solutions improves viability of tissues during normothermic perfusion (McAnulty and Huang 1997). Better understanding of the mechanisms by which tissues from hibernators avoid damaging effects of ROS during repeated cycles of torpor and arousal may identify cellular defense pathways that could be used to extend cold storage of organs for human and animal transplant procedures.

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