Myosin Isoform Expression and MAFbx mRNA Levels in Hibernating Golden-Mantled Ground Squirrels (*Spermophilus lateralis*)

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ABSTRACT

Hibernating mammals present many unexplored opportunities for the study of muscle biology. The hindlimb muscles of a small rodent hibernator (*Spermophilus lateralis*) atrophy slightly during months of torpor, representing a reduction in the disuse atrophy commonly seen in other mammalian models. How torpor affects contractile protein expression is unclear; therefore, we examined the myosin heavy-chain (MHC) isoform profile of ground squirrel skeletal muscle before and after hibernation. Immunoblotting was performed first to identify the MHC isoforms expressed in this species. Relative percentages of MHC isoforms in individual muscles were then measured using SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The soleus and diaphragm did not display differences in isoforms following hibernation, but we found minor fast-to-slow isoform shifts in MHC protein in the gastrocnemius and plantaris. These subtle changes are contrary to those predicted by other models of inactivity but may reflect the requirement for shivering thermogenesis during arousals from torpor. We also measured mRNA expression of the Muscle Atrophy F-box (MAFbx), a ubiquitin ligase important in proteasome-mediated proteolysis. Expression was elevated in the hibernating gastrocnemius and the plantaris but was not associated with atrophy. Skeletal muscle from hibernators displays unusual plasticity, which may be a combined result of the intense activity during arousals and the reduction of metabolism during torpor.

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Introduction

Mammalian muscle typically atrophies with disuse, which occurs following bed rest, immobilization, space flight, or denervation (Booth and Thomason 1991; Baldwin and Haddad 2001). Muscle fiber contractile properties, enzymatic properties, and protein composition are highly plastic and can be altered rapidly after mechanical unloading (Thomason et al. 1987; Caiozzo et al. 1998). In particular, adult rat and mouse skeletal muscle, which expresses four myosin heavy-chain (MHC) protein isoforms, changes the relative percentage of those isoforms following inactivity, together with reductions in oxidative enzyme capacity and fiber cross-sectional area. For instance, during conditions of mechanical unloading resulting from hindlimb suspension, myosin isoform expression generally shifts from slow to fast MHC isoforms, with accompanying increases in more glycolytic and fatigable fiber-type characteristics (Thomason et al. 1987; Caiozzo et al. 1998). Although many animal models exist for the study of muscle atrophy (Booth and Thomason 1991), they are confined to a relatively small number of species (e.g., mice, rats, rabbits, and dogs), and it is not clear whether findings from these animal models would apply to the skeletal muscle of hibernators during periods of torpor.

Many mammals from diverse orders have the ability to hibernate in some manner, entering a state of lowered metabolic expenditure, as an adaptive strategy for conserving energy during times of harsh environmental conditions or restricted food availability (Davis 1970; Lyman et al. 1982; Nedergaard and Cannon 1990). Characteristics differ greatly among groups, but these animals, including rodents, bats, marsupials, and bears, all experience bouts of torpor, which can last from a period of days to weeks. During torpor, there is greatly reduced ventilatory activity and a dramatic reduction in locomotor activity. This lack of skeletal muscle activity leads to mild atrophy in limb musculature (Yacoe 1983; Koebel et al. 1991; Tinker et al. 1998), while diaphragm and cardiac muscle in several hibernating rodents maintains mass and, in some instances, can even hypertrophy (Vyskocil and Gutmann 1977; Wickler et al. 1987, 1991; Reid et al. 1995). However, while overall muscle mass in hindlimb muscles decreases, oxidative metabolic capacity in

some tissues is conserved or increased, such as for citrate synthase activity (Koebel et al. 1991; Steffen et al. 1991; Storey 1997; Tinker et al. 1998). This blunted or even abated response to inactivity with respect to muscle mass and enzymatic properties differs starkly from that predicted in rat and human models. Thus, hibernating mammals present an opportunity to study the mechanisms and regulatory pathways by which certain muscle functions are defended by an organism, even with disuse and potential mechanical unloading. Moreover, it has been argued that the requisite hibernating machinery is ancestral to all mammalian orders (Geiser 1998; van Breukelen and Martin 2002*a*), and the number of species in which it remains extant is not trivial, numbering in the hundreds.

The relative percentages of MHC protein isoforms in a whole muscle have important functional consequences for muscle performance. One of the primary determinants of muscle function is the force-velocity relationship, which determines the mechanical work and power that a muscle can produce. Maximal shortening velocity of a given muscle or individual fiber is determined by the type of myosin isoform (slow or fast), which influences power across all shortening velocities; in addition, the economy of force production is dependent on the MHC isoform. Skeletal muscle fiber composition appears to be highly malleable and can be altered rapidly. Mechanical unloading of mammalian skeletal muscle typically produces atrophy and slow-to-fast shifts in MHC isoform composition, with sometimes complex influences on muscle power and efficiency (reviewed in Caiozzo 2002).

Little is known about the effects of hibernation on transcriptional, translational, and degradational processes responsible for determining the type and amount of contractile proteins. We examined the changes in MHC protein isoform composition of several hindlimb muscles and the diaphragm after 4 mo of torpor in a small mammalian hibernator, the golden-mantled ground squirrel (*Spermophilus lateralis*). On the basis of the findings from studies employing models of hindlimb unloading in mice and rats, we expected to find four MHC isoforms (types I, IIA, IIX, and IIB), and we hypothesized that even mild atrophy of hindlimb muscles would result in increased types IIB and IIX MHC expression, especially in the soleus muscle. The diaphragm muscle of hibernators was expected to have slight increases in type IIB protein, as predicted by a previous study (Reid et al. 1995). We also measured the mRNA expression of a newly identified and critical gene in other models of atrophy studied, the Muscle Atrophy F-box (MAFbx) component of the ubiquitin ligase-proteasome complex (Solomon et al. 1998; Lecker et al. 1999; Bodine et al. 2001). We hypothesized that MAFbx mRNA levels would be higher in the atrophied muscle of hibernating squirrels, leading to increased myofibrillar protein degradation and possibly providing a mechanistic explanation for the atrophy that does occur during hibernation.

Material and Methods

Animal Care and Tissue Collection

Adult golden-mantled ground squirrels (*Spermophilus lateralis*) were obtained in the summer of 2001 from a supplier in Redding, California, and housed in individual cages at the University of British Columbia, Vancouver. Animals, chosen for either the active or the hibernating experimental group, were matched as closely as possible for mass (190–230 g) and sex. All animals were held initially in a temperature-controlled room (21°C, 12L : 12D) and provided rodent chow and water ad lib., supplemented with sunflower seeds and fruit. Fall-active squirrels were killed in October 2001 after which the room temperature holding the remaining animals was reduced 3°C d^{-1} until the chamber reached 5°C, and the light cycle was changed to 2L : 22D. Squirrels entered hibernation within several weeks of the new temperature and photoperiod regime. Hibernators were held in captivity for 3–4 mo at 5°C and killed during hibernation at 5°–7°C. All animals were killed by sodium pentobarbital injection in accordance with the animal care guidelines of the University of British Columbia (A01-0093). Gastrocnemius, soleus, plantaris, and diaphragm muscles were isolated, wrapped in tinfoil, and quickly frozen in liquid nitrogen. Muscles were stored at −80°C until shipment on dry ice to the University of California, Irvine, in March 2002, where all subsequent analyses reported herein were carried out.

All muscle samples were prepared initially by homogenizing 30–35 mg of tissue at medium speed (PowerGen 125, with 5 × 95 mm bit; Fisher Scientific, Tustin, Calif.) for 15 s on ice in 15 vol of a sucrose solution (250 mM sucrose, 100 mM KCl, 5 mM EDTA). Aliquots were taken for total protein concentration, and the remaining solution was stored at −80°C. Total protein concentration was measured in triplicate on a microplate reader (Biotek, Winooski, Vt.) using a commercially available dye reagent (BioRad, Hercules, Calif.) and IgG as standard.

Myosin Heavy-Chain Protein Analyses

MHC isoforms were separated using a discontinuous gel electrophoresis technique (Talmadge and Roy 1993) that separates four known adult MHC isoforms in rat muscle (types I, IIA, IIX, and IIB). Homogenized whole muscle was placed into 30 μL of buffer (5% β-mercaptoethanal, 100 mM Tris-base, 5% glycerol, 4% SDS [sodium dodecyl sulfate], and 1% bromophenol blue); then each sample was heated to 95°C for 2 min and vortexed for 30 s. The separating gel of a two-stage gel was prepared (8% acrylamide, 0.6% bis-acrylamide, 29% glycerol, 0.4% SDS, 0.2 M Tris, and 0.1 M glycine) and polymerized using TEMED (0.05%) and ammonium persulfate (10%). After layering with ethanol, the gel was allowed to stand for 30 min. Once polymerization was complete, the alcohol was poured off and a stacking gel (4% acrylamide, 0.2% bis-acrylamide, 30% glycerol, 70 mM Tris [pH 6.7], 4 mM EDTA, and 0.4% SDS)

was added. Polymerization was again initiated using TEMED (0.05%) and ammonium persulfate (10%), and the gel was allowed to stand for 30 min. Ten microliters from each sample, or approximately 7–10 μg of total protein, was loaded into each well, and electrophoresis was carried out in a SG-200 vertical slab gel system (CBS Scientific, Del Mar, Calif.). The gels were immersed in a running buffer (0.1 M Tris, 0.15 M glycine, and 0.1% SDS) and run at a constant voltage of 275 V for 24 h at 4°C. The protein bands were then visualized using a Silver Stain Plus kit (BioRad, Hercules, Calif.) and scanned for MHC isoforms using a laser densitometer and image software (ImageQuant by Molecular Dynamics, Sunnyvale, Calif.).

Hybrid fibers, or muscle cells coexpressing two or more MHC isoforms, have been identified in many species (rats, rabbits, canines, and humans). To explore this phenomenon in ground squirrels, we performed single-fiber analyses by cutting two small 10-mg sections from different regions of frozen muscle, which were then placed into a glycerol-relaxing solution (2mM EDTA, 1 mM $MgCl_2$, 4 mM adenosine triphosphate, 10 mM imidazole, 100 mM KCl pH 7.0). The samples were stored overnight at −20°C and gradually warmed, first to 4°C and finally to room temperature. From each muscle, 40 single fibers, approximately 2 mm in length, were carefully dissected under a low-power microscope (× 50) using fine forceps. Each fiber was placed into sample buffer and electrophoresed as above.

Immunoblotting for Myosin Isoforms

Antibody detection of MHC isoforms was performed using Western blots of representative squirrel muscles. Whole muscle lysate was loaded at a concentration of 1 mg mL^{-1} (200 μg total protein loaded per gel) above a laneless stacking gel, electrophoresed as above, and then transferred to polyvinylidene fluoride membranes by blotting at 100 V for 2 h. Membranes were cut into strips, incubated in a blotting buffer (Blotto, Santa Cruz Biotechnology, Santa Cruz, Calif.) for 30 min, rinsed with phosphate-buffered saline, and then incubated for 1 h at room temperature with monoclonal antibodies specific for the rodent slow type I (BAD5, 1 : 100), fast type IIA (SC71, 1 : 100), or fast type IIB (BFF3, 1 : 100) MHC isoforms. In addition, an antibody that recognizes all three fast MHC isoforms was also used (Sigma fast myosin, 1 : 1,000). Blots were next incubated with a biotinylated secondary antibody (Vector ABC Elite, Burlingame, Calif.) for 1 h, and proteins were visualized using Vector VIP stain. All primary antibodies except for the Sigma primary were derived from cultures purchased from American Tissue and Cell Culture and originally developed by Schiaffino et al. (1989). Immunoblots were then compared with similar blots of rat vastus intermedius lysate, containing four adult MHC isoforms, and to silver-stained gels, containing the same squirrel lysates used in each blot. Immunoblots were repeated multiple times on the same muscle, and a total of 10 individual ground squirrel muscles were used for MHC typing by blotting.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Isolation of total RNA from whole muscle homogenates was performed using TriReagent LS and BCP reagent (Molecular Research Center, Cincinnati, Ohio). Isopropanol addition precipitated the RNA, which was washed twice with 75% ethanol and spun dry in a vacuum centrifuge. A small amount of water (10 μL) was then added to solubilize the mRNA, which was heated to 50°C for 2 min and frozen at −80°C before total RNA concentration was determined spectrophotometrically (Beckman DU 640B, Fullerton, Calif.) at 260 nm. One microgram total RNA from each sample was reverse transcribed to cDNA (SuperScript II, Invitrogen, Carlsbad, Calif.) for use in all subsequent PCR reactions and stored at −20°C.

Primers for the MAFbx gene (5′ AGACCGGCTACTGTGGA-AGAG, 3′ CCGTGCATGGATGGTCAGTG) were created using PrimerSelect software (LaserGene, DNASTAR, Madison, Wis.) and an available rat sequence (AY059628) providing an amplification product of 218 bp. Multiplex PCR reactions were carried out using a 327-bp ribosomal 18S amplification product (5′ AGGAATTGACGGAAGGGCAC, 3′ GTGCAGCCCCGGA-CATCTAAG) modified from a commercially developed primer application (Ambion, Austin, Tex.) as a control for variation in reaction efficiency and pipetting. A competing primer (5′ GAATTGACGGAAGGGCACTT, 3′ GCAGCCCCGGACATCT-AAGAA) was added to each reaction in a 4 : 1 ratio to adjust amplification levels of the ribosomal 18s gene. PCR reactions were carried out on an automated thermal cycler (Robocycler, Stratagene, La Jolla, Calif.) for 27 cycles at a primer annealing temperature of 56°C. The cycle number was carefully selected to be in the linear range by comparison with reaction products of varying cycle numbers (25–28 cycles). Amplification products were separated on 2% agarose gels, visualized using Sybr Green nucleotide stain (Molecular Probes, Eugene, Oreg.), and recorded on a digital camera (Nikon USA). Gels were analyzed using ImageQuant software, with the mRNA levels of the MAFbx gene expressed relative to the 18s product.

Statistical Analyses

Comparisons between hibernating and active tissues were made using one-way ANOVA carried out on a statistical package (Systat 10, Systat Software, Richmond, Calif.). Determinations of both whole muscle myosin protein and MAFbx mRNA were performed twice, and the average from each sample was used for all subsequent analyses. Numbers of observations reported represent the numbers of individual animals used. Outliers identified by the statistical program were discarded, and significance was determined as $P \leq 0.05$.

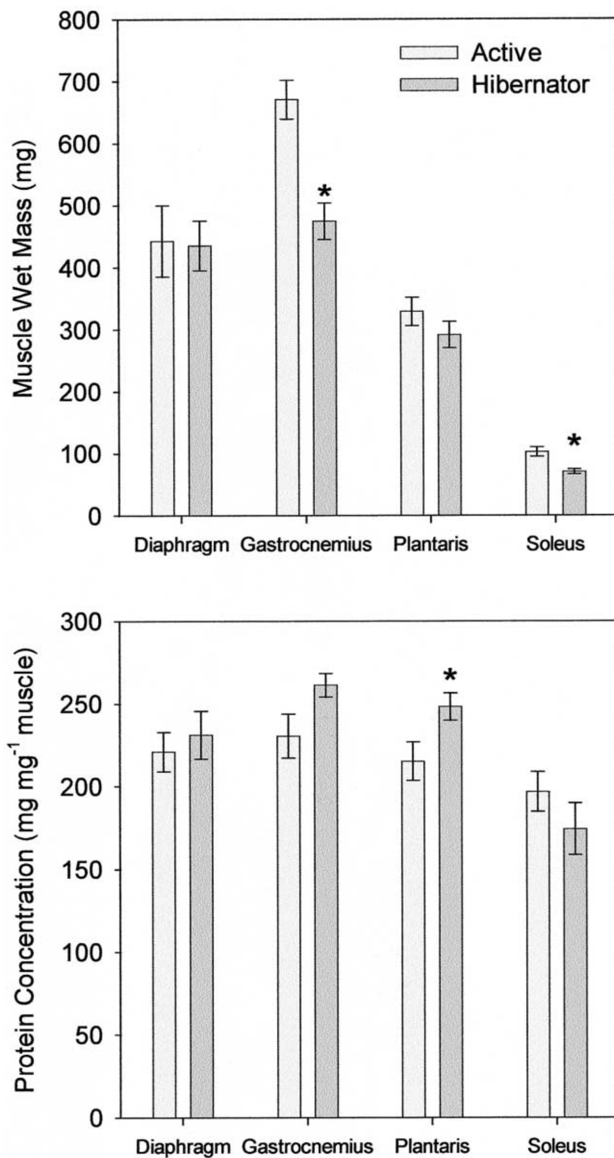

Figure 1. Muscle mass and protein. Wet mass (*top*) and total protein concentration (*bottom*) from muscles of active and hibernating squirrels. The diaphragm ($n = 18$) and plantaris ($n = 22$) masses do not vary, but gastrocnemius ($n = 26$) and soleus ($n = 27$) muscles atrophy by 30% ($P < 0.002$). Total protein concentration is increased significantly in hibernating plantaris ($P = 0.028$) muscles. Data are means ± SEM, and significance is denoted by an asterisk.

Results

Muscle Mass and Protein Concentrations

Gastrocnemius ($n = 26$, $P < 0.001$) and soleus ($n = 27$, $P = 0.002$) muscle wet masses were 30% lower in hibernating squirrels than in fall-active squirrels, while diaphragm ($n = 18$) and plantaris ($n = 22$) muscles did not vary (Fig. 1). Total protein concentration (Fig. 1) was increased in hibernating plantaris ($P = 0.028$) muscles and was unchanged in the diaphragm, soleus, and gastrocnemius.

SDS-PAGE and Immunoblot Analyses of MHC Isoform Composition

Squirrel MHC protein isoforms appear to migrate in an identical manner to rat myosin, with bands closely aligning to rat vastus intermedius muscle, which expresses all four MHC isoforms. SDS-PAGE (polyacrylamide gel electrophoresis) analysis of all four squirrel muscles found apparently only three MHC protein isoforms in whole muscle lysates (Fig. 2) and in single muscle fibers (Fig. 3), which were subsequently identified as types I, IIX, and IIB by immunoblotting (Fig. 4). The BAD5 antibody clearly identified the presence of the type I MHC isoform in squirrel muscle. The Sigma antibody used in the Western blotting analyses clearly identified two fast isoform bands. One of these corresponded to a band that also could be labeled with BF-F3, and, consequently, this was putatively labeled as the fast type IIB MHC isoform (see Fig. 4). The second fast band did not stain positive using the SC-71 antibody (specific for the fast type IIA MHC isoform), and, as a result, this band was determined to be the fast type IIX MHC isoform. This combination of MHC isoforms is highly consistent with the MHC isoform composition of hindlimb muscles in both rats and mice.

The relative percentage of types I, IIX, and IIB MHC protein measured in fall-active and hibernating muscles (Fig. 5) revealed modest 14% and 17% changes in IIX protein in two muscles, the gastrocnemius ($P = 0.043$) and plantaris ($P =$

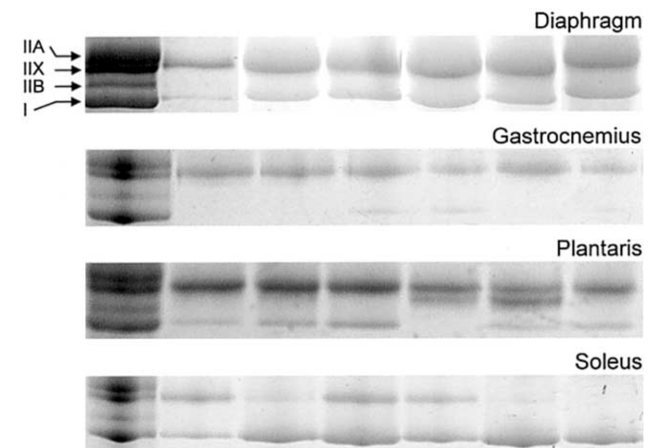

Figure 2. Whole muscle myosin heavy-chain (MHC) isoform. SDS-PAGE of MHC protein from active and hibernating animals, visualized with silver stain. The leftmost lane in each gel is rat vastus intermedius muscle, which expresses all four of the myosin isoforms and serves as a control marker. The three visible squirrel bands separate in a similar fashion to rat muscle, and MHC types I, IIX, and IIB were identified in lysates of whole squirrel muscle.

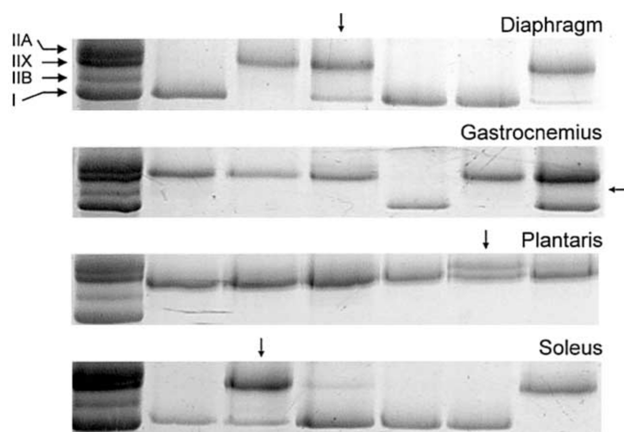

Figure 3. Single-fiber myosin heavy-chain (MHC) isoforms. SDS-PAGE samples from single fibers isolated from four fall-active squirrel muscles. Rat vastus intermedius control containing the four MHC isoforms is loaded at the left of each gel. Each lane represents myosin protein from a single muscle fiber, approximately 2 mm in length, microdissected from different regions of each muscle. Many fibers expressed only a single MHC isoform, but hybrid fibers expressing two MHC isoforms, such as types I and IIX together, are readily apparent (examples are marked by small arrow).

0.013). In the plantaris muscles, all three isoforms were detected, and the IIX protein was greater in hibernators, without apparent lower expression of types I and IIB protein. Hibernating gastrocnemius muscles also had greater levels of IIX MHC but showed no expression of IIB MHC, which had comprised roughly 10% in the active muscles. Diaphragm and soleus muscles, from both active and hibernating squirrels, expressed solely types I and IIX MHC protein and did not display differences in isoform percentages. None of the muscle groups showed any difference in the percentage of type I MHC protein expressed between active and hibernating animals.

Single-fiber analyses of one of each muscle from hibernating and active squirrels reveal many "hybrid" muscle fibers, expressing two different MHC isoforms (e.g., IIX and IIB together as "XB"; see Fig. 6), as well as fibers that expressed a single MHC protein. No hybrid fibers were observed that expressed more than two MHC isoforms. Because only a representative sample from each group was analyzed, no statistical comparisons were made between active and hibernating squirrels.

RT-PCR Analyses of MAFbx

RT-PCR measurement of MAFbx mRNA levels revealed some differences between active and hibernating muscle (Fig. 7). The diaphragm, which did not show evidence of atrophy in this study, showed detectable, but unchanging, MAFbx signal, while hibernating gastrocnemius ($P = 0.008$) and plantaris ($P = 0.021$) muscles showed a two- to fourfold greater mRNA signal compared with active muscles. The soleus, while significantly atrophied following hibernation, did not have increased MAFbx expression ($P = 0.089$). Expression levels were comparable between all muscles except for the soleus, which evidenced high expression even in active tissues.

Discussion

Few studies have looked at the effects of torpor on the properties or expression of skeletal muscle contractile proteins. Histochemical staining for skeletal muscle fiber type in hibernators has been used only in hamsters, bears, and ground squirrels (Vyskocil and Gutmann 1977; Koebel et al. 1991; Reid et al. 1995). Histochemical staining cannot detect subtle shifts in MHC expression, nor detect those masked by expression of hybrid fibers. The much higher resolution techniques of gel electrophoresis and immunoblots have only been used in bats, with the surprising finding of a new MHC isoform (Hermanson et al. 1998 and references therein), and in marsupials (Sciote and Rowlerson 1998; Zhong et al. 2001). Although species of both bats (McNab 1969; Henshaw 1970; Lyman 1970) and marsupials are known to hibernate, the studies did not address this aspect or the effects of torpor on myosin isoforms in any way. This study is therefore an important first application of gel electrophoresis and immunoblotting to hibernating muscle physiology, including single-fiber techniques hitherto unused in studies of hibernators. These latter techniques are capable

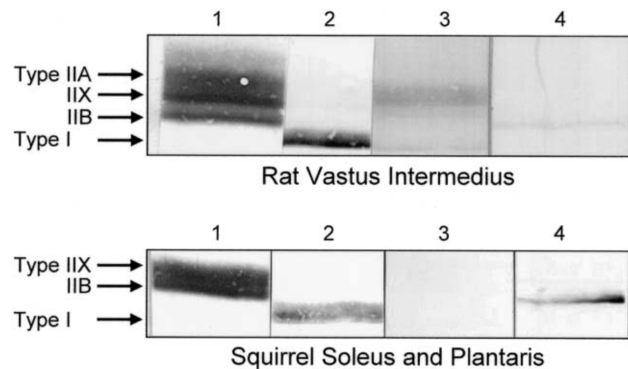

Figure 4. Antibody determination of myosin heavy-chain (MHC). Immunoblotting of MHC was performed to verify the MHC proteins initially detected by silver staining. Whole muscle lysate was electrophoresed in SDS-PAGE gels, then blotted to polyvinylidene fluoride, and probed with four antibodies against myosin isoforms. *Top panel*, Rat vastus intermedius whole muscle lysate containing I, IIA, IIX, and IIB protein; *bottom panel*, combined soleus and plantaris whole muscle lysate from active ground squirrels. The soleus and plantaris lysates were combined in this particular blot to give approximately equal amounts of the three MHC proteins, facilitating staining. Sigma antibody reacts with all fast types IIA, IIX, and IIB proteins (*lane 1*); BAD5 antibody (*lane 2*) reacts only with type I protein; SC71 (*lane 3*) reacts with type IIA; and BFF3 (*lane 4*) reacts with IIB alone. Squirrel samples predominantly demonstrate types I, IIX, and IIB protein based on the lack of staining for SC-71 antibody. Note that image contrast was adjusted individually for each strip.

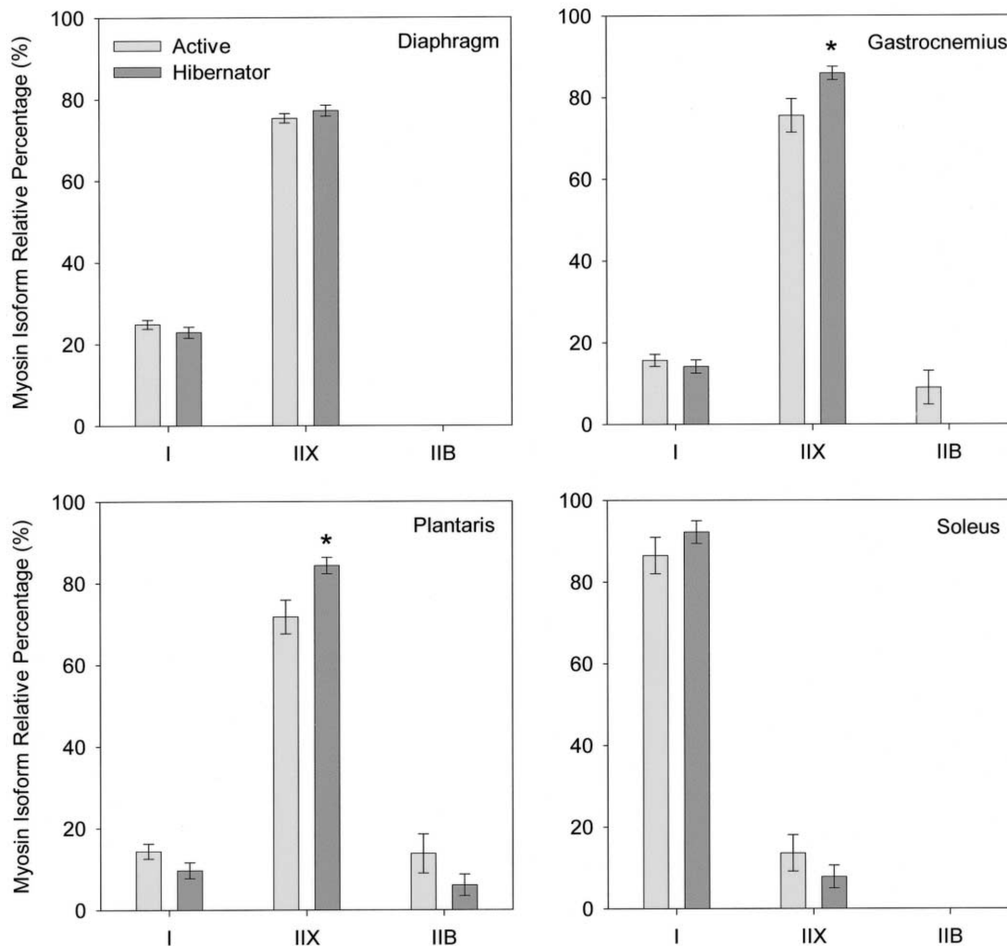

Figure 5. Whole muscle myosin heavy-chain (MHC) profiles. Relative myosin protein isoform percentages, as quantified by densitometry of silver-stained gels. Soleus muscle atrophies with hibernation but unusually displayed no shifts in MHC percentages ($n = 25$). Diaphragm muscles also showed no shifts in isoform percentages during hibernation ($n = 17$), contrary to earlier reports. Hibernating gastrocnemius ($P = 0.043$, $n = 26$) and plantaris ($P = 0.013$, $n = 21$) muscles showed an unusual fiber-type transformation by increasing IIX protein while both types I and IIB protein are unchanged. The gastrocnemius of hibernators lost all expression of type IIB MHC protein. Data are means ± SEM, and significance is denoted by an asterisk.

of clearly identifying types of muscle fibers and detecting subtle changes in MHC expression, which influence whole muscle performance. Second, we report MHC isoform transformations following torpor, which are unique in their characteristics and occurrence in atrophied and potentially unloaded skeletal muscle. Last, we address the molecular regulation of atrophy and protein degradation during torpor in ground squirrels.

Hindlimb Muscle Isoform Composition and Atrophy in Hibernators

Several studies have examined disuse atrophy in hibernating squirrels (Steffen et al. 1991; Wickler et al. 1991; Reid et al. 1995) and have noted the loss of protein in the hindlimb musculature. Lean mass has previously been shown not to vary significantly between hibernators and active squirrels (Wickler et al. 1991; Reid et al. 1995). Therefore, we did not determine carcass mass at death, but the animals were weight-matched at the outset, and we investigated changes to hibernating muscle protein composition on the assumption that muscle atrophy occurred during hibernation, particularly as noted in the soleus and gastrocnemius. As in previous studies, soleus and gastrocnemius masses were smaller in hibernating animals than in active animals (Fig. 1), and as in Reid et al. (1995), we measured no difference in diaphragm mass.

Protein concentration in the plantaris of hibernating squirrels was greater than active animals and was unchanged in the three other muscles (Fig. 1). An increase in protein concentration can represent a dehydrated muscle, which would reduce muscle mass without appreciably affecting contractile protein

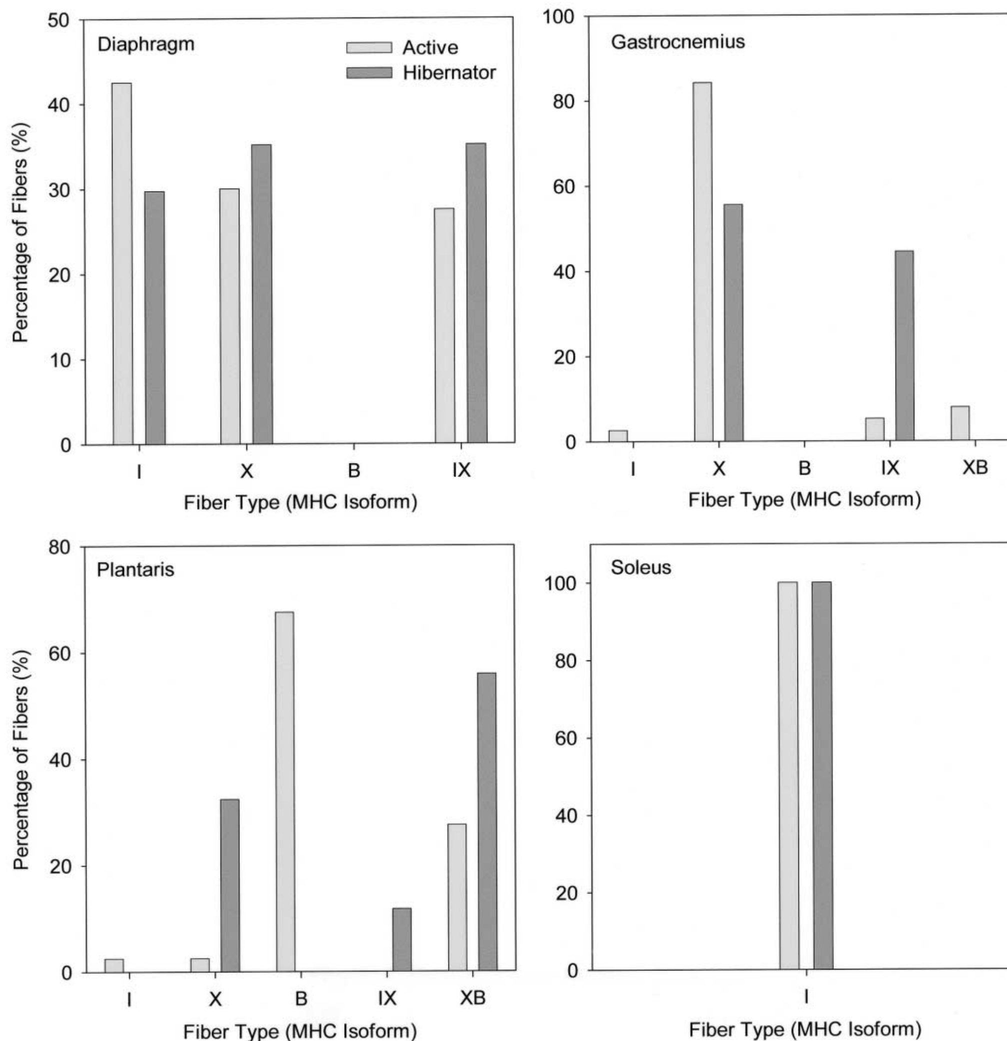

Figure 6. Examples of single-fiber myosin heavy-chain (MHC) profiles. Myosin protein isoform expression in single muscle fibers. Percentages represent sampling of 40 single fibers taken from one hibernating and one fall-active muscle each ($n = 8$ individual animals) and electrophoresed individually. All muscles except the soleus demonstrated the occurrence of hybrid fibers, coexpressing two MHC isoforms (e.g., IX or XB). No hybrid fibers were observed expressing more than two MHC isoforms.

content. However, while small increases in protein concentration (or loss of fluid) may account for a small proportion of muscle mass loss in hibernators, it cannot explain the >30% loss of gastrocnemius and soleus mass. We estimated the water content of gastrocnemius muscles from active and hibernating squirrels, using wet mass and mass following drying in a lyophilizer (data not shown) and found no differences between groups; this suggests that muscles were not dramatically dehydrated.

Other mammals, including the above-mentioned hibernators, and most rodents studied appear to express the same four basic MHC isoforms (type I, IIA, IIX, and IIB) in skeletal muscle (Rivero et al. 1996; Lucas et al. 2000). It is interesting that we observed only three MHC isoforms in the four squirrel muscles examined because the IIA protein was absent in most muscles. The silver-stained gels suggested that no type IIA protein was present, as three bands were observed, all of which aligned closely with other rat myosin I, IIX, and IIB isoforms. As the SC-71 antibody generally failed to detect IIA protein in immunoblots for whole muscle, we are confident in declaring that IIX and IIB are the predominant fast isoforms from these four squirrel muscle types. Many hybrid fibers were seen in hindlimb and diaphragm muscle, where two MHC isoforms are coexpressed in the same muscle cell. This phenomenon has been observed in a number of species (e.g., mouse, rat, rabbit, canines, and humans), including the existence of hybrid fibers expressing only types I and IIX MHC protein (Wu et al. 2000*a*, 2000*b*). Even if whole muscle MHC proportions are unchanged,

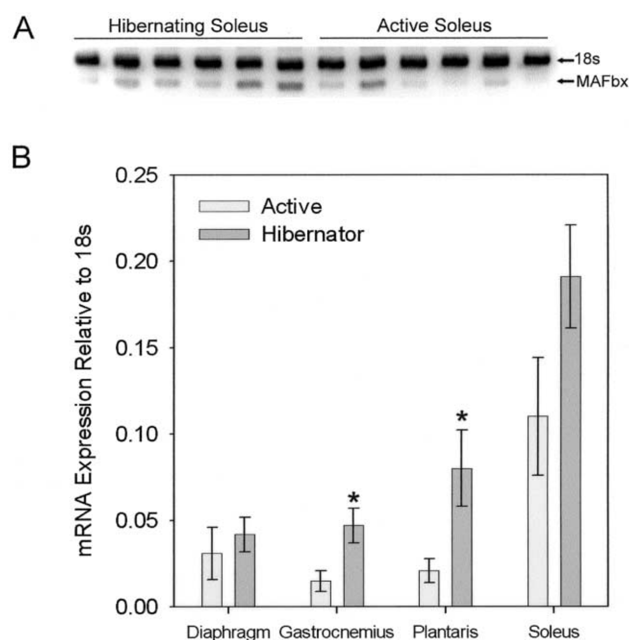

Figure 7. *A*, Muscle Atrophy F-box (MAFbx) mRNA levels were measured by RT-PCR in active and hibernating muscle. Ribosomal 18s was amplified in a multiplex reaction with MAFbx to control for variance in PCR efficiency, cDNA loading, and gel staining. Each lane represents the mRNA levels for 18s (*top*) and MAFbx (*bottom*) from an individual soleus muscle. Values for MAFbx expression were expressed relative to the 18s control. *B*, Hibernating gastrocnemius ($P = 0.008$, $n = 18$) and plantaris ($P = 0.021$, $n = 18$) muscles displayed significantly higher MAFbx levels than active muscles. Hibernating soleus ($P = 0.089$, $n = 22$) and diaphragm (NS, $n = 14$) muscle did not show an increase in MAFbx mRNA. Data are means ± SEM, and significance is denoted by an asterisk.

conversion of fibers to varying hybrid phenotypes has important consequences to contractile efficiency, which have not been explored further in these muscles.

In this study, we report highly unusual responses to atrophy in two hindlimb muscles of hibernating ground squirrels. In general, denervation or atrophy results in a slow-to-fast fiber conversion, increases the relative proportions of type IIX or IIB MHC protein isoforms, and produces a simultaneous decrease in oxidative characteristics (Thomason et al. 1987; Talmadge 2000; Baldwin and Haddad 2001). We do not have detailed electromyographic recordings, and the degree of neuromuscular activity and loading in each muscle during torpor and shivering is unclear. However, the atrophied soleus and gastrocnemius muscles that we examined were unusual in several respects. First, gastrocnemius muscles, while expressing minor (∼10%) amounts of IIB MHC in active animals, showed no IIB MHC in hibernating animals and had an increased proportion of IIX MHC protein during atrophy. This represents a surprising conversion from fast to slower MHC isoform with presumed mechanical unloading, contrary to rat and human responses. Second, atrophied soleus from hibernators did not show an increased proportion of type II protein at all but showed the same relative proportion of type I MHC as in active animals. This is in agreement with earlier studies noting the increase in oxidative capacity and capillarity of squirrel muscles during hibernation, possibly as a requirement for shivering thermogenesis during arousal (Steffen et al. 1991). This is, however, not universal in hibernators, as bats showed no differences in enzyme activity in flight muscle during hibernation (Fonda et al. 1983). Last, the plantaris, which typically is not as sensitive to mass loss or slow-to-fast MHC shifts with inactivity, also demonstrated increased IIX MHC proportions following hibernation. All three limb muscles examined showed no reductions in type I MHC, which may reflect a requirement for maintenance or enhancement of cross-bridge cycling efficiency during the 1–2 h of intense activity of rewarming.

Several reasons may exist for the observed squirrel MHC profiles and maintenance of oxidative fiber types in the face of disuse on the basis of the ecology of these animals and the above-mentioned requirement for heat production following torpor. Three hypotheses exist: first, the slow MHC phenotype may be preserved through an adaptive molecular mechanism in anticipation of use for thermogenesis and locomotion; second, the phenotype, which would erode similarly as in other mammalian skeletal muscle, may be halted from degradation because of the cessation of cellular reactions at low temperatures during torpor; third, the skeletal muscle activity during interbout arousals may be sufficient to maintain or to enhance oxidative muscle components. In certain northern populations of a *Spermophilus* congener, ground squirrels must forage widely for food (Kenagy and Hoyt 1989). Predation avoidance and foraging ability may be important factors related to running ability and endurance, which may be defended by the animals even during hibernation. It can thus be proposed that "oxidative" MHC isoforms are preserved to enable shivering thermogenesis during warming and to preserve locomotor function after full emergence from deep hibernation. However, while it is tempting to suppose that hibernating squirrels exhibit an adaptive, altered response to skeletal muscle inactivity, the combination of the other two potential explanations of low temperature effects and interbout activity may be important to the oxidative phenotype.

The months of hibernation in ground squirrels are alternating periods of 1–3 wk of torpor at near-freezing body temperatures of 2°–4°C, punctuated by interbout arousals and rewarming of the squirrels to euthermy for up to 24 h (Geiser and Kenagy 1988). The interbout arousals are accompanied by intense skeletal muscle shivering for a period of 1–2 h. It is possible that the very low body temperatures during torpor and accompanying Q_{10} reduction of metabolism to ∼1%–5% of basal values, simply halts degradation of MHC protein as a function of reduced reaction rates, even though the muscle is "inactive" or mechanically unloaded (Fig. 8). The return to

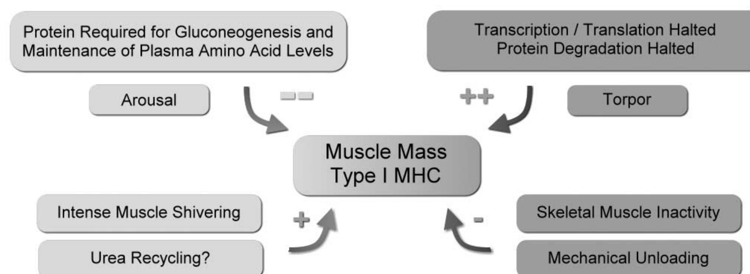

Figure 8. Skeletal muscle mass and myosin heavy-chain (MHC) isoform expression are determined by two distinct muscle states during ground squirrel hibernation. The 5–12-d bouts of torpor at 2°–4°C are periods of muscle inactivity and mechanical unloading, which may reduce muscle mass and type I MHC. However, Q_{10} effects during torpor also greatly reduce transcription, translation, and protein degradation and may largely prevent any mass or MHC isoform changes induced by inactivity. Periodic interbout arousals involve 1–2 h of intense muscle shivering, which can favor mass increases and preservation of type I MHC. Nevertheless, during the 24 h of arousal and euthermy, protein required for gluconeogenesis may be selectively removed from skeletal muscles, causing much of the observed atrophy. Minor use of urea recycling may offset this protein catabolism.

euthermy, when temperatures are high enough to allow a resumption of cellular reactions and any degradation or replacement of MHC isoforms would actually represent a period of relatively high skeletal muscle activity. This muscle use would then activate the molecular signaling pathways that preserve or enhance the oxidative MHC phenotype expression (Booth and Thomason 1991; Baldwin and Haddad 2001). However, hibernating squirrel muscle clearly loses protein and reduces fiber size (Steffen et al. 1991), so the effect is not completely analogous to exercise-induced enhancement of oxidative fiber complement. Temperature-mediated reductions in ground squirrel proteolysis may explain the minimal atrophy but cannot explain the observed shifts in MHC isoform to more oxidative isoforms. Ground squirrels also may recycle urea (Pengelley et al. 1971; Riedesel and Steffen 1980), which may offset the use of muscle protein for other metabolism. Hibernating ground squirrel skeletal muscle does not behave as rat or human muscle and presents tremendous opportunities for further study.

Diaphragm Muscle Isoform Composition in Hibernators

Reid et al. (1995) reported four fiber types by ATPase staining in the ground squirrel diaphragm, as well as shifts to IIB fibers and a hypertrophy of the diaphragm relative to carcass mass in hibernators, although total muscle mass did not increase. By immunoblotting and silver staining, we detected only two MHC isoforms, types I and IIX, in the diaphragm, and did not observe any significant isoform shifts. Some of the discrepancy in fiber typing may be related to the use of histochemical staining techniques by Reid et al. (1995), rather than the electrophoretic detection methods on whole muscle we employed. We detected many hybrid single muscle fibers expressing both types I and IIX MHC protein (see Figs. 3, 6), which would have an intermediate staining intensity, and would give the appearance of one or even two additional fiber types in the diaphragm. The subtle decrease in type I and increase in IIX protein that we did observe in the hibernating diaphragm was similar to the previously reported increase in IIB MHC isoforms, which was attributed to changes in pulmonary dynamics and lung compliance (Milsom and Reid 1995; Reid et al. 1995). We, however, conclude that no significant fiber-type transitions occurred, which is remarkable given the noted reduction in diaphragm activity.

Molecular Basis of Skeletal Muscle Protein Degradation and Atrophy in Hibernators

MHC protein expression and total contractile protein content of mammalian hindlimb muscle is controlled by a variety of signaling pathways, some of which target gene expression directly and some of which are important in phosphorylation and modification of existing proteins. We tested whether an increase in protein degradation during hibernation was mediated through the transcriptional upregulation of the MAFbx gene, an important ubiquitin ligase. Ubiquitin ligases ubiquitylate muscle proteins before entry into the proteasome complex, which is a critical effector of protein degradation (Solomon et al. 1998; Lecker et al. 1999; Bodine et al. 2001). Atrophy increases the rate of ubiquitin conjugation (Solomon et al. 1998), which marks myofibrillar proteins in particular for subsequent breakdown during atrophy or disease states (Lecker et al. 1999). In the gut and liver of *Spermophilus*, proteolysis is depressed during torpor and ubiquitylated proteins accumulate markedly, which then are degraded during the interbout arousals or periodic returns to euthermy (van Breukelen and Carey 2002). It is interesting that proteolysis is very sensitive to temperature, but ubiquitin conjugation persists even at very low temperatures and is perhaps even enhanced from shock-induced damage to proteins (van Breukelen and Carey 2002). Thus, even though many ubiquitylated proteins accumulate

during torpor, temperature-mediated reduction in proteolysis limits overall degradation of protein (van Breukelen and Carey 2002).

Translation is depressed during torpor in liver (Knight et al. 2000) and kidney, although brown adipose tissue does not show this (Hittel and Storey 2002); muscle was not examined. Transcription is similarly reduced, mostly because of a cessation of initiation at low body temperatures (van Breukelen and Martin 2002*b*); interbout arousals therefore may be critical periods when gene expression and a resumption of transcription are permitted. In hibernating bat pectoralis muscle, torpor also halted protein synthesis and degradation; yet, a loss of protein occurred on arousal, with degradation from gluconeogenesis (Yacoe 1983).

We found that atrophied gastrocnemius in hibernating squirrels expressed twofold higher MAFbx mRNA levels, relative to active muscles. The atrophied soleus, however, had unchanged MAFbx expression, while the plantaris showed a fourfold increase. The diaphragm, which did not atrophy, had unchanged MAFbx levels during hibernation. These data do not completely support the role of MAFbx in contributing to muscle atrophy but suggest that protein degradation is a key component of mass loss in hibernating squirrel muscle. However, if increased protein degradation is occurring as a result of MAFbx activation in the plantaris, it may be offset by greater protein synthesis through other pathways. Alternatively, MAFbx activation may not be a good predictor of degradation because the soleus atrophies without significant upregulation of expression. In light of the above discussion on the limits to transcription and translation during torpor, we postulate that the elevated MAFbx mRNA levels develop not during torpor but during the interbout arousals. This presents a potential paradox in the muscle physiology in that MAFbx pathways would appear to be upregulating myofibrillar atrophy through increased ubiqitylation, while putative upregulation or maintenance of oxidative enzyme activity and a small shift in myosin isoform protein is simultaneously occurring. An explanation may lie in the complex nature of rodent hibernation and conflicting metabolic demands on skeletal muscle during the two distinct muscle states of torpor and arousal (Fig. 8). Several factors during both torpor and arousal may exert independent influences on muscle mass and MHC isoform expression. A requirement for protein during hypophagia despite large stores of fat may cause slight skeletal muscle atrophy for gluconeogenesis and to maintain plasma amino acid levels (Riedesel and Steffen 1980), while activity during arousals influences the MHC isoform profile and oxidative enzyme properties. Inactivity during torpor itself may favor a reduction in muscle mass and type I MHC, but the simultaneous reduction in transcription and translation processes at low temperature may retard those effects. Thus, the hibernating condition in these rodents may not even represent a situation of "disuse" because no protein catabolism occurs at the low temperatures of torpor, and the euthermic condition is contemporaneous with intense shivering.

Conclusion

Our study demonstrates that ground squirrels can be an interesting organism for the study of the molecular basis of muscle atrophy. Important questions remain concerning whether the evident muscle plasticity is a passive result of the conditions of hibernation or is modulated for certain functional requirements. While these organisms do not respond in a fashion similar to classical human or rat models and should not be used as direct analogs, they provide an important alternative view of muscle function and regulatory pathways based on a different set of evolutionary constraints and history.

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Literature Cited

Baldwin K. and F. Haddad. 2001. Effects of different activity and inactivity paradigms on myosin heavy chain gene expression in striated muscle. J Appl Physiol 90:345–357.

Bodine S., E. Latres, S. Baumhueter, V. Lai, L. Nunez, B. Clark, W. Poueymirou, et al. 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294:1704–1708.

Booth F.W. and D.B. Thomason. 1991. Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. Physiol Rev 71:541–585.

Caiozzo V.J. 2002. Plasticity of skeletal muscle phenotype: mechanical consequences. Muscle Nerve 26:740–768.

Caiozzo V.J., M. Baker, and K.M. Baldwin. 1998. Novel transitions in MHC isoforms: separate and combined effects of thyroid hormone and mechanical unloading. J Appl Physiol 85:2237–2248.

Davis W.H. 1970. Hibernation: ecology and physiological ecology. Pp. 265–299 in W. Wimsatt, ed. Biology of Bats. Vol. 1. Academic Press, London.

Fonda M.L., G.H. Herbener, and R.W. Cuddihee. 1983. Biochemical and morphometric studies of heart, liver and skeletal muscle from the hibernating, arousing, and aroused big brown bat, *Eptesicus fuscus*. Comp Biochem Physiol B 76: 355–363.

Geiser F. 1998. Evolution of daily torpor and hibernation in birds and mammals: importance of body size. Clin Exp Pharmacol Physiol 25:736–740.

Geiser F. and G.J. Kenagy. 1988. Torpor duration in relation to temperature and metabolism in hibernating ground squirrels. Physiol Zool 61:442–449.

Henshaw R. 1970. Thermoregulation in bats. Pp. 188–232 in B. Slaughter and D. Walton, eds. About Bats. Southern Methodist University Press, Dallas.

Hermanson J.W., J.M. Ryan, M.A. Cobb, J. Bentley, and W.A. Schutt, Jr. 1998. Histochemical and electrophoretic analysis of the primary flight muscle of several phyllostomid bats. Can J Zool 76:1983–1992.

Hittel D.S. and K.B. Storey. 2002. The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*). Arch Biochem Biophysiol 401:244–254.

Kenagy G.J. and D.F. Hoyt. 1989. Speed and time-energy budget for locomotion in golden-mantled ground squirrels. Ecology 70:1834–1839.

Knight J., E. Narus, S.L. Martin, A. Jacobson, B.M. Barnes, and B.B. Boyer. 2000. mRNA stability and polysome loss in hibernating arctic ground squirrels (*Spermophilus parryii*). Mol Cell Biol 20:6374–6379.

Koebel D.A., P.G. Miers, R.A. Nelson, and J.M. Steffen. 1991. Biochemical changes in skeletal muscles of denning bears (*Ursus americanus*). Comp Biochem Physiol B 100:377–380.

Lecker S.H., V. Solomon, W.E. Mitch, and A.L. Goldberg. 1999. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J Nutr 129:227S–237S.

Lucas C.A., L.H.D. Kang, and J.F.Y. Hoh. 2000. Monospecific antibodies against the three mammalian fast limb myosin heavy chains. Biochem Biophys Res Commun 272:303–308.

Lyman C. 1970. Thermoregulation and metabolism in bats. Pp. 301–330 in W. Wimsatt, ed. Biology of Bats. Academic Press, New York.

Lyman C., J.S. Willis, A. Malan, and L.C.H. Wang. 1982. Who is who among the hibernators. Pp. 12–36 in C. Lyman, J.S. Willis, A. Malan, and L.C.H. Wang, eds. Hibernation and Torpor in Mammals and Birds. Academic Press, London.

McNab B.K. 1969. The economics of temperature regulation in neotropical bats. Comp Biochem Physiol 31:227–268.

Milsom W.K. and W.D. Reid. 1995. Pulmonary mechanics of hibernating squirrels (*Spermophilus lateralis*). Respir Physiol 101:311–320.

Nedergaard J. and B. Cannon. 1990. Mammalian hibernation. Philos Trans R Soc Lond B 326:669–686.

Pengelley E.T., S.J. Asmundson, and C. Uhlman. 1971. Homeostasis during hibernation in the golden-mantled ground squirrel, *Citellus lateralis*. Comp Biochem Physiol A 38:645–653.

Reid W.D., A. Ng, and W.K. Milsom. 1995. Characteristics of diaphragm muscle fiber types in hibernating squirrels. Respir Physiol 101:301–309.

Riedesel M.L. and J.M. Steffen. 1980. Protein metabolism and urea recycling in rodent hibernators. Fed Proc 39:2959–2963.

Rivero J., R. Talmadge, and V.R. Edgerton. 1996. Myosin heavy chain isoforms in adult equine skeletal muscle: an immunohistochemical and electrophoretic study. Anat Rec 246: 185–194.

Schiaffino S., L. Gorza, S. Sartore, L.D. Saggin, S. Ausoni, M. Vianello, K. Gundersen, and T. Lomo. 1989. Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 10:197–205.

Sciote J.J. and A. Rowlerson. 1998. Skeletal fiber types and spindle distribution in limb and jaw muscles of the adult and neonatal opossum, *Monodelphis domestica*. Anat Rec 251: 548–562.

Solomon V., V. Baracos, P. Sarraf, and A.L. Goldberg. 1998. Rates of ubiquitin conjugation increase when muscles atrophy, largely through activation of the N-end rule pathway. Proc Natl Acad Sci USA 95:12602–12607.

Steffen J.M., D.A. Koebel, X.J. Musacchia, and W.K. Milsom. 1991. Morphometric and metabolic indicies of disuse in muscles of hibernating ground squirrels. Comp Biochem Physiol B 99:815–819.

Storey K.B. 1997. Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. Comp Biochem Physiol B 99:815–819.

Talmadge R. 2000. Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. Muscle Nerve 23:661–679.

Talmadge R. and R. Roy. 1993. Electrophoretic separation of rat skeletal muscle myosin heavy chain isoforms. J Appl Physiol 75:2337–2340.

Thomason D.B., R.E. Herrick, D. Surdyka, and K.M. Baldwin. 1987. Time course of soleus muscle myosin expression during hindlimb suspension and recovery. J Appl Physiol 63: 130–137.

Tinker D.B., H.J. Harlow, and T.D.I. Beck. 1998. Protein use and muscle-fiber changes in free-ranging, hibernating black bears. Physiol Zool 71:414–424.

van Breukelen F. and H.V. Carey. 2002. Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels. J Comp Physiol B 172:269–273.

van Breukelen F. and S. Martin. 2002*a*. Molecular biology of thermoregulation invited review: molecular adaptations in mammalian hibernators—unique adaptations or generalized responses? J Appl Physiol 92:2640–2647.

———. 2002*b*. Reversible depression of transcription during hibernation. J Comp Physiol B 172:355–361.

Vyskocil F. and E. Gutmann. 1977. Contractile and histochemical properties of skeletal muscles in hibernating and awake golden hamsters. J Comp Physiol 122:385–390.

Wickler S.J., B.A. Horwitz, and K.S. Kott. 1987. Muscle function in hibernating hamsters: a natural analog to bed rest? J Therm Biol 12:163–166.

Wickler S.J., D.F. Hoyt, and K.S. Kott. 1991. Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis.* Am J Physiol 261:R1214–R1217.

Wu Y.Z., R.L. Crumley, W.B. Armstrong, and V.J. Caiozzo. 2000*a*. New perspectives about human laryngeal muscle: single-fiber analyses and interspecies comparisons. Arch Otolaryngol Head Neck Surg 126:857–864.

Wu Y.Z., R.L. Crumley, and V.J. Caiozzo. 2000*b*. Are hybrid fibers a common motif of canine laryngeal muscles? single-fiber analyses of myosin heavy-chain isoform composition. Arch Otolaryngol Head Neck Surg 126:865–873.

Yacoe M.E. 1983. Protein metabolism in the pectoralis muscle and liver of hibernating bats, *Eptesicus fuscus.* J Comp Physiol 152:137–144.

Zhong W.W.H., C.A. Lucas, L.H.D. Kang, and J.F.Y. Hoh. 2001. Electrophoretic and immunochemical evidence showing that marsupial limb muscles express the same fast and slow myosin heavy chains as eutherians. Electrophoresis 22:1016–1020.