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Hibernating squirrel muscle activates the endurance exercise pathway despite prolonged immobilization

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Abstract

Skeletal muscle atrophy is a very common clinical challenge in many disuse conditions. Maintenance of muscle mass is crucial to combat debilitating functional consequences evoked from these clinical conditions. In contrast, hibernation represents a physiological state in which there is natural protection against disuse atrophy despite prolonged periods of immobilization and lack of nutrient intake.

Even though peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1-α (PGC-1α) is a central mediator in muscle remodeling pathways, its role in the preservation of skeletal muscle mass during hibernation remains unclear. Since PGC-1α regulates muscle fiber type formation and mitochondrial biogenesis, we analyzed muscles of 13-lined ground squirrels. We find that animals in torpor exhibit a shift to slow-twitch Type I muscle fibers. This switch is accompanied

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Author Contributions

Conceived and designed the experiments: RDC EAM RX RM. Performed the experiments: RX EAM RM. Analyzed the data: RX EAM RM. Contributed reagents/materials/analysis tools: LAL DKM RHAF. Wrote the manuscript: RX EAM RDC EMM RM.

by activation of the PGC-1α-mediated endurance exercise pathway. In addition, we observe increased antioxidant capacity without evidence of oxidative stress, a marked decline in apoptotic susceptibility, and enhanced mitochondrial abundance and metabolism.

These results show that activation of the endurance exercise pathway can be achieved *in vivo* despite prolonged periods of immobilization, and therefore might be an important mechanism for skeletal muscle preservation during hibernation. This PGC-1α regulated pathway may be a potential therapeutic target promoting skeletal muscle homeostasis and oxidative balance to prevent muscle loss in a variety of inherited and acquired neuromuscular disease conditions.

Keywords

endurance exercise; peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1-α (PGC-1α); muscle fiber type; muscle atrophy; oxidative balance; hibernation

Introduction

Significant loss of muscle mass can occur as a result of several disuse conditions such as limb immobilization, bed rest, denervation, or microgravity. Aging, cachexia and many disease states can also lead to atrophy of the muscle fibers (Degens and Alway, 2006, di Prampero and Narici, 2003, Hornberger, et al., 2001, Jackman and Kandarian, 2004). In contrast, hibernation is a physiological state in which certain animal species overcome the challenges that arise from both prolonged immobilization and absence of feeding (Carey, et al., 2003). Despite facing several extreme conditions including long-term-immobilization, hypometabolism, hypoxia, and lack of food intake, these hibernators are capable of a remarkable preservation of skeletal muscle mass (Andres-Mateos, et al., 2012, Lee, et al., 2008).

Muscle remodeling in the absence of injury occurs as a response to environmental demands such as low caloric input or exercise (Bassel-Duby and Olson, 2006, Fluck and Hoppeler, 2003). Endurance exercise adaptations are mediated by the peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1-α (PGC-1α), which is a key regulator of mitochondrial biogenesis and fuel homeostasis in skeletal muscle (Calvo, et al., 2008, Lin, et al., 2005). PGC-1α has been shown to regulate the formation of Type I, oxidative slow-twitch fibers which are a hallmark of high endurance exercise (Lin, et al., 2002).

The PGC-1α-mediated signaling cascade includes AMP-activated Protein Kinase (AMPK) that acts as a sensor of the energy status of the cell and activates PGC-1α by phosphorylation (Greer, et al., 2007, Jager, et al., 2007, Jorgensen, et al., 2005). Members of the MAPK family such as p38 MAPK also increase PGC-1α activity in response to physical exercise (Akimoto, et al., 2005, Yu, et al., 2003). The Nuclear Respiratory Factors 1 and 2 (Nrf-1 and Nrf-2), key nuclear encoded proteins involved in mitochondrial respiration and function, are downstream targets of PGC-1α (Scarpulla, 2002, Wu, et al., 1999). By activating Nrf-1 and MEF-2A, PGC-1α coordinates the increase of GLUT4, which is associated with enhanced insulin-stimulated glucose transport (Baar, et al., 2003, Wende, et al., 2007).

PGC-1α also plays a vital role in increasing mitochondrial volume and function, and stimulating the antioxidant defense machinery by regulating numerous antioxidant proteins including the reactive oxygen species (ROS)-detoxifying enzymes Manganese superoxide dismutase (MnSOD), catalase, and uncoupling proteins (Lin, et al., 2005, St-Pierre, et al., 2006, St-Pierre, et al., 2003). PGC-1α can tune mitochondrial apoptotic susceptibility by modulating pro- and anti-apoptotic proteins (Adhihetty, et al., 2009). Overall, these PGC1-α

mediated muscle adaptations are not only protective against muscle wasting, but also against metabolic imbalance (Koves, et al., 2005, Minnaard, et al., 2005, Wang, et al., 2003).

Despite PGC-1α being critical for understanding the mechanisms of adaptive plasticity in skeletal muscle, its role in mediating the protection of skeletal muscles during hibernation is not known. In this study, we performed analyses of muscles before and during hibernation of the 13-lined ground squirrel, a natural hibernator that is able to survive prolonged periods of immobilization without significant loss of muscle mass.

Material and Methods

13-lined Ground Squirrels (*Ictidomys tridecemlineatus*)

All experimental procedures with 13-lined ground squirrels conformed to federal welfare guidelines and were pre-approved by the Institutional Animal Care and Use Committee (IACUC) of Johns Hopkins University School of Medicine. Hibernation-naïve euthermic 13-lined ground squirrels of both sexes were obtained from the captive breeding colony at the University of Wisconsin Oshkosh. Squirrels were supplied with food and water *ad libitum* during the summer period and after emerging from hibernation. When the squirrels evidenced periods of torpor, they were moved into 4°C and dark hibernaculum, and food and water was removed after several weeks without consumption (Vaughan, et al., 2006). During hibernation, the animals nest in shredded paper material, assume a fetal position, and lower their body temperature to ambient levels, often near freezing (Vaughan, et al., 2006). In addition, the hibernation period is characterized by a decline in heart rate from 300 b.p.m. to 5–10 b.p.m., and a concomitant decrease in ventilation rate and activity. The animals go through periodic interbout arousals every 3 weeks for a few hours, where shivering thermogenesis returns body temperature to normal, however, the animals do not show signs of food or water intake. (Van Breukelen and Martin, 2002).

As obligate hibernators, 13-lined ground squirrels enter hibernation in November/December and emerge in April/May. For the experimental hibernating group (n=10), squirrel muscle was collected 4–5 months after first immergence into torpor, while they were in full torpid, hypothermic state. When the squirrels emerged from hibernation, they were returned into a warm room and food and water was reinstated. 2–3 months hereafter, the squirrels were sacrificed and these comprised the control, non-hibernating group (n=6). A total of 16 squirrels went through hibernation, all of them survived and were healthy when sacrificed or emerged from hibernation. Animals were killed by decapitation after isoflurane anesthesia and the quadriceps muscle was quickly dissected from both hindlimbs and flash frozen.

Histology and Immunofluorescence

Skeletal quadriceps muscle was mounted in Tissue-Tek O.C.T Compound (Sakura Finetek) and flash frozen in cool isopentane. 10 μm sections of the tissue were cut with a cryostat. Sections were stained with hematoxylin and eosin following standard protocols. For immunofluorescence staining, sections were blocked with 3% goat serum/5% bovine serum albumin at room temperature and incubated with the following primary antibodies overnight at 4°C: BA-D5 myosin heavy chain I, BF-F3 myosin heavy chain IIB, Sc71 myosin heavy chain IIA (Developmental Studies Hybridoma Bank), followed by Alexa Fluor conjugated antibodies 350, 488 and 594 (Invitrogen) for 1 hour at room temperature. Sections were mounted with Fluoromount-G (SouthernBiotech). All images were acquired with an Eclipse i80 microscope (Nikon).

For mitochondrial staining, quadriceps sections were incubated with MitoTracker Green FM (Molecular Probes) 100–200 nM at 37°C for 15 min, washed with PBS and mounted with DAPI Hard media (Vector Laboratories). The LSM510 confocal laser-scanning microscope

(Zeiss) was used for confocal microscopy with a 63X lens objective. Focal series of 0.9 μm horizontal planes (Z-scan) spaced at 1 μm were registered.

Morphometry

The distribution percentage of Type I, Type IIA and Type IIB fibers was calculated by using Nikon NS elements BR 3.0 software (Laboratory Imaging, Nikon). A minimum of 1,500 muscle fibers per animal was analyzed.

Western Blot and Density Analyses

Quadriceps samples were homogenized in ice-cold lysis buffer (NP-40 1%, Glycerol 10%, NaCl 137 mM, TrisHCl 20 mM at pH=7.5) with the addition of protease (Complete Mini, Roche) and phosphatase (PhosSTOP, Roche) cocktail inhibitors and centrifuged at 14,000 rpm for 15 min at 4 °C. Protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Scientific). 20 μg of protein were electrophoresed using a Bis-Tris or Tris-Glycine Gel (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were incubated overnight at 4°C with the following primary antibodies diluted in blocking solution (5% milk/PBST): Catalase, Mfn-2, MnSOD, Nrf-1, UCP-2, UCP-3, VDAC-1/Porin (Abcam); Bcl-2 (BD Transduction Laboratories); Phospho-AMPKα (Thr^{172}), AMPKα, Cytochrome C, Phospho-p38 (Thr^{180}/Tyr^{182}), p38, SIRT3 (Cell Signaling); Fis1 (Enzo Life Sciences); tFAM (GenWay Biotech); ATP Synthase (Invitrogen); SIRT1 (Millipore); PGC-1α (Millipore and Novus Biologicals); Nrf-2 (R&D Systems); GAPDH, GLUT4, Mfn-1 (Santa Cruz). Horseradish Peroxidase–linked secondary antibodies (Amersham) were used to detect and SuperSignal West Dura or Femto Stable Peroxide Buffer (Thermo Scientific) to visualize bands. Quantification of all immunoblots was performed using ImageJ (National Institutes of Health). Fold changes were calculated against GAPDH for whole cell lysates and against VDAC-1 for mitochondrial fraction lysates.

Mitochondrial Fractionation

Mitochondrial proteins from skeletal muscle were isolated using a standard protocol (Frezza, et al., 2007). Briefly, quadriceps muscle (50–100 mg) was minced with scissors in 5 ml ice-cold dissection buffer (10 mM EDTA in PBS). Tissue was homogenized and resuspended in 5 ml digestion buffer (10 mM EDTA, 0.05% trypsin in PBS) for 30 min at 37°C, then centrifuged at 200 *g* for 5 min. The pellet was resuspended in ice-cold IB_m1 (67mM sucrose, 50mM Tris/Hcl, 50mM KCl, 10mM EDTA, 0.5% BSA at pH 7.4) and then homogenized using a Teflon pestle in precooled glassware. The resulting homogenate was centrifuged at 1600 *g* for 10 min at 4 °C. The supernatant was again centrifuged at 8000 *g* for 10 min at 4°C. The pellet was resuspended in 5 ml ice-cold IB_m2 (250 mM sucrose, 3mM EGTA/Tris, 10mM Tris/HCl at pH 7.4) and centrifuged at 8000 *g* for 10 min at 4°C. Finally, the resulting pellet of mitochondria was resuspended in the IB_m2 buffer.

Oxyblot

Oxyblots were performed using the OxyBlot Protein Oxidation Kit (Millipore), according to the manufacturer's recommendations. 50 mM DTT was added to the mitochondrial protein samples. Carbonyl groups were derivatized with 1X 2,4-dinitrophenylhydrazine DNPH solution. Immunoblot analysis was performed with an antibody against DNP and VDAC-1/Porin (Abcam). Quantification of oxidized proteins was then performed using ImageJ (National Institutes of Health).

Real-Time PCR

Total RNA isolation from ground squirrel quadriceps muscle was performed with TRIzol (Invitrogen) and treated with a Turbo DNA free Kit (Ambion). Purified RNA was then used to synthesize cDNA by reverse transcription using the TaqMan RT reaction (Applied Biosystems). PCR amplification was performed with an ABI PRISM 7900HT Sequence System (Applied Biosystems) using SYBR Green PCR (Applied Biosystems). Transcript expression was normalized by GAPDH. The primers used were: GAPDH forward: 5′caccatcttccaggagcgag3′, reverse: 5′ccttctccatggtggtgaagac3′; PGC-1α forward: 5′ccaaatgaccccaagggttc3′, reverse: 5′tatgaggaggagtggtgggtg3′.

Statistical analysis

All values are expressed as mean ± SEM. Significance between two groups was determined by the unpaired Student's t-test with a p-value ≤0.05 considered to be statistically significant.

Results

Muscle Fiber Switch to Slow Type I Fibers and Activation of the PGC-1α-mediated Endurance Pathway during Hibernation

Despite long periods of immobilization and caloric restriction, muscle morphology remains unchanged during hibernation (Andres-Mateos, et al., 2012). To further expand these observations, we performed fiber type staining for Type I, IIa and IIb muscle fibers in quadriceps muscles from both hibernating and non-hibernating squirrels. Non-hibernating muscle was composed of 69.2 ± 10.7% (mean ± SD) fast-twitch Type IIb fibers and 6.6 ± 3.1% slow-twitch Type I fibers. During the hibernating period however, there was a significant decrease of Type IIb fibers to 45.2 ± 2.9% and an increase of Type I fibers to 26.1 ± 4.3% (Fig. 1A and 1B), leading to a more oxidative phenotype.

Because PGC-1α has been shown to be a key regulator of mitochondrial biogenesis and muscle fiber type switching (Calvo, et al., 2008, Lin, et al., 2005, Lin, et al., 2002), we examined PGC-1α mRNA and protein in the quadriceps muscle and found significantly increased expression of PGC-1α and its protein level during hibernation (Fig. 2A and B). PGC-1α can be activated through phosphorylation by both AMP-activated Protein Kinase (AMPK) and members of the MAPK family such as p38 (Akimoto, et al., 2005, Jager, et al., 2007, Yu, et al., 2003). We found that hibernating squirrels showed a significant increase in the phosphorylated, active form of AMPK (Fig. 2B). Protein levels of total p38 MAPK were increased as well (Fig. 2B). Downstream targets of PGC-1α, including the Nuclear Respiratory Factors 1 and 2 (Nrf-1 and Nrf-2), also showed significant increases during hibernation (Fig. 2B) (Scarpulla, 2002, Wu, et al., 1999). By activating Nrf-1, PGC-1α also coordinates the increase of the GLUT4 isoform of the glucose transporter, which allows rapid glucose uptake into the muscle cell (Baar, et al., 2003, Ramachandran, et al., 2008, Wende, et al., 2007). We found that relative abundance of GLUT4 protein was enhanced during hibernation (Fig. 2B).

These results suggest that hibernating squirrels are able to activate the PGC-1α-mediated signaling cascade despite prolonged immobilization and absence of feeding.

Increased Mitochondrial Antioxidant Stress Response during Periods of Hibernation

PGC-1α activation is associated with antioxidant stress response and anti-apoptotic signaling. Mitochondrial proteins including the antioxidant enzymes Manganese superoxide dismutase (MnSOD) and catalase as well as uncoupling proteins UCP-2 and UCP-3 are regulated by PGC-1α upon oxidative stress (Puigserver, et al., 1998, St-Pierre, et al., 2006,

St-Pierre, et al., 2003). In order to examine mitochondrial changes, we isolated mitochondrial fractions from quadriceps muscle of hibernating and non-hibernating squirrels. We found that protein levels of PGC-1α, MnSOD, catalase, UCP-2 and UCP-3 were significantly increased in the mitochondrial fractions during hibernation (Fig. 3A). We also quantified oxidized proteins in the quadriceps mitochondrial fractions, yet found no signs of oxidative stress in hibernating squirrels (Fig. 3B). These results suggest that the 13-lined ground squirrels have evolved an efficient way to overcome the oxidative challenge of hibernation by overexpressing the mitochondrial antioxidant machinery.

Previous evidence suggests that PGC-1α can decrease mitochondrial apoptotic susceptibility as part of the cellular response to endurance exercise (Adhihetty, et al., 2007, Adhihetty, et al., 2009). We examined mitochondrial protein levels of apoptotic regulators including cytochrome C and Bcl-2, which has also been implicated as an antioxidant regulator (Hockenbery, et al., 1993, Kowaltowski, et al., 2004). We found significantly increased mitochondrial protein levels of cytochrome C and Bcl-2 (Fig. 3A) and, concurrently, a trend in decreased protein level of the pro-apoptotic regulator Apaf-1 (Fig. 3C). These findings are consistent with a marked decline in the apoptotic susceptibility of skeletal muscle during hibernation.

Enhanced Mitochondrial Abundance and Metabolism during Hibernation

Since we found increased mitochondrial oxidative capacity in skeletal muscle during hibernation, we used MitoTracker staining to explore mitochondrial abundance. The staining showed an increase of both cytoplasmic and subsarcolemmal mitochondria in the quadriceps muscle of hibernating animals (Fig. 4A). This increase was accompanied by higher protein levels of ATP Synthase and the mitochondrial transcription factor A (tFAM) (Fig. 4B and C), suggesting increased oxidative metabolism in the muscle of hibernating animals (Scarpulla, 2002).

Increased mitochondrial volume and function are associated with mitochondrial network dynamics and continuous remodeling – it is thought to require an elaborate equilibrium of the fusion and fission machinery (Chen and Chan, 2005, Rube and van der Bliek, 2004). These fusion and fission dynamics are important for the maintenance and integrity of functional mitochondria. Previous data have also demonstrated increased both fusion and fission protein levels in skeletal muscle after exercise (Cartoni, et al., 2005, Ding, et al., 2010). We tested the mitochondrial protein levels of Fis-1, Mfn-1 and Mfn-2, which are key regulators involved in mitochondrial fission and fusion. We found significantly increased relative abundance of these proteins in the mitochondrial fraction of quadriceps muscle during hibernation (Fig. 4B).

Increased Sirtuin Protein Levels during Hibernation—NAD^+-dependent protein deacetylases sirtuins are functional regulators of PGC-1α-associated mitochondrial activity (Gerhart-Hines, et al., 2007, Kong, et al., 2010) As metabolic sensors they are thought to link mitochondrial biogenesis with caloric restriction and cold exposure (D'Antona, et al., 2010, Haigis and Sinclair, 2010). Sirtuins have also been implicated in the exercise response: SIRT1 activates PGC-1α via deacetylation and is highly expressed after endurance exercise (Canto, et al., 2010, Gerhart-Hines, et al., 2007, Gurd, et al., 2010, Suwa, et al., 2008). Remarkably, we observed a 18-fold increase of SIRT1 protein levels in whole muscle lysates during hibernation (Fig. 5). SIRT3, which is localized to the mitochondria, has been shown to mediate PGC-1α effects on cellular ROS production and mitochondrial biogenesis (Kelly, 2010, Kong, et al., 2010). We also found a significant increase in SIRT3 in the mitochondrial fraction of quadriceps from hibernating squirrels when compared to the

non-hibernating animals (Fig. 5). Our data support increased mitochondrial protein abundance with enhanced metabolic and antioxidant capacity during hibernation.

Discussion

Extended periods of immobilization comprise a common and challenging clinical issue, ultimately having a deleterious impact on muscle function (Degens and Alway, 2006, di Prampero and Narici, 2003, Hornberger, et al., 2001, Jackman and Kandarian, 2004). Muscle inactivity not only results in atrophy and a decrease in muscle weight and strength, but also in mitochondrial dysbalance (Hortobagyi, et al., 2000, Nicks, et al., 1989). It is associated with mitochondrial loss, morphological changes, and impaired mitochondrial function (Powers, et al., 2012). However, mammalian hibernators are capable of preserving their muscle mass despite no caloric intake and long periods of immobilization (Andres-Mateos, et al., 2012). We have demonstrated that during hibernation the 13-lined ground squirrel activates PGC-1α signaling in ways that are very similar to endurance exercise. Previous studies in rats have shown that hindlimb immobilization or muscle unloading is associated with a slow-to-fast fiber type transformation, resulting in a muscle profile more susceptible to fatigue (Fitts, et al., 2001, Stevenson, et al., 2003, Thorlund, et al., 2011). In contrast, we demonstrate that hibernating skeletal muscle exhibits a fiber type switch towards slow Type I muscle fibers and a concomitant decrease in fast Type IIB fibers. The hibernating squirrels show not only a marked resilience to the morphological consequences of disuse atrophy but also a switch in muscle fiber type composition favoring a more oxidative, fatigue-resistant phenotypic profile.

PGC-1α has a pivotal role in regulating fiber type conversion and exercise training-induced skeletal muscle adaptations (Handschin, et al., 2007, Lin, et al., 2002). In the hibernating squirrel we observe the activation of the PGC-1α-mediated signaling cascade (Fig. 6): Not only do we detect increased levels of PGC-1α mRNA and protein itself, but we also show increased protein levels of its upstream activators p38 and pAMPK and its downstream targets Nrf-1, Nrf-2 and GLUT4 (Baar, et al., 2002, Daugaard, et al., 2000, Eddy, et al., 2005). Our findings reveal that the PGC-1α-mediated endurance exercise pathway can be activated *in vivo* despite the hibernators' extreme state of inactivity. PGC-1α also impacts mitochondrial homeostasis through enhanced antioxidant status, reduced ROS generation and altered pro- and antiapoptotic protein abundance. Specifically, recent studies showed a rise in oxidative stress enzymes during and after endurance exercise (Jiang, et al., 2009, Khassaf, et al., 2001). In addition, Bcl-2 protein levels are increased in exercised rodents and cytochrome C upregulation has been reported in both endurance exercise and high-intensity interval training (Leick, et al., 2010, Wright, et al., 2007). Both of these proteins are proposed to have a protective effect on the muscle against apoptosis. Our observations of muscle mitochondria in hibernating squirrels correspond with previously observed responses to exercise (Jiang, et al., 2009, Khassaf, et al., 2001, Leick, et al., 2010, Wright, et al., 2007). This is reflected by increased protein levels of the antioxidant proteins MnSOD, catalase and uncoupling proteins 2 and 3 as well as elevation of the apoptotic regulator proteins Bcl-2 and cytochrome C. Previous studies on hibernating ground squirrels have also reported enhanced antioxidant gene expression and protein abundance during hibernation (Allan and Storey, 2012, Morin, et al., 2008, Morin and Storey, 2007).

Since mitochondrial exercise adaptations are observed in hibernating squirrel tissue, the concern raises whether endurance exercise damages occur as well during hibernation, such as oxidative stress formation promoted through enhanced ROS formation (Powers and Jackson, 2008). In addition, long-term immobilization has been also associated with increased mitochondrial ROS production (Powers, et al., 2012). Unexpectedly, we did not find signs of increased oxidative stress formation during hibernation as revealed by

unchanged oxidized mitochondrial protein levels. Thus, given these increased basal protein levels of antioxidant enzymes during hibernation, hibernating skeletal muscle may ultimately be protected from oxidative stress and apoptosis.

During torpor/arousal periods, euthermic rewarming from the hypometabolic state occurs and requires an augmented capacity for shivering and nonshivering thermogenesis. In addition to their antioxidant property, uncoupling proteins have been proposed to play a role in thermal homeostasis and energy balance during hibernation (Boyer, et al., 1998). The activation of uncoupling proteins may play a dual role in atrophy-protective and thermoregulative mechanisms during the hibernating season.

It is well-established that endurance exercise increases the per-cell abundance of mitochondria and respiratory enzymes (Booth, 1977, Holloszy, 1967). Previous data have also shown that mRNA levels of mitochondrial fusion proteins Mfn-1/2 and fission protein Fis-1 are elevated significantly post-exercise (Cartoni, et al., 2005, Ding, et al., 2010). These fusion and fission proteins are highly involved in electrical and biochemical connectivity and protection of mitochondrial DNA (Berman, et al., 2008). Keeping a proper balance of mitochondrial network dynamics is essential to maintain functional mitochondria (Chen and Chan, 2005, Rube and van der Bliek, 2004). Disruption of dynamic remodeling regulated by proteins involved in mitochondrial fusion and fission can also lead to muscle atrophy (Romanello, et al., 2010). Our findings show increased abundance of mitochondria and enhanced protein levels of the respiratory enzymes ATP Synthase and cytochrome C in hibernating quadriceps muscle. Furthermore, we find increased protein levels of Mfn-1/2 and Fis-1 during hibernation. These results suggest that during hibernation the increase in mitochondrial number, as well as fusion and fission proteins, may enhance the capacity for ATP generation. This promotes metabolic protection to maintain functional and balanced mitochondrial dynamics.

It has been shown that the NAD^+-dependent protein deacetylases sirtuins are functional regulators of PGC-1α-associated mitochondrial biogenesis (Gerhart-Hines, et al., 2007, Kong, et al., 2010) Sirtuins are metabolic sensors that may link caloric restriction, cold exposure, and induction of mitochondrial metabolism (D'Antona, et al., 2010). Consistent with this hypothesis, we show that both SIRT1 and SIRT3 are significantly increased during hibernation. This finding not only substantiates the observed activation of the PGC-1α-mediated endurance pathway, it also highlights its clinical role in disuse atrophy: activators of SIRT1, such as Resveratrol, have been utilized as exercise mimetic in rodents to improve mitochondrial biogenesis and increase endurance capacity (Lagouge, et al., 2006, Momken, et al., 2011).

Besides the impact of endurance exercise on skeletal muscle, resistance exercise encompasses myofiber hypertrophy, increased protein and RNA content, and enhanced tension output (Adams, et al., 2004, Wong and Booth, 1988). The Akt/mTOR pathway is a central mediator of myofiber hypertrophy by regulating muscle cell growth and protein synthesis (Baar and Esser, 1999, Haddad and Adams, 2002, Kubica, et al., 2005). Previous work has shown that numerous components of this resistance exercise pathway also become activated during hibernation (Andres-Mateos, et al., 2012). The current paradigm suggests that the endurance and resistance pathways are mutually exclusive due to the so-called "AMPK-PKB" switch, which implies that phosphorylated AMPK inhibits the activity of mTOR and its downstream targets (Atherton, et al., 2005, Nader, 2006). In contrast to this model, we find a synchronization of both resistance and endurance pathways in the hibernating squirrel and demonstrate that a parallel activation of both pathways can occur *in vivo*. It is therefore reasonable to suggest that crosstalk of these two pathways might be more beneficial than activating solely one of them.

The co-existence of these two pathways might at first appear paradoxical. However, it is possible that the seemingly paradoxical activation of converse pathways may ultimately result in the same active change to an oxidative phenotype. In other adaptive instances, such as opposite changes in mechanical stress in the heart, an induction of similar patterns of gene expression has also been observed, namely the return to a “fetal gene program” (Depre, et al., 1998, Razeghi, et al., 2002). Therefore, these opposite pathways during hibernation may lead to the same direction of change – a fiber switch towards slow Type I muscle fibers.

Skeletal muscle is the largest physiological organ undergoing significant remodeling during exercise. The adaptations elicited by endurance exercise are complex and involve mitochondrial bioenergetics, redox homeostasis, and protein metabolism. These adaptations, such as enrichment of oxidative slow-twitch Type I fibers, are critical for maintaining skeletal muscle homeostasis when challenged with muscle wasting conditions (Minnaard, et al., 2005, Wenz, et al., 2009). Our results show that hibernating ground squirrels exhibit a remarkable plasticity in skeletal muscle that resembles both the myofiber transitions and protein level alterations that occur in the mammalian response to endurance exercise training. The mitochondrial homeostasis of antioxidant enzymes, apoptotic proteins, and the fusion and fission machinery may aid in the remodeling of muscle mass and combating the oxidative challenge during hibernation.

Many clinical conditions including inherited myopathies, disuse conditions (immobilization, bed rest, denervation, or microgravity), aging, and cachexia result in significant loss of muscle mass. In these circumstances, maintenance of muscle mass is crucial in combating debilitating functional consequences. However, there are no effective and safe treatment strategies available to prevent muscle atrophy (Glass, 2003, Narici and de Boer, 2011, Wagner, 2008). The 13-lined ground squirrel animal model is *physiologically* protected against disuse atrophy. This enables us to study underlying molecular pathways and potential pharmacological targets that effectively trigger skeletal muscle remodeling, regulate oxidative balance, and susceptibility to atrophy. Importantly, we have shown that activation of the endurance pathway can be achieved *in vivo* despite prolonged periods of immobilization. Future studies will be aimed at finding potential molecular targets for muscle wasting conditions that are so severe that they cannot withstand physical activity.

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Highlights

- Hibernating 13-lined ground squirrels exhibit a shift to slow-twitch Type I muscle fibers.
- The muscle fiber switch is accompanied by an activation of the PGC-1α-mediated endurance exercise pathway.
- Increased antioxidant capacity without evidence of oxidative stress is seen in hibernating skeletal muscle.
- We observe a marked decline in apoptotic susceptibility, and enhanced mitochondrial metabolism.
- An activation of the PGC-1α-mediated endurance exercise pathway can be achieved *in vivo* despite prolonged periods of immobilization during hibernation.

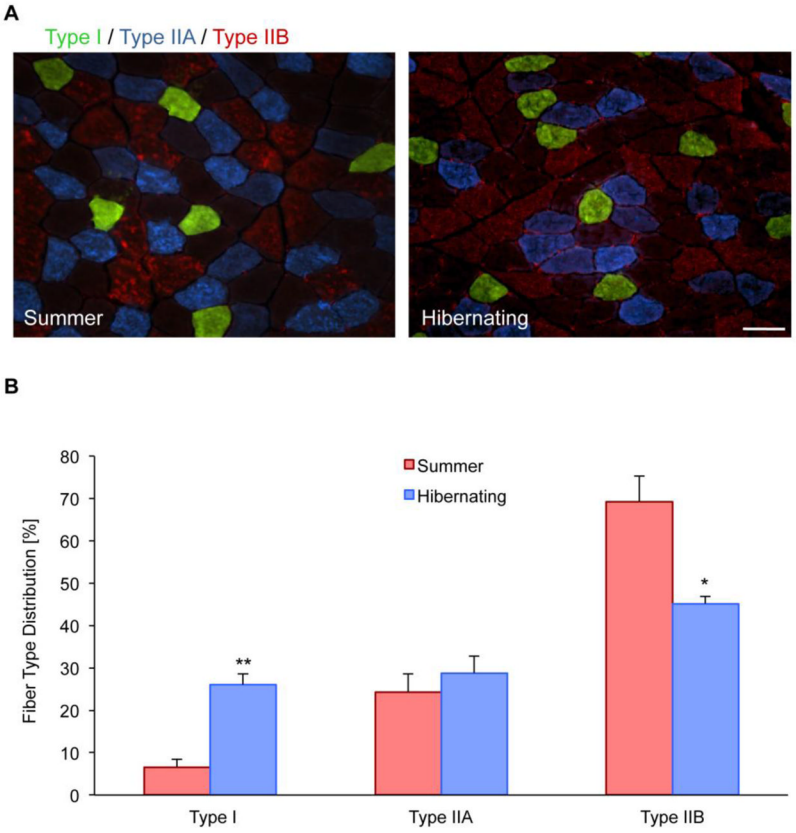

Figure 1. Slow-type muscle fiber switch during hibernation

A, Immunofluorescent staining of squirrel quadriceps muscle reveals the relative abundance of Type I, IIA, and IIB fiber types. Scale bar 100 μm. **B**, Comparison of muscle fiber type percentages between muscle from non-hibernating and hibernating squirrels. During hibernation there is a significant increase in slow-twitch, Type I muscle fibers and a decrease in fast-twitch, Type IIB fibers. (*P<0.05; **P<0.01)

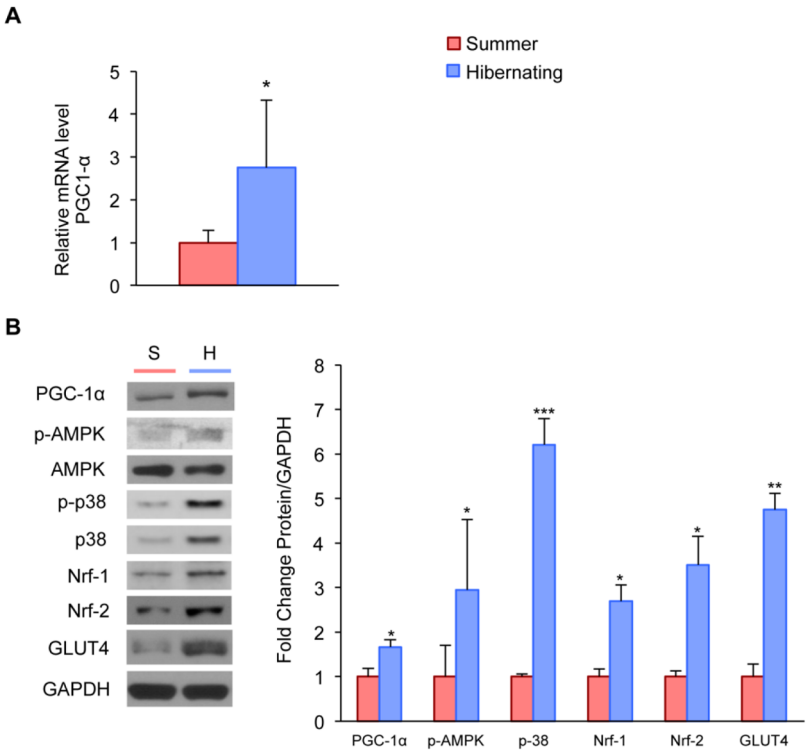

Figure 2. Activation of the PGC-1α-mediated endurance pathway in skeletal muscle (whole cell lysate) during hibernation

A, Real-time PCR of PGC-1α in whole muscle lysate reveals significantly enhanced mRNA levels during hibernation. **B**, Western blot and density analyses of PGC-1α and its down- and upstream targets: protein levels of p-AMPK, p-38, Nuclear Respiratory Factors 1 and 2 (Nrf-1 and Nrf-2), and GLUT4 are significantly upregulated in hibernating quadriceps muscle. (*P<0.05; **P<0.01; ***P<0.001)

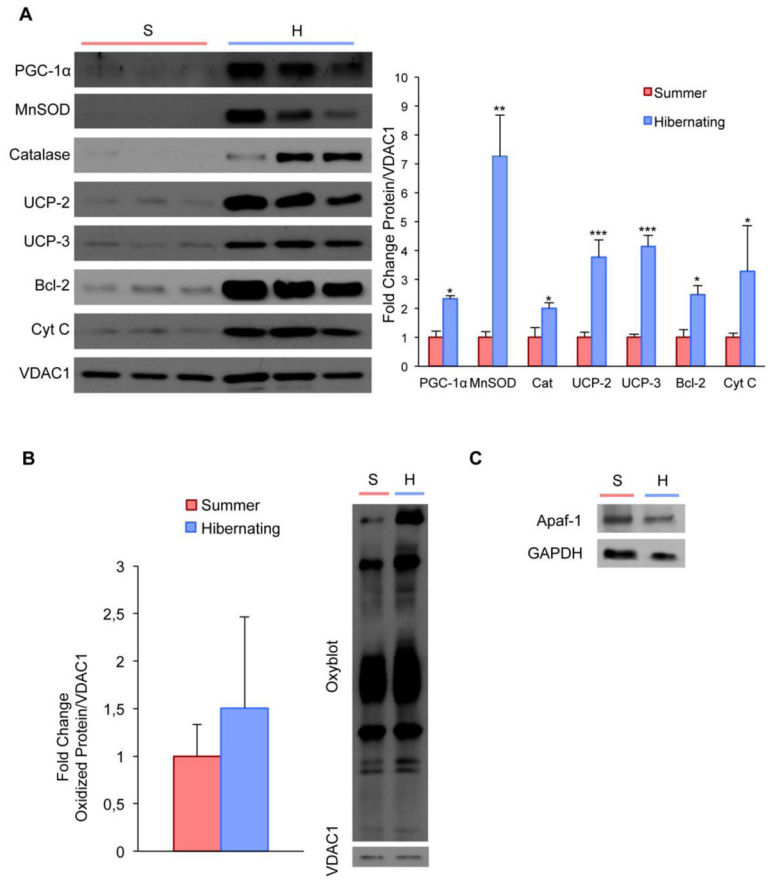

Figure 3. Upregulation of antioxidant, uncoupling, and antiapoptotic proteins in mitochondrial fractions from squirrel quadriceps muscle during hibernation without detection of oxidative stress

A, Western blot and density analyses in mitochondrial fractions from squirrel quadriceps muscle shows significant upregulation of PGC-1α, Manganese superoxide dismutase (MnSOD), catalase (Cat), uncoupling proteins 2 and 3 (UCP-2 and UCP-3), Bcl-2, and Cytochrome C (Cyt C). **B**, Despite increased oxidative stress proteins, OxyBlot analyses reveal unaltered oxidized protein levels of DNP-derivatized quadriceps muscle mitochondria. Quantification is normalized to the protein levels of VDAC1/Porin. **C**, In concomitance to increased antiapoptotic proteins during hibernation, Western blot shows a trend in decreased protein levels of the pro-apoptotic regulator Apaf-1 in the hibernating group. (*P<0.05; **P<0.01, ***P<0.001)

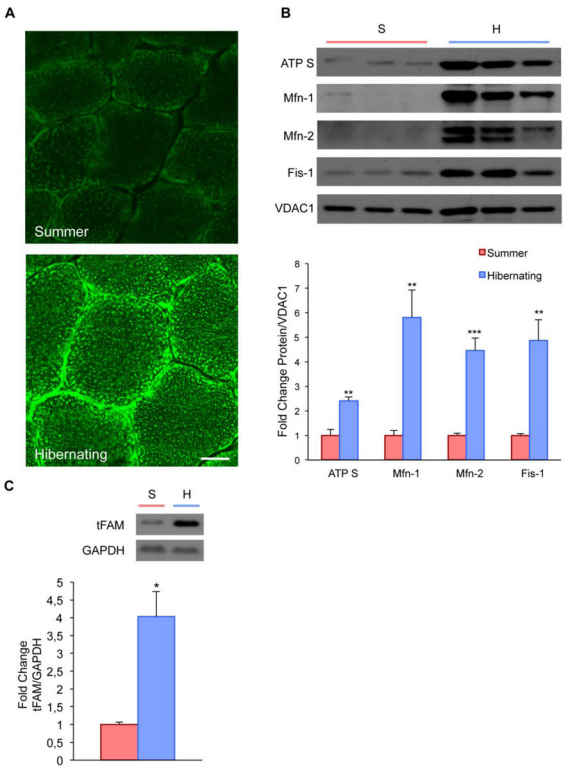

Figure 4. Mitochondrial biogenesis and dynamics during hibernation

A, MitoTracker staining shows accumulation of subsarcolemmal and cytoplasmic mitochondria in quadriceps muscle of hibernating squirrels. Scale bar, 10 μm. **B**, Western blot and density analyses of mitochondrial fractions shows significantly increased protein levels of ATP Synthase, Mitofusin-1 (Mfn-1), Mitofusin-2 (Mfn-2), and Fis-1, suggesting enhanced mitochondrial biogenesis during periods of hibernation. **C**, Relative increase of mitochondrial transcription factor A protein during hibernation. (**P<0.01, ***P<0.001)

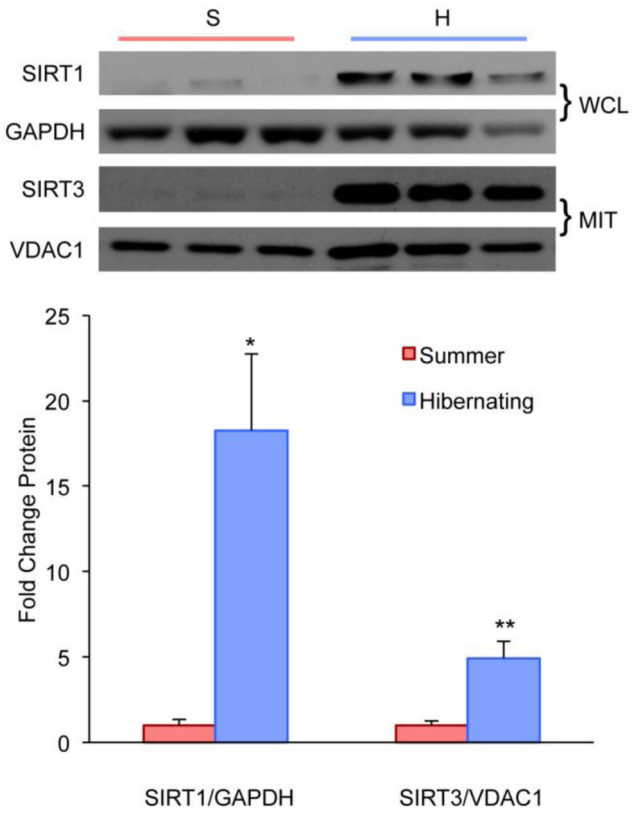

Figure 5. Increased sirtuin protein levels during hibernation

Western blot and density analyses of SIRT 1 in whole cell lysate (WCL) of quadriceps muscle shows an 18-fold upregulation during hibernation. SIRT3 is significantly increased in mitochondrial lysate fractions (MIT) of quadriceps muscle. (*P<0.05; **P<0.01)

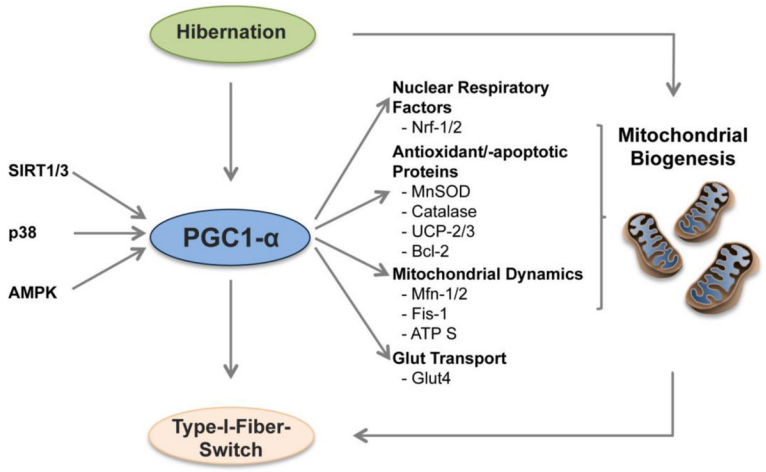

Figure 6. Mitochondrial signaling networks in hibernation

Activation of the PGC1α-mediated endurance pathway contributes to the enhanced mitochondrial biogenesis and Type I fiber switch seen during hibernation.