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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 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.

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., 2013; 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).

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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., 2003, 2006). 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.

Materials 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 were 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. Two to three 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. Ten micrometer 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 63× 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, Tris–HCl 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). Twenty micrograms of protein was 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 (67 mM sucrose, 50 mM Tris/Hcl, 50 mM KCl, 10 mM 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, 3 mM

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