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Inhibition of skeletal muscle atrophy during torpor in ground squirrels occurs through downregulation of MyoG and inactivation of Foxo4

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ABSTRACT

Foxo4 and MyoG proteins regulate the transcription of numerous genes, including the E3 ubiquitin ligases MAFbx and MuRF1, which are activated in skeletal muscle under atrophy-inducing conditions. In the thirteen-lined ground squirrel, there is little muscle wasting that occurs during hibernation, a process characterized by bouts of torpor and arousal, despite virtual inactivity. Consequently, we were interested in studying the regulatory role of Foxo4 and MyoG on ubiquitin ligases throughout torpor-arousal cycles. Findings indicate that MAFbx and MuRF1 decreased during early torpor (ET) by 42% and 40%, respectively, relative to euthermic control (EC), although MuRF1 expression subsequently increased at late torpor (LT). The expression pattern of MyoG most closely resembled that of MAFbx, with levels decreasing during LT. In addition, the phosphorylation of Foxo4 at Thr-451 showed an initial increase during EN, followed by a decline throughout the remainder of the torpor-arousal cycle, suggesting Foxo4 inhibition. This trend was mirrored by inhibition of the Ras-Ral pathway, as the Ras and Ral proteins were decreased by 77% and 41% respectively, at ET. Foxo4 phosphorylation at S197 was depressed during entrance and torpor, suggesting Foxo4 nuclear localization, and possibly regulating the increase in MuRF1 levels at LT. These findings indicate that signaling pathways involved in regulating muscle atrophy, such as MyoG and Foxo4 through the Ras-Ral pathway, contribute to important muscle-specific changes during hibernation. Therefore, this data provides novel insight into the molecular mechanisms regulating muscle remodeling in a hibernator model.

1. Introduction

In order to survive the long winter months in northern climates, resident animals have developed physiological adaptations which promote survival despite a lack of nutritional resources and frigid temperatures. Hibernation is one such survival mechanism; a cyclical phenomenon characterized by periods of deep torpor when body temperatures (T_b) drop to near ambient temperatures (0–5 °C) for days to weeks followed by brief periods of arousal where T_b returns to 37 °C [25,49,50,57]. During torpor, hibernating squirrels enter into a hypometabolic state whereby they reduce their metabolic rate to <5% of euthermic levels [57]. Metabolic rate depression allows the ground squirrel to save valuable ATP stores and redistribute energy expenditure to select, essential cellular functions [22,39,49].

Each organ and tissue needs to make specific adjustments in order for the organism to survive the periods of low T_b during torpor as well as fluctuations in T_b and metabolic rate as a result of the torpor-arousal cycles. Of particular interest, hibernators need to avoid significant disuse-induced skeletal muscle atrophy during hibernation, otherwise decreases in muscle mass and strength could compromise post-hibernation activities such as foraging for food and reproduction [3,13,35,44]. As a matter of fact, numerous studies have demonstrated a lack of significant muscle wasting in select muscles during hibernation despite the prolonged periods of inactivity and mechanical unloading that occur during hibernation [16,26,60]. Interestingly, it was demonstrated that the relative ratio of muscle mass/body weight actually increases throughout hibernation in ground squirrels due to significant losses in body weight as well as an effective mechanism of muscle preservation and remodeling that is unique to hibernators [26]. Therefore, uncovering the molecular mechanisms underlying this process of muscle

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maintenance, that occurs naturally in mammalian hibernators, have clinical relevance for therapeutic intervention of muscle wasting and assisting in physical rehabilitation.

The degree of muscle wasting is dependent on contrasting signals: those which promote cell growth and are predominately anabolic (hypertrophy) versus those which result in loss of muscle tissue with upregulated degradative pathways (atrophy). Recent studies in mammalian hibernators have begun to elucidate the molecular basis of muscle atrophy prevention, with findings implicating hypertrophy- and muscle remodeling-related factors such as the peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), the nuclear factor of activated T cells (NFAT) family of transcription factors, myocyte enhancer factor-2 (MEF2), and others in this process [54,60,62]. Similarly, some great progress has been made with respect to the involvement of catabolic pathways [17,44,58], although a deeper understanding of the involvement of protein degradation pathways, such as the transcriptional regulation of ubiquitin ligases, are still required.

The main signaling pathway that controls muscle-specific degradation pathways involves the forkhead box transcription factors of the O subclass (Foxo) as well as the myogenin (MyoG) transcription factor, and their regulation of the ubiquitin proteasome system (UPS) [38,46,48]. The UPS is an important mechanism for protein degradation, whereby substrates are ligated to ubiquitin via E3 ubiquitin ligases like Muscle Atrophy F-Box (MAFbx/atrogin-1) and Muscle Ring Finger 1 (MuRF1), which target these substrates for degradation in the proteasome [24,28,47]. In fact, calcineurin – an upstream regulator of NFAT, is ubiquitinated and targeted for degradation by MAFbx [2,34]. Therefore, this may be a mechanism by which the UPS promotes muscle atrophy. Also, the particular importance of MAFbx and MuRF1 in the context of muscle atrophy is demonstrated by denervation studies which showed significantly lower loss of muscle mass in MAFbx/MuRF1 null mice (MAFbx −/−; MuRF1 −/−) [6]. Also, multiple lines of evidence have shown a correlation between atrophy and the upregulation of MAFbx and/or MuRF1 [24]. Due to the importance of both MAFbx and MuRF1 for muscle atrophy, common regulators were found for both ligases. The Foxo family of transcription factors were the first of such factors [38,46,48]. Then, MyoG was shown to be a positive regulator of both E3 ligases as well; where its expression is upregulated in skeletal muscle following denervation and it regulates the expression of MAFbx and MuRF1. In addition, muscle atrophy was attenuated in MyoG-null mice [38]. MyoG expression is regulated in part by histone deacetylases (HDACs) 4 and 5 through transcriptional repression of Dach2, a negative regulator of MyoG [15,53]. Mice lacking these two HDACs failed to upregulate MyoG, leading to muscle mass preservation in denervated muscle [38]. Furthermore, the myogenic regulatory factor MyoD cooperates with NFATc2 and c3 at the MyoG promoter to regulate MyoG gene expression [1].

The mammalian Foxo family has four members: Foxo1, Foxo3a, Foxo4, and Foxo6, that are involved in various cellular processes in addition to muscle atrophy, such as antioxidant defense and apoptosis [5,27,58]. Foxo1, Foxo3a, and Foxo4 are all regulated by the Akt/protein kinase B (PKB) signaling pathway. Specifically, Akt blocks the function of all three Foxo proteins through phosphorylation at conserved residues that lead to cytoplasmic localization [9,36,51,52]. Previous work has been done to study the roles of Foxo1 and 3a, but not Foxo4 in the skeletal muscle of hibernators [18,58]; therefore, the current study compliments this work by focusing on the role of Foxo4 and its regulation of MAFbx and MuRF1 during hibernation. For Foxo4, Akt inhibits nuclear translocation by phosphorylating the Threonine (Thr)-32, Serine (Ser)-197, and Ser-262 residues [36,51]. However, Foxo4 transcriptional activation has been shown to be regulated through a separate pathway, involving the Ras and Ral GTPases [4,21,31,45], as summarized in Fig. 1. Ras and the Ral isoforms, RalA and RalB, are small GTPases that share very similar sequences, with Ral activation requiring the interaction with Ras in order to act as signaling molecules for a variety of downstream processes like transcription, DNA synthesis, and cellular differentiation [23,45]. Ral binding protein 1 (Ralbp1) is another integral part of the Ras-Ral pathway as it acts as a downstream effector of Ral and associates with Ral in a GTP-dependent manner [10,30]. Initially it was discovered that Foxo4 transcriptional activity was dependent upon activation of the Ras-Ral pathway as activation of the pathway results in phosphorylation of Foxo4 at Thr-447 and Thr-451 [31,45] (Fig. 1). Acting independently of Akt-mediated inhibition, RalA mediates Foxo4 phosphorylation via the Stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK). JNK is bound to the cellular scaffold protein, c-Jun-amino-terminal-interacting protein 1 (JIP1), where it is activated by RalA through phosphorylation [4] (Fig. 1). It was demonstrated that the activation of Foxo4 transcriptional activity through the Ras-Ral pathway and JNK was induced by tumor necrosis factor α (TNFα) [4,21]. Furthermore, the induction in MAFbx expression by TNFα is reliant on Foxo4 regulation and not Foxo1/3a [40]. These previous findings stress the importance of understanding Foxo4 regulation through both the Ras-Ral and Akt pathways, and studying how these pathways contribute to the control of muscle atrophy by Foxo4.

Given the unique ability of *I. tridecemlineatus* to avoid disuse-induced muscle wasting despite being inactive during periods of torpor, there is a need to study the molecular mechanisms underlying atrophy reduction during hibernation in this animal. We hypothesized that inhibition of muscle loss during torpor may be mediated in part by a downregulation of MyoG- and Foxo4-mediated MAFbx and MuRF1 expression, and this effect may be initiated by post-translational modifications acting on Foxo4. To test this hypothesis, the present study characterized the protein levels of total MyoG and Foxo4 as well as different phosphorylated forms of Foxo4, in addition to upstream regulatory factors such as Ras, RalA, and Ralbp1, and downstream ubiquitin ligases MAFbx, and MuRF1 over cycles of torpor and arousal in the skeletal muscles of *I. tridecemlineatus*.

2. Materials and methods

2.1. Animal treatment

Thirteen-lined ground squirrels (*I. tridecemlineatus*) weighing 150–300 g were captured by United States Department of Agriculture (USDA) licensed personnel (TLS Research, Bloomingdale, IL) and transported to the Animal Hibernation Facility at the National Institute of Neurological Disorders and Stroke (NINDS, Bethesda, MD). Experiments were performed on the animals during their natural hibernation phase in January and February by the laboratory of Dr. J.M. Hallenbeck, as previously described [37]. All animal protocols were approved by the NINDS Animal Care and Use Committee (NIH; animal protocol no. ASP 1223-05). Male and female squirrels were sampled equally in the study with a mixture of genders in each experimental condition, and all animals were 1–3 years of age, although the exact age of each animal was unknown since animals were captured from the wild. Squirrels were housed in individual cages within a room at an ambient temperature of 21 °C under a 12 h light – 12 h dark cycle. To monitor T_b, a sterile programmable temperature transponder was injected into the intrascapular area of the animals (IPTT-300, Bio medic Data Systems).

Torpor was induced in animals by transfer to an environmental chamber at 4–5 °C, and tissues were collected at six distinct stages of the torpor-arousal cycle as characterized by T_b, duration of torpor,