Brief communication

MOLECULAR METABOLISM

Osteocalcin is necessary and sufficient to maintain muscle mass in older mice

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ABSTRACT

Objective: A decrease in muscle protein turnover and therefore in muscle mass is a hallmark of aging. Because the circulating levels of the bone-derived hormone osteocalcin decline steeply during aging in mice, monkeys and humans we asked here whether this hormone might regulate muscle mass as mice age.

Methods: We examined muscle mass and strength in mice lacking osteocalcin (*Ocn*−/−) or its receptor in all cells (*Gprc6a*−/−) or specifically in myofibers ($Gprc6a_{Mck}$−/−) as well as in 9 month-old WT mice receiving exogenous osteocalcin for 28 days. We also examined protein synthesis in WT and *Gprc6a*−/− mouse myotubes treated with osteocalcin.

Results: We show that osteocalcin signaling in myofibers is necessary to maintain muscle mass in older mice in part because it promotes protein synthesis in myotubes without affecting protein breakdown. We further show that treatment with exogenous osteocalcin for 28 days is sufficient to increase muscle mass of 9-month-old WT mice.

Conclusion: This study uncovers that osteocalcin is necessary and sufficient to prevent age-related muscle loss in mice.

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Keywords Osteocalcin; Muscle mass; Aging

1. INTRODUCTION

With the increase of life expectancy our societies experience comes a slew of age-related diseases. The aging process is characterized for instance by changes in body composition that include a decrease in muscle mass and/or strength. This manifestation of aging known as muscle wasting [1,2], is a cause of a metabolic deregulation [3,4], and also contributes to the increased morbidity seen in the elderly because of the higher risk of falls and fractures. One of the proposed causes for this age-related decline in muscle mass is a reduced muscle protein turnover, defined as the balance between muscle protein synthesis and breakdown [5]. How protein turnover in muscle is regulated and can be affected by aging remains however, poorly understood. The recent demonstration that the bone-derived hormone osteocalcin favors muscle functions during exercise [6,7] raises the question of whether this hormone may also regulate muscle mass.

In support of this working hypothesis we note that osteocalcin favors physiological functions that like testosterone synthesis and male fertility [8,9], memory or adaptation to exercise [7,10], tend to decrease with age. At the same time the circulating levels of bioactive osteocalcin decrease rapidly and steeply in life in mice, monkeys and humans [7]. These findings were two additional incentives to ask whether osteocalcin signaling in myofibers also prevents another aspect of aging namely the decrease in muscle mass. Here we show that osteocalcin signaling in myofibers is necessary to maintain muscle mass in older mice because it promotes protein synthesis in muscle cells. More importantly, exogenous osteocalcin is also sufficient to increase muscle mass in 10-month-old mice. These results expand the importance of the regulation of muscle physiology by bone-derived hormones and suggest novel and adapted therapies to treat age-related muscle wasting.

2. MATERIALS AND METHODS

2.1. Animal studies

Osteocalcin (*Ocn*−/−) mice were maintained on 129-Sv genetic backgrounds. *Gprc6a*−/−, $Gprc6a_{Mck}$−/− and *Ocn*+/−;$Gprc6a_{Mck}$+/− mice were maintained on 129-Sv/C57/BL6 mixed genetic background. To minimize the possible confounding effect of a different genetic background all experiments were performed using control littermates. Generation of mice harboring a *Gprc6a* conditional allele has been described [9]. For osteocalcin treatment studies 9-month-old WT 129-Sv mice (Taconic) were implanted with subcutaneous osmotic pumps (Alzet, model 1004) delivering osteocalcin (90 ng/h) for 28 days. After this period, mice were euthanized and muscles dissected for analyses of muscle mass and histology examination. Recombinant osteocalcin was purified as previously described [10]. All procedures involving mice were approved by CUMC IACUC and conform to the relevant regulatory standards.

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2.2. Histology

Examination of muscle histology was done using 5-μm thick formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin (H&E) at the Molecular Pathology Core at UT Southwestern Medical Center.

2.3. Myoblasts culture and differentiation

Culture of mouse skeletal muscle myoblasts was performed as described [7,11], using 15- to 20-day-old mice. Myoblasts were cultured until 80% confluent and then differentiated into myotubes in DMEM supplemented with 5% horse serum.

For in vitro inactivation of *Gprc6a*, myoblasts from *Gprc6a*−/− mice and WT controls were isolated and differentiated as above.

2.4. Protein synthesis

Protein synthesis was determined as the incorporation of ^{3}H-tyrosine to cellular proteins. In brief, myotubes were differentiated for 4 days in 24-well plates. The day of the experiment cells were washed and incubated in serum free-high glucose DMEM 0.1% BSA for 4 h. Next, 2 μCi/well of ^{3}H-tyrosine with either vehicle or osteocalcin were added to each well. Cells were then incubated for 2 h following 2 washes with 1× PBS. Next, cellular proteins were precipitated with 10% TCA and radioactivity measured in the precipitated fraction. Results were corrected using the protein concentration in each well.

2.5. Western blot

To detect total and phosphorylated mTOR, S6K1, Raptor and GAPDH proteins were resolved on 8% acrylamide Tris gels. Western blot analyses were performed according to standard protocols. All antibodies used were from Cell Signaling: anti-phospho(Thr389)-S6K1 (#9205), anti-S6K1 (#2708), anti-phospho(Ser2448)-mTOR (#2971), anti-mTOR (#2972), anti-Raptor (#2280), anti-GAPDH (#2118).

2.6. Blood and urine measurements

Urine 3-methylhistidine (3MH) was measured as previously described in Ref. [12]. Serum osteocalcin levels were measured using an ELISA assay as previously described in Ref. [13].

2.7. Statistics

All data presented as mean ± SEM. Statistical analyses were performed using unpaired, two-tailed Student's test for comparison between two groups and ANOVA tests for experiments involving more than two groups.

3. RESULTS

3.1. Osteocalcin is necessary to maintain muscle mass in adult mice

The recent identification of osteocalcin as a regulator of muscle function during exercise [7] raised the question of whether this hormone regulates any other aspect of muscle biology. This question is also prompted by the fact that circulating osteocalcin levels plummet with age [7].

One of the most common manifestations of aging in muscle is the decrease in muscle mass and/or strength. To test whether osteocalcin is a regulator of muscle mass in adult mice, we analyzed the weight of hindlimb muscles (soleus, EDL, gastrocnemius and quadriceps) in 6-month-old *Ocn*−/− and WT littermates. Because osteocalcin promotes testosterone synthesis specifically in testes but not in ovaries, *Ocn*−/− male mice have lower testosterone levels than WT littermates [9]. Given the influence of testosterone in muscle mass [14] and to avoid this confounding factor all experiments were performed in female mice. These investigations revealed the existence of a significant decrease in the weight of soleus and gastrocnemius muscles and overall a decrease in body weight in *Ocn*−/− mice when compared to WT littermates (Figure 1A,B). This decrease in muscle mass in *Ocn*−/− mice was explained, at least in part, by a decrease in the cross-section area of the myofibers (Figure 1C). Since aging can decrease muscle strength we also analyzed this parameter in 12 month-old *Ocn*−/− mice. However, muscle strength measured as the peak amount of force applied by a mouse in grasping a pull bar, was the same in 12-month-old *Ocn*−/− and WT littermates (Figure 1D). This result suggests that osteocalcin favors maintenance of muscle mass but does not affect muscle strength in a measurable manner in older mice.

3.2. Osteocalcin signaling in myofibers is necessary to maintain muscle mass in older mice

The observation that osteocalcin is necessary to maintain muscle mass in adult mice raised the question as to whether this function of osteocalcin occurs following its signaling through its only identified receptor, GPRC6A [9]. When analyzed at 12 months of age, mice lacking *Gprc6a* in all cells (*Gprc6a*−/−) showed a significant decrease in muscle and body weight (Figure 2A,B). Because *Gprc6a* is expressed in myofibers and osteocalcin signals through this receptor to regulate muscle function during exercise [7], we next tested whether older mice lacking *Gprc6a* specifically in myofibers ($Gprc6a_{Mck}$−/−) presented any abnormality in the weight of their hindlimb muscles. The specificity and efficiency of *Mck*-Cre-mediated *Gprc6a* inactivation in $Gprc6a_{Mck}$−/− mice has been previously reported [7]. At 12 months of age, $Gprc6a_{Mck}$−/− mice showed a significant decrease in muscle weight when compared to control littermates, although their body weight was normal (Figure 2C,D). This latter result indicates that osteocalcin regulates muscle mass independently of its effect on energy metabolism [7,15]. That a similar decrease in muscle weight was observed in 12-month-old compound mutant mice lacking one allele of *Osteocalcin* and one allele of *Gprc6a* specifically in myofibers (*Ocn*+/−;$Gprc6a_{Mck}$+/−) (Figure 2E), provide a direct genetic evidence that osteocalcin is the main ligand of GPRC6A in myofibers responsible of its regulation of muscle mass.

3.3. Osteocalcin favors protein synthesis in myotubes

In an effort to understand the molecular bases of osteocalcin regulation of muscle mass in mice we tested whether osteocalcin regulates muscle protein synthesis or breakdown, because a decrease in muscle protein turnover has been previously associated with a loss of muscle mass [5,16–18].

Urinary elimination of 3MH, a byproduct of the degradation of structural proteins in muscle and a biomarker of protein breakdown [19], was similar in *Ocn*−/− and WT mice thus ruling out that osteocalcin regulates protein degradation in muscle (Figure 3A). That osteocalcin increased in a dose-dependent manner incorporation of ^{3}H-tyrosine into proteins of WT but not *Gprc6a*−/− myotubes, suggests that osteocalcin favors protein synthesis in these cells (Figure 3B–C). The importance of the mTOR complex for protein synthesis led us to ask whether the ability of osteocalcin to favor protein synthesis in myofibers relies in part on the activation of this complex [16,20]. We observed that osteocalcin induced phosphorylation of S6K1, an mTOR target protein, at Thr389 in WT myotubes (Figure 3C). This effect was abolished by Torin1, an inhibitor of mTOR signaling, or by the inactivation of the regulatory-associated protein of mTOR (Raptor) (Figure 3D,E). Taken together these data indicate that osteocalcin

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