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Increased mitochondrial turnover in the skeletal muscle of fasted, castrated mice is related to the magnitude of autophagy activation and muscle atrophy

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ABSTRACT

Androgen-deficiency promotes muscle atrophy in part by increasing autophagy-mediated muscle protein breakdown during the fasted state, but factors contributing to this remain undefined. To identify novel factors, mice were subjected to sham or castration surgery. Seven-weeks post-surgery, mice were fasted overnight, refed for 30 min, and fasted another 4.5 h before sacrifice. BNIP3-mediated turnover of mitochondria was increased within the atrophied tibialis anterior (TA) of castrated mice and related to the magnitude of muscle atrophy and autophagy activation (i.e. decreased p62 protein content), thus linking turnover of potentially dysfunctional mitochondria with autophagy-mediated atrophy. Autophagy induction was likely facilitated by AMPK activation as a stress survival mechanism since phosphorylation of AMPK (Thr172), as well as the pro survival kinases Akt (Thr308) and (ERK1/2 Thr202/Tyr204), were increased by castration. Together, these data identify a novel relationship between mitochondrial turnover in the fasted state with autophagy activation and muscle atrophy following androgen depletion.

1. Introduction

In males, androgens such as testosterone are important for maintaining skeletal muscle mass (Steiner et al., 2017; Ferrando et al., 2002; White et al., 2013a,b; Atkinson et al., 2010). For instance, the decrease in production and/or bioavailability of androgens induced by various pathological conditions, termed hypogonadism, contributes to muscle atrophy (Ferrando et al., 2002, 2003; White et al., 2013a,b; Ferrando et al., 2003). Androgen replacement therapy effectively reverses the loss of muscle due to androgen depletion, but this is not a universal therapeutic option, especially for those with established malignancies (Amos-Landgraf et al., 2014; Huggins and Hodges, 2002). Since maintaining muscle mass is directly linked to a favorable survival outcome during pathological conditions in which androgen production is reduced (Martin et al., 2013), there is a need to identify how androgens regulate muscle mass so that androgen-independent therapies can be generated to treat males unable to receive androgen replacement (Atkinson et al., 2010; Bassil et al., 2009).

Androgens regulate muscle mass largely through changes in protein balance, predominantly in the fasted metabolic state (Ferrando et al., 1998, 2003; Phillips et al., 2009; Ferrando et al., 1998). For instance, the loss of muscle mass in hypogonadal males was primarily attributable to increased rates of muscle protein breakdown following an overnight fast (Ferrando et al., 2002, 2003). Muscle atrophy in murine models of hypogonadism also coincided with increased markers of muscle protein breakdown, including autophagy activation (Steiner et al., 2017; Serra et al., 2013; Rossetti et al., 2017). For example, the content of lipidated microtubule-associated protein light chain 3 (LC3 II), the ratio of

Abbreviations: BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; TA, tibialis anterior; ERK 1/2, extracellular signal−regulated kinase 1/2; AMPK, 5′ AMP-activated protein kinase; LC3, lipidated microtubule-associated protein light chain 3; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; COX IV, cytochrome *c* oxidase subunit IV; VDAC, voltage-dependent anion channel; Bcl-xL, B-cell lymphoma extra-large; Pink1, PTEN induced putative kinase 1; BNIP3L/Nix, BCL2/Adenovirus E1B 19 kDa protein-interacting protein 3-like; PGC-1α, peroxisome proliferator activated receptor gamma coactivator 1 alpha; NRF1, nuclear respiratory factor 1; MuRF-1, muscle ring finger-1; MAFbx, muscle atrophy F-box; Bcl-2, B cell lymphoma 2 .

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lipidated to non-lipidated LC3 (LC3 II/I), and Cathepsin L activity were increased while the protein content of p62 was decreased in skeletal muscle of hypogonadal mice, indicating increased autophagy activation (Steiner et al., 2017; Serra et al., 2013; Rossetti and Gordon, 2017), a process known to promote muscle atrophy (Sandri, 2013).

Despite convincing evidence of enhanced autophagy in muscle following androgen depletion, the underlying mechanism(s) contributing to the elevation remain unresolved, and may be dependent upon the length of fast. For instance, we previously showed that markers of autophagy (p62 and LC3II/I ratio) within the atrophied tibialis anterior (TA) of castrated mice were increased after both an 18 h and 4.5 h fast (Steiner et al., 2017), but the factors regulating the induction of autophagy differed between these time points. After the 18 h fast, a reduction in signaling through the mechanistic target of rapamycin in complex 1 (mTORC1) and an increase in the expression of Regulated in Development and DNA Damage 1 (REDD1) likely induced autophagy, but after the 4.5 h fast, these two autophagy regulatory factors were not yet altered by castration (Steiner et al., 2017). Therefore, the elevation in autophagy after the shorter 4.5 h fast was mTORC1 and REDD1-independent and required further investigation. For that reason, the present study is a secondary analysis of those same muscle samples to identify factors contributing to the elevation of autophagy in the muscle of castrated mice after a 4.5 h fast (Steiner et al., 2017). Presently, we show that the castration-induced elevation in autophagy and the magnitude of muscle atrophy is related to increased mitochondrial turnover, providing a novel mTORC1 and REDD1-independent mechanism that is likely contributing to muscle atrophy following androgen depletion.

2. Materials and methods

2.1. Animals, castration surgery, and induction of post-absorptive metabolic state

The muscle samples used in the current study were from mice that were part of a larger study previously conducted by our laboratory in which outcome variables were measured in two groups of sham and castrated mice subjected to either an 18 h fast or a 4.5 h fast (Steiner et al., 2017). The specific muscle samples analyzed herein were from all of the sham and castrated mice subjected to the 4.5 h fast in that previous study. To generate these samples, 9-week old, male, C57BL/6NHsd mice were obtained from Envigo (Indianapolis, IN). Mice were housed individually upon arrival and placed on a 12:12-h light/dark cycle in a temperature controlled environment (25 °C) within the barrier containment vivarium at the Burnett School of Biomedical Sciences at the University of Central Florida. Mice were provided irradiated PicoLab 5053 rodent chow (LabDiet, St. Louis, MO) and water *ad libitum* throughout the study. Following a 1-week acclimation period, mice were subjected to a sham or castration surgery followed by a 7-week recovery period. Prior to sacrifice, the mice analyzed in the present study were subjected to an overnight fast (~14 h) followed by a 30 min refeeding period in which all mice ate similar quantities of food (~450 mg) (Steiner et al., 2017). Following this refeeding, all mice were fasted for 4.5 h with unrestricted water access. The TA muscle was initially analyzed in our previous report as this muscle is known to atrophy following androgen depletion (Ueberschlag-Pitiot et al., 2017). Thus, the data presented herein were obtained from a muscle whose mass exhibits the atrophy typically associated with post-pubescent, androgen depletion (Ferrando et al., 2002; White et al., 2013a; Serra et al., 2013; Ueberschlag-Pitiot et al., 2017). The Institutional Animal Care and Use Committee at the University of Central Florida approved the animal facilities and all experimental protocols.

2.2. Western blot analysis

Western blotting was conducted as previously described (Rossetti and Gordon, 2017; Gordon et al., 2015). Whole muscle protein of the TA was extracted by glass on glass homogenization in 10 vol of buffer (10 μl/mg of muscle) consisting of 50 mM HEPES (pH 7.4), 0.1% Triton-X 100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$, 100 mM β-glycerophosphate, 25 mM NaF, 5 mM Na_3VO_4, and 10 μl/ml protease inhibitor cocktail (Sigma Aldrich #P8340). Muscle extract was centrifuged for 10 min at 10,000 × *g*, and the supernatant fraction was quantified via the Bradford method. Proteins in the supernatant were fractionated on 4–20% Bio-Rad Criterion precast gels (Hercules, CA) and transferred to PVDF membranes. Effective transfer and equal protein loading were confirmed by Ponceau-S stain. Membranes were incubated overnight at 4 °C in TBS +0.1% Tween20 (TBST) with antibodies against Akt (Thr308) (cat. #9275), total Akt (cat. #9272), AMPK (Thr172) (cat. #2531), total AMPK (cat. #2532), Bcl-xL (cat. #2764), BNIP3 (cat. #3769), BNIP3L/NIX (cat. #12396), COX IV (cat. #4844), ERK1/2 (Thr202/Tyr204) (cat. #9101), Total ERK1/2 (cat. #9102), Parkin (cat. #2132), Beclin 1 (cat. #3738), Bcl-2 (cat. #3498), and VDAC (cat. #4866), which were all obtained from Cell Signaling Technology (Danvers, MA). Antibodies against Pink1 (cat. #ab23707) were obtained from Abcam (Cambridge, MA). Following incubation with secondary antibodies (Bethyl Laboratories) (cat. #A120-101P), the antigen-antibody complex was visualized by enhanced chemiluminescence using Clarity reagent (Bio-Rad) on a Bio-Rad ChemiDoc Touch imaging system. The pixel density from all blots was quantified using Image J software (NIH, Bethesda, MD), and expression of total protein values were normalized to the 45 kD band of the Ponceau-S stain.

2.3. RNA extraction, cDNA synthesis, and RT-PCR

TA muscle samples were homogenized in 600 μl of Zymo Tri Reagent (Irvine, CA), and RNA was isolated using a Zymo RNA Miniprep extraction kit (cat. #R2071) with on column DNase treatment (Irvine, CA). RNA quantity was determined spectrophotometrically by the 260-to-280 nm ratio, and cDNA was synthesized from RNA totaling 1.5 μg using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). RT-PCR was conducted on a QuantStudio5 thermal cycler (Thermo Fisher Scientific, Waltham, MA) using PowerUp Sybr Green Master Mix (Thermo Fisher Scientific, Waltham, MA). The conditions for RT-PCR with Sybr Green included an initial 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles which included a 15 s denature step at 95 °C, a 15 s annealing step at 55 °C, and a 1 min extension step at 72 °C within each cycle. A melt curve analysis was performed for each primer pair to ensure that a single product was efficiently amplified, and the product sizes for each primer pair were verified via agarose gel electrophoresis. The mRNA content of BNIP3 (Mm01275600_g1) and NRF1 (Mm01135606_m1) were determined using TaqMan predesigned probes (Thermo Fisher Scientific, Waltham, MA) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) following the manufacturer's recommended RT-PCR cycling conditions for the QuantStudio5 thermal cycler. Relative expression levels of each target gene were normalized by using the delta Ct method with GAPDH as the internal control. Primer sequences for MuRF-1, MAFbx, PGC-1α, and GAPDH have been described previously (Steiner et al., 2017; Gordon et al., 2017).

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