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Anabolic steroids activate calcineurin–NFAT signaling and thereby increase myotube size and reduce denervation atrophy

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ABSTRACT

Anabolic androgens have been shown to reduce muscle loss due to immobilization, paralysis and many other medical conditions, but the molecular basis for these actions is poorly understood. We have recently demonstrated that nandrolone, a synthetic androgen, slows muscle atrophy after nerve transection associated with down-regulation of regulator of calcineurin 2 (RCAN2), a calcineurin inhibitor, suggesting a possible role of calcineurin–NFAT signaling. To test this possibility, rat gastrocnemius muscle was analyzed at 56 days after denervation. In denervated muscle, calcineurin activity declined and NFATc4 was excluded from the nucleus and these effects were reversed by nandrolone. Similarly, nandrolone increased calcineurin activity and nuclear NFATc4 levels in cultured L6 myotubes. Nandrolone also induced cell hypertrophy that was blocked by cyclosporin A or overexpression of RCAN2. Finally protection against denervation atrophy by nandrolone in rats was blocked by cyclosporin A. These results demonstrate for the first time that nandrolone activates calcineurin–NFAT signaling, and that such signaling is important in nandrolone-induced cell hypertrophy and protection against paralysis-induced muscle atrophy.
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1. Introduction

Muscle atrophy is an important consequence of many medical conditions such as immobilization, burns, glucocorticoid administration, paralysis and aging. Androgens have been shown to reduce muscle loss due to burns (Wolf et al., 2006), glucocorticoids (Crawford et al., 2003; Zhao et al., 2008b), immobilization (Taylor et al., 1999), or paralysis due to nerve transection (Zhao et al., 2008a) or spinal cord transection (Wu et al., 2012). However, the underlying mechanisms are poorly understood (Gregory et al., 2003; Zhao et al., 2008a). Using denervation-induced muscle atrophy as a model, we have investigated the molecular basis for these beneficial actions of androgens in studies of the effects of the anabolic steroid nandrolone. Nandrolone lacks activity at tissues expressing 5-α-reductase, such as prostate, because 5-α reduction of nandrolone greatly reduces its affinity for the androgen receptor (AR) (Bergink et al., 1985). Thus, nandrolone preferentially exerts anabolic actions on skeletal muscle and bone while sparing the prostate. In studies of the effects of nandrolone on atrophy after sciatic nerve transection in which this agent was started at 29 days after neurectomy and administered together with a replacement dose of testosterone (NanTs), rates of atrophy were reduced (Zhao et al., 2008a). More recently, using gene expression profiling by DNA oligonucleotide microarrays, the genes affected by nandrolone at 35 days and potentially capable of regulating muscle size were identified (Qin et al., 2010a). One such gene was the regulator of calcineurin 2 (RCAN2), which inhibits calcineurin (Rothermel et al., 2000). Expression of RCAN2 was reduced in denervated muscle by nandrolone. Thus, it is possible that NanTs regulates calcineurin activity and signaling and thereby exerts beneficial actions on paralyzed skeletal muscle.

Calcineurin is a widely expressed serine/threonine phosphatase that is activated by sustained elevations in intracellular calcium concentrations (reviewed in Bassel-Duby and Olson, 2006). It is assembled from a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B). Calcineurin is believed to be an important factor in the determination of the fiber type of skeletal muscle (Al-Shanti and Stewart, 2009) and the size of myocardiocytes (Ni et al., 2006; Olson and Williams, 2000). However, the role of

Abbreviations: AR, androgen receptor; RCAN, regulator of calcineurin; MCIP, modulatory calcineurin interacting protein; NFAT, nuclear factor of activated T cells; MEF2, myocyte enhance factor 2.

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calcineurin in the regulation of skeletal muscle hypertrophy, or spontaneous recovery from muscle atrophy, has been controversial (Bodine et al., 2001; Bostrom et al., 2012; Dunn et al., 1999; Dupont-Versteegden et al., 2002; Mitchell et al., 2002; Olson and Williams, 2000; Zbreski et al., 2006). Administration of the calcineurin inhibitors cyclosporine A or FK506 blocked overload-induced hypertrophy of the plantaris in mice (Dunn et al., 1999). In a second study using the same animal model but lower doses of the calcineurin inhibitors, hypertrophy of the plantaris muscle that followed surgical removal of the soleus, medial, and lateral gastrocnemius muscles in the rat was blocked by rapamycin, an mTOR inhibitor, but not by the calcineurin inhibitors cyclosporin A or FK506 (Bodine et al., 2001; Glass, 2005). In separate studies, the overexpression of an activated calcineurin in mice did not result in muscle hypertrophy (Naya et al., 2000), and loss of either calcineurin A or calcineurin B did not reduce skeletal muscle size (Parsons et al., 2003). These studies focused primarily on properties of fast-twitch muscles. Several studies indicate that calcineurin supports hypertrophy of slow muscle fibers. In one such study, overexpression of calcineurin induced marked hypertrophy of the slow-type soleus muscle (Talmadge et al., 2004). Conversely, muscle-specific overexpression of RCAN1, an inhibitor of calcineurin, reduced the cross-sectional area of the soleus muscle (Oh et al., 2005). In a separate study, cyclosporin A blocked soleus muscle hypertrophy in response to mechanical overloading (Sakuma et al., 2008).

Downstream effectors of calcineurin signaling include the NFAT (nuclear factor of activated T-cells) family of transcription factors which translocate to the nucleus after dephosphorylation by calcineurin and form complexes with other transcription factors including GATA2 and myocyte enhancer factor 2 (MEF2) (Bassel-Duby and Olson, 2006). Once in the nucleus, NFATs regulate the transcription of specific muscle genes that are important for skeletal muscle development and differentiation as well as slow-twitch fiber determination. Four NFAT isoforms (NFATc1-NFATc4) are expressed in skeletal muscle. NFATc1 has been shown to be preferentially activated by low-frequency electrical stimulation that is a characteristic of slow-twitch motor neurons and to promote the expression of slow-twitch fiber genes (Calabria et al., 2009; McCullagh et al., 2004; Schiaffino, 2010). Activity of calcineurin is inhibited by a family of genes referred to as regulators of calcineurin (RCAN) (Davies et al., 2007). Three such genes are known, RCAN1, RCAN2 and RCAN3. The human RCAN1 gene comprises 7 exons, the first 4 of which are alternative first exons, resulting in different isoforms named for the first exon they include as RCAN1.1 to RCAN1.4 (previously called MCIP1.4); the isoforms show different patterns of expression and regulation (Fuentes et al., 1997). Nuclear localization of NFAT isoforms, and expression of NFAT-dependent genes, such as RCAN1.4, have been used as functionally relevant measures of calcineurin activity (e.g., Ni et al., 2006).

Based on our finding that the expression of RCAN2 is reduced by nandrolone in denervated muscle, we hypothesized that calcineurin activity and signaling in muscle were reduced by denervation and were increased by nandrolone. We also hypothesized that increased calcineurin activity induced by nandrolone would increase myotube size and be critical to the ability of nandrolone to slow denervation atrophy. Currently, there have been no studies evaluating the effects of androgens on calcineurin activity or signaling downstream of calcineurin, and the possibility that activation of calcineurin by androgens is a mechanism for slowing atrophy of denervated muscle has also not been examined. Therefore, we tested the effects of nandrolone on calcineurin activity and signaling through NFAT in an animal model of sciatic nerve-induced denervation atrophy and in androgen-induced hypertrophy of cultured L6 myoblasts. We assessed calcineurin phosphatase activity and total cellular calcineurin levels. Signaling through NFAT was determined by measuring nuclear translocation of NFATc4 and expression levels of RCAN1.4. We note that our choice of NFATc4 but not other isoforms (NFATc1-3) was based on a practical reason since this isoform was the most easily and reliably detected in the nuclear fractions of paralyzed muscle. Finally, the effects of nandrolone on expression levels of several determinants (e.g., RCAN2 and FOXO1) of calcineurin–NFAT signaling were investigated because these proteins are known to modulate calcineurin activity (Ni et al., 2006; Rothermel et al., 2000). MEF-2C levels were tested as well given the critical role of MEF2 transcription factors in transcriptional control by NFAT family transcription factors (Dunn et al., 2001).

2. Materials and methods

2.1. Animals, sciatic nerve transection and drug administration

The animal studies were reviewed and approved by the James J. Peters VA Institutional Animal Care and Use Committee. The studies employed a sciatic nerve transection of denervation atrophy. Sham-denervated groups were also included to control for non-specific effects of the surgery and anesthesia. The procedures used to create the animal model and characteristics of the muscle from the denervated and sham-denervated hindlimbs have been described previously (Qin et al., 2010a; Zhao et al., 2008a). Two studies were performed.

In one study, male Wistar rats underwent left sciatic nerve transection or sham sciatic nerve transection followed by administration of nandrolone (0.75 mg/kg/week) and a replacement dose of testosterone (2.8 mg/day) or vehicle (propylene glycerol), which were begun at 29 days after surgery and continued for an additional 28 days. Testosterone was included to control for possible reductions in circulating testosterone levels resulting from central actions of nandrolone on gonadotropin release from the hypothalamus and pituitary, as described in our previous reports (Qin et al., 2010a; Zhao et al., 2008a). The dose chosen has been previously shown to provide high–normal testosterone levels in rats (Borst et al., 2005). The combination of nandrolone and testosterone is referred to as 'NanTs' hereafter. Muscle tissue was weighted and flash-frozen at sacrifice and stored at –80 °C.

In a separate study, male Wistar rats underwent left sciatic nerve transaction followed by administration of NanTs or vehicle alone, or in combination with cyclosporin A (Toronto Research Chemicals Inc., North York, ON, Canada) administered by subcutaneous injection at a dose of 25 mg/kg once daily. These agents were initiated at 29 days after nerve transection and continued for 28 days. To control for possible effects of Cremophor (Sigma-Aldrich, St. Louis, MO), the vehicle used to solubilize cyclosporin A, animals not administered cyclosporine A were administered 10% cremophor.

2.2. Cell culture and measurements of cellular size and protein content

A line of rat L6 myoblasts stably expressing human AR (L6.AR cells) under a retroviral transgene was passaged as described (Wu et al., 2010b). Cells were seeded into wells of 6-well plates using 1×10^6 cells per well in DMEM containing 10% fetal bovine serum supplemented with antibiotics (1% penicillin/streptomycin) and incubated overnight. Cells were then incubated for 48 hours in media in which fetal bovine serum had been replaced by 2% horse serum to initiate differentiation. Hormones dissolved in ethanol, or ethanol alone, were added and cells were incubated for an additional 48 hours.

To measure cellular protein content, differentiated L6.AR cells were lysed with 1 × Lysis Buffer (Cell Signaling Technology, Inc., Danvers, MA). Protein concentrations were determined using the Bio-Rad Protein assay (BioRad, Hercules, CA) and expressed as μg protein per well. To test for changes in cell size, L6.AR cells were