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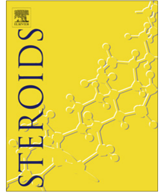

Transcriptional regulation of myotrophic actions by testosterone and trenbolone on androgen-responsive muscle

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A B S T R A C T

Androgens regulate body composition and skeletal muscle mass in males, but the molecular mechanisms are not fully understood. Recently, we demonstrated that trenbolone (a potent synthetic testosterone analogue that is not a substrate for 5-alpha reductase or for aromatase) induces myotrophic effects in skeletal muscle without causing prostate enlargement, which is in contrast to the known prostate enlarging effects of testosterone. These previous results suggest that the 5α-reduction of testosterone is not required for myotrophic action. We now report differential gene expression in response to testosterone versus trenbolone in the highly androgen-sensitive levator ani/bulbocavernosus (LABC) muscle complex of the adult rat after 6 weeks of orchiectomy (ORX), using real time PCR. The ORX-induced expression of atrogenes (Muscle RING-finger protein-1 [MuRF1] and atrogin-1) was suppressed by both androgens, with trenbolone producing a greater suppression of atrogin-1 mRNA compared to testosterone. Both androgens elevated expression of anabolic genes (insulin-like growth factor-1 and mechano-growth factor) after ORX. ORX-induced increases in expression of glucocorticoid receptor (GR) mRNA were suppressed by trenbolone treatment, but not testosterone. In ORX animals, testosterone promoted WNT1-inducible-signaling pathway protein 2 (WISP-2) gene expression while trenbolone did not. Testosterone and trenbolone equally enhanced muscle regeneration as shown by increases in LABC mass and in protein expression of embryonic myosin by western blotting. In addition, testosterone increased WISP-2 protein levels. Together, these findings identify specific mechanisms by which testosterone and trenbolone may regulate skeletal muscle maintenance and growth.

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1. Introduction

Loss of muscle mass and bone mineral density (BMD) with aging predisposes individuals to increased risk of disability, falls, and dependency in the elderly [1,2]. Many factors contribute to this deleterious process, such as decreased physical activity, nutritional deficits, and reductions in anabolic stimuli, including growth hormone, insulin-like growth factor-1 (IGF-1), and testosterone [3]. In particular, the decline in testosterone that occurs with age may contribute to reduced muscle strength in men [4]. In addition, androgen deprivation has been shown to prevent muscle regeneration in aging mice [5]. As such, androgen therapy for older hypogonadal men holds promise for maintaining and increasing muscle mass [3], and for improving clinical outcomes and quality of life [6].

Recent research has focused on the safety and efficacy of testosterone replacement therapy [7] and of treatment with other androgens or selective androgen receptor modulators (SARMs) that are designed to prevent both sarcopenia and osteopenia [8]. Ideally, these treatments would produce beneficial responses in tissues of interest (e.g., muscle and bone), while avoiding androgenic

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activity in other tissues (e.g., prostate) [8]. In this regard, trenbolone (a highly potent non-5α-reducible and non-estrogenic synthetic testosterone analogue) holds great promise. The affinity of trenbolone for the human and rodent androgen receptor is 3-fold higher than that of testosterone and approximately equal to that of dihydrotestosterone (DHT) [8]; however, trenbolone produces less potent effects than testosterone in the prostate. As evidence, our laboratory has reported that low-dose trenbolone produces myotrophic action in muscle and prevents hypogonadism-induced bone loss in orchiectomized rats in a magnitude equal to that of supraphysiologic testosterone, without inducing prostate enlargement [9,10]. However, the mechanisms by which testosterone and trenbolone increase muscle mass remain poorly understood.

Skeletal muscle consists primarily of postmitotic fibers, which undergo constant remodeling and regeneration throughout the life span. The phosphoinositide 3-kinase (PI3K)-Akt1 pathway is considered a primary signaling pathway regulating muscle protein synthesis [11]. This pathway leads to the regulation of mTOR kinase, which is sufficient to induce muscle hypertrophy by enhancing integral components of the protein synthesis machinery [12]. IGF-1 is a key activator of the PI3K-Akt1 pathway [11]. The importance of local muscle-produced IGF-I in hypertrophy is emphasized by the normal growth seen in mice that lack the circulating 'liver' form of IGF-I [13], and by increased IGF-I mRNA levels in the overloaded muscles of surgically hypophysectomized rats [14]. In humans, testosterone deficiency is associated with reduced circulating IGF-1 [15] and intramuscular IGF-1 expression [16]. The ubiquitin–proteasome pathway increases protein breakdown during skeletal muscle atrophy. Two ubiquitin ligases, Muscle Ring Finger1 (MuRF1) and Muscle Atrophy F-box (MAFbx) also called Atrogin-1 [17,18], serve as markers of skeletal muscle atrophy under a multitude of catabolic perturbations, such as fasting, diabetes, cancer, renal failure, and experimental sepsis [17,19,20].

Testosterone induces expression of the myogenic regulatory factor MyoD and of myosin heavy chain, suggesting that testosterone may promote muscle regeneration [21]. Wingless/Int (Wnt) is a family of secreted glycoproteins that regulate cell proliferation and differentiation [22]. Testosterone activates Wnt signaling, which allows β-catenin to translocate to the nucleus where it activates transcription of Wnt regulated target genes and regulates the differentiation of satellite cells [23]. In adult skeletal muscle, Wnt signaling influences satellite cells, but the precise downstream effect is still under debate. A common target for both androgen and estrogen receptors, Wnt1-inducible signaling protein 2 (WISP2) has been demonstrated to be activated by both androgens and estrogens in cattle and sheep muscle [24].

The purpose of the current study was to examine the long-term effects of orchiectomy and androgen (testosterone or trenbolone) treatment on expression of anabolic and catabolic genes in an androgen sensitive muscle in mature male rats. Furthermore, we characterized the effects of both androgens on muscle regeneration by measuring the protein levels of embryonic myosin. In addition, we aimed to detect potential differences in mechanisms of action between testosterone and trenbolone in regulating muscle mass.

2. Materials and methods

2.1. Animal care

All experimental procedures conformed to the ILAR Guide to the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Gainesville VA Medical Center. Barrier-raised and viral pathogen-free Fischer F344/Brown Norway male rats aged 10 months were obtained from Charles River Laboratories (Wilmington, MA). Animals were individually housed in an accredited animal facility at the Gainesville VA Medical Center under a 12 h light–12 h dark cycle. All rats underwent a one week acclimatization period prior to beginning experimental interventions. Rats were fed a diet of Purina rodent chow containing 3.3 kcal/g, distributed as 58.9% carbohydrate, 12.4% fat and 28.7% protein (No. 5001, Purina Mills, St. Louis MO) and tap water *ad libitum*.

2.2. Experimental design

Rats were divided into 4 groups ($n = 10$/group), including: sham surgery plus vehicle (SHAM), orchiectomy (ORX), ORX+(7.0 mg·week-1) testosterone-enanthate (TE) (ORX + TE), and ORX+(1.0 mg·week-1) trenbolone-enanthate (ORX + TREN). All treatment groups except SHAM group underwent bilateral orchiectomy and removal of epididymal fat under isoflurane anesthesia (5% induction, 1.5–2.5% maintenance), and received buprenorphine analgesia to reduce pain. A nutritional supplement (Jell-O cube with added protein and fat) (NIH protocol diet) was provided following the surgery for one week in order to minimize weight loss resulting from surgery. One week after the surgery, rats received weekly intramuscular (quadriceps) injections of vehicle (sesame oil) or androgen alternatively for five weeks. At day 42, rats were euthanized via intraperitoneal pentobarbital (120 mg/kg) injection. The levator ani/bulbocavernosus (LABC) muscle was removed, weighed, and snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Previously, we have reported serum androgen levels, prostate weight, bone morphological and muscle adaptations, and hemoglobin and adipocytokine changes [25].

2.3. Hormone delivery

Testosterone-enanthate (Savient Pharmaceutical, East Brunswick, NJ), trenbolone-enanthate (Steraloids, Newport, RI), and vehicle were dissolved in sesame oil and administered (0.1 mL) once every seven days, under isoflurane anesthesia, into the quadriceps musculature. Injections were alternated between legs to reduce possible discomfort of repeated injections. Previous studies from our laboratory have reported that once-weekly supraphysiological (7.0 mg/week) TE administration [26–28] or once weekly TREN (1.0 mg/week) [10] administration successfully prevents skeletal muscle and bone loss in growing ORX rodents (aged 3 months).

2.4. Real-time PCR assessment of the gene expression

Real-time PCR was performed on the LABC muscle complex from a subset ($n = 6$) of animals from each group. Samples were chosen that best approximated the mean LABC mass of each group in order to obtain a representative sampling from each group. Total RNA was extracted from the LABC muscle with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA). The RNA was solubilized in nuclease-free H_2O, incubated with DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove any DNA present in the sample. Total RNA quantity was determined by absorbance at 260 nm using a NanoVue spectrophotometer (GE Healthcare, USA). For each sample, cDNA was synthesized from 1 microgram of RNA using a RETRO-script first-strand synthesis kit (Ambion, Austin, TX, USA). cDNA was used as a template for real-time PCR using the primers listed below and a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). TaqMan probe-based chemistry was used to detect PCR products, and quantification was performed using a relative standard curve. Primers were purchased from Life technologies: IGF-1 (Rn00710306_m1), mechano-growth Factor (MGF, Rn01503688_m1), androgen receptor (AR, Rn00560747_m1), atro-

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