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Androgen-Mediated Regulation of Skeletal Muscle Protein Balance

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Abstract

Androgens significantly alter muscle mass in part by shifting protein balance in favor of net protein accretion. During various atrophic conditions, the clinical impact of decreased production or bioavailability of androgens (termed hypogonadism) is important as a loss of muscle mass is intimately linked with survival outcome. While androgen replacement therapy increases muscle mass in part by restoring protein balance, this is not a comprehensive treatment option due to potential side effects. Therefore, an understanding of the mechanisms by which androgens alter protein balance is needed for the development of androgen-independent therapies. While the data in humans suggest androgens alter protein balance (both synthesis and breakdown) in the fasted metabolic state, a predominant molecular mechanism(s) behind this observation is still lacking. This failure is likely due in part to inconsistent experimental design between studies including failure to control nutrient/feeding status, the method of altering androgens, and the model systems utilized.

Keywords

Hypertrophy; Atrophy; Anabolic; Catabolic; Testosterone; Autophagy

3.1 INTRODUCTION

The importance of maintaining skeletal muscle mass during various catabolic conditions is becoming increasingly recognized since muscle wasting into older age is predictive of an unfavorable survival outcome (Martin, Birdsell et al., 2013). In males, reduced production or bioavailability of androgens, termed hypogonadism, directly contributes to muscle atrophy

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since androgens play a major role in the maintenance or restoration of muscle mass (Ferrando, Sheffield-Moore et al., 2003, Steiner, Fukuda et al., 2016, White, Gao et al., 2013, White, Puppa et al., 2013, Atkinson, Srinivas-Shankar et al., 2010). While a therapy such as resistance exercise is effective at increasing muscle mass during hypogonadal conditions (Sullivan, Roberson et al., 2005), there is also evidence that resistance exercise cannot increase mass to the same absolute value achieved by those with circulating androgen levels in the physiological range (Kvorning, Andersen et al., 2006). This highlights the important physiological role of androgens, in conjunction with other factors such as physical activity, in the overall maintenance of muscle mass.

Androgen-mediated changes in muscle mass are due in part to alterations in muscle protein balance with hypogonadism shifting this balance in favor of net protein breakdown (Ferrando et al., 2003, Ferrando, Tipton et al., 1998, Sheffield-Moore, Urban et al., 1999). Several studies have examined the molecular factors implicated in androgen-mediated changes in muscle size and protein metabolism (i.e. (Hughes D.C., 2012); however, numerous experimental inconsistencies preclude a definitive conclusion from being made about the predominant factors/pathways contributing to this change in protein balance. This is important because mimicking the effects of androgens pharmacologically to increase muscle mass is required for those individuals in which androgen replacement is not a treatment option due to potentially negative side effects (Atkinson et al., 2010, Bassil, Alkaade et al., 2009). For example, androgens may augment the growth of a cancer tumor, making androgen replacement a non-viable option for those with established cancer tumors and suffering from cancer cachexia (Huggins and Hodges, 2002, Amos-Landgraf, Heijmans et al., 2014)Therefore, the goal of this review is to critically discuss the molecular factors thought to contribute to the effects of androgens on skeletal muscle protein balance and to identify critical areas of future research required for the continual progression towards the development of androgen-independent therapies.

4.1 ANDROGENS

Androgens represent a class of hormones predominantly responsible for the development of male secondary sex characteristics including increased muscle mass (White et al., 2013, Guyton Ac, 2006). While females also synthesize androgens, circulating concentrations are much lower (Guyton AC, 2006), likely contributing to their smaller muscle mass. In males, androgens are synthesized in the Leydig cells of the testes using cholesterol as a precursor (Guyton AC, 2006). The adrenal cortex also produces androgen hormones, though the contribution of this alternative source to overall levels in males is thought to be negligible (Guyton AC, 2006). In contrast, this non-gonadal source in females accounts for a much larger portion of total androgen production (Guyton AC, 2006). Testosterone and its reduced metabolite, 5α-dihydrotestosterone (DHT), are the two most prominent anabolic androgens and can be produced locally in skeletal muscle from precursor androgens (i.e. Dehydroepiandrosterone; DHEA) via the enzymes 3β-hydroxy-steriod dehydrogenase, 17β-hydroxy-steriod dehydrogenase, or 5α-reductase (Sato, Iemitsu et al., 2008, Aizawa, Iemitsu et al., 2007). However, the *in vitro* concentrations of DHEA and/or testosterone precursors needed to induce this conversion were in the micromolar range and well above normal physiological concentrations, indicating that further *in vivo* studies are required to confirm

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whether this occurs when circulating concentrations are much lower than those used *in vitro* (Hopper and Yen, 1975, Velders and Diel, 2013). In general, circulating total testosterone values between 17 and 35 nmol/l are considered to be in the normal physiological range for males (Velders and Diel, 2013, Sader, Griffiths et al., 2003). Despite circulating concentrations of androgens being most frequently reported, concentrations within the tissues may also be important. For instance, evidence suggests that concentrations of androgens in skeletal muscle, rather than in circulation, is more predictive of strength and muscle cross sectional area at least in older men (Sato, Iemitsu et al., 2014). Further, while not conducted in muscle cells, intracellular androgen concentrations in cultured prostate cancer cells differ from those values observed in the surrounding culture media. When extrapolated to skeletal muscle, this suggests that measurement of hypogonadal or physiological concentrations of androgens in circulation may not be representative of those levels within skeletal muscle (Sedelaar and Isaacs, 2009, Wu, Godoy et al., 2013).

The most recognized androgen mechanism of action is through binding to the cytosolic androgen receptor (AR) (Guyton AC, 2006). Upon androgen binding, the AR translocates to the nucleus where it interacts with the androgen response element (ARE) of target genes to alter gene transcription (both positively and negatively) (Guyton AC, 2006). However, the role of this mechanism *in vivo* has been questioned since the dissociation constant (K_d) of testosterone or DHT for the androgen receptor was estimated to be ~2–5 nM (Wilson and French, 1976), which can be lower than androgen concentrations found in hypogonadal males (i.e. <17 nmol/l) (Velders and Diel, 2013). Thus, the receptor could be saturated even in a hypogonadal state, suggesting that alternative androgen-mediated mechanisms exist. Indeed, testosterone administration to L6 myoblasts in culture altered signaling events within 20 minutes of exposure (Wu, Bauman et al., 2010); a time frame which is likely to be shorter than the traditional AR-mediated changes in gene transcription; illustrating the presence of alternative mechanisms of action. However, these alternative mechanisms remain poorly defined and require further attention representing an avenue for pharmacological intervention.

5.1 REGULATION of PROTEIN BALANCE

Skeletal muscle mass is regulated in part by the coordinated balance between rates of muscle protein synthesis and muscle protein breakdown. In healthy individuals, where muscle mass is maintained, these two processes wax and wane throughout the diurnal cycle in response to anabolic (i.e. nutrient consumption) and catabolic (i.e. fasting) stimuli (Phillips, Glover et al., 2009). Conversely, a long-term shift in this balance favoring net protein synthesis results in muscle hypertrophy while a long-term shift favoring net protein breakdown results in muscle atrophy (Phillips et al., 2009). These concepts and the molecular regulation of each have been reviewed elsewhere and therefore are only briefly summarized (Gordon, Kelleher et al., 2013, Hornberger, 2011, Kimball and Jefferson, 2010, Laplante and Sabatini, 2009, Ma and Blenis, 2009, Milan, Romanello et al., 2015, Sandri, 2010, Sandri, 2013, Goodman and Hornberger, 2014).

In general, the increase in protein synthesis following anabolic stimuli requires signaling through the mechanistic target of rapamycin in complex 1 (mTORC1) (Dickinson, Fry et al.,

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2011, Drummond, Fry et al., 2009). Signaling through mTORC1 regulates mRNA translation initiation as well as peptide chain elongation through phosphorylation of at least two known substrates termed the 70 kD ribosomal protein S6 kinase 1 (p70S6K1) and the eukaryotic initiation factor 4E (eIF4) binding protein 1 (4E-BP1) (Kimball and Jefferson, 2010). Various upstream effectors regulate the magnitude of mTORC1 activity. Positive effectors include phosphorylation and activation of Akt (a.k.a. protein kinase b) by hormones such as insulin and IGF-1, which promotes mTORC1 activity through phosphorylation and subsequent inhibition of proteins including Tuberous Sclerosis 2 (TSC2; a.k.a Tuberin) and proline-rich Akt substrate of 40 kD (PRAS40) (Dennis, Baum et al., 2011, Dennis, Coleman et al., 2014). Amino acids also activate mTORC1 signaling through a mechanism that is distinct from Akt (Dennis et al., 2011, Sancak, Bar-Peled et al., 2010, Sancak, Peterson et al., 2008). Conversely, mTORC1 signaling is inhibited by activation of the 5′ AMP-activated protein kinase (AMPK) and expression of Regulated in Development and DNA Damage 1 (REDD1) (Sullivan et al., 2005, Dennis et al., 2014, Gordon, Steiner et al., 2014, Gordon, Williamson et al., 2015, Bolster, Crozier et al., 2002). While mTORC1 is required for the increase in protein synthesis following anabolic stimuli, its role in regulating protein synthesis during the basal, non-stimulated condition is less clear. For example, treating humans with rapamycin to inhibit mTORC1 did not alter global rates of muscle protein synthesis in the fasted metabolic condition (Dickinson, Drummond et al., 2013) though it was sufficient to completely block the nutrient-induced stimulation of this process (Dickinson et al., 2011). Evidence also indicates mTORC1-independent mechanisms are involved in the regulation of muscle protein synthesis following anabolic stimuli (West, Baehr et al., 2016), although this concept is less well-defined.

Protein breakdown encompasses two general processes; the selective protease-mediated breakdown of poly ubiquitylated proteins via the ubiquitin proteasome system (UPS), and the lysosomal-mediated breakdown of bulk or specific cellular components/proteins termed autophagy (Sandri, 2013). The activity of the UPS has been linked to changes in E3 ubiquitin ligase expression (Sandri, 2013). The two most prominent skeletal muscle specific E3 ligases, collectively termed the atrogenes, are Muscle RING-finger protein-1 (MuRF1) and Muscle Atrophy F-box (MAFbx/atrogin-1) (Bodine, Latres et al., 2001). Their expression is markedly increased during various atrophic conditions (White et al., 2013, Bodine et al., 2001, Kelleher, Gordon et al., 2014, Kelleher, Kimball et al., 2013), and deletion of these atrogenes can attenuate muscle atrophy (Bodine et al., 2001). However, expression of these atrogenes does not necessarily correlate with UPS activity as global deletion of MuRF1 increased UPS activity in the skeletal muscle of aged mice, suggesting that other factors contribute to its activation (Hwee, Baehr et al., 2014). Indeed, additional E3 ligases have recently been identified in muscle. For example, expression of Muscle ubiquitin ligase of SCF complex in atrophy 1 (MUSA1), FbxO21 (a.k.a. SMART), and FbxO31 accompanied the loss of skeletal muscle mass induced by fasting (Milan, Romanello et al., 2015). The expression of these E3 ligases, as well as atrogene expression, is regulated in part by the Forkhead box (FoxO) and Smad transcription factors (Sandri, Sandri et al., 2004, Sartori, Milan et al., 2009). FoxO signaling is inhibited by post translational modification (e.g. phosphorylation) from upstream effectors such as Akt (Sandri et al., 2004). Conversely, phosphorylation and subsequent activation of Smad

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transcription factors is increased following stimulation of the activin receptors by the transforming growth factor beta (TGFβ) family of cytokines (Goodman and Hornberger, 2014, Sartori et al., 2009). In particular, Myostatin, a TGFβ family member, is well known to negatively regulate muscle mass in mice, cattle, dogs, and humans (Lee, 2004) through upregulation of atrogene expression although other mechanisms have been suggested as the changes in atrogenes are inconsistent (Mcfarlane, Plummer et al., 2006, Trendelenburg, Meyer et al., 2009). In this regard, myostatin was shown to negatively affect muscle satellite cell number and activation, while muscle hypertrophy induced by myostatin inhibition occurred independent of myonuclear accretion (Welle, Mehta et al., 2011). Further details pertaining to the role of myostatin in skeletal muscle can be found elsewhere (Rodriguez, Vernus et al., 2014).

Initiation of autophagy is regulated by various signals including mTORC1 signaling, AMPK activation, and expression of regulatory proteins such as REDD1 and BCL2/Adenovirus E1B 19kDa Interacting Protein 3 (BNIP3) (Gordon et al., 2014, Kim, Kundu et al., 2011, Qiao, Dennis et al., 2015, Zhang, Xue et al., 2016). For instance, mTORC1 phosphorylates several proteins such as uncoordinated like kinase 1 (ULK1) to inhibit the initiation steps of autophagy (Kim et al., 2011). Conversely, AMPK phosphorylates ULK1 on different residues to promote the initiation of autophagy (Kim et al., 2011). Expression of REDD1 or BNIP3 also alters this metabolic process. For example, mouse embryonic fibroblasts (MEFs) lacking the REDD1 gene were resistant to autophagy induction in what appeared to be an mTORC1-independent manner (Qiao et al., 2015). Likewise, expression of BNIP3 is sufficient to induce the autophagic removal of mitochondria; a process termed mitophagy (Zhang et al., 2016).

6.1 ANDROGEN REGULATION OF MUSCLE PROTEIN SYNTHESIS

In humans, androgens alter protein synthesis when measured in the fasted, but not the fed, metabolic state. For example, short term (i.e. <5 days) and long term (i.e. 6 months) androgen treatment each increased rates of protein synthesis following an overnight fast in both young and aged male subjects (Ferrando et al., 1998, Sheffield-Moore et al., 1999, Griggs, Kingston et al., 1989, Urban, Bodenburg et al., 1995, Brodsky, Balagopal et al., 1996). However, no differences were observed following amino acid stimulation (Ferrando et al., 2003, Sheffield-Moore, Wolfe et al., 2000). Similarly, supraphysiological testosterone administration to post-menopausal women increased muscle protein synthesis following an overnight fast (Smith, Yoshino et al., 2014). This is not a universal phenomenon as treating aged males with androgens in which androgens were raised to the normal physiological range did not alter protein synthesis in the fasted state (Ferrando et al., 2003, Ferrando, Sheffield-Moore et al., 2002). A major factor promoting these discrepant findings related to protein synthetic rate may be due to the androgen concentrations prior to the measurement (hypogonadal vs. supraphysiological). For instance, synthetic rate was increased at 5–7 days following a testosterone enanthate (TE) injection even though testosterone levels were in the normal physiological range by the time of the synthetic measurement (Ferrando et al., 1998, Griggs et al., 1989, Urban et al., 1995). It is possible that the increased synthetic rate observed was a residual effect from when testosterone levels were in the supraphysiological range at earlier time points (i.e. 1–3 days post injection) (Snyder, 1984). Conversely,

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increasing androgen levels from the lower physiological/hypogondal range to the physiological range did not have this same effect (Ferrando et al., 2003, Ferrando et al., 2002), suggesting that only supraphysiological concentrations of androgens alter proteins synthesis or lead to a greater magnitude of change (Fig. 1). The androgen administered may have also contributed to the discordance in synthetic rate. For example, one study showed that testosterone cypionate (TC) increased muscle protein synthesis 14 days post injection while others using TE did not (Ferrando et al., 2003, Brodsky et al., 1996, Ferrando et al., 2002). Importantly, the circulating testosterone concentrations within these studies were all similar at the time of the synthetic measurement. This lone study reporting an increase in synthetic rate following TC administration may be an anomaly, or it may indicate that the cypionate ester in the testosterone molecule enhanced its anabolic effect since the pharmacokinetics of TE or TC is equivalent (Schultebeerbuhl and Nieschlag, 1980). In future studies, utilizing a consistent method of altering androgens such as implantable, timed release pellets, rather than a bolus method such as injections, will help resolve these issues.

Studies in animal or *ex vivo* models have yielded conflicting results regarding androgen-mediated regulation of skeletal muscle protein synthesis. In one study, castration decreased rates of protein synthesis in the gastrocnemius of mice, while restoration of androgens through weekly injections of nandrolone decanoate normalized this measure (White et al., 2013). Likewise, *ex vivo* treatment of isolated extensor digitorum longus (EDL) and soleus muscle fiber bundles from elderly female mice (~700 days old) with 2 nM DHT increased rates of protein synthesis (Wendowski, Redshaw et al., 2016). Alternatively, castration did not change rates of protein synthesis in the tibialis anterior (TA) of mice or the gastrocnemius of rats following an overnight fast (Steiner et al., 2016, Jiao, Pruznak et al., 2009). Synthetic rates in the TA were also not different between castrated and sham mice 4 hr following refeeding, nor did castration affect the increase in protein synthesis following a bout of high frequency muscle contractions (Steiner et al., 2016). Conversely, castration prevented the leucine-induced stimulation of protein synthesis in the gastrocnemius of rats, suggesting mediation of the contributing pathways (Jiao et al., 2009). Several methodological issues may explain the discordant findings between animal studies including the feeding paradigm, model system employed, and muscle or animal species used. For example, the metabolic status of the mice at sacrifice was not specified in the study by (White et al., 2013) whereas the others utilized overnight fasting and timed refeeding (Steiner et al., 2016, Jiao et al., 2009). Thus, future studies should consider using timed refeeding or overnight fasting to bypass this likely contributing factor. Measuring protein synthesis *ex vivo* could have also contributed to this discrepancy as the Ringer's solution in which the muscles were incubated may not have mimicked the *in vivo* cellular environment (Wendowski et al., 2016). Differences in the muscle analyzed (gastrocnemius vs. TA) or the species of the animals (rats vs. mice) may have further contributed. Regardless, the observation that castration did not alter muscle protein synthesis in the fasted state is consistent with the human studies in which global rates of muscle protein synthesis only appeared to be affected by supraphysiological androgen concentrations (Fig. 1). Thus, future studies should consider using timed refeeding or overnight fasting to ensure consistent findings across studies.

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6.2 MOLECULAR REGULATION OF MUSCLE PROTEIN SYNTHESIS BY ANDROGENS

The prominent molecular mechanism(s) behind the androgen-mediated increase in protein synthesis remain undefined although increased signaling through mTORC1 via upstream effectors such as IGF-1/Akt and/or extracellular signal-regulated kinase 1/2 (ERK1/2) have been hypothesized to contribute. In humans, IGF-1 mRNA and protein content were increased in the muscle of humans following androgen administration while the mRNA content of IGF-1 binding protein 4 (IGF-1BP-4) was decreased (Urban et al., 1995, Ferrando et al., 2002). However, blocking the IGF-1 receptor did not impair the supraphysiological testosterone-induced increase in C_2C_{12} myotube diameter (Hughes, Stewart et al., 2016). Conversely, AR blockade in these studies severely blunted the testosterone-induced increase in myotube formation and diameter in addition to reducing the mRNA content of the IGF-1 receptor, phosphorylation of Akt (Ser473) and phosphorylation of ERK1/2, suggesting that signaling through the AR precedes these downstream effects and is a more potent regulatory factor (Hughes et al., 2016). Additional indications of the importance of mTORC1 are garnered from animal and cell culture experiments including the finding that administration of supraphysiological concentrations of testosterone (100 nM) to primary rat myotubes increased myotube size in an mTORC1-dependent manner (Basualto-Alarcon, Jorquera et al., 2013). This report also showed that the androgen-mediated increase in mTORC1 signaling was preceded by activation of Akt (Basualto-Alarcon et al., 2013). Of interest, blocking the AR prior to testosterone treatment negated the increase in myotube size, but it was not determined whether Akt/mTORC1 signaling was affected by this blockade (Basualto-Alarcon et al., 2013). In further support of an mTORC1 dependent mechanism, administration of testosterone (100 nM) to L6 myoblasts increased cell diameter and protein content, and co-incubation with rapamycin negated this effect (Wu et al., 2010). ERK1/2 was implicated as an upstream activator as phosphorylation of ERK1/2 preceded the testosterone-induced mTORC1 activation (Wu et al., 2010). However, only inhibition of AR or phosphatidylinositol-3 kinase (PI3K), but not ERK1/2, prevented the testosterone induced increase in protein content. Additional support for a role of PI3K was also observed in control C_2C_{12} myotubes and C_2C_{12} myotubes that were subjected to population doubling to mimic “aged” skeletal muscle. Here, inhibition of PI3K reduced the testosterone (100 nM) induced increase in myotube differentiation and diameter in both cell types (Deane, Hughes et al., 2013). Despite these findings, the relationship between activation of either AR or PI3K and mTORC1 signaling remains elusive. Alternatively, the changes in mTORC1 signaling following androgen treatment may be due to enhanced availability of amino acids as *ex vivo* incubation of isolated soleus and EDL muscle fiber bundles from aged female mice with DHT increased the expression of the sodium-coupled neutral amino acid transporter (SNAT) 2 and L-type amino acid transporter (LAT) 2 channels (Wendowski et al., 2016). Collectively, these data support a role of mTORC1 in the regulation of muscle size and protein accretion by supraphysiological concentrations of androgens. However, protein synthesis and long-term changes in muscle mass were not measured in any of these mechanistic studies, which is important for defining the role of these molecular signals on androgen-mediated changes in muscle size. Furthermore, whether *in vitro/ex vivo* findings translate to humans is unclear considering that androgens appear to only increase protein

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synthetic rates under fasted conditions, which may not be accurately reproduced *in vitro/ex vivo* (Ferrando et al., 2003, Ferrando et al., 1998, Sheffield-Moore et al., 1999, Griggs et al., 1989, Urban et al., 1995, Brodsky et al., 1996, Sheffield-Moore et al., 2000, Smith et al., 2014, Ferrando et al., 2002).

Though shifting androgen levels from the physiological range to the hypogonadal range has yielded conflicting results regarding rates of protein synthesis, multiple studies show that mTORC1 signaling is reduced by this shift. For example, castration of mice reduced phosphorylation of mTOR (Ser2448), p70S6K1 (Thr389) and 4E-BP1 (Thr37/46) while androgen administration sufficiently restored these to sham levels (White et al., 2013). In this study, the changes in mTORC1 signaling were accompanied by corresponding changes in Akt and PRAS40 phosphorylation as well as REDD1 mRNA content, suggesting a regulatory role for these upstream factors (White et al., 2013). Of note, no change in the phosphorylation of AMPK (Thr172) was observed in this study following castration (White et al., 2013). In another study, castration of mice increased the phosphorylation of regulated associated protein of mTOR (Raptor) on Ser792 and decreased phosphorylation of TSC2 (Thr1462) in the levator ani and triceps brachii muscles (Serra, Sandor et al., 2013). Though these events would likely suppress mTORC1 signaling, no downstream measures of the activity of this signaling complex were assessed (e.g. phosphorylation of p70S6K1) (Serra et al., 2013). Using an overnight fasting model, phosphorylation of ribosomal protein s6 (rps6) on Ser235/236, a putative p70S6K1 substrate, was reduced in the gastrocnemius of castrated rats relative to sham values (Jiao et al., 2009). Notably, in this study, castration negated the leucine-induced stimulation of mTORC1 signaling, though a potential mechanism for this observation was not described (Jiao et al., 2009). Using a similar feeding paradigm, castration reduced the phosphorylation of mTORC1 substrates p70S6K1 (Thr389) and 4E-BP1 (Ser65) in the TA (Steiner et al., 2016). The protein content of REDD1 and phosphorylation of AMPK (Thr172) were also increased, likely contributing to the repression of mTORC1 signaling (Steiner et al., 2016). Interestingly, and in contrast to previous reports, the phosphorylation of Akt (Thr308) trended to increase ($P = 0.06$) in the muscle of castrated mice in the fasted state despite the repressed mTORC1 signaling (Steiner et al., 2016). This report went on to show that refeeding previously fasted mice negated the castration-induced repression in mTORC1 signaling (i.e. p70S6K1 (Thr389) and 4E-BP1 (Ser65)) as well as the increase in REDD1 protein content (Steiner et al., 2016). Collectively, these data suggest that mTORC1 signaling is sensitive to changes in androgen concentration, although alterations in the rate of muscle protein synthesis do not necessarily correspond (Table 1). Thus, the role of mTORC1 in the regulation of muscle protein synthesis, or one of its many other metabolic actions following changes in androgen levels, requires further investigation. Using genetic mouse models (i.e. gene knockout mice) or chemical inhibitors (i.e. rapamycin) in models of androgen administration will help define the role of mTORC1.

Lastly, there is evidence that androgens alter muscle translational capacity (i.e. ribosome content), which would impact protein balance. Here, castration of rats decreased the RNA content within the levator ani/bulbocavernosus muscle supporting a reduction in ribosome number. This paralleled reductions in markers of ribosome biogenesis including the content of the 47S pre-rRNA and the mRNA content of genes that regulate the transcription and

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processing of the pre-rRNA such as v-myc avian myelocytomatosis viral oncogene homolog (c-Myc), nuclear protein 56 (Nop56), block of proliferation1 (Bop1), and nucleolin (Ncl) (Mobley, Mumford et al., 2016). Interestingly, TE treatment restored the RNA content of the muscle to sham values, but the markers of ribosome biogenesis remained suppressed (Mobley et al., 2016), perhaps in an attempt to limit uncontrolled muscle growth.

7.1 ANDROGENS AND MUSCLE PROTEIN BREAKDOWN

In humans, the role of endogenous and exogenous androgens on muscle protein breakdown in the fasted state has yielded conflicting results. For example, TE administration in aged males reduced protein breakdown in two separate studies (Ferrando et al., 2003, Ferrando et al., 2002). Conversely, a single TE injection did not alter breakdown 5 days following administration in young males, though sufficient statistical power may have been lacking to measure the small change in breakdown that was observed (Ferrando et al., 1998). Further, 5 days of oxandrolone treatment to young males also failed to modulate rates of breakdown (Sheffield-Moore et al., 1999). Age, and thereby endogenous testosterone levels, may have contributed to the discrepant findings. For example, reduced protein breakdown occurred only in the aged (~67 years) subjects whose endogenous pretreatment testosterone levels were in the hypogonadal/lower physiological range (Ferrando et al., 2003, Ferrando et al., 2002). Meanwhile, endogenous pretreatment testosterone values in the young subjects were in the normal physiological range (Ferrando et al., 1998, Sheffield-Moore et al., 1999), suggesting that a shift in androgen levels from the hypogonadal range to the physiological range likely has a greater impact on protein breakdown compared to a shift from the physiological to the supraphysiological range (Fig. 1).

Androgen-mediated changes in markers of protein breakdown are also observed in animal models. For instance, 8 weeks of castration increased 20S proteasome activity within the gastrocnemius of rats following an overnight fast as well as in the levator ani muscle of mice 7 days post castration (Serra et al., 2013). Conversely, 20S proteasome activity was not increased in the triceps brachii at 50 days post castration surgery indicating a time and/or muscle dependent effect (Serra et al., 2013). Castration also reduced the content of ubiquitylated proteins in the TA of mice following an overnight fast relative to sham levels, while refeeding negated this effect (Steiner et al., 2016). Though speculative, it was concluded that the change in ubiquitylated proteins in this study following the overnight fast might have been reflective of an increase in UPS activity; however, UPS activity was not measured to confirm this possibility.

In addition to UPS activity, markers of autophagy activation are also sensitive to changes in androgen concentration. For example, activity of Cathepsin L and the microtubule-associated protein 1A/1B-light chain 3 (LC3) II/I ratio were elevated in the levator ani muscle within 7 days of castration, and administration of TE restored these markers (Serra et al., 2013). Similar findings were observed in the triceps brachii muscle in that Cathepsin L and the LC3 II/I ratio were increased in mice 50 days after castration surgery (Serra et al., 2013). However, at this time point, TE administration was unable to normalize the LC3 II/I ratio while Cathepsin L activity returned to sham values (Serra et al., 2013). In agreement with that study, the LC3 II/I ratio was increased and p62 protein content was decreased in

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the TA of castrated mice following an overnight fast relative to the values in sham mice (Steiner et al., 2016). Further, refeeding previously fasted castrated mice failed to restore these markers to levels observed in the refed sham mice (Steiner et al., 2016). The reason for the sustained elevation in autophagy markers in refed castrated mice is unknown, but it may indicate autophagic removal of specific muscle proteins or organelles.

7.2 MOLECULAR REGULATION OF MUSCLE PROTEIN DEGRADATION BY ANDROGENS

The prominent molecular mechanisms by which hypogonadism regulates protein breakdown are ill defined and based largely upon associative studies. In regards to the UPS, castration increased the mRNA content of both atrogenes (MuRF1 and MAFbx) within the levator ani, suggesting a role for the E3 ligases (Serra et al., 2013, De Naeyer, Lamon et al., 2014). A corresponding decrease in the phosphorylation of FoxO3a (Ser318/321) implied that the activation of this transcription factor promoted atrogene expression (Serra et al., 2013). These events were testosterone and AR sensitive as testosterone administration to castrated mice normalized these measures while AR blockade via flutamide negated the androgen-mediated restoration (Serra et al., 2013). In contrast, atrogene mRNA content was not increased 50 days post castration in the triceps brachii muscle, suggesting that the regulation of atrogene expression and UPS activity is either muscle type or time point specific (Serra et al., 2013). MuRF1 and MAFbx mRNA content were also increased in the levator ani/bulbocavernosus and gastrocnemius muscle of castrated rats and mice, respectively (White et al., 2013, Ye, Mccoy et al., 2014). Consistent with this change, the phosphorylation of FoxO3a (Ser253) was decreased in the gastrocnemius of castrated mice providing further support for this transcription factor in the atrogene expression during hypogonadism (White et al., 2013).

In contrast, reductions in atrogene mRNA content have also been reported following castration including a decrease in MuRF-1 and MAFbx in the gastrocnemius of castrated rats following an overnight fast compared to sham values (Jiao et al., 2009). Similarly, an overnight fast led to a non-significant decrease in atrogene mRNA content within the TA of castrated mice relative to sham values, and this was accompanied by a non-significant increase in FoxO3a (Ser253) phosphorylation (Steiner et al., 2016). Lastly, supraphysiological concentrations of testosterone (100 nM) failed to alter the mRNA content of MuRF-1 and MAFbx in primary rat myotubes while nandrolone decanoate treatment failed to alter atrogene expression in the soleus of mice (Basualto-Alarcon et al., 2013, Camerino, Desaphy et al., 2015). The reason(s) for the discrepant findings between animal studies is unknown, but like many of the other processes highlighted herein, it may be due to the feeding parameters and metabolic state of the animal when muscles were isolated. For instance, the later studies (i.e. (Steiner et al., 2016, Jiao et al., 2009) utilized overnight fasting to control the metabolic state at sacrifice while the others did not indicate the nutritional status of the animals (White et al., 2013, Serra et al., 2013). Additionally, the duration of androgen manipulation (7 days vs. 50 days) and/or the muscle analyzed (TA vs. Gastrocnemius vs. Triceps Brachii vs. Levator Ani) may also contribute. Future studies

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would benefit from using controlled feeding while also analyzing these molecular events in various muscle groups from the same animal.

Activin/Smad signaling also exhibited time-dependent effects following changes in androgen levels. Specifically, the content of myostatin, Activin A, Activin B, and Activin AB were all transiently increased throughout a 10-week castration time course in both the gastrocnemius and triceps brachii muscles (Pan, Singh et al., 2016). Further, the expression of growth differentiation factor 11 (GDF11), an activin receptor ligand, exhibited a transient expression pattern in these muscles following castration (Pan et al., 2016). Despite this transient pattern in TGFβ cytokine expression, phosphorylation of the downstream activin receptor substrate, Smad3 (Ser423/425), was only increased at the 4-week post castration time point (Pan et al., 2016). In contrast, another study showed phosphorylation of Smad2/3 and expression of mature myostatin protein were unaltered in the levator ani bulbocavernosus muscle 43 days following castration (Dalbo, Roberts et al., 2016). Administration of TE or trenbolone to previously castrated rats increased the expression of the mature myostatin protein and activin IIB mRNA content without altering Smad2/3 phosphorylation, suggesting that androgens may prevent Smad2/3 activation despite the increased myostatin expression (Dalbo et al., 2016). Despite these contradictory data, blocking activation of the activin receptors in castrated mice increased muscle mass to a value that was greater than those observed in sham mice (Pan et al., 2016). A caveat to this observation was that there was no mention of the effect of receptor blockade on the muscle mass of sham mice, precluding definitive conclusion(s) from being made.

While those studies show changes in activin/Smad signaling, other work has focused on androgen-mediated changes in the upstream mediator, myostatin. For example, myostatin expression in the gastrocnemius/plantaris complex and the soleus were repressed by testosterone in a dose dependent manner (Shigeo Kawada, 2006). Similarly, castration increased the expression of myostatin in the levator ani and EDL muscles, and this effect was reversed by testosterone administration (De Naeyer et al., 2014, Mendler, Baka et al., 2007). Testosterone administration to aged mice also decreased the expression of the mature myostatin peptide (Kovacheva, Hikim et al., 2010). This effect of testosterone on myostatin may be mediated via AR signaling inhibiting androgen binding to the AR increased myostatin mRNA content in both control and population doubled C_2C_{12} myotubes (Hughes et al., 2016). The suppressive effect of androgens on myostatin may also be related to satellite cell function as expression of Notch and Proliferating Cell Nuclear Antigen (PCNA) were increased in the gastrocnemius of aged mice following testosterone administration (Kovacheva et al., 2010, Sinha, Sinha-Hikim et al., 2014).

In direct contrast, myostatin expression was decreased in the levator ani muscle 30 days post castration, and testosterone administration increased this measure back to sham levels (De Naeyer et al., 2014). In line with this discordant finding, the myostatin gene was identified as a direct target of the AR in skeletal muscle (Dubois, Laurent et al., 2014). However, contrary to the putative atrophic role of myostatin, this report showed that increased AR signaling enhanced myostatin expression (Dubois et al., 2014). While these studies were in murine and cell culture models, treating post-menopausal women with supraphysiolocial concentrations of testosterone failed to alter myostatin or follistatin mRNA expression in the

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fasted state (Smith et al., 2014). Collectively, the data indicate that myostatin expression is altered by androgens, although only one report has investigated the direct role of myostatin on androgen-mediated growth. In this regard, administration of supraphysiological concentrations of testosterone or DHT to previously castrated myostatin null mice appeared to enhance the growth promoting effects of the androgen compared to castrated, wild type mice (i.e. an interaction between genotype and androgens). While this finding is consistent with the previously described role of the AR in the promotion of myostatin expression, the actual changes in muscle weight observed in this study were not reported, but rather, muscle weight corrected for body weight was described, which may be why this outcome was observed. The reason(s) for discrepancies between studies in regards to myostatin expression and androgens are not clear, but it may be due in part to the failure to control for feeding as myostatin has been shown to be sensitive to nutrient consumption (Carneiro, Gonzalez et al., 2013), the difference in the muscle analyzed (levator ani vs. gastrocnemius), or the species analyzed (rodents vs. humans). Future androgen related studies using a controlled feeding experimental paradigm, while at the same time analyzing various muscle groups, will help to further define the role of the TGFβ/myostatin/activin/Smad signaling pathway.

Similar to the UPS, the molecular regulation of autophagy by androgens is also largely based upon associative studies. For example, castration increased phosphorylation of the autophagy activator, AMPK (Thr172), as well as the mRNA content of autophagy related genes including BNIP3, Beclin1, and Transcription factor EB (Tfeb) in the levator ani and triceps brachii of mice (Serra et al., 2013). Similarly, castration increased REDD1 protein content and phosphorylation of AMPK (Thr172) as well as decreased phosphorylation of ULK1 (Ser757) in the TA of castrated mice following an overnight fast (Steiner et al., 2016). However, in contrast to the previous study (i.e. (Serra et al., 2013)), castration did not alter BNIP3 protein content in the TA when measured in the fasted metabolic state (Steiner et al., 2016). While REDD1 protein content and phosphorylation of AMPK and ULK1 were altered in a manner consistent with elevated autophagy in the TA following an overnight fast, only REDD1 protein content was found to be significantly correlated with the LC3 II/I ratio autophagy marker, suggesting a predominant role of this protein in the fasting-induced regulation of autophagy (Steiner et al., 2016). Of note, refeeding castrated mice prevented the castration-induced changes in REDD1 protein content and ULK1 (Ser757) phosphorylation observed in the fasted state even though autophagy markers remained elevated, suggesting an unknown mediator(s) of autophagy contributed to this elevation (Steiner et al., 2016). Overall, the preponderance of data indicated that a lack of androgens altered various autophagy regulatory factors in favor of increased autophagy. As with other factors discussed herein, discrepant findings may be largely due to feeding paradigms utilized. However, a major shortcoming of this body of knowledge is the lack of mechanistic experiments utilizing the manipulation of the expression/activation of the proposed regulatory factors. Also, the use of autophagy inhibitors such as colchicine will help define the contribution of autophagy to the overall shift in protein balance following androgen deprivation.

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8.1 CONCLUSION

It is well-accepted that androgens influence muscle mass, and this change is thought to occur in part through alterations in protein balance (Phillips et al., 2009). Accordingly, the data presented herein suggest that in the fasted metabolic state, supraphysiological concentrations of androgens increase muscle protein synthesis while hypogonadism increases protein breakdown (Fig. 1). Despite this concept being fairly well described in humans, the molecular factors thought to contribute to this effect (Fig. 2) are inconsistent between studies (both animal and cell culture). These inconsistencies are likely due in large part to the different nutrient/feeding paradigms utilized, especially as these methodological details were often not reported. As the human data overwhelmingly show that androgens alter muscle protein balance selectively in the fasted metabolic state (Sheffield-Moore et al., 2000), future mechanistic studies need to be cognizant of this concept. For example, the use of an overnight fast and/or refeeding paradigm will certainly help alleviate these discrepancies as this enables feeding status measurement at both ends of the metabolic spectrum (fasted vs. refed). However, this type of paradigm comes at the cost of making accurate muscle phenotype measurements (e.g. cross sectional area) due to the significant loss of body weight caused by the overnight fast. The translatability of cell culture systems to human models of either hypogonadism or androgen supplementation also needs to be addressed. For example, recapitulating the diurnal fluctuations in nutrient exposure that occur throughout the day in humans would be extremely time consuming and difficult in a cell-based system. Thus, it might be useful to perform serum or nutrient deprivation treatments prior to harvesting cells in this system in an attempt to more closely mimic the fasted metabolic state. Additionally, future work needs to be cognizant of how different androgen concentrations (i.e. hypogonadal vs. physiological vs. supraphysiological) appear to modulate protein balance (Fig. 1). While it is also important to initially identify molecular signals/events associated with androgen-mediated changes in protein synthesis and breakdown, future mechanistic work needs to delineate the role of these events by modifying their activation/expression. Using genetic knockout models (e.g. MuRF-1 and MAFbx null mice) or chemical inhibitors (e.g. rapamycin) would further our understanding of the androgen-mediated contribution of these factors/pathways in the regulation of muscle morphology and protein metabolism. Additionally, specificity of muscle groups/fiber types in response to androgen levels should also be determined including direct comparison of more androgen sensitive muscles, such as the levator ani, to less androgen sensitive muscles, such as the triceps brachii, within the same animal. Addressing these issues will help identify the most prominent mechanisms through which androgens regulate skeletal muscle protein balance, and therefore, expedite the development of new, androgen-independent therapies to offset muscle atrophy for those unable to undergo standard replacement therapy.

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Abbreviations

mTORC1	mechanistic target of rapamycin in complex 1
AR	androgen receptor
DHT	5α-dihydrotestosterone
TE	testosterone enanthate
TC	testosterone cypionate
ARE	androgen response element
p70S6K1	70 kD ribosomal protein S6 kinase 1
4E-BP1	eukaryotic initiation factor 4E binding protein 1
TSC2	tuberous sclerosis 2/tuberin
PRAS40	proline-rich Akt substrate of 40 kD
AMPK	5′ AMP-activated protein kinase
REDD1	regulated in development and DNA damage 1
UPS	ubiquitin proteasome system
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
MuRF-1	muscle RING-finger protein-1
MAFbx/atrogin-1	muscle Aatrophy F-box
FoxO	forkhead box
IGF-1BP-4	IGF-1 binding protein 4
PI3K	phosphatidylinositol-3 kinase
ERK1/2	extracellular signal-regulated kinase 1/2
SNAT2	sodium-coupled neutral amino acid transporter 2
LAT2	L-type amino acid transporter 2
rps6	ribosomal protein s6
EDL	extensor digitorum longus
TA	tibialis anterior
Raptor	regulated associated protein of mTOR
c-myc	v-myc avian myelocytomatosis viral oncogene homolog
Nop56	nuclear protein 56

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Bop1	block of proliferation1
Ncl	nucleolin
TGFβ	transforming growth factor beta
GDF11	growth differentiation factor 11
Tfeb	transcription factor EB
LC3	microtubule-associated protein 1A/1B-light chain 3
ULK1	uncoordinated like kinase 1
PCNA	proliferating cell nuclear antigen
DHEA	Dehydroepiandrosterone
MUSA1	Muscle ubiquitin ligase of SCF complex in atrophy 1

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Highlights

- In humans, androgens alter protein balance in the fasted metabolic state
- Predominant molecular factors altering protein balance are inconclusive
- Inconclusiveness likely due to many methodological differences between studies

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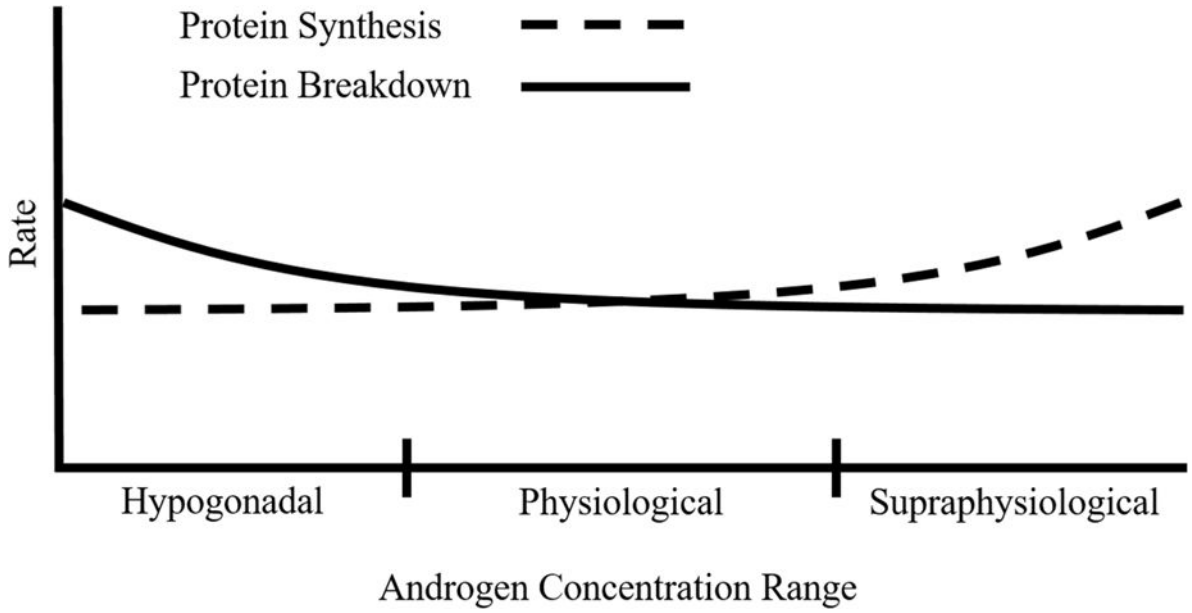

FIGURE 1.
Theoretical model by which androgens alter muscle protein balance at differing androgen concentrations.

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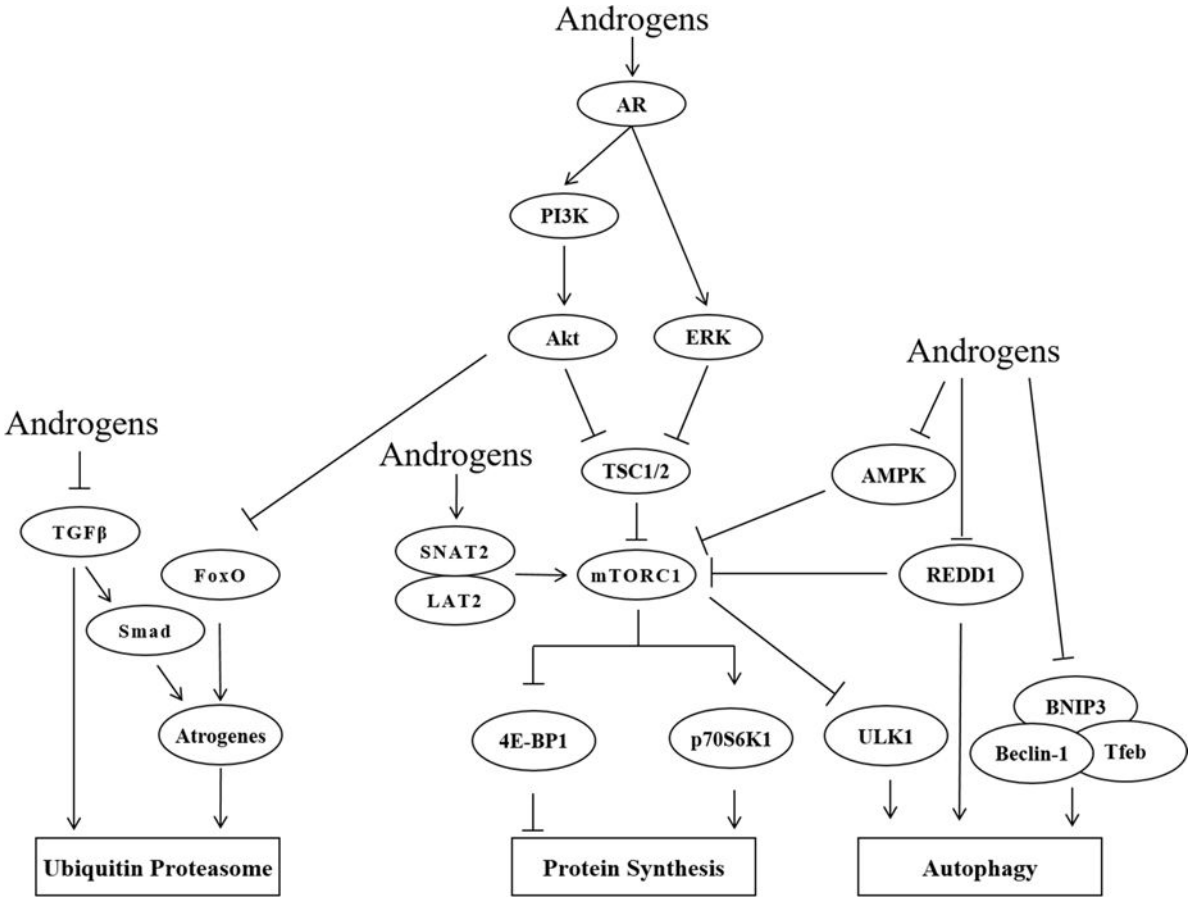

FIGURE 2.
Summary of the proposed mechanisms by which androgens alter muscle protein balance.

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TABLE 1

Summary of metabolic and molecular alterations following androgen manipulation.

Ref	Species/Sex	Muscle Analyzed	Androgen Given	T Conc. at Protein Metabolic Measure (nmol/l)	Metabolic State at Protein Metabolic Measure	Protein Synthesis	Protein Breakdown	Molecular Events Associated with Change in Androgen
67	Human/Male	VL	TE	17–28	Fasted	↔	↓	↑ IGF-1 protein
65	Human/Male	VL	Ox	Not Indicated	Fasted/AA Infusion	↑ Fasted, ↔ AA Infusion	↔	N/A
63	Human/Male	VL	TE	17.4–34.7	Fasted	↑	Not Indicated	↑ IGF-1 mRNA content; ↓ IGF-1BP-4 mRNA content
2	Human/Male	VL	TE	~23	Fasted/AA Infusion	↔	↓	N/A
9	Human/Male	VL	TE	~33	Fasted	↑	↔	N/A
10	Human/Male	VL	Ox	~8	Fasted	↑	↔	N/A
64	Human/Male	VL	TC	10.4–13.9	Fasted	↑	Not Indicated	N/A
62	Human/Male	VL	TE	>40	Fasted	↑	Not Indicated	N/A
66	Human/Female	Quad	T	~8.4	Fasted	↑	Not Indicated	↔ FOX03, MSTN, FST mRNA concentrations
71	Rat/Male	Gast	N/A	0.1	Fasted/Leucine Gavage	↔	↑	Fasted State: ↓ MuRF1 and MAFbx mRNA content, ↓ rps6 (Ser235/236). Following Leucine Gavage: ↓ rps6 (Ser235/236), 4E-BP1 phosphorylation, eIF4E bound to eIF4G; ↑ eIF4E bound to eIF4G
81	Rat/Male	LABC	TE/Tren	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Castration: ↔ Myostatin Protein, Smad 2/3 phosphorylation (site not indicated); ↓ MyostaBn, AcBvin Receptor IIb mRNA Content. Castration + Testosterone Enanthate and Trenbolone: ↑ Myostatin Protein; ↑ MyostaBn, Activiin Receptor IIb mRNA Content ; ↔ Smad 2/3 phosphorylation
76	Rat/Male	LABC	TE	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Castration: ↓ c-Myc, Nop56, Bop1, Ncl mRNA; ↓ 45S pre-rRNA. Castration + TE: Did not alter measures.

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Ref	Species/Sex	Muscle Analyzed	Androgen Given	T Conc. at Protein Metabolic Measure (nmol/l)	Metabolic State at Protein Metabolic Measure	Protein Synthesis	Protein Breakdown	Molecular Events Associated with Change in Androgen
78	Rat/Male	LABC	TE/Tren	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Castration: ↑ MuRF-1, MAFbx mRNA. Castration + TE or Tren: Normalized these measures.
83	Rat/Male	LA	TP	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Castration: ↑ myostatin protein. Castration + Testosterone: ↓ myostatin protein
4	Mouse/Male	Gast	ND	Not Indicated	Not Indicated	↓ by Castration ↑ by ND	Not Indicated	Castration: ↓ Akt (Ser473), mTOR (Ser2448), p70S6K (Thr389), 4E-BP1 (Thr37/46), PRAS40 (Thr246), Fox03A (Ser253); ↑ MuRF1, MAFbx, and REDD1 mRNA content. ND: Normalized all measures
80	Mouse/Male	Gast/Triceps Brachii	N/A	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Castration: ↑Myostatin, Activin A, Activin B, Activin AG, GDF-11 in a transient manner throughout 2–10 week timecourse. ↑ Smad2 phosphorylation only at 4 week time point.
75	Mouse/Male	LABC	TP	Not Indicated	Not Indicated	Not Indicated	↑ with Castration	Castration (Levator Ani): ↑MuRF1 and MAFbx mRNA; ↑LC3B, Bnip3, Beclin1, and Tfeb mRNA; ↓ Fox03A (Ser318/321), TSC2 (Thr1462); ↑ AMPKα (Thr172), Raptor (Ser792). Castration (Triceps Brachii): ↓ MuRF1 and ↔ MAFbx mRNA; ↑ LC3 II/I; ↑ Tfeb mRNA; ↓ Fox03A (Ser318/321), TSC2 (Thr1462); ↑ AMPKα (Thr172), Raptor (Ser792)
3	Mouse/Male	TA	N/A	<0.1	Fasted/Refed	↔	↑	Fasted State: ↓ p70S6K1 (Thr389), 4E-BP1 (Ser65), ULK1 (Ser757); ↑ REDD1 protein content, AMPK (Thr172); ↔ MAFbx or MuRF-1 mRNA content. Refed State: ↔ p70S6K1 (Thr389), 4E-BP1 (Ser65), ULK1 (Ser757), REDD1 Protein Content
79	Mouse/Male	Soleus	ND	Not Indicated	Not Indicated	Not Indicated	Not Indicated	↔ MuRF-1 mRNA Content

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Ref	Species/Sex	Muscle Analyzed	Androgen Given	T Conc. at Protein Metabolic Measure (nmol/l)	Metabolic State at Protein Metabolic Measure	Protein Synthesis	Protein Breakdown	Molecular Events Associated with Change in Androgen
86	Mouse/Male	LA	T/DHT	Not Indicated	Not Indicated	Not Indicated	Not Indicated	AR KO and Castration: ↓ myostatin mRNA, ↑ activin IIB mRNA.
82	Mouse/Male	Gast-Plant and Soleus	T	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Testosterone: ↓ myostatin expression in a dose dependent manner
84	Mouse/Male	Gast	T	20–28	Not Indicated	Not Indicated	Not Indicated	Testosterone: ↓ mature myostatin protein, ↑ Notch and PCNA protein
85	Mouse/Male	Gast	T	<0.1	Not Indicated	Not Indicated	Not Indicated	Testosterone: ↑ Notch and PCNA protein
77	Mouse/Male	LA, Soleus, EDL	T	Not Indicated	Not Indicated	Not Indicated	Not Indicated	Castration: ↑ MuRF-1, MAFbx, myostatin mRNA. Castration + Testosterone: ↓ MuRF-1, MAFbx, myostatin mRNA
72	Mouse	C2C12	T	N/A	DMEM + 2% Horse Serum	Not Indicated	Not Indicated	Testosterone: ↑ myotube diameter and myotube number in a n AR dependent manner. ↑ AR content
74	Mouse	C2C12	T	N/A	DMEM + 2% Horse Serum	Not Indicated	Not Indicated	Testosterone: ↑ myotube diameter in a PI3K dependent manner. ↓ myostatin mRNA content
25	Rat	L6 Myoblasts	T	N/A	DMEM + 2% Horse Serum	Not Indicated	Not Measured	↑ ERK1/2 (Thr202/Try204), p70S6K1 (Thr389), mTOR (Ser2481), rps6 (Ser235/236)
73	Rat/N/A	N/A	TE	N/A	DMEM-Ham's F-12 without serum	Not Indicated	Not Measured	↑ Phosphorylation of p70S6K1 (Thr412/389), ERK1/2 (Thr202/Try204), Akt (Ser473); ↔ MAFbx or MuRF-1 mRNA Content.
70	Mouse/Female	Soleus and EDL	DHT	N/A	Ringer's Solution	↑	Not Measured	With Age: ↓ Protein Synthesis (both EDL and Soleus); ↓ SNAT2 and LAT2 protein in EDL only. With Age + DHT: ↑ Protein Synthesis (both EDL and Soleus), SNAT2 and LAT2 protein (both EDL and Soleus)

T, Testosterone; TE, Testosterone Enanthate; TC, Testosterone Cypionate; ND, Nandrolone Decanoate; DHT, 5α-dihydrotestosterone; Gast, Gastrocnemius; Plant, Plantaris; EDL, Extensor Digitorum Longus; LABC, Levator Ani/Bulbocavernosus; N/A, Not Applicable; Quad, Quadriceps; Tren, Trenbolone; AA, Amino Acid; VL, Vastus Lateralis; Ox, Oxandrolone; TP, Testosterone Propionate; AR, Androgen Receptor; KO, Knockout; PCNA, Proliferating Cell Nuclear Antigen; PI3K, Phosphatidylinositol-3 Kinase