Stimulation of both estrogen and androgen receptors maintains skeletal muscle mass in gonadectomized male mice but mainly via different pathways

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Abstract

Testosterone is a major regulator of muscle mass. Little is known whether this is due to a direct stimulation of the androgen receptor (AR) or mediated by aromatization of testosterone to estradiol (E_2), the ligand for the estrogen receptors (ERs), in peripheral tissues. In this study, we differentiated between the effects mediated by AR and ER by treating orchidectomized (orx) male mice for 5 weeks with E_2 or the non-aromatizable androgen dihydrotestosterone (DHT). Both E_2 and DHT increased muscle weight and lean mass, although the effect was less marked after E_2 treatment. Studies of underlying mechanisms were performed using gene transcript profiling (microarray and real-time PCR) in skeletal muscle, and they demonstrated that E_2 regulated 51 genes and DHT regulated 187 genes, with 13 genes (=25% of E_2 -regulated genes) being regulated by both treatments. Both E_2 and DHT altered the expression of *Fbxo32*, a gene involved in skeletal muscle atrophy, affected the IGF1 system, and regulated genes involved in angiogenesis and the glutathione metabolic process. Only E_2 affected genes that regulate intermediary glucose and lipid metabolism, and only DHT increased the expression of genes involved in synaptic transmission and heme and polyamine biosynthesis. In summary, ER activation by E_2 treatment maintains skeletal muscle mass after orx. This effect is less marked than that of AR activation by DHT treatment, which completely prevented the effect of orx on muscle mass and was partly, but not fully, mediated via alternative pathways.

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Introduction

Testosterone is a major regulator of body composition. Androgen deficiency is associated with decreased muscle mass, and testosterone supplementation increases muscle mass in hypogonadal men, HIV-infected men, and older men with low testosterone concentrations (Snyder *et al.* 1999, 2000, Kong & Edmonds 2002). Also, in orchidectomized (orx) mice, testosterone treatment dose dependently increases the mass of individual muscles (Axell *et al.* 2006).

Both testosterone and the non-aromatizable androgen dihydrotestosterone (DHT) bind to and activate the androgen receptor (AR; MacLean *et al.* 1997). The AR gene is expressed widely in muscle including myoblasts, myofibers, and satellite cells (Chen *et al.* 2005). The AR is also expressed in motor neurons which may contribute to the regulation of muscle mass and function (Yang & Arnold 2000). Several different AR knockout (ARKO) mouse models have been reported. In one of these models, muscle mass was unchanged, whereas in another model, decreased

muscle mass and impaired muscle function were observed in male ARKO mice but not in female ARKO mice (Lin *et al.* 2005, MacLean *et al.* 2008). Ophoff *et al.* (2009) recently reported that a myocyte-specific knockout of the AR in male mice resulted in decreased lean mass and a conversion of fast towards slow muscle fibers, without affecting muscle strength or fatigue. In their study, similar results were obtained in male mice with ubiquitous ARKO.

Testosterone can exert its effect either directly by stimulation of the AR or via aromatization in target tissues to estradiol (E_2), the ligand for the estrogen receptors (ERs) α and β (also known as ESR1 and ESR2 respectively). Muscle from both men and women contains aromatase enzyme activity (Matsumine *et al.* 1986). The extent to which the actions of testosterone in muscle are a consequence of AR or ER activation or both is not clear.

Skeletal muscle myoblasts, myotubes, and mature fibers all express functional ERs, indicating a direct effect of estrogen in muscle (Kahlert *et al.* 1997, Barros *et al.* 2006). Women after menopause have decreased lean body mass, which can be reversed by estrogen

replacement therapy (Sorensen *et al.* 2001). In animals, estrogen has been shown to regulate skeletal muscle mass in developing livestock, rats, and mice (Trenkle 1976, Kobori & Yamamuro 1989, McCormick *et al.* 2004, Moran *et al.* 2007). Furthermore, ovariectomy decreases rat skeletal muscle mass recovery, and estrogen replacement benefits atrophied muscle mass recovery (Brown *et al.* 2005, Sitnick *et al.* 2006). Male mice lacking ER β also exhibit altered muscle function (Glenmark *et al.* 2004).

By studying the effects on lean mass and weight of individual muscles and comparing gene expression after ER- and AR-mediated stimulation in muscle of gonadectomized mice, this study aimed to investigate whether the effect of testosterone on lean tissue could be due to a direct stimulatory effect on the AR or due to aromatization of testosterone to E₂.

Materials and methods

Animals and study design

Mice were on a C57BL/6 background, and had free access to fresh water and soy-free food pellets (R70, Lactamin AB, Stockholm, Sweden or 2016, Harlan Teklad, UK). The ethics committee at the University of Gothenburg approved this study.

At 12 weeks of age, male mice were orx, and then treated for 5 weeks with DHT (45 μ g/day), E₂ (0.05 μ g/day), or vehicle (veh) administered via subcutaneous silastic implants (Silclear Tubing; Degania Silicone, Ltd, Jordan Valley, Israel) in the cervical region (Vandenput *et al.* 2002). Gonadectomy and implantation of pellets were performed during the same surgical session for all experimental groups. At the end of the treatment period, dual X-ray absorption (DXA) measurements were performed *in vivo* ($n=6-8$ in each group). Then, m. quadriceps and m. gastrocnemius together with various organs were dissected, and their wet weights were determined. Blood was collected for analyses of serum insulin-like growth factor 1 (IGF1) concentration, and distal femur trabecular volumetric bone mineral density (vBMD) was determined *ex vivo* using peripheral quantitative computerized tomography (pQCT) ($n=6-8$ in each group). In addition, total RNA was isolated from snap-frozen m. gastrocnemius for analyses using microarray ($n=5$ in each group).

To determine the short-term effects of the hormone treatments on the expression of selected genes in muscle, a similar experiment as that described above was performed, but with a treatment period of only 1 week. Total RNA was isolated from snap-frozen m. gastrocnemius and m. levator ani for analyses using real-time PCR (RT-PCR).

DXA analysis

Body composition of mice was measured by DXA using the Lunar PIXImus Mouse Densitometer (Wipro GE Healthcare, Madison, WI, USA) with the mice under inhalation anesthesia with isoflurane (Forane; Abbot Scandinavia).

Peripheral quantitative computerized tomography

Distal femur trabecular vBMD was measured *ex vivo* using the Stratec pQCT XCT Research M (software version 5.4B; Norland Medical Systems Inc., White Plains, NY, USA) operating at a resolution of 70 μ m (Windahl *et al.* 1999). The pQCT scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 3% of the total length of the femur, and the trabecular bone region was defined as the inner 45% of the total cross-sectional area.

DNA microarray analysis

Total RNA was isolated from snap-frozen m. gastrocnemius using RNeasy Mini Kit including an on-column DNase digestion step using the RNase-free DNase set (Qiagen). The mRNA samples derived from each individual mouse were reverse transcribed into cDNA, labeled, and analyzed using DNA microarray (mouse expression set 430; Affymetrix, Santa Clara, CA, USA) ($n=5$ in each group). Preparation of labeled cRNA, hybridization, and staining were done according to the Affymetrix Gene Chip expression analysis manual. The stained probe array was scanned, and the resultant image was captured as a data image (.CEL) file. The signal intensities for the β -actin (*Actb*) and the *Gapdh* genes were used as the internal quality controls. The ratio of fluorescent intensities for the 5' end the 3' end of these housekeeping genes was <3 . The microarray data can be accessed at EMBL-EBI ArrayExpress repository, ArrayExpress accession: E-MEXP-2192.

Bioinformatics

To correct for variation between GeneChips, the signal data of CEL files of Affymetrix mouse expression set 430 chips were quantile normalized, with probe set intensities calculated using the Robust Multiarray Average (Irizarry *et al.* 2003). For each gene, a *t*-test was used to estimate the effect of treatment. A gene was considered regulated if it demonstrated a fold change ≥ 1.5 and $P \leq 0.05$ in response to E₂ or DHT treatment compared with the veh. The mouse expression set 430 annotation file dated August 2008 was downloaded

from Affymetrix, from which the gene title, gene symbol, gene ontology (GO) biological process, GO cellular component, and GO molecular functions of the regulated genes were identified. The genes were then grouped by their unique GO classifications (biological process, cellular component, and molecular function).

Quantitative RT-PCR analysis

Total RNA was isolated from snap-frozen m. gastrocnemius. The RT-PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden). The mRNA abundance of each gene was calculated using the 'standard curve method' (User Bulletin 2; PE Applied Biosystems), and was adjusted for the expression of 18S. Primer and probe sequences are available upon request.

Serum assay

Serum IGF1 level was measured by a double-antibody IGF-binding protein-blocked RIA (Mediagnost, Tübingen, Germany).

Statistical analyses

All the descriptive statistical results are presented as the means $\pm$ S.E.M. Between-group differences were calculated using unpaired *t*-tests. Comparisons between multiple groups were done using a one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. A two-tailed $P \leq 0.05$ was considered significant.

Results

Comparison of the effects of AR and ER stimulation in male mice

To compare the importance of AR versus ER stimulation in muscle in male mice, 12-week-old male orx mice were treated for 5 weeks with either veh, the non-aromatizable androgen DHT, or E_2 .

Seminal vesicles and bone

To test whether the DHT and E_2 doses were physiological, we studied the effects on seminal vesicles and trabecular vBMD. The DHT dose used (45 μ g/day) was physiological, since it completely prevented the orx-induced loss of weight of the seminal vesicles (Fig. 1A). As expected, E_2 (0.05 μ g/day) had no effect on the weight of the seminal vesicles (Fig. 1A). Both E_2 and DHT prevented the orx-induced reduction in trabecular vBMD almost completely as measured using pQCT (Fig. 1B).

Figure 1 Seminal vesicle weight (A), trabecular volumetric bone mineral density (B), and serum IGF1 (C) in 12-week-old male mice that were either sham-operated or orchidectomized (orx) and then treated for 5 weeks with vehicle (veh), dihydrotestosterone (DHT) (45 μ g/day), or 17 β -estradiol (E_2) (0.05 μ g/day). Values are given as means $\pm$ S.E.M., $n = 6-8$ in each group. * $P \leq 0.01$ versus sham; † $P \leq 0.01$ versus orx + veh; ‡ $P \leq 0.05$ versus sham; § $P \leq 0.05$ versus orx + veh; P values are based on a one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test.

Serum IGF1

Orx had no effect on serum IGF1 levels. E₂ treatment increased serum IGF1 by 18·4% ($P \leq 0\cdot05$ versus sham, Fig. 1), whereas DHT treatment did not differ significantly from the sham-operated controls (Fig. 1).

Lean tissue and muscle weight

As measured using DXA, there was a loss of whole-body lean tissue mass by 9·4% after orx ($P \leq 0\cdot01$ versus sham) that was prevented partly by E₂ and completely by DHT (Fig. 2). E₂ treatment resulted in an increased lean mass by 3·9% ($P \leq 0\cdot05$ versus orx+veh), and DHT treatment resulted in even further increased lean mass (5·6% over orx+E₂, $P \leq 0\cdot05$, and 9·7% over orx+veh, $P \leq 0\cdot01$). These results were confirmed by dissection and weighing of m. quadriceps and m. gastrocnemius (Fig. 2).

The effects of E₂ and DHT treatments on gene expression in skeletal muscle

Microarray analyses were performed on gastrocnemius muscle samples from individual mice ($n=5$ in each group) to compare global gene expression after treating orx mice for 5 weeks with either DHT or E₂. Genes regulated by E₂ or DHT (fold change $\geq 1\cdot5$ and $P \leq 0\cdot05$ versus veh) were assigned to several functional categories (Table 1). A complete catalog of these genes is published as supplementary data (Supplementary Table 1, see section on supplementary data given at the end of this article). DHT regulated 187 (133 up-regulated and 54 down-regulated) genes in muscle (Table 1). Fewer genes ($n=51$) were regulated after E₂ treatment (33 up-regulated and 18 down-regulated, Table 1). Although a treatment period of 5 weeks would mean that several of these genes may reflect secondary effects of hormone treatment, we refer to them as E₂/DHT-regulated genes for simplicity.

Regulated genes in common for DHT and E₂

DHT and E₂ regulated different sets of genes, except 13 genes (=25% of E₂-regulated genes) that were regulated by both treatments (5·8% of the total number of regulated genes, Table 2). Among them was *Fbxo32*, a gene encoding a muscle-specific F-box protein implicated in muscle atrophy that was up-regulated by both treatments. Five of the regulated genes had unknown function (Table 2).

IGF1 signalling

In muscle, E₂ decreased the expression of *Igf1* (Table 3). The IGF1 signaling pathway includes many signaling molecules that are also important for

signaling through the insulin receptor. E₂ up-regulated one of these signaling molecules, *Tbc1d1* (Table 3). DHT decreased the expression of *Grb10*, an inhibitor of IGF1 signaling, and *Socs2*, a suppressor of cytokine

Figure 2 Lean tissue that was measured by DXA (A) and wet weight of dissected quadriceps muscle (B) and gastrocnemius muscle (C) from 12-week-old male mice that were either sham-operated or orchidectomized (orx) and then treated for 5 weeks with vehicle (veh), dihydrotestosterone (DHT) (45 µg/day), or 17β-estradiol (E₂) (0·05 µg/day). Values are given as means $\pm$ S.E.M., $n=6-8$ in each group. * $P \leq 0\cdot01$ versus sham; † $P \leq 0\cdot01$ versus orx+veh; ‡ $P \leq 0\cdot05$ versus orx+veh; § $P \leq 0\cdot05$ versus orx+E₂; # $P \leq 0\cdot01$ versus orx+E₂. P values are based on a one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test.

signaling. Furthermore, there was an increased expression of *Shp2*, a phosphatase important for IGF1 signaling (Table 3).

Fuel metabolism

In muscle, E₂ increased the expression of two genes involved in glucose metabolism: *Pcx*, which encodes an enzyme converting pyruvate to oxaloacetate, and *Fbp2*, which encodes an enzyme active in glyconeogenesis. There was an increased expression of *Ces3*, which encodes a lipase mediating hydrolysis of triglycerides in muscle, and a decreased expression of *Scd1*, which encodes an enzyme active in triglyceride synthesis (Table 3). After DHT treatment, there was a decreased expression of *Oxct1*, which encodes an enzyme active in the catabolism of ketone bodies. DHT increased the expression of two genes involved in cholesterol

metabolism: *Npc1*, which encodes a protein that regulates the transport of cholesterol in the cell, and *Vldl* receptor, which encodes a peripheral lipoprotein receptor. Furthermore, DHT increased the expression of two genes encoding proteins involved in mitochondrial ATP synthesis-coupled proton transport, *Ndufs4* and *Cbr2* (Table 3).

Both E₂ and DHT increased the expression of genes in the glutathione metabolic pathway that functions in the detoxification of hydrogen peroxide, protecting cells from oxidative damage (Table 3).

Angiogenesis

Both E₂ and DHT affected the expression of genes involved in angiogenesis. E₂ up-regulated *Angpt1*, which is a promoter of angiogenesis, and down-regulated *Thbs1*, which is a negative regulator of

Table 1 Number of regulated genes in mouse skeletal muscle assigned to functional categories

	GO ID	Regulated genes E ₂			Regulated genes DHT		
		Total	Up	Down	Total	Up	Down
Classification							
Transcription	0006350	3	2	1	18	12	6
Signal transduction	0007165	8	4	4	23	17	6
Metabolic process							
Glucose	0006006	3	2	1	—	—	—
Lipid	0006629	2	1	1	—	—	—
Cholesterol	0008203	—	—	—	2	2	—
Ketone body	0046950	—	—	—	1	—	1
Glutathione	0006749	1	1	—	3	2	1
Others		1	1	—	3	—	3
Mitochondrial respiratory chain	0005746	—	—	—	2	2	—
Protein metabolism and modification							
Synthesis		—	—	—	4	1	3
Degradation	0030163	5	4	1	11	8	3
Post-translation modification	0043687	2	2	—	3	2	1
Ion/substrate transport	0022892	3	3	—	17	15	2
Cell adhesion	0007155	—	—	—	5	4	1
Apoptosis	0006915	—	—	—	2	2	—
Cell cycle	0007049	2	1	1	4	2	2
Microtubule, cytoskeleton organization	0000226	—	—	—	2	2	—
Angiogenesis	0001525	2	1	1	1	1	—
Muscle contraction	0006936	1	—	1	10	—	10
Polyamine biosynthetic process	0006596	—	—	—	3	3	—
Extracellular matrix constituent	0005201	—	—	—	8	6	2
Immune response	0006955	2	1	1	2	2	—
Actin binding	0003779	—	—	—	4	3	1
Cellular iron ion homeostasis	0006879	—	—	—	1	1	—
Heme biosynthetic process	0006783	—	—	—	1	1	—
Cellular calcium ion homeostasis	0006874	—	—	—	1	1	—
Synaptic transmission	0007268	—	—	—	3	3	—
Multicellular organism development	0007275	3	—	3	4	3	1
Unknown function		8	5	3	35	28	7
Unknown identity		5	5	—	14	10	4
Total responsive genes		51	33	18	187	133	54

Microarray analyses of m. gastrocnemius from 12-week-old male mice that were either sham-operated or orchidectomized (orx) and treated for 5 weeks with vehicle (veh), dihydrotestosterone (DHT) (45 µg/day), or 17β-estradiol (E₂) (0.05 µg/day). A gene was considered regulated if it demonstrated a fold change ≥ 1.5 and *P* ≤ 0.05 (*t*-test) in response to E₂ or DHT treatment compared with the vehicle, *n* = 5 in each group. GO ID, gene ontology biological process identity nos.

Table 2 Regulated genes in common for 17β-estradiol (E₂) and dihydrotestosterone (DHT) treatments in mouse skeletal muscle

Gene	Symbol	Fold change		
		E ₂	DHT	Function
Up-regulated genes in common after E ₂ and DHT treatments				
FK506-binding protein 5	<i>Fkbp5</i>	1·8*	1·8*	Protein post-translational modification
F-box protein 32	<i>Fbxo32</i>	1·6 [†]	2·2*	Protein degradation
Potassium voltage-gated channel, subfamily Q, member 5	<i>Kcnq5</i>	2·6*	2·3*	Ion/substrate transport
Ribosomal protein S6 kinase, polypeptide 5	<i>Rps6ka5</i>	1·5*	1·6 [†]	Transcription
RIKEN cDNA 5330406M23 gene	<i>5330406M23Rik</i>	1·6*	1·6 [†]	Unknown
ARP3 actin-related protein 3 homolog B	<i>Actr3b</i>	1·5*	2·1*	Unknown
Nudix-type motif 18	<i>Nudt18</i>	1·6*	1·9*	Unknown
CDKN2A interacting protein N-terminal like	<i>Cdkn2aipnl</i>	1·6*	1·6 [†]	Unknown
Phosphodiesterase 4D interacting protein	<i>Pde4dip</i>	1·6*	1·5 [†]	Unknown
Down-regulated genes in common after E ₂ and DHT treatments				
Gremlin 2	<i>Grem2</i>	1·6 [†]	2·4*	Signal transduction
Myosin, light polypeptide 3	<i>Myl3</i>	1·5 [†]	1·8*	Muscle contraction
HtrA serine peptidase 4	<i>Htra4</i>	1·6 [†]	1·5*	Protein degradation
Tumor necrosis factor, α-induced protein 2	<i>Tnfai2</i>	1·9 [†]	1·5 [†]	Multicellular organismal development

Microarray analyses of m. gastrocnemius from 12-week-old male mice that were either sham-operated or orchidectomized (orx) and treated for 5 weeks with vehicle (veh), DHT (45 µg/day), or E₂ (0.05 µg/day). Values are given as fold change, *n* = 5 in each group. **P* ≤ 0.01 versus vehicle; †*P* ≤ 0.05 versus vehicle. ‡*t*-test.

angiogenesis (Table 3). DHT up-regulated *Agtr1l*, also known as the apelin receptor, which is active during angiogenesis (Table 3).

Synaptic transmission

Skeletal muscle fibers are innervated by motor neurons, and DHT up-regulated three genes involved in synaptic transmission in muscle: *Musk*, *Lynx1*, and *Dlgap4* (Table 3).

Heme biosynthetic process

The transferrin receptor (*Tfrc*), which maintains cellular iron ion homeostasis by importing iron into the cell, and *Alas1*, a rate-controlling mitochondrial heme biosynthetic enzyme, were both up-regulated after DHT treatment (Table 3).

Muscle contraction and extracellular matrix structural constituent

The gene expression of ten myofibrillar proteins was down-regulated after DHT treatment, and that of one myofibrillar protein was down-regulated after E₂ treatment (Table 3). Among the genes that encode the structural proteins that constitute the extracellular matrix including tendons, six different collagen genes were up-regulated after DHT treatment, and one gene was down-regulated. A collagen-binding protein lumican was also down-regulated after DHT treatment (Table 3).

Polyamine biosynthesis

Three rate-limiting polyamine biosynthetic enzymes, *Amd1*, *Odc1*, and *Smox*, were highly up-regulated after DHT treatment (Table 3).

Verification of microarray results

To verify the microarray data, the transcript levels of six genes (*Igf1*, *Fbxo32*, *Grb10*, *Gpx3*, *Odc1*, and *Myl3*) were quantified by RT-PCR. Analysis was performed on individual muscle samples, with 18S included as an internal control. As shown in Fig. 3, the regulation of all genes, except that of *Myl3*, was confirmed.

For the RT-PCR experiment, we also included the sham-operated control group which added some further information on the regulation of these genes. The expression of *Fbxo32* was decreased by 42.3% after orx (*P* < 0.01 versus sham, Fig. 3), and it was normalized by both E₂ and DHT treatments to orx mice (Fig. 3). *Igf1* expression was decreased in orx mice treated with E₂ (−54.8%, *P* < 0.01 versus sham and −48.5%, *P* < 0.05 versus orx), while orx by itself or DHT treatment to orx mice had no effect (Fig. 3). Orx resulted in an increase in *Grb10* expression (43.3%, *P* < 0.01 versus sham, Fig. 3), which was normalized by DHT treatment but not by E₂ treatment to orx mice. Orx and DHT had no effect on *Gpx3* expression, but E₂ treatment to orx mice resulted in a dramatic increase (251%, *P* < 0.01 versus sham and 225%, *P* < 0.01 versus orx, Fig. 3). Orx resulted in a decrease in *Odc1* expression (−52%, *P* < 0.01 versus sham, Fig. 3), which was normalized by DHT treatment but not by E₂ treatment to orx mice.

Table 3 Regulated genes in mouse skeletal muscle after 17 β -estradiol (E₂) and dihydrotestosterone (DHT) treatments in selected functional categories of importance for skeletal muscle function and development

Gene title	Gene symbol	Fold change	Down/up	P
<i>Genes regulated after E₂ treatment</i>				
IGF1 signalling				
Insulin-like growth factor 1	<i>Igf1</i>	1.6	Down	<0.01
TBC1 domain family, member 1	<i>Tbc1d1</i>	2.3	Up	<0.01
Fuel metabolism				
Pyruvate carboxylase	<i>Pcx</i>	3.1	Up	<0.01
Fructose bisphosphatase 2	<i>Fbp2</i>	1.6	Up	<0.01
Carboxylesterase 3	<i>Ces3</i>	1.6	Up	<0.01
Stearyl-coenzyme A desaturase 1	<i>Scd1</i>	1.8	Down	<0.01
Glutathione metabolic process				
Glutathione peroxidase 3	<i>Gpx3</i>	2.2	Up	<0.01
Angiogenesis				
Angiopoietin 1	<i>Angpt1</i>	1.6	Up	0.046
Thrombospondin 1	<i>Thbs1</i>	1.5	Down	0.021
Muscle contraction				
Myosin, light polypeptide 3	<i>Myl3</i>	1.5	Down	0.025
<i>Genes regulated after DHT treatment</i>				
IGF1 signalling				
Growth factor receptor-bound protein 10	<i>Grb10</i>	1.5	Down	<0.01
Suppressor of cytokine signaling 2	<i>Socs2</i>	1.8	Down	0.038
Protein tyrosine phosphatase, non-receptor type 11	<i>Ptpn11</i>	1.5	Up	<0.01
Fuel metabolism				
3-Oxoacid CoA transferase 1	<i>Oxct1</i>	1.8	Down	<0.01
Niemann–Pick type C1	<i>Npc1</i>	1.8	Up	0.012
Very low-density lipoprotein receptor	<i>Vldlr</i>	1.6	Up	<0.01
Mitochondrial ATP synthesis-coupled proton transport				
NADH dehydrogenase (ubiquinone) Fe-S protein 4	<i>Ndufs4</i>	1.6	Up	<0.01
Carbonyl reductase 2	<i>Cbr2</i>	2.2	Up	<0.01
Glutathione metabolic process				
Glutathione S-transferase mu 2	<i>Gstm2</i>	2.5	Down	<0.01
Microsomal glutathione S-transferase 3	<i>Mgst3</i>	1.8	Up	<0.01
Microsomal glutathione S-transferase 1	<i>Mgst1</i>	1.9	Up	<0.01
Angiogenesis				
Angiotensin receptor-like 1	<i>Agtr1</i>	1.6	Up	0.017
Synaptic transmission				
Muscle, skeletal, receptor tyrosine kinase	<i>Musk</i>	1.6	Up	0.011
Ly6/neurotoxin 1	<i>Lynx1</i>	1.6	Up	<0.01
Discs, large homolog-associated protein 4	<i>Dlgap4</i>	1.6	Up	<0.01
Heme biosynthetic process				
Transferrin receptor	<i>Tfrc</i>	1.8	Up	<0.01
Aminolevulinic acid synthase 1	<i>Alas1</i>	1.8	Up	<0.01
Polyamine biosynthesis				
S-Adenosylmethionine decarboxylase	<i>Amd1</i>	2.3	Up	<0.01
Ornithine decarboxylase, structural 1	<i>Odc1</i>	2.8	Up	<0.01
Spermine oxidase	<i>Smox</i>	2.6	Up	<0.01
Muscle contraction				
Myosin, light polypeptide 3	<i>Myl3</i>	1.6	Down	<0.01
Myosin, heavy polypeptide 6	<i>Myh6</i>	1.7	Down	<0.01
Troponin I	<i>Tnni1</i>	2.1	Down	<0.01
Myosin, light polypeptide 2	<i>Myl2</i>	2.2	Down	<0.01
Troponin C	<i>Tnnc1</i>	2.2	Down	<0.01
Myosin, heavy polypeptide 7	<i>Myh7</i>	2.6	Down	<0.01
Actin, α , cardiac muscle 1	<i>Actc1</i>	3.8	Down	<0.01
Myosin-binding protein H	<i>Mybph</i>	11.9	Down	<0.01
Troponin T1	<i>Tnnt1</i>	2.8	Down	<0.01
Tropomyosin 3	<i>Tpm3</i>	2.2	Down	0.017
Extracellular matrix structural constituent				
Collagen, type I, α 1	<i>Col1a1</i>	1.6	Up	0.044
Collagen, type V, α 2	<i>Col5a2</i>	1.7	Up	0.015
Collagen, type I, α 2	<i>Col1a2</i>	1.7	Up	0.049

(continued)

Table 3 Continued

Gene title	Gene symbol	Fold change	Down/up	P
Collagen, type III, α 1	<i>Col3a1</i>	1.8	Up	0.013
Collagen, type IV, α 2	<i>Col4a2</i>	1.9	Up	<0.01
Collagen, type IV, α 1	<i>Col4a1</i>	2.2	Up	<0.01
Lumican	<i>Lum</i>	1.7	Down	<0.01
Collagen, type XIV, α 1	<i>Col14a1</i>	1.7	Down	<0.01

Regulated genes after E₂ and DHT treatments in IGF1 signaling, fuel metabolism, mitochondrial ATP synthesis-coupled proton transport, glutathione metabolic process, angiogenesis, synaptic transmission, heme biosynthetic process, polyamine biosynthesis, muscle contraction, and extracellular matrix structural constituents. Microarray analyses of m. gastrocnemius from 12-week-old male mice that were either sham-operated or orchidectomized (orx) and treated for 5 weeks with vehicle (veh), DHT (45 µg/day), or E₂ (0.05 µg/day). Values are given as fold change, n=5 in each group. *t*-test.

Time course study of regulated genes

To determine the short-term effects of the hormone treatments on the expression of selected genes in muscle, an experiment with a treatment period of only 1 week was performed. After 1-week treatment, the expression of *Fbxo32* was increased in m. gastrocnemius (72%, *P*<0.01 versus sham, Fig. 4A). There was a tendency to normalization of *Fbxo32* expression levels with E₂ and DHT, but it did not reach statistical

significance (Fig. 4A). For comparison, we analyzed m. levator ani, a fast-twitch muscle known to be testosterone dependent in rodents. The effect on *Fbxo32* expression was much more dramatic in m. levator ani, although in the same direction as in m. gastrocnemius, and DHT by itself could completely normalize *Fbxo32* expression levels after orx (Fig. 4B). The higher levels of *Fbxo32* in m. gastrocnemius after orx were only transient, and after 5-week treatment, the levels were similar for the orx group compared with the orx

Figure 3 To verify the microarray data, the transcript levels of six genes (*Igf1*, *Fbxo32*, *Grb10*, *Gpx3*, *Odc1*, and *Myl3*) were quantified by RT-PCR. Analysis was performed on individual muscle samples from 12-week-old male mice that were either sham-operated or orchidectomized (orx) and then treated for 5 weeks with vehicle (veh), dihydrotestosterone (DHT) (45 µg/day), or 17 β -estradiol (E₂) (0.05 µg/day). 18S was included as an internal control. Data are expressed as percentage of Orx, and are presented as means $\pm$ S.E.M., n=4–8 in each group. **P*≤0.01 versus sham; †*P*≤0.01 versus orx + veh; ‡*P*≤0.05 versus sham; §*P*≤0.05 versus orx + veh; #*P*≤0.01 versus orx + E₂; €*P*≤0.05 versus orx + E₂. Microarray data are shown for comparison.

Figure 4 Time course study for *Fbxo32* expression in muscle. RT-PCR analysis was performed on individual muscle samples from 12-week-old male mice that were either sham-operated or orchidectomized (orx) and then treated for 1 (A and B) or 5 (C) weeks with vehicle (veh), dihydrotestosterone (DHT) (45 µg/day), or 17β-estradiol (E₂) (0.05 µg/day). *Fbxo32* expression in m. gastrocnemius (A) and m. levator ani (B) is presented after 1-week treatment for comparison and in m. gastrocnemius (C) after 5-week treatment. *Fbxo32* mRNA levels are corrected for the expression of 18S, and are presented as means ± S.E.M., $n=4-8$ in each group. * $P \leq 0.01$ versus sham; † $P \leq 0.01$ versus orx + veh; ‡ $P \leq 0.05$ versus sham; § $P \leq 0.05$ versus orx + veh; # $P \leq 0.01$ versus orx + E₂.

group after 1-week treatment but had increased for all other groups (Fig. 4C). For the other four genes that were analyzed (*Igf1*, *Grb10*, *Gpx3*, and *Odc1*), there was no major difference in expression pattern between 1- and 5-week treatments (Table 4). There was no weight difference in m. gastrocnemius between different treatment groups in the 1-week experiment (data not shown).

Discussion

Testosterone treatment of orx mice has previously been shown to induce a dose-dependent increase in the mass of individual muscles (Axell *et al.* 2006). However, how much of this effect is through aromatization of testosterone to E₂ is unknown. To differentiate between the direct effects of testosterone and those of testosterone aromatized to E₂ on muscle mass, we treated orx mice with E₂ and the non-aromatizable androgen DHT. Although the effect of DHT was more marked, both treatments prevented loss of muscle mass after gonadectomy, indicating that AR- and ER-mediated signaling has effects on muscle mass that are independent of each other. The doses used were likely in the physiological range as DHT completely prevented the orx-induced loss of seminal vesicle weight, and E₂ and DHT almost completely prevented the orx-induced reduction in trabecular vBMD.

To investigate the mechanism underlying the effect on muscle mass, we performed microarray and RT-PCR analyses on skeletal muscle samples. In line with a more profound effect of DHT on muscle mass, the microarray analyses demonstrated that a greater number of genes were regulated after DHT treatment ($n=187$) than after E₂ treatment ($n=51$). Thirteen genes were regulated by both E₂ and DHT, and these could represent mechanisms in common for how activation of AR and ER signaling stimulates skeletal muscle mass. However, the results indicate that most of the effects of E₂ and DHT in skeletal muscle of male mice are mediated by different mechanisms. Even when E₂ and DHT affected the same pathways, such as IGF1 signaling, increased angiogenesis, and glutathione oxidation, it was done by regulating different sets of genes.

Regulated genes in common for DHT and E₂

Among the eight genes with known function that were regulated by both DHT and E₂, increased expression was observed for *Kcnq5*, which encodes a potassium channel that is involved in skeletal muscle cell proliferation by triggering changes in membrane potential and regulating cell volume (Roura-Ferrer *et al.* 2008). Surprisingly, *Fbxo32*, a gene encoding a muscle-specific F-box protein implicated in muscle

Table 4 Time course study for the expression of regulated genes in muscle. Data are expressed as percentage of orchidectomized (Orx), and are presented as means ± S.E.M., n=4–8 in each group

	One-week treatment				Five-week treatment			
	Sham	Orx veh	E ₂	DHT	Sham	Orx veh	E ₂	DHT
<i>Igf1</i>	137 ± 15	100 ± 16 [‡]	42 ± 5 ^{*,§}	93 ± 12 ^{‡,}	114 ± 10	100 ± 14	52 ± 7 ^{*,§}	82 ± 11 [‡]
<i>Grb10</i>	71 ± 4	100 ± 4 [*]	70 ± 3 [†]	57 ± 4 ^{‡,†,}	70 ± 6	100 ± 7 [*]	98 ± 7 [*]	55 ± 4 ^{‡,}
<i>Gpx3</i>	112 ± 11	100 ± 10	274 ± 11 ^{*,†}	75 ± 12	92 ± 10	100 ± 7	325 ± 24 ^{*,†}	89 ± 4
<i>Odc1</i>	159 ± 12	100 ± 5 [*]	80 ± 5 [*]	193 ± 30 ^{‡,}	210 ± 29	100 ± 6 [*]	90 ± 6 [*]	226 ± 33 ^{‡,}

Time course study for the expression of five genes (*Igf1*, *Fbxo32*, *Grb10*, *Gpx3*, and *Odc1*) by RT-PCR. Analysis was performed on individual muscle samples from 12-week-old male mice that were either sham-operated or orx and then treated for 1 or 5 weeks with vehicle (veh), dihydrotestosterone (DHT) (45 µg/day), or 17β-estradiol (E₂) (0.05 µg/day). 18S was included as an internal control. *P≤0.01 versus sham; †P≤0.05 versus orx+veh; ‡P≤0.01 versus orx+E₂; §P≤0.05 versus orx+veh; ||P≤0.01 versus orx+E₂.

atrophy, was up-regulated by both treatments. The RT-PCR analyses demonstrated that this up-regulation was a normalization of *Fbxo32* expression to the same levels as in the sham-operated controls. In multiple models of skeletal muscle atrophy, *Fbxo32* is up-regulated, and it appears to be essential for accelerated muscle protein degradation in a variety of experimental models of catabolism, including diabetes, cancer, AIDS, fasting, renal failure, hindlimb suspension, immobilization, oxidative stress, and sepsis (Sacheck *et al.* 2007). In a recent study done by Pires-Oliveira *et al.* (2010), *Fbxo32* expression was high in rat m. levator ani over a week after castration, and this increase could be reversed by treatment with testosterone. Interestingly, in another recent study done by Rogers *et al.* (2010), mice showed decreased expression of *Fbxo32* 12 weeks after castration similar to what was observed in our study. In a short-term experiment of only 1 week, we could show that these seemingly contradictory results depend on the fact that the increase of *Fbxo32* with gonadectomy is only transient and could be partly reversed by treatment with E₂ and fully reversed by treatment with DHT in m. levator ani. After a 5-week treatment period, the levels of *Fbxo32* had increased in all groups but stayed on similar levels in the orx groups. The transient increase of *Fbxo32* probably reflects that accelerated muscle loss occurs early and transiently after castration, and that at least in m. levator ani, which is a fast-twitch muscle, this loss can be partly prevented by E₂ and fully prevented by DHT.

The size of pre-existing muscle fibers is decreased in skeletal muscle atrophy, and in line with this, one myofibrillar protein was decreased after both DHT and E₂ treatments and ten myofibrillar proteins were decreased after DHT treatment alone, despite the observed prevention of loss in muscle mass. Our hypothesis is that a decrease in muscle mass probably occurs very early after orx, and that the biosynthesis and breakdown of myofibrillar proteins have begun to enter, or already reached, a new steady state after 4 weeks.

Extracellular matrix structural constituents

Collagens are the main proteins of the extracellular matrix including tendons that surround and support muscle fibers. The expression of six different collagen genes was up-regulated after DHT treatment, indicating that synthesis of more extracellular matrix was an ongoing process. The GH/IGF1 axis exerts, like testosterone and E₂, anabolic effects on lean body mass. In a previous study, in which muscle biopsies were analyzed using microarray before and after 2-week GH treatment to hypopituitary men with hypogonadism, we also observed increased synthesis of collagens and a decreased expression of several myofibrillar proteins (Sjogren *et al.* 2007).

IGF1 signaling

IGF1 is a potent anabolic agent in muscle (Musaro *et al.* 2001). After E₂ treatment, the importance of the observed changes in the IGF1 system for muscle function was not clear as serum levels of IGF1 were increased, whereas the local gene expression of *Igf1* in muscle was reduced. However, increased serum IGF1 levels have also been observed in postmenopausal women treated with transdermal E₂ (Weissberger *et al.* 1991, Ho *et al.* 2003).

DHT treatment decreased the expression of *Grb10*, a negative regulator of IGF1 signaling (Dufresne & Smith 2005). In line with IGF1 being an anabolic agent in muscle, mice with a disrupted *Grb10* gene have increased muscle and lean mass (Smith *et al.* 2007). The RT-PCR analyses showed that DHT but not E₂ could normalize *Grb10* expression after orx, indicating that this is an AR-mediated effect. Interestingly, the expression of *Grb10* in muscle has been shown to be gender dependent with higher expression in women than in men, suggesting a role for *Grb10* in the sexual dimorphism of skeletal muscle mass (Welle *et al.* 2008). Furthermore, DHT reduced the expression of the *Socs2* gene in muscle. SOCS2 reduces GH signaling; therefore, a down-regulation of *Socs2* expression in muscle

likely increases the effect of GH/IGF1 locally in muscle. Together these indicate that the AR-mediated effects on muscle mass include increased local effect of GH/IGF1 by a down-regulation of *Grb10* and *Socs2*.

Angiogenesis

Increased angiogenesis would result in improved microcirculation in muscle, and hence would provide an increased oxygen and nutrient supply as well as lead to the removal of waste products. E₂ up-regulated *Angptl1*, an angiogenesis promoter during embryonic development, essential to endothelial cell survival, vascular branching, and pericyte recruitment (Carmeliet 2000). Furthermore, E₂ down-regulated *Thbs1*, a potent angiogenesis inhibitor (Lawler 2002). *Agtrl1* or the apelin receptor was up-regulated by DHT. Apelin through its receptor AGTRL1 is involved in the regulation of blood vessel diameter during angiogenesis, and apelin-deficient mice showed narrow blood vessels during embryogenesis (Kidoya *et al.* 2008). Together these results point to increased angiogenesis in muscle after both E₂ and DHT treatments.

Glutathione metabolism

Both E₂ and DHT increased the expression of genes in the glutathione metabolic pathway that protects cells from oxidative damage. One of these genes, *Gpx3*, was markedly up-regulated in muscle by E₂. *Gpx3* has earlier been shown to be directly regulated by E₂ in white adipose tissue (Lundholm *et al.* 2008). *Gpx3* improves skeletal muscle insulin sensitivity by mediating the antioxidant effect of PPAR γ (PPARG; Chung *et al.* 2009), and increased glutathione peroxidase activity could mediate the improvement of muscle strength by selenium treatment in selenium-deficient patients (Brown *et al.* 1986).

Fuel metabolism

E₂ had effects on genes regulating intermediary glucose and lipid metabolism. Pyruvate carboxylase, a mitochondrial protein that is important for intermediary metabolism by catalyzing the carboxylation of pyruvate to form oxaloacetate, was strongly up-regulated by E₂. Oxaloacetate is an intermediate of the citric acid cycle and glyconeogenesis. *Fbp2*, expressing an enzyme that catalyzes the hydrolysis of fructose-1,6-bisphosphate into fructose-6-phosphate and is critical in glyconeogenesis pathway, was also up-regulated after E₂ treatment, indicating increased glycogen synthesis in muscle. *Ces3*, which mediates some or all of the lipolysis that is independent of hormone-sensitive lipase (Soni *et al.* 2004), was up-regulated in muscle by E₂, indicating increased utilization of free fatty acids as fuel.

Synaptic transmission

DHT up-regulated several genes involved in synaptic transmission in muscle, among them was *Musk*, which encodes a receptor tyrosine kinase that is essential for synapse formation and the development of new neuromuscular junctions (Kim & Burden 2008), indicating a role for AR-mediated signaling in this process.

Heme biosynthetic process

DHT also affected the expression in muscle of genes important for the supply of oxygen. Iron, which is essential for oxygen transport because it is incorporated in the heme of the oxygen-binding proteins hemoglobin and myoglobin, is delivered to cells via binding to the Tfrc (Xu *et al.* 2005). *Tfrc* together with *Alas1*, a rate-controlling mitochondrial heme biosynthetic enzyme, was up-regulated after DHT treatment, suggesting increased heme biosynthesis.

Polyamine biosynthesis

Three rate-limiting polyamine biosynthetic enzymes, *Amd1*, *Odc1*, and *Smox*, were highly up-regulated after DHT treatment. In a study measuring the short-term effects of DHT on gene expression in muscle of castrated mice, genes involved in polyamine synthesis were also up-regulated, indicating a direct effect of DHT on this process (Yoshioka *et al.* 2006). Data suggest that polyamines play a role in muscle hypertrophy, although their mechanisms of action are still unknown (Abukhalaf *et al.* 2002). Furthermore, male ARKO mice have impaired skeletal muscle development and function, which were associated with decreased expression of polyamine biosynthetic enzymes (MacLean *et al.* 2008). This indicates a role for these enzymes in the AR-mediated effects on muscle.

In conclusion, activation of both ER and AR preserves muscle and lean mass after gonadectomy in male mice, although the effect was more marked after AR activation. Few genes were regulated by both E₂ and DHT, and when E₂ and DHT regulated the same pathways (IGF1 signaling, increased angiogenesis, and glutathione oxidation), it was done by affecting different sets of genes. However, short-term treatment with E₂ partly prevented and with DHT fully prevented the transient increase in *Fbxo32* expression observed after gonadectomy. E₂ alone had effects on genes involved in glucose and lipid metabolism, likely increasing glycogen synthesis and utilization of FAs for fuel in muscle. DHT alone regulated the expression of genes influencing synaptic formation and transmission, oxygen transport, and polyamine biosynthesis. Based on these findings, it could be hypothesized that the metabolic effects of testosterone on skeletal

muscle of male mice are to a large extent dependent on ER activation, whereas other effects of testosterone in skeletal muscle are more dependent on AR activation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JME-09-0165>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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