Effect of testosterone on markers of mitochondrial oxidative phosphorylation and lipid metabolism in muscle of aging men with subnormal bioavailable testosterone

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Abstract

Objective: Recent studies have indicated that serum testosterone in aging men is associated with insulin sensitivity and expression of genes involved in oxidative phosphorylation (OxPhos), and that testosterone treatment increases lipid oxidation. Herein, we investigated the effect of testosterone therapy on regulators of mitochondrial biogenesis and markers of OxPhos and lipid metabolism in the skeletal muscle of aging men with subnormal bioavailable testosterone levels.

Methods: Skeletal muscle biopsies were obtained before and after treatment with either testosterone gel ($n = 12$) or placebo ($n = 13$) for 6 months. Insulin sensitivity and substrate oxidation were assessed by euglycemic–hyperinsulinemic clamp and indirect calorimetry. Muscle mRNA levels and protein abundance and phosphorylation of enzymes involved in mitochondrial biogenesis, OxPhos, and lipid metabolism were examined by quantitative real-time PCR and western blotting.

Results: Despite an increase in lipid oxidation ($P < 0.05$), testosterone therapy had no effect on insulin sensitivity or mRNA levels of genes involved in mitochondrial biogenesis (*PPARGC1A*, *PRKAA2*, and *PRKAG3*), OxPhos (*NDUF51*, *ETFA*, *SDHA*, *UQCRC1*, and *COX5B*), or lipid metabolism (*ACADVL*, *CD36*, *CPT1B*, *HADH*, and *PDK4*). Consistently, protein abundance of OxPhos subunits encoded by both nuclear (*SDHA* and *UQCRC1*) and mitochondrial DNA (*ND6*) and protein abundance and phosphorylation of AMP-activated protein kinase and p38 MAPK were unaffected by testosterone therapy.

Conclusion: The beneficial effect of testosterone treatment on lipid oxidation is not explained by increased abundance or phosphorylation-dependent activity of enzymes known to regulate mitochondrial biogenesis or markers of OxPhos and lipid metabolism in the skeletal muscle of aging men with subnormal bioavailable testosterone levels.

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Introduction

A large proportion of elderly men have low testosterone levels. Population-based studies have shown that an age-related decline in testosterone levels is associated with an adverse metabolic profile, which includes abdominal obesity, insulin resistance and unfavorable changes in body composition with decreased muscle mass and

increased fat mass (1, 2). Consistently, there is observational evidence that low testosterone increases the risk of developing the metabolic syndrome and type 2 diabetes (1, 2). Accordingly, an increase in muscle mass and a reciprocal decrease in fat mass have been reported in randomized, placebo-controlled trials of testosterone

therapy in elderly nondiabetic men (1, 2, 3, 4). However, the changes in body composition are modest, and this may explain why only a few, but not all, of these studies have found a positive effect on the measures of insulin sensitivity (1, 4, 5). A better understanding of the mechanisms involved in androgen action is needed to establish to what extent testosterone deficiency causally contributes to insulin resistance and type 2 diabetes in elderly men, and to clarify potential beneficial effects of testosterone replacement therapy on insulin sensitivity.

Skeletal muscle is the major site of insulin resistance in obesity, type 2 diabetes, and related metabolic disorders (6). The association of low testosterone with insulin resistance and reduced muscle mass suggests that common factors may play a role in these metabolic disorders in men. In aging, obesity, and type 2 diabetes, insulin resistance in skeletal muscle has been linked to a number of abnormalities in mitochondrial oxidative metabolism (7, 8, 9, 10). This includes transcriptomic and proteomic evidence of a coordinated downregulation of genes and proteins involved in oxidative phosphorylation (OxPhos) in insulin-resistant skeletal muscle (11, 12, 13, 14) as well as reduced expression of the peroxisome proliferator-activated receptor gamma coactivator 1 alpha gene (PGC1 α), which plays a key role in mitochondrial biogenesis (13, 14). The decrease in mitochondrial oxidative capacity has been proposed to cause reduced lipid oxidation with subsequent accumulation of lipids and lipid metabolites, which in turn can inhibit insulin signaling to glucose transport and glycogen synthesis (6, 15), as observed in skeletal muscle in obesity and type 2 diabetes (16, 17, 18). Of interest, a recent study has reported that low testosterone was associated with low insulin sensitivity, low maximal aerobic capacity (VO₂max), and reduced expression of OxPhos genes in a mixed population of men with normal glucose tolerance, impaired glucose tolerance, and type 2 diabetes (19). This indicates that low testosterone may contribute to the link between reduced mitochondrial oxidative capacity and insulin resistance (2, 19). Moreover, we have recently shown that testosterone therapy promoted a shift in substrate partitioning toward an increased lipid oxidation and decreased glucose oxidation in elderly men with subnormal bioavailable testosterone levels (4). Consistently, other studies have reported that testosterone therapy stimulates lipid oxidation in hypopituitary men (20, 21), and that testosterone deficiency, induced by a gonadotropin-releasing hormone analog in younger men, increases adiposity and decreases fat oxidation and resting energy expenditure (22). The possible effect of testosterone on energy metabolism is

supported by a recent microarray study of orchidectomized mice, in which testosterone replacement in addition to the known positive effects on muscle mass and insulin-like growth factor 1/Akt signaling also normalized the expression of OxPhos genes (3, 23). Finally, a recent study of myotubes from male donors has shown that a testosterone-induced increase in palmitate oxidation was associated with increased activation of AMP-activated protein kinase (AMPK) and p38 MAPK (24), which are known regulators of mitochondrial biogenesis (25). Taken together, these findings suggest that the beneficial effect of testosterone therapy on muscle mass, lipid oxidation, and possibly insulin sensitivity in men with low testosterone may, in part, be mediated by an increased mitochondrial biogenesis leading to increased abundance of molecular markers of OxPhos and lipid metabolism in skeletal muscle.

To test this hypothesis, we investigated the effect of testosterone therapy on muscle transcript levels, protein abundance, and phosphorylation of enzymes involved in mitochondrial biogenesis, OxPhos, and lipid metabolism in skeletal muscle biopsies obtained from well-matched groups of elderly men with subnormal bioavailable testosterone levels (<7.3 nmol/l) randomized to treatment with transdermal testosterone or placebo for 6 months.

Subjects and methods

Study design

We have recently reported the effect of testosterone therapy for 6 months on body composition, insulin sensitivity, and substrate metabolism in a single center, randomized, placebo-controlled, double-blinded study of men aged 60–78 years ($n=38$), with subnormal bioavailable testosterone levels and increased BMI (4). The cutoff level of bioavailable testosterone (7.3 nmol/l) was defined as the lower reference interval limit by observations in a large population of young (20–29 years) males ($n=685$) with a low risk for secondary androgen deficiency. The corresponding lower reference interval limit for total testosterone was 11.7 nmol/l (26). Herein, we report the effect of testosterone therapy or placebo for 6 months on selected muscle transcripts and enzymes involved in mitochondrial biogenesis, OxPhos, and lipid metabolism in a subcohort of the participants ($n=25$), who completed the above-mentioned study (4), and from whom a muscle biopsy was obtained before and after treatment. Subjects were randomly assigned to receive testosterone (5 g gel/50 mg Testim, Ipsen, Boulogne, Bilancourt,

France) or placebo, and safety parameters and compliance were evaluated as described previously (27). If bioavailable testosterone levels after 3 weeks of treatment were <7.3 nmol/l, the dose was increased to 10 g gel (100 mg testosterone or placebo). The dose was increased in all participants in the placebo group and in six of 12 participants in the testosterone group (bioavailable testosterone after 3 weeks treatment was 4.4 ± 0.4 nmol/l in the placebo group vs 10.2 ± 2.4 in the testosterone group; $P < 0.02$). The subjects were advised to refrain from all self-initiated resistance exercise training and intense endurance training, but were allowed to continue other habitual activities throughout the study. Subjects were informed not to change their diet. For further details about the participants please refer to Frederiksen *et al.* (4). The study was approved by the Local Ethics Committee and declared in [ClinicalTrials.gov](https://clinicaltrials.gov) (identifier: NCT00700024). All participants gave written informed consent (4).

Euglycemic–hyperinsulinemic clamp and indirect calorimetry

The euglycemic–hyperinsulinemic clamp studies were performed after an overnight fast as described previously (4). Participants were instructed to refrain from strenuous physical activity for a period of 48 h before the experiment. In brief, a 120 min basal tracer equilibration period was followed by the infusion of insulin at a rate of 40 mU/m^2 per min for 180 min. A primed-constant $[3\text{-}^3\text{H}]\text{-glucose}$ infusion was used throughout the 300 min study, and $[3\text{-}^3\text{H}]\text{-glucose}$ was added to the glucose infusates to maintain plasma-specific activity constant at baseline levels during the 180 min clamp period as described previously (28). As reported, euglycemia was maintained using a variable infusion of 20% glucose, and serum insulin at ~ 400 pmol/l was obtained during the insulin-stimulated period. Serum insulin and plasma glucose concentrations were measured as described previously (29). Steele's non-steady-state equations adapted for labeled glucose infusates were used to calculate total glucose disposal rates (Rd), assuming a glucose distribution volume of 200 ml/kg body weight and a pool fraction of 0.65 (28). The studies were combined with indirect calorimetry using a ventilated hood system (TrueOne 2400 Metabolic Measurement System, ParvoMedics, Sandy, UT, USA) to assess the rates of glucose and lipid oxidation and the respiratory exchange ratio (RER) as described previously (4, 30). A muscle biopsy from each subject was obtained before and after the 6-month treatment period from the vastus lateralis in the resting, basal state using a modified

Bergström needle with suction under local anesthesia. Muscle samples were immediately frozen in liquid nitrogen and stored at -130°C until further analysis.

Assays

Safety parameters and analysis of plasma free fatty acids (FFAs), serum levels of testosterone, sex-hormone-binding globulin (SHBG), free testosterone, bioavailable testosterone, and adiponectin were assessed as described previously (4, 27). Of importance, serum total testosterone was measured by liquid chromatography tandem mass spectrometry after extraction using diethyl ether. Bioavailable and free testosterone were calculated using validated formula (31). For testosterone measurements, the intra-assay coefficient of variation (CV) was $< \pm 10\%$ for total testosterone (TT) > 0.2 nmol/l and CV was $< \pm 30\%$ in the range between 0.1 and 0.2 nmol/l (4).

Body composition

Total fat mass and lean body mass (LBM) were measured by dual X-ray absorptiometry using a Hologic Discovery device (Hologic, Waltham, MA, USA) with CV as reported (4).

RNA extraction

Total RNA was extracted from muscle using TRIzol reagent (Life Technologies) and chloroform/isopropyl alcohol (Sigma–Aldrich) according to the manufacturer's instructions. Briefly, ~ 50 mg of muscle was removed from the freezer and immediately immersed in 1 ml of TRIzol reagent. The muscle was homogenized using the bead beater system Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France). The aqueous and organic phases were separated using $200 \mu\text{l}$ of chloroform, and total RNA was precipitated using $500 \mu\text{l}$ of isopropyl alcohol, washed with RNase-free 75% ethanol, briefly air dried, and resuspended in $50 \mu\text{l}$ RNase-free H_2O (Sigma–Aldrich) and stored at -80°C . The quantity and purity of the RNA was determined using a NanoDrop device.

RT and quantitative real-time PCR

Briefly, $5 \mu\text{g}$ of total RNA was reverse transcribed to cDNA using a commercially available kit (High Capacity cDNA RT Kit, Applied Biosystems) according to the manufacturer's protocol including RNase inhibitor. Real-time PCR was carried out on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using the pre-designed

commercially available Human Endogenous Control Array (Applied Biosystems, catalog no. 4367563) and the TaqMan Custom Arrays (Applied Biosystems) for determination of suitable reference genes with no regulation upon testosterone treatment and regulation of gene expression of the assessed target genes respectively. Quantitative real-time PCR (qRT-PCR) for 16 reference genes was carried out on pre- and post-treatment samples from eight individuals and run in triplicates (data not shown). Three suitable reference genes (*IPO8*, *POLR2A*, and *PPIA*) were chosen based on geNorm (32) analyses and included in the TaqMan Custom Arrays that were carried out in triplicates for qRT-PCR of the target genes (Table 1). The resulting data were analyzed using the qBase Biozelle Software (Zwijnaarde, Belgium) (32, 33) with normalization to the geometric mean of the three reference genes.

Muscle lysate

Muscle tissue was freeze-dried and dissected free of visual blood, fat, and connective tissues. Muscle lysate was prepared by homogenization of muscle tissue (1:80, weight:vol) in a buffer containing the following: 50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 20 mmol/l Na-pyrophosphate, 20 mmol/l β -glycerophosphate, 10 mmol/l NaF, 2 mmol/l Na-orthovanadate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Noidet P-40, 10% glycerol, 2 mmol/l phenylmethylsulphonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 3 mmol/l benzaminidine. Homogenates were rotated end-over-end for 1 h at 4 °C, and then cleared by centrifugation at 17 000 *g* at 4 °C for 20 min. Protein content in the supernatant was measured by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA).

Table 1 Gene symbols, protein names, and qRT-PCR primer and probe information of genes studied. Assay ID: Applied Biosystems/Life Technologies TaqMan Array assay ID.

Gene symbol	Protein name	Assay ID
Reference genes		
<i>IPO8</i>	Importin-8	Hs00183533_m1
<i>POLR2A</i>	DNA-directed RNA polymerase II subunit RPB1	Hs00172187_m1
<i>PPIA</i>	Cyclophilin A	Hs99999904_m1
Target genes		
Lipid metabolism (adiponectin–AMPK signaling)		
<i>ADIPOR1</i>	Adiponectin receptor 1	Hs00360422_m1
<i>ADIPOR2</i>	Adiponectin receptor 2	Hs00226105_m1
<i>PRKAA2</i>	AMPK subunit alpha-2	Hs00178903_m1
<i>PRKAG3</i>	AMPK subunit gamma-3	Hs00179660_m1
Lipid metabolism (fusion of lipid droplets)		
<i>SNAP23</i>	Synaptosomal-associated protein 23	Hs00187075_m1
<i>STX5</i>	Syntaxin-5	Hs00908470_m1
Lipid metabolism (transport of lipids, regulation of β -oxidation)		
<i>ACADVL</i>	Very long-chain specific acyl-CoA dehydrogenase	Hs00817723_g1
<i>CD36</i>	Fatty acid translocase	Hs00169627_m1
<i>CPT1B</i>	Carnitine <i>O</i> -palmitoyltransferase 1, muscle isoform	Hs00992664_m1
<i>HADH</i>	Trifunctional enzyme subunit alpha	Hs00193428_m1
<i>PDK4</i>	Pyruvate dehydrogenase kinase 4	Hs01037712_m1
Glycolysis		
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
<i>PGAM2</i>	Phosphoglycerate mutase 2	Hs00165474_m1
TCA cycle and oxidative phosphorylation		
<i>ATP5A1</i>	ATP synthase subunit alpha	Hs00900735_m1
<i>COX5B</i>	Cytochrome c oxidase subunit 5B	Hs00426948_m1
<i>CS</i>	Citrate synthase	Hs02574374_s1
<i>ETFA</i>	Electron transfer flavoprotein subunit alpha	Hs00164511_m1
<i>NDUFS1</i>	NADH-ubiquinone oxidoreductase 75 kDa subunit	Hs00192297_m1
<i>SDHA</i>	Succinate dehydrogenase (ubiquinone) flavoprotein subunit	Hs00188166_m1
<i>UQCRC1</i>	Cytochrome <i>b</i> – <i>c</i> 1 complex subunit 1	Hs00163415_m1
<i>PPARGC1A</i>	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Hs01016719_m1

TCA cycle, tricarboxylic acid cycle.

SDS–PAGE and western blot analyses

Muscle lysate proteins were boiled in Laemmli buffer and separated by SDS–PAGE on self-cast Tris–HCl (7–12%) gels. Proteins were transferred onto a PVDF membrane (Immobilon Transfer Membranes; Millipore, Bagsværd, Denmark) by semidry blotting. The membrane was blocked in a TBST milk (2–5%) or BSA (3%) solution and afterwards probed with primary antibodies and appropriate secondary antibodies (see below). Protein bands were visualized using a Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany) after probing with enhanced chemiluminescence (Merck Millipore, Billerica, MA, USA). Bands were quantified using Science Lab Multi-Gauge, version 3.0 (LifeScience FujiFilm, Tokyo, Japan).

Antibodies used for immunoblotting

The antibodies used for detection of specific phosphorylation sites and protein expressions were as follows: ND6 protein (complex I), Molecular Probes (Eugene, OR, USA), Invitrogen (#A31857), USA; SDHA protein (complex II), Molecular Probes, Invitrogen (#A11142); UQCRC1 protein (complex III), Molecular Probes, Invitrogen (#A21362); p38 MAPK protein: p38 MAPK, Cell Signaling Technology (#9212), Danvers, MA, USA; p38 MAPK phosphorylation: phospho-p38 MAPK (Thr180/Tyr182 antibody, Cell Signaling Technology (#9211)); AMPK α phosphorylation at Thr172: phospho-AMPK α Thr172 antibody, Cell Signaling Technology (#2531); AMPK α 2 protein: antibodies raised in sheep as previously described (34) was kindly donated

by D G Hardie (University of Dundee, Scotland, UK); and AMPK γ 3 protein: anti-gamma3, Zymed Laboratories (#52-5717), San Francisco, CA, USA.

Statistical analyses

Statistical analyses were performed using the PASW Statistics 18 Package (SPSS, Inc.). Differences between the groups and within groups were analyzed by the Student's *t*-tests for paired and unpaired data of clinical and metabolic parameters, and with Mann–Whitney *U*-test for unpaired and Wilcoxon's signed-rank test for paired data of mRNA levels, protein abundance, and phosphorylation data. Univariate correlation analysis was performed using Spearman's ρ correlation coefficient. Data are presented as mean $\pm$ S.E.M. *P* values below 0.05 were considered significant. For the effect of testosterone therapy on expression of the 21 genes, a Bonferroni's corrected *P* value $<0.05/21=0.0024$ was applied to adjust for multiple testing.

Results

Clinical and metabolic characteristics

Before treatment, there were no differences in biochemical measures, body composition (LBM or percent body fat), insulin sensitivity, or substrate metabolism (RER, glucose, or lipid oxidation) between the groups (Table 2 and Fig. 1). In the participants included in the present study,

Table 2 Effect of testosterone on body composition and metabolic parameters. Data are mean $\pm$ S.E.M.

	Placebo before	Placebo after	Testo before	Testo after
<i>n</i>	13	13	12	12
Age (years)	69 $\pm$ 1.6	69 $\pm$ 1.6	69 $\pm$ 1.3	69 $\pm$ 1.3
Lean body mass (kg)	64.3 $\pm$ 1.9	64.4 $\pm$ 2.0	62.4 $\pm$ 2.3	64.0 $\pm$ 2.4 ^{*,¶}
Total fat mass (kg)	24.2 $\pm$ 1.9	24.0 $\pm$ 1.8	25.9 $\pm$ 1.5	23.9 $\pm$ 1.5 ^{‡,a}
Percent body fat (%)	26.2 $\pm$ 1.4	26.0 $\pm$ 1.3	28.4 $\pm$ 1.0	26.3 $\pm$ 1.0 ^{‡,¶}
BMI (kg/m ²)	29.4 $\pm$ 1.1	29.3 $\pm$ 1.1	30.2 $\pm$ 1.1	30.5 $\pm$ 1.2
Total testosterone (nmol/l)	12.6 $\pm$ 1.2	9.5 $\pm$ 0.6 [†]	11.4 $\pm$ 1.0	21.2 $\pm$ 2.8 ^{‡,§,b}
Bioavailable testosterone (nmol/l)	4.8 $\pm$ 0.4	3.8 $\pm$ 0.3 [†]	4.8 $\pm$ 0.3	10.0 $\pm$ 1.1 ^{‡,¶,b}
Free testosterone (nmol/l)	0.25 $\pm$ 0.02	0.20 $\pm$ 0.02 [†]	0.25 $\pm$ 0.02	0.51 $\pm$ 0.06 ^{‡,§,b}
SHBG (nmol/l)	47 $\pm$ 5	43 $\pm$ 5 [†]	40 $\pm$ 6	38 $\pm$ 5
Plasma adiponectin (mg/l)	9.2 $\pm$ 1.2	9.4 $\pm$ 1.1	8.3 $\pm$ 1.0	7.5 $\pm$ 1.0 ^{‡,¶}
Serum insulin (pmol/l)	45 $\pm$ 6	45 $\pm$ 6	59 $\pm$ 11	56 $\pm$ 11
Rd basal (mg/min per m ²)	85 $\pm$ 5	73 $\pm$ 4 [*]	83 $\pm$ 3	78 $\pm$ 2
Rd clamp (mg/min per m ²)	260 $\pm$ 36	283 $\pm$ 44	213 $\pm$ 25	230 $\pm$ 23
FFA basal (mmol/l)	0.48 $\pm$ 0.04	0.49 $\pm$ 0.04	0.52 $\pm$ 0.03	0.52 $\pm$ 0.04
FFA clamp (mmol/l)	0.07 $\pm$ 0.01	0.04 $\pm$ 0.01	0.10 $\pm$ 0.04	0.06 $\pm$ 0.01

**P* < 0.05, [†]*P* < 0.01, and [‡]*P* < 0.001 vs before. [§]*P* < 0.05 and [¶]*P* < 0.001 Testo after vs Placebo after. ^{*}*P* < 0.05, ^a*P* < 0.01, and ^b*P* < 0.001 treatment-induced changes (delta-effects) Testo vs Placebo.

Figure 1

Whole-body (A) respiratory exchange ratio (RER), (B) glucose oxidation (mg/min per m²), and (C) lipid oxidation (mg/min per m²) measured by indirect calorimetry in the basal state in elderly men with subnormal testosterone levels before (white bars) and after (black bars) treatment with either placebo ($n=13$) or testosterone (Testo; $n=12$) for 6 months. Data are mean $\pm$ S.E.M. # $P<0.05$ vs before; * $P<0.05$ and (*) $P<0.10$ treatment-induced changes (delta-effects) Testo vs Placebo.

testosterone therapy caused a twofold increase in serum levels of bioavailable, free, and total testosterone (all $P<0.01$), but no change in serum SHBG. In the placebo group, a small decrease in the measures of circulating

testosterone ($P<0.01$) was accompanied by a small decrease in SHBG ($P=0.007$). Moreover, testosterone therapy ($n=12$) but not placebo ($n=13$) for 6 months decreased percent body fat ($P<0.001$) and increased LBM ($P=0.011$). In the testosterone group, plasma adiponectin decreased ($P=0.004$), while no change was seen in the placebo group. Consistent with the findings earlier, the treatment-induced changes (delta-values) in these parameters of body composition, testosterone levels, and adiponectin were significantly larger in the testosterone than the placebo group (all $P<0.05$). As reported in the entire study cohort (4), testosterone therapy had no effect on insulin-stimulated glucose disposal rates (Rd) (Table 2), but it increased basal lipid oxidation and decreased basal glucose oxidation and RER (Fig. 1). These effects were also significant when evaluated as treatment-induced changes compared with the placebo group.

Effect of testosterone on muscle genes-regulating metabolism

To test whether the testosterone-induced increase in lipid oxidation was explained by changes in related biological processes within skeletal muscle, we examined transcript levels of genes involved in mitochondrial biogenesis, OxPhos, tricarboxylic acid (TCA) cycle, glycolysis, and lipid metabolism (Table 1). There were no differences in transcript levels of these genes between the groups before treatment (Fig. 2). Testosterone therapy had no effect on muscle mRNA levels of genes involved in OxPhos, TCA cycle, glycolysis, or lipid metabolism, but caused a downregulation of *ADIPOR2* ($P=0.041$). Moreover, post-treatment mRNA levels of *PRKAG3* ($P=0.039$) were higher in the testosterone-treated group than in the placebo group. However, these differences were not significant after correction for multiple testing. When evaluating fold-changes in response to treatment, there were no differences in response between the testosterone and the placebo groups.

Effect of testosterone on regulators of mitochondrial biogenesis and OxPhos protein levels

To validate the transcriptional findings, we tested the effect of testosterone therapy on protein levels of OxPhos subunits and known regulators of mitochondrial biogenesis. Protein levels of OxPhos genes encoded by both nuclear (*SDHA* and *UQCRC1*) and mitochondrial DNA (*ND6*) (Fig. 3), protein abundance of AMPK α 2 and AMPK γ 3 subunits, and p38 MAPK, as well as

Figure 2

Transcript levels (mRNA) of genes involved in (A) AMPK signaling and lipid metabolism and (B) genes involved in oxidative phosphorylation (OxPhos), glycolysis, and TCA cycle in skeletal muscle biopsies obtained in the resting, fasting state from elderly men with subnormal bioavailable testosterone levels before and after treatment with either placebo ($n = 13$) or testosterone (Testo; $n = 12$) for 6 months. Data are mean $\pm$ S.E.M., and are given as fold-changes. # $P < 0.05$ vs before and * $P < 0.05$ vs placebo after.

phosphorylation of AMPK α -subunits and p38 MAPK (Fig. 4) in skeletal muscle were unaffected by testosterone therapy for 6 months.

Changes in muscle transcripts related to changes in body composition, metabolism, and testosterone

We next examined the relationship between testosterone-induced changes in body composition, basal substrate metabolism, and circulating testosterone and changes

in muscle transcript levels. The increase in LBM correlated positively with changes in mRNA levels of *ACADVL*, *CD36*, *HADH*, *CPT1B*, *ETFA*, and *COX5B* ($r = 0.64$ – 0.78 ; all $P < 0.05$). Consistently, the decrease in percent body fat correlated inversely with changes in mRNA levels of *ACADVL*, *CD36*, and *HADH* ($r = 0.58$ – 0.73 ; all $P < 0.05$). Moreover, the testosterone-induced decrease in plasma adiponectin correlated positively with changes in mRNA levels of the same three genes: *ACADVL*, *CD36*, and *HADH* ($r = 0.61$ – 0.68 ; all $P < 0.05$). The increase in basal glucose oxidation correlated negatively with the change in *STX5* expression ($r = -0.59$, $P < 0.05$). No other significant correlations were found (data not shown).

Baseline muscle transcripts related to substrate metabolism, testosterone levels, and body composition

To test to what extent we could reproduce the reported association of low testosterone and insulin resistance with expression of genes involved in OxPhos (19), we examined the relationship between the examined muscle transcripts and whole-body substrate metabolism, insulin sensitivity, testosterone levels, and body composition at baseline. In the total cohort, basal glucose oxidation and RER correlated

Figure 3

Protein abundance of OxPhos subunits encoded by (A and B) nuclear DNA (*SDHA* and *UQCRC1*) and (C) mitochondrial DNA (*ND6*) in skeletal muscle biopsies obtained in the resting, fasting state from elderly men with subnormal bioavailable testosterone levels before (white bars) and after (black bars) treatment with either placebo ($n = 12$) or testosterone ($n = 12$) for 6 months. Findings are shown in representative immunoblots (D). Results are expressed as mean $\pm$ S.E.M. * $P < 0.05$ vs before.

Figure 4

Protein abundance of (A and B) AMPKα2 and AMPKγ3 subunits and (C) p38 MAPK; phosphorylation of (D) AMPK (Thr172) and (E) p38 MAPK in skeletal muscle biopsies obtained in the resting, fasting state from elderly men with subnormal bioavailable testosterone levels before (white bars) and after (black bars) treatment with either placebo (n=12) or testosterone (n=12) for 6 months. Findings are shown in representative immunoblots (F). Results are expressed as mean ± S.E.M. *P < 0.05 vs before.

positively with muscle mRNA levels of most genes involved in OxPhos and TCA cycle and a few genes involved in lipid metabolism (Table 3). Basal lipid oxidation showed an inverse relationship with mRNA levels of four OxPhos muscle transcripts. Insulin-stimulated Rd was not significantly associated with muscle transcripts of any genes involved in OxPhos, TCA cycle, or glycolysis, but correlated negatively with mRNA levels of the AMPK subunit, *PRKAG3*, and positively with *SNAP23* and *CPT1B* (Table 3). Pretreatment levels of bioavailable testosterone and to a lesser extent total testosterone correlated positively with the expression of some genes involved in OxPhos, TCA cycle, and lipid metabolism. Fasting plasma FFA correlated inversely with transcript levels of *CS*, *ATP5A1*, *SDHA*, and *UQCRC1*, and positively with expression of *PDK4* (Table 3). Percent body fat was negatively associated with muscle transcript levels of several genes involved in OxPhos, TCA cycle, and different aspects of lipid metabolism, and positively associated with *PRKAG3* expression. LBM only correlated with expression of *CD36*.

Discussion

Recent studies have indicated a link between testosterone, insulin sensitivity, and mitochondrial oxidative capacity, including lipid oxidation (4, 19, 20, 21, 22, 23, 24). We hypothesized that testosterone therapy, which is known to cause favorable changes in body composition, would stimulate mitochondrial biogenesis and lead to increased abundance of molecular markers of OxPhos and lipid metabolism in skeletal muscle. However, we here report that transdermal testosterone therapy for 6 months did not change the expression of *PGC1α* or genes involved in OxPhos, TCA cycle, or lipid metabolism in the skeletal muscle of elderly men with subnormal bioavailable testosterone levels. Consistently, we demonstrate that testosterone therapy has no effect on protein levels of representative OxPhos subunits or protein abundance and phosphorylation of two enzymes, AMPK and p38 MAPK, known to regulate mitochondrial biogenesis through *PGC1α* (25). While elderly men with increased adiposity, low-normal testosterone, and low insulin sensitivity may have impaired mitochondrial oxidative capacity in skeletal muscle (19), our results strongly indicate that therapy with physiological doses of testosterone does not improve mitochondrial biogenesis or insulin sensitivity in aging men with subnormal bioavailable testosterone levels. This suggests that other factors, which are not corrected by testosterone therapy, may play a greater role for the decreased insulin sensitivity and reduced oxidative capacity in these male individuals.

Androgen mediates many of its actions in skeletal muscle by binding and activating its receptor, the androgen receptor (AR), which leads to transcription of target genes (3). A recent study of myotubes from male donors has shown that the testosterone-mediated increase in palmitate oxidation was attenuated in the presence of an AR antagonist (24). This suggested that the positive effect of testosterone on lipid oxidation reported in humans (4, 20, 21, 22) could be mediated by the AR in skeletal muscle. Accordingly, it has been reported that the testosterone-mediated stimulation of whole-body lipid oxidation is not due to an increase in liver lipid oxidation, but rather takes place in peripheral tissues such as skeletal muscle (20). As reported in other clinical trials (1, 2, 3, 4), we found a modest but significant increase in muscle mass (LBM) and a reciprocal decrease in adiposity in response to testosterone therapy. These favorable changes in body composition, which were not accompanied by increased insulin sensitivity, were associated with a shift in substrate partitioning toward increased lipid oxidation. The main finding of the present study is that

Table 3 Muscle transcripts related to substrate metabolism, insulin sensitivity, testosterone levels, and body composition. Spearman's ρ correlation coefficients are given.

Gene name	RER basal	GOX basal	LOX basal	Rd clamp	Bioavailable testosterone	Total testosterone	FFA basal	Fat %	LBM
<i>ADIPOR1</i>	0.23	0.21	-0.18	0.13	0.22	0.18	-0.09	-0.38	0.23
<i>ADIPOR2</i>	0.03	0.13	0.11	-0.001	0.18	0.25	-0.09	-0.23	0.05
<i>PRKAA2</i>	0.01	0.06	0.08	0.20	-0.06	-0.04	-0.02	-0.16	-0.14
<i>PRKAG3</i>	0.11	0.11	-0.15	-0.56 [†]	0.10	-0.14	0.20	0.46*	0.08
<i>SNAP23</i>	-0.07	0.04	0.20	0.45*	0.04	0.32	-0.08	-0.65 [†]	-0.01
<i>STX5</i>	-0.17	0.02	0.36	0.30	0.15	0.46*	0.12	-0.54 [†]	0.08
<i>ACADVL</i>	0.26	0.45*	-0.05	0.06	0.43*	0.43*	-0.05	-0.45*	0.25
<i>CD36</i>	-0.02	0.16	0.24	0.04	0.31	0.50*	-0.15	-0.47*	0.40*
<i>CPT1B</i>	0.07	0.16	0.04	0.50*	0.11	0.33	-0.21	-0.69 [†]	0.17
<i>HADH</i>	0.43*	0.47*	-0.26	0.29	0.21	0.06	-0.20	-0.36	0.15
<i>PDK4</i>	-0.30	-0.29	0.31	-0.19	-0.26	0.10	0.66 [†]	-0.08	-0.31
<i>GAPDH</i>	0.29	0.23	-0.21	-0.08	-0.04	-0.26	-0.13	0.12	0.02
<i>PGAM2</i>	0.31	0.19	-0.25	-0.06	-0.09	-0.18	-0.08	-0.043	0.10
<i>ATP5A1</i>	0.56 [†]	0.58 [†]	-0.43*	0.06	0.43*	0.11	-0.44*	-0.37	0.30
<i>COX5B</i>	0.54 [†]	0.52 [†]	-0.46*	0.03	0.47*	0.12	-0.37	-0.23	0.34
<i>CS</i>	0.43*	0.43*	-0.25	0.34	0.41*	0.29	-0.44*	-0.63 [†]	0.10
<i>ETFA</i>	0.40*	0.51 [†]	-0.19	0.26	0.32	0.29	-0.27	-0.61 [†]	0.25
<i>NDUFS1</i>	0.38	0.46*	-0.20	0.25	0.44*	0.33	-0.32	-0.55 [†]	0.30
<i>SDHA</i>	0.47*	0.51 [†]	-0.28	0.23	0.30	0.22	-0.56 [†]	-0.59 [†]	0.22
<i>UQCRC1</i>	0.64 [†]	0.65 [†]	-0.47*	0.21	0.46*	0.15	-0.46*	-0.45*	0.16
<i>PPARGC1A</i>	0.49*	0.44*	-0.43*	0.34	0.32	0.14	-0.16	-0.51 [†]	-0.14

RER, respiratory exchange ratio; GOX, glucose oxidation; LOX, lipid oxidation; LBM, lean body mass. * $P < 0.05$ and [†] $P < 0.01$.

the testosterone-induced increase in lipid oxidation is not mediated by changes in transcriptional levels of genes involved in mitochondrial biogenesis, OxPhos, TCA cycle, or lipid metabolism in skeletal muscle.

Mitochondrial biogenesis is a strongly coordinated response, which involves the activation of a key co-transcription factor, PGC1 α , and several down-stream factors (25). In our study, the absence of increase in the expression of PGC1 α and of mRNA and protein levels of OxPhos subunits encoded by both nuclear and mitochondrial DNA provide evidence that testosterone therapy does not improve mitochondrial oxidative capacity by increasing mitochondrial biogenesis in human skeletal muscle. In accordance, testosterone therapy did not change the protein abundance or phosphorylation-dependent activation of AMPK or p38 MAPK, which are known to regulate mitochondrial biogenesis through transcriptional and phospho-dependent control of PGC1 α in skeletal muscle (25).

AMPK plays a critical role in skeletal muscle metabolism by regulating a number of metabolic pathways in response to changes in cellular energy status (35, 36). In addition to its effect on PGC1 α -mediated mitochondrial biogenesis, AMPK stimulates lipid oxidation by inhibition of acetyl-CoA carboxylase- β (ACC β) (35). Recombinant adiponectin has been shown to promote fatty acid oxidation by mechanisms that not only involve stimulation of AMPK and inhibition

of ACC β (37) but also include activation of p38 MAPK (38). Moreover, plasma adiponectin has been reported to correlate with both insulin sensitivity and markers of mitochondrial content in human skeletal muscle (39, 40). However, despite a significant decrease in plasma adiponectin in response to testosterone, we observed no changes in the abundance or phosphorylation-dependent activities of AMPK or p38 MAPK, and there was no change in the expression of PGC1 α or genes involved in OxPhos or lipid metabolism. Based on findings in myotubes from male donors, we expected that testosterone would increase lipid oxidation in skeletal muscle by increasing the activity of AMPK and p38 MAPK (24). Our results suggest the possibility that the decrease in plasma adiponectin counteracted any direct effect of increasing testosterone levels on muscle levels and activity of AMPK and p38 MAPK. Another possibility is that neither the decrease in plasma adiponectin nor the increase in circulating testosterone affects these enzymes. These findings demonstrate that the testosterone-induced increase in lipid oxidation does not involve changes in AMPK or p38 MAPK signaling in skeletal muscle. This suggests that other peripheral tissues than skeletal muscle may be more responsible for the increase in whole-body lipid oxidation. For example, recent animal studies have demonstrated a positive effect of testosterone on adipocyte dysfunction and differentiation capacity (41). Thus, other tissues including different types of adipose tissue and the

liver are other interesting sites of action in response to testosterone therapy, not only with respect to the regulation of lipid metabolism and insulin sensitivity, but also the decrease in fat mass as well as reduced plasma adiponectin. Further studies are warranted to establish the mechanisms by which testosterone stimulates lipid oxidation in specific peripheral tissues, and to what extent this involves skeletal muscle.

In our study, aging men with subnormal bioavailable testosterone below 7.3 nmol/l were included. A number of these men had total testosterone levels slightly above or within the range (8–12 nmol/l) defined as equivocal by recent recommendations (42, 43). Therefore, the effects of testosterone therapy on markers of mitochondrial OxPhos and lipid metabolism in the skeletal muscle of aging men with unequivocally low total testosterone levels (<8 nmol/l) remain to be investigated. In addition, our study reports the chronic effects of testosterone therapy, and therefore we cannot exclude transient acute effects of testosterone on expression, abundance, or phosphorylation of the genes and proteins studied. Another potential limitation of our study was the lack of assessment of physical activity level in the study participants. Finally, our sample size was sufficient to detect a response to testosterone therapy larger than 20–30% with a power >80% for most of the genes and proteins investigated. This, however, suggests the possibility that effects lower than this may have escaped identification, although the biological relevance of such changes may be questioned.

Although we report that no effect on genes involved in OxPhos and lipid metabolism was seen, the favorable changes in body composition induced by testosterone therapy correlated positively with changes in muscle transcripts of certain genes involved in lipid metabolism (*CD36*, *ACADVL*, and *HADH*). In contrast, the changes in the expression of these three genes correlated negatively with the decrease in plasma adiponectin induced by testosterone. This suggests that in men who respond to testosterone with an improved body composition but without a decrease in plasma adiponectin, the expression of certain genes involved in energy metabolism may increase. These findings also indicate that the decrease in plasma adiponectin seen in response to testosterone in many studies (4, 44, 45), but not all studies (46), may counteract the positive effects on body composition and contribute to some of the discrepancies reported in clinical trials. It was recently reported that oxidative capacity correlated with testosterone levels and insulin sensitivity in a mixed population of men (19). In our cohort of nondiabetic men with a narrow range of

bioavailable testosterone in subnormal concentrations, we found that pretreatment expression of some genes involved in OxPhos and lipid metabolism levels correlated positively with testosterone and inversely with adiposity. However, there was no significant correlation between insulin sensitivity and expression of genes involved in OxPhos or lipid metabolism. This suggests that in men with the lowest testosterone levels and highest degree of adiposity, mitochondrial OxPhos and lipid metabolism may be further compromised in skeletal muscle.

In summary, we conclude that testosterone therapy has no significant effect on known regulators of mitochondrial biogenesis or markers of OxPhos and lipid metabolism in the skeletal muscle of aging men with subnormal bioavailable testosterone levels, despite a favorable effect on muscle mass, adiposity, and basal lipid oxidation. Our results show that a clinically relevant increase in bioavailable testosterone in a randomized, placebo-controlled trial is not accompanied by improvements in insulin sensitivity or markers of mitochondrial oxidative capacity in skeletal muscle of elderly men.

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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Author contribution statement

S J Petersson, J M Kristensen, R Kruse, and K Højlund were responsible for the conception and design of the study, analysis, and interpretation of data, and drafting the article. L L Christensen and M Andersen were responsible for the clinical parts of the study. S J Petersson, L L Christensen, J M Kristensen, R Kruse, M Andersen, and K Højlund were responsible for revising the article critically for important intellectual content and final approval of the version to be published.

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