Testosterone or 17 β -estradiol exposure reveals sex-specific effects on glucose and lipid metabolism in human myotubes

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Abstract

Changes in sex hormone levels with aging or illness may lead to metabolic disorders. Moreover, the ratio changes in men versus women may have distinct pathological responses. Since little is known about sex hormone action on muscle metabolism, we examined the role of testosterone or 17 β -estradiol (E₂) in metabolism and investigated whether either hormone may mediate a sex-specific effect. Myotubes from postmenopausal women and age-matched male donors were treated with 10 nM testosterone or E₂ for 4 days, and assays were performed to measure metabolic readouts, signal transduction, and mRNA expression. Testosterone and E₂ treatment enhanced insulin-stimulated glucose incorporation into glycogen and AKT phosphorylation in myotubes from female donors, highlighting a sex-specific role of sex hormone in glucose metabolism. Testosterone treatment increased palmitate oxidation in

myotubes from both female and male donors, while E₂ enhanced palmitate oxidation in myotubes from male donors only. Testosterone-mediated increase in palmitate oxidation was attenuated at the presence of androgen receptor antagonist, which may indicate a role of nuclear steroid receptor in muscle lipid oxidation. Testosterone treatment increased mRNA expression of the insulin receptor substrate 2 in myotubes from male and female donors, whereas it increased mRNA expression of glycogen synthase 1 only in myotubes from male donors. E₂ treatment increased pyruvate dehydrogenase kinase 4 mRNA expression in myotubes from female donors. Thus, our data suggest that testosterone or E₂ modulates muscle glucose and lipid metabolism and may play a role in metabolism in a sex-dependent manner.

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Introduction

The incidence of metabolic syndrome, also called insulin resistance syndrome, increases substantially during menopause (Polotsky & Polotsky 2010) and advancing age. The free levels of sex hormones were decreased during aging, which may indicate an important role of sex hormones in metabolic homeostasis. Thus, the influence of sex hormones and relative risks for metabolic disease are commonly related to the circulating plasma level of the different sex hormones (Shoupe & Lobo 1984, Haffner *et al.* 1994, 1996, van den Beld *et al.* 2000, Abate *et al.* 2002, Tivesten *et al.* 2007). Declined testosterone serum levels correlate to incidence of type 2 diabetes in men and is associated with aging (Grossmann *et al.* 2010), indicating an important role of testosterone in metabolism. Interestingly, estradiol replacement therapy in postmenopausal women may partially provide cardiovascular protection and is suggested to be both estrogen receptor (ER) dependent and independent (for review, Dubey & Jackson (2001)). Moreover, cardiovascular disease is less common in premenopausal women when compared with age-matched men, and this observed sex difference is abolished after

menopause in women (Mercuro *et al.* 2003), a finding attributed to altered levels of sex hormones with decreased female sex hormones.

Many lines of evidence point to gender aspects as a risk factor for metabolic disorders and highlight distinct roles of androgen and estradiol on whole-body metabolism (Roepstorff *et al.* 2002, Mercuro *et al.* 2003, Murphy & Steenbergen 2007, Regitz-Zagrosek *et al.* 2007). Testosterone levels decline with age in men (Tenover *et al.* 1987), therefore testosterone replacement therapy in older men has become more of interest, and it is gaining an increasing interest and attention in the treatment of insulin resistance. Previously, the total normal circulating levels (free and bound) of testosterone and estrogen (measured at 8.86 and 0.12 nM respectively) exhibited a strong correlation to body composition and muscle strength in elderly men (van den Beld *et al.* 2000).

Low-dose testosterone treatment in castrated male rats reduces blood glucose levels and increases skeletal muscle glycogen stores, whereas 17 β -estradiol (E₂) has no effect. Thus, testosterone and E₂ have differential effects on glucose metabolism in skeletal muscle (Ramamani *et al.* 1999). In addition to the role of sex hormones in glucose

metabolism, these mediators also play a role in fat metabolism. Estradiol treatment reduces the expression of lipogenic genes in adipocytes from ovariectomized mice (D'Eon *et al.* 2005) and reduces lipid oxidation in skeletal muscle in ovariectomized rats (Campbell & Febbraio 2001), while testosterone replacement enhances lipid metabolism and decreases fat depots in men (Zitzmann 2008). Estradiol hormone replacement in postmenopausal women exhibited positive changes on a wide range of metabolic parameters, including improved peripheral vascular reactivity, increased high-density lipoprotein levels (Al-Khalili *et al.* 1998, Godsland 2001), reduced abdominal obesity, enhanced overall insulin sensitivity (Andersson *et al.* 1997), lower lipid levels, lower blood pressure (Salpeter *et al.* 2006, Lobo 2008), as well as prevention of new type 2 diabetes cases. Similarly, testosterone treatment of obese men with hypogonadism (Marin *et al.* 1992, Miner *et al.* 2008) decreased plasma insulin and glucose levels, improved the waist/hip ratio, and enhanced muscle strength (Marin *et al.* 1992).

Recent therapeutic strategies targeting sex hormones have been proposed (Casey & Barkin 2008, Miner *et al.* 2008, Zitzmann 2008) for the treatment of type 2 diabetes through administration of sex steroids to reduce cardiovascular risk in both young and elderly patients. Clinical studies provide evidence for sex differences in insulin sensitivity and the pathogenesis of type 2 diabetes due to the specific effects of circulating testosterone and E₂ (for review, Haffner (2000)). For example, a low testosterone level in men predicts insulin resistance and progression to type 2 diabetes, whereas an elevated testosterone level in women is associated with metabolic impairments (Haffner 2000, Oh *et al.* 2002). Since skeletal muscle is a major target organ for insulin-regulated glucose metabolism, it is possible that sex hormones may play a significant role in skeletal muscle metabolism and may have distinct sex-dependent metabolic effects on skeletal muscle derived from men versus women.

Thus, hormonal replacement therapy has a beneficial effect on total body metabolism and substrate utilization for aged subjects (Baltgalvis *et al.* 2010). The androgen receptor (AR) is expressed higher in young men compared with old men (Poole *et al.* 2011). As in aged subjects, estrogen is suggested to improve cellular stress via ER (Baltgalvis *et al.* 2010), and the AR mRNA expression may change in response to exercise with respect to aging (Poole *et al.* 2011). Therefore, we speculate that sex hormone treatment of aged muscle should improve total cell metabolism and substrate turnover.

Currently, however, little is understood regarding the outcome of hormone replacement therapy with respect to the direct role of sex hormones on insulin action on skeletal muscle, the subsequent consequences on local lipid and glucose metabolism, and importantly, the impact on type 2 diabetes (Singh *et al.* 2002). Clearly, the role of sex hormones and intrinsic sex differences in skeletal muscle cell metabolism via sex hormone treatment is not well elucidated, and further investigations are warranted to unravel the role of sex hormones.

In this study, we isolated and cultured skeletal muscle cells derived from male and female donors in the presence of either testosterone or E₂ and determined the effects on metabolism and gene expression. We have recently demonstrated that cultured skeletal muscle myotubes derived from postmenopausal women and age-matched male donors do not retain intrinsic sex differences in basal glucose and lipid metabolism (Rune *et al.* 2009). Given this finding, we hypothesized that sex hormone treatment plays an important role in metabolism in skeletal muscle cell, and intrinsic sex-related differences in metabolism may become apparent following sex hormone exposure. In this study, we provide evidence that testosterone and E₂ have differential effects on lipid and glucose metabolism and the mRNA expression of metabolic genes, as well as a sex-dependent influence on glucose incorporation into glycogen in myotubes from female and male donors. Thus, testosterone or E₂ is involved in skeletal muscle metabolism, and the skeletal muscle cells retain a sex-dependent response to sex steroids in an exogenous *in vitro* environment.

Materials and Methods

Material

DMEM, DMEM-F-12, fetal bovine serum (FBS), penicillin/streptomycin, and fungizone were obtained from Gibco (Invitrogen). Testosterone, E₂, and CDX (Bicalutamide, AR antagonist) were purchased from Sigma-Aldrich. Charcoal/dextran-treated FBS was from HyClone (Logan, UT, USA). Radioactive reagents ([9-10(*n*)-³H] palmitate was purchased from Amersham or ¹⁴C-palmitate and ¹⁴C-glucose were purchased from Perkin-Elmer (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA).

Subjects

A cohort of healthy postmenopausal women and age-matched (60±1 years) male volunteers (11 men and 10 women), with no family history of diabetes or heart disease, was selected based on clinical characteristics of body mass index for male (27±0.6) and for female (30±1.3) subjects (not significant and fasting glucose (5.3±0.1 mmol/l) as reported previously (Rune *et al.* 2009)). Urinalysis, blood chemistry, and measurements of blood pressure and anthropometric parameters were assessed at Karolinska University Hospital, Stockholm, Sweden, and were found to be normal. None of the male donors were under hormone replacement therapy. Of the female participants, one woman received estrogen replacement therapy, Vagifem 0.25 mg 2x/w, a vaginal tablet that releases small amounts of estradiol locally into the vaginal tissues and is used at short term. This individual was included in all data analysis, since the obtained values for this study fell within a normal range. Skeletal muscle biopsies from *vastus lateralis* were obtained from 11

male and 10 female donors of the cohort. The ethical committee at Karolinska Institutet approved all protocols and informed consent was received.

Satellite cell culture

Satellite cells were isolated from skeletal muscle biopsies by trypsin-EDTA digestion and cultured as described (Al-Khalili *et al.* 2003), with some modifications. Biopsies were washed in PBS supplemented with 1% penicillin/streptomycin and 1% fungizone. Isolated satellite cells were propagated and differentiated into myotubes as described (Al-Khalili *et al.* 2003). Cells were studied at passage 3–5. Myotubes were cultured in DMEM supplemented with 2% charcoal/dextran-treated FBS and treated with 0.1% ethanol as control, 10 nM testosterone, or 10 nM E₂ for 3 days. Media were replaced once a day and serum starvation occurred on the 4th day of treatment. An additional concentration of the testosterone or E₂ (1 nM) was also used to determine the dose–response of sex hormones on palmitate oxidation. When the sex hormone receptor antagonist 10 nM CDX (AR antagonist) was used, myotubes were treated with 0.1% DMSO or CDX for at least 30 min before the addition of testosterone.

Skeletal muscle cell differentiation

Myotubes were grown on six-well plates for Giemsa–Wright staining as described previously (Al-Khalili *et al.* 2004). To assess the extent of myotubes differentiation by Giemsa–Wright staining, myotubes were fixed in methanol (10 min), 1:10 Giemsa (15 min), and 1:10 Wright (20 min). Cells were washed with double-distilled H₂O, and mono- or multi-nucleated cells were observed under a phase contrast inverted light microscope. Cells were placed over a Bürker chamber, and total number of myotubes were counted in 20 squares (0.04 mm²) using the 40× objective. Myotube formation and fusion rate after sex hormone stimulation were measured as percentage >5 nuclei/total myotubes/μm² over control. Moreover, the *GLUT4* mRNA expression was chosen to be the specific skeletal muscle cell marker for cell differentiation (Al-Khalili *et al.* 2003).

Quantitative real-time PCR

mRNA was extracted from human myotubes of seven male and seven female subjects following 4 days of hormone treatment using a Qiagen RNeasy Mini Kit (Qiagen), and cDNA was synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). mRNA expression was analyzed by real-time PCR with the ABI PRISM 7000 Sequence Detector System (Applied Biosystems Life Technologies, Sweden). mRNA expression of genes involved in metabolism was determined, including glycogen synthase 1 (*GYS1*), insulin receptor substrate 1 (*IRS1*), TBC1 domain family member 1 (*TBC1D1*), glucose transporter 4; *GLUT4* (*SLC2A4*) and pyruvate

dehydrogenase kinase isozyme 4 (*PDK4*), carnitine palmitoyltransferase 1 (*CPT1*), *IRS2*, stearoyl-CoA desaturase (*SCD*), peroxisome proliferator-activated receptor delta (*PPARD*) and gamma (*PPARG*), fatty acid translocase (*CD36*), sterol regulatory element-binding protein 1 (*SREBP1*; *SREBF1*), uncoupling protein 2 (*UCP2*), *UCP3*, and AMP-activated protein kinase alpha 2 (*AMPKα2*; *PRKAA2*). Sex hormone-specific genes including the enzyme P450 aromatase (P450arom; *CYP19A1*), the sex hormone-binding globulin (*SHBG*), ERα (*ER1*), ERβ (*ER2*), steroid-5-α-reductase, alpha polypeptide 1 (*SRD5A1*), *SRD5A2*, and *AR* were also measured. The primer/probes for all genes were purchased from ABI, with a FAM reporter. The relative abundance of the target transcript was calculated from duplicate samples after data were normalized against a housekeeping gene using CT comparative method. We tested three internal controls (housekeeping genes 18s, *GAPDH*, and β2-microglobulin). The housekeeping gene 18s was chosen for normalization to ensure highest accuracy of analysis and to standardize expression from myotubes.

Free fatty acid oxidation

³H palmitate assay The assay was performed as described previously (Rune *et al.* 2009). In brief, myotubes obtained from female (*n*=3) or male (*n*=3) donors were grown in 12-well plates, differentiated for 3 days, and treated for 4 days with either sex hormone in the presence or absence of the 0.1% DMSO as control or CDX (Bicalutamide). After serum starvation overnight, myotubes were supplemented with 0.2% fatty acid-free albumin from bovine serum and treated with 0.5 μCi palmitic acid [9–10(*n*)-³H] + 10 μM cold palmitate in the presence or absence of 1 or 10 nM testosterone or steroid receptor inhibitor (CDX) for 4–5 h (pre-incubated with CDX for 30 min and thereafter 30 min with testosterone). To absorb non-metabolized palmitate, 0.2 ml cell supernatant was mixed with 0.8 ml charcoal slurry (0.1 g charcoal powder in 1 ml 0.02 M Tris–HCl buffer, pH 7.5) in a 2 ml Eppendorf tube and shaken for 30 min. Samples were subjected to centrifugation for 15 min at 20 000 g, after which 0.3 ml supernatant with tritium-bound water was withdrawn, and radioactivity was determined in a liquid scintillation counter (Win-Spectral 1414 liquid scintillation counter; Wallac, Turku, Finland). Each experiment was performed in duplicate.

¹⁴C palmitate assay Assessment of free fatty acid oxidation was determined in myotubes from seven female and seven male subjects as described (Al-Khalili *et al.* 2006). Myotubes were grown in a 25 cm² cell culturing flasks. At day 4 of treatment with either 10 nM E₂ or 10 nM testosterone, myotubes were serum-starved overnight and thereafter treated with palmitate (0.4 μCi). Myotubes were incubated in the presence or absence of either sex hormone, with or without 120 nM insulin for 4.5 h (pre-incubated with either sex hormone for 30 min). Following the addition of 35% perchloric acid to the media, released ¹⁴CO₂ was trapped in a filter soaked with

solvable reagent (benzethonium hydroxide). The amount of released ¹⁴CO₂ was measured in a liquid scintillation counter (WinSpectral 1414 liquid scintillation counter, Wallac). Each experiment was performed on duplicate wells.

Glucose incorporation to glycogen

Glucose incorporation to glycogen was determined in myotubes from seven female and seven male subjects, as described previously (Al-Khalili *et al.* 2003). After 3 days of treatment with either 10 nM E₂ or 10 nM testosterone, myotubes were serum-starved overnight in the presence of either hormone. To assess glucose incorporation to glycogen, myotubes were incubated in the absence or presence of sex hormones and treated with 120 nM insulin for 30 min before adding D-[U-¹⁴C] glucose (1 μCi/ml) for the last 90 min at day 5. [¹⁴C]-labeled glycogen was counted in a liquid scintillation counter (WinSpectral 1414 liquid scintillation counter, Wallac). Each experiment was performed in duplicate.

Western blot analysis

Overnight serum-starved myotubes were treated with either sex hormone with or without 120 nM insulin for 20 min. Cells were harvested, pooled, and processed for western blot analysis as described previously (Al-Khalili *et al.* 2003). Total protein antibodies and phospho-specific antibodies AMPK^{thr172} (CHEMICON/Upstate/Linco, Sweden), AKT^{ser473}, pIRS1^{ser636}, p70^{s6k}, p38, and ERK 1/2 mitogen-activated protein kinase (MAPK) (all from Cell Signaling Technology, Sweden) were used. Total expression and phosphorylation of each target protein were quantified on the same membrane. A GAPDH (Cell Signaling Technology) antibody was used to confirm equal loading of proteins. Proteins were separated by 12–6% gradient SDS–PAGE,

transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), and blocked in 7.5% non-fat dried milk in Tris-buffered saline with 0.02% Tween (TBST) for 2 h at room temperature. Membranes were incubated overnight at 4 °C with total or phospho-specific antibodies (1:1000 dilution). After washing in TBST, the membranes were incubated with a HRP-labeled anti-rabbit IgG secondary antibody for all target proteins (1:25 000, Bio-Rad) for 1 h at room temperature, followed by additional washing in TBST. Proteins were visualized by enhanced chemiluminescence (Amersham) and quantified by densitometry (Al-Khalili *et al.* 2003).

Statistical analysis

Data are presented as mean ± S.E.M. Changes for comparison for factor ‘sex’ (female versus male at each condition) or factor ‘hormone treatment’ (testosterone versus control or E₂ versus control) were analyzed using two-way ANOVA, one factor repeated measurement for the basal group and for the insulin-stimulated group for signaling and metabolic data. A similar test was used for mRNA expression data analysis. A value of *P* < 0.05 was considered as significant. The analysis was performed using SigmaStat 3.5.

Results

Sex hormone effect on differentiation

Exposure to 10 nM testosterone or E₂ for 4 days did not lead to alterations in cell growth, differentiation, or appearance of myotubes, as assessed by the Giemsa–Wright staining of the myotubes under light microscopy (data not shown), compared with the untreated cells. To minimize the effect of external sex hormones, which might present in FBS,

Table 1 Unaltered mRNA expression of selected genes assessed in myotubes obtained from female and male donors after sex hormone exposure. Myotubes were treated with vehicle as control (0.1% ethanol), 10 nM 17β-estradiol, or testosterone for 4 days. Results are mean ± S.E.M. for *n* = 7 female subjects and *n* = 7 male subjects. Results were normalized to 18s rRNA, and all the values are multiplied by 10^{−6}

	Control		17β-Estradiol		Testosterone	
	Female	Male	Female	Male	Female	Male
Genes						
AMPKα2	0.64 ± 0.12	0.64 ± 0.11	0.60 ± 0.09	0.47 ± 0.09	0.55 ± 0.08	0.66 ± 0.17
CD36	101.96 ± 7.74	109.25 ± 29.10	83.23 ± 17.98	120.18 ± 27.09	93.95 ± 15.12	74.4 ± 24.26
ERα	0.67 ± 0.20	0.83 ± 0.04	0.46 ± 0.16	0.35 ± 0.05	0.14 ± 0.01	0.45 ± 0.06
FABP3	2.09 ± 0.44	2.23 ± 0.42	1.91 ± 0.29	2.43 ± 0.51	1.88 ± 0.20	2.24 ± 0.45
GLUT4	0.05 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.005	0.03 ± 0.01
IRS1	3.37 ± 0.32	2.43 ± 0.50	3.41 ± 0.44	2.33 ± 0.32	3.52 ± 0.69	4.21 ± 0.77
TBC1D1	0.74 ± 0.13	0.57 ± 0.05	0.64 ± 0.07	0.62 ± 0.01	0.63 ± 0.03	0.71 ± 0.09
UCP2	25.37 ± 8.71	23.22 ± 10.33	16.42 ± 7.63	5.54 ± 2.50	14.98 ± 6.22	5.75 ± 3.04
UCP3	0.65 ± 0.06	0.54 ± 0.08	0.81 ± 0.23	0.44 ± 0.09	1.01 ± 0.03	0.57 ± 0.04
PGC1α	0.3 ± 0.06	0.20 ± 0.06	0.37 ± 0.04	0.20 ± 0.10	0.27 ± 0.07	0.29 ± 0.10
SRD5A1	565.79 ± 90.23	491.83 ± 48.21	983.16 ± 317.22	442.01 ± 37.16	667.92 ± 220	427.40 ± 94.71
SREBP1	7.91 ± 1.89	12.58 ± 1.01	8.71 ± 1.23	11.06 ± 1.2	12.12 ± 1.03	12.44 ± 1.66

Figure 1 Dose-dependent effects of testosterone or 17β-estradiol on palmitate oxidation. Myotubes were treated for 4 days with or without 0 (0.1% ethanol), 1, and 10 nM testosterone, or 0 (0.1% ethanol), 1, and 10 nM E₂. Results are expressed as mean ± S.E.M. for myotubes derived from six subjects; three male and three female. [#]*P* < 0.05, basal sex hormone treatment versus basal control.

myotubes were cultured in 2% charcoal/dextran-treated FBS during sex hormone treatment. At baseline, the media supplemented with 2% charcoal/dextran-treated FBS showed no further changes on myotube differentiation when compared with myotubes differentiated at the presence of 2% normal FBS, as assessed by phenotypic changes after staining with Wright–Giemsa or by *GLUT4* mRNA expression levels (data not shown). Moreover, the mRNA expression of *GLUT4* was similar in myotubes exposed to 10 nM testosterone or E₂, when compared with the control group for 4 days (Table 1).

Dose–response of sex hormone exposure on lipid metabolism

To explore the effects of hormone treatment on lipid oxidation, human skeletal muscle cells obtained from female and male donors were differentiated for 3 days and exposed, thereafter to 0, 1, and 10 nM testosterone or E₂ for 4 days, and palmitate oxidation was assessed. It was found that 10 nM testosterone treatment significantly increased palmitate oxidation. No effects of E₂ treatment on palmitate oxidation were observed (Fig. 1). However, 10 nM E₂ treatment enhanced palmitate oxidation in cells derived from male donors (Fig. 2). Therefore, 10 nM of sex hormones were chosen for further experiments.

Sex hormone-mediated effects on palmitate oxidation

To determine whether sex hormones differentially influence lipid metabolism in human myotubes derived from male or female donors, basal and insulin-stimulated palmitate oxidation were assayed following 4 days of hormone treatment. Testosterone treatment enhanced basal palmitate oxidation by 82 and 98% (*P* < 0.05) in myotubes from female and male donors respectively (Fig. 2). Treatment with testosterone also enhanced insulin-mediated inhibition of palmitate oxidation by 20% (*P* < 0.05) in myotubes from male donors. Conversely, E₂ treatment enhanced basal palmitate oxidation

in myotubes from male donors and decreased insulin action on palmitate oxidation by 45% (*P* < 0.05) in myotubes derived from female donors (Fig. 2).

Effect of testosterone treatment on palmitate oxidation is mediated by classical nuclear receptor

To determine the molecular mechanism of testosterone effect on palmitate oxidation, we investigated whether this effect is mediated by classical nuclear receptor, using the specific sex hormone receptor antagonist, CDX, at the final concentration of 10 nM. CDX did not affect basal palmitate oxidation in human myotubes, and the testosterone-mediated increase in palmitate oxidation was abolished in the presence of 10 nM CDX (Fig. 3).

Testosterone or E₂ treatment enhances insulin-stimulated glucose incorporation into glycogen in cells derived from female donors

To explore the effects of sex hormones on glucose metabolism in human myotubes derived from male or female donors, the basal and insulin-stimulated rates of glucose incorporation into glycogen were measured following hormone treatment. Testosterone treatment enhanced insulin-stimulated glucose incorporation into glycogen by 60% (*P* < 0.05) in myotubes derived from female donors (Fig. 4). Conversely, testosterone treatment did not alter insulin-stimulated glucose incorporation into glycogen in myotubes derived from male donors (Fig. 4). Moreover, E₂ treatment also increased insulin-stimulated glucose incorporation into glycogen in human myotubes derived from female donors. Overall, a strong sex-dependent response is observed in glucose incorporation to glycogen in response to hormone treatment.

Figure 2 Palmitate oxidation. Myotubes were treated for 4 days with or without 10 nM testosterone or 10 nM 17β-estradiol in the absence (white bar) or presence (black bar) of 120 nM insulin. Results are expressed as mean ± S.E.M. for myotubes derived from female *n* = 7 and male *n* = 7 donors. [□]*P* < 0.05, sex hormone treatment with insulin versus control with insulin; [#]*P* < 0.05, basal sex hormone treatment versus basal control; **P* < 0.05, insulin versus basal in each condition.

Figure 3 Effect of steroid receptor antagonist on testosterone-mediated palmitate oxidation: myotubes were treated for 4 days with 10 nM testosterone with (black bar) or without (white bar) 10 nM CDX (three males and three females). Results are expressed as mean $\pm$ S.E.M., $n=6$. ^a $P<0.05$ testosterone + CDX versus testosterone + 0.1% DMSO; [#] $P<0.05$, sex hormone treatment versus control (0.1% ethanol + 0.1% DMSO).

Sex hormone treatment mediates protein phosphorylation

To investigate the impact of sex hormone treatment on signal transduction in myotubes derived from female and male donors, the level of protein phosphorylation of prominent muscle metabolic signaling factors was assayed under basal and insulin-stimulated conditions following hormone treatment. Testosterone or E_2 treatment increased AKT^{Ser473} phosphorylation by 64 and 43% ($P<0.05$) respectively in myotubes derived from female donors in sex-dependent manner (Fig. 5A). Total AKT protein expression was unaltered.

Testosterone treatment increased AMPK^{Thr172} phosphorylation by 50% ($P<0.05$) in myotubes from male donors and tended to increase AMPK^{Thr172} phosphorylation in female donors by 49% ($P=0.06$; Fig. 6B). Total AMPK protein expression was unaltered. E_2 did not alter either AMPK^{Thr172} phosphorylation or protein content in human myotubes (Fig. 6A and B).

Testosterone treatment enhanced basal and insulin-stimulated p38 phosphorylation in myotubes derived from female donors by 309% ($P<0.05$) and 217% ($P<0.05$) respectively (Fig. 7A). In myotubes derived from males, basal and insulin-stimulated p38 phosphorylation were enhanced by 198% ($P<0.05$) and 145% ($P<0.05$) respectively (Fig. 7B). In contrast, E_2 did not alter either p38 phosphorylation or protein content in human myotubes (Fig. 7A and B). Phosphorylation and total protein levels of ERK1/2 MAPK, pIRS1^{Ser636}, and p70^{S6k} were unaltered after a 4-day exposure to either sex hormone (data not shown).

Differential effects of testosterone and E_2 on mRNA expression

To determine the influence of sex hormone treatment on mRNA expression of genes involved in lipid and glucose metabolism, human myotubes derived from female or male donors were treated with testosterone or E_2 for 4 days and gene expression was determined. The treatment with either hormone had a differential effect on several genes (Table 2). *SHGB* mRNA expression was increased by 53% for E_2 -treated and 60% for testosterone-treated versus control ($P<0.05$) in myotubes from female donors (Table 2). The mRNA expression of *PDK4* was increased with E_2 treatment in myotubes from female donors by 123% ($P<0.05$; Table 2). The mRNA level of *PDK4* in the estradiol-treated state was significantly higher in myotubes from female donors than in myotubes from male donors, which may indicate a sex-dependent response. *PPARG* gene expression was reduced in cultured cells from female donors by 42 and 41% in myotubes from male donors for testosterone-treated versus control ($P<0.05$). E_2 treatment reduced *PPARG* mRNA expression by 21% in myotubes from male donors ($P<0.05$) and showed a tendency to decrease in myotubes from female donors ($P=0.08$). In a sex-dependent manner, the effect of E_2 on *PPARG* expression is significantly higher in myotubes from male donors (Table 2). Treatment with testosterone, but not E_2 , increased *GYS1* mRNA expression by 29% ($P<0.05$) for testosterone versus control in cultured cells from male subjects (Table 2).

The *IRS2* mRNA expression was increased by 233 and 200% for testosterone-treated versus control ($P<0.05$) in cultured cells from female and male donors respectively. Interestingly, the effect of E_2 on *IRS2* mRNA expression showed a sex-specific difference (Table 2). *PPARG* gene

Figure 4 Glucose incorporation into glycogen. Myotubes were treated for 4 days with or without 10 nM testosterone or 10 nM 17β-estradiol in the absence (white bar) or presence (black bar) of 120 nM insulin. Results are expressed as mean $\pm$ S.E.M. for myotubes derived from female $n=7$ and male $n=7$ donors. ^a $P<0.05$, sex hormone treatment with insulin versus control with insulin and ^{*} $P<0.05$, insulin versus basal in each group; S $P<0.05$, male versus female, indicates sex-specific differences.

Figure 5 AKT^{Ser473} phosphorylation and protein abundance. Myotubes were treated for 4 days with or without 10 nM testosterone or 10 nM 17 β -estradiol in the absence (white bar) or presence (black bar) of 120 nM insulin. AKT^{Ser473} phosphorylation and protein expression were measured in myotubes derived from female (A; $n=7$) or male (B; $n=7$) donors. Results are expressed as mean $\pm$ S.E.M. $^{\square}P<0.05$, sex hormone treatment with insulin versus control with insulin; $^*P<0.05$, insulin versus basal in each condition; $^{\square}P<0.05$, male versus female, indicates sex-specific differences.

expression was increased by 16.5% with E₂ treatment versus control ($P<0.05$; Table 2). Moreover, *PPARD* mRNA level showed a significant sex-related difference, with a higher expression pattern in myotubes from female donors than from male donors in the control group.

Exposure of either sex hormone reduced the mRNA expression of *AR* by 43 and 50% for E₂ and testosterone treatment versus control respectively in myotubes from male donors ($P<0.05$). In addition, the mRNA expression of *AR* is significantly higher in myotubes from male donors when compared with myotubes from female donors. The *CPT1* mRNA expression showed a sex-dependent expression pattern, as higher expression was observed in myotubes from male donors than from female donors in the control group ($P<0.05$; Table 2). The mRNA expression of *SCD*, in a sex-dependent manner, was significantly lower in myotubes obtained from female donors than from male donors in the control group (Table 2). Several other metabolic genes were also analyzed and were not altered by either E₂ or testosterone treatment (Table 1). The mRNA expression of *P450arom*, *ER β* , and *SRD5A2* was undetectable in human myotubes.

Discussion

Androgen deficiency syndromes and hypogonadism, clinical conditions characterized by a deregulation of sex hormones, are associated with aging, obesity, type 2 diabetes, and cardiovascular disease (Soler *et al.* 1989, Haffner *et al.* 1996, Stellato *et al.* 2000, Oh *et al.* 2002, Farrell *et al.* 2008, Traish *et al.* 2009). We tested the hypothesis that testosterone or E₂ sex hormone treatment plays an important role in metabolism in primary skeletal muscle cells, and that an intrinsic sex difference may become apparent following exogenous sex hormone exposure on metabolism.

In this study, we show evidence that both testosterone and E₂ play a role in glucose and lipid metabolism, and intrinsic

sex differences in metabolism may reveal after sex hormone exposure in human skeletal muscle cell. There is much evidence showing that estradiol plays a role in regulating substrate metabolism, and it had been shown that treatment with estradiol in young men increased skeletal muscle β -oxidation capacity (Maher *et al.* 2010a). Consistent with this, we have observed an enhanced palmitate oxidation mediated by E₂ in myotubes from male donors only, while testosterone exposure enhanced palmitate oxidation in myotubes obtained from both sexes. The effect of testosterone on lipid oxidation occurred in concordance with increased AMPK^{Thr172} phosphorylation, a key factor in the regulation of lipid metabolism (Hardie 2004). IRS2 is also an important regulator of skeletal muscle lipid metabolism in cultured myotubes (Bouzakri *et al.* 2006), and in this study, we demonstrate that the *IRS2* mRNA expression was increased in response to testosterone treatment in both female and male donors, which may explain the enhanced fatty acid oxidation. In addition, administration of testosterone in elderly men has shown to increase muscle mass and muscle strength in a dose-dependent relationship (Storer *et al.* 2008) which may indicate the important role of testosterone in metabolism and muscle growth (Wolfe *et al.* 2000). However, any clear changes in the skeletal muscle growth and differentiation were not found in this study.

To investigate the molecular mechanism of testosterone effect on lipid oxidation, we exposed cultured myotubes to the specific AR antagonist, CDX. This inhibitor has been used in cancer research in breast or prostate cancer (Bhattacharyya *et al.* 2006, Auricchio *et al.* 2008). Moreover, sex hormone receptor inhibitors have been used to investigate the molecular mechanism of sex hormone action in skeletal muscle (Inoue *et al.* 1994). Interestingly, we show that the positive effect of testosterone on palmitate oxidation was abolished at the presence of the AR antagonist (CDX). This effect may indicate that testosterone-enhanced palmitate oxidation is via the classical steroid receptor. However, the effect of E₂ differs between female and male subjects, and further investigation is warranted to determine the cause of sex-related differences on estradiol-mediated palmitate oxidation.

Figure 6 AMPK^{Thr172} phosphorylation and protein abundance. Myotubes were treated for 4 days with or without 10 nM testosterone or 10 nM 17 β -estradiol in the absence (white bar) or presence (black bar) of 120 nM insulin. AMPK^{Thr172} phosphorylation and protein expression were determined in myotubes derived from female (A; $n=7-9$) or male (B; $n=7-9$) donors. Results are expressed as mean $\pm$ S.E.M. $^{\#}P<0.05$, hormone treatment versus basal control.

Figure 7 p38 phosphorylation and protein abundance. Myotubes were treated for 4 days with or without 10 nM testosterone or 10 nM 17β-estradiol in the absence (white bar) or presence (black bar) of 120 nM insulin. p38 phosphorylation and protein abundance in myotubes derived from female (A; n=7–8) or male (B; n=7–8) donors. Results are expressed as mean±s.e.m. #P<0.05, sex hormone treatment with insulin versus control with insulin; *P<0.05, hormone treatment versus basal control.

Previous studies provide evidence for a pivotal role of testosterone in skeletal muscle glucose metabolism. Testosterone treatment increased glycogen content in extensor digitorum longus and soleus muscles of hyperglycemic sedentary female rats (Ramamani *et al.* 1999, van Breda *et al.* 2003). In this study, treatment with either hormone increased insulin-stimulated glucose incorporation into glycogen and phosphorylation of AKT^{Ser473} in myotubes from female donors only. This may indicate a specific sex-related role of sex hormones on glucose storage via insulin action in skeletal muscle cells.

Testosterone treatment of skeletal muscle cells triggered the activation of known signaling factors such as p38 MAPK, which is associated with insulin resistance (Liu & Cao 2009). Although enhanced p38 activation may suggest a direct link to insulin resistance in skeletal muscle cells, our data do not provide evidence for any impairment in the insulin response. Rather we found that testosterone treatment enhances insulin-stimulated glucose incorporation into glycogen in myotubes derived from female donors. Interestingly, a p38 MAPK inhibitor prevents glucose transport in 3T3 L1

adipocytes and L6 muscle cell cultures (Sweeney *et al.* 1999), which may suggest an important positive role of p38 MAPK in glucose transport. This may partly support our findings that enhanced p38 phosphorylation after testosterone treatment may have a positive effect on glucose incorporation into glycogen. However, this effect is not found on the basal or insulin-stimulated level of testosterone in the myotubes obtained from male donors. Thus, the role for testosterone inducing p38 phosphorylation requires further investigation.

Sex hormone binding globulin (SHBG) mRNA expression was detected in human skeletal muscle myotubes, but its function has not previously been evaluated in myotubes. SHBG is produced in the liver, brain, uterus, placenta, and testes (Hammond & Bocchinfuso 1996), based on the present observations, in human cultured skeletal muscle. Interestingly, the upregulation of the SHBG mRNA expression after the exposure with either sex hormone was only found in myotubes from female subjects. This effect of testosterone is in accordance with earlier results, whereby treatment with 10 nM testosterone increased the intra- and extracellular SHBG content in HepG2 cell cultures (Loukovaara *et al.* 1995).

We were unable to obtain any differences or changes in the ER mRNA expression levels, while the mRNA expression of the AR was higher in myotubes from male donors at baseline in a sex-dependent manner, and the expression was decreased after the exposure to either sex hormone. It is well established that AR and ER expression are sensitive to circulating hormones *in vivo* (Baltgalvis *et al.* 2010). In a study done on stromal cells, estradiol reduced the expression of both AR and ER (Smith *et al.* 2002), in line with our finding on the AR mRNA expression, but differs with the ER mRNA expression data, which was not altered after sex hormone treatment or between sexes. In addition, AR expression was increased after chronic testosterone treatment in a C2C12 skeletal muscle cell line and during differentiation (Wannenes *et al.* 2008), and this effect was also observed in mesenchymal,

Table 2 Differential mRNA expression of metabolic genes assessed in myotubes from female and male donors after sex hormone exposure. Myotubes were treated with vehicle as control (0.1% ethanol), 10 nM 17β-estradiol, or testosterone for 4 days. Results are mean±s.e.m. for n=7 female subjects and n=7 male subjects. Results were normalized to 18s mRNA, and all the values are multiplied by 10⁻⁶

Genes	Control		17β-Estradiol		Testosterone	
	Female	Male	Female	Male	Female	Male
AR	*0.13±0.03	*1.79±0.28	0.66±0.02	†1.02±0.23	0.48±0.07	*0.89±0.03
CPT1	*0.13±0.02	*0.08±0.01	0.1±0.01	0.09±0.004	*0.14±0.01	*0.07±0.01
GYS1	16.92±1.11	17.16±3.4	16.59±1.08	18.42±2.85	18.28±0.87	*22.07±3.79
IRS2	0.03±0.003	0.03±0.01	*0.06±0.01	*0.02±0.003	*0.10±0.02	*0.09±0.02
PDK4	0.46±0.15	0.40±0.07	*,†1.03±0.21	*0.57±0.13	0.68±0.15	0.53±0.10
PPARD	*106.69±16.04	*69.85±4.63	†86.38±10.12	62.30±3.7	75.98±4.82	65.10±11.24
PPARG	*27.58±5.28	*51.88±4.01	*14.86±1.38	*,†40.78±7.02	†16.08±1.06	*30.47±6.4
SHGB	0.15±0.04	0.19±0.01	†0.23±0.04	0.24±0.03	*0.24±0.04	0.20±0.01
SCD	*135.6±13.34	*234.77±27.61	101.36±5.75	154.76±24.77	160.05±2.23	179.71±46.65

*P<0.05, male versus female per condition and indicates the sex-specific differences; †P<0.05, 17β-estradiol treatment versus control; *P<0.005, testosterone treatment versus control.

pluripotent cell line after exposure to testosterone (Singh *et al.* 2003). However, our finding may indicate a negative feedback loop of chronic testosterone exposure on its own receptor in human myotubes.

Interestingly, we have previously reported that the sex of the donor does not influence glucose and lipid metabolism in human myotubes in steroid hormone-free media, with evidence against 'metabolic memory' of the external factors such as hormones and cytokines known to influence glucose and lipid metabolism in cell cultures grown *in vitro* (Rune *et al.* 2009). However, we previously observed a trend towards a sex dependency in the mRNA expression of some genes such as *PPARG* at basal, hence our earlier study was likely underpowered to detect the previous data. However, in this study, significant sex differences in baseline mRNA expression of *CPT1*, *PPARD*, *PPARG*, and *SCD* were shown. Interestingly, in line with our findings, there is evidence showing that the mRNA of *CPT1* and *PPARD* was higher in skeletal muscle biopsies from young women, when compared with men (for review, Maher *et al.* (2010b)). These data may indicate that women in comparison with men have greater capacity for fatty acid metabolism in skeletal muscle. Moreover, a sex difference was observed at the mRNA level of *IRS2* and *PDK4* after E_2 treatment, where it was higher in myotubes from female donors when compared with male donors.

Our data showed a clear effect of either hormone on the mRNA expression of several proteins involved in glucose metabolism such as *GYS1* and *PDK4* or lipid metabolism *PPARD*, *PPARG*, and *IRS2*. In addition to an involvement in the regulation of glucose metabolism (Rosa *et al.* 2003, Wende *et al.* 2005), *PDK4* plays an important role in lipid metabolism (Rosa *et al.* 2003), and it is also known to be regulated by pyruvate production. It has been shown previously that *PDK4* activity is enhanced at high rates of fatty acid oxidation, which causes a consequent inhibitory effect on glucose oxidation (for review, Sugden & Holness (2006)). Consistent to our mRNA expression data, E_2 treatment increased *PDK4* gene expression in ovariectomized female rats, (Campbell *et al.* 2003), suggesting a regulative role of E_2 in skeletal muscle lipid metabolism. In contrast to this, our data showed that E_2 treatment-mediated fatty acid oxidation was only observed in myotubes from male donors and not in the female donors. Whether *PDK4* is directly involved in skeletal muscle cell palmitate oxidation or insulin-stimulated glucose incorporation into glycogen after sex hormone exposure remains to be investigated.

PPARG is a gene involved in lipid metabolism (reviewed in Zhang *et al.* (2004) and Jay & Ren (2007), and our data regarding the effect of testosterone on *PPARG* mRNA expression is similar to earlier findings in rat primary neuronal cultured cells, where testosterone treatment inhibited *PPARG* activity and fat mass control (Du *et al.* 2009). We have also found a sex difference of *PPARG* mRNA expression at baseline. Whether the downregulation of the *PPARG* gene observed in this study is due to a negative feedback in response to sex hormone treatment is unknown. Thus, our current

data suggest a 'sex memory' at the level of mRNA, which is further pronounced following exposure to sex hormones.

Nevertheless, the mRNA expression of several genes crucial to lipid oxidation (*UCP2*, *UCP3*, *FABP3*, *PGC1alpha* and *SREBP*) was unaltered in control or sex hormone-treated myotubes from female and male donors. The mRNA expression of several regulatory genes involved in glucose metabolism such as the *TBC1D1*, *GLUT4* and *IRS1*, was not changed after sex hormone treatment or between sexes. However, many of these genes such as *SREBP* (Simpson & McInnes 2005) and *GLUT4* (Muthusamy *et al.* 2009) were shown by others to be regulated by either testosterone or E_2 .

We have investigated whether testosterone can be aromatized to E_2 via the *P450arom* or into its 'bioactive' metabolite dihydrotestosterone, DHT, via the 5- α reductase *SRD5A1* and *SRD5A2*, and our data show an almost undetectable *P450arom* and *SRD5A2* mRNA expressions, but a clear mRNA expression of *SRD5A1* was found in human myotubes, which may speculate a local effect of the DHT on skeletal muscle metabolism.

In summary, testosterone and E_2 have differential effects on the regulation of glucose and lipid metabolism in human skeletal muscle cells. This is clear through observed changes in gene expression and metabolic readouts. Testosterone and E_2 treatment enhanced glucose incorporation to glycogen via increased insulin-stimulated AKT^{Ser473} in a sex-dependent difference. Moreover, treatment with testosterone increased palmitate oxidation in cultured myotubes derived from female and male donors, which is potentially mediated via classical nuclear sex hormone receptors. Taken together, our data suggest that testosterone and E_2 exert differential metabolic outcomes in a sex-dependent manner, and this intrinsic sex hormone-specific difference is maintained in cultured myotubes and consequently uncovered by exogenous sex hormone exposure.

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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