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ORIGINAL ARTICLE

Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women

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Summary

Objective To examine the effects of aromatizable or nonaromatizable androgens on abdominal subcutaneous (SC) and omental (OM) adipose tissue lipid metabolism and adipogenesis in men and women.

Design and subjects Primary organ and preadipocyte cultures were established from surgical samples obtained in men ($n = 22$) and women undergoing biliopancreatic diversions ($n = 12$) or gynaecological surgeries ($n = 8$). Cultures were treated with testosterone, dihydrotestosterone (DHT) and methyltrienolone (R1881).

Measurements Heparin-releasable lipoprotein lipase (HR-LPL) activity, glycerol release, adiponectin secretion, glycerol-3-phosphate dehydrogenase activity and lipid accumulation were measured.

Results In organ cultures from men, DHT had a statistically significant inhibitory effect on HR-LPL activity in the OM compartment. Testosterone significantly inhibited HR-LPL activity in SC and OM cultures. In women, high DHT concentrations tended to inhibit HR-LPL activity in OM cultures. Minor androgenic effects were observed for basal and isoproterenol-stimulated lipolysis as well as adiponectin release in men. On the other hand, adipocyte differentiation was significantly and dose-dependently inhibited by DHT, testosterone and R1881 in SC and OM cultures from both sexes. These effects did not differ according to adipose tissue depot but appeared to be more pronounced in women than in men.

Conclusions Androgens slightly decreased HR-LPL activity in adipose tissue organ cultures, but markedly inhibited adipogenesis in SC and OM primary preadipocyte cultures in both sexes. Androgenic effects on adipose tissue in men *vs.* women may not differ in terms of direction but in the magnitude of their negative impact on adipogenesis and lipid synthesis.

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Introduction

There is a marked sex difference in regional fat distribution in humans. Men tend to accumulate adipose tissue in the abdominal region whereas women generally accumulate fat in the gluteofemoral region. For a similar fat mass, men have a twofold higher intra-abdominal or visceral adipose tissue accumulation compared with women.[1] This unique sex dimorphism in visceral fat accumulation, which has been closely linked to insulin resistance and the risk for type 2 diabetes,[2] strongly suggests a role for sex steroid hormones.

In men, abdominal obesity has usually been associated with low plasma testosterone levels in cross-sectional[3] and in longitudinal studies.[4] A large number of studies which have measured abdominal fat areas using imaging techniques such as computerized tomography or magnetic resonance have confirmed that low total plasma testosterone concentrations are often found with elevated visceral fat accumulation.[5–7] Moreover, androgen treatment in hypogonadal men generally leads to a decrease in abdominal fat accumulation.[8] These effects appear to be dose-dependent[9] and lead to concomitant improvements of glucose-insulin homeostasis[8,10] while having neutral effects on the lipid profile.[11] Such effects are observed when the androgen levels reached during treatment remain within the physiological range.[12] Supra-physiological androgen treatment in anabolic steroid users increases cardiovascular risk partly due to alterations of the lipid–lipoprotein profile.[13]

In women, the association between circulating androgens and abdominal obesity is more complex. In contrast to men, it is generally thought that abdominally obese women are hyperandrogenic.[14,15] This belief is largely based on the observation that women with the polycystic ovary syndrome (PCOS) show hyperandrogenism that is often associated with abdominal obesity and hyperinsulinaemia.[14] Interesting developments in our understanding of the pathophysiology of PCOS indicate that elevated androgens in these women may not be the cause of abdominal obesity, but the

result of a hyperinsulinaemic/insulin resistant state often associated with excess visceral fat.[14] Except for PCOS-related hyperandrogenism, the association between circulating androgen levels and abdominal obesity in women remains unclear. Some studies that included abdominal obese women with and without clinical androgen excess still show high plasma testosterone (total or free),[15–17] but fewer studies are available and results are not always consistent.[18,19] Some studies actually found negative associations between plasma testosterone levels and visceral fat accumulation.[20–22] However, androgen treatment leading to supra-physiological circulating levels in female-to-male transsexuals reportedly increases visceral fat accumulation.[23,24]

The presence of androgens, of the androgen receptor (AR) and of androgen binding in adipose tissue has long been established.[25–29] One of the most consistent effects of androgens observed on fat cell function is a stimulation of lipolysis that appears to be receptor-dependent and that may affect the lipolysis cascade at different levels.[12] These data are concordant with observations in androgen-receptor null mice, which show a late-onset obesity phenotype likely attributable to impaired lipolysis.[30] However, a recent study in postmenopausal women showed that testosterone administration for 3 months downregulated hormone-sensitive lipase protein and increased phosphodiesterase-3B mRNA expression in subcutaneous (SC) adipose tissue, suggesting that testosterone favoured fat storage in this depot.[31] Additional data seem to indicate that testosterone inhibits adipose tissue lipoprotein lipase (LPL) activity in human fat cells,[32] which is consistent with an inhibitory effect of androgens on lipid accumulation. Regarding the effect of androgens on adipogenesis (preadipocyte differentiation), discordant results have been obtained.[12] In rats[33] or in the 3T3-L1 and C3H 10T1/2 cell lines,[34,35] androgens [testosterone and dihydrotestosterone (DHT)] were found to inhibit preadipocyte differentiation. Castration was found to increase differentiation in preadipocytes from the perirenal fat depot in rats.[36] However, castration was also previously found to inhibit differentiation of epididymal preadipocytes.[37] The responsiveness to androgens was found to be more pronounced in deep fat depots (visceral) in comparison to SC adipose compartments in other studies.[27,33,37] Overall, previous data generally suggest that androgens may decrease fat mass, possibly through reduced adipogenesis and increased lipolysis.

It was reported in human fat cells that testosterone had no effect, in any region, on glycerol-3-phosphate dehydrogenase (G3PDH) activity, a late marker of differentiation[38] but a recent study has shown that DHT inhibited adipocyte differentiation in abdominal SC, mesenteric and omental (OM) primary cultures, in men.[39] No significant difference was observed between men and women for the number of androgen binding sites in intra-abdominal (OM and mesenteric) compared with SC preadipocytes,[27,40] and the effect of androgens on adipogenesis has not been tested in women.

Several lines of evidence suggest sex differences in adipose tissue metabolism between men and women (reviewed in[41]). The effects of androgens may also be influenced by sex. However, very few studies compared the effects of androgens on adipose tissue according to sex in humans. There was no effect of DHT on preadipocyte proliferation in either sex in one study.[42] Leptin secretion is known to be influenced by androgens in a different manner in men *vs.* women.[43] It was also shown that prereceptor regulation of androgen, oestrogen and glucocorticoid action by steroid-converting enzymes may differ according to sex.[44,45] However, we do not know whether there is a sex difference in the effect of androgens on human fat cells. Considering the apparently opposite effects of androgens on abdominal adiposity in men and women, more experimentation is necessary to better understand the impact of androgens on adipose tissue function in humans.

The first objective of this study was to examine the effects of aromatizable (testosterone) or nonaromatizable (DHT) androgens on abdominal SC and OM adipose tissue lipid metabolism in organ cultures from men and women. The second objective was to study the effects of androgens (testosterone, DHT and methyltrienolone, R1881) on adipocyte differentiation in primary preadipocyte cultures isolated from SC and OM adipose tissue of men and women. We tested the hypothesis that androgens would stimulate lipolysis, inhibit adiponectin release and lipoprotein lipase (LPL) activity in organ cultures and inhibit adipogenesis in preadipocytes. We also postulated that these effects would be sex- and depot-specific.

Materials and methods

Subjects

Men and women of this study were recruited through the elective surgery schedule of the Laval University Medical Center and through the bariatric surgery schedule of the Laval Hospital. The study included men (n = 23, aged 30–66 years, BMI 56·6 ± 8·1 kg/m^2, range 48·9–69·8 kg/m^2) and women (n = 12, aged 30–59 years, BMI 52·1 ± 9·6 kg/m^2, range 37·0–70·5 kg/m^2) undergoing biliopancreatic diversion surgery for the treatment of morbid obesity and women undergoing gynaecological surgery (n = 13, aged 39–53 years, BMI 23·8 ± 6·1 kg/m^2, range 18·2–37·6 kg/m^2). None of the women were taking oral contraceptives or had a diagnosis of PCOS. Women undergoing gynaecological surgery were all premenopausal. Six women undergoing biliopancreatic diversion were premenopausal, three were postmenopausal and data were missing for one woman. Clinical information was missing for three men. Ethics approval was obtained from the medical committees of Laval University, Laval Hospital and Laval University Medical Center. All subjects provided written informed consent before their inclusion in the study. In addition to primary preadipocytes isolated in our laboratory, primary preadipocytes were also obtained commercially (n = 6 for SC and n = 3 for OM, from women aged 33–51 years) (Zen Bio, Research Triangle Park, NC, USA).

Adipose tissue sampling

Omental and SC adipose tissue samples were collected during the surgical procedure and immediately carried to the laboratory in phosphate-buffered saline (PBS) preheated at 37 °C.

Organ culture

Adipose tissue explants were kept in culture according to the procedure of Fried *et al.*[46] Briefly, fresh adipose tissue was cut

into 5–10 mg pieces and placed in serum-free Medium 199. A proportion of 30 mg of adipose tissue per millilitre of medium was maintained in all experiments. Organ cultures were kept at 37 °C under a 5% CO_2 atmosphere. For heparin-releasable lipoprotein lipase (HR-LPL) activity experiments, explants were incubated in Medium 199 containing 7 nM insulin and 25 nM dexamethasone for 10 days in the presence of a wide range of DHT and testosterone concentrations (from 0 to 1 μM). Adipose tissue was harvested and kept at −80 °C until the measurement of HR-LPL activity and the medium was kept for the adiponectin assay using ELISA (Otsuka Pharmaceuticals, Tokyo, Japan). For lipolysis experiments (assessed by glycerol release in the medium), explants were incubated with 200 μM adenosine and androgens at various concentrations, but without insulin or dexamethasone. After 24 h, the medium was changed for fresh medium with 1 U/ml adenosine desaminase with or without 1 μM isoproterenol and androgens. The medium was harvested at baseline and after 3, 6 and 24 h to obtain the area under the curve of glycerol release.

HR-LPL activity

Heparin-releasable lipoprotein lipase activity was determined in organ culture samples by the method of Taskinen *et al.*[47] Tissue eluates were obtained by incubating the samples in Krebs Ringer Phosphate buffer and heparin (5 U/ml) at 28 °C for 90 min. The eluates were then incubated with excess concentrations of unlabeled and[14] C-labelled triolein in arabic gum-TRIS-albumin buffer (7% albumin) emulsified with ultrasound. The reaction was carried out at 37 °C for 60 min with agitation. The resulting free fatty acids liberated from triolein by the LPL reaction were isolated by the Belfrage extraction procedure. Porcine plasma (10%) was used as a source of Apo-CII to stimulate LPL activity, and unpasteurized cow's milk as an internal LPL activity standard for inter-assay variations. Activity results were calculated in nmol oleate/h/mg tissue and expressed as percentage of control.

Glycerol release

Glycerol release in the medium was measured by bioluminescence using the NADH-linked bacterial luciferase assay,[48] an EG&G Berthold Microlumat plus bioluminometer (LB 96 V), and the WINGLOW software. The average coefficient of variation for duplicate glycerol release measurements was 14·1%. Glycerol release was calculated as μM/mg tissue and results were expressed as percentage of control.

Preadipocyte isolation and primary cultures

Fresh tissue samples were digested with collagenase type I in Krebs-Ringer-Henseleit (KRH) buffer for 45 min at 37 °C according to a modified version of the Rodbell method.[49] Adipocyte suspensions were filtered through nylon mesh and washed three times with KRH buffer. Preadipocytes were isolated using a modification of the method previously described by Van Harmelen *et al.*[50] Briefly, the residual KRH buffer of the adipocyte isolation was centrifuged and the pellet was washed in DMEM-F12 supplemented with 10% foetal bovine serum, 2·5 μg/ml amphotericin B and 50 μg/ml gentamicin. Cells were treated with erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM K_2HPO_4 and 0·1 mM EDTA pH 7·5) and DMEM-F12 was added. Preadipocytes were then subsequently filtered through 140 and 30 μm nylon mesh to remove endothelial cells, placed in culture plates and cultured at 37 °C under a 5% CO_2 atmosphere. Serum-free preadipocyte medium (DMEM-F12 supplemented with 66 nM insulin, 1 nM triiodo-L-thyronine, 10 μg/ml transferrin, 10 nM hydrocortisone, 1 nM basic fibroblast growth factor, 17 μM pantothenate, 33 μM biotin and antibiotics) and passages allowed to eliminate most of the remaining mesothelial cells present in OM cultures. Medium was changed every 2–3 days.

Induction of adipocyte differentiation and treatments

Cells subcultured 1–5 times in DMEM-F12 containing 10% foetal bovine serum were seeded at a density of 3·5 to 5×10^4 cells/cm^2 in 96-well plates to obtain full confluence within 48 h. The day after, androgens (DHT, testosterone and R1881 at various concentrations) were added and cultures were incubated for 24 h prior to the induction of adipocyte differentiation. The choice of a large range of concentrations including high androgen concentrations in our experiments was to ascertain that we did not miss stimulation of adipogenesis at high doses. Flutamide at 1 μM was incubated with preadipocytes 2 h before the addition of androgens wherever appropriate. Cells were differentiated in 12-well plates for real-time RT-PCR quantifications and Western blots. Differentiation medium consisted of DMEM-F12 supplemented with a PPAR-γ agonist, insulin, dexamethasone and 3-isobutyl-1-methylxanthine. Cells were differentiated and maintained for 16–20 days, according to a standard protocol (Zen Bio).

Glycerol-3-phosphate dehydrogenase activity measurements

Glycerol-3-phosphate dehydrogenase activity was measured according to Sottile *et al.*[51] with some modifications. Differentiated cells from three separate wells were washed with PBS. Cold homogenization solution (100 μl/well; 20 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7·3) was added to harvest cells, and samples were kept at −80 °C until analysis. After thawing, samples were assayed for G3PDH activity in 96-well plates. To 100 μl of the sample, 90 μl of reaction mix (100 mM triethanolamine, 2·5 mM EDTA, 0·1 mM β-mercaptoethanol, 353 μM NADH, pH 7·7) was added and incubated for 10 min at 37 °C. The assay was initiated by the addition of dihydroxyacetone phosphate (10 μl/well of a 8-mM stock solution), and a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure optical density at 340 nm at repeated intervals during 5 min. Purified G3PDH enzyme was used to generate a standard curve to calculate G3PDH activity in mU of purified enzyme. Proteins were quantified in duplicate by the BCA method in two separate wells and used to normalize for the amount of biological material.

G3PDH activity was expressed as percentage of control wells, which were submitted to differentiation in the presence of vehicle (i.e. ethanol).

Lipid accumulation

Oil red O staining followed by spectrophotometric analysis was performed to measure lipid accumulation as previously described.[52] Cells from three different wells were washed with PBS and fixed with formalin for 1 h. An Oil red O solution at 4·9 mM in isopropanol was added to the wells and incubated for 2 h. After washing three times with dH_2O, Oil red O retained by lipid droplets was eluted with isopropanol containing 4% Igepal CA-630. Optical density at 490 nm was measured and results were expressed as percentage of control.

Real-time PCR measurements of enzyme mRNA abundance

Total RNA was isolated from differentiated primary cultures using Rneasy Lipid Tissue Mini Kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's recommendations. RNA quality was assessed with a bioanalyser (Agilent Technologies, Mississauga, Ontario, Canada). First strand cDNA synthesis was accomplished using 0·75–1·5 μg of the isolated RNA in a reaction containing 200 units of Superscript III Rnase H- reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA), 50 ng of random hexamers, 300 ng of oligo dT_{18}, 500 μM dNTP, 5 mM DTT and 40 units of Protector RNase inhibitor (Roche Diagnostics, Laval, Quebec, Canada) in a final volume of 50 μl. Resulting cDNA was then treated with 1 μg of Rnase A for 30 min at 37 °C and purified thereafter with Qiaquick PCR purification kits (Qiagen). For quantitative PCR analyses, a Light-Cycler PCR (Roche Diagnostics) was used to measure the mRNA abundance of peroxisome proliferator-activated receptor-γ_2 (PPAR-γ_2) and acyl-CoA: diacylglycerol acyltransferase (DGAT2). The sets of primers used were: 5′-ATTACAGCAAACCCCTATTCCATG-3′/5′-TTGCAGACAGTGTATCAGTGAAGGAAT-3′ for PPAR-γ_2 and 5′-CCGATGGGTCCAGAAGAAGTT-3′/5′-TCACCAGGGCCTCCATGTACA-3′ for DGAT2. The FastStart DNA Master Plus SYBRGreen I kit (Roche Diagnostics) was used in a final reaction volume of 20 μl containing 3 mM $MgCl_2$, 20 ng of each primer and 20–200 ng of the cDNA template. The PCR was carried out according to the following conditions: 50 cycles of (95 °C/10 s, 60–62 °C/10 s, 72 °C/14 s and reading at 76 °C/5 s). A universal standard curve was generated with ATP synthase from an amplification with perfect efficiency (i.e. efficiency coefficient $E = 2{\cdot}00$) using cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies. The crossing points (*C*p) to calculate the amount of copies in initial cDNA specimens were determined using the double derivative method.[53] For each sample, the Cp value was divided by that of the housekeeping gene. To further minimize inter-assay variability, this Cp ratio was then multiplied by the average Cp generated for housekeeping gene amplifications of all samples examined in this experiment. PCR data were expressed in number of copies per μg total RNA.

Western blot

Cultures were harvested in lysis buffer [20 mM Tris (pH 7·3), 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Igepal CA-630 and 10% glycerol] containing protease inhibitors. For immunoblotting, 30 μl protein homogenate in SDS buffer 4× [0·8 mM Tris (pH 6·8), 8% SDS, 5 mM EDTA, 40% glycerol, 0·2 mM DTT] were heated at 37 °C for 30 min and separated on a 10% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (1 h at 100 V) and unspecific sites were blocked with 5% nonfat milk diluted in wash solution [50 mM Tris (pH 7·4), 150 mM NaCl, 0·1% Tween 20] for 1 h. Membranes were then incubated overnight at 4 °C with the primary antibody against G3PDH (Abcam Inc., Cambridge, MA, USA) or FABP4 (Cayman Chemicals, Ann Arbor, MI, USA), washed 3 × 10 min and incubated 1 h with anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. Finally, membranes were washed 3 × 10 min and proteins were visualized by chemiluminescence. Tubulin was used as the control protein. Densitometric analysis was performed with IMAGEQUANT TL software (GE Healthcare, Piscataway, NJ, USA).

Cytotoxicity and apoptosis measurements

Preadipocyte cultures were treated with DHT and testosterone. Cytotoxicity was assessed by the measurement of adenylate kinase release in the medium using the ToxiLight nondestructive cytotoxicity bioassay kit (Lonza, ME, USA). Nuclear staining with Hoescht 33258 for the detection of nuclear condensation/fragmentation and measurement of caspases 3–7 activities using Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA) were used as indices of apoptosis. Cytotoxicity was also assessed in differentiated cultures using the Toxilight assay. Androgens were present either for the last 24 h only or during the entire differentiation experiment including preincubation, as described.

Statistical analyses

As a result of large inter-individual variation in the level of fat cell differentiation reached, results of this study were expressed as percentage of vehicle-treated cells (control). The pattern observed in most cultures taken individually when results were expressed in mU G3PDH/μg protein was similar to results expressed in percentage of control. In addition, subsets of G3PDH activity measurements were expressed in mU G3PDH/μg protein to illustrate the extent of inhibition observed. Analyses were performed on $\log_{10}$-transformed or Box Cox-transformed values when variables were not normally distributed. When variances were unequal based on the Levene test ($P < 0{\cdot}05$), the Welch ANOVA was used to test differences between treatments because it is robust in conditions of unequal variances. Otherwise, the ANOVA was considered. When a significant model effect was observed, the Tukey–Kramer test was used for the comparison of individual means. When normality could not be reached, *a posteriori* mean contrast analyses were performed. A *P*-value <0·05 was considered statistically significant. Statistical analyses were performed with the JMP software (SAS Institute, Cary, NC, USA).

Results

Organ cultures

In men, DHT had a statistically significant effect on HR-LPL activity in the OM fat depot (Fig. 1b). We observed a slight stimulation at the 0·025 nM DHT dose, whereas inhibition was observed at higher concentrations. A statistically significant effect was found only between the latter dose and 1 μM DHT in OM cultures. Testosterone significantly inhibited HR-LPL activity in SC and OM organ cultures in men (Fig. 1c,d). The effects of testosterone were observed at lower concentrations in the OM depot compared with SC adipose tissue. In women, DHT at 1 μM had a significant inhibitory effect on HR-LPL activity in the OM depot (Fig. 1f). Only the nonaromatizable androgen DHT was used in women given our focus on androgenic effects.

In men, basal and isoproterenol-stimulated lipolysis tended to increase with increasing DHT and testosterone concentration, but the effect did not reach statistical significance (data not shown). Adiponectin release was not affected by DHT or testosterone. The effects of androgens on lipolysis were not tested in women.

Preadipocyte differentiation

Figure 2 illustrates the inhibitory effect of androgens on adipocyte differentiation in a representative SC culture from men. The inhibitory effect was clearly dose-dependent for DHT, testosterone and R1881. Figure 3 shows the effects of DHT and testosterone on

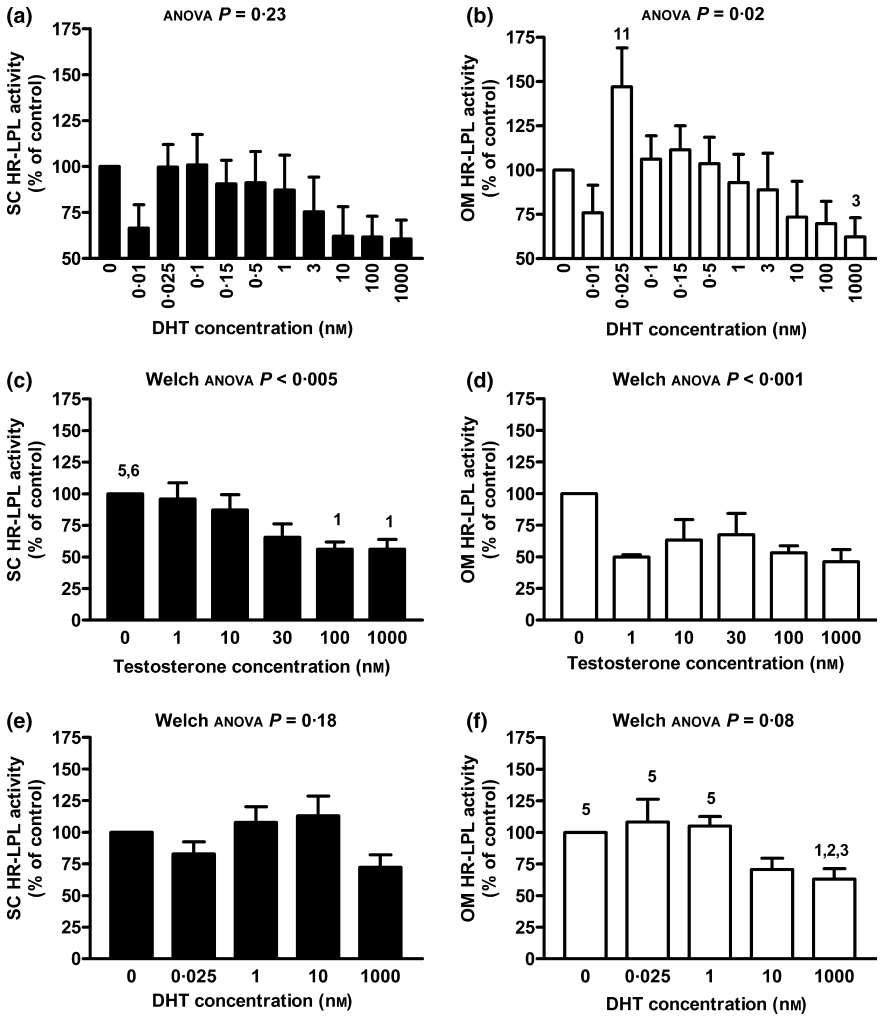

Fig. 1 Effects of androgens on heparin-releasable lipoprotein lipase (HR-LPL) activity in organ cultures obtained from men and women: (a) Effects of dihydrotestosterone (DHT) on HR-LPL activity in subcutaneous (SC) explants from men ($n = 7$ for 1 nM, $n = 8$ for other concentrations). (b) Effects of DHT on HR-LPL activity in OM explants from men ($n = 7$ for 1 nM, $n = 8$ for 0·5, 3, 10, 100, 1000 nM and $n = 9$ for other concentrations). (c) Effects of testosterone on HR-LPL activity in SC explants from men ($n = 4$). (d) Effects of testosterone on HR-LPL activity in omental (OM) explants from men ($n = 3$). (e) Effects of DHT on HR-LPL activity in SC explants from women ($n = 4$). (f) Effects of DHT on HR-LPL activity in OM explants from women ($n = 3$ for 10 nM and $n = 4$ for other concentrations). All cultures performed in duplicate. Mean ± SEM are shown. Numbers above bars indicate a statistically significant difference with the corresponding bar using the Tukey–Kramer test.

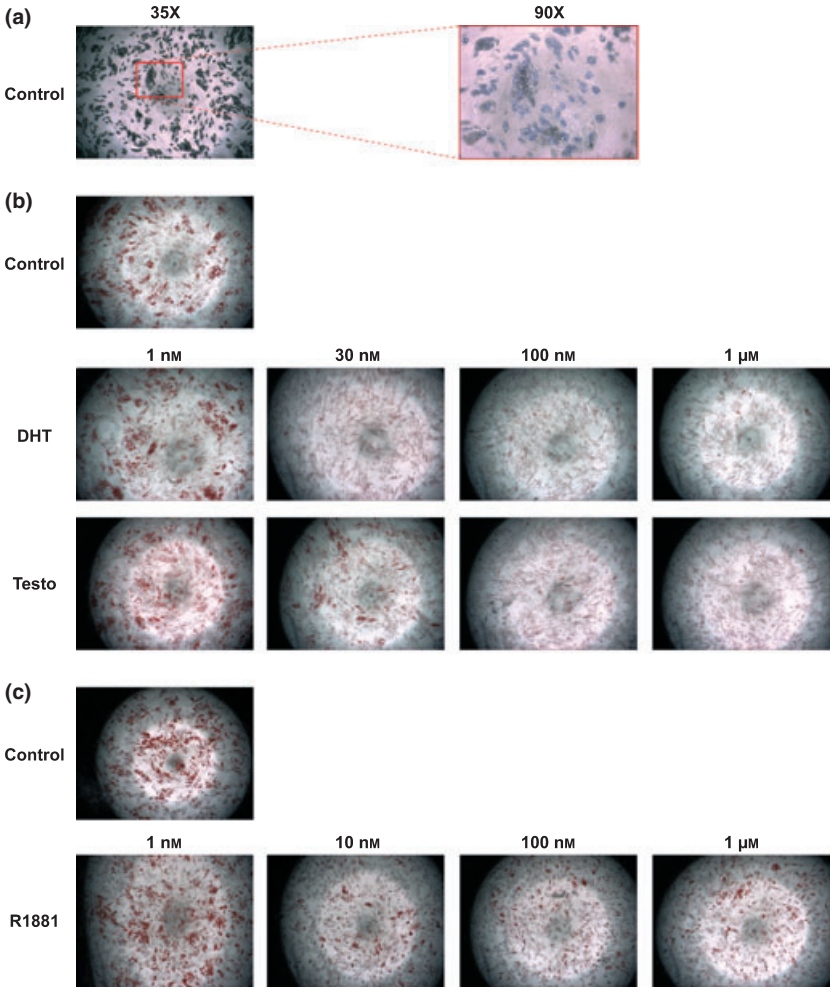

Fig. 2 Photomicrographs showing a subcutaneous culture from women after adipocyte differentiation in the presence of several androgen concentrations. (a) Phase contrast microscopy at 35 × magnification and at 90 × magnification of the zone delineated in red to show accumulation of lipid droplets. (b) Phase contrast microscopy at 35 × magnification showing the inhibitory effect of dihydrotestosterone and testosterone on adipogenesis. (c) Phase contrast microscopy at 35 × magnification showing the inhibitory effect of R1881 on adipocyte differentiation.

G3PDH activity and lipid accumulation in women. DHT strongly and significantly inhibited G3PDH activity and lipid accumulation in SC and OM preadipocyte cultures (Fig. 3a–d). The effects of androgens did not differ significantly between depots. Thus, inhibitory constant (IC_{50}) values were determined in combined SC and OM samples. The IC_{50} of DHT on G3PDH activity and lipid accumulation in pooled SC and OM data were 3·5 and 5·3 nM respectively. Similar to DHT, testosterone had a significant dose-dependent inhibitory effect on adipogenesis measured by G3PDH activity and lipid accumulation in SC and OM cultures (Fig. 3e–h). The IC_{50} of testosterone on G3PDH activity and lipid accumulation in pooled SC and OM data were 9·0 and 5·9 nM respectively.

When analysed by fat depot, percentage inhibitions by androgens were not significantly different between SC and OM cultures in men. Consequently, data were analysed using pooled data from the SC and OM depots. Figure 4 shows the effects of DHT and testosterone on G3PDH activity and lipid accumulation in SC and OM cultures, in men. DHT significantly inhibited adipogenesis as measured by G3PDH activity (Fig. 4a) and lipid accumulation (Fig. 4b). The IC_{50} of DHT on G3PDH activity and lipid accumulation in pooled SC and OM data were 8·2 and 9·8 nM respectively. Testosterone significantly inhibited adipogenesis in a dose-dependent manner as measured by G3PDH activity (Fig. 4c). The IC_{50} of testosterone on G3PDH activity and lipid accumulation in pooled SC and OM samples were 8·9 and 6·2 nM respectively.

In Fig. 5, G3PDH activity was expressed in mU/μg protein in subsets of cultures obtained from men and women. In agreement with the results expressed as percentage of control, DHT (Fig. 5a–d) and testosterone (Fig. 5e) significantly inhibited or tended to inhibit G3PDH activity. This effect was particularly clear in SC cultures (black bars), and was also more pronounced in women (Fig. 5a,b and e,f) compared to men (Fig. 5c,d and g,h).

The synthetic, nonmetabolizable androgen R1881 also had a statistically significant inhibitory effect on adipogenesis as measured by G3PDH activity and lipid accumulation (Fig. 6a,b). Results are presented for pooled SC and OM cultures from both men and women. A concentration of 1 nM R1881 was sufficient to inhibit G3PDH activity to approximately 50% of the control (Fig. 6a,b).

The inhibitory action of DHT on adipocyte differentiation was partially attenuated by treatment with 1 μM flutamide (Flu) in primary cultures from men (Fig. 6c,d). Flutamide restored G3PDH activity from 51 ± 16 to 65 ± 6% of the control for DHT 30 nM and from 39 ± 8 to 63 ± 8% for DHT 100 nM (Fig. 6d). Flutamide partially attenuated the inhibitory effect of testosterone on adipogenesis from 34 ± 6 to 59 ± 13% of the control for 30 nM

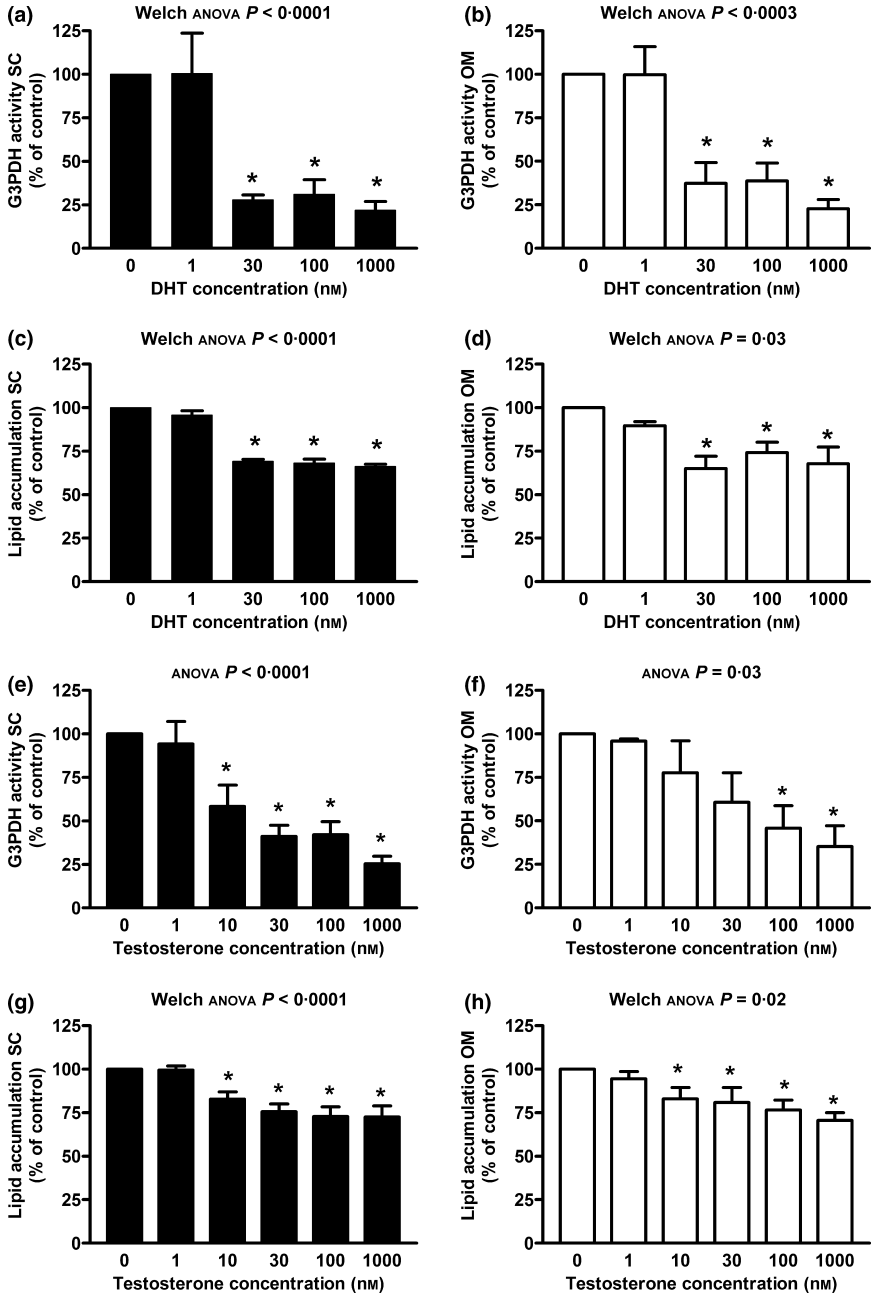

Fig. 3 Effects of dihydrotestosterone (DHT) (a–d) and testosterone (e–h) on adipocyte differentiation in preadipocyte cultures obtained from women: (a) Effects of DHT on glycerol-3-phosphate dehydrogenase (G3PDH) activity in subcutaneous (SC) cultures ($n = 2$ for 100 nM, $n = 3$ for 30 nM, $n = 4$ for 1 nM and $n = 5$ for other concentrations). (b) Effects of DHT on G3PDH activity in omental (OM) cultures ($n = 3$ for 1 nM and 100 nM, $n = 4$ for 30 nM, $n = 5$ for other concentrations). (c) Effects of DHT on lipid accumulation as measured by oil red O staining in SC cultures ($n = 3$ for 30 nM and 100 nM, $n = 7$ for 1 nM, $n = 8$ for other concentrations). (d) Effects of DHT on lipid accumulation as measured by oil red O staining in OM cultures ($n = 2$ for 1 and 30 nM, $n = 3$ for other concentrations). (e) Effects of testosterone on G3PDH activity in SC cultures ($n = 3$ for 1 nM and $n = 8$ for other concentrations). (f) Effects of testosterone on G3PDH activity in OM cultures ($n = 2$ for 1 nM and $n = 3$ for other concentrations). (g) Effects of testosterone on lipid accumulation as measured by oil red O staining in SC cultures ($n = 4$ for 1 nM and $n = 7$ for other concentrations). (h) Effects of testosterone on lipid accumulation as measured by oil red O staining in OM cultures ($n = 2$ for 1 nM and $n = 4$ for other concentrations). Mean ± SEM are shown. *: significant difference from the control ($P < 0·05$) by *a posteriori* mean contrast.

testosterone, but this effect was not observed at 100 nM testosterone. *Post hoc* analyses revealed a trend toward flutamide inhibiting the effects of 100 nM DHT ($P = 0·10$) and 30 nM testosterone ($P = 0·08$).

Figure 7 shows the effects of androgens on late markers of adipogenesis. Treatment with 100 nM testosterone or R1881 significantly decreased the expression of DGAT2 mRNA levels. DHT, testosterone and R1881 at 100 nM significantly inhibited the production of G3PDH protein measured by western blot. Treatment with 100 nM testosterone or R1881 significantly inhibited the production of FABP4 protein.

Dihydrotestosterone and testosterone had no cytotoxic effect as measured by the release of adenylate kinase in the medium (data not shown). The activity of caspases 3/7 was not different in control *vs.* DHT- and testosterone-treated cells (data not shown), suggesting that androgen treatment did not induce apoptosis in human preadipocytes. No cytotoxic effect of androgens as evaluated by adenylate kinase release was observed in differentiated cultures under acute or long-term androgen treatment. Excluding subjects who were taking prescribed medications that could affect metabolism did not alter the major findings of this study. Passage number of preadipocyte cultures did not explain the effects observed in this study.

Discussion

In this study, we tested the hypothesis that androgens would stimulate lipolysis, inhibit HR-LPL activity and inhibit adipocyte

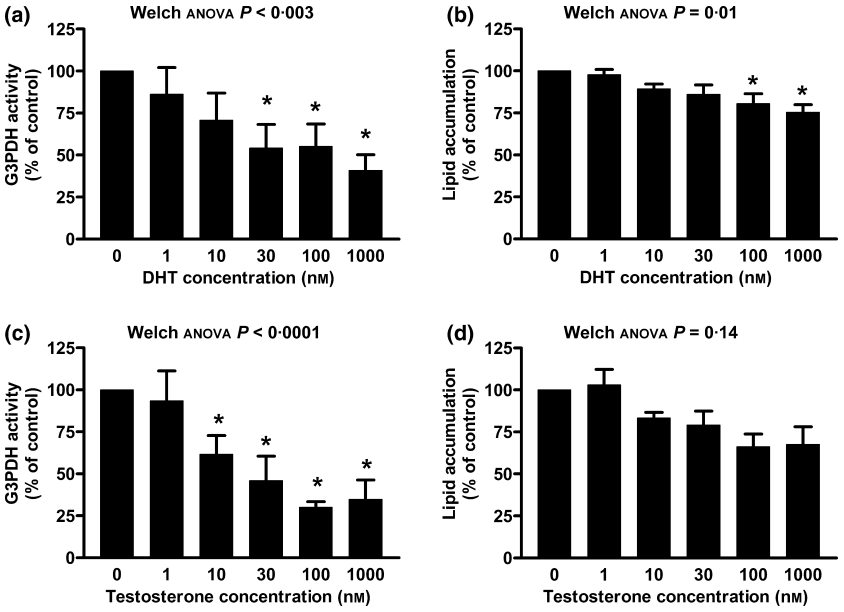

Fig. 4 Effects of dihydrotestosterone (DHT) and testosterone on adipocyte differentiation in preadipocyte cultures obtained from men: (a) Effects of DHT on glycerol-3-phosphate dehydrogenase (G3PDH) activity in pooled subcutaneous (SC) and omental (OM) cultures ($n = 4$ for 10, 30 and 100 nM, $n = 5$ for 1 nM and $n = 7$ for other concentrations). (b) Effects of DHT on lipid accumulation as measured by oil red O staining in pooled SC and OM cultures ($n = 2$ for 30 nM, $n = 5$ for 1, 10 and 100 nM and $n = 7$ for other concentrations). (c) Effects of testosterone on G3PDH activity in pooled SC and OM cultures ($n = 4$). (d) Effects of testosterone on lipid accumulation as measured by oil red O staining in pooled SC and OM cultures ($n = 2$ for 1 nM and $n = 3$ for other concentrations). Mean ± SEM are shown. *: significant difference from the control ($P < 0{\cdot}05$) by *a posteriori* mean contrast.

differentiation. In organ cultures from men, DHT had a statistically significant inhibitory effect on HR-LPL activity in OM cultures. We observed a slight stimulation at the lowest DHT dose whereas an inhibition was observed at the highest DHT concentrations. In women, DHT tended to inhibit HR-LPL activity in the OM depot. In men, testosterone significantly inhibited HR-LPL activity in SC and OM cultures. Only modest, nonsignificant androgenic effects were observed for basal, isoproterenol-stimulated lipolysis and adiponectin release in men. On the other hand, adipocyte differentiation was strongly and significantly inhibited by DHT, testosterone and R1881, in a dose-dependent manner, in SC and OM cultures from both sexes. These effects did not differ according to adipose tissue depot but appeared to be more pronounced in women and SC adipocytes. To our knowledge, this study is the most extensive characterization of the effects of androgens on adipose tissue lipid metabolism and adipocyte differentiation in abdominal SC and OM cultures from men and women.

The major finding of this study is that DHT and testosterone markedly inhibited adipocyte differentiation in SC and OM primary cultures, in both men and women. This is in agreement with recent results obtained in men by Gupta *et al.*,[39] showing that DHT significantly inhibited adipogenesis in primary preadipocyte cultures from abdominal SC, mesenteric and OM adipose tissue. One of the most original features of this study is that we observed that such inhibition was present in both sexes, with no dramatic dimorphism nor pronounced depot differences. It has to be kept in mind that results were expressed in percentage of control and that the absolute differentiation rate was lower in OM than in SC cultures, as observed in previous studies.[54] When G3PDH activity was expressed in mU/μg protein for a subset of experiments, results were essentially similar, but a trend for better inhibition was found in SC *vs.* OM cultures of women. Large inter-individual variations as well as lower differentiation rates in OM cells prevent us from commenting on possible depot differences in men. To reconcile the apparent discordance between these effects of androgens at the adipocyte level and the finding that high doses of androgens in female-to-male transsexuals lead to increased visceral fat accumulation,[23] we propose that androgens inhibit fat cell differentiation where it is predominant in women, that is, in SC fat, thereby shifting the preferential site of fat storage from the periphery to more central depots. The sex dimorphism in the response to androgens could be the result of differences in the expression of or binding to the AR, or in the expression of local steroid-converting enzymes including androgen-, oestrogen- or glucocorticoid-converting enzymes. Further studies are needed to confirm this hypothesis, but this study shows that the anti-adipogenic effects of androgens on adipose cells are clearly observed in both sexes.

The main effect of androgens in organ culture was a trend toward inhibition of HR-LPL activity at high concentrations. This is in agreement with association studies which have found that elevated plasma androgen levels are associated with a lower fat accumulation in men.[5–7] Testosterone therapy significantly decreased lipid accumulation and LPL activity in abdominal SC adipose tissue in healthy men.[55,56] This effect was observed only in visceral fat (OM, mesenteric and retroperitoneal) in another study by the same group.[32] Accordingly, we found that the inhibitory effects of DHT were observed in both sexes and were slightly more pronounced in OM adipose tissue. This suggests that elevated DHT and testosterone concentrations in adipose tissue would lead to reduced free fatty acid delivery through reduced LPL activity. This effect may be particularly important in the OM fat compartment.

In contrast with previous studies[12] and our hypothesis, androgens did not stimulate lipolysis in our experiments. There was a slight trend for stimulation in some of the concentrations tested, but none reached significance. However, we must consider that the *in vitro* experimental procedure used in this study may not be optimal to detect androgenic effects on lipolysis. Rebuffé-Scrive *et al*[56] showed that testosterone treatment in men increased the lipolytic responsiveness to norepinephrine in SC abdominal fat. Thus, we

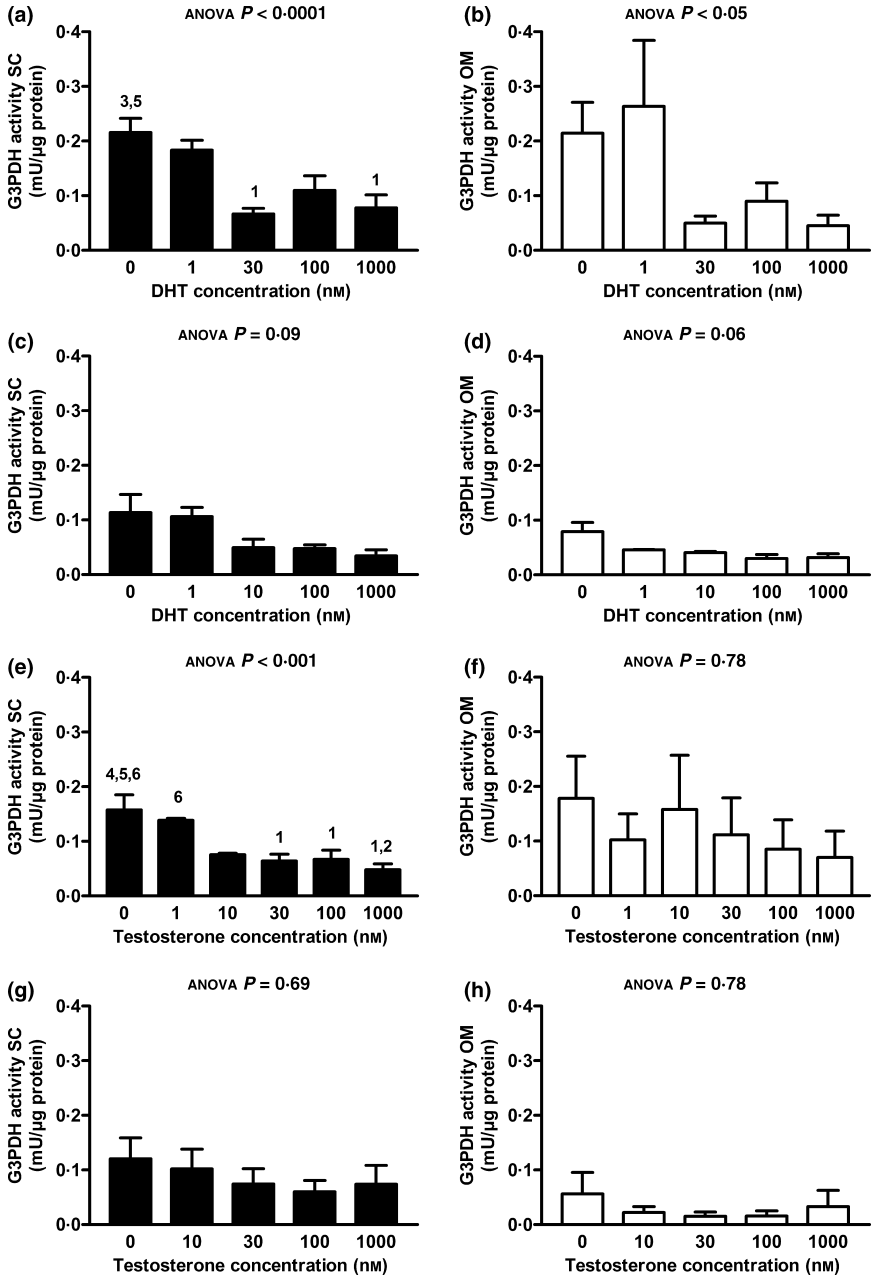

Fig. 5 Effects of dihydrotestosterone (DHT) and testosterone on adipocyte differentiation in subsets of preadipocyte cultures obtained from men and women. Glycerol-3-phosphate dehydrogenase (G3PDH) activity was used to quantify adipogenesis and was expressed as mU G3PDH/µg protein. (a) Effects of DHT in subcutaneous (SC) cultures from women ($n = 4$ for 1 nM, $n = 3$ for 30 nM and 100 nM and $n = 7$ for other concentrations). (b) Effects of DHT in omental (OM) cultures from women ($n = 2$ for 1 and 30 nM and $n = 3$ for other concentrations). (c) Effects of DHT in SC cultures from men ($n = 3$ for 1 nM, $n = 2$ for 10 and 100 nM and $n = 4$ for other concentrations). (d) Effects of DHT in OM cultures from men ($n = 2$). (e) Effects of testosterone in SC cultures from women ($n = 2$ for 1 nM and $n = 5$ for other concentrations). (f) Effects of testosterone in OM cultures from women ($n = 2$ for 1 nM and $n = 3$ for other concentrations). (g) Effects of testosterone in SC cultures from men ($n = 3$). (h) Effects of testosterone in OM cultures from men ($n = 2$). Mean ± SEM are shown. Numbers above bars indicate a statistically significant difference with the corresponding bar using the Tukey–Kramer test.

cannot exclude a positive effect of androgens on lipolysis. Nishizawa *et al.*[57] previously demonstrated that androgens decrease adiponectin secretion by 3T3-L1 adipocytes and plasma adiponectin levels in mice. In this study, we did not find a significant inhibition of adiponectin secretion by androgens, possibly because of the lipogenic culture conditions, which would rather stimulate adiponectin release. Another possible explanation for the absence of androgenic effects on adiponectin secretion may be that androgens could have different effects on oligomeric forms of adiponectin. The assay used in this study did not allow to separate these forms. Accordingly, Xu *et al.*[58] previously observed that testosterone significantly decreased the high molecular weight form, whereas the low molecular weight form of adiponectin was rather significantly increased in differentiated rat adipocytes. Together, our results indicate that the effects of androgens on the adipose tissue explant measures of HR-LPL, lipolysis and adiponectin release were not very pronounced.

Although DHT levels were suggested to be higher in adipose tissue than plasma,[25] DHT concentrations necessary to inhibit adipogenesis in this study are relatively high compared with plasma levels found in men and women.[59] Testosterone is generally found to be less potent at the receptor level compared with DHT.[59] However, testosterone had significant inhibitory effects on adipocyte differentiation at concentrations (10 nM) that are commonly observed in hypogonadal men (serum total testosterone below 10·4–12·1 nmol/l).[59] Interestingly, adipocyte differentiation was decreased by more than 50% after testosterone treatment at a concentration typically found in the plasma of young, healthy men (30 nM). It is important to point out that these effects of testosterone are androgenic.

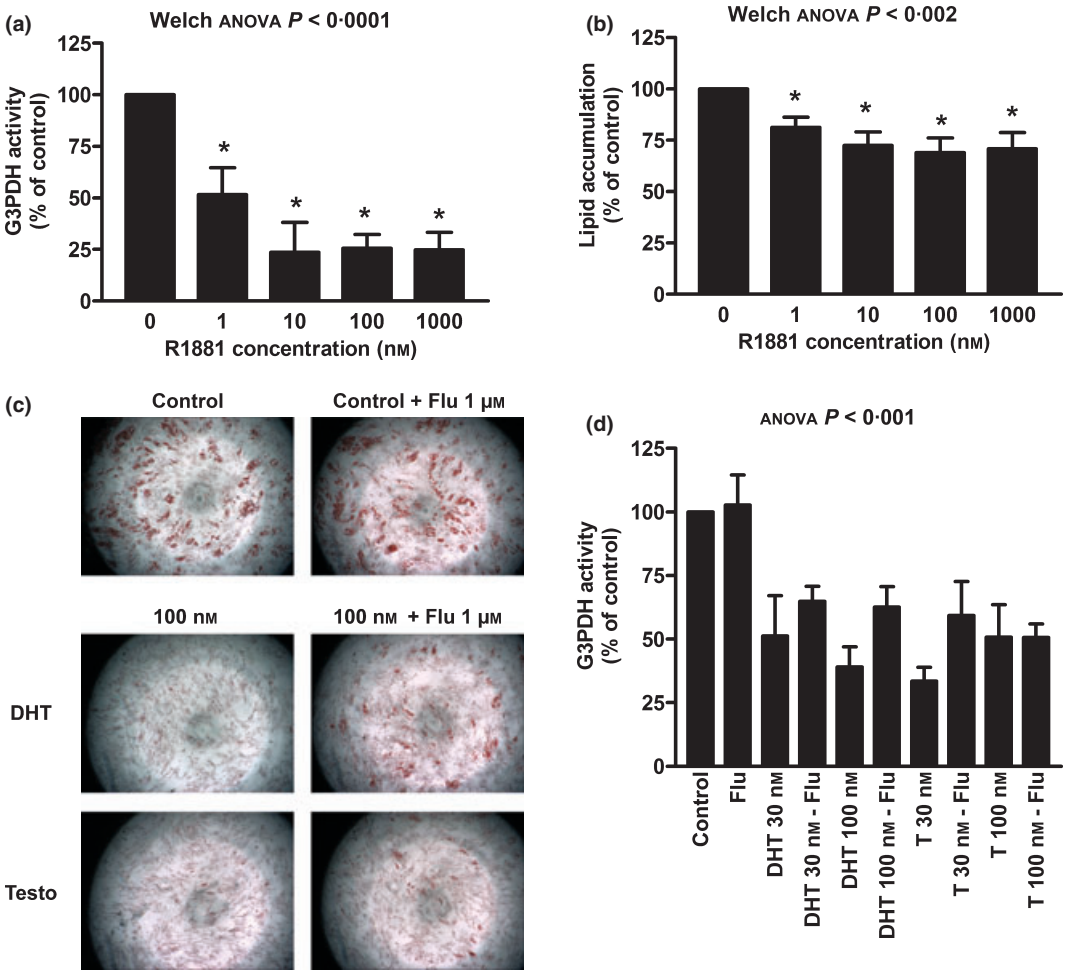

Fig. 6 Effects of the synthetic, nonmetabolizable androgen R1881 on adipocyte differentiation in primary cultures obtained from men and women (total, $n = 5$) [subcutaneous (SC) cultures from women ($n = 3$), omental (OM) cultures from women ($n = 1$) and SC culture from men ($n = 1$)]. (a) Glycerol-3-phosphate dehydrogenase (G3PDH) activity. (b) Lipid accumulation. (c) Photomicrographs showing that the inhibitory effect of dihydrotestosterone and testosterone (T) on adipocyte differentiation was partially attenuated by treatment with 1 μM flutamide (Flu) in a SC primary cultures from a woman. Phase contrast microscopy at 35 × magnification. (d) Effects of androgens and flutamide on G3PDH activity (total, $n = 3$) [SC cultures ($n = 2$), OM culture ($n = 1$)], all culture conditions except flutamide only were significantly different from the control by *a posteriori* mean contrast, $P < 0{\cdot}05$.

Indeed, the synthetic, nonmetabolizable androgen R1881 also significantly inhibited adipogenesis in this study.

Regarding the mechanism of androgenic action, Singh *et al.*[34,35] observed similar inhibitory effects of DHT and testosterone on adipogenesis in murine C3H 10T1/2 pluripotent cells and in 3T3-L1 adipocytes. These authors have shown that the effects of androgens on adipocyte differentiation were mediated, at least in part, through AR-mediated nuclear translocation of β-catenin and induction of the Wnt signalling pathway.[35] Consistent with the latter reports, the AR antagonist flutamide only partially restored adipogenesis, indicating that other AR-independent mechanisms may be involved. Dieudonné *et al.*[33] observed that DHT and testosterone inhibited adipocyte differentiation in epididymal but not in SC rat primary cultures and suggested that the observed androgen-mediated decrease in insulin-like growth factor 1 receptor expression may have been responsible for this effect. Another potential mechanism explaining the effects of androgens may be a possible cross-talk between AR and PPAR-γ through common coregulators. Heinlein *et al.*[60] have shown that ARA70 is a ligand-dependent coactivator of both AR and PPAR-γ induced at the early stage of differentiation.[61] In the presence of androgens, AR may compete with PPAR-γ for the coactivator ARA70 and may decrease PPAR-γ target gene expression and adipogenesis.

Although DHT cannot be transformed to estrogens by P450 aromatase, it can be metabolized to less potent metabolites. Previous studies have shown that DHT can be converted to the inactive steroids 5α-androstan-3α,17β-diol (3α-diol) and 5α-androstan-3β,17β-diol by aldo-keto reductases (AKR) 1C1, 1C2 and 1C3.[29,62] AKR1C2 in particular shows a very strong 3α-ketosteroid reductase activity (DHT inactivation into 3α-diol), especially in mature adipocytes.[29] This may represent a potential explanation for the lower sensitivity to DHT compared with testosterone observed in this study. Accordingly, the nonmetabolizable androgen R1881 also significantly inhibited adipogenesis and was approximately 10–30 times more potent than DHT in the inhibi-

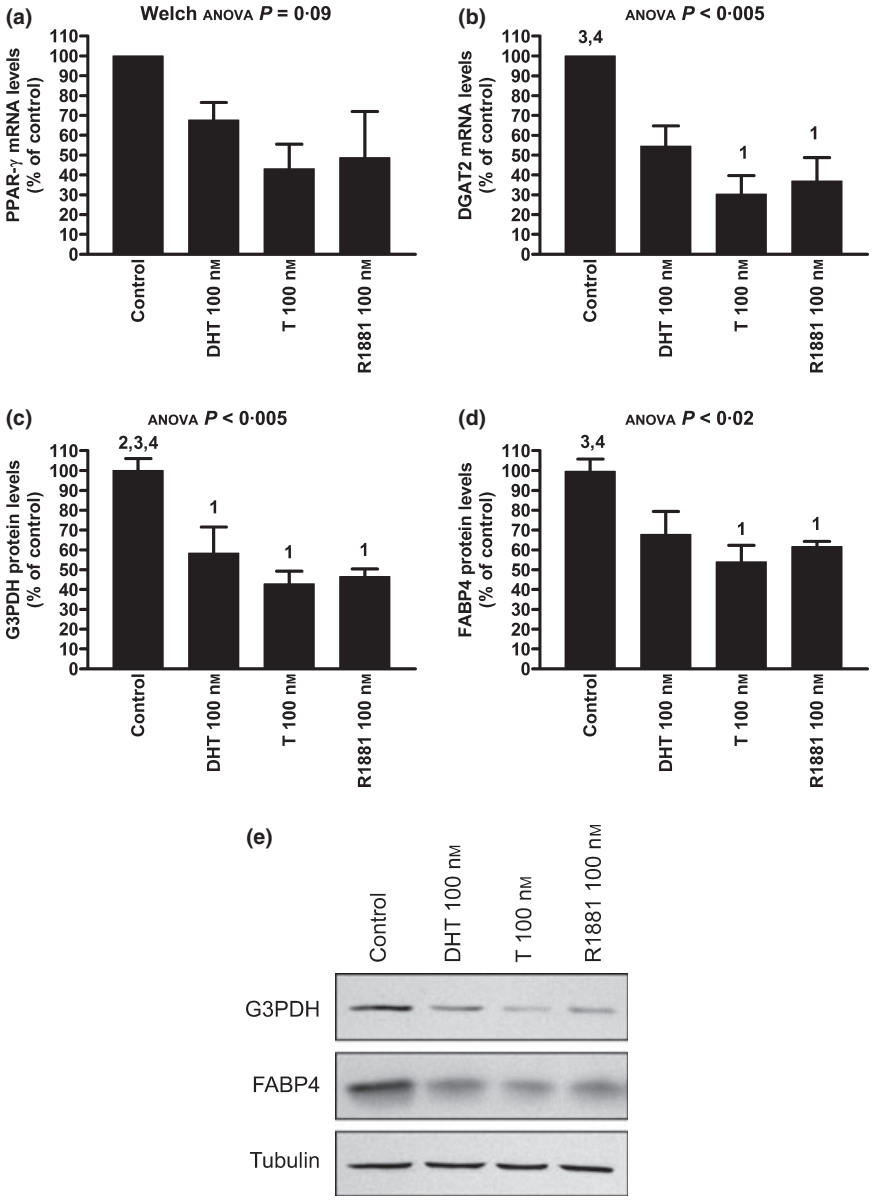

Fig. 7 Expression of late adipocyte markers in cultures differentiated with androgens or vehicle (control). (a, b) mRNA levels ($n = 4$), (c, d) protein levels ($n = 3$), (e) representative Western blot from a SC culture. Mean ± SEM are shown. Numbers above bars indicate a statistically significant difference with the corresponding bar using the Tukey–Kramer test.

tion of adipocyte differentiation. This cannot be explained by a lower binding of DHT to the AR compared with R1881 in adipose tissue.[63] Bélanger *et al.*[28] have demonstrated that adipose tissue DHT levels were significantly higher in OM *vs.* SC adipose tissue of morbidly obese men, and we hypothesized that AKR1C enzymes may be responsible for this depot difference.[29] Our observations suggest that increased androgen inactivation may promote fat observations storage and modulate body fat patterning in humans.

In conclusion, androgens had moderate inhibitory effects on HR-LPL activity in adipose tissue organ cultures, but markedly inhibited adipogenesis in abdominal SC and OM primary cultures, in men and women. No clear sexual dimorphism was observed regarding the direction of the androgenic effect. These data demonstrate for the first time that androgen effects on fat cells from human males *vs.* females may not differ in terms of directionality, but in the magnitude of their negative impact on adipogenesis and lipid synthesis.

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Competing interests/financial disclosure

Nothing to declare.

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